Handbook of Toxicology of Chemical Warfare Agents

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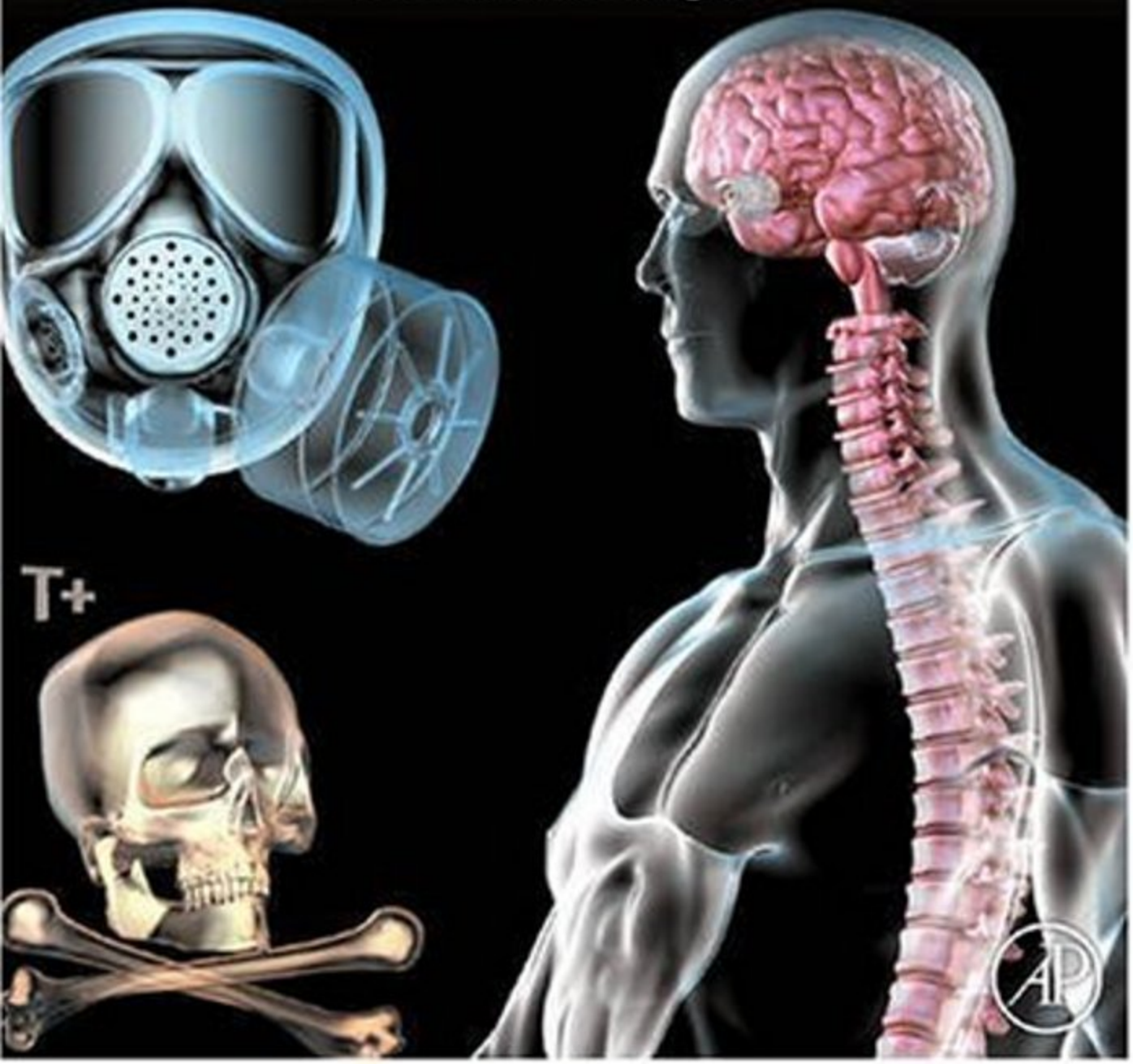
Ramesh C. Gupta



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This book is dedicated to my beloved wife Denise, daughter Rekha,
and parents the late Chandra and Triveni Gupta

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Foreword

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A highly toxic chemical intended to harm, kill, incapacitate, or control adversaries in warfare is called a chemical warfare agent (CWA). Throughout the world, some 70 different chemicals have been developed and produced, and many of them have been stockpiled as CWAs or chemical weapons of mass destructions (CWMD) during the 20th and 21st centuries. The use of biotoxins (botulinum toxin, ricin, saxitoxin, anthrax, etc.) is also sometimes referred to as chemical warfare.

Although the use of CWAs dates back to the 5th century BC, modern CWAs were not used at full scale until World War I. Since then, a variety of CWAs have been developed and deployed in many wars, conflicts, terrorist attacks, hostage crises, and riots. These chemicals came into the limelight particularly due to Gulf War Syndrome (aftermath of Gulf War I, 1991) and the Tokyo Subway terrorist attacks in 1994 and 1995. In the present world situation, the intentional use of highly toxic chemicals as CWAs/CWMD is a growing concern for government officials and civilians alike in developing as well as industrialized countries. The terrorist attacks in New York City on September 11, 2001 led to an increased awareness of protecting national monuments, landmarks, federal and state buildings, and workplaces, in addition to civilians, as possible terrorist targets.

Performing a comprehensive analysis of the inadequate database on chemical warfare agents is often a highly demanding task for toxicologists, risk assessors, regulatory agencies, and policy and decision makers at both state and federal levels. Due to the lack of adequate control, legislation, regulations, and knowledge, the term ''chemical warfare'' is often misunderstood or misused. In the recent past, in order to protect people and educate them about terrorist attacks, a variety of Standards and Exposure Guidelines have been made available, and on January 23, 2008, the U.S. Department of Labor published ''Safety and Health Topics, Chemical Terrorism''.

Handbook of Toxicology of Chemical Warfare Agents is the first book on chemical warfare agents that provides a plethora of knowledge on the historical perspective, epidemiology, detailed toxicology profile of CWAs/CWMD, target organ toxicity, analytical methodologies, biosensors, biomarkers, prophylactic and therapeutic countermeasures, and decontamination and detoxification procedures. In addition, the book serves as a significant source of information for nuclear and biological warfare agents. Therefore, this book appears to be an extremely useful reference source for academicians and regulatory authorities for risk and safety assessment, and management of chemical terrorism. The information provided in this book will draw immense attention from federal and state agencies, as well as political decision makers.

The editor, contributors, and publisher faced tremendous challenges in order to cover comprehensively all possible aspects of the toxicology of CWAs. Presently, there are at least two dozen chemicals that can be used as CWAs. Organophosphate (OP) nerve agents and mustards have been most frequently used and are most likely to be used in the future by terrorists and dictators throughout the world, because of their easy access and delivery systems. As a result, these chemicals have been extensively studied, and books, monographs, reviews, and papers are widely published. Recently, Academic Press/Elsevier published a most comprehensive book entitled *Toxicology of Organophosphate and Carbamate Compounds*, and *Handbook of Toxicology of Chemical Warfare Agents* appears to be unique in providing a thorough assessment of all possible aspects of toxicology, risk assessment, and remedial measures of CWAs in humans, animals, and wildlife.

The contributors of this book from around the globe are leading scientists and internationally recognized for their expertise in particular areas of toxicology of CWAs/CWMD and countermeasures. The book is divided into nine sections that deal with different aspects of CWAs. Section I deals extensively with the historical perspective, epidemiology and global impact of CWAs. Section II covers the broad array of chemical agents that can be weaponized and deployed as CWMD. In this section, toxicity profile, mechanism of action, risk assessment, and prophylactic/therapeutic measures of the individual chemicals are described in an in-depth manner. Section III provides an exhaustive coverage of target organ toxicity, which is indeed a novel aspect of this book. Several chapters on special topics about OP nerve agents, including molecular/

cellular mechanisms and neuropathological modulations, are discussed in section IV. Section V describes the risks to animals and wildlife associated with CWAs and chemicals of terror contaminating feed and water reservoirs, which can have a serious impact on human and animal health and the environment. Section VI deals with the metabolism, toxicokinetics and physiologically based pharmacokinetics of CWAs. A novel section (VII) is introduced with six chapters that provide discussion of analytical methodologies, biosensors, and biomarkers of CWAs. These topics will aid researchers in determining the extent of human/animal exposure, risk/safety assessment of CWAs, and management of poisoning. Section VIII covers extensively the unique approaches and strategies involved in prophylactic and therapeutic management and countermeasures. Many novel topics are included in this section, such as medical management of not only military personnel but civilians (more importantly the pediatric population), physiologically based pharmacokinetic modeling in countermeasures, catalytic and non-catalytic bioscavenging enzymes, and novel oximes. Prophylaxis and therapeutics for other CWAs are discussed in section II dealing with individual CWAs. The final section (IX) deals with information on decontamination and detoxification of CWAs.

In essence, this book is a landmark publication in the field of toxicology of CWAs/CWMD, as it provides comprehensive coverage of these chemicals and emphasizes current and novel issues that have not previously been addressed. It is hoped that this book will aid not only academicians but lay persons in community preparedness at local, state, and federal levels to protect civilians, military personnel, animals, wildlife, and the environment from chemical attacks by terrorists, dictators, and other adversaries. This book will be an invaluable source of information for homeland security, the Department of Defense, the Department of Veteran Affairs, the Department of Defense Research Establishment, diagnostic labs, poison control centers, federal, state and local authorities, forensic scientists, pharmacologists, toxicologists, chemists, biologists, environmentalists, teachers, students, and libraries.

CHAPTER 1

Introduction

RAMESH C. GUPTA

For centuries extremely toxic chemicals have been used in wars, conflicts, terrorists', extremists' and dictators' activities, malicious poisonings, and executions. One of the earliest forms of chemical warfare agents (CWAs) were natural toxins from plants or animals, which were used to coat arrowheads, commonly referred to as ''arrow poisons''. Ancient use of some CWAs and riot control agents (RCAs) dates back to the 5th century BC, during the Peloponnesian War, when the Spartans used smoke from burning coal, sulfur, and pitch to temporarily incapacitate and confuse occupants of Athenian strongholds. The Spartans also used bombs made of sulfur and pitch to overcome the enemy. The Romans used irritant clouds to drive out adversaries from hidden dwellings. In the 15th century, Leonardo da Vinci proposed the use of a powder of arsenic sulfide as a chemical weapon. Modern use of CWAs and RCAs or incapacitating agents dates back to World War I (WWI).

With advancements in science and chemistry in the 19th century, the possibility of chemical warfare increased tremendously. The first full-scale use of chemical warfare agents began in April of 1915 when German troops launched a poison gas attack at Ypres, Belgium, using 168 tons of chlorine gas, killing about 5,000 Allied (British, French, and Canadian) soldiers. During WWI, the deployment of CWAs, including toxic gases (chlorine, phosgene, cyanide, and mustard), irritants, and vesicants in massive quantities (about 125,000 tons), resulted in about 90,000 fatalities and 1.3 million non-fatal casualties. The majority of the deaths in WWI were a result of exposure to chlorine and phosgene gases. During the Holocaust, the Nazis used carbon monoxide and the insecticide Zyklon-B, containing hydrogen cyanide, to kill several million people in extermination camps. Poison gases were also used during the Warsaw Ghetto Uprising in 1943. Again, in November 1978, religious cult leader Jim Jones murdered over 900 men, women and children with cyanide.

Prior to, during, and after World War II, anticholinesterase organophosphate (OP) nerve agents/gases were developed in Germany, the USA, the UK, and Russia, and produced in large volumes in many other countries. They were maximally produced and stockpiled during the ''Cold War'' period. These nerve agents have been used in wars and by dictators, extremists, cult leaders, and terrorist groups as chemical weapons of mass destruction (CWMD) on many occasions. In 1980, Iraq attacked Iran, employing mustard and OP nerve gases. During the period of the Iraq and Iran conflict (1980–1988), Iran sustained 387 attacks and more than 100,000 troops were victims along with significant numbers of civilians. Thousands of victims still suffer from long-term health effects. Shortly after the end of the Iraq–Iran war in 1988, the brutal dictator of the Iraqi regime, Saddam Hussein, used multiple CWAs against the Kurdish minorities in Halbja village, killing more than 10% of the town's 50,000 residents. To date, mustards have been used in more than a dozen conflicts, killing and inflicting severe injuries in millions of military personnel and civilians.

During the Persian Gulf War, exposure to OP nerve agents occurred from the destruction of munitions containing 8.5 metric tons of sarin/cyclosarin housed in Bunker 73 at Khamisyah on March 4, 1991, and additional destruction of these nerve agents contained in rockets in a pit at Khamisyah on March 10, 1991. Although exposure levels to nerve agents were too low to produce signs of acute toxicity, the serving veterans in and around the Khamisyah area still suffer from long-term adverse health effects, most notably ''Gulf War Syndrome''. In 1996, about 60,000 veterans of the Persian Gulf War claimed to suffer from ''Gulf War Syndrome'' or ''Gulf Veterans' Illnesses'', possibly due to low-level exposure of nerve agents, mustard, pyridostigmine bromide and pesticides. Exposed veterans had an increased incidence of chronic myelocytic leukemia and increased risk of brain cancer deaths compared to unexposed personnel.

In the mid-1990s, two terrorist attacks by a fanatic religious cult Aum Shinrikyo (Supreme Truth), known as Aleph since 2000, took place in Japan (Matsumoto, 1994 and Tokyo Subway, 1995). In both incidents, the OP nerve agent sarin was used as a CWA. An estimated 70 tons of sarin was manufactured by Aum Shinrikyo in Kamikuishiki, Japan. Although the total fatality count involved not more than 20 civilians, injuries were observed in more than 6,000 and millions were terrified. These acts of chemical terrorism were unprecedented and the impact propagated not only throughout Japan, but the entire world. In the past few decades, many incidents have also occurred with biotoxins such as ricin and anthrax. Publicity surrounding frequent recent use due to easy access, and

copycat crimes increase the possibility of future use of these chemicals and biotoxins, which warrants advancement in emergency preparedness planning at the federal, state, and local government levels.

It is interesting to note that toxic chemicals have been used by governmental authorities against rebels, or civilians. In 1920s, Britain used chemical weapons in Iraq ''as an experiment'' against Kurdish rebels seeking independence. Winston Churchill strongly justified the use of ''poisoned gas against uncivilized tribes''. The Russian Osnaz Forces used an aerosol containing fentanyl anesthetic during the Moscow theater hostage crisis in 2002. RCAs were frequently used in the USA in the 1960s to disperse crowds in riot control.

At present, more than 25 countries and possibly many terrorist groups possess CWAs, while many other countries and terrorist groups are seeking to obtain them, due to their easy access. Some of these agents are stockpiled in enormous quantities and their destruction and remediation are not only expensive but associated with significant health risks. There is also the possibility of accidental release of CWAs or CWMD at the sites of their production, transportation, dissemination, and deployment. The intentional or accidental release of highly toxic chemicals, such as nerve agent VX (Dugway Proving Ground, Utah, 1968), Agent Orange (Vietnam, 1961–1972), PBB (Michigan, USA, 1973), dioxin (Seveso, Italy, 1976), and methyl isocyanate (Bhopal, India, 1984), has caused injuries in more than a million people, and deaths in several thousands. A 1968 accident with VX nerve gas killed more than 6,000 sheep in the Skull Valley area of Utah.

After September 11, 2001, the chances are greater than ever before for the use of CWMD by extremist and terrorist groups like Al Qaeda, which presents great risks to humans, domestic animals, and wildlife in many parts of the world. On 26 November 2008, Pakistani Islamic terrorists attacked Mumbai city in India at 10 different sites, including two luxury hotels, a Jewish center, a train station, and hospitals and cafes. Approximately 200 innocent people died and about 300 people were injured by bullets and fire smoke. It is more likely that these terrorist groups may use toxic industrial chemicals (agents of opportunity) either as such or as a precursor for more deadly CWMD. At present, many countries have established Defense Research Institutes with two major missions: (1) to understand the toxicity profile of CWAs/CWMDs, and (2) to develop strategic plans for prophylactic and therapeutic countermeasures. By the turn of the 21st century, the USA established the Department of Homeland Security. Many other countries also developed similar governing branches and agencies at the state and national level to protect people and properties from terrorist attacks. Among chemical, biological, and radiological weapons, the possibility of CWMD is more likely because of their easy access and delivery system. It is important to mention that understanding the toxicity profile of CWAs/CWMD is very complex, as these chemical compounds are of a diverse nature, and as a result, treatment becomes very difficult or in some cases impossible.

In the past, many accords, agreements, declarations, documents, protocols, and treaties have been signed at the international level to prohibit the development, production, stockpiling, and use of CWAs, yet dictators and terrorists produce and/or procure these chemicals to harm or kill enemies, create havoc, and draw national and international attention. In 1907, The Hague Convention outlawed the use of chemical weapons, yet during WWI, many countries used these chemicals. The first international accord on the banning of chemical warfare was agreed upon in Geneva in 1925. Despite the General Protocol, the Japanese used chemical warfare against China in 1930. In 1933, the Chemical Weapon Convention banned the development, possession, and use of CWAs. The document was signed and implemented by more than 100 countries. Yet, during WWI many chemicals of warfare were developed, produced, and used by many countries. In 1993, another global convention banning the production and stockpiling of chemical warfare agents was signed by more than 100 countries.

In the present world situation, it is highly likely that these agents will be used in wars, conflicts, terrorist attacks, and with malicious intent. In such scenarios, these extremely toxic agents continuously pose serious threats to humans, animals, and wildlife.

This *Handbook of Toxicology of Chemical Warfare Agents* was prepared in order to offer the most comprehensive coverage of every aspect of the deadly toxic chemicals that can be used as CWAs/CWMD. In addition to the chapters on radiation, several chapters are included on deadly biotoxins (ricin, abrin, strychnine, anthrax, and botulinum toxins) that can be weaponized in chemical, radiological, and biological warfares. Many special and unique topics are offered that have not been covered in previous books. This is the first book that offers detailed target organ toxicity in this area of toxicology. In every chapter, all factual statements are substantiated with appropriate references.

This book meets the needs not only of academicians but lay persons as well. The format employed is user friendly and easy to understand. Standalone chapters on individual chemicals, target organ toxicity, biosensors and biomarkers, risks to man, animal and wildlife, and prophylactic and therapeutic countermeasures are just a few of the many novel topics covered in this book. The chapters are enriched with the historical background as well as the latest information and up-to-date references. With more than 70 chapters, this book will serve as a reference source for toxicologists, pharmacologists, forensic scientists, analytical chemists, local/state/federal officials in the Department of Homeland Security, Department of Defense, Defense Research Establishments, Department of Veterans Affairs, physicians at medical and veterinary emergency care units of hospitals, poison control centers, medical and veterinary diagnostic

labs, environmentalists and wildlife interest groups, researchers in the area of nuclear, chemical, and biological warfare agents, and college and university libraries.

Contributors of the chapters in this book are the most qualified scientists in their particular areas of chemical and biological warfare agents. These scientists are from around the globe and regarded as authorities in the field of pharmacology, toxicology, and military medicine. The editor sincerely appreciates each author for his/her dedicated hard work and invaluable contributions to this volume. The editor gratefully acknowledges Robin B. Doss and Kristie A. Rohde for their technical assistance, Alexandre M. Katos for cover design and Denise M. Gupta for indexing. Finally, the editor remains indebted to Renske Van Dijk, Rebecca Garay, and William Brottmiller, the editors at Elsevier, for their immense contributions to this book.

CHAPTER 2

Historical Perspective of Chemical Warfare Agents

NATHAN H. JOHNSON, JOSEPH C. LARSEN, AND EDWARD MEEK

The opinions or assertions contained herein are the private views of the authors, and are not to be construed as reflecting the views of the Department of Defense, the Defense Threat Reduction Agency, or the US Air Force.

I. INTRODUCTION

The employment of chemicals in warfighting has a long history (Silvagni *et al.*, 2002; Romano *et al.*, 2008). Just as the utilization of chemicals brought about tremendous advances in society, the concept of using chemicals as a contributing factor in winning wars has been pursued for centuries (Joy, 1997; Smart, 1997). There are many examples of the exploitation of chemicals in warfare and conflict dating back to antiquity. Primitive man may have been the first to use chemical compounds in hunting and in battle. The use of smoke from fires to drive animals or adversaries from caves may have been the earliest use of chemical weapons. Natural compounds from plants, insects, or animals that were observed to cause sickness or death were likely used by our distant forefathers in attempts to gain or maintain superiority (Hammond, 1994). Natural toxins from plants or animals on arrowheads or the poisoning of water or food could increase casualties and cause fear in opposing military forces or civilian populations. These early uses of chemicals would pave the way for more lethal chemical weapons. For example, in the fourth century BC, smoke containing sulfur was used in the war between Sparta and Athens (Joy, 1997). Chinese manuscripts indicate arsenical-based compounds were used in conflict (Joy, 1997). A few hundred years later, toxic smoke was used by the Romans in Spain (Coleman, 2005). During the second siege of Constantinople, the Byzantine emperor Leo III used ''Greek fire'' in his quest for military victory (Coleman, 2005). During the ensuing years, there were many instances of the limited and attempted use of chemicals and toxins on the battlefield. Many of these examples may have been influenced by the intentional poisonings occurring in civilian settings (Joy 1997; Smart, 1997; Newmark, 2004; Coleman, 2005). The earliest known treaty to ban poisons in warfare was signed between the French and Germans in the 17th century (Smart, 1997). In the siege of Groningen, incendiary devices were used by European armies to release belladonna, sulfur, and other compounds. This led to the Strasbourg Agreement in 1675 (Coleman, 2005). This agreement prohibited poison bullets (Smart, 1997).

As science and chemistry advanced in the 19th century, the possibilities of chemical warfare increased exponentially. Advancements were made in industrial applications of sulfur, cyanide, and chorine (Joy, 1997). In addition, the concept of chemicals in projectiles was introduced. During the Crimean War, the British refused to use cyanide-based artillery shells against the Russians on the grounds that it was a ''bad mode of warfare'' (Smart, 1997). This was an early example of the ethical questions surrounding chemical use in warfare that continued into the 20th century (Vedder and Walton, 1925). During the American Civil War, both the Northern and Southern armies seriously considered using various chemicals in their pursuit of operational victories (Smart, 1997). Early attempts at international treaties were met with mixed results. The USA prohibited any use of poison in the Civil War. The Brussels Convention on the Law and Customs of War of 1874 prohibited poisons or poison-related arms (Smart, 1997). The first Peace Conference at The Hague prohibited projectiles filled with asphyxiating or deleterious gases (Smart, 1997). Some countries, including the USA, were not signatories to this agreement. The employment of chemicals as asphyxiating warfare agents was vigorously discussed at The Hague convention (Joy, 1997). Arguments were again made against chemicals based on moral grounds. However, counterarguments were made based on the assumption that chemicals lead to a death devoid of suffering (Vedder and Walton, 1925; Joy, 1997; Coleman, 2005). Individuals who advocated chemicals did not see their use as an unfair advantage; rather, it was just one in a series of technological advances, which if mastered could provide strategic, operational, and tactical advantages on the battlefield. The second Peace Conference at The Hague 8 years later prohibited poisons or poisoned weapons (Smart, 1997). The British use of picric acid-filled shells during the Boer War and the Japanese use

of arsenical rag torches in the Russo-Japanese War further illustrate that chemical warfare was considered by some a legitimate form of warfare at the turn of the 20th century (Smart, 1997). During the early 20th century, technological advancements in the chemical industry made the possibility of sustained military operations using chemicals a realistic possibility. The murder of Archduke Francis Ferdinand at Sarajevo set the stage for what would become the first widespread use of chemical weapons to date (Harris and Paxman, 2002).

II. THE FIRST SUSTAINED USE OF CHEMICALS AS AGENTS OF WARFARE

The talk and rhetoric of the late 19th century should have prepared the countries involved in World War I for chemical warfare. However, that was not case (Smart, 1997). World War I clearly demonstrated the deadly and destructive nature of chemicals in modern warfare. Both alliances in the war experimented with novel forms of warfare, to include chemical weapons, and followed the lead of their advisory (Hay, 2000). It is little wonder this war is known as the ''chemist's war'' (Fitzgerald, 2008). Initially, the French used gas grenades with little effect and were followed by the German use of shells filled with tear gas (Joy, 1997). The Germans, capitalizing on their robust chemical industry, produced shells filled with dianisidine chlorosulfate (Smart, 1997). These shells were used in October of 1914 against the British at Neuve-Chapelle but had little effect. In the winter of 1914–15, the Germans fired 150 mm howitzer shells filled with xylyl bromide (Smart, 1997). The xylyl bromide shells were fired on both the eastern and western fronts with disappointing effects. Despite the inauspicious start of chemical warfare on both fronts, efforts were continued to develop new uses. It would soon be evident that chemical warfare would be devastating on the battlefield (Coleman, 2005; Tucker, 2006). Fritz Haber, a German scientist who later won the Nobel prize in Chemistry, had proposed the possibility of releasing chlorine gas from cylinders (Joy, 1997). Chemical warfare was attractive to Germans for two reasons: the shortage of German artillery shells and the ability to defeat the enemy trench system (Smart, 1997). After consideration and debate, the Germans released chlorine in April 1915 at Ypres, Belgium (Coleman, 2005). The German military was not prepared for the tremendous operational advantage the chlorine release provided. It did not take long for the British and French forces to respond in kind to the German offensive (Vedder and Walton, 1925; Joy, 1997; Smart, 1997; Coleman, 2005). In the fall of 1915, a British officer, William Livens, introduced a modified mortar (Figure 2.1) that could project gas-filled shells of chlorine or phosgene, the two agents of choice at that time (Joy, 1997). Both chlorine and phosgene caused extreme respiratory problems to those soldiers who were exposed (Vedder and Walton, 1925; Joy, 1997; Smart, 1997; Coleman, 2005; Hurst *et al.*, 2007) (Figure 2.2).

As the USA entered the war in the spring of 1917, an obvious concern of the military command was the effect of chemical warfare on standard operations. Chemistry departments at universities were tasked with investigating and developing novel chemical agents (Joy, 1997). Protective equipment (Figure 2.3) and basic studies of the biological effects of chemical agents were assigned to the US Army Medical Department (Joy, 1997). In the fall of 1917, the Army began to build an industrial base for producing

FIGURE 2.1. British Livens Projector, Western Front, World War I.
Source: United Kingdom Government (http://en.wikipedia.org/wiki/Image:Livens_gas_projector_loading.jpg)

FIGURE 2.2. Australian infantry in trench with gas masks donned, Ypres, Belgium, September 1917. Photo by Captain Frank Hurley (http://en.wikipedia.org/wiki/Image:Australian_infantry_small_box_respirators_Ypres_1917.jpg)

FIGURE 2.3. US Army captain wearing a gas mask in training, 1917.
Source: Library of Congress.

chemical agents at Edgewood Arsenal, Maryland (Joy, 1997). As the effects of chlorine and phosgene became diminished by the advent of gas masks (Figure 2.4), the Germans turned to dichlorethyl sulfide (mustard) at Ypres against the British (Joy, 1997). As opposed to the gases, mustard remained persistent in the area and contact avoidance was the major concern (Joy, 1997). It is worth noting that almost 100 years after it was first used on the battlefield, mustard still has no effective treatment and research continues for effective therapeutics (Babin and Ricketts *et al.*, 2000; Baskin and Prabhaharan, 2000; Casillas and Kiser, 2000; Hay, 2000; Schlager and Hart, 2000; Hurst *et al.*, 2007; Romano *et al.*, 2008). It has been estimated that there were over one million chemical casualties (Figure 2.5) of World War I with almost 8% being fatal (Joy, 1997). The Russians on the eastern front had a higher percentage of fatalities when compared with other countries in the war, primarily due to the later introduction of a protective mask (Joy, 1997). The relatively low mortality rate of chemical casualties in World War I demonstrated the most insidious aspect of their use, the medical and logistical burden it placed on the affected army. The eventual Allied victory brought a temporary end to chemical warfare. In 1919, the Treaty of Versailles prohibited the Germans from production and use of chemical weapons.

III. INITIAL COUNTERMEASURES

The conceptualization of a protective mask dates back over 500 years to Leonardo da Vinci (Smart, 1997). By the mid-19th century, protective masks were proposed in the USA and Europe for both industrial and military use. The modern ''gas mask'' was developed by the Germans with sodium thiosulfate and bicarbonate soaked pads and used in World War I (Joy, 1997). The French and English soon followed with their own versions of gas masks (Joy, 1997). In 1916, the Germans introduced a mask that incorporated a canister through which the soldiers breathed (Joy, 1997). Initially, the American forces in World War I used gas masks obtained from allies already fighting in the war (Smart, 1997). In 1918, the Americans introduced their RFK mask, a modified version of the British mask. Masks were also developed for the animals that supported the war fighting efforts. Decontamination efforts during World War I were rudimentary and included chemical neutralization and aeration of clothing and equipment. Although the need for

FIGURE 2.4. World War I soldier and horse wearing gas mask. *Source*: National Archives and Records Administration (http://commons.wikimedia.org/wiki/Image:Gasmask_for_man_and_horse.jpeg)

detection of chemical agents was clearly identified, very little progress was made during World War I. Medical treatment included removal of the patient from the source, decontamination, and palliative care (Smart, 1997).

IV. EVENTS AFTER WORLD WAR I

At the conclusion of World War I, the world had been introduced to chemical warfare on an unprecedented level. While there were groups that thought that humanity had learned a lesson from the cruel nature of chemical warfare, others prudently went to work on improved chemical defense (Vedder and Walton, 1925). The thoughts of many professional military officers were that future wars would be fought under the new paradigm of chemical warfare (Vedder and Walton, 1925; Vedder, 1926; Smart, 1997). New gas masks were developed and training in chemical environments was introduced (Vedder and Walton, 1925; Vedder, 1926; Joy, 1997). Textbooks and manuals, such as those written by US Army Colonel Edward B. Vedder (Figure 2.6), were introduced to the military medical community (Vedder and Walton, 1925). In addition, the civilian medical community gained valuable insight into toxicology and animal models from the events of World War I (Vedder, 1929; Johnson, 2007). Despite the first-hand experience with chemical warfare, some countries, including the USA, struggled to adequately fund their offensive and defensive programs during demobilization (Smart, 1997). It did not take long for chemical warfare to appear in other conflicts. Chemical agents were used to subdue rioters and suppress rebellions. The British used chemical agents to suppress uprisings in Mesopotamia by dropping bombs in cities throughout the area in the early 1920s (Coleman, 2005). The Soviet Union used chemical agents to quell the Tambov rebellion in 1921, and France and Spain used mustard gas bombs to subdue the Berber rebellion during the 1920s (Werth *et al.*, 1999). Italy and Japan used mustard in small regional conflicts (Joy, 1997). The Italian conflict in Ethiopia was noteworthy because

FIGURE 2.5. British soldiers temporarily blinded by tear gas awaiting treatment at the Battle of Estaires, April 1918. Photo by 2nd Lt T.L. Aitken.
Source: United Kingdom Government (http://en.wikipedia.org/wiki/Image:British_55th_Division_gas_casualties_10_April_1918.jpg)

FIGURE 2.6. Captain Edward Vedder, ''the father'' of USAMRICD. Photo courtesy of Mrs Martha Vedder.

mustard was sprayed and dropped from planes and the agent's use was considered by some to be significant to the Italian victory (Smart, 1997). This use demonstrated the contemporary thought that allowed chemicals to be viable alternatives to traditional combat. The Japanese also used chemical weapons during the 1930s against regional foes. Mustard gas and the vesicant Lewisite were released on Chinese troops and were also used in South East Asia (Coleman, 2005). Lewisite is an arsine which was usually produced as an oily brown liquid that was said to have the odor of geraniums (Spiers, 1986; Hammond, 1994). It was developed in the USA by Winford Lee Lewis in 1918 and was found to be effective at penetrating clothing. The USA produced approximately 20,000 tons of Lewisite but only used small quantities of the chemical in World War I (Coleman, 2005). Dimercaprol, more commonly called British anti-Lewisite, was developed as an effective treatment for the vesicant (Goebel, 2008). In the inter-war period, mustard was a key concern in defensive planning (Coleman, 2005). New stores of mustard were produced in many countries. Work continued on many fronts to improve protective equipment. For example, the US Chemical Warfare Service introduced the M1A2 mask, an improvement of the M1 mask (Smart, 1997). In the Geneva Protocol of 1925, 16 of the world's major nations pledged never to use gas as a weapon of warfare; it was not ratified in the USA until 1975 (Hammond, 1994). There has long been vigorous debate on the merits of treaties with nations balancing the military needs versus the potential irrational concept of chemical warfare (Vedder, 1926).

V. WORLD WAR II

In the lead up to World War II, the Germans forever changed chemical warfare with the discovery of the organophosphorus nerve agents (Goebel, 2008). These organophosphorus-containing nerve agents inhibit cholinesterase enzyme in the nerve synapse responsible for the breakdown of the neurotransmitter acetylcholine (ATSDR, 2008). This results in the accumulation of the neurotransmitter in the synapse and overstimulation of the nervous system. This can result in subsequent respiratory failure and death (ATSDR, 2008).

In 1936, Gerhard Schrader, a German chemist working on the development of insecticides for IG Farben, developed a highly toxic organophosphate compound which he named ''tabun'' (Hersh, 1968; Hammond, 1994). Schrader and an assistant became a casualty of their discovery when a drop of the neurotoxicant was spilled in the lab exposing both of them (Tucker, 2006). Had the amount of tabun spilled been greater both researchers would have certainly succumbed to the effects of the poison. Tabun was the first member in a series of compounds termed ''nerve gases'' (Coleman, 2005). The correct terminology is ''nerve agents'' as these agents are not gases, but liquids dispersed as fine aerosols. Tabun was extremely toxic in small amounts and invisible and virtually odorless (Tucker, 2006). The compound could be inhaled or absorbed through the skin. These characteristics made it too dangerous to be used as an insecticide by farmers. German law required that any discovery having military application be reported to military officials (Tucker, 2006). Schrader was not overly excited about producing chemical agents for the military; however, the Germans placed him in a secret military research facility with the emphasis on producing these nerve agents and discovering new agents (Tucker, 2006). Subsequently, Schrader and his team of researchers discovered a more lethal organophosphate compound similar to tabun, which he named ''sarin'' in honor of the team members: Schrader, Ambrose, Rudriger, and van der Linde (Coleman, 2005).

At the onset of World War II, both the Allies and the Germans anticipated chemical agents would be deployed on the battlefield (Tucker, 2006). This expectation intensified research into the development of new agents, delivery systems, and methods of protection (Figures 2.7 and 2.8). The Allied forces were unaware of the Germans' new nerve agent, tabun, at the beginning of the war. The rapidly advancing German army offered very little opportunity to use chemical agents, as it could prevent the rapid movement of the German troops into an area after being released (Tucker, 2006). Nevertheless, the Germans produced and stockpiled large amounts of nerve agents throughout the war (Spiers, 1986). The production of these organophosphate agents was complex, required custom equipment, and was hazardous to those involved in production (Tucker, 2006). If exposed, the workers would be dunked in a bath of sodium bicarbonate (Harris and Paxman, 2002; Goebel, 2008). It is also interesting to note that some members of the German workforce were given rations containing higher percentages of fat (Harris and Paxman, 2002). This was done because

FIGURE 2.7. Gas mask production – Detroit, Michigan, 1942. *Source*: Library of Congress.

FIGURE 2.8. World War II: a private trains using protective gear. Photo courtesy of the US Army Medical Research Institute of Chemical Defense.

authorities observed that workers with higher quality rations seemed protected against exposure to low levels of tabun. Many detainees were used in the manufacture and testing of chemical agents in Germany (Harris and Paxman, 2002; Tucker, 2006). It is not known how many chemical casualties there were in prisoners of war due to their forced labor in nerve agent production, but documented fatalities were recorded. The discovery of tabun and sarin was followed by the discovery of soman by Richard Kuhn and Konrad Henkel at the Kaiser Wilhelm Institute for Medical Research in 1944 (Tucker, 2006). This class of nerve agents is collectively termed ''G'' agents; the G is for German, since German researchers discovered this class of compounds. A second letter is included as the specific identifier of each compound: GA (tabun), GB (sarin), GD (soman), and GF (cyclosarin) (ATSDR, 2008). These agents were mass produced by the Nazi regime throughout the war but were not used (Tucker, 2006). There has been considerable debate questioning why the Germans did not employ their chemical weapons in World War II. While it may never be conclusively known, several potential reasons include a lack of intelligence regarding the German superiority in chemical weapons discovery, fear of retaliation, and Adolph Hitler's personal exposure to chemical agents on the battlefield in World War I (Harris and Paxman, 2002; Tucker, 2006).

Other chemical agents that had been produced during and following World War I were still being produced. On December 2, 1943, German planes sank several American ships off the coast of Italy and at least one of the ships contained mustard that was to be used as a retaliatory response if the Germans unleashed a large-scale chemical weapons attack (Tucker, 2006). Casualties resulted from exposure to the mustard, some of which were inflicted on civilian merchant seamen (US Navy, 2008). The presence of the agent on the ship was classified and resulted in incorrect treatment of many of the exposed by physicians (Tucker, 2006).

VI. POST-WORLD WAR II

By the conclusion of World War II, both the Allies and Germany had stockpiled large amounts of chemical agents (Tucker, 2006). The Allied forces divided up the stockpiles of agents discovered in German facilities. Following the end of the war, many of the Allied countries continued to conduct research on the German nerve agents. The rise of the Soviet Union as a power and adversary prompted the USA and other countries to continually search for novel chemical and biological warfare agents (Tucker, 2006). The research and resources that were allotted for these efforts were not trivial even though they were often overshadowed by the research and development of thermonuclear weapons (Hersh, 1968; Goebel, 2008).

The post-World War II era ushered in the nuclear age. Some felt the age of chemical warfare was past (Smart, 1997). Events would prove this to be a hasty conclusion. In the USA, research of the G-series agents and medical countermeasures against these agents was accomplished by

the late 1940s. Research and intelligence gathering was further hastened by the impressive gains the Soviet Union made in chemical warfare capability in the years after World War II. By the early 1950s, production of sarin had been initiated in the USA (Smart, 1997). At nearly the same time, Ranajit Ghosh, a chemist at the British Imperial Chemical Industries plant, developed a new organophosphate compound as a potential insecticide (Tucker, 2006). Like Gerhard Schrader before him, this compound was deemed too toxic to be used in the field as a pesticide. The compound was sent to researchers in Porton Down, England, synthesized and developed into the first of a new class of nerve agents, the ''V'' agents (Goebel, 2008). Like the ''G'' agents the ''V'' agents also have a second letter designation: VE, VG, VM, and VX (Coleman, 2005). Of these agents, VX was the most commonly produced. The ''V'' series of agents are generally more toxic than the ''G'' agents (ATSDR, 2008). In a deal brokered between the British and US governments, the British traded the VX technology for thermonuclear weapons technology of the USA (Tucker, 2006). The USA produced and stockpiled large quantities of VX (Hersh, 1968; Hammond, 1994).

Throughout the 1950s and 1960s, advancements were made in production and delivery of chemical weapons to include sarin and VX (Smart, 1997). While work on improved masks continued, a renewed concern was the inability to detect nerve agents. Several prototypes were developed in the mid-1950s. Great advancements were made in therapeutics of agents that inhibited the enzyme acetylcholinesterase (Gupta, 2006; Taylor, 2006; Klaassen, 2008). Atropine was introduced in the early 1950s. Oximes were added as an adjunct to speed up reactivation of the enzyme (Smart, 1997). The autoinjector was developed to overcome user fear of self-injection of atropine. Major advances were made in utilization of chemical weapons in artillery (Figure 2.9). For example, the USA developed both short and long range rockets filled with chemical agent. The USA disposed of stockpiles of its chemical weapons in the late 1960s in an operation termed CHASE (cut holes and sink em) in the sea (Coleman, 2005). In 1969, nerve agent stockpiles were discovered in US depots in Japan after several US military servicemen became ill while doing maintenance (Tucker, 2006). This stockpile had been kept secret from the Japanese and created an uproar that resulted in the later disposal of the agents in the Johnston Atoll in the Pacific Ocean.

Defensive equipment such as improved field alarms and drinking tubes for gas masks were introduced in the 1960s (Smart, 1997). Great strides were also made in collective protection in the 1960s and 1970s. Although not used extensively since World War I, chemical agents have nonetheless been used for military purposes. The Egyptians allegedly used mustard and possibly nerve agents in the Yeman civil war (Joy, 1997; Smart, 1997). This was the first reported use of nerve agent in armed conflict. There were allegations that chemical agents were used by the Vietnamese in Laos and Kampuchea in the late 1970s (Coleman, 2005). In the Vietnam War, the USA used defoliants and tear gas (Joy, 1997). The Soviet Union was accused of using chemical agents in their war in Afghanistan (Joy, 1997).

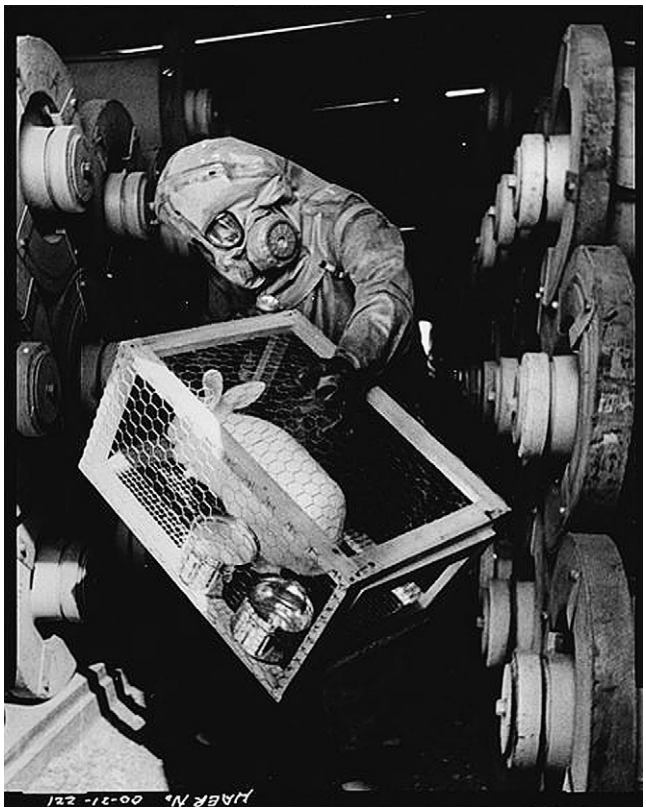

FIGURE 2.9. Testing for leaks at Sarin production plant, 1970. *Source*: Library of Congress (http://memory.loc.gov/pnp/habshaer/co/co0100/co0168/photos/316333pr.jpg).

VII. INCAPACITANTS AND TOXINS

Incapacitating agents have long been considered an intermediate between chemical and traditional warfare. The Germans investigated the military use of lacrimators in the 1880s followed shortly thereafter by the French (Smart, 1997). The English and French considered using lacrimators in World War I (Smart 1997). Japanese forces used tear gas against the Chinese in the late 1930s. The US Army used riot control agents and defoliants in the Vietnam War (Smart, 1997). The defoliant ''Agent Orange'' was later potentially linked to several forms of cancer (Stone, 2007). During the 1950s and 1960s, the USA had an active incapacitant program (Smart, 1997). These agents were thought of as more humane than traditional chemical agents because the intent was not lethality. These agents were designated ''K-agents'' and included tetrahydrocannabinol and lysergic

acid (Smart, 1997). One of the most extensively studied incapacitating agents was 3-quinuclidinyl benzilate, designated BZ by the US Army (Ketchum, 2006). Like many incapacitating agents, BZ was not adopted due to difficulty producing reproducible effects, unwanted side effects, latency to produce effects, and difficulty in producing a dissemination that was free of smoke (Smart, 1997; Ketchum, 2006).

There have been multiple attempts to use the toxins from plants and living organisms to develop viable weapon systems. Two that are noteworthy are ricin and botulinum toxin. Ricin has been recognized as a potential biological weapon since World War I. While the British were developing the V agents, US military researchers patented a procedure for purifying ricin, a very potent toxin from the castor bean plant (Harris and Paxman, 2002). The development of a method of dissemination of ricin as a chemical weapon proved problematic thus making its use very limited. In 2003, ricin was detected on an envelope processed in a Greenville, South Carolina, postal facility. Postal workers did not develop symptoms of ricin exposure and the individual who mailed the letter remains at large (Shea, 2004). The development and use of botulinum neurotoxin as a biological weapon was initiated at least 60 years ago (Smart, 1997; Arnon, 2001). In the 1930s, during occupation of Manchuria, the Japanese biological warfare group, Unit 731, purportedly fed cultures of *Clostridium botulinum* to prisoners causing human lethality. The US Army biological weapons program produced botulinum neurotoxin during World War II in response to Germany's biological weapons program (Coleman, 2005). In fact, more than 100 million toxoid vaccine doses were prepared and forward positioned in time for the D-Day invasion of Normandy (Arnon, 2001).

VIII. RECENT EXPERIENCES

The 1980s proved to be very significant in the employment of chemical weapons on the battlefield. In 1980, Iraq invaded Iran (Smart, 1997). The Iraqi armed forces, who were advised by the Soviet Union, possessed chemical agents and were trained in their use. The war was unequivocally barbarous and neither side gained an advantage. In many ways, this war had similarities to World War I. By 1983, Iran formally protested to the United Nations about the Iraqi use of chemical agents. The general consensus was that Iraq used mustard agent and possibly tabun in this war (Figure 2.10). It is estimated that 5% of Iranian casualties, totaling approximately 45,000, can be attributed to chemical warfare agents (Smart, 1997). The same author also reported that the Iraqi Army used chemical agents against the Kurdish minority in northern Iraq. Lybia was also suspected of using chemical agents when Chad was invaded in 1986 (Smart, 1997).

FIGURE 2.10. Aftermath of Iraqi chemical weapon attack (1980s). Photo by Sayeed Janbozorgi; image used under the terms of the GNU free documentation license (http://en.wikipedia.org/wiki/Image:Chemical_weapon2.jpg).

The late 1980s also saw improvements in defensive equipment such as the M40 gas mask developed by the USA (Smart, 1997). Other advancements were made in collective protection, decontamination, and detection. In 1984, US President Ronald Reagan issued a statement calling for an international ban on chemical weapons (Tucker, 2006). Subsequently, on June 1, 1990, President George H.W. Bush and Soviet Union leader Mikhail Gorbachev signed a treaty banning the production of chemical weapons and initiated the destruction of the stockpiles of both nations (Tucker, 2006). In 1993, the Chemical Weapons Convention was convened and signed. It was implemented in 1997 (Hammond, 1994). As of 2008, the vast majority of United Nations member states have joined the Chemical Weapons Convention (OPCW, 2008).

In 1990, the Iraqi Army invaded neighboring Kuwait. Subsequently, the USA and eventually a coalition sent forces to the area at the request of Saudi Arabia (Smart, 1997). Because of the broad knowledge of Iraqi chemical use on the battlefield in the 1980s, coalition forces were the largest force to operate in a potential chemical environment since World War I. Forces moved into the area of operation were provided with atropine autoinjectors, an acetylcholinesterase reactivator, and a nerve agent pretreatment (pyridostigmine bromide). Fortunately, chemical weapons were not apparently used in this conflict, although multiple false alarms were reported. The failure of the Iraqi military to use chemical weapons could be attributed to fear of retaliation, breakdown of communication, changing wind patterns, the surprising speed of the coalition attack, or the fact that Iraqi chemical infrastructure was attacked during the initial portion of the conflict. There have been many coalition veterans who report a myriad of symptoms that

have been commonly referred to as ''Gulf War Syndrome''. The etiology of this syndrome is unclear despite multiple epidemiological studies (Coleman, 2005).

IX. TERRORIST USE

One of the reasons why chemical weapons have been used relatively infrequently in combat over the past century is the fear of retaliation by opposing countries. In less organized asymmetrical conflicts, the fear of retaliation is of less concern. The potential exploitation of chemical weapons by terrorists is of great worldwide concern. The appeal of these weapons to terrorists is centered on the fact that many of the chemical agents are cheap and relatively easy to produce, transport, and release. These characteristics, along with the fear associated with the idea of a chemical attack, make chemicals an ideal weapon for creating terror (Romano and King, 2001). In 1974, Muharem Kurbegovic attacked several public buildings with firebombs in California and claimed to have developed sarin and some other nerve agents (Tucker, 2006). The search of his home resulted in the discovery of various precursor materials for chemical agents and a large amount of sodium cyanide. In 1994, the Aum Shinrikyo, a Japanese religious cult, carried out several attacks using sarin produced by the cult's members (Tucker, 2006). The attacks included a residential and subway exposure. A total of 19 people were killed and over 6,000 sought medical attention. Some of those seeking medical attention may be attributed to a fear of exposure. Psychological stress is a common aftermath of a chemical or biological attack (Romano and King, 2001). In the 21st century, formerly used chemicals of military interest have reemerged as contemporary threats. In the fall of 2006, Al Qaeda and associated groups used chlorine combined with traditional car and truck bombings to spread panic in Iraq (Garamone, 2007). These attacks were followed by similar attacks in the subsequent months.

X. CONCLUDING REMARKS AND FUTURE DIRECTION

As long as there are legitimate uses for chemicals in our society, the risk of chemical agents in conflict and terrorist activity will always be present. Research across the globe continues for better detection, protection, and treatment of chemical warfare agents. While many countries have denounced and are signatories to various treaties to limit the use and production of chemical warfare agents, non-state and terror organizations are under no such restrictions. Luckily, chemical weapon use has been limited in warfare and conflict. As we progress into the 21st century, the use of established chemical warfare agents is a real possibility. The potential use of legitimate industrial chemicals (e.g. the Iraqi burning of petroleum fields in the first Gulf War) and the potential synthesis of new agents should also be recognized. History has demonstrated that chemicals have been used in both organized and asymmetrical conflicts and preparations for defense and therapy for such encounters is prudent. Chemicals represent a unique force multiplier that simply cannot be ignored in the 21st century.

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CHAPTER 3

Global Impact of Chemical Warfare Agents Used Before and After 1945

JIRI BAJGAR, JOSEF FUSEK, JIRI KASSA, KAMIL KUCA, AND DANIEL JUN

I. INTRODUCTION

The threat of chemical weapons (CWs), used either by States or Parties to the Chemical Weapons Convention (Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on their Destruction) or by terrorists, has never attracted so much public attention as in the past 10 years. In spite of the existing legal documents dealing with prohibition of CWs, e.g. Geneva Protocol 1925, and Chemical Weapons Convention (CWC), some incidents of the use of CWs in different conflicts and terroristic attacks have been observed. Moreover, alleged use of CWs was noted during the period from 1925 to the present time. It must be emphasized that the theoretical and practical basis for production, storage, and employment of CWs still exists. Also, it must be clearly stated that CWs are applicable at any time, in any place, and in large quantities.

CWs consist of the chemical warfare agents (CWAs) and the means to deliver to the target. They are characterized by high effectivity and large targets and are known as area weapons or silent weapons. They are relatively low cost and with their use it is possible to achieve destruction of everything that is living but avoid destruction of materials and buildings. They are also called the nuclear weapons of poor countries – ''poor man's nuclear weapon''. It should be pointed out that the use of CWs is connected with the use or release of toxic chemicals, thus, chemical warfare can be considered part of generally observed situations where toxic chemicals are used or released and influence the environment and humankind.

There exist a number of causal reasons for these events but apart from accidents connected with the release of toxic chemicals from a natural source (e.g. volcanoes), the factors shown in Figure 3.1 or their combinations can be involved.

For military purposes, a number of chemicals were tested, but only a small number are contained in military arsenals. However, according to the definition contained in the CWC, any toxic chemical intended for military use must be considered a chemical weapon, i.e. the aim is to limit the designation of the compound in question for use as a CW. On the other hand, all toxic chemicals of high toxicity can be chosen by terrorists.

II. BACKGROUND

The use of toxic chemicals against humankind is as old as any warfare conflict. The use of the poisoned arrow against man – not animal – can be considered as the beginning of chemical warfare and would be characterized as the intentional use of chemicals.

At the very beginning, chemical warfare was more closely connected with fire. ''Greek fire'' was an excellent naval weapon because it would float on water and set fire to the wooden ships. There are other examples from history: for example, toxic smoke was used in in China in 2000 BC. In Thucydides' *History of the Peloponnesian War*, the 5th century BC war between Athens and Sparta, we find the first description of chemical warfare – the formation of toxic sulfur oxide by burning sulfur. In the year 184 BC, Hannibal of Carthage used baskets with poisonous snakes against his enemy. Both Socrates and Hamlet's father were poisoned with koniin. Aqua Toffana containing arsenic was also a known poison in ancient Italy. Leonardo da Vinci proposed a powder of arsenic sulfide in the 15th century. There are many more examples of the use of chemical warfare agents (Bajgar *et al.*, 2007b). Modern history shows us that terrorists have used other chemicals, such as ricin (Bulgarian G. Markov was poisoned in 1978) or dioxin (the President of Ukraine Viktor Andriyovych Yushchenko was poisoned in 2004).

In a region of Bohemia, a ''form'' of CW was used as early as 600 years ago. It was in the year 1422 when the castle of Karlstein, the property of King Charles IV, was beseiged and 1,822 kegs containing the cesspools of the streets of Prague were hurled into the castle. Allegedly, the stench in the castle was unbearable. According to historical sources, the castle defenders were probably intoxicated with hydrogen sulfide released from the contents of the cesspools, therefore showing typical symptoms of poisoning (Bajgar, 2006).

Necessary condition: existence of toxic agent
production, processing, stockpiling, transport
(both for intentional and unintentional use)
↓
USE, RELEASE
↓ ↓
intentional/unintentional
military or local conflicts, unrestrained catastrophes, incidental events
terrorism or sabotage, failure of human factor or techniques

FIGURE 3.1. Possible reasons for a release/use of toxic chemicals.

There were some attempts to prohibit CWs by international agreement or law. Most of the early attempts were bilateral or unilateral agreements directed at the use of poisons. These included the 1675 agreement between France and Germany, signed in Strasbourg, to ban the use of poison bullets.

The first international attempt to control chemical and biological weapons took place in Brussels in 1874, when the International Declaration was signed and included a prohibition against poison and poisoned arms. In spite of the Brussels and Hague Conventions – first and second – (1899 and 1907 – signatories agreed not to use projectiles that could spread asphyxiating or deleterious gases), the world witnessed the application of chemicals in warfare to an unprecedented extent during World War I (WWI). A brief summarization of the events connected with the use/release of toxic chemicals is given in Table 3.1.

III. MILITARY USE OF CWs

The intentional use of CWs for military purposes can be found in both global and local conflicts. A typical example is the warning ''Gas! Gas!'' This was common in WWI and it is well known from the E.M. Remarque novel *All Quiet on the Western Front* where Remarque suggestively describes a chemical attack with chlorine.

During WWI, many chemicals were used including mustard, asphyxiating and irritant agents. About 45 types (27 more or less irritating and 18 lethal) of toxic chemicals were used. During the latter part of 1914, irritants were used by Germany and France; the effect was insubstantial. In late 1914, Nobel prize winner Fritz Haber of the Kaiser Wilhelm Physical Institute in Berlin (chemical synthesis of ammonium in 1918) came up with the idea of creating chlorine, although this idea of using toxic chemicals in war was expressed by Admiral Dundonald as early as 1855. Chemical warfare really began in 1915, when German troops launched the first large-scale poison gas attack at Ypres, Belgium, on April 22, using 6,000 cylinders to release 168 tons of chlorine gas, killing 5,000 British, French, and Canadian soldiers. The date is recognized as ''the birthday of modern chemical warfare'' and thereafter the belligerent parties frequently used chemical gases against each other. Phosgene was introduced by Germany late in 1915. Shortly after the first chlorine attack, the Allies had primitive emergency protective masks. In May 1916, the Germans started using diphosgene, while the French tried hydrogen cyanide 2 months later and cynogen chloride the same year. The first time mustard gas was used by German troops was July 12, 1917, and after its use near Ypres it was also called yperite.

By the end of the WWI, some 124,200 tons of chemical warfare agents (chlorine, phosgene, mustard, etc.) had been released, causing at least 1.3 million casualties of which more than 90,000 were fatal. The threat of the use of CWAs led to the development of protective means not only for humans, but also for horses and dogs. The effectivity of CWs in comparison with classic munition was evident: 1 ton of classic explosives caused 4.9 casualties; 1 ton of chemical munition caused 11.5 casualties; and 1 ton of yperite caused 36.4 casualties (Bajgar, 2006).

IV. THE PERIOD BETWEEN WWI AND WWII

The terrible casualties of CWs used during WWI, and the dangerous consequences on humans and the environment, led to the signing in June 17, 1925 of the ''Geneva Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous and other Gases and Bacteriological Methods of Warfare''. This is recognized as one of the unique and famous international treaties on the prohibition of CWs. However, it neither comprises provisions for effective verification nor prohibits development, stockpiling, and transfer of CWs. Moreover, no definition of CWs was included. Despite the provisions of the Geneva Protocol, in 1935–1936 Italian troops employed CWs during their invasion of Abyssinia (Ethiopia). This first major use of CWs after WWI came after October 3, 1935, when Mussolini launched an invasion of this country. Despite the Geneva Protocol (Italy had ratified in 1928) the Italians used mustard gas with horrible effects. Later, CWs were used between Japan and China in 1937–1945. The Japanese attacked Chinese troops with mustard gas and lewisite. The Japanese, in adition to their biological program, had an extensive CWs program and were producing agent and munitions in large quantities by the late 1930s.

V. WWII

Despite the storing and stockpiling of CWs by the great powers engaged in WWII, these fatal weapons were not practically used (except small examples) during WWII (probably because of the fear of massive retaliatory use of CWs). An example of intentional use but not in military conflict was the killing of prisoners in concentration camps in Nazi Germany. The agent first used in the camps was

TABLE 3.1. Some milestones related to the use/release of CWs and toxic chemicals

Year(s)	Event
2000 BC	Toxic smoke in China inducing sleep
4th century BC	Spartacus – toxic smoke
184 BC	Hannibal – baskets with poison snakes
1168	Fustat (Cairo) – use of ''Greek fire''
1422	Bohemia region – cesspools (H_2S)
1456	Beograde – rats with arsenic
19th century	Admiral Dundonald – proposed the use of chemicals in war
1914–1918	WWI – start of chemical war
1918–1939	Development of new CWs and protective means
June 17, 1925	Geneva Protocol
December 23, 1936	Lange and Kruger – synthesis of tabun
1940–1945	Concentration camps – cyanide
1943	Synthesis of sarin
1943	Hoffmann and Stoll – synthesis of LSD-25
1945	Kuhn – synthesis of soman
1950	V agents are begun
1961–1968	Production of VX
1961–1971	Vietnam War – herbicides (impurity dioxin)
1962	BZ was introduced into military arsenals
1970	Bicyclic phosphates considered as potential CWAs
1976	Seveso – release of dioxin
1980	Some rumors on intermediate volatility agent
1984	Bhopal incident – release of methyl isocyanate
1985	Decision on production of binary CWs
1986, 1987	Demonstration of USA CWs (Tooele) and Soviet Union CWs (Shikhany) to the CD in Geneva
1987	Production of binary CWs
1988	Halabja – use of mustard
1980–1990	Rumors of new nerve agent Novichok
1989	Conference on chemical disarmament, Paris
1991	Persian Gulf War – veteran's syndrome
1992	BZ military stocks of the USA were destroyed
1992	Finalization of the rolling text of the CWC at the CD – Geneva
1993	Signing CWC in Paris
1993	Preparatory Commission on OPCW
1994	CWs of Iraq were destroyed
1994	Aum Shinrikyo – sarin attack in Matsumoto
1995	Aum Shinrikyo – sarin attack in Tokyo
April 29, 1997	CWC – entry into force; establishment of OPCW in The Hague
2000	Research on nonlethal weapons intensified
2002	Moscow theater – Fentanyl derivatives used against terrorists
April 29, 2012	CWs of the State Parties to the CWC will be destroyed but will be prolonged

carbon monoxide, followed by the more ''effective'' hydrogen cyanide released from Zyklon B. Some experiments with aconitine-impregnated shells and some other toxic compounds including biological agents were tested on prisoners.

However, during WWII, an important step in the preparation of the most dangerous CWA was observed in Germany. In Schrader's group, organophosphates (OPs) were synthesized, primarily with the aim of obtaining more effective insecticides. Between 1934 and 1944, Schrader's team synthesized approximately 2,000 OPs including two well-known OP compounds, parathion and paraoxon. As early as 1935, the government of Nazi Germany insisted that Schrader switch the primary aim from OP insecticides to CWAs. At present, OPs are widely used in agriculture, medicine (human and veterinary), and industry. These compounds also include nerve agents (the most toxic compounds of the OP group). Nerve agents such as sarin, tabun, soman, and VX are the main compounds of CWAs. The Germans were also the greatest producers of nitrogen mustard and produced about 2,000 tons of HN-3.

Tabun was synthesized in 1936, followed by others (sarin, at the end of WWII, followed by soman) and production of these agents for the military in large quantities and their stockpiling were recognized after WWII in Dyhernfurth, Poland (e.g. stocks of tabun and some quantities of sarin). The technology was subsequently transferred to Russia and research and development of new OP nerve agents was continued. During this period British and American scientists were evaluating the toxic properties of DFP.

VI. THE PERIOD AFTER WWII AND THE COLD WAR

At the end of WWII, many Allied nations seized the chemical weapons. Most of the CW manufacturing plants in Germany were taken over and moved to Russia to new sites, e.g. the military area of Shikhany. This ''takeover'' prompted other states to begin even more research on CWs. Despite the Allies' own research into CWs, very important technologies and ''know how'' were obtained from Nazi Germany for both the USA and the former Soviet Union.

The interest in CW technology was probably one reason for the change of the future border: according to Churchill's history of WWII the proposed future boundary between Poland and Germany had been primarily agreed to consist in part of the Oder river flowing to the Baltic Sea, and its tributary, the Neisse river. Before their confluence, the Neisse consists of two branches, the East Neisse and the West Neisse. The East Neisse should be the boundary, resulting in slightly more territory for Germany. Stalin held for the West Neisse and progress was delayed. No one knows why Stalin was so insistent in this matter. The reason was probably very simple: the small town of Dyhernfurth (now Brzeg Dolny), a few kilometers north of Breslau (Wroclaw) in the disputed territory and a factory for the production of nerve agents. It was estimated that when Dyhernfurth was captured it contained stockpiles of 12,000 tons of tabun, 600 tons of sarin, and an unknown amount of soman. Presumably, the factory was dismantled, and along with their stockpiles, transported to the Soviet Union (Koelle, 1981). It has been documented that the Soviets were ready to conduct a chemical attack and their research and development of CWs was very intensified.

In the USA, during the 1950s, the chemical corporations concentrated on the weaponization of sarin. At the same time, they became interested in developing CWs that incapacitated rather than killed the targets. Mescaline and its derivatives were studied but without practical output. Five years later, a new project ''Psychochemical Agents'' (later K-agents) was established. The objective was to develop a nonlethal but potent incapacitant. Nonmilitary drugs like LSD-25 and tetrahydrocannabinol were also examined. None of these agents were found to be of military importance. The first and only incapacitant was BZ, developed in 1962; however, its stocks were destroyed in 1992 as declared by the US delegation to the Conference on Disarmament in Geneva (Document of CD, 1991). These agents, intended not to kill but to induce incapacitance, are covered under the class of nonlethal weapons (Hess *et al.*, 2005).

In the former Soviet Union, as a whole in 1940–1945 approximately 110,000 tons of first generation toxic chemicals were produced and most of them were yperite and lewisite, and irritating agents. Second generation CWs were composed of nerve agents such as sarin, soman, V agents, and to a lesser degree tabun. The development of new CWs of the third generation comprised traditional and nontraditional CWs, e.g. blister and irritant agents, and nerve gases including new types, e.g. Novichok 5, whose exact chemical structure is unknown though some assessments have been made (Bajgar, 2006); it could be a nerve agent having high toxicity. Its effects are difficult to treat using common antidotes.

An example of the unintentional use of CWs has also been observed. In March 1968, thousands of dead sheep were discovered in the Skull Valley area, Arizona, USA. This area was adjacent to the US Army's Dugway open-air testing site for CWs. Nerve gas had drifted out of the test area during aerial spraying and killed the sheep. One year later, on July 8, 1969, the Army announced that 23 US soldiers and one civilian had been exposed to sarin in Okinawa during the clearing of sarin-filled bombs (Sidell and Franz, 1997).

There are a number of examples of localized conflicts where CWs have been intentionally used but cannot be verified: e.g. in 1951–1952 in the Korean War; in 1963 the Egyptians used mustard bombs against Yemeni royalists in the Arabian peninsula; in the Indo-China War (see Vietnam War); in 1970, in Angola antiplant agents were almost certainly used; and in former Yugoslavia, there were rumors of the use of psychotomimetic agents.

A. Iraq–Iran and Afghanistan War

On September 22, 1980, Iraq launched its invasion against Iran. There has been mention of the large-scale use of CWAs in the Iran–Iraq war. In November 1983, Iran informed the United Nations that Iraq was using CWs against Iranian troops. Soon after, the use of CWs was unleashed. In addition, mustard and tabun were used. It is well known that the Iraqi Government used these agents against its own citizens, more conspicuously at Halbja in March 1988. The CWs attack was the largest against a civilian population in modern times. More than 100,000 Iranians were poisoned with CWAs; sulfur mustard was the most frequently used and has induced a number of delayed complications in Iranian veterans (pulmonary, dermal, ocular, immune system depression, reproduction, malignancy, etc.) (Afshari and Balali-Mood, 2006). Other localized conflicts involving alleged use of CWs are described in detail in an extensive review (Robinson, 1971).

The Soviet Union probably used mustard (and nerve gas) in Afghanistan. The Afghanistan war was considered the Soviet Union's ''Vietnam''. The use of CWs was described by Sidell and Franz (1997). The use of CWs by Soviet forces was also significant and has been confirmed against unprotected subjects. Despite the use of CWs, the withdrawal of Soviet troops from Afghanistan was realized at the beginning of 1989.

B. Vietnam War

After WWII, the main employment of CWs is recorded in 1961–1972 when the US Army used defoliants. The herbicide Agent Orange was used during the Vietnam War and led to the injury of more than one million Vietnamese and Americans. Agent Orange (a mixture of 2,4-dichlorophenoxy acetic acid and 2,4,5-trichlorophenoxy acetic acid) contained the chemical contaminant dioxin as an impurity which caused many deaths on both sides. There were other herbicide mixtures such as Agent White (2,4-D and picloram) and Agent Blue (cacodylic acid). The biological effects of dioxin were described by Sofronov *et al.* (2001). The first major operation of this type was conducted over the Ca Mau peninsula in September–October 1962. The area sprayed with defoliants during 1965 had been five times larger than in 1965 and in 1967 ten times larger. The scale of the use of defoliants was roughly in proportion to the overall involvement of US troops. In 1970, herbicides and defoliants were used in tens of tons, especially 2,4,5-T. The area sprayed enlarged from 23 km^2 in 1962 to 22,336 km^2 in 1969. The area exposed to spraying was assessed to be 58,000 km^2 and the number of people exposed was assessed to be more than one million including more than 1,000 deaths. In addition to defoliants used to destroy vegetation concealing the North Vietnamese, the USA used tear gas for clearing tunnels and bunkers. The irritants CS, CN, and DM were reported to be used. The total CS procured was approximately 7,000 tons from 1963 to 1969.

C. Development of VX Agent

VX was synthesized in the 1960s on the basis of the results of Tammelin and Aquilonius (Aquilonius *et al.*, 1964; Tammelin, 1957). The manufacturing of VX began in the USA in 1961. Construction of the USA's VX agent production plant at Newport, Indiana, was completed in 1961, when the first agent was produced. The production facility only operated for 7 years, and was placed on standby in 1968 (Smart, 1997).

During the same period, Soviet scientists developed the so-called Russian VX (VR, RVX, R 033) (Kassa *et al.*, 2006; Kuca *et al.*, 2006). The chemical structure of VX was unknown for a long time. Therefore some attempts to resolve this question have been made (Bajgar, 1968). Because of these ambiguities and difficulties in synthesis, model V agent [EDMM, *O*-ethyl *S*-(2-dimethylaminoethyl) methylphosphonothioate] was initially used in the Eastern Block to study antidotal treatment. Another structural analog of VX known as Chinese VX (CVX) was also developed and studied (Eckert *et al.*, 2006).

A very important step in the development in CWs has been the production of ''binary munition'', in which the final stage of synthesis of the agent from precursors is carried out in the munition (bomb, shell, or warhead) immediately before or during delivery to the target. In the 1950s, armed forces had begun looking at binary weapons. Until this time, CWs were unitary, i.e. the toxic agent was filled in the munition and then stored ready to be used. The binary concept – mixing or storing two less toxic chemicals and creating the nerve agent within the weapon – was safer during storage. The production of binary projectiles began on December 16, 1987 at the Pine Bluff Arsenal, Arkansas, USA.

D. Persian Gulf War

On August 2, 1990, Saddam Hussein sent Iraqi troops into Kuwait – allegedly in support of Kuwaiti revolutionaries who had overthrown the emirate. Iraq was known to have a large stockpile of CWs during its conflict with Iran and confirmed that they would use CWs.

President George Bush ordered US forces to be sent to Saudi Arabia at the request of the Saudi Government (Operation Desert Shield) – this was the buildup phase of the Persian Gulf War. As a consequence, in 1996, almost 60,000 veterans of the Persian Gulf War claimed certain medical problems related to their war activities, some caused by exposure to nerve agents (released after the bombing and desctruction of the sarin production facility). Unexplained ''Gulf War Syndrome'' with low-dose exposure to CWAs was suggested as a possible cause. Extensive research failed to find any single case of the problem.

However, some health effects, including alterations to the immune system 3 months after the exposure to low concentrations of sarin, were demonstrated (Kassa *et al.*, 2001, 2003). In the desert, during the fall and winter of 1990–1991, the threat of chemical warfare became very real to allied military personnel. It was demonstrated by the UN Commission that major Iraqi agents were mustard, tabun, sarin, and cyclosarin. Mustard agent was relatively pure but nerve agents were a complex mixture of the agent and degradation products. Over the period from June 1992 to June 1994, the Commission's Chemical Destruction Group destroyed 30 tons of tabun, 70 tons of sarin, and 600 tons of mustard, stored in bulk and in munitions.

Suddenly, it became clear to the whole world that there were countries that have CWs and biological weapons, and there were other countries that might obtain or produce them.

VII. UNINTENTIONAL USE OF TOXIC CHEMICALS

There are two main accidents connected with the release of toxic chemicals. In July 1976, in Seveso, Italy, more than 40,000 people were exposed to dioxin, a persistent and highly toxic chemical. The first signs were skin lesions appearing on children, and after some months there was evidence of chloracne. Health consequences have been observed from that time to the present. The Seveso accident was possibly the most systematically studied dioxin contamination incident. A similar contamination of one building of the Spolana company in Neratovice (a town in the former Czechoslovakia) was also observed (Bajgar *et al.*, 2007a). Another example, the Bhopal accident, is probably the greatest industrial disaster in history. On the night of December 2–3, 1984, water inadvertently entered the methylisocyanate storage tank (containing about 40 tons of this chemical). As a result, methylisocyanate was released into the sorrounding area. There was no warning. Many people who inhaled high concentrations of toxic gas were asphyxiated because of extensive lung damage. About 150,000 people were intoxicated (50,000 seriously poisoned) and more than 2,500 people died (Bajgar, 2006).

VIII. TERRORIST USE OF CW

Terrorists have expressed an interest in nerve agents and have deployed them in attacks on unprotected civilians (Rotenberg and Newmark, 2003). A Japanese religious cult, Aum Shinrikyo, independently manufactured numerous chemical and biological agents. The first such attack with sarin occurred in Matsumoto in 1994 and the Tokyo subway in 1995. Thousands of people were affected and dozens of people died (Nagao *et al.*, 1997; Ohtomi *et al.*, 1996; Okumura *et al.*, 1998; Yokoyama *et al.*, 1998). In Matsumoto (1994), 600 people were poisoned and hospitalized, and seven died (Morita *et al.*, 1995; Nakajima *et al.*, 1997; Yoshida, 1994). The attack in the Tokyo subway (1995) resulted in 5,500 people seeking hospital evaluation and 12 deaths (Bajgar, 2006). An interesting terroristic act was described by Tsuchihashi *et al.* (2005) – a fatal intoxication with VX administered percutaneously.

Nerve agents belong to the group of OPs. These compounds in the form of pesticides are commercially available, and are used in agriculture which can lead to professional, suicidal, or accidental intoxication. The mechanism of action, diagnosis, and treatment of intoxication with OP pesticides and nerve agents is a very hot topic at present. Moreover, some principles of the effects, diagnosis, and therapy are very similar for OP and highly toxic nerve agents, and therefore the principle of action and effective treatment can be applied in general for the civilian sector too.

The use of these chemicals was observed in Moscow in 2002. The Moscow theater hostage crisis was the seizure of a crowded theater on October 23, 2002 by about 40 armed Chechen militants who claimed allegiance to the separatist movement in Chechnya. They took 850 hostages and demanded the withdrawal of Russians from Chechnya and an end to the Chechnya war. The leader of the terrorists was 22-year-old Movsar Baraev. After two and half days of waiting, Russian forces used an unknown gas pumped into the ventilation system. Officially, 39 terrorists and at least 129 of the hostages (nine of them foreigners) were killed. Some estimates have put the civilian death toll at more than 200. It was thought that the security services used an aerosol of a chemical warfare agent, first assessed as BZ, but later it was specified that an aerosol anesthetic of the Fentanyl type was used (Bajgar and Fusek, 2006).

In the hospitals, the survivors were cut off from any communications with the outside and their relatives were not allowed to visit them. An incorrect list of hospitals for victims was released. The main problem was the lack of information about those dealing with the identification and characterization of the chemical used and the unavailability of known antidotes (e.g. naloxon) by medical staff treating the victims (Bajgar *et al.*, 2007a). It appeared from this event that there were compounds not explicitly enumerated in the CWC and therefore not controlled by this Convention. Fentanyl can be considered as a nonlethal weapon (a group of so-called calmatives) and these chemicals can also be used to incapacitate animals; of course, its use against humans is not excluded (Bajgar, 2006; Hess *et al.*, 2005).

IX. NEGOTIATIONS

Though the Cold War was continuing, the political situation led to increased activities in international negotiations. At the Conference on Disarmament in Geneva, some attempts to negotiate a ban of CWs was begun, first as the Ad hoc

Working Group, and later as the Ad hoc Committee on Chemical Weapons with the mandate to negotiate the text of a convention banning CWs.

The discussions in Geneva were more intensive from 1987 and, in 1992, the elaboration of the so-called rolling text of future CWCs was finished. During these negotiations, the text of future Conventions (''rolling text'') was enlarged: the final report (CD/342) of February 2, 1983 contained 23 pages; the same report of August 23, 1985 (CD/636) had 46 pages; and CD/952 of August 18, 1989 contained 134 pages. Simultaneously with the Geneva negotiations, in September 1989, the Memorandum of Understanding between the Governments of the United States and USSR regarding a bilateral verification experiment, data exchange related to prohibition of CWs otherwise known as the Wyoming, MT, started negotiations between two main possessors of CWs. These countries also contributed to the negotiations in Geneva: they demonstrated their CWs to the Conference on Disarmament in the USA in November 1986 (Tooele) and the USSR in October 1987 (Shikhany). The final document of the Convention is approximately 200 printed pages. The Convention was then agreed in New York at the UN General Assembly and signed in Paris in 1993. The CWC (Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on their Destruction) entered into force on April 29, 1997, 180 days after the deposit of the 65th instrument of ratification of the Convention by Hungary. At this time, 87 countries ratified the CWC and became original States Parties to the Convention. Simultaneously, the Organization for Prohibition of Chemical Weapons (OPCW) in The Hague started its work of supervising the destruction of CW stocks and monitoring the world's chemical industry to prevent future misuse. There are many activities of the OPCW, e.g. training of the inspectors for control of destruction of CWs including their medical protection, research, and supported activities, solving problems due to practical implementation of the CWC, control of chemical and military facilities and other activities. Russia and the USA are unlikely to meet the final stockpile destruction deadline of April 29, 2012. By the middle of 2008, 183 Signing States and 194 recognizing States had adhered to the Convention (Davey, 2008). However, there are still States nonsignatories to the Convention. CWs have a long and ancient history. A lack of CW employment in WWII suggested that ''gas warfare'' had ended. However, further development and the utility of chemicals in Vietnam and in terrorist attacks have maintained a military interest in chemical weapons.

It is clear that the use (incidental or otherwise) of toxic chemicals has impacts in different spheres of human existence such as state structures and infrastructure, economics, psychic and public behavior, and the environment. Toxic chemicals are a great consumer of natural sources, both renewable and nonrenewable. They also consume raw materials and energy, and as a consequence cause pollution of the environment and lead to deficiency of raw materials throughout the world and therefore an unequal distribution of the world's natural sources. The impact on the psychology of humankind is also important, following either chemical wars (both global and local) or use of these chemicals by terrorists. The development of new technologies is equally important because they influence positively and negatively further human development. Research in this direction can not only contribute to ''improvement'' of chemicals to obtain more effective CWAs but also improve our knowledge in basic sciences (toxicology, neuropharmacology, etc.) and allow us to better understand physiological functions in general. It is appropriate to recall the history of cholinesterases and their inhibitors. The existence of cholinesterases was predicted by H.H. Dale in 1914, i.e. 14 years before acetylcholine was demonstrated as a natural constituent of animal tissues. This research approach was changed during WWII and cholinesterases acquired a special significance in the context of chemical warfare and nerve agents (Silver, 1974). Another publication in this area (Koelle, 1963) can be considered as the first to deal with anticholinesterase agents including CWAs – nerve agents. One can only hope that in the future the only physiological and pharmacological research will be performed in a nonmilitary framework, but that may not be the case.

X. CONCLUDING REMARKS AND FUTURE DIRECTION

The threat of the use (either military or terroristic) of CWAs (and other toxic chemicals) still exists. The military use of these agents is limited but their terroristic use is unlimited. The spectrum of these agents is very large and the ability to be prepared against the use of toxic chemicals is necessary.

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CHAPTER 4

The Tokyo Subway Sarin Attack: Toxicological Whole Truth

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Humankind has not yet experienced a full-scale sarin attack in a major modern city.

I. INTRODUCTION

The Tokyo subway sarin attack occurred in 1995, following the Matsumoto sarin attack, and served as a ''wake-up call'' for anti-NBC (nuclear, biological, and chemical) terrorism policy throughout the world. In the 10 years since the attack, efforts to combat NBC terrorism have focused on rapid and effective measures to respond to attacks employing nerve agents such as sarin.

II. SARIN TOXICITY AND MECHANISM OF ONSET

Sarin is an organophosphate compound. Within the context of chemical weapons, organophosphates are collectively referred to as ''nerve agents'', of which sarin, tabun, soman, and VX are examples. Organophosphates inhibit the enzyme acetylcholinesterase (AChE), which degrades acetylcholine (ACh), a neurotransmitter substance that acts locally on nerve synapses. Once the organophosphates bind to and phosphorylate AChE to inhibit its activity, ACh accumulates at nerve terminals, resulting in enhanced ACh activity at receptor sites. ACh effects can be functionally classified based on their site of action and can have muscarinic, nicotinic, and central nervous system (CNS) effects. These effects cause the major symptoms associated with an acute organophosphate toxicity. Muscarinic effects increase parasympathetic nerve activity and cause miosis, visual disturbances (accommodation disorder), increased salivary and bronchial secretions, bronchospasm, bradycardia, and increased gastrointestinal peristaltic activity (e.g. abdominal pain, nausea, vomiting, and diarrhea). Nicotinic effects, due to hyperstimulation of neuromuscular junctions, cause fasciculations, muscle weakness, and respiratory paralysis, and increased sympathetic nerve activity leads to miosis, sweating, tachycardia, and hypertension. CNS effects due to ACh, when severe, include anxiety, headaches, excitement, ataxia, somnolence, disorientation, coma, and seizures.

Well-known symptoms of sarin toxicity include ''miosis'', ''hypersecretion'', ''bradycardia'', and ''fasciculations''. However, the mechanism of organophosphate toxicity seems to involve conflicting actions. For example, mydriasis or miosis, and bradycardia or tachycardia may occur. Acute respiratory insufficiency is the most important cause of immediate death. Early symptoms include (1) tachypnea due to increased airway secretions and bronchospasm (muscarinic effect), (2) peripheral respiratory muscle paralysis (nicotinic effect), and (3) inhibition of respiratory centers (CNS effect), which all lead to severe respiratory insufficiency. If left untreated at this stage, death will result. Cardiovascular symptoms may include hypertension or hypotension. In more severe cases, hypotension and shock develop. Various arrhythmias can also occur, and caution is required when the QT interval is prolonged. In particular, if hypoxemia is present, fatal arrhythmias may occur with intravenous administration of atropine sulfate, which means that this drug should be given intramuscularly to victims of sarin poisoning in the ''field''. Common gastrointestinal symptoms include nausea, vomiting, and diarrhea.

An ''intermediate syndrome'' lasting 1–4 days after sarin exposure is said to exist. This is due to prolonged AChE inhibition and is associated with acute respiratory muscle paralysis, motor nerve paralysis, and cervical flexor and proximal muscle paralysis. Recumbent patients who have difficulty raising their head and neck require particular care. However, the intermediate syndrome has not been reported with nerve agent toxicity in animals or humans (Sidell, 1997), and some experts even doubt that an intermediate syndrome actually exists (De Bleecker, 1992). Others believe that the cause may be due to oral toxicity or inadequate treatment (intestinal decontamination, antidote administration, and respiratory management). In organophosphate-induced delayed neuropathy (OPIDN), seen two to three weeks after exposure, and characterized by distal muscle weakness without fasciculations, the pathophysiology is not well understood. OPIDN was first reported in the 1930s due to

contamination of Jamaican ''ginger jake'' by organophosphates. This incident (so-called ''ginger paralysis'') caused lower limb paralysis in about 20,000 victims. OPIDN symptoms have also recently been reported in Matsumoto and Tokyo subway sarin victims (Himuro *et al.*, 1998; Sekijima *et al.*, 1997). Inhibition of neuropathy target esterase (NTE) plays a role in OPIDN, but despite several basic research studies, the detailed pathophysiology has not yet been established, thus making OPIDN difficult to treat.

III. OVERVIEW OF THE TOKYO SUBWAY SARIN ATTACK

The attack took place during the morning rush hour, about 8:00 am, on March 20, 1995, the day before a holiday. The attack was carried out by members of Aum Shinrikyo to distract police from carrying out a raid on the cult's headquarters. The terrorist target was government buildings in Kasumigaseki in the heart of Tokyo. Most offices in Kasumigaseki open for business at 9:30 am, but the early morning rush hour was heavy because this was a Monday. Some believe that the time of 8:00 am was because some cult members had inside information about the government offices. The police, based on an undercover investigation, suspected that Aum Shinrikyo was manufacturing sarin for use in a terror attack, but few people, even within the police department, were aware of this. The police did not have personal protective equipment (PPE), which meant that they had to borrow PPE and receive training on use of the equipment from the Self-Defense Forces. Members of the Self-Defense Forces were alerted to some of Aum Shinrikyo's planned activities, but the general public, including healthcare providers and fire department personnel, knew nothing about their activities.

According to a subsequent police report, the terrorists placed sarin in five subway trains. Approximately 600 grams of sarin at a concentration of 33% was mixed with hexane and *N*, *N*-diethylaniline and placed in a nylon/polyethylene bag. Five terrorists then wrapped the bags in newspaper, punctured the bags with the tips of their umbrellas and left the bags on the subways. In this way the sarin seeped out of the bags and vaporized, but no other active means of dispersal were used, and in this sense, the Tokyo subway sarin attack was not really a ''full-scale'' attack.

Thus, the way in which we use the lessons learned from this attack will affect our ability to adequately deal with future terrorist attacks using sarin, which could be even greater and more serious with respect to the number of victims. Can we really assume that only 12 of the approximately 5,500 victims died because the Japanese medical system was particularly well prepared for such an eventuality? Probably not. It is more likely that the relatively low number of fatalities was due to the low concentration of sarin and passive means of dispersing it. From this perspective, the Matsumoto sarin attack one year previously was more aggressive than the Tokyo subway sarin attack. In a trial after the Matsumoto incident, it was revealed that a 70% concentration of sarin was actively volatilized using an electric heater and dispersed using an electric fan. Seven victims died and 660 were injured, giving a fatality rate of 1%. In other words, if the Tokyo subway sarin attack had been conducted using the same means as those employed in the Matsumoto sarin attack, the number of fatalities may have risen to 50 or 60. Fortunately, only 12 victims died, but this suggests that the Tokyo subway sarin attack was not a full-scale attack. In other words, mankind has not yet experienced a full-scale sarin attack in a major city.

Of the bags of sarin used in the attack, two bags were not punctured. These bags were returned to the police laboratory for analysis. At one of the subway stations on the

FIGURE 4.1. Scene from a sarin attack at Tsukiji station.

Chiyoda line, Kasumigaseki, two station employees collapsed and died on the platform after they cleaned and removed the as yet unidentified object using gloves. The actual number of victims varies depending on the source, but all confirm that 12 people died in the attack and it is generally believed that at least 5,500 victims suffered mild to serious injuries; fire fighting agencies estimate 5,642 victims, and the police, 3,796 victims. Official figures released by the subway company put the total number of victims at 5,654. This includes the 12 who died (ten passengers, two employees), those hospitalized (960 passengers, 39 employees), and those treated for minor injuries (4,446 passengers, 197 employees).

This incident was the first chemical terrorist attack in a large city. There were few first responders who could even have conceived of such an attack and would have been prepared to rapidly evacuate victims from the subway station premises. Many passengers who had difficulty walking rushed out of the trains and onto the subway platform and fell down, which in effect would have increased their exposure to sarin in the subway station. In addition, the site to which many of the victims were finally evacuated at ground level where they could lie down was in close proximity to an air exhaust vent from the subway below.

Cult members arranged to puncture all the bags containing sarin at 8:00 am, and the first call for an ambulance came at 8:09 am with the first report of a ''victim with seizures at Kayabacho Station''. After 8:15 am, the reports of victims started to increase. Around this time, the fire department received a report from Tsukiji Station stating that ''an explosion occurred and several people were injured''. Calls for ambulances eventually came from 19 subway stations, and after 8:30 am, victims, either by walking or being picked up by passing vehicles, began to pour into local clinics and hospitals. According to the Tokyo Fire Department, 5,493 people were treated at 267 medical institutions in Tokyo, 17 people were treated at 11 medical institutions outside Tokyo, and among the victims, 53 were seriously injured (Ieki, 1997). Another source states that a total of 6,185 people were treated at 294 medical institutions (Chigusa, 1995). The discrepancy in the number of victims reported by different agencies attests to some of the confusion at the time. St Luke's Hospital received the largest number of victims (640 on the day of attack). The reason for this was because of its close proximity to the Hibiya line, where there were many victims, and because of a report on television which stated that ''St Luke's Hospital has the antidote for treatment''.

IV. EMERGENCY TREATMENT OF SARIN TOXICITY

In victims of the Tokyo subway sarin attack, endotracheal intubation was not difficult. The Japanese medical literature describes the standard treatment for sarin toxicity as (A) maintain the airway, (B) assist breathing, and (C) support circulation. However, in the Matsumoto sarin attack, endotracheal intubation was more difficult in many victims because of airway hypersecretion and bronchospasm. This difference in symptoms is attributable to the higher 70% concentration and active means by which the sarin was dispersed at Matsumoto, as opposed to the 33% concentration and passive means of dispersal employed in Tokyo. Dr Fredrick Sidell (now deceased), an expert on chemical terrorism in the USA, advocated decontamination, drugs, airway, breathing, and circulation (DDABC) as the basic treatment for nerve agent poisoning. Even if the so-called ABCs of emergency treatment are followed, initial efforts to achieve adequate ventilation may be in vain. Efforts to achieve adequate ventilation should be made after at least initial administration of atropine to control airway secretions and bronchoconstriction (Sidell, 1997). If healthcare professionals learn from the Matsumoto attack, they can better recognize early parasympathetic nervous symptoms, including miosis, hypersecretion, and rhinorrhea as common symptoms of chemical terrorism due to nerve agents and institute appropriate treatment with antidotes. In large-scale disasters with many victims, treatment is often deferred in those with cardiopulmonary arrest (so-called ''black tag''). However, at St Luke's Hospital, one in three persons with cardiopulmonary arrest and two patients with respiratory arrest made a full recovery and were discharged. This high rate of recovery and return to the community is unlike that seen in other types of disasters. Therefore, if medical resources are available, all victims of a sarin attack should be aggressively treated, including cardiopulmonary resuscitation (CPR) when necessary.

The global standard for the treatment of sarin toxicity is the administration of: (1) atropine, (2) an oxime agent like PAM, and (3) diazepam (Medical Letter, 2002).

Recommended doses of atropine are 2 mg in patients with mild symptoms, primarily ocular, but without respiratory symptoms or seizures; 4 mg in patients with moderate symptoms, including respiratory symptoms such as dyspnea; and 6 mg in patients with severe symptoms, including seizures and respiratory arrest, the standard administration route for which should be intramuscular. As mentioned previously, intravenous administration of atropine in the setting of severe symptoms such as hypoxemia can induce ventricular fibrillation; thus, intramuscular administration is advised. Oxime agents such as PAM (pralidoxime methiodide, or 2-formyl-1-methylpyridinium iodide oxime) should also be given. The recommended dose for PAM in moderate and severe cases of inhalation, or for liquid exposure to a nerve agent, is 1 g by intravenous infusion over 20 to 30 min. Further continuous administration of 500 mg per hour may also be required in severe cases. Since the rate of aging of nerve agent–enzyme bond is correlated with time until administration of PAM, if the aging half-life of sarin is 5 h, then PAM must be administered before this time. The oxime of choice for sarin and VX is PAM, but HI-6 should be used

FIGURE 4.2. Sarin victims at St Luke's International Hospital.

for soman and obidoxime for tabun. Seizures are treated with diazepam. This three-drug combination, atropine, PAM, and diazepam, is the global recommendation for sarin toxicity and autoinjectors are available in several countries (Vale *et al.*, 2006).

After the Tokyo subway sarin attack, St Luke's Hospital, which treated 640 victims, used about 700 ampules of PAM and 2,800 ampules of atropine (Okumura *et al.*, 1998). This calculates out to 550 mg of PAM and 2.2 mg of atropine for each victim. The route of administration was intravenous in all cases with a total dose of atropine in severe cases 1.5 mg to 9 mg (Okumura *et al.*, 1996); doses which reflect the low concentration and passive means of sarin dispersal used in the Tokyo attack.

However, in Tokyo, no one was saved by administration of PAM, and conversely, no one died because they did not receive PAM. In other words, if ''living or dying'' was the endpoint, there was no clinical evidence that PAM was effective. The only reported finding was a more rapid return of plasma pseudocholinesterase levels to normal in some patients who received PAM as compared to those who did not. But in terms of long-term prognosis, this does not rule out the effectiveness of oxime therapy. Ideally, detailed studies are needed to evaluate the efficacy of PAM, including for long-term prognosis, but there was no sophisticated study designed in victims of the Tokyo subway sarin attack.

One piece of evidence supporting the efficacy of PAM in sarin toxicity has been the clinical benefit associated with PAM in toxicity due to organophosphorous agrochemicals. However, some experts now doubt whether such a benefit really exists. For example, Peter *et al.* (2006), using meta-analytic techniques, reevaluated the effects of oxime therapy in organophosphate poisoning. Not only did they find no beneficial effects, they also reported possible adverse effects. The Cochrane reviews for clinical evidence-based medicine reported no risk/benefit evidence for the use of oxime agents in organophosphate poisoning, but they did conclude that further detailed investigations are necessary (Buckley *et al.*, 2005).

Based on the experience of Iranian physicians who treated sarin toxicity during the Iran–Iraq war (Newmark, 2004), PAM was not available on the front lines and atropine alone was used for treatment. The doses of atropine used were considerably higher than those used in the Tokyo subway sarin attack, or that are generally recommended in the USA (Medical Letter, 2002). The Iranian protocol called for initial administration of 4 mg intravenously. If no atropine effects (improvement in dyspnea or decrease in airway secretions) were seen after 1 to 2 min, 5 mg was then administered intravenously over 5 min while heart rate was monitored. A rise in heart rate of 20 to 30 beats per min was regarded as an atropine effect. In severe cases, 20 mg to 200 mg was given. Regardless of dose, the key to saving lives, in their opinion, was how soon the atropine was administered.

Thus, treatment without the use of an oxime agent is possible. Of course, ideally, in countries where this is economically possible, treatment should use the three recommended drugs: (1) atropine, (2) an oxime agent like PAM, and (3) diazepam, and the use of autoinjectors for administration is also helpful. Unfortunately, terrorist attacks using sarin are also carried out in less economically developed countries and even if the drugs are available, considerations related to cost performance need to be considered. In this sense, preference should be given to the availability of atropine and diazepam. In other words, unless it is economically feasible, funds should be used to obtain atropine and diazepam, rather than oxime agents, whose cost–benefit ratio is still inconclusive.

V. ACUTE AND CHRONIC SYMPTOMS OF SARIN TOXICITY

Based on available data from 627 victims treated at St Luke's Hospital, symptoms in order of occurrence were: miosis 568 (90.5%), headache 316 (50.4%), visual darkness 236 (37.6%), eye pain 235 (37.5%), dyspnea 183 (29.2%), nausea 168 (26.8%), cough 118 (18.8%), throat pain 115 (18.3%), and blurred vision 112 (17.9%) (Okumura *et al.*, 1998). Cases were defined as severe for seizures or respiratory arrest requiring mechanical ventilation, moderate for respiratory distress or fasciculations, and mild for eye symptoms only. Of 640 cases reported by St Luke's Hospital, degree of intoxication was severe in five, moderate in 107, and mild in 528 victims with nicotinic effects observed in those with moderate or severe symptoms.

In the Tokyo subway sarin attack, decontamination was not performed on site, and first responders and healthcare workers initially did not wear personal protective equipment (PPE). As a result, of 1,364 fire department personnel, 135 (9.9%) became secondary victims. Official reports for police department personnel were not released, but the number of secondary exposure victims was probably similar. At St Luke's Hospital, 23% of the hospital staff became secondary victims (Okumura *et al.*, 1998). The percentage of secondary victims by hospital occupation was: nurse assistants (39.3%), nurses (26.5%), volunteers (25.5%), doctors (21.8%), and clerks (18.2%). Thus, increased contact with a primary victim increased the risk of becoming a secondary victim, with the percentage of secondary victims by hospital location being the chapel (45.8%), ICU (38.7%), outpatient department (32.4%), general ward (17.7%), and the emergency department (16.7%). The high rate of secondary victims in the chapel was attributed to poor ventilation and the large number of victims sheltered there. Because it was during the winter, victims entered the chapel fully clothed. When they removed their coats, and every time they moved, some of the sarin trapped inside the clothing probably escaped, causing secondary exposure. Fortunately, none of the secondary victims died. However, in the event that a higher concentration of sarin and more effective means of dispersion had been employed in the Tokyo attack, such as that used in Matsumoto, for example, then it is likely that fatalities would have been encountered among secondary victims.

Within the context of risk communication, the so-called ''worried-well'' patients who are concerned about having been exposed to the nerve agent, and those complaining of symptoms, even though actual exposure was unlikely, also flock to hospitals seeking treatment (Bloch *et al.*, 2007). Among patients treated at St Luke's Hospital on the day of the attack, 90.5% (568/627) had miosis (pupillary constriction), an objective finding due to sarin exposure. The remaining 9.5% were considered to be ''worried-well'' patients. As days passed by, the number of ''worried-well'' patients appeared to increase, but no actual data for this is available.

The reason for the small number of ''worried-well'' patients in the Tokyo subway sarin attack is unclear. Given the extensive coverage by the news media who mentioned that victims were flocking to St Luke's Hospital, persons without definitive symptoms, or those who were unsure whether they had been exposed but who did not want to add to the confusion, avoided going to St Luke's Hospital creating a kind of natural selection process. In addition, the target of the attack was the government buildings in Kasumigaseki at heart of Tokyo, which would have meant that many of the victims were well educated. This may also have contributed to the small number of ''worried-well'' patients. Conversely, unfamiliarity with sarin and toxic gases in general may also have contributed to the low number of ''worried-well'' patients. In either case, these observations should be reviewed from the perspective of risk communication.

Fortunately, only one victim from the Matsumoto and Tokyo subway sarin attacks has still not regained consciousness and remains in a vegetative state due to anoxic brain damage (Yanagisawa *et al.*, 2006). Sarin victims treated at St Luke's Hospital were regularly followed for the development of chronic symptoms. One year after the incident a survey was conducted, and 303 of 660 victims responded (Ishimatsu *et al.*, 1996). Forty-five percent of the respondents reported that they still experienced symptoms. Regarding physical symptoms, 18.5% of the victims still complained of eye problems, 11.9% of easy fatigability, and 8.6% of headaches. Regarding psychological symptoms, 12.9% complained of fear of subways, and 11.6% still had fears related to escaping the attack. In another survey after 3 years, 88% of the respondents reported several sequelae (Okumura *et al.*, 1999). Unfortunately, these surveys may lack objectivity and may suffer from bias. For example, the response rate may have been higher among victims still complaining of symptoms.

Murata *et al.* (1997) performed a controlled comparison study in victims 6 to 8 months after the attack, with evaluations of event-related and visual-evoked potentials (P300 and VEP), brainstem auditory evoked potentials, electrocardiographic R-R interval variability (CVRR), and scores on a posttraumatic stress disorder (PTSD) checklist. In the sarin victims, P300 and VEP (P100) latencies were significantly prolonged, and the CVRR was abnormal, indicating depression of cardiac parasympathetic nervous activity. The findings suggested persistent effects of sarin in the higher and visual nervous systems. In another study, Yokoyama *et al.* (1998a) reported a delayed effect on the vestibulocerebellar system induced by acute sarin poisoning. Yokoyama *et al.* (1998b) also reported a chronic effect on psychomotor performance. In addition, Miyaki *et al.* (2005) described the chronic effects associated with psychomotor and memory function up to 7 years after exposure.

As mentioned previously, two victims with OPIDN were reported (Sekijima *et al.*, 1997; Himuro *et al.*, 1998).

As part of a series of scientific studies sponsored by the Japanese Ministry of Health, Labor, and Welfare, Matsui *et al.* (2002) conducted two studies 7 years after the sarin attack. The first study was a case control study comparing victims treated at St Luke's Hospital with a control group. Statistical analysis showed significantly higher rates of chest pain, eye fatigue, presbyopia, eye discharge, nightmares, fear, anxiety, difficulty concentrating, and forgetfulness in the victim group. Moreover, in the victim group, there were even significantly higher rates of visual blurring, myopia, problems with focal convergence, abnormal eye sensations, flashbacks, fear of returning to the attack site, and not wanting to watch news about the attacks. The rate of PTSD, as evaluated by several diagnostic criteria, was also higher in the victim group. The second study was a cohort study comparing a group who required medical intervention after the attack with a group who did not. For lethargy, diarrhea, myopia, presbyopia, problems with focal convergence, eye discharge, and apathy, there were no significant differences between the groups, but for other evaluated parameters, scores were significantly higher in the nonintervention group. Comparison of PTSD incidence based on whether intervention was received showed that the nonintervention group had a significantly higher rate of masked PTSD. There was a higher incidence of eye symptoms in the victim group than in the nonvictim group, but there was no difference between the intervention group and nonintervention group. Thus, eye symptoms are probably long-term physical sequelae of sarin exposure. In some Matsumoto cases, persistent EEG changes without seizure activity have been reported up to 5 years (Yanagisawa *et al.*, 2006).

The results of these studies suggest some long-term effects of sarin toxicity and careful follow-up and observation are indicated in these victims.

VI. LABORATORY FINDINGS IN SARIN TOXICITY

According to inpatient records from St Luke's Hospital, the most common laboratory finding related to sarin toxicity was a decrease in plasma cholinesterase (ChE) levels in 74% of patients. In patients with more severe toxicity, plasma ChE levels tended to be lower, but a more accurate indication of ChE inhibition is measurement of erythrocyte ChE, as erythrocyte ChE (AChE) is considered ''true ChE'' and plasma ChE is ''pseudo ChE''. However, erythrocyte ChE is not routinely measured, whereas plasma ChE is included in many clinical chemistry panels; thus, it can be used as a simple index for ChE activity. In both the Matsumoto and Tokyo subway sarin attacks, plasma ChE served as a useful index of sarin exposure. In 92% of hospitalized patients, plasma ChE levels returned to normal on the following day. In addition, inpatient records from St Luke's Hospital showed an elevated creatine phosphokinase (CPK) and leukocytosis in 11% and 60% of patients, respectively. In severe cases in the Matsumoto attack, hyperglycemia, ketonuria, and low serum triglycerides due to toxicity of sarin on the adrenal medulla were observed (Yanagisawa *et al.*, 2006).

VII. CONCLUDING REMARKS AND FUTURE DIRECTION

This chapter has discussed sarin toxicity based on experiences of the attacks in Matsumoto and the Tokyo subway, and also the Iran–Iraq war. This section provides some conclusions drawn from the toxicological issues related to sarin.

Given the low concentration and means of dispersal, the Tokyo subway sarin attack can be referred to as a ''passive'' attack. The implication of such an assumption is therefore that mankind has not yet witnessed a ''full-scale'' sarin attack in any major city. While valuable information can certainly be gained from the Tokyo subway sarin attack, the experience obtained from the more aggressive Matsumoto sarin attack and the Iran–Iraq war should also be considered when developing initiatives directed at dealing with a potential ''full-scale'' attack in the future where the effects will be more serious.

Importantly, reliable epidemiologic data is lacking regarding the long-term effects of sarin toxicity, whether low dose exposure to sarin has any long-term effects, and specific effects on children, pregnant women, and fetuses (Sharp, 2006). The sporadic and limited epidemiologic surveys undertaken to date suggest that some long-term effects are present. Thus, well-designed international epidemiologic studies should be conducted in victims exposed to sarin in Japan, Iran, and during the Persian Gulf War.

There are several issues regarding treatment that need to be resolved. Before the Tokyo subway sarin attack in 1995, treatment of chemical weapons victims was exclusively regarded as a military issue; however, since then, the deliberate release of nerve agents against the general public has become a serious public safety issue. Treatment of chemical weapon injuries in a military setting assumes that one is dealing with healthy males, who have received basic and ongoing training, and who are wearing PPE. In an attack on the general public, however, we are dealing with a heterogeneous population from different backgrounds, and the victims will include women, pregnant women, children, and persons who are elderly, sick, and disabled. Furthermore, the public is defenseless against chemical weapons because of their lack of knowledge of dangerous chemical substances, or lack of experience with wearing PPE. Taken together, there is thus the potential to have thousands of victims in the event that there is a deliberate release of nerve agents against ordinary citizens.

Therefore, the medical treatment required for responding to a chemical terrorist attack on the general public will require a different strategy than that employed for such attacks in a military setting. This is because, even though there are numerous lessons that can be learned from military experience, there will be measures that may not be applicable to an attack on the public. An important issue is the means by which appropriate drugs can be safely and reliably supplied to a large number of victims. In addition, it is unrealistic to expect that first responders wearing PPE will be able to establish intravenous lines in large numbers of victims at the scene of a terrorist attack and the use of autoinjectors for intramuscular or intraosseous access is more realistic (Ben-Abraham *et al.*, 2003). In this regard, what is needed are not standardized autoinjectors issued to military personnel, but rather, a variety of autoinjectors that are uncomplicated and easy to use by victims. Research on the drugs used to treat chemical terrorism victims crosses the military/private sphere and is being conducted in several countries. However, unlike drugs that are designed for treating diseases, clinical trials cannot be performed in humans due to ethical concerns. Conducting a randomized control study is also difficult because of an insufficient number of cases of organophosphate poisoning to establish a reliable sarin toxicity model. A prime example is the oxime agent HI-6. Despite being developed more than 10 years previously, considerable time elapsed before its widespread use. From the standpoint of international security, collaborative research on drugs for treating chemical terrorism and a global agreement on standard treatment are needed. These are important issues in clinical toxicology that require international collaboration.

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CHAPTER 5

Epidemiology of Chemical Warfare Agents

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I. INTRODUCTION

While chemical warfare agents have been used for decades in military conflict, it is only in the last two decades that increasing attention has been placed on the acute and chronic health effects associated with exposure to these agents. The Gulf War of 1991 and the subsequent reports of ill-defined illnesses in the veterans of that conflict, followed by the 1995 sarin terrorist event in the Japanese subway system, placed increased attention on the capacity of deliberate or accidental exposure to chemical warfare agents resulting in significant human death and subsequent disability.

Epidemiological studies of chemical warfare agents have suffered problems in determining exposure. Other than epidemiological investigations following the Japanese terrorist event, little objective epidemiological evidence is available. In this chapter, the major studies that have been conducted on populations exposed to the chemical warfare agents are discussed and methodological issues summarized.

II. PRE-WORLD WAR II

The first full-scale deployment of chemical warfare agents was during World War I in 1915, when the Germans used chlorine gas against French, Canadian, and Algerian troops. Deaths were light, though casualties relatively heavy. A total of 50,965 tons of pulmonary, lachrymatory, and vesicant agents were deployed by both sides of the conflict, including chlorine, phosgene, and mustard gas. Official figures declare about 1,176,500 nonfatal casualties and 85,000 fatalities directly caused by chemical warfare agents during the course of the war (Heller, 2005). In 1925, 16 of the world's major nations signed the Geneva Protocol, pledging never to use gas in warfare again; however, there were subsequent reports of its use. In 1935 Italy used mustard gas during the invasion of Ethiopia in the Second Italo-Abyssinian War with 15,000 chemical casualties reported. In this military conflict and subsequent wars in which chemical agents were used, no systematic attempt was made to accurately describe the epidemiology of the exposures, nor were any accurate data established to follow the health of exposed populations after the acute exposure.

Concern regarding potential long-term effects of these exposures continued to be an issue and in 1975 a longitudinal follow-up study of the mortality experience of three samples of World War I veterans was conducted to determine if a single exposure to mustard gas with respiratory injury was associated with increased risk of lung cancer in later life (Norman, 1975). Rosters of men born between 1889 and 1893 [2,718 exposed to mustard gas, 1,855 hospitalized with pneumonia in 1918, and 2,578 with wounds of the extremities (controls)] were traced via the Veterans Administration's death records. The 4,136 deaths reported were 95% of that expected. Observed deaths from lung cancer numbered 69, or 2.5%, for the mustard-gas group as compared to 33, or 1.8%, for the pneumonia group and 50, or 1.9%, for the controls. The risk of death from lung cancer among men gassed relative to that for the controls was estimated as 1.3, with 95% confidence limits of 0.9–1.9.

III. WORLD WAR II

In 1938, the chemical structure of sarin nerve gas was discovered by the Germans, followed by the discovery of the nerve agent soman in the spring of 1944 (Schmaltz, 2006), but chemical warfare was not extensively used by either side due in part to fear of a devastating Allied retaliatory attack. There was one account of an exposure to mustard gas among Allied troops when several American ships were sunk by the Germans in 1943, including one carrying mustard gas intended for use in retaliation by the Allies if German forces initiated gas warfare. Because the presence of the gas was highly classified, authorities ashore, treating casualties, had no idea that they were seeing the effects of mustard gas and prescribed improper treatment. This incident was not uncovered for many years and military records account that 69 deaths were attributed in whole or part to mustard gas, out of 628 mustard gas military casualties (US Naval Historical Center, 1943). The due impact of the gas exposure to military and civilian populations was not accurately reported due to the high secrecy regarding the exposure and the difficulty discerning the effect of gas exposure from other types of injuries.

During the Holocaust, the Nazis used the insecticide Zyklon B containing hydrogen cyanide to kill several

million people in extermination camps and reportedly used poison gases during the Warsaw Ghetto Uprising in 1943. Human experiments were conducted on concentration camp prisoners using mustard gas and phosgene.

In 1994 a United States Senate Report, entitled ''Is military research hazardous to veterans health? Lessons spanning a half century'' reported that US military personnel were used as human subjects in the 1940s to test the chemical agents mustard gas and lewsite. This testing was done to determine how to best protect military troops from the effects of chemical warfare agents (Pechura and Rall, 1993).

During the war, the US military conducted a secret research program aimed at determining how best to protect military personnel against the effects of mustard gas and a similar compound, lewisite (Pechura and Rall, 1993). Up to 4,000 men took part in the program which required participants to wear gas masks and clothing that had been treated in an attempt to block the gas from reaching the skin. Men were required to remain in the sealed test room from 1 to 4 h. Some men were tested in the field where they were required to stay in an area that had been bombed with mustard gas anywhere from 1 h to 3 days. In 1992, the US Department of Veterans Affairs (VA) began to allow compensation for seven conditions that can result from mustard gas exposure: laryngitis, chronic bronchitis, emphysema, asthma, chronic conjunctivitis, chronic keratitis, and corneal opacities. Following publication of a report by the National Academy of Sciences (Pechura and Rall, 1993), the VA extended the list to include respiratory cancers (nasopharyngeal, laryngeal, and lung except for mesothelioma), skin cancer, chronic obstructive pulmonary disease, and acute nonlymphocytic leukemia.

In 2000, Bullman and Kang (2000) reported a 50-year mortality follow-up study of veterans exposed to low levels of mustard gas. They conducted a retrospective mortality follow-up study of World War II Navy veterans who received low-level nonlethal exposures to mustard gas while participating in mustard gas chamber tests at Bainbridge, Maryland, between 1944 and 1945. These veterans were exposed to mustard gas while wearing protective clothing and masks. Control veterans consisted of 2,663 Navy veterans who served at the same location and time as the exposed, but did not participate in chamber tests. The investigators found no excess of any cause-specific mortality associated with varying levels of mustard gas exposures that were sufficient to cause skin reactions. A significant strength of this study was that the length of time in the exposure chamber, the dose of exposure, and documentation of any observative acute effect were available for each of the exposed subjects so that a dose–response analysis could be done.

In a 2000 report, Schnurr *et al.* (2000) reported on the prevalence of current post-traumatic stress disorder (PTSD) associated with participation in these secret military tests of mustard gas exposure. Using the registry established by the VA, 363 male military veterans were randomly sampled and found to have a current prevalence of 32% for full PTSD and 10% for partial PTSD. Prevalence of PTSD varied as a function of risk and protective factors, including volunteering, physical symptoms during the tests, and prohibited disclosure. Veterans with full PTSD reported poorer physical health, a higher likelihood of several chronic illnesses and health-related disability, greater functional impairment, and higher likelihood of healthcare use than those with no PTSD. Veterans with partial PTSD also had poorer outcomes than did veterans with no PTSD in a subset of these domains.

Schnurr *et al.* (1996) postulate that these exposures involved elements of ''contamination stressors'' in which information about the exposure is the stressor rather than the tangible event. The late disclosure of the dangerous nature of these tests served as an additional stressor for many of the exposed men. Lack of information during the test, leaning to vague or diffuse fear with unknown consequences, could also contribute to the development of PTSD. The contamination stressor led to a future orientation; a worry about what problems will develop as a result of the previous exposure.

IV. POST-WORLD WAR II

Development of other agents such as the VX nerve agent continued during the 1950s and in 1961 the USA was producing large amounts of VX and performing its own nerve agent research. In 1952 the US Army patented a process for developing the powerful toxin ricin.

In 1969, 23 US servicemen and one US civilian stationed in Okinawa, Japan, were exposed to low levels of the nerve agent sarin while repainting the depot's buildings. When the exposure was publicized, the USA moved the weapons in 1971 to Johnston Atoll. Between 1951 and 1969 at the Dugway Proving Ground, various chemical and biological agents were tested. From 1962 to 1973 more than 5,800 military personnel participated in a series of tests on the vulnerability of warships to biological and chemical attacks. Only some of the involved military personnel consented to the tests. Many of the tests used chemical warfare simulants, thought at the time to be harmless. The results of the tests were reported in classified documents (SHAD report). In 2000, the Department of Defense released the names of the participants and information about the testing that occurred. In 2002 the Institute of Medicine agreed to undertake a scientific study of potential long-term health effects associated with these exposures. The IOM assembled a comparable control group and conducted a telephone health survey. Mortality records were also examined. The primary outcomes of interest were mortality, general health, and medical conditions. The SHAD participants were divided into four groups:

- Group A consisted of 3,000 participants whose exposure was limited to either *Bacillus globigii* (BG) or methylacetoacetate (MAA);
- Group B consisted of 850 participants whose only potential exposure was to trioctyl phosphate (TEHP or TOF) and contained a large number of marine participants;
- Group C consisted of 720 participants who were in tests where active chemical warfare agents were used;
- Group D consisted of 850 subjects potentially exposed to simulants who were not in groups A, B, or C.

Control groups were assembled for each of the exposed groups. Of the nearly 12,500 Navy and Marine subjects, 9,600 were assumed alive and were surveyed. The response rate for the SHAD participants was 60.8% and 46.6% for controls. No differences were observed in all-cause mortality between SHAD participants and controls, although the SHAD participants had a statistically significant higher risk of death due to heart disease. Lack of cardiovascular risk factor data makes this difference difficult to interpret. SHAD participants also reported statistically significantly worse health than controls, but no specific patterns of illness were found. Group C, the only group with potential exposure to active chemical or biological agents, reported the smallest differences in overall health compared to controls. Small differences in memory and attention as well as somatization were observed and SHAD participants had higher levels of neurogenerative conditions. SHAD participants also reported higher rates of symptoms, thought to be related to reporting bias. There were no significant differences in self-reported hospitalizations.

This report was significant in that it was the first epidemiological investigation of a military population with documented exposure to chemical agents or stimulants. The survey was conducted, however, 30 years after the exposure and with the exception of mortality records was limited to self-reported measures of health.

V. IRAN–IRAQ WAR

Saddam Hussein received chemical weapons from many countries, including the USA, West Germany, the Netherlands, the UK, France and China (Lafayette, 2002). In 1980 Iraq attacked Iran and employed mustard gas and tabun with 5% of all Iranian casualties directly attributable to the use of these agents. Iran sustained approximately 387 chemical attacks during the eight-year war (Shemirani *et al.*, 1993). About 100,000 Iranian solders were chemical warfare victims along with significant numbers of civilians. Nerve gas agents killed about 20,000 Iranian soldiers immediately. Shortly after the war ended in 1988, the Iraqi Kurdish village of Halabia was exposed to multiple chemical agents resulting in the death of 10% of the town's 50,000 residents. Hashemian *et al.* (2006) reported on the results of a cross-sectional randomized survey of 153 civilians in three towns exposed to military conflict in northwestern Iran; Oshnaviveh (low-intensity conventional warfare), Rabat (high-intensity conventional warfare), and Sardasht (both high-intensity conventional warfare and chemical weapons). The surveys measured full or partial PTSD diagnosis, anxiety symptoms, and depressive symptoms. The authors reported a 93% response rate from respondents (mean age of 45 years) and all were of Kurdish ethnicity. Compared with individuals exposed to low-intensity warfare, those exposed to high-intensity warfare and chemical weapons were at a higher risk for lifetime PTSD [odds ratio (OR), 18.6; 95% confidence interval (CI), 5.8–59.4], current PTSD (OR, 27.4; 95% CI, 3.4–218.2), increased anxiety symptoms (OR, 14.6; 95% CI, 6.0–35.6), and increased depressive symptoms (OR, 7.2; 95% CI, 3.3–15.9). Exposure to high-intensity warfare but not to chemical weapons was also significantly associated with lifetime PTSD (OR, 5.4; 95% CI, 1.7–17.6), compared with those in the low-intensity warfare group. Further, compared with individuals exposed to high-intensity warfare alone, those exposed to both high-intensity warfare and chemical weapons were at higher risk for lifetime PTSD (OR, 3.4; 95% CI, 1.5–7.4), current PTSD (OR, 6.2; 95% CI, 2.0–20.1), increased anxiety symptoms (OR, 5.6; 95% CI, 2.5–12.6), and increased depressive symptoms (OR, 3.7; 95% CI, 1.8–7.2).

This study was the first epidemiological study to document the long-term negative mental health sequelae of exposure to war and chemical weapons among civilians. The authors argue that exposure to chemical weapons is an extreme traumatic event that can result in acute helplessness and anxiety, loss of perceived safety, and chronic physical disabilities. The study had a number of limitations including the reliance on self-reported data; however, self-reported chemical exposure was verified with medical records.

VI. GULF WAR 1991

Given the past use of chemical weapons of Iraq on its own citizens, there was much concern that Saddam Hussein would again employ these weapons during the conflict against coalition forces. The only known exposure to anticholinesterase chemical warfare agents during the Gulf War was the destruction of munitions containing 8.5 metric tons of sarin/cyclosarin housed in Bunker 73 at Khamisyah, Iraq, on March 4, 1991, and additional destruction of sarin/cyclosarin rockets in a pit at Khamisyah on March 10, 1991. The US Department of Defense (DOD) reported that the exposure levels were too low to activate chemical alarms or to cause symptoms at the time of the detonation; however, several studies have been conducted to assess long-term health effects associated with this exposure. The DOD conducted modeling of the air plume that resulted from the detonation and estimated the extent of troops potentially exposed to the plume.

McCauley *et al.* (1999) conducted a computer-assisted telephone survey of 2,918 Gulf War veterans from Oregon, Washington, California, North Carolina, and Georgia to evaluate the prevalence of self-reported medical diagnoses and hospitalizations among this potentially exposed population and among comparison groups of veterans deployed and nondeployed to the Southwest Asia theater of operations. Troops reported to be within 50 km of the Khamisyah site did not differ from other deployed troops on reports of any medical conditions or hospitalizations in the nine years following the Gulf War. Hospitalization rates among deployed and nondeployed troops did not differ. Deployed troops were significantly more likely to report diagnoses of high blood pressure (OR = 1.7); heart disease (OR = 2.5); slipped disk or pinched nerve (OR = 1.5); PTSD (OR = 14.9); hospitalization for depression (OR = 5.1); and periodontal disease (OR = 1.8) when compared to nondeployed troops. There was a trend for deployed veterans to report more diagnoses of any cancer (OR = 3.0).

Smith *et al.* (2003) investigated postwar morbidity for Gulf War veterans, contrasting those who may have been exposed to low levels of nerve agents at Khamisyah and those unlikely to have been exposed. Cox regression modeling was performed for hospitalizations from all causes and hospitalizations from diagnoses within 15 categories during the period March 10, 1991 through December 31, 2000, for the duration of active-duty status. Veterans possibly exposed to nerve agents released by the Khamisyah demolition were not found to be at increased risk for hospitalizations from most chronic diseases nearly 10 years after the Gulf War. Only two of 37 models suggested that personnel possibly exposed to subclinical doses of nerve agents might be at increased risk for hospitalization from circulatory diseases, specifically cardiac dysrhythmias.

In 2005, Bullman *et al.* (2005) reported the results of a mortality study of troops exposed to chemical warfare agents based on the air plume models that were developed after the detonation. The cause-specific mortality of 100,487 exposed veterans was compared with that of 224,480 unexposed US Army Gulf War veterans. The risks for most disease-related mortality were similar for exposed and unexposed veterans. However, exposed veterans had an increased risk of brain cancer deaths (relative risk = 1.94; 95% CI = 1.12, 3.34). The risk of brain cancer death was larger among those exposed 2 or more days than those exposed 1 day when both were compared separately to all unexposed veterans.

This same team of investigators also conducted a study to examine the association of exposure to the Khamisyah plume with subsequent self-reported morbidity (Page *et al.*, 2005). The study sample included 1,056 deployed Army Gulf War veterans who responded to the 1995 National Health Survey of Gulf War Era Veterans and who were resurveyed in 2000. One-half of the subjects had been notified of potential exposure to chemical warfare agents and one-half had not. Comparing notified and nonnotified subjects, there were no statistically significant differences with respect to bed days, activity limitations, clinic visits, or hospital visits. Among 71 self-reported medical conditions and symptoms, there were five statistically significant differences, four of which were for lower rates of illness among notified subjects.

Page and colleagues also published a similar study undertaken to investigate whether possible chemical warfare exposure was associated with morbidity among Army Gulf War veterans using morbidity data for 5,555 Army veterans who were deployed to the Gulf region (Page *et al.*, 2005). Responses to 86 self-assessed health measures, as reported in the 1995 Department of Veterans Affairs National Health Survey of Gulf War Era Veterans, were evaluated. They found little association between potential exposure and health, after adjustment for demographic variables. The investigators concluded that potential exposure to sarin or cyclosarin at Khamisyah did not seem to have adversely affected self-perceived health status, as evidenced by a wide range of health measures.

More recently, Heaton examined the association between modeled estimates of sarin/cyclosarin exposure levels and volumetric measurements of gross neuroanatomical structures in 1991 Gulf War veterans with varying degrees of possible low-level sarin/cyclosarin exposure (Heaton *et al.*, 2007). Twenty-six GW-deployed veterans recruited from the Devens Cohort Study participated. Magnetic resonance images of the brain were acquired and analyzed using morphometric techniques, producing volumetric measurements of white matter, gray matter, right and left lateral ventricles, and cerebrospinal fluid. Volumetric data were analyzed using exposure estimates obtained from refined models of the 1991 Khamisyah presumed exposure hazard area. No differences were observed in the 13 ''exposed'' veterans when compared to 13 ''non-exposed'' veterans in volumetric measurements of discrete brain tissues. However, linear trend analyses showed a significant association between higher levels of estimated sarin/cyclosarin exposure and both reduced white matter (adjusted parameter estimate = 4.64, $p < 0.0001$) and increased right lateral ventricle (adjusted parameter estimate = 0.11, $p = 0.0288$) and left lateral ventricle (adjusted parameter estimate = 0.13, $p < 0.0001$) volumes. These findings suggest subtle but persistent central nervous system pathology in Gulf War veterans potentially exposed to low levels of sarin/cyclosarin.

This investigative team also compared previous neurobehavioral performance results collected prior to notification of veterans who were potentially exposed in the Khamisyah detonation (Proctor *et al.*, 2006). They hypothesized the exposure to sarin and cyclosarin would be associated with poorer performances on objective neurobehavioral tasks in specific functional domains (particularly in visuospatial abilities and psychomotor functioning) in a dose-dependent manner. They found that sarin and cyclosarin exposure was significantly associated with less

proficient neurobehavioral functioning on tasks involving fine psychomotor dexterity and visuospatial abilities 4–5 years after exposure. They concluded that the findings suggest a dose–response association between low-level exposure to sarin and cyclosarin and specific functional central nervous system effects 4–5 years after exposure.

VII. TERRORISM

Two terrorist attacks with the nerve agent sarin affected populations in Matsumoto and Tokyo, Japan, in 1994 and 1995 killing 19 and injuring more than 6,000. Morita *et al.* (1995) described the acute effects including instantaneous death by respiratory arrest in four victims in Matsumoto. In Tokyo, two died in station yards and another ten victims died in hospitals within a few hours to 3 months after poisoning. Six victims with serum cholinesterase (ChE) below 20% of the lowest normal were resuscitated from cardiopulmonary arrest (CPA) or coma with generalized convulsion. Five recovered completely and one remained in a vegetative state due to anoxic brain damage. EEG abnormalities were observed for up to 5 years in certain victims. Miosis and copious secretions from the respiratory and gastrointestinal tracts (muscarinic effects) were common in severely to slightly affected victims. Weakness and twitches of muscles (nicotinic effects) appeared in severely affected victims. Neuropathy and ataxia were observed in a small number (less than 10%) of victims, in which findings disappeared between 3 days and 3 months. Leukocytosis and high serum CK levels were common. Hyperglycemia, ketonuria, low serum triglyceride, and hypopotassemia were observed in severely affected victims, in which abnormalities were attributed to damage of the adrenal medulla.

The Matsumoto Japanese government assembled a committee of city government, local hospitals and physicians from Shinsu University to monitor the immediate and long-term effects of the exposure, resulting in the most comprehensive epidemiological studies of acute and residual effects of exposure to chemical warfare agents. Three weeks after the attack, community residents ($n = 2{,}052$) residing in an area within 1,000 to 850 m of the attack were surveyed and categorized as severely affected (admitted to the hospital), moderately affected if treated in outpatient clinics, and slightly affected if they had symptoms but did not seek medical attention. At the time of this follow-up survey, 28% of the affected residents remained symptomatic (69% of the severely affected, 42% of the moderately affected, and 14% of the slightly affected). The most frequent persisting symptoms were fatigue, dysesthesia of extremities, and ocular pain. Visual problems continued in about 10% of severely affected victims (Yanagisawa *et al.*, 2006).

In the Tokyo subway attack, 640 victims were seen within hours of the incident. Five were critically injured and required mechanical ventilation. One hundred and seven were moderately injured with systemic symptoms and signs of respiratory, digestive, and/or neurological systems in addition to ocular signs. The large majority ($n = 528$) had only eye signs or symptoms and were released after several hours of observation (Yanagisawa *et al.*, 2006).

There have been a number of investigations of the health of the survivors of the Tokyo subway attack. Yokoyama *et al.* (1998) conducted a study of 18 victims 6–8 months after the attack. At that time the mean plasma ChE was 72.1, lower than the ''normal'' range of 100–250 IU/l. In neurobehavioral testing at that time, sarin cases had significantly lower scores on the digit symbol test than the control group. Scores on the General Health Questionnaire and fatigue were significantly higher in the victims and PTSD scores were also increased. Postural balance was also different in victims suggesting that integration of visual input might have been impaired. P300 and VEP (P100) latencies in the sarin cases were significantly prolonged compared with the matched controls (Murata *et al.*, 1997). In the sarin cases, the CVRR was significantly related to serum ChE levels determined immediately after exposure; the PTSD score was not significantly associated with any neurophysiological data despite the high PTSD score in the sarin cases. These findings suggest that asymptomatic sequelae to sarin exposure, rather than PTSD, persist in the higher and visual nervous systems beyond the turnover period of ChE.

The National Police Academy (1999) conducted a survey of 1,247 residents who reported to the Police Department that they had contact with sarin at the incident. More than half complained of physical symptoms, such as asthnopia and decrease in visual acuity. Seventeen percent reported psychological trauma from the event with 14% still unable to ride on subways 3 years after the incident.

There continued to be follow-up studies indicating the residual effects of the attack. Ohtani *et al.* (2004) followed 34 victims 5 years after the attack. Not only PTSD but also nonspecific mental symptoms persisted in the victims at a high rate. A total of 11 victims were diagnosed with current or lifetime PTSD. Victims with PTSD showed higher anxiety levels and more visual memory impairment.

Yamasue *et al.* (2007) conducted a 5 year follow-up study to identify persistent morphological changes subsequent to the attack. Thirty-eight victims of the sarin attack, who had been treated in the emergency department for sarin intoxication and 76 matched health control subjects underwent weighted and diffusion tensor magnet resonance imaging. ChE values were compared to levels immediately after the attack. The voxel-based morphometry exhibited smaller than normal regional brain volumes in the insular cortex and neighboring white matter, as well as in the hippocampus in the victims. The reduced regional white matter volume correlated with decreased serum cholinesterase levels and with the severity of chronic somatic complaints related to interoceptive awareness.Voxel-based

analysis of diffusion tensor magnetic resonance imaging further demonstrated an extensively lower than normal fractional anisotropy in the victims. These findings suggest that sarin intoxication might be associated with structural changes in specific regions of the human brain.

Rescue and safety workers have also been studied. Nishiwaki *et al.* (2001) studied 27 male rescue team staff and 30 police officers, 3–45 months after the event. The study subjects showed decreased performance on the digit span test; however, no effects on stabilometry and vibration perception threshold were found. Li *et al.* (2004) followed 27 male firefighters and 25 male police officers three years after the attack for genotoxic effects. They found an elevated frequency of sister chromatid exchanges in lymphocytes of the victims which were related to the percentage of ChE inhibition observed just after the attack.

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

This chapter described the major epidemiological studies of populations who have been exposed to chemical warfare agents. Many of the studies of military populations have suffered from inaccurate exposure assessment and lack of clinical data. The studies in the past decade of the survivors of the sarin terrorist attacks provide the most comprehensive data to date on the scope of health outcomes associated with these exposures. These reports point to the need for long-term follow-up studies of victims following such events. The data from the terrorist events and the Gulf War when many troops believed they were exposed to chemical agents point to the prevalence of PTSD associated with real or threatened exposure.

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CHAPTER 6

Organophosphate Nerve Agents

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I. INTRODUCTION

The chemical warfare (CW) nerve agents primarily addressed in this chapter include the anticholinesterase nerve agents tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), and VX, all of which are, or have been, part of the US domestic munitions inventories (Carnes, 1989; NRC, 1999; Opresko *et al.*, 1998). Russian VX (often represented as VR) will be evaluated in the following chapter by Radilov *et al.* (2009). Other, less well-characterized nerve agents such as compound GE, VG (Amiton™) or V_x will be evaluated as data allow.

These agents are potent anticholinesterase compounds deliberately formulated to induce debilitating effects or death during wartime hostilities and have been used by military authorities of several nations to develop munitions (e.g. Germany during the Nazi era, the USA, the former Soviet Union). US military stockpiles of CW munitions manufactured as a Cold War deterrent decades ago await demilitarization at designated stockpile sites and have been the subject of extensive emergency preparedness and response planning. Additional planning has been necessary at other current and formerly used military sites where containers, buried munitions, etc., have been inventoried (the ''nonstockpile'' sites) (NRC, 2003, 1999; Opresko *et al.*, 1998). More recent global events have focused attention on the potential threat of chemical terrorism, especially at transportation hubs (Tucker and Raber, 2008). The deliberate release of nerve agent sarin at lethal concentrations in the Japanese cities of Matsumoto (1994) and Tokyo (1995) by a Japanese domestic terrorist group has illustrated that such attacks can be a reality and require advance emergency preparedness planning (Morita *et al.*, 1995; Okumura *et al.*, 1996, 2007; Sidell, 1996; Yangisawa *et al.*, 2006; Cannard, 2006; Tu, 2007).

Information provided on agent toxicity, risk assessment, treatment options, and other related topics will be useful to communities and facilities developing and updating emergency preparedness plans for accidental or intentional release of nerve agents; this information can also be used to support environmental decision-making where nonstockpile materiel - military gear has been found. In response to various Public Laws and international agreements such as the Chemical Weapons Convention (PL 99-145, 1986; PL 102-484, 1993; CWC, 1997), existing emergency guidance and military policy documents currently reflect the identified criteria and information (e.g. CSEPP, 2003, 2006a, b; NRT, 2008; OASA, 1999).

While there are different applications for the information provided in this evaluation, in general the toxicological focus for emergency preparedness and response applications is that of acute exposures associated with a one-time release. Typically, the scenarios considered include a single-source airborne release either from an intentional terrorist attack or accident involving an agent container or munition from a military site. It is widely recognized that vapor inhalation is the exposure route of greatest concern for such an event (ATSDR, 2007; Sidell, 1997). In contrast, the toxicological focus of environmental site remediation plans for military installations and formerly used defense sites where buried chemical warfare agent residues may occur requires consideration of long-term release and potential incidental ingestion of media such as soil particles or water with relatively low levels of contamination. To reflect these critical applications and information needs, the current evaluation will also primarily focus on

- single-source, one-time nerve agent releases and exposure routes involving agent vapor inhalation or direct ocular vapor exposures, and
- long-term (chronic or subchronic) exposure from residual nerve agent contamination.

It is acknowledged that there exists a rich and valuable body of repeat-exposure studies employing serial vapor or serial injection exposures for the nerve agents soman (GD), sarin (GB) and VX (please see recent excellent reviews and analyses in Shih *et al.*, 2006; McDonough and Romano, 2008 as well as recent experimental studies by Dabisch *et al.*, 2005, 2007a); the interested reader is encouraged to examine these and related resources, as the current evaluation does not highlight experiments that apply serial exposure protocols.

II. BACKGROUND

A. Development of Organophosphate Formulations as Chemical Warfare Agents

The G-series nerve agents evaluated are all toxic ester derivatives of phosphonic acid containing either a cyanide

(GA) or fluoride (GD, GE, GF) substituent and are commonly termed ''nerve'' agents as a consequence of their anticholinesterase properties as well as their effects on both the peripheral nervous system (PNS) and central nervous system (CNS). The ''G'' series military nomenclature used by NATO member nations has historically been considered to be an abbreviation for ''German'', with the second letter of the code (''A'', ''B'', etc.) identifying the order in which these compounds were found and analytically identified by Allied forces investigating materials found in captured German military facilities at the close of WWII (Sidell, 1997). Agent VX, a phosphonic acid ester with a sulfur substituent, was industrially synthesized in the United Kingdom in the early 1950s; the code letter ''V'' is a reported reference to ''venom'' (Sidell, 1997). Other, less well-characterized V-series compounds include V_x, VE, VM, and VG (trade name Amiton™ when commercially introduced as a miticide in the mid-1950s).

As Cold War deterrents, nerve agents began to be manufactured and weaponized by the USA in the 1950s. When the US chemical warfare agent production program was terminated by the Nixon presidential ''Statement on Chemical and Biological Defense Policies'' of November 1969 (National Security Decision Memorandum 35), the US stockpile of unitary munitions included bulk (''ton'') containers, underwing spray tanks, projectiles, rockets, bombs, land mines, and rockets (Carnes, 1989; Sidell, 1997). Nerve agent unitary munitions contained either GA, GB, or VX. The US chemical warfare agent munition stockpile is obsolete, and is presently undergoing destruction and disposal by the US Army Chemical Materials Agency (see www.cma.army.mil) at each of the several unitary stockpile sites for compliance with the Chemical Weapons Convention (CWC) and to eliminate the risk of continued storage for these aging CW munitions; current nerve agent stockpile sites are located in Alabama, Arkansas, Indiana, Kentucky, Oregon, and Utah. In December 2008, the US Army Chemical Materials Agency announced that 58% of the US unitary chemical munitions stockpile had been successfully destroyed.

B. Physical and Chemical Properties of Nerve Agents

The G-agents are all viscous liquids of varying volatility (vapor density relative to air between 4.86 and 6.33) with faint odors (''faintly fruity'', or ''spicy'', ''odor of camphor''). Agent VX is an amber-colored liquid with a vapor density of 9.2, and is considered odorless. Thus, nerve agent vapors possess little to no olfactory warning properties (Table 6.1).

The vapor pressures and acute toxicity of these agents are sufficiently high for the vapors to be rapidly lethal. Within the G-series, GB is considered to present the greatest vapor hazard (order of vapor hazard approximates GB > GD > GF > GA). Agent VX was deliberately formulated to possess a low volatility; VX is approximately 2,000 times less volatile than nerve agent GB (DA, 1990a, b). As a consequence, agent VX is considered a persistent, ''terrain denial'' military compound with the potential to be a contact hazard or generate off-gas toxic vapor concentrations over a period of days following surface application particularly under cold weather conditions or when bulk-release quantities of liquid agent are involved. While not readily volatile, VX vapors (if allowed to accumulate) are nevertheless considered more acutely potent than those of agent GB or the other G-series agents (Mioduszewski *et al.*, 1998).

As a consequence of the volatilities exhibited by G-series nerve agents (Table 6.1), the most likely exposure route (and source of primary hazard) is via direct vapor exposure to the eyes and upper respiratory tract tissues, and vapor inhalation (with consequent systemic absorption) (Dabisch *et al.*, 2008a; Cannard, 2006); G-agents are considered ''nonpersistent'' as per definitions employed by the US Department of Defense (DOD, 2008). Nerve agent VX is widely considered to present a greater threat from the percutaneous exposure route (when compared to the G-series agents) as well as a vapor inhalation threat at elevated ambient temperatures (e.g. >40°C; Benton *et al.*, 2005, 2006a; Craig *et al.*, 1977; Sidell, 1997).

Nerve agent V_x exhibits volatility (76.4 mg/m^3 at 25°C) intermediate to that of agents GA and VX, and a vapor density (7.3) intermediate to that of agents GF and VX; V_x is also considered ''persistent''. There are few data from which to characterize nerve agents VE (*O*-Ethyl-*S*-[2-(diethylamino) ethyl]ethylphosphonothioate, CAS No. 21738-25-0) or VM (*O*-Ethyl-*S*-[2-(diethylamino)ethyl]-methylphosphonothioate, CAS No 21770-86-5).

III. MECHANISM OF ACTION

All of the nerve agents under consideration are anticholinesterase compounds and induce accumulation of the neurotransmitter acetylcholine (ACh) at neural synapses and neuromuscular junctions by phosphorylating acetylcholinesterase (AChE). Depending on the route of exposure and amount absorbed, the PNS and/or CNS can be affected and muscarinic and/or nicotinic receptors may be stimulated. Interaction with other esterases may also occur, and direct effects to the nervous system have been observed.

Exposure to acutely toxic concentrations of nerve agents can result in excessive bronchial, salivary, ocular and intestinal secretions, sweating, miosis, bronchospasm, intestinal hypermotility, bradycardia, muscle fasciculations, twitching, weakness, paralysis, loss of consciousness, convulsions, depression of the central respiratory drive, and death (Grob and Harvey, 1953; Grob, 1956; Marrs, 2007; Sidell, 1997; Yanagisawa *et al.*, 2006; many others). Minimal effects observed at low vapor concentrations

TABLE 6.1. Physical and chemical properties of organophosphorous nerve agents

Parameter	GA	GB	GD	GF	VX	GE	V_x
CAS Registry No.	77-81-6	107-44-8	96-64-0	329-99-7	50782-69-9	1189-87-3	20820-80-8
Chemical name[a]	Ethyl dimethylamido cyanophosphate	Isopropyl methyl-phosphonofluoridate	Pinacolyl methyl-phosphonofluoridate	*O*-cyclohexyl methyl-phosphonofluoridate	*S*-(2-diisopropyl-aminoethyl) *O*-ethyl methyl phosphonothiolate	Isopropyl ethylphosphono-fluoridate	*O*-ethyl *S*-(2-dimethyl-aminoethyl) methyl-phosphonothiolate
Common name[a,b]	Tabun	Sarin	Soman	Cyclosarin	VX	NA	NA
Chemical formula[a]	$C_5H_{11}N_2O_2P$	$C_4H_{10}FO_2P$	$C_7H_{16}FO_2P$	$C_7H_{14}FO_2P$	$C_{11}H_{26}$ NO_2PS	$C_5H_{12}FO_2P$	$C_7H_{18}NO_2PS$
Molecular weight[a]	162.13	140.10	182.178	180.2	267.38	154.12 (calc)	211.26
Physical state[a,c]	Liquid, vapor	Liquid, vapor	Liquid, vapor	Liquid, vapor	Oily liquid, vapor	Vapor	Liquid
Vapor pressure (mm Hg)[a]	0.037 (20°C)	2.10 (20°C)	0.40 (25°C)	0.056 (20°C)	0.0007 (25°C)	NA	6.73×10^{-3} (25°C)
Volatility (mg/m^3 at 25°C)[a,c]	610	22,000	3,900	548 at 20°C; 817 at 25°C	10.5	11.6 mg/l at 25°C (saturated concentration)	76.4
Liquid density (g/ml)[a]	1.073 (25°C)	1.102 (20°C)	1.0222 (25°C)	1.1327 (20°C)	1.006 (20°C)	1.0552 (25°C)	1.06 (25°C)
Vapor density (air = 1)[a]	5.63	4.86	6.33	6.2	9.2	NA	7.3 (calculated)
Melting point (°C)[a,b,c]	−50	−56	−42	−30	−39 (calculated)	NA	NA
Boiling point (°C)[a,b,c]	245	158, 150	198	239	298	67–68	256 (extrapolated)
Water solubility[a,c]	98 g/l (25°C); 72 g/l (20°C)	Miscible	21 g/l (20°C)	0.37% (20°C)	30 g per 100 g (25°C)	NA	Slightly
Hydrolysis half-life[d] (20°C and pH 7)	8.5 h	39–41 h; 80 h	80–83 h; 45 h at pH 6.65	42 h[a]	400–1000 h	NA	NA
Log K_{ow}[e]	1.18	0.15	1.02	NA	NA	NA	NA
Odor[a,b,c,f]	Faintly fruity; no odor when pure	Odorless when pure	Fruity, odor of camphor when impure	Perceptible; fruity; no agreement on odor description; odorless when pure	Odorless when pure	NA	Odorless
Odor threshold (mg/m^3)[a,b,c,f]	Undefined	<1.5	~1.5 to ~7.0	~10.4 to ~14.8	Odorless when pure	Undefined	Undefined
Henry's law constant[e,g] (atm m^3/mol)	1.52×10^{-7}	5.34×10^{-7}	4.56×10^{-6}	NA	3.5×10^{-9} (est.)	NA	NA
Conversion factors[h] in air	ppm = (0.15) × mg/m^3 mg/m^3 = (6.6) × ppm	ppm = (0.17) × mg/m^3 mg/m^3 = (5.7) × ppm	ppm = (0.13) × mg/m^3 mg/m^3 = (7.5) × ppm	ppm = (0.14) × mg/m^3 mg/m^3 = (7.4) × ppm	mg/m^3 = (10.936) × ppm ppm = (0.0914) × mg/m^3	NA	NA

Adapted from NRC (2003) with permission by the National Academy of Sciences, courtesy of the National Academies Press, Washington DC

[a]DA (1990a, b); Gates and Renshaw (1946); Buckles (1947); Tevault *et al.* (2003); Abercrombie (2003)

[b]DA (1992)

[c]DA (1974); Yang (1999)

[d]Clark (1989); DA (2005)

[e]Britton and Grant (1988)

[f]McGrath *et al.* (1953); Dutreau *et al.* (1950); DA (2005)

[g]Opresko *et al.* (1998); Small (1984)

[h]Calculated from molecular weight

include miosis (contraction of the pupils of the eye, with subsequent decrease in pupil area), tightness of the chest, rhinorrhea, and dyspnea (Dunn and Sidell, 1989; Dunn *et al.*, 1997). Pupillary contraction, resulting in varying degrees of miosis characterized by measures of pupil diameter, is consequent to local inhibition of ocular AChE activity with pupillary sphincter contraction (Dabisch *et al.*, 2007b, 2008a, b).

Reactivation of inhibited cholinesterase by dephosphorylation is not possible once the nerve agent–cholinesterase complex undergoes ''aging'', which is thought to be the consequence of the loss of an alkyl or alkoxy group. Agent GD ages very rapidly when bound to red blood cell cholinesterase (RBC–ChE), with a $t_{1/2}$ (time required for 50% of the enzyme to become resistant to reactivation) of 1.3 min (Harris *et al.*, 1978). The aging half-time for agent GA with RBC–ChE is 46 h (calculated; De Jong and Wolring, 1978), and the $t_{1/2}$ for agent GB with RBC–ChE is 5 h (Sidell and Groff, 1974). The complex formed between RBC–ChE and agent VX does not age significantly (half-life of approximately 48 h) (Sidell and Groff, 1974; Dunn *et al.*, 1997).

A. Direct Nervous System Effects

Although nerve agents exert toxic effects on the CNS and PNS indirectly through AChE inhibition (Koelle, 1975, 1981), nerve agents may also affect nerve impulse transmission by additional mechanisms at neuromuscular junctions (see reviews by Somani and Husain, 2001; Marrs, 2007) and at neurotransmitter receptor sites in the CNS. Rao *et al.* (1987) reported that VX caused an increase in ACh release at neuromuscular junctions in the frog by an interaction with the nicotinic ACh receptor–ion channel complex. Aas *et al.* (1987) reported alterations in muscarinic receptors in rat bronchi and lung tissue after subacute inhalation exposures to agent GD. In the CNS, nerve agents may act directly on muscarinic, nicotinic, and glutamate receptors in manners unrelated to cholinesterase inhibition (Bakry *et al.*, 1988; Chebabo *et al.*, 1999; Lallement *et al.*, 1991a, b; Rocha *et al.*, 1998, 1999). Chebabo *et al.* (1999) reported that 0.3–1 nM of agent GB reduced the amplitude of GABA-mediated postsynaptic currents (GABA; neurotransmitter γ-aminobutyric acid), but had no effect on the amplitude of glutamatergic-mediated postsynaptic currents; this selective reduction in action potential-dependent release of GABA might account for GB-induced seizures. Lallement *et al.* (1991a, b) had earlier suggested that GD-induced overstimulation of glutamatergic receptors contributed to maintenance of seizures.

Although these electrophysiological data indicate that nerve agents may have direct effects on the nervous system unrelated to AChE inhibition, the data do not provide a means of determining a dose conversion to an integrative whole-body endpoint such as lethality or qualitative/quantitative comparisons directly relevant to adverse effects.

It should be further noted that the effects of nerve agents on GABAergic transmission in the CNS may have implications for behavioral effects in laboratory animals and humans, and may also contribute to the induction of convulsions at higher doses (Bakshi *et al.*, 2000). Nevertheless, given the present undefined application of non-cholinergic data to whole-body estimations, reliance on the primary assumption of AChE action is consistent with recognized opinion (Bakshi *et al.*, 2000).

B. Binding with Blood Cholinesterases

The activity of red blood cell cholinesterase (RBC–ChE), as well as that of plasma cholinesterase (plasma–ChE, BuChE or butyrylcholinesterase), has been used to monitor exposure to, and recovery from, anticholinesterase pesticides as well as nerve agents. There is some historical evidence that RBC–ChE can be as sensitive as brain–ChE to the anticholinesterase effects of nerve agents; Grob and Harvey (1958) reported that *in vitro* concentrations producing 50% activity depression of brain–ChE and RBC–ChE were equivalent in the case of GA (1.5×10^{-8} mol/l), and comparable in the case of GB (3.0×10^{-9} vs 3.3×10^{-9} mol/l). The *in vivo* animal studies conducted by Jimmerson *et al.* (1989) disagree, which is further supported by the fact that blood ChE activity may not always be correlated with exposure or with signs and symptoms of toxicity (Holmstedt, 1959; Sidell, 1992, 1997) (Table 6.2). This was also observed during clinical treatment of cases following the Matsumoto and Tokyo chemical terrorist incidents of GB exposure to the public (Yanagisawa *et al.*, 2006; Nozaki *et al.*, 1997).

It is generally considered that systemic effects in humans following acute nerve agent exposures are likely when RBC–ChE is inhibited by 75–80% (e.g. to 20–25% of normal activity levels) (Sidell, 1992). Nevertheless, it is well known that local signs and symptoms of the eye and nose in humans and animals (e.g. miosis, rhinorrhea) can occur in the absence of any measurable change from baseline ChE activity in the blood following vapor or aerosol nerve agent exposure (Harvey, 1952; Craig and Woodson, 1959; Sidell, 1992) and are attributable to the local and direct effects of agent on tissues of the eye and upper respiratory tract (Grob, 1956; Dabisch *et al.*, 2008a) (Table 6.2). When systemic exposure (e.g. other than direct ocular or direct nasal) occurs, miosis and rhinorrhea are not usually observed as first noticeable effects (Dabisch *et al.*, 2008a; NRC, 2003).

EPA science policy guidelines regarding use and application of cholinesterase activity inhibition data generally consider blood ChE activity inhibition to be an imperfect measure, and there appears to be no fixed percentage of blood ChE activity change that can distinguish adverse from nonadverse effects (USEPA, 2000; Storm *et al.*,

2000). A number of investigations have noted the poor association between blood (RBC and plasma) cholinesterase activity and nerve agent intoxication (Koelle, 1994; Sidell, 1992, 1997; Rubin and Goldberg, 1957; Mioduszewski *et al.*, 2002a; Yanagisawa *et al.*, 2006; Cannard, 2006); minimal blood ChE activity has been observed in association with normal tissue function (Sidell, 1992). In a clinical situation, measurement of blood ChE activity has forensic utility and is helpful as a measure of recovery, but is not a quantitative measure of absorbed dose (Cannard, 2006).

C. Binding with Other Enzymes

Nerve agents also interact with detoxification enzymes such as carboxylesterases (CarbE) and A-esterases (e.g. arylesterase and paraoxonase), and the degree of such interaction can alter the magnitude and extent of the toxic cascade following AChE inhibition (Pope, 1999; Pope and Liu, 2002; Fonnum and Sterri, 2006) as well as species-specific characteristics. Observed spontaneous reactivation of soman-inhibited plasma CarbE in the rat indicates that ''aging'' does not occur for the GD–plasma CarbE complex (in contrast to that observed for GD and RBC–ChE; Dunn *et al.*, 1997) and further suggests that endogenous plasma CarbE may be a principal functional scavenger for agent GD (Maxwell and Brecht, 2001). Recent studies indicate that full characterization of the OP-protective capabilities of CarbEs requires assessment not only of the amount, but also of the affinity exhibited by CarbEs for the inhibitor, as well as the total CarbE activity unlikely to be inhibited (inhibitor resistant esterase activity, or IRE) (Chanda *et al.*, 2002). The detoxification potential of CarbEs is multifaceted, and is an area requiring further experimental characterization (Fonnum and Sterri, 2006).

IV. TOXICITY

A. Effects

Nerve agents are toxic anticholinesterase compounds by all routes of exposure, and exhibit a steep dose–response. Detailed descriptions of nerve agent toxicity may be found in reviews by Bakshi *et al.* (2000), NRC (1999, 2003), Mioduszewski *et al.* (1998), Marrs (2007), Opresko *et al.* (1998), Sidell (1997), Somani and Husain (2001), Munro *et al.* (1994), and others.

Anticholinesterase effects of nerve agent exposure can be characterized as muscarinic, nicotinic, or CNS. Muscarinic effects occur in the parasympathetic system and, depending on the amount absorbed, can be expressed as conjunctival congestion, miosis, ciliary spasm, nasal discharge, increased bronchial secretion, bronchoconstriction, anorexia, emesis, abdominal cramps, sweating, diarrhea, salivation, bradycardia, and hypotension. Nicotinic effects are those that occur in somatic (skeletal/motor) and sympathetic systems, and can be expressed as muscle fasciculations and paralysis. CNS effects may be manifested as confusion, reflex loss, anxiety, slurred speech, irritability, forgetfulness, depression, impaired judgment, fatigue, insomnia, depression of central respiratory control, and death (Sidell, 1992, 1997; Sidell and Groff, 1974; Opresko *et al.*, 1998; Bakshi *et al.*, 2000). Minimal effects observed at low concentrations in human subjects include miosis, a feeling of ''tightness'' in the chest, rhinorrhea, and dyspnea (Dunn and Sidell, 1989) (Table 6.2).

While RBC–ChE inhibition in the blood is considered an operationally acceptable surrogate for CNS inhibition, plasma ChE is more labile and is a less reliable reflection of enzyme activity change at neuro-effector sites (USEPA, 2000; Young *et al.*, 1999).

In the whole-body agent vapor exposure studies of Mioduszewski *et al.* (2002a; SD rat single exposures to GB vapor) and Benton *et al.* (2006a; SD rat single exposures to VX vapor), miosis was usually not correlated with or accompanied by reductions of circulating AChE, BuChE, or CarbE. For the VX vapor exposure study of Benton *et al.* (2006a) and among those rats exhibiting only one sign (either whole-blood AChE activity inhibition or miosis), miosis developed in the absence of blood AChE activity depression ''90% of the time''. The findings of Mioduszewski *et al.* (2002a) for SD rats are consistent with those for human volunteers exposed to GB vapor in the study of Rubin and Goldberg (1957). These results further document the fact that miosis alone, and in the absence of signs such as ChE or CarbE activity inhibition, is a local effect, and reflects an exposure much less than that required for generation of systemic clinical effects. Thus, consideration of a local effect such as miosis as a critical endpoint for decision criteria and exposure guideline determination allows a useful margin of protection against the potential for agent exposures sufficiently large so as to generate systemic effects.

B. Minimal Potential for Delayed Neuropathy

A continuing area of public concern regarding nerve agent exposure is the possibility of chronic neurological effects, particularly delayed neuropathy, given that neuropathic effects have been observed following high levels of occupational exposure to the lipophilic agricultural pesticides. Exposure to some OP anticholinesterase compounds results in delayed neurotoxic effects (ataxia, distal neuropathy, paralysis), which are collectively described as organophosphate-ester induced delayed neuropathy (OPIDN). OPIDN is characterized by myelin sheath and axon degeneration and was once thought to be the consequence of inhibition and aging of neuropathy (or neurotoxic) target esterase (NTE) (Abou-Donia, 1993; Ehrich and Jortner, 2002). With greater knowledge and recent data pointing out that NTE-knockout mice may also develop OPIDN

TABLE 6.2. Human experimental data for single exposures to GB vapor[a]

Concentration (mg/m^3)	Exposure duration	Ct ($mg\text{-}min/m^3$)	Signs and symptoms	Reference
0.05	20 min	1	Headache, eye pain, rhinorrhea, tightness in chest, cramps, nausea, malaise	Harvey (1952)
0.05	20 min	1	Threshold (<1 mm pupil diameter decrease) to mild (1–2 mm pupil diameter decrease) miosis[b] in test subjects	Johns (1952)
0.06	20 min	1.2	No reported effects	McKee and Woolcott (1949)
0.06	40 min	2.4	Miosis; slight tightness in chest ($n = 4$)	McKee and Woolcott (1949)
0.3	0.5 min	0.15	Rhinorrhea in 16/16; chest tightness in 7/16	Fairley and Mumford (1948)
0.5	30 min	15.0	Miosis, dyspnea, photophobia, 40% inhibition of RBC–ChE, subclinical SFEMG[c] changes	Baker and Sedgwick (1996)
0.6	1 min	0.6	Miosis and slight tightness in chest	McKee and Woolcott (1949)
2	2 min	4	Miosis ''moderate''; no other signs of ChE inhibition	Rubin *et al.* (1957)
NA	10 min to 5 h	3.13	50% pupil area decrement	Callaway and Dirnhuber (1971)
NA	10 min to 5 h	13.85	90% pupil area decrement	Callaway and Dirnhuber (1971)
4.19 (average)	2 min	8.38	Average 47% inhibition of RBC–ChE; no other effects (breathing rate 5.6–8.4 l/min through nose or mouthpiece)	Oberst *et al.* (1968)
20.7 (average)	2 min	41.4	Average 49% inhibition of RBC–ChE; no other effects (breathing rate 47–65 l/min through nose or mouthpiece)	Oberst *et al.* (1968)
2.8–4.3	1–2.25 min	4.5–5.0	Miosis of unprotected (unbandaged)[d] eyes of 10 military servicemen; min pupil size of 1.8 mm	Sim (1956)
4.0–4.5	2–2.25 min	8.3–9.8	Miosis of unprotected (unbandaged)[d] eyes of 22 military servicemen; min pupil size of 1.6 mm	Sim (1956)
9.5	1 min, 3 s	10	Miosis of unprotected (unbandaged)[d] eyes of 12 military servicemen; min pupil size of 1.7 mm	Sim (1956)
5.5–7.6	1.75–2.5 min	13.1–15.4	Miosis of unprotected (unbandaged)[d] eyes of 54 military servicemen; min pupil size of 1.5 mm	Sim (1956)
12.8–15.3	1–1.2 min	14.4–15.0	Miosis of unprotected (unbandaged)[d] eyes of 38 military servicemen; min pupil size of 1.5 mm	Sim (1956)

[a]Adapted from NRC (2003) with permission by the National Academy of Sciences, courtesy of the National Academies Press, Washington DC
[b]Mild miosis defined by Johns (1952) as ''decrease of 1 to 2 mm'' in pupil diameter; reversible within 24 h.
[c]Single fiber electromyography (SFEMG)
[d]Note that a similar experimental exposure protocol employed by Sim (1956) for subjects with bandaged eyes (''protected'') resulted in no clinical miosis in any subject

(Abou-Donia, 2003; Winrow *et al.*, 2003; O'Callahan, 2003), the NTE theory has been replaced with one involving a noncholinergic, proteolytic mechanism involving cytoskeletal proteins found in neurofilaments (De Wolff *et al.*, 2002). The resulting proteolysis, accompanied by perturbed ionic gradients, cellular edema, and myelin debris, can generate neuropathy.

A number of well-conducted studies employing USEPA guidelines for experimental determination of delayed neurotoxicity (USEPA, 1998) have been performed for the G-agents and agent VX (Gordon *et al.*, 1983; Willems *et al.*, 1984; Goldman *et al.*, 1988; Wilson *et al.*, 1988). The USEPA protocol requires toxicological testing with the domestic hen, an OPIDN-sensitive laboratory animal. In general, exposure to the standard threat nerve agents (e.g. GA, GB, GD, GF, VX) is not considered neuropathic in humans (Marrs, 2007) given that (1) agent VX is not neuropathic in standard challenge tests with hens and (2) G-agent concentrations necessary to induce OPIDN would be supralethal, and human survival would be highly unlikely.

C. Evaluation of Other Potential Effects

Animal data from vapor, oral, and injection exposure studies for the G-series nerve agents and agent VX indicate that these agents do not induce reproductive or developmental effects in mammals (Denk, 1975; LaBorde and Bates, 1986; LaBorde *et al.*, 1996; Bucci *et al.*, 1993; Bates *et al.*, 1990; Schreider *et al.*, 1984, 1988; Goldman *et al.*, 1988; Van Kampen *et al.*, 1970). Incidental data from the Tokyo subway incident (Ohbu *et al.*, 1997) documenting the birth of healthy children to women who had received exposures to toxic GB concentrations at 9–36 weeks' gestation support this finding.

Neither agent GB nor agent VX was genotoxic in a series of microbial and mammalian assays (Goldman *et al.*, 1987, 1988; Crook *et al.*, 1983), while agent GA has been reported to be weakly mutagenic in similar cellular assays (Wilson *et al.*, 1994). Experimental results indicate that agents GB, GA, and VX have no carcinogenic potential (Weimer *et al.*, 1979; Bucci *et al.*, 1992a, b; Goldman *et al.*, 1988).

D. Inhalation/Ocular Toxicity in Human Subjects

It is noted that the most complete experimental data set for the nerve agents evaluated in all species is that for agent GB; the following analysis reflects that emphasis (Table 6.2). Human study reports evaluated have been previously judged by the US Environmental Protection Agency National Advisory Committee for Acute Exposure Guideline Levels for Hazardous Substances and the National Research Council (NRC) Committee on Toxicology to be consistent with acceptable criteria and procedures regarding informed consent and appropriate clinical supervision (NRC, 2001, 2003).

1. Agent GB

Fairley and Mumford (1948) exposed 16 male volunteers to 0.3 mg GB/m^3 for 0.5 min. Nine of the test subjects reported that they could detect the agent by smell; seven reported tightness of the chest and 16 reported rhinorrhea.

McKee and Woolcott (1949) evaluated the effects of low concentrations of agent GB on 14 male volunteers. A single exposure to 0.6 mg GB/m^3 for 1 min, or 0.06 mg GB/m^3 for 40 min resulted in miosis and slight tightness of the chest; within 24 h, signs and symptoms resolved in subjects exposed for 1 min, while more than 48 h was required for resolution in subjects exposed for 40 min.

In a study reported by Harvey (1952), 128 adult male volunteers were exposed in a chamber to GB concentrations ranging from 0.05 to 3.0 mg/m^3 for 2 to 20 min. The corresponding cumulative exposures ranged from 1.0 to 6.0 mg-min/m^3. The most common signs and symptoms resulting from the GB exposures were headaches, eye pain, rhinorrhea, tightness in the chest, cramps, nausea, and concentration difficulties.

When evaluating data from the Harvey (1952) study, Johns (1952) reported on the occurrence of miosis in exposed individuals. Regression analysis of 150 observations, including 55 controls, indicated that the concentration at which a 50% decrease in pupil diameter would be attained was approximately 4.1 mg-min/m^3, with 90% confidence limits of about 2.7 and 5.7 mg-min/m^3. Johns (1952) defined ''mild miosis'' as a ''decrease of 1 to 2 mm'' in pupil diameter, which usually disappeared within 24 h. While mild miosis as defined above was observed in some subjects at the lowest Ct tested (Ct = 1.0 mg-min/m^3), other subjects exhibited mean maximal pupil decreases of <1 mm, indicating attainment of a response threshold at this level of exposure. Untreated controls exhibited a pupil diameter decrease of ≥ 0.33 mm; Johns (1952) attributed this difference to observer bias and pointed out that there was still a relative difference between the control group and the exposed groups.

Oberst *et al.* (1968) conducted inhalation studies in which 125 volunteers were exposed to low concentrations of GB vapor in order to measure levels of GB retention and changes in RBC–ChE activity. In one series of tests in which resting subjects were exposed to GB for 2 min, the calculated retained dose was 3.4–3.8 µg/kg and the percent inhibition of RBC–ChE activity was 39–63% (average 49%). In a second series of tests, in which exercising men were exposed to GB for 2 min, the calculated retained dose was 3.2–4.0 µg/kg and the percent inhibition of RBC–ChE activity was 29–58% (average 47%). The reported 2 min ChE_{50} dose for all 125 subjects (grouped data) was 3.95 µg GB/kg. From these data, the 2 min EC_{50} for cholinesterase inhibition can be estimated as approximately 21 mg/m^3 for resting men breathing about 7 l/min and about 4 mg/m^3 for exercising men breathing about 50 l/min.

Baker and Sedgwick (1996) exposed eight human volunteers to 0.5 mg GB/m^3 for 30 min in a chamber; test subjects walked at a rate of 96 paces per minute while breathing normally. The exposure resulted in a 60% inhibition of RBC–AChE activity; subjects exhibited miosis, some photophobia and mild dyspnea. Respiratory symptoms resolved within minutes and the ocular effects within 48 h post-exposure. There were no clinical neuromuscular signs or symptoms; however, small and non-clinical changes in single fiber electromyography (SFEMG) of the forearm were measured at 3 h and 3 days post-exposure; SFEMG changes were not detectible 15–30 months post-exposure.

The results of agent GB vapor exposure studies conducted with human volunteers indicate that the threshold for miosis and other minimal toxic effects falls in the range of 0.05–0.5 mg/m^3 for 10–30 min exposures (see Table 6.2 and summaries above).

Rubin *et al.* (1957) evaluated the effects of agent GB on the visual threshold of three adult volunteers. The test individuals were exposed to 2 mg GB/m^3 for 2 min with the eyes exposed or protected. With the eyes unprotected, the exposure resulted in moderate miosis with no other obvious signs of cholinesterase activity inhibition, but with a significant elevation of the absolute visual threshold in the dark-adapted eye.

Callaway and Dirnhuber (1971) evaluated the ''miotogenic potency'' of GB vapor in humans (62 miosis responses in 26 human volunteers). Exposure time periods ranged from 10 min to 5 h. Callaway and Dirnhuber reported 50% and 90% decrements in pupil area (Table 6.2). There are acknowledged weaknesses in the protocol and data of Callway and Dirnhuber (1971), such as limited 1970s-era capabilities for measuring agent vapor concentrations, semi-subjective protocols for measuring miosis in human eyes, and incomplete documentation of miosis incidence.

Based on human and animal data, McNamara and Leitnaker (1971) estimated that the EC_{50} for miosis in humans would be 0.0083 mg/m^3 for 8 h exposure duration or 0.0028 mg/m^3 for 24 h exposure duration. McNamara and Leitnaker (1971) did not expect miosis to occur at 0.001 mg/m^3 for 8 h or 0.0003 mg/m^3 for 24 h.

2. Agents VX and V_x

No experimental data are available for direct characterization of acute VX vapor toxicity in humans following inhalation exposure. Based on lethality data for several animal species, Bide and Risk (2000, 2004) estimated the 10 min LCt_{50} value for a VX aerosol to be 7 mg-min/m^3 for a 70 kg man breathing 15 l/min for 10 min.

One of the few experimental attempts to evaluate human exposure to VX vapor for durations greater than a few minutes is the historically important study of Bramwell *et al.* (1963) in which eight individuals were exposed to VX vapor concentrations ranging from 0.23 mg/m^3 to 5 mg VX/m^3 for durations ranging from 2.25 s to 24 min (Cts = 0.7 to 25.6 mg-min/m^3). The Bramwell *et al.* (1963) study is not considered credible because of its seriously flawed exposure protocol; both C and t were varied (resulting in no replicate cumulative exposures), and the organic solvent benzene was used to help disperse the agent in the exposure (carrier solvent may have altered agent absorption) (Reutter *et al.*, 2000).

Koon *et al.* (1959) evaluated the minimum odor detection limits of VX in 16 volunteers. Each subject sniffed the agent both in the morning and afternoon on two successive days (presumably only one sniff at each time point). The estimated total doses for the four exposures ranged from 0.01 to 0.13 μg/kg. No significant changes in RBC or plasma ChE activity were observed in the test subjects. Three subjects reported headaches the evening of the last test, and three other subjects reported slight chest tightness, dryness of the mouth, and nasal irritation for 30 min following the test.

Recent multiservice (Army, Marine Corps, Navy, and Air Force) guidance on agent-specific exposure limits estimates the VX ECt_{50} for mild toxicity in humans (miosis, rhinorrhea) to be 0.10 mg VX-min/m^3 for 2–360 min exposures (DA, 2005). The inhalation/ocular ECt_{50} for severe effects in humans (i.e. muscular weakness, tremors, breathing difficulty, convulsions, paralysis) was estimated to be 10 mg-min/m^3 for 2–360 min exposures for a respiratory minute volume of 15 l/min (DA, 2005).

Agent V_x is considered toxic via inhalation exposure or direct contact with the eye and/or skin (DA 2005), but has been poorly studied. Due to lack of data suitable for analysis, DA (2005) has determined that no toxicity estimates for V_x can be developed at this time.

E. Inhalation/Ocular Toxicity in Laboratory Species

1. G-Series Agents

a. Lethal Levels

There are considerable data on the acute lethality of G-series agents for short-term exposures (Table 6.3; see also NRC (2003) for a detailed review).

In studies conducted by Mioduszewski *et al.* (2001, 2002a), acute lethality of agent GB to male and female SD rats was evaluated for time periods of 10, 30, 60, 90, 240 and 360 min in a whole-body dynamic chamber. GB concentrations ranged from about 2 to 56 mg/m^3, and lethality was assessed at 24 h and at 14 days post-exposure. Female rats were reported to be significantly ($p < 0.01$) more sensitive than males to GB vapor toxicity over the range of exposure concentrations and durations studied.

In studies conducted by Bide and Risk (2004), male CD-1 strain mice were exposed whole body to GB for time periods ranging from 20 to 720 min. LC_{50} values for 3–12 h were progressively higher (toxicity lower) than that predicted by either Haber's rule or the Ten Berge relationship (Ten Berge *et al.*, 1986). In studies conducted by Anthony *et al.* (2004), male and female SD rats were exposed whole

body to agent GF for 10, 60 or 240 min, and lethality was assessed 24 h and 14 days post-exposure (Table 6.3). Females were more sensitive than males.

Hulet *et al.* (2006b) exposed male and female Göttingen minipigs whole body to lethal concentrations of agent GF vapor for 10, 60, or 180 min (Table 6.3). No significant gender differences were observed in the GF lethality values.

In the latter years of WWII, agent GE underwent acute inhalation toxicity characterization at a number of research facilities managed by the Office of Scientific Research and Development (National Defense Research Committee). These results, for which the research protocols and exposure concentrations are not available for comparison, were summarized by Gates and Renshaw (1946) and are provided in Table 6.3 as LCt_{50} values.

b. Sublethal Levels

A consistent endpoint for sublethal effects determination is miosis; this information is summarized in Table 6.4.

Van Helden *et al.* (2003, 2004a, b) exposed male and female marmosets (*Callithrix jacchus*, Harlan, UK) (whole body) to mean GB vapor concentrations of 0.27–0.91 μg/m^3 and male Dunkin–Hartley guinea pigs to 0.02–0.43 μg/m^3 for 5 h. The lowest cumulative exposure at which the internal dose became measurable (based on fluoride-regenerated GB from blood BuChE) was 0.04 ± 0.01 mg-min/m^3 in marmosets and 0.010 ± 0.002 mg-min/m^3 in guinea pigs.

Miosis, EEG effects, and visual evoked response (VER) were examined following 5 h exposures at concentrations ranging from 7.5 to 150 μg GB/m^3. Significant miosis (as measured by the ratio of pupil diameter to iris diameter; $p < 0.05$) was attained for marmosets and guinea pigs (Table 6.4) (Van Helden *et al.*, 2003; 2004a). Significant ($p < 0.05$) threshold change in EEG parameters for marmosets occurred at 0.2 mg-min/m^3, while significant threshold VER changes occurred at 25 mg-min/m^3 (Van Helden *et al.*, 2004b).

Mioduszewski *et al.* (2002a, b) exposed young adult (8–10 week) male and female Sprague–Dawley (SD) rats whole body to GB vapor concentrations of 0.01 to 0.48 mg/m^3 for three exposure periods of 10, 60, and 240 min in a dynamic airflow inhalation chamber. Rat pupil diameters were assessed and blood samples were also collected for RBC–AChE, butyrylcholinesterase (BuChE), and plasma carboxylesterase (CarbE) activity determinations. Animals were also observed for development of clinical signs during a 7-day post-exposure period; EC_{50} values for miosis were reported (Table 6.4; miosis EC_{50} points defined as the statistical concentration required for post-exposure pupil diameters of 50% or less of the pre-exposure pupil diameter in 50% of the exposed population) (Mioduszewski *et al.*, 2002a, b). Gender differences (females more susceptible) were observed. Whole-body exposure to GB vapor did not result in significant activity inhibition for any blood enzyme monitored (RBC–AChE, plasma–BuChE, or CarbE) for any GB vapor concentration and duration tested. Mioduszewski *et al.* (2002a, b) concluded that clinical signs associated with whole-body GB vapor exposure were limited to miosis.

Kassa *et al.* (2001) exposed male albino Wistar rats for 60 min in an inhalation chamber once, or repeatedly to GB concentrations of 0.8, 1.25, or 2.5 mg/m^3. Animals exposed to the lowest concentration (Level 1) were asymptomatic based on clinical and laboratory measurements. Animals exposed to the second concentration (Level 2) were asymptomatic based on clinical signs, but experienced significant inhibition of RBC–AChE activity (by 30%). The highest test concentration (Level 3) was reported to be a nonconvulsive symptomatic exposure. Three months following exposure, control and exposed animals were evaluated for GB-induced effects using biochemical, hematological, neurophysiological, behavioral, and immunotoxicological methods. None of the exposed animals showed any clinical signs of intoxication; their body weight did not differ significantly from control values, and there were no changes in hematological or biochemical parameters, including blood and brain cholinesterase activity. The only significant effect ($p < 0.05$) observed in rats exposed once to 1.25 mg GB/m^3 (Level 2) was an increase in stereotypical behavior. In a continuation of these studies, Kassa *et al.* (2004) reported that at 3 months after exposure, the Level 3 animals showed significant increases in two biochemical markers of stress, plasma corticosterone, and liver tyrosine aminotransferase activities. The latter was also significantly increased in Level 2 test animals. In spatial discrimination tests, animals tested at all three GB concentrations showed significant increases in reaction time up to 2 h after exposures. In the Level 3 animals, the effects lasted for 3 weeks.

Walday *et al.* (1991) exposed male Wistar rats to 0.05 or 0.2 mg GD/m^3 for a single 40 h period. No clinical signs of toxicity were seen during the exposures. AChE, BuChE, and CarbE activities were significantly inhibited in airway and lung tissue at both doses. Brain BuChE and CarbE activity exhibited significant effects at either dose; brain AChE activity did not significantly change from baseline at 0.05 mg GD/m^3, but did so at 0.2 mg GD/m^3.

Genovese *et al.* (2004) evaluated cognitive and general performance effects of GB on adult male SD rats. The test animals received a single whole-body exposure for 60 min once to 1.7–4.0 mg GB/m^3. Cognitive and behavioral performance testing began 48 h after inhalation exposure, and were conducted during 55 sessions occurring over approximately 11 weeks following exposures. Single exposures did not significantly affect performance and no delayed performance onset was observed.

Genovese *et al.* (2008) characterized the miosis EC_{50} for sarin in a nonhuman primate (African green monkey; *Chlorocebus aethiops*) after 10 min exposures (Table 6.4). Evaluation of potential behavioral change by performance on a serial probe recognition test indicated no change from baseline for all subjects. No other clinical sign was observed.

TABLE 6.3. Acute inhalation lethality (LC_{50}; LCt_{50}) for nerve agent vapor in laboratory animals

Agent	Species	LC_{50} (mg/m^3)	LCt_{50} (mg-min/m^3)	Duration (h)	Reference
GB	Rat (f)	18.1	–	0.16[a]	Mioduszewski *et al.* (2001, 2002a)
GB	Rat (m)	22.6	–	0.16[a]	Mioduszewski *et al.* (2001, 2002a)
GB	Rat (f)	8.51	–	0.50[a]	Mioduszewski *et al.* (2001, 2002a)
GB	Rat (m)	8.84	–	0.50[a]	Mioduszewski *et al.* (2001, 2002a)
GB	Rat (f)	6.39	–	1[a]	Mioduszewski *et al.* (2001, 2002a)
GB	Rat (m)	7.55	–	1[a]	Mioduszewski *et al.* (2001, 2002a)
GB	Rat (f)	4.46	–	1.5[a]	Mioduszewski *et al.* (2001, 2002a)
GB	Rat (m)	4.81	–	1.5[a]	Mioduszewski *et al.* (2001, 2002a)
GB	Rat (f)	3.03	–	4[a]	Mioduszewski *et al.* (2001, 2002a)
GB	Rat (m)	4.09	–	4[a]	Mioduszewski *et al.* (2001, 2002a)
GB	Rat (f)	2.63	–	6[a]	Mioduszewski *et al.* (2001, 2002a)
GB	Rat (m)	2.89	–	6[a]	Mioduszewski *et al.* (2001, 2002a)
GB	Mouse (m)	21.5	–	0.33	Bide and Risk (2004)
GB	Mouse (m)	9.0	–	1	Bide and Risk (2004)
GB	Mouse (m)	5.0	–	3	Bide and Risk (2004)
GB	Mouse (m)	3.4	–	6	Bide and Risk (2004)
GB	Mouse (m)	3.1	–	12	Bide and Risk (2004)
GB	Guinea pig (m)	3.99	–	1	Whalley *et al.* (2007)
GE[b]	Rat	–	260 to <350	0.16	Gates and Renshaw (1946)
GE[b]	Mouse	–	245	0.08	Gates and Renshaw (1946)
GE[b]	Mouse	–	330–1,000	0.16	Gates and Renshaw (1946)
GE[b]	Mouse	–	570	0.5	Gates and Renshaw (1946)
GE[b]	Guinea pig	–	>210–1,000	0.16	Gates and Renshaw (1946)
GE[b]	Rabbit	–	230–1,000	0.16	Gates and Renshaw (1946)
GE[b]	Cat	–	170	0.16	Gates and Renshaw (1946)
GE[b]	Dog	–	230	0.16	Gates and Renshaw (1946)
GE[b]	Monkey	–	210	0.16	Gates and Renshaw (1946)
GF	Rat (f)	25.2	–	0.16[a]	Anthony *et al.* (2003, 2004)
GF	Rat (m)	36.9	–	0.16[a]	Anthony *et al.* (2003, 2004)
GF	Rat (f)	5.49	–	1[a]	Anthony *et al.* (2003, 2004)
GF	Rat (m)	6.60	–	1[a]	Anthony *et al.* (2003, 2004)
GF	Rat (f)	2.2	–	6[a]	Anthony *et al.* (2003, 2004)
GF	Rat (m)	2.48	–	6[a]	Anthony *et al.* (2003, 2004)
GF	Minipig (f)	8.69	–	0.16	Hulet *et al.* (2006b)
GF	Minipig (m)	7.25	–	0.16	Hulet *et al.* (2006b)
GF	Minipig (f)	2.12	–	1	Hulet *et al.* (2006b)
GF	Minipig (m)	1.76	–	1	Hulet *et al.* (2006b)
GF	Minipig (f)	0.97	–	3	Hulet *et al.* (2006b)
GF	Minipig (m)	1.01	–	3	Hulet *et al.* (2006b)
VX	Rat (f)	5.44	–	0.16	Benton *et al.* (2006b, 2007)
VX	Rat (m)	4.85	–	0.16	Benton *et al.* (2006b, 2007)
VX	Rat (f)	0.74	–	1	Benton *et al.* (2006b, 2007)
VX	Rat (m)	0.65	–	1	Benton *et al.* (2006b, 2007)
VX	Rat (f)	0.16	–	4	Benton *et al.* (2006b, 2007)
VX	Rat (m)	0.16	–	4	Benton *et al.* (2006b, 2007)

[a]Lethality assessed over 14 days
[b]LCt_{50} values summarized from numerous obscure sources by Gates and Renshaw (1946)
m = male, f = female

TABLE 6.4. Experimental ocular toxicity (miosis) values for G-agents and VX vapor exposures

Species	Toxicity value						Reference
	GB (mg/m^3)	GB (mg-min/m^3)	GD (mg-min/m^3)	GF (mg/m^3)	GF (mg-min/m^3)	VX (mg/m^3)	
Human (10 min–5 h, ECt_{90})	–	13.85	–	–	–	–	Callaway and Dirnhuber (1971)
Human (20 min, ECt_{50})	–	4	–	–	–	–	Johns (1952)
Human (10 min–5 h, ECt_{50})	–	2.33	–	–	–	–	Callaway and Dirnhuber (1971)
Human (20 min, no effect)	–	1.2	–	–	–	–	McKee and Woolcott (1949)
Marmoset (5 h, threshold)	–	2.5	–	–		–	Van Helden *et al.* (2004a)
Rabbit (10 min–5 h, ECt_{50})	–	1.32	0.59	–	0.75[a]	–	Callaway and Dirnhuber (1971)
Rabbit (10 min–5 h, ECt_{90})	–	2.71	2.19	–	1.79[a]	–	Callaway and Dirnhuber (1971)
Guinea pig (5 h, threshold)	–	1.8	–	–	–	–	Van Helden *et al.* (2004a)
Rat (m) (10 min, EC$_{50}$)	0.087	–	–	0.184	–	0.01	Benton *et al.* (2005, 2006a)
Rat (m) (60 min, EC$_{50}$)	0.030	–	–	0.042	–	0.004	Benton *et al.* (2005, 2006a)
Rat (m) (240 min, EC$_{50}$)	0.024	–	–	0.029	–	0.002	Benton *et al.* (2005, 2006a)
Rat (f) (10 min, EC$_{50}$)	0.068	–	–	0.080	–	0.007	Benton *et al.* (2005, 2006a)
Rat (f) (60 min, EC$_{50}$)	0.020	–	–	0.024	–	0.002	Benton *et al.* (2005, 2006a)
Rat (f) (240 min, EC$_{50}$)	0.012	–	–	0.017	–	0.001	Benton *et al.* (2005, 2006a)
Göttingen minipig (m) (10 min, EC$_{50}$)	0.244	–	–	0.161	–	–	Hulet *et al.* (2006a)
Göttingen minipig (m) (60 min, EC$_{50}$)	0.043	–	–	0.047	–	–	Hulet *et al.* (2006a)
Göttingen minipig (m) (180 min, EC$_{50}$)	0.032	–	–	0.022	–	–	Hulet *et al.* (2006a)
Göttingen minipig (f) (10 min, EC$_{50}$)	0.214	–	–	0.190	–	–	Hulet *et al.* (2006a)
Göttingen minipig (f) (60 min, EC$_{50}$)	0.044	–	–	0.058	–	–	Hulet *et al.* (2006a)
Göttingen minipig (f) (180 min, EC$_{50}$)	0.022	–	–	0.037	–	–	Hulet *et al.* (2006a)
African green monkey (f) (10 min, EC$_{50}$)	0.469	–	–	–	–	–	Genovese *et al.* (2008)

[a]Data for T2715 (2-methylcyclohexyl methyphosphonfluoridate) analog for agent GF
m = male, f = female

Genovese *et al.* (2006) also evaluated cognitive and general performance effects of GF on adult male SD rats using the same protocol as that for GB. The test animals were exposed for 60 min to 0, 1.6, 3.7, or 5.2 mg GF/m^3. The highest test concentration resulted in a significant decrease in the response rate in the behavioral task for the first two post-exposure sessions; however, the deficit was not persistent as recovery occurred rapidly. None of the exposures to GF caused a significant effect on completion time in the maze (cognitive) task.

In tests conducted by Allon *et al.* (2005), freely moving male albino SD rats were exposed whole body to 34.2 ± 0.8 µg GB/L for 10 min, after which electrocardiograms (ECG) of exposed and control animals were monitored every 2 weeks for 6 months. One and 6 months post-exposure, rats were challenged with epinephrine under anesthesia and the threshold for cardiac arrhythmia was determined. Surviving, treated, rats displayed agitation, aggression, and weight loss compared to nonexposed rats and about 20% experienced sporadic convulsions. GB-challenged rats with severe signs demonstrated QT segment prolongation during the first 2–3 weeks after exposure. Epinephrine-induced arrhythmias (EPIA) appeared at a significantly lower blood pressure in the treated group in the first month after exposure, and lasted for up to 6 months.

Callaway and Dirnhuber (1971) evaluated the miotogenic potency of GB vapor in rabbits exposed to GB under goggles (43 miosis responses in ten albino rabbits). Exposure time periods ranged from 10 min to 5 h (Table 6.4).

Bartosova-Sevelova and Bajgar (2005) exposed rats to agent GB vapors for 4 h at four different concentrations (0.30, 0.43, 0.58, and 0.82 mg/m^3) in a whole-body exposure chamber. Convulsions and hypersalivation were observed in one animal exposed to 0.82 mg/m^3. There was a significant decrease in blood AChE activity in all but the low-dose test groups and the control. AChE activity in the brain was significantly decreased only in animals exposed to 0.58 mg/m^3, and only in the pontomedullar area. No significant alterations in AChE activity were seen in the frontal cortex or in the basal ganglia. AChE activity in the pontomedulla was lowest at the greatest dose (0.82 mg/m^3), but the data were too variable for statistical significance.

Sekowski *et al.* (2004) evaluated gene and protein level changes in the brain of male and female SD rats exposed to low-level doses (0.004–0.033 mg/m^3) of aerosolized agent GB and GF via whole-body inhalation for 4 h. Preliminary results indicate that exposure to nerve agent results in differential expression of a number of neuronal genes, including a group that affects cellular processes critical to neurological injury and regeneration, and gender-associated differences in the level and type of gene expression response were significant.

Whalley *et al.* (2004) exposed adult male and female SD rats whole body to a series of agent GF vapor concentrations for 10, 60, or 240 min. Miosis (defined as a 50% reduction in pupil area compared to baseline) measured approximately 30 min after exposure indicates that females were significantly more sensitive than males ($p < 0.05$) (Table 6.4).

In studies conducted by Hulet *et al.* (2006a, c, 2007), male and female Göttingen minipigs were exposed whole body to agent GB or GF for 10, 60, or 180 min (Table 6.4). Male minipigs were significantly ($p = 0.022$) more sensitive to the effects of GF exposure than females.

Conn *et al.* (2002) exposed male F344 rats to 0, 0.2, or 0.4 mg GB/m^3 for 1 h/day for one or more days. Animals were maintained at either 25°C or 32°C to evaluate the effects of heat stress. Body temperature and activity were monitored by telemetry continuously during exposure and for one month following the exposures. Although RBC–ChE activity was reduced in the exposed animals (quantitative data not provided), the test protocol did not significantly alter temperature regulation or locomotive activity of the rats.

2. Agent VX

a. Lethal Levels

Benton *et al.* (2006b, 2007) experimentally determined the LCt_{50} and LC_{50} in male and female adult SD rats exposed whole body to VX vapor for 10, 60, and 240 min in a dynamic exposure chamber (Table 6.3); study protocol was similar to that for agent GB in the studies conducted by Mioduszewski *et al.* (2001, 2002a). Experiments testing the role of decontamination less than 24 h post-exposure provided clear evidence for percutaneous toxicity induced by whole-body vapor exposure to the persistent nerve agent VX. For severe and lethal VX vapor exposure effects, females were not more susceptible than males for the exposure durations examined.

Bide and Risk (2000) exposed outbred male CD1 (SD) BR rats, outbred male CD1 (ICR) BR mice, and outbred male (HA) BR guinea pigs to NaCl aerosols containing entrained VX in a nose-only inhalation system for an exposure time of 12 min. Observed LCt_{50} values are summarized in Table 6.3.

Bide and Risk (2000, 2004) also cite several earlier studies in which LCt_{50} values for mice, guinea pigs, rabbits, hamsters, and dogs were reported (Table 6.3).

For exposure to VX vapors, Koon *et al.* (1960) reported 10 min LCt_{50} values for mice exposed either whole body or head only, as well as for goats. Carroll *et al.* (1957) also reported female mouse LCt_{50} values for nose-only and whole-body protocols. However, Carroll *et al.* (1957) reported that the concentration of VX in the exposure chamber was not measured directly but was estimated from the mortality level which was correlated with the LD_{50} for IV injection.

b. Sublethal Level

Benton *et al.* (2005, 2006a, 2007) have characterized miosis as well as severe effects (severe tremors and/or prostration, convulsions and/or gasping) in male and female SD rats exposed whole body to VX vapor (0.00037 to 0.016 mg VX/m^3) under study protocols similar to those for agent GB in

the studies conducted by Mioduszewski *et al.* (2001, 2002a). Miosis EC_{50} endpoints were derived for VX vapor exposure durations of 10, 60 and 240 min (Table 6.4). At the highest VX concentrations tested for each exposure duration, significant (>99.9% confidence) differences between control and experimental whole-blood AChE activity were observed; no other signs (e.g. tremors, salivation, etc.) were observed and delayed pupil effects were minimal. For the miosis endpoint, female rats are considered more susceptible than males to VX vapor exposure.

For severe effects (tremors, prostration, etc.), the ECt_{50} values (mg-min/m^3) reported by Benton *et al.* (2007) were as follows: 10 min, 40.9 (female) and 35.2 (male); 60 min, 30.0 (female) and 31.2 (male); 240 min, 31.5 (female) and 29.9 (male). EC concentrations were not reported.

Following single 60 min VX vapor exposures in the range of 0.016 to 0.45 mg VX/m^3, Genovese *et al.* (2007) examined blood AChE activity, dose estimation by regeneration assay, transient miosis, and behavior parameters in adult male SD rats. Behavioral evaluation included a radial maze task and a variable-interval schedule-of-reinforcement task. At all concentrations tested, transient miosis and AChE activity inhibition were observed and some subjects exhibited transient ataxia and slight tremor. Following 3-month post-exposure evaluations of behavior, the authors concluded that performance deficits were minor and transient at these concentrations. Further, no delayed effects were observed.

V. RISK ASSESSMENT

Application of standard risk assessment methods by numerous authorities and agencies to the toxicological data summarized above has generated exposure guidelines that provide objective and health-based foundations for responsible and efficient response following nerve agent release as well as a basis for site recovery and decontamination decision criteria. The health-based nerve agent exposure guidance summarized here has been derived in an open and transparent manner and judged scientifically valid and protective (NRC, 1999, 2001, 2003; Krewski *et al.*, 2004, Opresko *et al.*, 1998, 2001; Watson *et al.*, 2006a, b; see also www.epa.gov/oppt/aegl/). While initially developed to facilitate disposal of the US stockpile of CW munitions and to support remediation or closure at sites where CWs were historically processed, nerve agent exposure guidance became a subject of interest for homeland defense applications after the events of September 2001.

For reasons described earlier, the air exposure pathway has been a primary focus of risk assessment activity for nerve agents (NRC, 2003; ATSDR, 2007; Cannard, 2006; Dabisch *et al.*, 2008a; others). In situations where long-term agent release is a concern, and where agent residuals may be found, potential exposure to relatively low levels of ingested agent is a priority.

In all cases, it is important to appropriately safeguard public health without defaulting to overly conservative actions (e.g. to ''nondetect'') that would divert limited resources without significant benefit. The following sections summarize toxicological support and developmental rationale for the two primary criteria of interest to community decision-makers managing response to an intentional or accidental release of nerve agent(s) to the environment.

A. Acute Exposure Guideline Levels (AEGLs)

Credible short-term nerve agent exposure limits designed to aid state and local government agencies in developing emergency response plans in the event of accidental or deliberate atmospheric release have been derived. These short-term values have also proved useful in deployed force health protection and in establishing health-based CWA performance goals for detection system development (USACHPPM, 2004, 2008).

Acute Exposure Guideline Levels (AEGLs; expressed in units of mg/m^3 or ppm) are vapor exposure guideline values for numerous hazardous compounds (primarily toxic industrial compounds) that have been published by the National Academy Press (e.g. NRC, 2003, 2007). For each hazardous compound, guideline levels are developed for vapor exposure durations of 10 and 30 min, 1 h, 4 h, and 8 h as well as for three gradations of toxic effect severity. AEGL-1 concentrations are the mildest effect category while AEGL-3 concentrations represent the most severe effect category (NRC, 2001). The point above the AEGL-3 concentration at which ''Level-3'' effects would initiate for any given human exposure duration is not identified in the AEGL assessment protocol.

Typically, the AEGL concentration established for any given effect level is often less than the known experimental concentration at which such toxicological effects occur. This protective nature of the AEGL process and values was demonstrated for each of the nerve agents, where observed human thresholds for reversible effects occur at air concentrations greater than AEGL-1 levels (Watson *et al.*, 2006a, b).

Selection protocols for critical effects and studies, AEGL derivation, time scaling, use and selection of uncertainty and modifying factors, and a description of the lengthy and deliberative review process employed are all described in NRC (2001) as well as in recent papers by Krewski *et al.* (2004) and Bruckner *et al.* (2004). Development of AEGL values includes consideration of uncertainty factors as well as the need for any modifying factors. Because exposure–response data are usually not available for each AEGL-specific exposure duration (NRC, 2001), temporal extrapolation is employed in the development of values for some AEGL-specific time periods. The concentration–exposure time relationship for many systemically acting vapors and gases may be described by $C^n \times t = k$, where the exponent n ranges from 0.8 to 3.5 (Ten Berge *et al.*, 1986;

NRC, 2001). Some investigators refer to the Ten Berge extrapolation as the ''toxic load model'' and *n* as the ''toxic load exponent'' (Dabisch *et al.*, 2008a; Sommerville *et al.*, 2006). The excellent data collected by investigators at Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD) characterizing nerve agent vapor exposure miosis and lethality endpoints for multiple agents and species has allowed agent-specific determination of *n* (summarized in Dabisch *et al.*, 2008a). In the case of swine exposed to G-agents and rats exposed to VX, the experimentally determined *n* values for these endpoints are $\leq$1.6, less than the *n* of 2 assumed during AEGL development for these same compounds in 2001–2003 (NRC, 2003), and based on then-available data for SD rats exposed to agent GB (Mioduszewski *et al.*, 2002a, b). It thus appears that G-agent and VX dose response for the miosis and lethality endpoints are less steep than previously indicated and that the published nerve agent AEGL values (Table 6.5) are more protective than originally considered (NRC, 2003; Watson *et al.*, 2006a, b).

For comparison, it is useful to consider other common guideline sources applicable to short-term nerve agent release events. The US Department of Energy, in their development of 1 h Protective Action Concentrations/Temporary Emergency Exposure Levels (PAC/TEELS), has chosen to replicate the published nerve agent-specific 1 h AEGL-1, -2, and -3 values as Tier 1, Tier 2, and Tier 3 PAC/TEEL values, respectively (see http://hss.energy.gov/HealthSafety/WSHP/chem_safety/teel.html).

1. Application of AEGL Values

The AEGL process does not include specific implementing or application guidance, and specific approaches for using the values are left to the discretion of risk managers and appropriate authorities (NRC, 2001). Nevertheless, AEGL application is already broad, and continues to expand.

The utility of AEGL values for chemical warfare agent emergency preparedness planning was recognized by the Chemical Stockpile Emergency Preparedness Program (CSEPP) when FEMA and Army representatives adopted final nerve agent AEGL concentrations to replace previous agent toxicity criteria for emergency response decision-making (CSEPP, 2003). As of February 2003, standing CSEPP policy guidance for each of the communities hosting agent demilitarization facilities in the US recommends application of AEGL-2 concentrations as the protective action level for evacuation or shelter-in-place, and AEGL-1 concentrations as notification levels (CSEPP, 2003). Since publication of final AEGL levels by NRC (2003) and enactment of the above CSEPP Policy Paper (CSEPP, 2003), multiple stockpile states and counties have incorporated the Policy Paper recommendations into their individual community emergency response plans and employed them in making regulatory decisions permitting agent munition disposal operations (CSEPP, 2006a, b).

In February 2008, the US National Response Team (NRT) posted *Quick Reference Guides* for the G-series nerve agents and VX for public access on its website (www.nrt.org) (NRT, 2008). These *Quick Reference Guides* are useful summaries of agent characteristics and advisories, and are provided as national guidance. While acknowledging that site-specific cleanup decision criteria will be the result of multi-agency agreements and site-specific factors, the NRT considers that attainment of agent-specific air concentrations $<$8 h AEGL-1 is an acceptable criterion for verification of site decontamination.

In general, agent concentrations $<$AEGL 2 are considered to be in a range that poses relatively negligible health consequences for acute exposures.

Other AEGL applications performed or recommended include use as hazard assessment plume modeling criteria for the US Non-Stockpile Chemical Material Program and Homeland Defense scenarios, as testing criteria for personal protective equipment intended for use by first responders in a single weapon-of-mass-destruction scenario, as detection performance goals for advanced equipment acquisition and development, and as a tool for assessing potential exposures during military missions such as peacekeeping (USACHPPM, 2008).

B. Estimated Oral Reference Doses (RfD_e)

Development of nerve agent-specific reference dose estimates is critical to remediation and restoration at existing and closing military sites, which is a priority activity for DOD (Opresko *et al.*, 1998, 2001).

A reference dose (RfD; mg/kg/day) was originally designed for estimating noncancer health risks at CERCLA (Comprehensive Environmental Response, Compensation, and Liability Act of 1980) Superfund sites (USEPA, 1989). As such, it is an essential component of the site risk assessment used to assess potential long-term exposures to contaminated media such as soil, where RfDs address the pathway of incidental ingestion of soil particles (see Dourson, 1994; Cicmanec *et al.*, 1996; Abernathy *et al.*, 2004; USEPA, 1989).

Methods used to derive oral RfDs for nerve agents follow standard USEPA protocols (USEPA, 1989; Dourson, 1994), employ appropriate toxicological data and uncertainty factors, and have undergone review for consistency by the National Research Council (NRC, 1999; Bakshi *et al.*, 2000; Opresko *et al.*, 1998, 2001). Because EPA has not officially verified the derived values for nerve agents, they are identified as estimated RfDs (RfD_e) (Table 6.6).

These criteria are selected by the Office of the Army Surgeon General as the most appropriate oral toxicity reference values for use in environmental risk assessments, and represent the Army's position (Opresko *et al.*, 2001). The RfD_e values have been input to USEPA risk models along with accepted chronic vapor exposure limits (as cited in Watson and Dolislager, 2007) to generate agent-specific

TABLE 6.5. Summary of AEGL values for G-series nerve agents and VX (mg/m^3)[a]

Agent	Classification	10 min	30 min	1 h	4 h	8 h
GA	AEGL-1	0.0069	0.0040	0.0028	0.0014	0.0010
	AEGL-2	0.087	0.050	0.035	0.017	0.013
	AEGL-3	0.76	0.38	0.26	0.14	0.10
GB	AEGL-1	0.0069	0.0040	0.0028	0.0014	0.0010
	AEGL-2	0.087	0.050	0.035	0.017	0.013
	AEGL-3	0.38	0.19	0.13	0.070	0.051
GD	AEGL-1	0.0035	0.0020	0.0014	0.00070	0.00050
	AEGL-2	0.044	0.025	0.018	0.0085	0.0065
	AEGL-3	0.38	0.19	0.13	0.070	0.051
GF	AEGL-1	0.0035	0.0020	0.0014	0.00070	0.00050
	AEGL-2	0.044	0.025	0.018	0.0085	0.0065
	AEGL-3	0.38	0.19	0.13	0.070	0.051
VX	AEGL-1	0.00057	0.00033	0.00017	0.00010	0.000071
	AEGL-2	0.0072	0.0042	0.0029	0.0015	0.0010
	AEGL-3	0.029	0.015	0.010	0.0052	0.0038

[a]Adapted from NRC (2003) with permission by the National Academy of Sciences, courtesy of the National Academies Press, Washington DC

Health Based Environmental Screening Levels (HBESLs). These HBESLs are endorsed by military policy as criteria to assess potentially contaminated soils (Table 6.6) (USACHPPM, 1999; OASA, 1999; Watson and Dolislager, 2007).

VI. TREATMENT

A. Critical Role of Decontamination

Prior to discussion of antidotes and treatment regimens, it is noted that affected individuals should be removed from the site of agent exposure as quickly as possible and undergo rapid decontamination to remove potential for continued personal exposure and to prevent secondary exposure to responders, healthcare workers, medical transport vehicles, and treatment facilities (Okumora *et al.*, 2007, Cannard, 2006; ATSDR, 2007; Sidell, 1997; Pulley and Jones, 2008). Decontamination of CW agents is discussed more fully by Gordon (2009) in a later chapter of this volume.

B. Signs and Symptoms Guiding Medical Management

Depending on concentration and duration of exposure, cases of nerve agent intoxication can exhibit a dose-dependent ''constellation'' of clinical signs and symptoms representing a variety of parasympathetic effects, functional change at neuromuscular junctions, and altered CNS function (Cannard, 2006). As a consequence, critical care personnel and others responsible for developing and administering treatment protocols should take into account the totality of the case presentation. A good example is drawn from observations made by medical personnel treating subway passengers presenting at Tokyo area hospitals and clinics on Day 1 of the sarin release incident. In decreasing order of frequency, the following clinical signs and symptoms were noted: miosis (observed in most patients), headache, dyspnea, nausea, vomiting, muscular weakness, cough, rhinorrhea, chest oppression, muscular fasciculations, and psychological disturbances such as anxiety (Lillibridge, 1995). Similar distributions were observed among subway passengers treated at St Luke's International Hospital (Okumura *et al.*, 1996, 2007) as well as residents affected in the Matsumoto incident of 1994 (Yanagisawa *et al.*, 2006), and are consistent with classic descriptions of nerve agent intoxication (Sidell, 1997; Leikin *et al.*, 2002; Cannard, 2006). Miosis was found to be a more responsive exposure index than RBC–ChE activity inhibition (Nozaki *et al.*, 1997) or serum cholinesterase activity (Yanagisawa *et al.*, 2006) in cases of sarin vapor exposure during the Tokyo and Matsumoto incidents, respectively.

Yanagisawa *et al.* (2006) classified individuals exhibiting a pupil diameter >3.0 mm as without miosis and not affected by nerve agent vapor exposure given that simultaneous serum ChE activity measurements taken during treatment of the Matsumoto cases displayed no inhibition (e.g. largely $\geq 100\%$ of normal serum ChE activity with two cases exhibiting activity $<90\%$ of normal). This, and additional miosis data from Matsumoto, can be employed in determining appropriate treatment for individuals without known or confirmed nerve agent vapor exposure (Yanagisawa *et al.*, 2006; Cannard, 2006); for these individuals, examination, observation without treatment, and discharge are appropriate actions and were successfully implemented during medical responses to the Tokyo subway incident (Lillibridge, 1995).

TABLE 6.6. Estimated reference doses (RfD_e), RfD uncertainty factors, and health-based environmental screening levels for nerve agents[a]

Nerve agent	RfD_e (mg/kg/day)	Composite uncertainty	Residential soil preliminary remediation goal (mg/kg)	Industrial soil preliminary remediation goal (mg/kg)
VX	6E-7	100	0.042 (est.)	1.1 (est.)
GA	4E-5	3,000	2.8 (est.)	68.0 (est.)
GB	2E-5	3,000	1.3 (est.)	32.0 (est.)
GD	4E-6	3,000	0.22 (est.)	5.2 (est.)

[a]From Opresko *et al.* (1998, 2001), USACHPPM (1999), OASA (1999), Watson and Dolislager (2007)

C. Nerve Agent Antidotes

The choice of appropriate treatment for nerve agent intoxication depends on the agent as well as extent and route(s) of exposure. Very mild exposure to nerve agent vapor may necessitate only decontamination and observation; severe exposure to vapor or liquid requires immediate decontamination, antidote administration, artificial respiration, monitoring, and supportive therapy over hours to multiple days (ATSDR, 2007; Sidell, 1997; Vale *et al.*, 2007; Pulley and Jones, 2008). Convenient triage classifications have been developed by ATSDR (2007) in collaboration with the US Army Medical Research Institute of Chemical Defense.

In cases of massive inhalation exposure, immediate care is vital to prevent death from respiratory failure and because the agent–AChE complex becomes resistant to reactivation by oxime-type antidotes. ''Aging'' is compound-specific; aging half-times range from minutes (agent GD) to days (agent VX) (Sidell and Groff, 1974; Sidell, 1997). Standard antidotes clinically available in the US are atropine (anticholinergic) and pralidoxime (Protopam or 2-PAM-Cl). In addition, CNS active drugs such as diazepam (Valium) are strongly recommended if convulsions occur; anticonvulsant treatment is critical for protection against lethality and brain pathology (Shih *et al.*, 2003).

Individuals exposed to vapor and exhibiting miosis only or miosis and rhinorrhea only do not usually require antidote treatment and will resolve without medical intervention (ATSDR, 2007; Cannard, 2006), but should be observed. If rhinorrhea is problematic in these vapor-only cases, ATSDR (2007) advises atropine IM (0.05 mg/kg pediatric; 2.0 mg adult) to relieve signs, followed by patient discharge. If eye pain/headache or nausea is problematic in vapor-only cases, ATSDR (2007) further advises administration of topical atropine or homatotropine to the eye for relief, eye protection from bright light, and discharge.

If liquid/droplet exposure is known or suspected in an individual exhibiting miosis only or miosis and rhinorrhea only, it is recommended that the individual receive no antidote treatment but be closely observed for at least 18 h given that toxic effects of liquid percutaneous exposure may not manifest for several hours (Sidell, 1997; Cannard, 2006). Toxic effects from vapor-only exposure usually occur quickly (within minutes; Sidell, 1997). Current medical management guidelines and recommended medication protocols are summarized in ATSDR (2007), Pulley and Jones (2008) and Vale *et al.* (2007). The ATSDR antidote treatment protocol for civilian emergency management is summarized in Table 6.7.

D. Ongoing Antidote Development

Given that termination of seizure activity protects against development of neuropathological lesions (especially neuronal necrosis) in brain tissues of experimental animals (Marrs and Sellström, 2007; Martin *et al.*, 1985; Shih *et al.*, 2003), focus on anticonvulsant therapy is critical. Reduced potential for permanent brain damage in human cases by preventing or limiting the duration of *status epilepticus* is a primary goal. In challenge tests against multiple LD_{50} doses of agents GA, GB, GD, GF, VX, and VR in guinea pigs, the anticonvulsants midazolam and trihexyphenidyl were more effective than diazepam for seizure control with midazolam the most rapidly effective (Shih *et al.*, 2003). The diazepam pro-drug avizafone is also effective (Lallement *et al.*, 2000, 2004) and is available via autoinjector administration (as is diazepam). For more rapid seizure control during acute treatment phases, Marrs and Sellström (2007) recommend midazolam administered IM due to its more rapid intramuscular absorption.

While pralidoxime is an effective and well-tolerated reactivator, it is not very potent. Search has thus continued for oximes that would combine high reactivator effectiveness against nerve agents with low toxicity and good chemical stability; several promising drugs (the oxime HI6; trimedoxime, or TMB-4; and obidoxime, or LüH-6) have emerged, but are not equally effective against all agents and forms of exposure (Dawson, 1994; Eyer and Worek, 2007; Marrs *et al.*, 2006; Wetherell *et al.*, 2007; Antonijevic and Stojiljkovic, 2007). Marrs *et al.* (2006) point out that, in the absence of pyridostigmine pretreatment, there are at present ''no clinically important differences'' between the standard oxime pralidoxime and the alternative oximes HI-6 and obidoxime in the treatment of nerve agent intoxication.

As a combinatorial drug with atropine, galantamine has been effective and safe in counteracting lethal GD and GB doses in the guinea pig (Albuquerque *et al.*,

2006; Pereira *et al.*, 2008); galantamine also protects against neurodegeneration at $\geq LD_{50}$ doses in the guinea pig, and shows promise as a pretreatment prior to GD or GB exposures.

Lethality was prevented by treatment with nasal atropine (atropine methyl nitrate) and post-exposure treatment with atropine methyl bromide instillation in combination with pulmonary therapeutic surfactants or liquevents in guinea pigs exposed to approximate LC_{50} concentrations of VX aerosol (Nambiar *et al.*, 2007); this concept shows promise for operational application in emergency response.

E. Pretreatment When Exposure is Likely

In certain military deployment settings when threat of nerve agent exposure exists, pretreatment with an anticholinesterase carbamate compound has been fielded to protect personnel. The pretreatment carbamate of current choice, pyridostigmine bromide (PB), reversibly sequesters (and thereby protects) a fraction of AChE from bonding with circulating nerve agents; the carbamate moiety spontaneously hydrolyzes from the AChE molecule within hours and allows AChE to again become available for normal physiological function. Such a pretreatment concept and drug enhances the effectiveness of atropine and oximes in treating lethal doses of GA, GB, and VX (Gall, 1981; Inns and Leadbeater, 1983). US combat units already supplied with atropine and pralidoxime have been equipped with 30 mg PB tablets for oral administration every 8 h; the current maximum pretreatment period is 21 days (Sidell, 1997; Scott, 2007).

A related carbamate, physostigmine, has been shown to protect animals against not only nerve agent lethality, but also incapacitation (Leadbeater *et al.*, 1985). When co-administered with hyoscine, physostigmine effectively reduced incapacitation and prevented death in guinea pigs exposed to agent GD (Wetherell, 1994) and prevents lethality and reduced/prevented incapacitation in guinea pigs exposed to GA, GB, GD, GF, and VX (Wetherell *et al.*, 2002). Transdermal patch administration of physostigmine and hyoscine, or physostigmine alone, in the guinea pig has protected against GD intoxication (Meshulam *et al.*, 1995).

Other developmental pretreatment options include pre-exposure loading with an excess of circulating ChE or BuChE (Van der Schans *et al.*, 2008; Bajgar *et al.*, 2007; Saxena *et al.*, 2008; Lenz *et al.*, 2008; Podoly *et al.*, 2008) or CarbEs (Maxwell *et al.*, 1987) to bind nerve agent before the agent can reach tissue AChE sites.

VII. CONCLUDING REMARKS AND FUTURE DIRECTION

A good deal of nerve agent toxicity research performed and published since the mid-1990s has served to bring a more widespread awareness of the knowledge that detectable (either by means of devices or manifestation of signs) low-level nerve agent exposures under a variety of scenarios are

TABLE 6.7. Recommended antidote protocol for emergency nerve agent exposure therapy[a]

	Antidotes		
Patient age	**Mild/moderate signs and symptoms[b]**	**Severe signs and symptoms[c]**	**Other treatment**
Infant (0–2 yr)	Atropine: 0.05 mg/kg IM or 0.02 mg/kg IV 2-PAM-Cl: 15 mg/kg IV slowly	Atropine: 0.1 mg/kg IM or 0.02 mg/kg IV 2-PAM-Cl: 15 mg/kg IV slowly	Assisted ventilation as needed Repeat atropine (2 mg IM or 1 mg IM for infants) at 5–10 min intervals until secretions have diminished and breathing is comfortable or airway resistance has returned to near normal Phentolamine for 2-PAM induced hypertension (5 mg IV for adults; 1 mg IV for children) Diazepam for convulsions (0.2 to 0.5 mg IV for infants up to 5 yr; 1 mg IV for children >5 yr; 5 mg IV for adults)
Child (2–10 yr)	Atropine: 1.0 mg IM 2-PAM-Cl: 15 mg/kg IV slowly	Atropine: 2.0 mg IM 2-PAM-Cl: 15 mg/kg IV slowly	
Adolescent (>10 yr)	Atropine: 2.0 mg IM; 2-PAM-Cl: 15 mg/kg IV slowly	Atropine: 4.0 mg IM 2-PAM-Cl: 15 mg/kg IV slowly	
Adult	Atropine: 2.0–4.0 mg IM 2-PAM-Cl: 15 mg/kg (1 g) IV slowly	Atropine: 6.0 mg IM 2-PAM-Cl: 15 mg/kg IV slowly	
Elderly, frail	Atropine: 1.0 mg IM 2-PAM-Cl: 5–10 mg/kg IV slowly	Atropine: 2.0 mg IM 2-PAM-Cl: 5–10 mg/kg IV slowly	

[a]Contents reproduced from ATSDR (2007) (public domain)
[b]Mild/moderate signs and symptoms include localized sweating, muscle fasciculations, nausea, vomiting, weakness, dyspnea
[c]Severe signs and symptoms include unconsciousness, convulsions, apnea, flaccid paralysis

both sublethal and also potentially without significant long-term adverse health impact. Although such points are also inherent in the development of LD_{50}s and related values, much of the original focus of nerve agent toxicological work was, not surprisingly, on lethal or severe toxicological endpoints. To the extent that it adds detail and rigor to the estimation of exposure levels for nonlethal effects as well as insightful information on specifics of the mechanisms of nerve agent action, the more recent research has been highly useful in validating past conclusions on the subject. These recent data also further direct attention towards responsible consideration of the consequences for transient presence of residual, low-level agent concentrations in a number of scenarios. Having a transparent, robust and strongly data-based framework within which to evaluate likely consequences of potential low-level nerve agent exposures will greatly aid in planning and evaluating response operations and reducing the magnitude of disruption to affected communities and facilities.

It is hoped that additional species and agents will be evaluated under comparable experimental protocols for the endpoints of miosis and lethality as well as for more intermediate toxicological endpoints.

Standard treatment and pretreatment guidance is also summarized, as well as consideration of novel antidote development and promising approaches. Additional clinical trials will assist in developing protocols and practices for expanding available treatment options. Fielding of improved seizure management drugs and protocols should be expedited. The detoxification potential of CarbEs is multifaceted and is an area that would benefit from further characterization.

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CHAPTER 7

Russian VX

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I. INTRODUCTION

One of the most abundant and most toxic chemical warfare agents in the chemical arsenals of the USA and Russia is VX and Russian VX, respectively, whose development in the middle of the 20th century signified the peak of warfare chemistry. V-gases are low-volatile liquids with high boiling points and, therefore, they are much more persistent than higher volatility organophosphorus (OP) agents of the G-series, such as sarin, soman, or tabun. V-series compounds are more toxic that OP nerve agents of the G-series. For example, in comparison with sarin (GB), VX is estimated to be approximately twice as toxic by inhalation, ten times as toxic by oral administration, and approximately 170 times as toxic after skin exposure (Munro *et al.*, 1994). V-series nerve agents are quite effective when exposed through skin contact, especially as tiny drops, and commonly cause death. Poisoning occurs irrespective of route of exposure; specifically inhalation, ingestion of vaporous and liquid agents through intact or injured skin or eye mucosa, and on contact with contaminated surfaces. This chapter describes in detail the chemistry, analysis, toxicity, monitoring and regulatory hygiene, and therapy of Russian VX nerve agent.

II. BACKGROUND

The arbitrary name VX relates to a group of *O,S*-diesters of methylphosphonic acid $ROPO(CH_3)S(CH_2)_2N(R1)_2$. *O*-Isobutyl *S*-2-(diethylamino)ethyl methylphosphonothioate (R = *i*Bu, R1 = Et), produced since 1972 exclusively in the former Soviet Union, was generally referred to as Russian VX or RVX (CAS #159939-87-4). The synonyms are: VR; VA; phosphonothioic acid methyl-, *S*-[2-(diethylamino)ethyl] *O*-(2-methylpropyl) ester; *O*-isobutyl *S*-2-(diethylamino)ethyl methylthiophosphonate; *O*-isobutyl *S*-(*N,N*-diethylaminoethyl) methylphosphonothioate; and Russian V-gas. The brutto formula of RVX is $C_{11}H_{26}SNPO_2$ (MW 276.37). The structural formula of RVX is presented in Figure 7.1.

RVX is a colorless transparent liquid resembling glycerol in mobility, boiling point 300.0°C, melting point 35.0°C, ρ^{20}1.0083 g/cm^3, and volatility C_{max}^{20} 0.0105 mg/dm^3. The agent is poorly soluble in water (less than 5% at 20°C) and readily soluble in organic solvents. Technical product can be colored from yellow to dark brown and the odor is fried sunflower seeds.

Research into environmental behavior of highly toxic chemicals is an important branch of analytical toxicology. We take the word ''behavior'' to mean persistence, mechanisms of possible transformation, composition of such transformation products and their toxicity. Among known toxicants, V-series compounds, in view of their unique structure, tend to undergo perhaps the most diverse transformations. Possessing a high reactivity on the one hand and polyfunctionality on the other, these compounds incorporated in multicomponent matrices are capable of concurrently reacting with several components. The reactions may involve different active centers in one and the same molecule. The routes and results of such reactions cannot be predicted in advance. The situation is complicated by the fact that active components of a natural or technogenic matrix are not always known. In view of this, research aimed at identifying transformation products of VX in various media is of particular importance. The degradation of such compounds is suggested to be initiated by electron addition to phosphorus via reaction with anionic nucleophiles (Yang, 1999), such as hydroxide ion, water, alcohols, amines, or unsaturated organic compounds. The problem of determination of RVX in complex matrices and identification of RVX transformation products is still more complicated by the scarcity of available reference information.

Cholinesterases (ChE) are well-known targets for organophosphates (OPs), and RVX is no exception. Much less information is available about other enzymes that could be primary targets upon exposure to low doses of OP, and on biochemical markers of possible delayed effects of OP intoxication when the level of ChE activities is the same as the control. However, this problem is very important due to various reasons, among which is fulfillment of chemical weapon agents (CWAs) nonproliferation conventional programs and inherent possibility of accidental exposure of

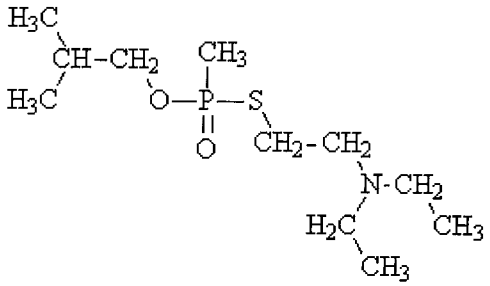


FIGURE 7.1. Structural formula of Russian VX.

personnel to RVX, as well as the chronic effects of subsymptomatic concentrations of RVX that could arise from the stockpiles of chemical agents. Among other reasons, the terrorist threat is a well-recognized problem relevant to the scope of our concern.

A. Ambient Monitoring and Environmental Persistence of Russian VX

The composition of VX degradation products and admixtures has been studied in detail by hybrid chromatography–mass spectrometry methods. The objects of admixture studies were the contents of containers in which VX had been stored for a long time. Dozens of admixtures and stabilizers could be identified. There has been much work on identification of admixtures in VX and its degradation products. A systematic review of VX transformation products is presented by Munro *et al.* (1999). There are no such systematic data for RVX. The degradation of RVX in various media always produces complex mixtures of products that commonly contain dozens of both volatile and nonvolatile organic compounds. The prevalent volatile products are phosphorus-free alkyl diethylaminoethyl mono- and polysulfides. The electron impact (EI) mass spectra of these compounds are quite similar to each other and most commonly contain a single strong peak at *m/z* 86, formed by the $[(Et)_2NCH_2]^+$ ion. Among phosphorus-containing products, methylphosphonic acid (MPA) and its mono- and diisobutyl esters (iBuMPA and iBu_2MPA) are almost always detected in certain quantities. These compounds are also present as admixtures in technical RVX samples in varied contents (from tenths of a percent to several percent). The P–S bond cleavage in RVX forms diethylaminoethanethiol and iBuMPA. The latter slowly hydrolyzes to form MPA. Conditions favoring P–O bond cleavage to form the highly toxic *S*-2-(diethylamino)ethyl methylphosphonothioic acid are unknown.

The most hazardous known VX hydrolysis products are persistent bis[2-(diisopropylamino)ethyl] disulfide, and highly toxic and persistent *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate. The decomposition of RVX gives rise to structural analogs of the above products: bis[2-(diethylamino)ethyl] disulfide and *S*-[2-(diethylamino)ethyl] methylphosphonothioate. The latter is commonly called by its trivial name ''monothiol''. Systematic data on the behavior of these products in the environment, as well as on their acute and chronic toxicity to humans and mammals and ecotoxicity, are lacking. Components of the complex mixture of RXV decomposition products feature as low-informative EI mass spectra and, therefore, these compounds are hardly possible to identify on the basis of mass spectral data solely. Chemical-ionization (CI) mass spectra are much more characteristic, but CI mass spectra, in view of their irreproducibility and lack of databases, are of limited use for identification. Russian VX, like VX, does not possess a strong electron-acceptor center favoring decomposition, but still is not a complete analog of VX in this respect. In dilute aqueous solutions, RVX proved to be much more persistent: half-life 12.4 days against 4.8 days for VX (Crenshaw *et al.*, 2001). The mechanism of the neutralization of VX and RVX with an equimolar amount of water was first described by Yang *et al.* (1996). It was found that autocatalytic hydrolysis is possible exclusively in V-series nerve agents, since it should involve the protonated amino group.

We performed experimental research on stability assessment of RVX and identification of its transformation products under the action of equimolar or excess amounts of water. The method for analysis was GC-EIMS. In a dilute aqueous solution (10 mg/ml) in the presence of 5% phosphoric acid, the concentration of RVX after exposure for 20 days at room temperature without stirring was 2.7 mg/ml or 27% of the initial amount. Since among RVX hydrolysis products both volatile and nonvolatile compounds could be expected, we chose three schemes for sample preparation: (a) evaporation to dryness followed by silylation; (b) organic solvent extraction; and (c) silylation of the extract obtained by procedure (b).

Table 7.1 lists the principal products of RVX hydrolysis with excess water in an acid medium, with specified analytical fractions with the highest contents of each compound. It should be noted that even after 100-day exposure we could detect in the solution 1% of the initial amount of RVX, which implied a fairly uniform hydrolytic degradation.

Experiments on RVX hydrolysis with an equimolar amount of water were performed as follows. A mixture of 74 µl of RVX and 5.6 µl of water was exposed at room temperature for 3.5 months without stirring. An ash-gray thick uniform material formed after hydrolysis and completely dissolved in 5 ml acetonitrile. The solution was diluted 100 times with acetonitrile and analyzed by GC-EIMS (sample D). An aliquot of this sample was mixed with an equal volume of bis-trimethylsilyltrifluoroacetamide (BSTFA), and the mixture was heated at 70°C for 30 min and then analyzed by GC-EIMS (sample E). The autocatalytic hydrolysis of RVX was almost complete by the end of the experiment, since the RVX content in the sample was no more than 0.01%. Qualitatively, the reaction mixture in the latter case was much poorer than in the hydrolysis with

TABLE 7.1. Products of RVX hydrolysis with excess water in an acid medium

RT (min)	Compound	Formula	Fraction	Content in the reaction mixture[a], %[b]
6.709	*N*,*N*-Diethylformamide	$HCONEt_2$	**B**[c]	1
7.480	2-(Diethylamino)ethanethiol	$Et_2NCH_2CH_2SH$	**B**, C	10
7.917	*N*,*N*-Diethylacetamide	$MeCONEt_2$	**B**	<1
10.275	Methylphosphonic acid (as bis(trimethylsilyl) derivative)	$MePO[OSi(Me)_3]_2$	**A**, C	5
11.442	Isobutyl hydrogen methylphosphonate (as TMS derivative)	$CH_3PO(OiBu)OSi(Me)_3$	**A**, C	22
12.217	*O*-Isobutyl *S*-hydrogen methylphosphonothioate (as S-TMS derivative)	$CH_3PO(iBu)SSi(Me)_3$	A, **C**	11
12.674	Diisobutyl methylphosphonate	$MePO(iBuO)_2$	**B**, C	3
16.733	Bis(2-diethylaminoethyl) sulfide	$(Et_2NCH_2CH_2)_2S$	**B**, C	10
18.50	RVX	$MePO(iBu)SCH_2CH_2NEt_2$	**A**, B, C	26
18.898	Bis(2-diethylaminoethyl) disulfide	$(Et_2NCH_2CH_2)_2S_2$	**B**, C	1

[a]Hydrolysis time 20 days
[b]Dozens of minor and insignificant components altogether amounted to close to 10% and are not shown here
[c]Bold = the fraction in which quantitative analysis for the component was performed

excess water. Among the volatile hydrolysis products, the following were detected by GC-EIMS in sample D: 2-(diethylamino)ethanethiol (4%), diisobutyl methylphosphonate (2%), bis[2-(diethylamino)ethyl] sulfide (2%), RVX (<0.01%), and bis[2-(diethylamino)ethyl] disulfide (80%). Of nonvolatile products in sample E, methylphosphonic acid (MPA) (3%) and isobutyl ester of MPA (isobutyl MPA) (96%) were identified as trimethylsilyl ester. Isobutyl MPA which catalyzes RVX degradation was detected as the major components of the reaction mixture. As the second most abundant component we expected, according to Yang *et al.* (1996), 2-(diethylamino)ethanethiol but found that it almost completely converted into bis[2-(diethylamino)ethyl] disulfide. This result seems feasible, since once the autocatalytic degradation of RVX was complete nothing would prevent thiol from being converted into disulfide. In a dilute aqueous solution of RVX, this conversion occurs less rapidly, and even after 100 days the solution contains much less 2-(diethylamino)ethanethiol than bis(2-diethylaminoethyl) disulfide.

The Convention requires Signatory States to completely destroy not only their stockpiled chemical weapons, but also the corresponding industrial facilities, including buildings and other constructions. Toxicity and hazard assessment of demolition wastes of former VX production facilities is quite a challenging problem in view of the high absorption capacity of building materials for VX-series agents. Whether V-series agents are possible to detect in one or another material is difficult to predict because of the paucity of data on their persistence in various media. An ion-trap secondary ionization mass spectrometry study showed that VX undergoes complete degradation when in contact with concrete surfaces (Groenewold *et al.*, 2002). The process follows first- or pseudo-first-order kinetics, and the half-life of VX is about 3 h. Affinities and persistence of VX in certain materials was studied by Love *et al.* (2004). The test materials included powdered activated charcoal, as well as natural iron and aluminum oxyhydroxide minerals: goethite (yellow ochre) and montmorillonite (clay), respectively. It was shown that VX has a high affinity for charcoal, a moderate affinity for montmorillonite, and a very low affinity for goethite. The adsorption on goethite was increased in the presence of dissolved organic matter. VX degraded more rapidly on dry goethite than in the presence of water. We performed experiments with RVX applied on soil, concrete, bricks, and a polymeric material (polyisobutylene used in hydroinsulation) at concentrations over the range 10^{-6}–10^{-5} mg/kg which corresponded to the tentative maximum allowable concentrations accepted in the Russian Federation. Conditions for effective extraction of RVX could not be found for any of the materials tested. The extremely low recoveries of RVX from materials of various nature can be explained by both irreversible sorption and degradation. The problem of simulating the behavior of V-series nerve agents, including RVX, in various materials is complicated by the fact that such factors as the presence of water and/or organic compounds strongly affect both RVX sorption and degradation kinetics. In any case, in alkaline materials (concrete, cement, plaster, or lime) RVX undergoes fast degradation, especially in the presence of water, whereas in organic hydrophobic media (polymers, lubricants) it can persist for a long time.

During operation of an RVX production or destruction facility, its inner surfaces might have been treated with various reagents, and it is therefore quite difficult to predict the transformation routes and degradation products of the toxic agent. These products may include previously unknown toxic compounds. We faced such problems in

assessing toxicities and hazards of samples, specifically wall and ceiling fragments, from a former RVX production facility (Radilov *et al.*, 2007). This research was required for developing technical regulations for demolition of the facility. Table 7.2 lists compounds isolated from the samples of building materials, taken in working areas of the former RVX production facility, which might have contacted RVX. Aqueous and organic extracts of the samples were prepared according to the Organization for the Prohibition of Chemical Weapons (OPCW) Recommended Operational Procedures (Rautio, 1994). Analysis was performed by GC-MS in the electron impact and positive chemical ionization (reactant gas methane) modes. According to the procedures, aliquots of all extracts were subjected to BSTFA silylation and diazomethane methylation.

Along with RVX transformation products, the samples contained more than 100 organic compounds which are matrix components of building materials, solvents, plasticizers, reaction products of degassing agents with matrix components, etc. In brick and concrete samples we detected, in high concentrations (up to 10 g/kg), so-called fyrols, components of foam fire-extinguishing compositions. In terms of chemical composition, fyrols are mixtures of tris(dibromoalkyl) phosphates with phosphoric acid. Among the RVX transformation products in Table 7.2, components with RI 1685, 1888, and 2241 were relatively abundant (up to 5 mg/kg) and were assigned two almost equally probable structures (printed in bold). Such products could not be expected in advance and, as a result, were not detected by target analysis. However, their detection can be considered as evidence that the samples in hand were contaminated with RVX in the past.

B. Biomonitoring and Toxicokinetics of Russian VX

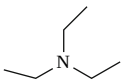
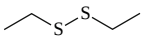
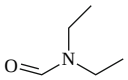
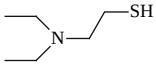
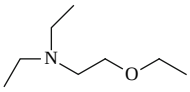
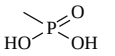
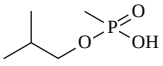
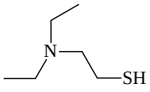
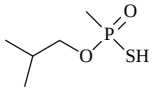
1. GC-MS and HPLC-MS Analyses of Russian VX Metabolites

Identification and quantitative assessment of toxic chemicals and their metabolites in biomedical samples can be performed by the following scenarios: (1) establishment of the fact and factor of exposure of humans and animals to chemical accidents; (2) clinical diagnosis of poisoning; (3) forensic expertise; and (4) biomonitoring of people who deal with highly toxic chemicals. RVX, like other highly toxic readily metabolizing chemicals, is difficult to detect in body fluids and tissues even a very short time after exposure. Successful identification and quantitative assessment of the toxicity factor in biomedical samples is possible under the following conditions (Savelieva *et al.*, 2003): right choice of biological matrix (object for analysis); right choice of a biomarker pertinent to the level and nature of exposure; measurable biochemical or biological effect; availability of a reliable and sufficiently selective and sensitive analytical procedure; availability of reference compounds and criteria for correct data interpretation. Analytical procedures applied to diagnosis and retrospective verification of exposure to OP include (Worek *et al.*, 2005): (1) biochemical determination of ChE activity; (2) identification of unbound OP; (3) identification of decomposition products; (4) fluoride-induced reactivation of inhibited ChE, followed by analysis of the inhibitor; and (5) analysis of phosphyl–protein adducts after tryptic digestion of the protein. The last procedure is regarded to be the most specific and sensitive, but has the drawback of being strongly dependent on the analysis of butyrylcholinesterase (BChE), the most abundant plasma serine esterase with a half-life of about 16 days.

It is well known that hydrolysis is the major metabolism pathway of G-series agents (DeFrank *et al.*, 1993; Beck and Hadad, 2008). The enzymatic hydrolysis of these agents primarily involves phosphorylyl phosphatases and produces *O*-alkyl methylphosphonic acids (*O*-alkylMPA). V-series agents (VX, RVX) are not typical substrates for phosphorylyl phosphatases, which probably explains the higher persistence of these agents in the organism. Free *O*-alkyl methylphosphonic acids formed by hydrolysis of OP agents can be detected in body fluids and tissues and are used as markers of exposure to these agents. The final hydrolysis product is MPA, but its fraction is smaller compared to *O*-alkylMPA. The low-molecular RVX hydrolysis products are actively excreted within the first days after intoxication and can serve as suitable targets for retrospective analysis for no longer than 2–3 weeks. However, 2–3 days after exposure the body levels of *O*-alkylMPA and MPA become much more demanding in terms of detection sensitivity. We have developed a series of procedures for identification and quantitative assessment of *O*-isobutyl MPA as a marker of exposure to RVX, in urine and blood plasma. Characteristics of the procedures are presented in Table 7.3.

The GC-MS procedure involves sample deproteinization, purification, evaporation to dryness, *tert*-butyldimethylsilylation of redissolved dry residue, and GC-EIMS analysis in the SIM mode. The solid-phase microextraction (SPME)-GC-MS procedure involves extraction of *O*-alkyl MPA from urine on microfiber, their *tert*-butyldimethylsilylation directly on microfiber, and thermodesorption of the resulting derivatives in a GC injector. SPME of *O*-alkyl MPA on microfiber is an effective approach for urine, since it allows one to avoid the stage of sample desalination associated with inevitable losses of target compounds. However, SPME is much less effective with plasma because of its high protein content. The HPLC-MS procedure involves sample centrifuging, solid-phase extraction on Diapak C16M cartridges, elution with an acidic (pH 3.0–4.0) methanol, evaporation and redissolution of the eluate, and HPLC-MS analysis in the chemical ionization mode at atmospheric pressure with registration of negative ions (Rodin *et al.*, 2006, 2007). The latter procedure has been used in toxicokinetic experiments for measuring the concentration of *O*-alkyl MPA in rat plasma 1, 6, 24, and 48 h after

TABLE 7.2. RVX-related organic compounds identified in samples of building materials (Radilov *et al.*, 2007)

RT (min)	Compound	Structural formula, molecular weight (MW)	RI
4.19	Triethylamine	 MW 101	
7.35	Diethyl disulfide	 MW 122	929
7.64	*N*,*N*-Diethylformamide	 MW 101	946
7.76	Ethylamine (as TMS derivative)	 MW 45	952
8.44	2-(Diethylamino)ethanethiol	 MW 133	996
8.72	(2-Ethoxyethyl)diethylamine	 MW 145	1014
10.79	Methylphosphonic acid (as bis-TMS derivative)	 MW 96	1147
11.75	Methylphosphonothioic acid (as TMS derivative)	 MW 112 (256)	1210
11.92	Isobutyl hydrogen methylphosphonate (as TMS and M derivatives)	 MW 152	1223
12.46	2-(Diethylamino)ethanethiol (as TMS derivative)	 MW 133	1261
12.58	*O*-Isobutyl *S*-hydrogen methylphosphonothioate (as TMS derivative)	 MW 165 (240)	1269

(*continued*)

TABLE 7.2 (*continued*)

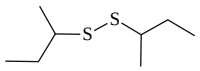
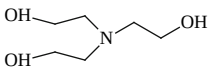
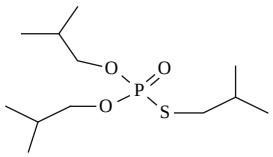
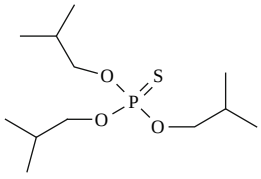
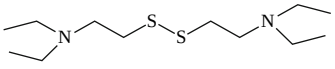
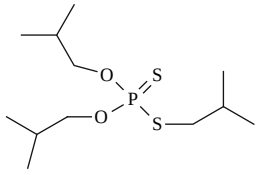
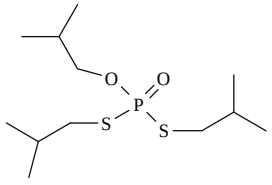
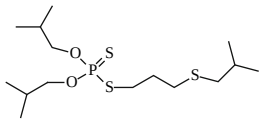
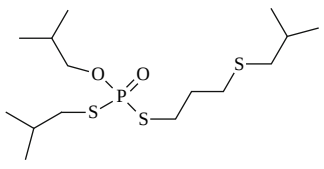
RT (min)	Compound	Structural formula, molecular weight (MW)	RI
12.76	Diisobutyl disulfide	 MW 178	1281
17.19	Tris(2-hydroxyethyl)amine (as TMS derivative)	 MW 149	1635
17.79	*O,O,S*-Triisobutyl phosphorothioate or *O,O,O*-triisobutyl phosphorothioate	 or  MW 282	1685
19.26	Bis(2-diethylaminoethyl) disulfide	 MW 264	1822 1828
19.94	*O,O,S*-Triisobutyl phosphorodithioate or *O,S,S*-triisobutyl phosphorodithioate	 or  MW 298	1888
23.20	*O,O*-Diisobutyl *S*-[(3-isobutylsulfanyl)propyl] phosphorodithioate or *O,S*-Diisobutyl *S*-[(3-isobutylsulfanyl)propyl] phosphorodithioate	 or  MW 372	2241

TABLE 7.3. Characteristics of the procedures for determination of *O*-isobutyl MPA as a marker of exposure to RVX, in urine and blood plasma

Method	Detection limit (ng/cm^3)	Matrix	Total analysis time per sample (h)
GC-MS	10	Plasma	10
SPME-GC-MS	5	Urine	1.5
GC-MS-MS	1	Plasma	10
HPLC-MS	1.0	Plasma	1.5
HPLC- MS-MS	0.8	Plasma	1.5

intramuscular injection of OP agents at a dose of $0.8 \times LD_{50}$ (0.0144 mg/kg). The toxicokinetic curve for *O*-isobutyl MPA is presented in Figure 7.2.

The HPLC-MS (quadrupole analyzer) and HPLC-MS-MS (medium-class ion-trap analyzer) procedures for the determination of OP metabolites in blood plasma were found to be comparable in terms of sensitivity, accuracy, and performance, but HPLC-MS is preferred in terms of availability and cost of equipment and maintenance.

2. MS/MS Analysis of Human Albumin

Albumin has been demonstrated to be an OP hydrolase (Erdos and Boggs, 1961; Sogorb *et al.*, 1998). Bovine albumin binds diisopropylfluorophosphate (DFP) in equimolar proportions (Murachi, 1963), and binding of DFP to Tyr was shown simultaneously (Sanger, 1963). Mass spectrometry identified the active site to be Tyr410 for bovine albumin and Tyr411 for human albumin (Schopfer *et al.*, 2005; Li *et al.*, 2007). A characteristic feature of this Tyr is a low pKa near 8, in contrast to pKa near 10 for other tyrosines (Means and Wu, 1979), which is believed to be due to a pocket with nearby basic residues Arg410 and Lys414 in human albumin (Sugio *et al.*, 1999; Li *et al.*, 2007). Various warfare agents were shown to readily bind to Tyr411, though VX needs more time and a higher concentration for this chemical binding, which is explained by a lower potential of the $SCH_2CH_2N(iPr)_2$ substituent in VX to leave in a noncatalyzed reaction, as compared with F^- or CN^- (Williams *et al.*, 2007).

In order to find out the specificity of RVX binding with albumin, we first obtained a mass spectrum of human albumin after *in vitro* incubation of blood serum of one of the authors (V.B.) with RVX (Figure 7.3). The shift in the spectrum of about 400 Da suggested two to four sites of RVX binding to albumin.

To search for the sites we incubated RVX with commercially available human albumin (Sigma), and then digested it with trypsin to obtain MS/MS peptide spectra. Two sites of binding RVX to human albumin were revealed, one of them being the well-known Tyr411 (not shown here).

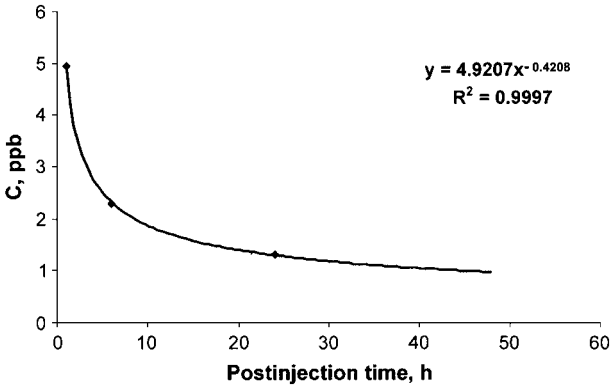


FIGURE 7.2. Concentration of *O*-isobutyl MPA in blood plasma versus time after intramuscular injection of RVX in rats at a dose of $0.8 \times LD_{50}$.

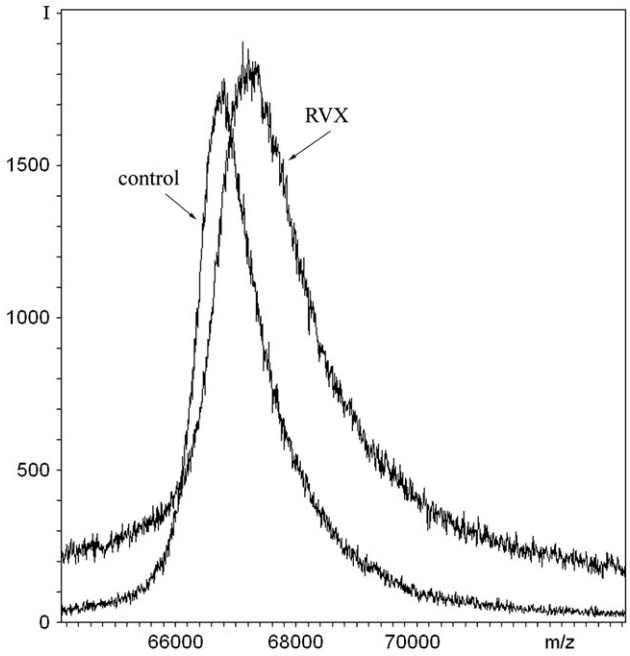


FIGURE 7.3. Mass spectrum of human blood serum albumin in linear mode after *in vitro* incubation with 0.1 mg/ml RVX at 37°C for 1 h.

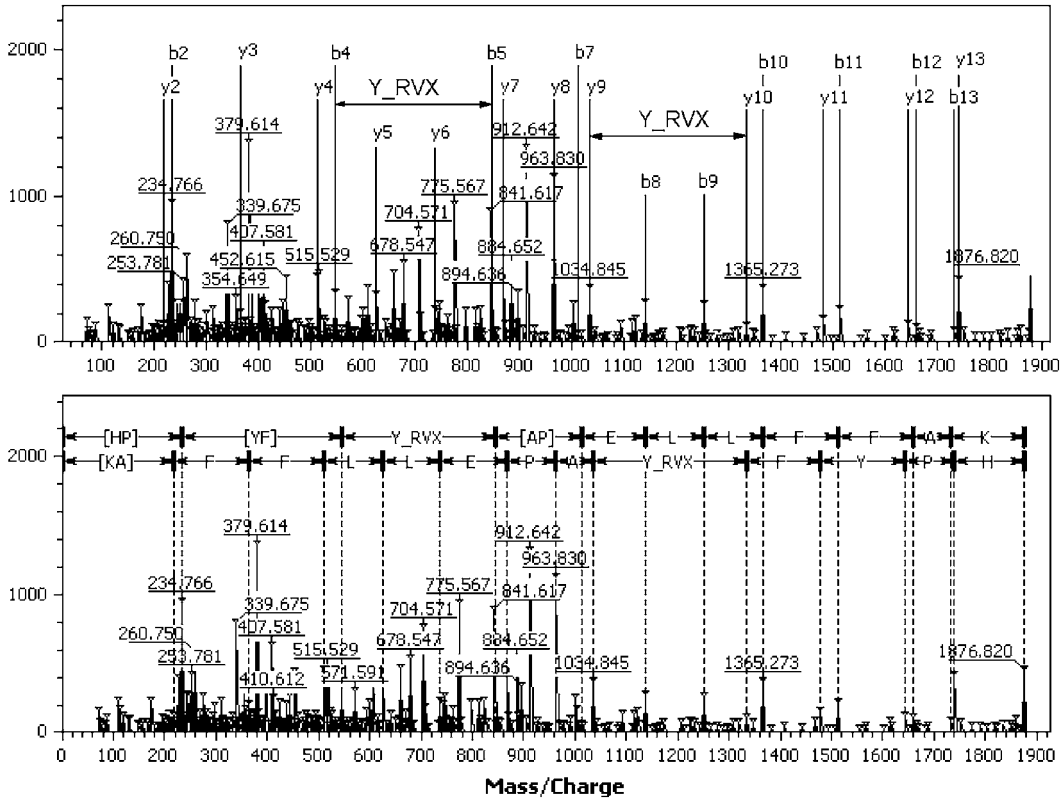


FIGURE 7.4. MS/MS spectrum of peptide HPYFYAPELLFFAK with phosphonylated Tyr150, obtained after trypsinolysis of human albumin incubated *in vitro* with RVX 0.1 mg/ml at 37°C for 1 h. The parent ion has MH+ 1876.820. Ions y and b (up) and deciphered sequence of the peptide (bottom). Tyr150 of the phosphonylated RVX is designated (Y_RVX).

The second site was an unexpected Tyr150 (Figure 7.4). Taking into consideration that 144–160 amino acids in human albumin are RRHPYFYAPELFFAKR (http://myhits.isb-sib.ch/cgi-bin/motif_scan), we suggest that Tyr150 could be activated by positively charged RRH and KR, which could serve to lower its pKa. However, these data and suggestion need *in vivo* experimental evidence.

III. MECHANISMS OF ACTION AND TOXICITY

Three types of damage induced by OPs have been identified: acute poisoning, the so-called intermediate syndrome (IS), and OP-induced delayed polyneuropathy (OPIDP) (Ray, 1998). The IMS symptoms and signs usually occur after apparent recovery from the acute cholinergic syndrome but before OPIDP is developed (Karalliedde *et al.*, 2006). OPIDP also occurs almost exclusively in patients with preceding acute cholinergic toxicity related to severe acute exposure to an OP compound; neuropathy target esterase (NTE) is considered to be the principal molecular target for OPIDP (Lotti, 1991; Ehrich and Jortner, 2001; Lotti and Moretto, 2005). However, there is no data to indicate that VX has any potential at high or low doses for the induction of OPIDP in its classic manifestation in human beings or other species either with acute or long-term exposure. The reason could be that the ability of VX to inhibit NTE is near 1000-fold less than that of GB (Vranken *et al.*, 1982; Gordon *et al.*, 1983). Single intramuscular, injections of VX at 5 × LD_{50} in atropine-protected chickens did not produce inhibition of NTE and histological or behavioral evidence of OPIDP (Wilson *et al.*, 1988a). There was no ability of VX at subchronic exposure (0.04 mg/kg for 90–100 days) to induce OPIDP in antidote-protected chickens (Wilson *et al.*, 1988b). According to other data, NTE activity in brain areas and soleus muscle of rats was significantly depressed after they were subacutely exposed to VX in the absence of supporting therapy for 14 days at doses at the LD_{50} level and higher, and surviving animals exhibited muscle myopathy in the soleus muscle (Lenz *et al.*, 1996). Blood AChE activity was depressed to zero throughout the experiment, so there is no contradiction on the lack of clear OPIDP signs under severe intoxication with VX.

Thus, molecular mechanisms of delayed and chronic effects of VX are still poorly understood, and scientific data on these effects under RVX intoxication is much scarcer. It seems that chronic intoxication should be a separate type of damage with OPs. The problem requires a search for new criteria of intoxication. One should differentiate at least two aspects of this problem: molecular and functional. The former concerns revealing new molecular targets of OP

action. For instance, plasma and liver carboxylesterases (CarbEs) may be more sensitive targets compared to AChE in cases of chronic action of OP low concentrations (Ray and Richards, 2001). Since CarbEs are responsible for the metabolism of a variety of xenobiotics, this fact has been ignored over a long period of time. Another esterase is involved in testosterone biosynthesis metabolizing cholesterin esters in the testis (Jewell and Miller, 1998), one more (surfactant convertase) is also involved in cholesterin metabolism in the lung (Krishnasamy *et al.*, 1997), and a number of brain CarbEs and other hydrolases are also sensitive to OPs (Poulson and Aldridge, 1964; Chemnitius and Zech, 1983; Richards *et al.*, 2000; Nomura *et al.*, 2005). Various OPs are potent inhibitors of fatty acid amide hydrolase and monoacylglycerol lipase (Quistad *et al.*, 2001, 2006), the principal enzymes of the endocannabinoid system.

The second (functional) aspect of this problem concerns the development of pathologic symptoms, the molecular causes of which are not due to AChE inhibition. One of the first cases of OP nonanticholinesterase effects (when the association between the OP molecular target and the functional disturbance has been proved) involved inhibition of kinurenin formamidase of the hen egg yolk sac membrane responsible for teratogenic effects (Seifert and Casida, 1978). Another example of OP teratogenic effect is abnormal development of the conjunctival tissue of *Xenopus* embryos due to inhibition of lysyloxidase and incomplete post-translational modification of collagen (Snawder and Chambers, 1993).

A. Acute Intoxication with RVX

A contributing factor to the high toxicity of VX may be its preferential reaction with AChE. Moreover, unlike the G agents, VX depresses AChE activity significantly more than BChE in humans (Sidell and Groff, 1974); the result is that more VX is available to react specifically with the target enzyme, AChE. At the same time, indirect primary (connected with AChE inhibition) and secondary (not connected with AChE inhibition) effects of RVX have also been described. Development of after-intoxication immunodeficient or immunotoxic states relates to the first group of effects (Germanchuk and Zabrodskii, 2005). In experiments with rats that were administered RVX at a dose $0.75 \times LD_{50}$ a suppression of immune reactions was found: Th-1 cells significantly lost their functions, and T-dependent immune reactions were depressed. In addition, an optimal balance of cAMP and cGMP in lymphocytes necessary for their proliferation and differentiation was disturbed (Zabrodskii *et al.*, 2003, 2007). On the other hand, after a severe intoxication with RVX of laboratory rats ($2 \times LD_{50}$ with therapeutic treatment) there was no after-effects concerning the capability of the central nervous system to produce conditioned reflex reactions, either in early post-intoxication period (2 weeks) or at remote terms (1–6 months) (Novikova *et al.*, 2007). Four different effects of RVX were ascribed to the second group (Prozorovskii and Chepur, 2001): (1) sensitization and desensitization of cholinoceptors to acetylcholine (ACh) that is characteristic for M-cholinoceptors; (2) influence on ACh release by nerve terminals: an inhibition under stimulation of M- and facilitation under stimulation of N-cholinoceptors; (3) direct interaction with cholinoceptors, mainly with nicotinic ones, leading to either their activation or inactivation; and (4) interaction with ionic channels, mainly with that of N-cholinoceptors.

Hypoxic syndrome is one of the principal clinical manifestations under acute intoxication with RVX. It is triggered by disturbance of ventilation, which in its turn is caused by bronchospasm, bronchorrhea, convulsions, and central deregulation of respiration. A series of pathological mechanisms leads to reduction of circulating blood volume, decrease of blood vessel tone, and deregulation of vessel wall permeability. Abnormalities of blood rheology, caused by the loss of body fluids (salivation, bronchorrhea, etc.), and aggregation of blood cells significantly increase dynamic viscosity of blood and aggravate disturbances of microcirculation. Disturbances of oxygen transport and its delivery to tissues induce secondary metabolic disorders and involvement in pathological process of related biological systems which provide oxygen utilization by the tissues (Shestova and Sizova, 2005). Imbalance of electrolytes has also been described: in blood plasma under acute intoxication there was a decrease of sodium and potassium, the latter being more expressed than the former (Rybalko *et al.*, 2005). Indirect secondary effects of OP agents including RVX can be induced by excessive amounts of ACh in blood, with its action on cells having no cholinergic innervations. This can lead to deformation of red blood cells and endothelial cells, activation of basophils, and degranulation of mast cells (Prozorovskii and Chepur, 2001). In this context, one should keep in mind that endothelial cells have all the attributes of autonomic cholinergic regulation. Not only M- but also N-cholinoceptors have been revealed (Hsu *et al.*, 2005), as well as activity of AChE (Carvalho *et al.*, 2005; Santos *et al.*, 2007), the system of synthesis of acetylcholine (choline acetyltransferase), and vesicular system of ACh transport out of the cells (Kirkpatrick *et al.*, 2001, 2003).

B. Chronic and Subchronic Intoxication with RVX, and Delayed Effects

In the context of realization of the conventional programs for chemical weapon (CW) destruction, chronic effects of subsymptomatic concentrations of RVX are of particular interest, since risk of exposure of personnel of the CW destruction facilities to chemical agents cannot be completely ruled out. In addition, members of the rescue service engaged in the decontamination process are regarded as a group at high risk of exposure. The difficulty of diagnosis of delayed effects and chronic intoxication with RVX relates to polymorphism of the clinical manifestations

(Savateev *et al.*, 2001). Long-term monitoring of personnel staff engaged in production of RVX revealed slowly progressing signs of chronic intoxication (Gur'eva *et al.*, 1997). The clinical signs of this may consist of functional and organic disorders of the central nervous system together with vegetative dysregulations, such as peripheral angiodystonic syndrome, vegetosensory polyneuropathy or complex motor-sensory-vegetative pathology of axonopathic or myelinopathic type. Neuromuscular affects, visual and gastrointestinal disorders, immunodeficiency and metabolic disorders have also been described (Gur'eva *et al.*, 1997; Yanno *et al.*, 2000). Cessation of contact with RVX does not lead to involution of the clinical picture of chronic intoxication. Along with nervous, gastrointestinal, and motor disorders, there can be visual and cardiovascular diseases. Moreover, the chronic occupational pathology of the former workers of the facilities may develop in 3–6 years even though they were exposed to no more than tenfold the maximum permissible concentrations of RVX and had no acute intoxications in their anamneses (Filippov *et al.*, 2005).

Experimental results were reported on exposing male and female rats to VX (0.00025, 0.001, or 0.004 mg/kg, s.c.) daily for 30, 60, and 90 days (Goldman *et al.*, 1988). RBC-AChE activity was significantly depressed in rats at all VX doses for 30, 60, and 90 days. BChE was significantly depressed in rats given 0.001 mg/kg VX for 30 days and in both genders of the high-dose group at all exposure periods. No dose-related changes were reported in clinical chemistry and histopathology. The authors concluded that VX exposure sufficient to significantly depress RBC-AChE activity produced no subchronic toxic effects.

Taking into consideration these clinical manifestations of delayed and chronic effects of RVX, an experimental search was undertaken to seek new possible mechanisms of the pathogenesis and novel functional signs of intoxication (Goncharov *et al.*, 2001, 2002, 2003; Mindukshev *et al.*, 2005b). To model chronic intoxication in experiments with animals, RVX was dissolved daily in drinking water to concentrations of 5×10^{-8}, 5×10^{-7}, and 5×10^{-6} g/100 ml. A group of five rats consumed 20 ml of RVX aqueous solution daily. So, during the 3 month test, animals of the first group consumed daily RVX with drinking water in a dose 10^{-5} mg (**I**), animals of the second group 10^{-4} mg (**II**), and those of the third group 10^{-3} mg (**III**) per 1 kg body weight. Measurement of AChE activity in red blood cells (RBC-AChE) was performed by Ellman's method (Ellman *et al.*, 1961). The functional activity of platelets was investigated by a novel method of low angle light scattering that allows all stages of the platelet transformation to be assessed (Mindukshev *et al.*, 2005a, b). Investigation of monosynaptic miotatic reflex and conduction rate through the peripheral nerve fiber was conducted with N. tibialis.

Comparative analysis of biochemical and physiological parameters studied is indicative of the complete absence of significant changes of RBC-AChE activity in rats of all three groups relative to the control, after exposure to the RVX doses given above (not shown here). On the contrary, platelets of test animals exposed to RVX differed from the control by their pronounced instability, an indication of which was development of their spontaneous activation and aggregation (Figure 7.5).

After 3 months of intoxication, kinetic parameters of aggregation – normalized maximal rate U_{max} and effective concentration EC_{50} – were significantly increased (in groups **II** and **III**). Two months after cessation of the chronic intoxication with RVX (rehabilitation period) significant differences of both kinetic parameters were found only for group **III**. Groups **II** and **I** showed significant increases of EC_{50}, but only a tendency ($p < 0.1$) to increase U_{max}. The final estimation of kinetic parameters of aggregation made after 6 months of rehabilitation showed significant decrease of U_{max} in group **III** and increase of EC_{50} in all three

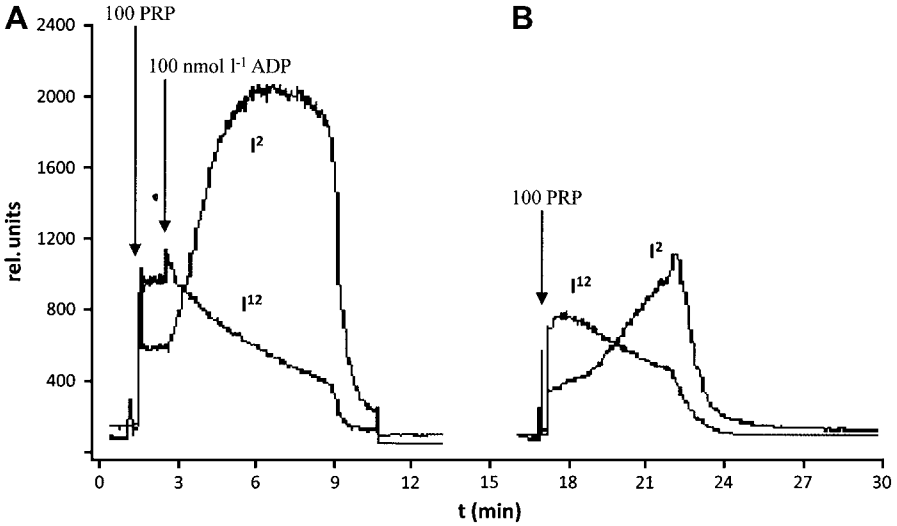


FIGURE 7.5. (A) ADP-induced activation and aggregation of blood platelets of control rats. (B) Spontaneous activation and aggregation of rat blood platelets immediately after 3 months' RVX exposure at a dose of 10^{-4} mg/kg. 100 PRP = 100 µl of Platelet-Rich Plasma; I^{12} and I^{2} indicate low angles of 12° and 2° at which light-scattering was recorded. After Mindukshev *et al.* (2005a). *Spectroscopy Int. J.* **19**: 247–57. Copyright (reprinted with permission from IOS Press).

intoxicated groups (Figure 7.6). Significant increase of U_{max} suggests the sensitization of platelets with the primary activation of signaling ways via protein kinases, the action of which tends to increase the expression of GPIIb/IIIa receptors (Geiger *et al.*, 1994; Shattil *et al.*, 1998). The increase of the EC_{50} parameter with further elevation of U_{max} points to a partial desensitization of $P2X_1$ and $P2Y_1$ receptors, as does the growing activity of the above-mentioned kinases.

Stimulation of the peripheral nerve trunk of intact animals leads to generation of muscle action potentials of three types. According to the duration of latent periods, they fall into the following order: M-response (the result of the direct stimulation of α-motor neuron axons), H-response (the monosynaptic response), and polysynaptic responses with the variable latent period from 8–12 up to about 40 ms. In test animals of the **III** group, the changes of temporal parameters refer mainly to the latent period and duration of M-response (Table 7.4). Polysynaptic responses occur at all intensities of excitation and have a more pronounced character than in intact rats. A marked level and more distinct differentiation of the peaks of the complex action potential were noted.

The results obtained after examining rats of **II** group differ from the control more significantly. Along with normal action potentials (of the ''spike'' type) there were slow waves of depolarization of up to 30 ms duration. Another significant difference is the absence of the ''subthreshold border'', that is, graduation of the increase of the amplitude of the action potential when the irritation stimulus enhances (Figure 7.7). Such an event can usually take place in newborn animals and is caused by slight differentiation of motorneurons (Bursian, 1983). The latent period of M-response, the rate of rising action potentials, and their duration increased significantly. The results of testing the rats of group **I** did not differ from the control. At the same time, some animals had the entire spectrum of pathologic reactions observed in rats of the **II** and **III** groups: fasciculations, the presence of slow (local, depolarized) potentials, and paradoxical discharges. The opposite character of the changes in velocity of nerve impulse conduction within **III** and **II** groups could be caused by different intensity of demyelinization of nerve fibers with different diameters.

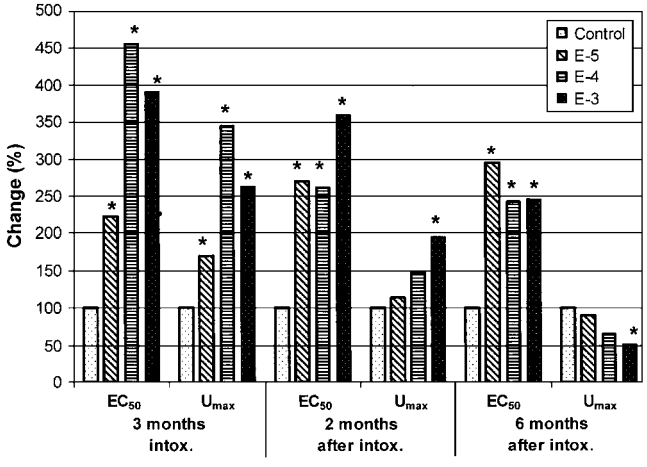


FIGURE 7.6. Kinetic parameters of rat platelet aggregation immediately after 3 months' RVX exposure, and at 2 and 6 months after cessation of the intoxication (* = $p < 0.05$ relative to control). E-5, E-4, and E-3 relate to doses of RVX ($1*10^{-5}$, $1*10^{-4}$, and $1*10^{-3}$ mg/kg) consumed by rats daily with drinking water. After Mindukshev *et al.* (2005b). Reprinted from *Spectroscopy Int. J.* **19**: 247–57. Copyright (reprinted with permission from IOS Press).

Earlier, there were contradictory functional disorders under exposure to OPs described, for which no appropriate interpretations were suggested from the point of view of molecular sources. For example, daily administration of DFP to rats for 20 days at a dose of 17% of LD_{50} caused an increase of velocity of nerve impulse conduction (Anderson and Dunham, 1985), though dimethoate or dichorvos treatment at subsymptomatic doses for at least 8 weeks caused reduction of peripheral nerve conductivity (Desi and Nagimajtenyi, 1999).

C. Studies of Peptide Fractions in Blood Plasma After Subchronic Exposure to RVX

To further clarify the pathogenesis of RVX chronic intoxication, and to seek new approaches to monitoring and estimating the effects of exposure of living organisms to RVX, we focused our efforts on analysis of the peptide fraction of blood plasma. In the past few years, a field of proteomics named peptidomics has been actively developed (Richter *et al.*, 1999; Villanueva *et al.*, 2006). Investigations in this field are focused on biomarkers of various pathological states mainly in oncology, and we have suggested that this scientific methodology could be of value in case of intoxication with OPs. A set of low molecular weight peptides is regarded as a ''molecular imprint'' of the state of an organism, or its molecular ''signature''. The pattern of peptide spectrum (''peptidome'', ''degradome'') reflects the state of certain proteins–enzymes, whose activity or even very existence is extremely hard to measure by direct biochemical methods (Villanueva *et al.*, 2006). With regard to specificity and sensitivity, the most convenient method for peptidome analysis should be mass spectrometry, and when considering a cohort analysis the most appropriate method should be matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS).

The mass spectra analysis of peptides was performed on an Ultraflex-TOF-TOF instrument (Bruker Daltonics, Germany) with a MALDI source equipped with UV laser (337 nm) operated with positive ion detection (*m/z* 700–2,000) and in reflection mode. The spectra were processed using Flex Analysis 2.4 software. A SwisProt database search was performed using the MASCOT program package (Matrix Science, UK). The exact monoisotope masses and

TABLE 7.4. Electrophysiological parameters of the peripheral nervous system of rats after RVX 3 months' chronic exposure

	Control	I	II	III
t_M	1.36 ± 0.29	1.50 ± 0.19	1.72 ± 0.33**	1.07 ± 0.14*
$t_{peak\ M1}$	2.33 ± 0.54	2.79 ± 0.38	3.06 ± 0.42**	1.74 ± 0.44*
T_M	5.02 ± 1.87	6.73 ± 2.26	7.29 ± 1.70**	3.62 ± 0.96*
t_H	4.00 ± 0.41	3.51 ± 0.20	3.78 ± 0.20	4.02 ± 0.58
$t_{peak\ H1}$	4.60 ± 0.74	4.12 ± 0.33	3.95 ± 0.56*	4.35 ± 0.70

Symbols: t_M – M-response latent period, $t_{peak\ M1}$ – latent period of the 1st maximum component of M-response, T_M – M-response duration, t_H – H-response latent period, $t_{peak\ H1}$ – latent period of 1st maximum component of H-response, *differences are significant with $p < 0.05$, **differences are significant with $p < 0.01$

isotope distributions were obtained using the MassPro program, and MS/MS data were represented by means of the Sequence Viewer 2.0 program (Institute of Analytical Instrument Making, Russian Academy of Sciences). For procedure development and testing, the whole venous blood from healthy donors was taken into vacuum tubes charged with EDTA (Becton Dickinson). The low-molecular fraction of rat blood plasma peptides was obtained by precipitating plasma proteins with a 1:2 mixture of acetonitrile and 2% acetic acid for 30 min at 4°C followed by centrifuging at 10,000*g* for 30 min. The precipitate was then dissolved in 0.1% TFA, and the undissolved fraction of proteins unprecipitated at the preceding stage was removed by centrifuging at 10,000*g* for 10 min. The sample was desalinated with a membrane with a cross-linked C18 phase, placed in a microcolumn, after which the peptides retained by the membrane were eluted onto a target with a 60% solution of acetonitrile in 0.1% TFA, containing α-cyano-4-hydroxycinnamic acid (10 mg/ml).

The analysis undertaken revealed that several components of the peptide spectrum grew to become the major ones (Figure 7.8, right). Moreover, during the process of identification all the major peptides were referred to fibrinopeptide A that was truncated at the N-end. A new peptide GEGDFLAEGGGVR (MH^+ 1263.6 Da) came into the picture, which also had a fibrinogenic nature being characterized by the absence of three amino acids as compared to human fibrinopeptide ADSGEGDFLAEGGGVR.

A subchronic intoxication was then conducted by administration of aqueous solutions of RVX at doses $1/100LD_{50}$, given to rats with drinking water every day for 3 weeks. The period of exposure was followed by a 1 month recovery period during which further analyses were carried out. Activities of AChE and BChE in whole blood and plasma were measured according to Ellman *et al.* (1961), and activity of NTE was measured according to Johnson (1969). There were no significant changes in activity of AChE, BChE, and NTE under our experimental conditions (data not shown). Comparing the peptide spectra, we found no qualitative difference between the peptide pools in the mass range of 700–2000 Da. At the same time, several signals which were not notable in the control spectra became major ones in the spectra of intoxicated animals (Figure 7.9, right). Moreover, a new signal arose corresponding to peptide with MH^+ 1452.77 Da, which was absent in control spectra. For every peptide relating to these signals MS/MS analysis was carried out, which revealed that all of them originated from the N-end truncated fibrinopeptide A.

Thus, characteristic changes can be revealed in the low molecular weight fraction of blood plasma, and these could serve as a new molecular marker of intoxication with RVX and other OPs. The peptides of rats and humans begin with Gly from the N-end, and the preceding truncated amino acid is a neutral and hydroxyl-bearing one (Thr in rat fibrinopeptide and Ser in human fibrinopeptide) (Table 7.5).

It seems that under exposure to RVX an inhibition of some exopeptidases (aminopeptidases) takes place, resulting in a shift of the quantitative ratio of the low molecular weight peptide components of blood plasma. Specifically, an enhancement of the fibrinopeptide fragments' signaling occurs as compared to other signals in the spectrum, as well as the appearance of novel peptides related to fibrinogen. Thus, in addition to the above-mentioned peptide with MH^+ 1263.6 Da, another peptide with MH^+ 1616.6 Da emerged in the samples of human blood plasma exposed to RVX. According to the MS/MS analysis this signal was also due to fibrinopeptide A, though phosphorylated at a serine residue. Fibrinopeptide A is a peptide cut off from fibrinogen by thrombin during activation of blood clotting system (Blomback, 1967), and changes in the peptide spectra of fibrinogen origin signify a possible impact of RVX on blood vessels and hemostasis.

In addition, we have discovered another peptide SFSYKPRAPSAEVEMTAYVL that was present in plasma of control rats, but absent in plasma of subchronically intoxicated rats just after intoxication and 1 month after cessation of the subchronic intoxication (Figure 7.10). Identification of the peptide revealed that it was a part of

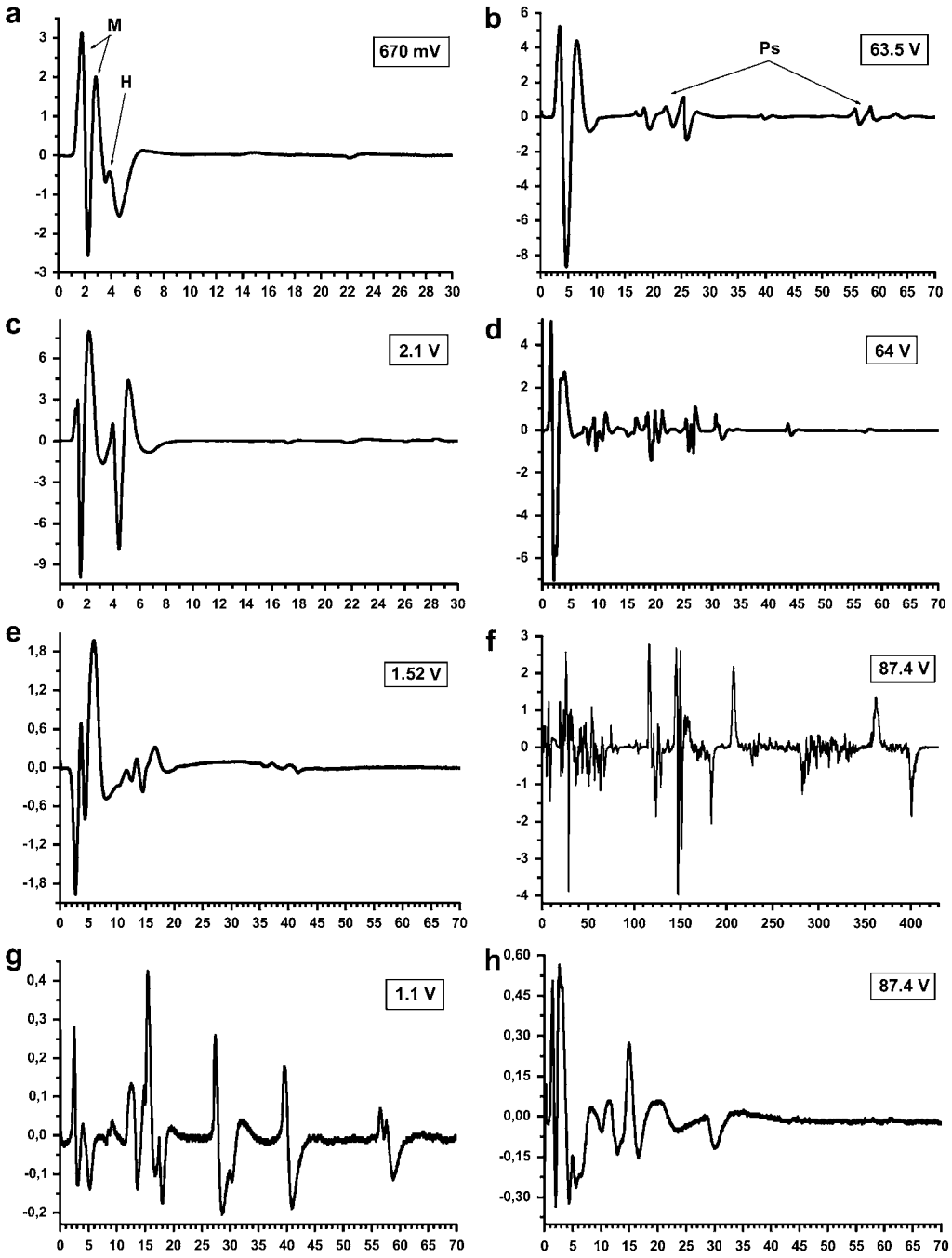


FIGURE 7.7. Action potentials of the rat gastrocnemius muscle under stimulation of N. tibialis: effect of direct stimulation of the muscle (M-response, M), mono- (H-response, H) and polysynaptic (Ps) responses of control rats (a, b), rats of group III (c, d), II (e, f), and I (g, h). Ciphers within frames indicate value of the stimulus. *x*-axis shows time of registration, msec; *y*-axis shows amplitude of action potential, mV.

alpha-1-macroglobulin of rat plasma. Furthermore, some other peptides were found that could arise from chymotryptic activity of proteinases of rat blood plasma and which could be fragments of alpha-1-macroglobulin (not shown here). As for possible sources of chymotryptic activity in blood plasma, several chymotrypsin-like proteinases have been described, the most common of which are chymase and cathepsin G (Schoenberger *et al.*, 1989; Bisaro *et al.*, 2005). Their targets have been shown to be C1-inhibitor, angiotensin I, extracellular matrix, TGF-beta1, IL-1beta, and alpha-2-macroglobulin (Schoenberger *et al.*, 1989; Doggrell and Wanstall, 2004; Bisaro *et al.*, 2005; Bacani and Frishman, 2006). Considering data obtained in experiments with rats, one should keep in mind that alpha-1-macroglobulin is the main macroglobulin in rat blood plasma or serum, its concentration being at the level of 2–4 mg/ml, whereas the concentration of alpha-2-macroglobulin is less than 50 μg/ml (Lonberg-Holm *et al.*, 1987; Sottrup-Jensen, 1989). In human plasma, alpha-1-macroglobulin is completely absent (Lonberg-Holm *et al.*, 1987), but the concentration of human alpha-2-macroglobulin is similar to that of rat alpha-1-macroglobulin, and it has been suggested that these two macroglobulins have similar functions in humans and rats (Tsuji *et al.*, 1994).

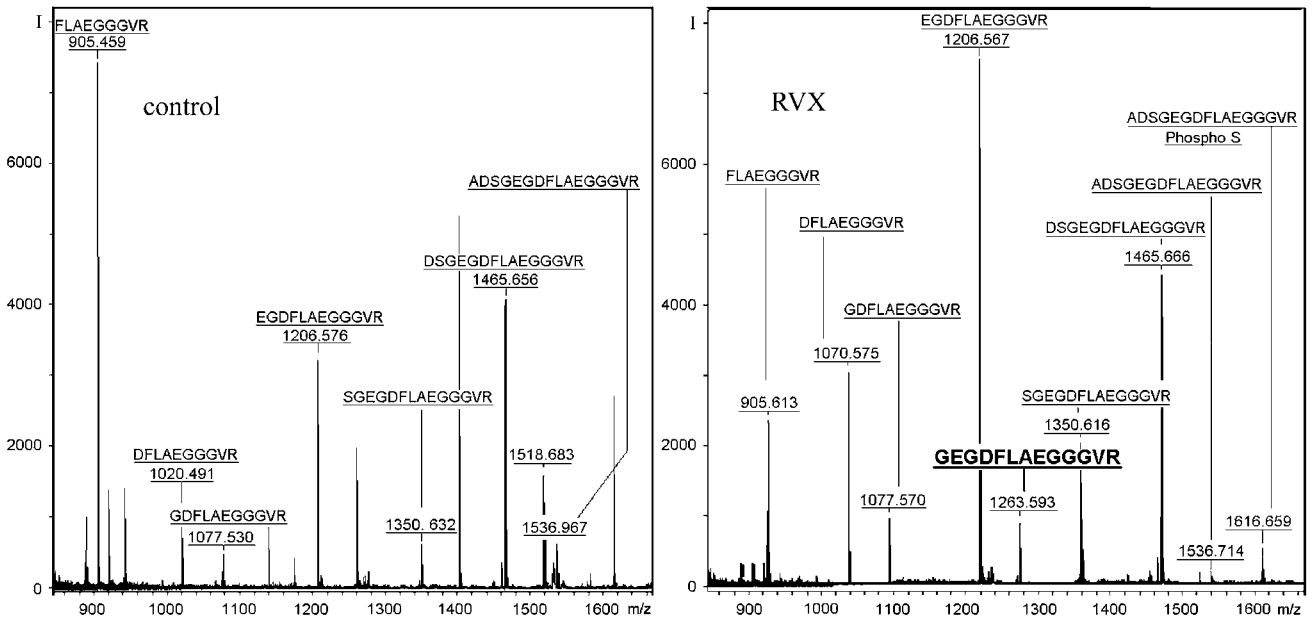


FIGURE 7.8. Mass spectra of peptide fraction of human blood plasma: control (left) and after *in vitro* incubation of whole blood with RVX 0.1 mg/ml at 37°C for 1 h (right).

D. Embryotoxicity, Gonadotoxicity, Mutagenesis, and Carcinogenesis

To study the embryotoxicity of RVX, it was administered to pregnant female rats perorally at a dose $1/100LD_{50}$ (Kiryukhin *et al.*, 2007). RVX had a toxic effect in the females judged by decrease of their RBC-AChE activity; at the same time, AChE activity in tissues of fetoplacental complex complied with age-specific control levels. These results may be regarded as evidence for impossibility or low probability of transplacental transfer of RVX from maternal organism to fetus. However, manifestation of intoxication symptoms by pregnant rats indicates that RVX can induce an embryotoxic effect, which is apparent from disorders of

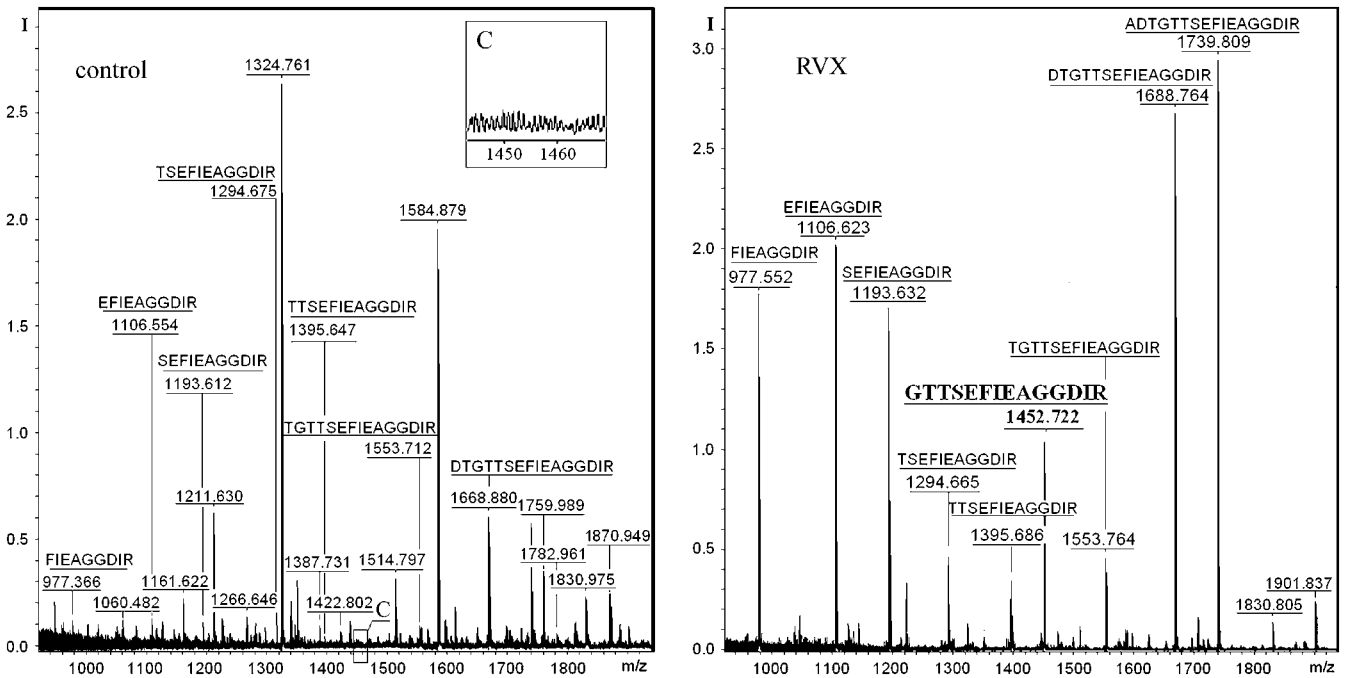


FIGURE 7.9. Mass spectra of peptide fraction of blood plasma of control rats (left) and that of rats after subchronic exposure to RVX (right). Lack of peptide with MH+ 1452.77 Da is demonstrated on the spectrum of control rats (C).

TABLE 7.5. Fragments of fibrinopeptide A found in the peptide fraction of rat blood plasma in control and after subchronic exposure to RVX, and those found in human blood plasma in normal state and after *in vitro* exposure to RVX. Phosphorylated peptide ADTGTTSEFIDEGAGIR (analogous to peptide ADSGEGDFLAEGGGVR found in human blood plasma) was not found in rat blood plasma either in control or after exposure to RVX

Rat				Human			
#	Peptide (MH^+ Da)	Control	RVX	#	Peptide (MH^+ Da)	Control	RVX
1	ADTGTTSEFIDEGAGIR + Phospho ST	–	–	1	ADSGEGDFLAEGGGVR + Phospho ST (1616.6515)	–	+
2	ADTGTTSEFIDEGAGIR (1739.809)	+	+	2	ADSGEGDFLAEGGGVR (1536.6912)	+	+
3	DTGTTSEFIDEGAGIR (1668.772)	+	+	3	DSGEGDFLAEGGGVR (1465.6481)	+	+
4	TGTTSEFIDEGAGIR (1553.745)	+	+	4	SGEGDFLAEGGGVR (1350.6212)	+	+
5	GTTSEFIDEGAGIR(1452.697)	–	+	5	GEGDFLAEGGGVR (1263.5891)	–	+
6	TTSEFIDEGAGIR (1395.676)	+	+	6	EGDFLAEGGGVR (1206.5677)	+	+
7	TSEFIDEGAGIR (1294.628)	+	+	7	GDFLAEGGGVR (1077.5251)	+	+
8	SEFIDEGAGIR (1193.580)	+	+	8	DFLAEGGGVR (1020.5036)	+	+
9	EFIDEGAGIR (1106.548)	+	+	9	FLAEGGGVR (905.4767)	+	+

pre-natal and post-natal ontogenesis (Tochilkina and Kiryukhin, 2007). It should be mentioned that embryotoxicity of VX to rat fetuses was also shown after single LD_{50} doses to the mother (Guittin, 1988), and after repeated doses of 0.005 mg/kg (near $1/5LD_{50}$) at varying times during fetal development (Guittin *et al.*, 1987). On the other hand, no teratogenic potential of VX was found in sheep, rats, and rabbits (Van Kampen *et al.*, 1970; Goldman *et al.*, 1988).

Gonadotoxicity of RVX was investigated in chronic experiments for different ways of introduction. Dermal exposure of male rats to RVX demonstrated that general toxic effects were the governing factor of gonadotoxicity. There were no gonadotoxic effects at the level of threshold dose, which was estimated to be $4*10^{-6}$ mg/kg (Maslennikov and Kiryukhin, 2003). Other research has not revealed changes in weight parameters of left and right testicles and epididymis, nor as their total and specific weight parameters (Shabasheva *et al.*, 2007). It has been concluded that RVX poses no danger concerning development of specific disorders of male reproductive function, and this is in agreement with available data on VX: neither acute nor chronic VX exposure had deleterious effects on reproductive potential (Van Kampen *et al.*, 1970; Goldman *et al.*, 1987).

Studies of mutagenicity of RVX in the Ames test revealed no point mutations in the indicative bacteria. However, studies conducted by a micronuclear test have

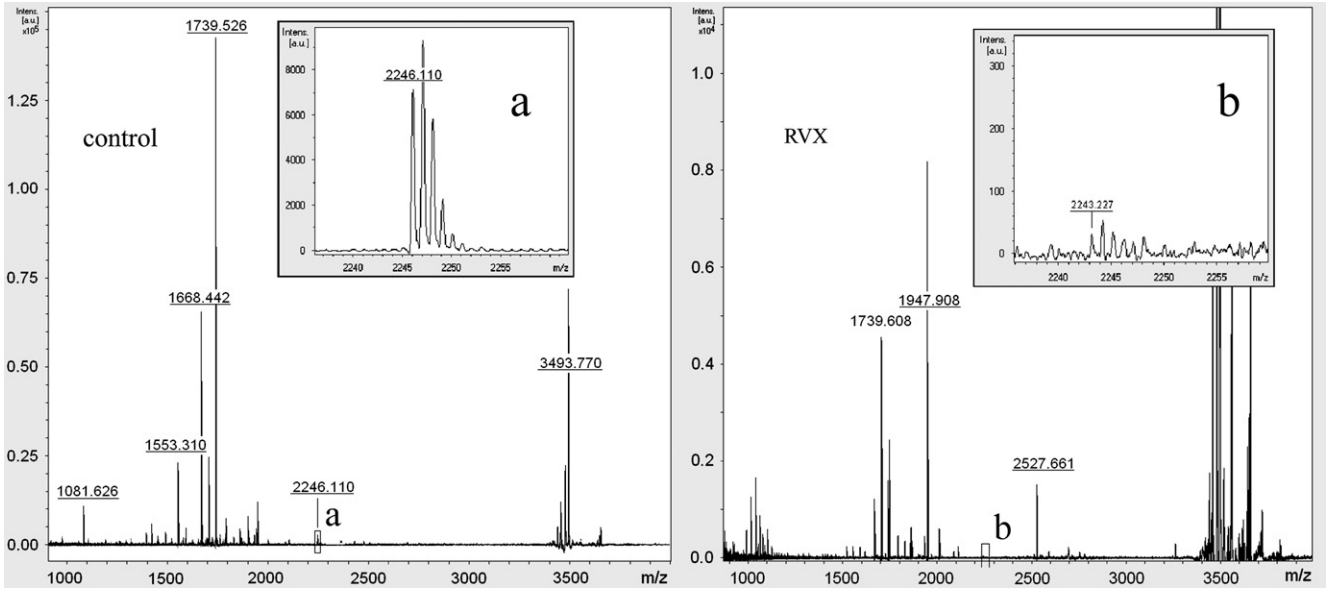


FIGURE 7.10. Peptide SFSYKPRAPSAEVEMTAYVL. (a) Blood plasma of control rats, (b) Blood plasma of rats after subchronic exposure to RVX.

demonstrated that a single intragastric introduction of RVX at a dose $1/10LD_{50}$ stimulated in rats an enhancement of frequency of polychromatophilic erythrocytes with extranuclear inclusions. In the case of absence of the cytotoxic effects, these alterations could be interpreted as being of a mutagenic nature (Maslennikov and Ermilova, 2005). At the same time, the threshold dose of general toxic effect of RVX did not have mutagenic as well as embryotoxic and gonadotoxic effects. Therefore, the delayed effects of RVX can appear at doses exceeding those that induce general toxic effects, indicating nonspecific character of its action.

In studies involving bacteria or yeast, VX was tested both with and without metabolic enzyme activation to determine if VX metabolites might be mutagenic; the results were negative (Crook *et al.*, 1983; Goldman *et al.*, 1988). In addition, negative results were obtained for VX in the *Drosophila* mutagenicity assay (Crook *et al.*, 1983), and VX was considered to be a nonmutagen in a test with mouse lymphoma cells (Goldman *et al.*, 1988).

In the available literature, we could not find experimental data on carcinogenicity of RVX. Nevertheless, epidemiologic data on tumor and pre-tumor diseases of the people who were engaged in production of RVX did not reveal an increase of oncological morbidity as compared to control humans (Fedorchenko *et al.*, 2003). In agreement with this, McNamara *et al.* (1973) reported that there was no increase in cancer in personnel working daily with VX.

IV. TOXICOMETRY AND HYGIENIC REGULATIONS

In countries dealing with destruction of chemical warfare agents, control limits for exposure via surface contact of drinking water are needed, as are detection methods for their low levels in water, soil, or foodstuffs. Some of the toxicity parameters of RVX for humans and animals are available in the text *A Book of Instructions and Technical Documentation on the Problem of Chemical Weapon Destruction* (Anon, 2001), and are given in Table 7.6.

In the event of contact of human skin with the fabric of a protective suit, there have been toxicometric parameters of RVX experimentally estimated with laboratory rats (Zhukov *et al.*, 2007): $LD_{50} = 0.55 \pm 0.09$ mg/kg, or $6.9*10^{-3}$ mg/cm^2; Lim ac (integr.) $= 0.056 \pm 0.001$ mg/kg, or $7.0*10^{-4}$ mg/cm^2; Lim ac (sp) $= 0.0051 \pm 0.001$ mg/kg, or $6.4*10^{-5}$ mg/cm^2; Lim ch $= 4.75*10^{-5}$ mg/kg, or $5.9*10^{-7}$ mg/cm^2. The maximum concentration limit of RVX for the fabric of protective suits has been estimated to be $3.1*10^{-8}$ mg/cm^2, taking into consideration the reserve coefficient, an average body weight, and total area of the skin.

For the purposes of sanitary regulations, it has been estimated that RVX within the range of concentrations 0.01–1.0 mg/l has no negative influence on the natural purification of aquatic reservoirs, on growth and decay of saprophytic and pathogenic microflora, and on nitrification processes; the noneffective dose of RVX has been estimated to be $1*10^{-7}$ mg/kg, the threshold dose $1*10^{-6}$ mg/kg, and the effective dose $1*10^{-5}$ mg/kg (Maslennikov and Ermilova, 2006). Studies on RVX effects on the soil microflora have revealed that actinomyces and micromycetes proved to be the most vulnerable, whereas *Nitrobacteria* was the least vulnerable species (Gorbunova and Maximova, 2003). The hygienic regulations for RVX are presented in Table 7.7.

V. PRINCIPLES OF THERAPY

Acute toxicity of OPs in general and RVX in particular has been much more extensively investigated than chronic toxicity, so it is not surprising that an effective therapy has been developed for acute intoxications only. The most effective antidote complex for treating acute intoxications with RVX consists of an antagonist of M-cholinoceptors, a reversible inhibitor of cholinesterase, and a reactivator of cholinesterase; in addition, anticonvulsants can be used in cases where convulsions occur. In experiments with guinea pigs, they were pretreated with pyridostigmine (0.026 mg/kg, i.m.), then immediately after RVX intoxication ($2 \times LD_{50}$) animals were given pralidoxime chloride (25 mg/kg, i.m.) and atropine sulfate (2, 8, or 16 mg/kg, i.m.); diazepam (5 mg/kg, i.m.) was administered to animals that displayed seizures and convulsions (Chang *et al.*, 2002). It was also

TABLE 7.6. Parameters of RVX toxicity for animals and humans

Parameter	Route of exposure	Dose
LCt_{50} (human)	Inhalation	0.04 mg/min/l
CL_{50} (mouse)	Inhalation	$1.8–4.5*10^{-5}$ mg/kg
LCt_{50} (mouse)	Inhalation	0.011 mg/min/l
LD_{50} (rabbit)	Percutaneous	0.014 mg/kg
LD_{50} (cat)	Percutaneous	0.01 mg/kg
LD_{50} (dog)	Percutaneous	0.0157 mg/kg
LD_{50} (mice)	Percutaneous	0.016 mg/kg
LD_{50} (human)	Percutaneous	0.1–0.01 mg/kg

TABLE 7.7. RVX safety standards in the Russian Federation (Uiba *et al.*, 2007)

MAC for working air (mg/m^3)	$5*10^{-6}$
MAC for reservoir water (mg/m^3)	$2*10^{-6}$
ASEL for ambient air (mg/m^3)	$5*10^{-8}$
MPL for equipment surface (mg/dm^2)	$2*10^{-6}$
MPL for human skin (mg/dm^2)	$3*10^{-8}$
MAC for soil (mg/kg)	$5*10^{-5}$
MEL for ambient air (mg/m^3)	
after 1 h	$1.6*10^{-5}$
after 4 h	$4.1*10^{-6}$
after 8 h	$2.0*10^{-6}$
after 24 h	$6.6*10^{-7}$

Abbreviations: MAC – Maximal Allowable Concentration
ASEL – Approximate Safety Exposure Level
MPL – Maximal Permissible Level of Contamination
MEL – Maximal Emergency Level

shown that in case of acute intoxication with RVX, higher doses of atropine should be administered as compared to those for VX, and of several oximes (pralidoxime, obidoxime, HI-6) the most effective was HI-6, as it was in the case of VX, sarin, cyclosarin, or soman poisonings (Kassa *et al.*, 2006).

Looking for therapy to treating chronic low-dose intoxication, one should keep in mind that the VX-AChE complex has been found to undergo a significant degree of spontaneous reactivation in humans (at a rate of about 1%/h over the first 70 h after i.v. administration of VX); another feature of VX toxicity is the lack of aging or stabilization of the VX-AChE complex and the relative ease of reactivation of VX-poisoned enzyme by oxime antidotes in humans (Sidell and Groff, 1974). Because there are no major differences in the reactivation process of both VX and RVX-inhibited cholinesterase (Kuca *et al.*, 2006), natural inactivation with plasma and liver paraoxonase (PON1) of RVX, VX, and other warfare agents could play a bigger role in prophylactic therapy of acute and chronic intoxications and their delayed effects (Li *et al.*, 1993; Costa *et al.*, 2005). PON1 is a catalytic bioscavenger, in contrast to stoichiometric bioscavenger BChE. A constituent of red wine, resveratrol, has been shown to be a natural and rather effective up-regulator of PON1, and it has good potential for protecting living cells against chemical warfare agents (Curtin *et al.*, 2008).

VI. CONCLUDING REMARKS AND FUTURE DIRECTION

The ambient monitoring and biomonitoring of RVX and its destruction products within the areas of chemical weapon storage and destruction facilities is an important task for the State Sanitary Inspection Units of the Russian Federation. In this regard, development of chemical analytical and bioanalytical methods and procedures should be among the main purposes of applied sciences. At the same time, estimation of probability of exposure to RVX and other warfare agents calls for fundamental problems of molecular and functional diagnostics of the pathogenesis to be solved, as well as development of effective therapy or prophylaxis.

In this chapter, we presented the principal products of RVX hydrolysis with excess water in an acid medium. It has been shown that after the 3 months' exposure, close to 1% of the initial amount of RVX in the solution could be detected. According to data on RVX hydrolysis with an equimolar amount of water, the autocatalytic hydrolysis of RVX was almost completed after 3 months, since the RVX content in the sample was no more than 0.01%. Moreover, the reaction mixture was much poorer than in the case of hydrolysis with excess water. Isobutyl MPA, which catalyzes RVX degradation, was detected as the major component of the reaction mixture. Yang *et al.* (1996) found 2-(diethyl amino)ethanethiol as the second major component of the mixture, but in our experiment it was almost completely converted into bis[2-(diethylamino)ethyl]disulfide.

We have not found conditions for effective extraction of RVX in experiments with its application on soil, concrete, bricks, and polyisobutylene at concentrations of 10^{-6}–10^{-5} mg/kg. The extremely low recoveries of RVX from materials of various natures can be explained by both irreversible sorption and degradation. A summarized list of compounds isolated from samples of building materials was presented, which were taken from some areas of a former RVX production facility and might have contacted RVX. RVX degradation products were found, along with organic components of building materials, solvents, plasticizers, reaction products of degassing agents with matrix components, etc. High concentrations of fyrols were detected in some brick and concrete samples.

Procedures for identification and quantitative assessment of *O*-isobutyl MPA (a marker of exposure to RVX) in urine and blood plasma have been developed, and toxicokinetic experiments conducted to measure *O*-alkyl MPA in rat plasma after intramuscular injection of RVX at a dose of $0.8 \times LD_{50}$. In addition, we have obtained a mass spectrum of human albumin after *in vitro* incubation of blood serum with RVX. Incubation of commercially available human albumin with RVX with subsequent trypsinolysis and MS/MS analysis has revealed two sites of binding RVX to human albumin, Tyr 411 and Tyr150. These data could contribute to development of sensitive and specific diagnostic methods.

Having analyzed our own experimental data and available scientific literature we came to the conclusion that the toxicological features of RVX were not properly studied. On the one hand, pathogenesis of acute intoxication has been described, and toxicometric parameters and hygienic regulations have been developed. On the other hand, mechanisms of nonspecific effects and chronic intoxication, and the pathogenesis of delayed manifestations, need further

clarification. This is why there is a lack of effective prophylactic and therapeutic means of treating the delayed effects of RVX (and many other OPs) that cannot be reduced to development of OPIDP. The search for non-cholinesterase targets is still far from its final stage and not all the functional consequences of an OP exposure have been revealed. Various OPs can covalently bind to tyrosine residues of tubulin, near the GTP binding sites or within loops that interact laterally with protofilaments (Grigoryan *et al.*, 2008), and this could partly explain an impairment of fast axonal transport and neuropsycological effects of low-dose chronic exposure to OPs (Stephens *et al.*, 1995; Terry *et al.*, 2007). Previously, assumptions have been made about a direct influence of OPs on the humoral components of hemostasis and the complement system in terms of abundance of serine proteases in these systems (O'Neill, 1981; Ray, 1998). An interrelation of hemostasis and neuromuscular conduction is of undoubted interest considering the low percentage of endoneural capillaries in the structure of nerve fibers, and dependence of the trophism of any tissue upon the integrity of the endothelial monolayer and upon adequate functioning of hemostatic mechanisms. An idea about dependence of the trophism of nerve fibers upon the vascular bed comes from data on their ratio: the volume of capillaries amounts to just 2–4% of the total volume of nerve fiber (Odman *et al.*, 1987). Moreover, peripheral nerves have no lymphatic vessels, which could provide an effective outflow of the capillary infiltrate and prevent development of edema (Low, 1984; Olsson, 1984).

Our data support suggestions on a key role of nonsynaptic mechanisms of developing effects under chronic exposure to RVX. The morphofunctional changes at the level of the microcirculatory bed that influence the functional state of platelets may prove to be significant factors in the etiology of delayed effects of chronic intoxication. Since the life span of blood platelets is about 10 days (Stuart *et al.*, 1975), the changes observed are likely to reflect multiple micro-angiopathies, when clinically pronounced manifestations are prevented by compensatory mechanisms, such as receptor desensitization, endothelial and plasmatic proteases, and molecular effectors providing the feedback regulation.

It is well known that OPs cause inhibition of several major enzymes, and this fact can be easily proved by simple methods of laboratory and clinical biochemistry. On the other hand, diagnostics of a low-dosed chronic action of OPs, or their delayed effects after subacute intoxication, is a big problem because of lack of inhibition or recovery of the enzymes' activity. Determination of a peptide spectrum of blood plasma or serum could serve as an alternative and more sensitive method for diagnosing the intoxications. We have shown with MS/MS analysis that peptide components of plasma or serum affected by RVX are represented by fragments of fibrinopeptide A, and this fact could signify an inactivation of exopeptidases (aminopeptidases) under exposure to RVX. It is interesting that earlier an N-acyl-peptide hydrolase was found in brain that was covalently bound with some OP pesticides in subsymptomatic concentrations (Richards *et al.*, 2000). Various aminopeptidases relevant to our research field have been found in plasma or serum. Aminopeptidase A (L-alpha-aspartyl-(L-alpha-glutamyl)-peptide hydrolase, EC 3.4.11.7) hydrolyzes only acidic amino acid derivatives (Asp and Glu in rat and human fragments of fibrinopeptide A) (Lalu *et al.*, 1986). Aminopeptidase N (CD13/APN, EC 3.4.11.2) is a multifunctional protein and the principal aminopeptidase in plasma; it is present as a membrane-bound enzyme on myeloid cells including mature monocytes and neutrophils (Favaloro *et al.*, 1993). It is also expressed on epithelial cells, fibroblasts, and endothelial cells; angiogenic growth or signaling factors induce CD13/APN expression (Pasqualini *et al.*, 2000; Bhagwat *et al.*, 2001; Bauvois and Dauzonne, 2006). On the other hand, alphastatin, a 24-amino acid peptide derived from the amino terminus of the α-chain of human fibrinogen, has been found to possess the anti-angiogenic activity that was originally observed to be present in fibrinogen degradation product E (Staton *et al.*, 2004). Numerous other proteolytic degradation products have been shown to be endogenous inhibitors of angiogenesis (Van Hinsbergh *et al.*, 2006). The MS/MS analyses conducted in our laboratories have revealed another enzyme activity affected by RVX, and it is likely to be a chymotrypsin-like activity. We certainly have to directly measure cathepsin G and chymase activities to prove our hypothesis; moreover, it is interesting in view of the fact that cathepsin B can be activated with cathepsin G to further enhance angiogenesis (Van Hinsbergh *et al.*, 2006).

An adequate interpretation of the experimental data would lead to a proper understanding of the therapeutic approaches, which could prevent or even reverse development of delayed effects of RVX and other warfare or pesticide OPs. Resveratrol is now being extensively studied in different laboratories as a possible remedy, being an effective up-regulator of PON1 (Curtin *et al.*, 2008). In view of our understanding of the pathogenesis of delayed effects, it is important to mention another property of resveratrol as a protector of endothelial progenitor cells, which contribute to renovation of injured blood vessels (Gu *et al.*, 2006; Xia *et al.*, 2008).

In conclusion, we outline some present and future directions in the studies on analytical chemistry, biochemistry, and toxicology of RVX:

- Improvement of the methodology of chemical and biochemical monitoring in environmental objects and human organisms;
- Development of prognostic modeling for probable risk assessment in case of elevation of the allowable levels of RVX in various media;
- Studies on the quantitative relations ''dose-effect'', ''time-effect'', and ''dose-time-effect'' for cholinesterase and noncholinesterase effects at both acute and chronic exposure to RVX;
- Studies on novel molecular and functional effects under acute and chronic exposure to RVX;

- Studies on mechanisms of development of delayed effects after intoxication with RVX;
- Development of novel effective means for prophylaxis and treatment of delayed effects of intoxications with RVX.

We believe that fulfillment of these studies would surely contribute to fundamental and applied knowledge far beyond the toxicology of RVX.

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CHAPTER 8

Mustards and Vesicants

ROBERT A. YOUNG AND CHERYL BAST

I. INTRODUCTION

In the simplest terms, vesicants are chemicals that cause tissue blistering. Their toxic activity is, however, not limited to the skin and their mode of action is complex. These cytotoxic alkylating agents were initially developed as chemical weapons used to induce ocular, dermal, and respiratory damage resulting in immediate casualties, reduction in fighting efficiency, and demoralization. Depending on the exposure, injury may be local or systemic. This chapter will focus on sulfur mustards, nitrogen mustards, and lewisite. Although occasionally classified as a vesicant due to its action as a skin, eye, and respiratory tract irritant, phosgene oxime (CX) is more appropriately considered an urticant or nettle agent and is not discussed in this chapter. Extensive information regarding the chemistry and toxicology of vesicants is available in several publications (Papirmeister *et al.*, 1991; Somani, 1992; USACHPPM, 1996; ATSDR, 2003; Romano *et al.*, 2008). In response to a request by the Department of Veterans Affairs, a panel of experts extensively reviewed and evaluated the medical and scientific literature on mustard agents and lewisite, and the military testing programs relative to these agents (IOM, 1993).

The sulfur mustards include distilled mustard [bis(2-chloroethyl)sulfide; HD, SM], Levenstein mustard (H), agent HT [a mixture of HD and bis(2-chloroethylthioethyl)ether], a sulfur mustard–lewisite mixture (HL), and sesqui mustard (Q). Distilled mustard (HD) is relatively pure (97%) bis(2-chloroethyl) sulfide and results from the vacuum distillation of HD. Generic reference to sulfur mustard usually implies HD. Levenstein mustard (H) is a mixture of HD and sulfur impurities (generally in a 70:30 ratio) and, as its name implies, was produced by the Levenstein process involving reacting ethylene with sulfur chloride. Agent H may contain sulfur impurities imparting a yellowish color and sweet garlic-like odor.

Agent HT is generally a mixture of 60% HD and 40% bis(2-chloroethylthioethyl)ether (T), although this ratio may vary. Agent HL is a mixture of sulfur mustard (HD) and lewisite (L) that was developed for cold weather or high-altitude use due to its lower freezing point. Sesqui mustard (Q) is 1,2-bis(2-chloroethylthio) ethane and is considered a more potent vesicant than HD but its very low vapor pressure limits its effectiveness as a warfare agent, a condition remedied by combining it with HD to form agent HQ. Removal of one chlorine from sulfur mustard results in ''half-mustard'' (2-chloroethyl ethyl sulfide; CEES), a monofunctional sulfur mustard analog. Although retaining some alkylating properties, half-mustard is not as highly regulated as is sulfur mustard and is frequently used in sulfur mustard research. Most of the discussion of sulfur mustards in this chapter will be in reference to distilled sulfur mustard (HD).

Nitrogen mustards include HN1 [bis(2-chloroethyl) ethylamine], HN2 (2,2′-dichloro-*N*-methyldiethylamine), and HN3 [tris(2-chloroethyl)amine hydrochloride]. As will be discussed later, some of these found application in therapeutic arenas rather than in warfare.

Lewisite [L or L-1; dichloro(2-chlorovinyl) arsine] is an arsenical vesicant developed early in the 20th century. Lewisite occurs as *cis*- and *trans*-isomers; the typical ratio being 10:90. Several impurities including *bis*(2-chlorovinyl) chloroarsine (L-2) and *tris*(2-chlorovinyl)arsine (L-3) are typically present. The chemical and physical properties of the *cis*- and *trans*-isomers are similar.

A. Sulfur Mustards

Sulfur mustards are chemical vesicants capable of causing severe skin and eye damage at very low concentrations. The chemical name, synonyms, identification codes, chemical formula, and structural formula for sulfur mustard are shown in Table 8.1.

Although frequently referred to as ''mustard gas'', the chemical is a liquid at normal ambient temperatures. Due to its oily consistency and low aqueous solubility, sulfur mustard is persistent in the environment. Information on the half-life of HD in air is unavailable. As previously noted, impurities may impart a garlic-like odor to sulfur mustard. Odor thresholds ranging from 0.15 to 0.6 mg/m^3 have been reported for sulfur mustard (Dudley and Wells, 1938; Bowden, 1943; Fuhr and Krakow, 1945; Ruth, 1986).

Watson and Griffin (1992) have summarized information on the distribution of unitary chemical weapon stockpiles in the USA. The chemical and physical properties of sulfur mustard (agent HD) are shown in Table 8.2.

The water solubility of sulfur mustard has been reported as 0.092 g per 100 g water at 22°C (DA, 1974), and

TABLE 8.1. Nomenclature, chemical formulae, and chemical structure of sulfur mustard

Sulfur mustard (HD)	
Synonyms	*bis*(2-chloroethyl)sulfide; 1,1′-thiobis(2-chlorethane); 1-chloro-2-(2-chloroethylthio)ethane; SM; distilled mustard; agent HD; mustard gas; yperite; yellow cross
CAS No.	505-60-2
Chemical formula	$C_4H_8Cl_2S$
Chemical structure	C_2H_4-Cl / S \ C_2H_4-Cl

5×10^{-3} M at room temperature (MacNaughton and Brewer, 1994). In dilute aqueous solutions sulfur mustard hydrolyzes almost completely to thiodiglycol and hydrochloric acid (Papirmeister *et al.*, 1991). For dissolved HD, the hydrolysis half-life ranges from about 4 to 15 min for temperatures of 20–25°C. Bulk HD may persist in water for up to several years (Small, 1984). It has been estimated that it would take 15 days for the mass of a 1 cm droplet of HD in quiescent water to decrease by one half (Small, 1984). The Henry's law constant for HD has been estimated to be 2.1×10^{-5} atm m^3/mol (MacNaughton and Brewer, 1994), indicating a moderate potential for evaporation from water.

The persistence of sulfur mustard in soil depends on the soil type, pH, moisture content, and whether the agent is at the soil surface or buried. Small (1984) reported that HD applied to the soil surface volatilized and would likely be the main route of HD loss (half-life about 30 min). However, if the soil was wet, hydrolysis would be the primary loss pathway. When sprayed onto soil, a vesicant action may persist for about 2 weeks but when the agent continually leaks into the soil vesicant action may be present after 3 years (DA, 1974). Rosenblatt *et al.* (1995) state that the persistence of sulfur mustard in soil is due to the formation of oligomeric degradation products that coat the surface of the mustard agent and that are resistant to hydrolysis. This may greatly enhance the environmental persistence of sulfur mustard. Sulfur mustard has a log K_{ow} of 1.37 and a K_{oc} of 133, indicating that binding to soil organics would limit transport through soil to groundwater (MacNaughton and Brewer, 1994). MacNaughton and Brewer (1994) also calculated a leaching index of 7.2 for HD (i.e. the number of leachings required to reduce the HD soil concentration to one-tenth of the original amount, assuming that for each leaching one kilogram of soil is in equilibrium with one liter of water).

TABLE 8.3. Nomenclature, chemical formulae, and chemical structures of nitrogen mustards

HN-1	
Synonyms	ethyl-*bis*(2-chloroethyl)amine; *bis*-(2-chloroethyl)ethylamine
CAS No.	538-078
Chemical formula	$(ClCH_2CH_2)_2NC_2H_5$
HN-2	
Synonyms	methyl-bis(-chloroethyl)amine; 2,2′-dichloro-*N*-methyldiethylamine; ''S''; mechlorethamine
CAS No.	51-75-2
Chemical formula	$(ClCH_2CH_2)_2NCH_3$
HN-3	
Synonyms	*tris*(-chloroethyl)amine; [*tris*(2-chloroethyl)amine hydrochloride]
CAS No.	555-77-1
Chemical formula	$N(CH_2CH_2Cl)_3$

B. Nitrogen Mustards

Nitrogen mustards are tertiary *bis*(2-chloroethyl)amines with vesicant activity (NDRC, 1946). All are active alkylating agents. The nomenclature, chemical and physical properties of HN1, HN2, and HN3 are summarized in Tables 8.3 and 8.4. Due to their toxicity and various

TABLE 8.2. Selected physical and chemical properties of sulfur mustard

	Value	Reference
Physical state	Oily liquid	MacNaughton and Brewer (1994)
Molecular weight	HD: 159.08	DA (1996)
Density	5.4	DA (1996)
Boiling point	HD: 215–217°C	DA (1996); Budavari *et al.* (1989)
Freezing point	HD: 14.5°C	DA (1996)
Vapor pressure (mm Hg)	HD: 0.072 mm Hg at 20°C; 0.11 mm Hg at 25°C	DA (1996)
Water solubility (g/l)	Sparingly soluble in water; soluble in organic solvents	DA (1996); Budavari *et al.* (1989)

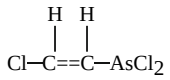
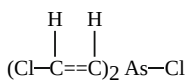
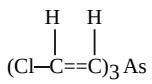
TABLE 8.4. Physical and chemical properties of nitrogen mustards

	Value	Reference
Physical state	HN-1: oily liquid HN-2: oily liquid HN-3: oily liquid	USACHPPM (1996)
Molecular weight	HN-1: 170.08 HN-2: 156.07 HN-3: 204.54	USACHPPM (1996)
Boiling point[a]/Freezing point	HN-1: 194°C/−34°C HN-2: 75°C/−60°C HN-3: 256°C*/−3.7°C	USACHPPM (1996)
Vapor pressure (mm Hg)	HN-1: 0.25 mm @ 25°C HN-2: 0.43 mm @ 25°C HN-3: 0.01 mm @ 25°C	USACHPPM (1996)
Water solubility (g/l)	HN-1: limited; miscible with organic solvents HN-2: limited; miscible with organic solvents HN-3: limited; miscible with organic solvents	USACHPPM (1996)

[a]Decomposes prior to reaching boiling point

physical–chemical properties, initial interest in these chemicals as warfare agents came about shortly before or during World War II. Although HN2 and HN3 were specifically developed as military agents, HN1 was originally developed as a pharmaceutical. HN2 (mechlorethamine) later found use as an antineoplastic agent. Nitrogen mustards and derivatives such as melphalan, chlorambucil, and cyclophosphamide are alkylating agents used as cancer therapeutic agents (Somani, 1992).

TABLE 8.5. Nomenclature, chemical formulae, and chemical structure of lewisites

Lewisite (L)	
Synonyms	2-chlorovinyldichloroarsine; (2-chlorovinyl)arsenous dichloride; beta-chlorovinyldichloroarsine; dichloro(2-chlorovinyl) arsine; chlorovinylarsine dichloride; EA 1034
CAS No.	541-25-3
Chemical formula	$ClCH{=}CHAsCl_2$
Chemical structure	
L-2	
Synonym	Lewisite-2
CAS No.	40334-69-8
Chemical formula	$(ClCH{=}CH)_2AsCl$
Chemical structure	
L-3	
Synonym	Lewisite-3
CAS No.	40334-70-1
Chemical formula	$(ClCH{=}CH)_3As$
Chemical structure	

C. Lewisite

Lewisite is composed of *cis*- and *trans*-isomers in the ratio of 10:90 and several impurities including *bis*(2-chlorovinyl)chloroarsine (L-2) and *tris*(2-chlorovinyl)arsine (L-3) (Rosenblatt *et al.*, 1975). The chemical and physical properties of the *cis*- and *trans*-isomers are similar. The nomenclature and chemical and physical properties of L-1, L-2, and L-3 are presented in Tables 8.5 and 8.6, respectively. In pure form, lewisite is colorless and odorless but usually occurs as a brown oily liquid with a distinct geranium-like odor. Gates *et al.* (1946) reported an odor threshold of 14–23 mg/m^3 for lewisite.

Information regarding the atmospheric tranformation of lewisite is limited. MacNaughton and Brewer (1994) reported that some photodegradation may take place and that hydrolysis may also occur in the gas phase. Lewisite is only sparingly soluble in water; 0.5 g/l (Rosenblatt *et al.*, 1975). Hydrolysis of lewisite results in the formation of lewisite oxide and HCl, and may occur rapidly. The hydrolysis of lewisite is complex and includes several reversible reactions (Epstein, 1956; Rosenblatt *et al.*, 1975; Clark, 1989; MacNaughton and Brewer, 1994). Under slightly acidic conditions, lewisite initially undergoes rapid and reversible conversion to dihydroxy arsine, 2-chlorovinyl arsine oxide and two equivalents of hydrogen chloride:

$$Cl{-}CH{=}CH{-}AsCl_2 + 2H_2O \;\Omega\; Cl{-}CH$$
$${=}CH{-}As(OH)_2 + 2HCl$$

The production of two equivalents of chloride occurs within 3 min at 20°C; at 5°C the reaction is 90% complete within

TABLE 8.6. Physical and chemical properties of lewisite

	Value	Reference
Lewisite		
Physical state	Oily, amber brown liquid	Lindberg *et al.* (1997)
Molecular weight	170.08	USACHPPM (1996)
Boiling point	190EC	Trammel (1992)
Freezing point	−18°C; varies ∀ 0.1°C depending on purity	Watson and Griffin (1992)
Vapor pressure (mmHg)	0.34 mm Hg at 25°C; 0.22 mm Hg at 20°C	USACHPPM (1996)
Water solubility (g/l)	0.5 g/l in water; soluble in most organic solvents	USACHPPM (1996)

2 min, and the completion of the reaction requires several hours. The hydrolysis rate constant is reported as 1 min^{-1} at 20°C. Hydrolysis of 2-chlorovinyl arsine oxide is slower, resulting in lewisite oxide (chlorovinyl arsenous oxide) and polymerized lewisite oxide:

$$\mathrm{Cl{-}CH{=}CH{-}As(OH)_2\ \Omega\ H_2O + Cl{-}CH{=}CH{-}AsO + (Cl{-}CH{=}CH{-}AsO)_n}$$

The forward reaction is favored because lewisite oxide and polymerized lewisite oxide are insoluble. In a basic solution, the *trans*-lewisite isomer is cleaved by the hydroxyl ion to give acetylene and sodium arsenite; this reaction may occur even at low temperatures (Rosenblatt *et al.*, 1975; Clark, 1989). *Cis*-lewisite heated to over 40°C reacts with sodium hydroxide to yield vinyl chloride, sodium arsenite, and acetylene (Rosenblatt *et al.*, 1975). In aqueous solution, the *cis*-isomer undergoes a photoconversion to the *trans*-isomer (Rosenblatt *et al.*, 1975). Epstein (1956) reported that the toxic trivalent arsenic of lewisite oxide in standing water is converted to the less toxic pentavalent arsenic.

Lewisite in soil may rapidly volatilize or may be converted to lewisite oxide due to moisture in the soil (Rosenblatt *et al.*, 1975). The low water solubility suggests intermediate persistence in moist soil (Watson and Griffin, 1992). Both lewisite and lewisite oxide may be slowly oxidized to 2-chlorovinylarsonic acid (Rosenblatt *et al.*, 1975). Possible pathways of microbial degradation in soil include epoxidation of the C=C bond and reductive dehalogenation and dehydrohalogenation (Morrill *et al.*, 1985). Due to the epoxy bond and arsine group, toxic metabolites may result. Additionally, residual hydrolysis may result in arsenic compounds. Lewisite is not likely to bioaccumulate. However, the arsenic degradation products may bioaccumulate (Rosenblatt *et al.*, 1975).

II. HISTORY AND BACKGROUND

A. Sulfur Mustards

Sulfur mustard was developed in Germany and initially used as a warfare agent (as Levenstein mustard) during World War I. More recent use occurred in Middle East conflicts. Its oily nature makes it persistent on surfaces it contacts. Because sulfur mustard exerts toxic effects following dermal, ocular, and inhalation exposure, its use necessitated full body protection which, in turn, required the development of protective clothing and significant changes in warfare operations.

Minute quantities of sulfur mustard are used by various military and contract laboratories for defense research purposes, and for verification of Chemical Weapons Convention compliance. Bulk quantities of sulfur mustard are no longer manufactured in the USA. Military stockpiles of sulfur mustard are awaiting destruction or are in the process of being destroyed. Some sulfur mustard may also be found buried or abandoned at former defense sites. Sulfur mustard was frequently loaded into artillery shells and aerial bombs (often with lewisite). Various quantities of sulfur mustard also exist in other countries. Large amounts of sulfur mustard have been disposed of at sea.

Outside of military conflicts, exposure to sulfur mustard has occurred or may occur in work environments associated with chemical weapon materiel (e.g. storage depots, demilitarization facilities, research laboratories), during emergency response operations or remediation and decontamination activities, or during treaty verification activities in support of the Chemical Weapons Convention. Chemical weapons such as the vesicants are still considered potential military threats and terrorist targets. The most likely route of exposure to sulfur mustard is via aerosol/vapor exposure of the skin, eyes, and respiratory tract.

B. Nitrogen Mustards

Due to their toxicity and various physical–chemical properties (they are structurally similar to sulfur mustard), initial interest in nitrogen mustards as warfare agents came about shortly before or during World War II. Like sulfur mustard, all are alkylating agents. This document is limited to the nitrogen mustards referred to as HN1, HN2, and HN3, selected chemical and physical properties of which are summarized in Tables 8.3 and 8.4. Although HN2 and HN3 were initially investigated as military agents, HN1 was

originally developed as a pharmaceutical. HN2 (mechlorethamine) later found use as a pharmaceutical. Nitrogen mustards and derivatives such as melphalan, chlorambucil, and cyclophosphamide are alkylating agents used as cancer therapeutic agents (Somani, 1992). HN1 and HN3 are among the chemical agents found in Chemical Agent Identification Sets (CAIS) which are considered a component of nonstockpiled material. Generally, the nitrogen mustards have not had the interest or high profile associated with sulfur mustard and lewisite.

C. Lewisite

Lewisite, an organoarsenic compound, was developed in an attempt to create a more effective blister agent than sulfur mustard. Its development is generally credited to Winford Lewis at the Catholic University, Washington DC, and is based upon a thesis by Julius Nieuwland who described the synthesis of lewisite from arsenic trichloride, acetylene, hydrochloric acid, and mercuric chloride. Early on, the compound was frequently referred to by the vividly descriptive term, ''Dew of Death''. Like sulfur mustard, it is both a vesicant and systemic poison with target tissues not limited to the skin. In an attempt to develop an antidote to lewisite, British Anti-lewisite (BAL; dimercaprol) was developed (Peters *et al.*, 1945) which later became invaluable in the treatment of arsenic poisoning. Late in World War I and into World War II, large quantities of lewisite were manufactured by Germany, the US, Italy, the Soviet Union, and Japan (reviewed by Trammell, 1992). Large amounts of lewisite were manufactured (up to 2 tons/day by Japan) and stored prior to and during World War II (Tanaka, 1988; Trammell, 1992). Lewisite was frequently a component (often mixed with sulfur mustard) in artillery shells and aerial bombs. Like sulfur mustard, there are reports of large amounts of the compound being disposed of at sea (Spiers, 1968). With the possible exception of its use against Iranian soldiers during the Iraq–Iran conflict (Perera, 1985), there has been little or no use of lewisite in battle situations. Goldman and Dacre (1989) have reviewed the chemistry and toxicology of lewisite.

III. TOXICOKINETICS

A. Sulfur Mustards

With its high lipophilicity, toxicologically relevant amounts of sulfur mustard are absorbed into epithelial tissue (Papirmeister *et al.*, 1991). Dermal absorption is dependent on the thickness of the epidermis and on the presence of moisture, which enhances penetration. Absorption tends to be greater at the base of hair shafts and in the hair follicle where the epithelial tissue is thinner than the surrounding surface area (Papirmeister *et al.*, 1991). Approximately 20% of sulfur mustard applied to skin may be rapidly absorbed while 12–50% of this may react and remain at the application site (Somani and Babu, 1989). About 12% of that absorbed remains at the contact site and the remaining 88% enters the circulation (Renshaw, 1947). Renshaw (1947) noted that the rate of penetration is 1–4 μg/cm^2/min at 75°F.

For dermal exposure, penetration rates over 2–8 h ranged from 2.9 to 6.7% and rates of absorption from 1.2 to 4.0% following application of 400 μg of radiolabeled sulfur mustard per cm^2 of isolated perfused porcine skin (Riviere *et al.*, 1995). The average total recovery of the radiolabel was 9.3% (3.8–17.7%) suggesting substantial loss due to volatilization.

Relative to dermal absorption, little is known about absorption in the respiratory tract. Cameron *et al.* (1946) calculated the absorption of sulfur mustard vapor in the noses of rabbits and rhesus monkeys. The concentration of the agent in the nasal passages was 10–30% of the chamber concentrations (40, 100, and 500 mg/m^3), implying an absorption of approximately 70–90%.

Several studies using radiolabeled sulfur mustard have shown that sulfur mustard and its metabolites may be widely distributed in the body after percutaneous or intravenous exposure. Maximum levels of radioactivity were detected in the kidney, lungs, and liver of rabbits following intravenous administration (Boursnell *et al.*, 1946). At 15 min following percutaneous exposure of rats, sulfur mustard derived radioactivity was found in all examined tissues except the eyes (Young *et al.*, 1944). Similarly, Clemedson *et al.* (1963) noted uniform distribution of radioactivity in mice after either percutaneous or intravenous exposures, with most radioactivity occurring in the nasal region, kidneys, liver, and intestine. Hambrook *et al.* (1993) reported on the uptake and distribution of radiolabeled sulfur mustard in the skin and blood of rats after cutaneous application. It was found that much of the agent entering the blood binds to hemoglobin and, to some extent, with glutathione. Results of studies with rabbits showed that sulfur mustard concentrated in the cornea and to a lesser extent in the iris, lens, and conjunctiva within 5 min after application (Axelrod and Hamilton, 1947).

The biotransformation of sulfur mustard after intravenous or intraperitoneal injection of radiolabeled compound in rats has been examined. Following intravenous injection, the major urinary metabolite was glutathione-bis-chloroethyl sulfide conjugates (45% of total urinary radioactivity) and smaller amounts of sulfone conjugates (7%) and thiodiglycol and its conjugates (14.4%) (Davison *et al.*, 1961). Roberts and Warwick (1963) found the major urinary product of cysteine-bis-(β-chloroethyl)sulfone after intraperitoneal injection of sulfur mustard in rats. Papirmeister *et al.* (1991) concluded that hydrolysis to thiodiglycol and reaction with glutathione are the most important routes of detoxification. This is supported by human data showing that thiodiglycol is present in the urine for one week or more after exposure (Wils *et al.*, 1987).

B. Nitrogen Mustards

The effects of time, temperature, and humidity on the vapor penetration of HN1 and HN3 into the forearm skin of human male volunteers were reported by NDRC (1945). Results of this work showed similar effects of temperature and humidity as observed for sulfur mustard, e.g. greater absorption with increased temperature and humidity. The penetration of HN1 and HN3 was found to be linear with time (5 to 20 min for HN1 and 30–60 min for HN3). At 71–72°F and 50–52% relative humidity, HN1 penetration rate was 2.8 μg/cm^2/min and for HN3 was 0.18 μg/cm^2/min at 72–73°F and 45–48% relative humidity. At 86–87°F and 47–49% relative humidity, HN1 penetration rate increased to 5.2 μg/cm^2/min and HN3 penetration rate increased to 0.3 μg/cm^2/min at 85°F and 47–48% relative humidity. Excretion via the urine is likely a major route of elimination, especially due to the water solubility of the immonium ion (see section IV).

C. Lewisite

Little information is available regarding the toxicokinetics of lewisite. Lewisite is readily absorbed by mucous membranes and, because of its lipophilicity, dermal absorption is significant (HSDB, 2004). Dermal absorption is reportedly more rapid than for sulfur mustard (Hurst and Smith, 2008). Axelrod and Hamilton (1947) reported that radiolabeled (^{74}As) lewisite applied to a 0.43 cm^2 area of human skin was primarily fixed on the epidermis and that very little was found in the dermis; most was detected in hair and hair follicles. In experiments with guinea pigs, histological examination revealed that lewisite applied to skin entered epidermis within 2 min and penetrated into the dermis within 10 min (Ferguson and Silver, 1947). Only trace amounts were detectable in the dermis at 24 h post-application.

IV. MECHANISM OF ACTION

A. Sulfur Mustards

The mechanism of action of sulfur mustard is multifaceted and complex, and has been reviewed in some detail by Papirmeister *et al.* (1991), Hurst and Smith (2008), and Smith *et al.* (2008). Efforts to understand the mechanisms of sulfur mustard toxicity are ongoing. Basically, sulfur mustard disrupts the interface of the epidermis and basement membrane causing blistering between the epidermis and dermis. Both immediate (immediate cell membrane damage) and delayed phases (secondary effects resulting from inflammatory responses, DNA damage, vascular leakage) have been described for sulfur mustard-induced dermal effects (Somani and Babu, 1989). Many of the toxic effects of sulfur mustard can be attributed to oxidative stress.

Among the most studied mechanisms of sulfur mustard toxicity are thiol depletion resulting in intracellular calcium imbalance and subsequent cell death, alkylation of DNA and other cellular macromolecules, lipid peroxidation resulting from sulfur mustard-induced gluthathione depletion, and induction of an inflammatory response. The overall mechanism of sulfur mustard toxicity likely involves an interlinking of the aforementioned processes which are briefly described below.

A key component of sulfur mustard toxicity is the formation of a sulfonium ion and resulting episulfonium intermediate which may react with sulfhydryl-containing macromolecules. Damage may include Ca^{2+} translocases (Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase) which are dependent on thiol groups to maintain cellular Ca^{2+} homeostasis, and microfilamentous proteins. The resulting increase in intracellular calcium levels ultimately causes a decrease in cellular integrity and induction of apoptosis. Oxidative stress in sulfur mustard toxicity has been reviewed by Smith *et al.* (2008).

The role of DNA alkylation and the poly(ADP-ribose) polymerase (PARP) hypothesis theory for sulfur mustard toxicity has been reviewed by Papirmeister *et al.* (1991). In this mechanism, DNA is the initial target of the mustard agent. Alkylated DNA purines are enzymatically depurinated creating apurinic sites which are cleaved by apurinic endonucleases resulting in DNA strand breaks. The accumulation of DNA breaks leads to activation of the chromosomal enzyme PARP, which utilizes NAD^+ causing severe lowering of cellular NAD^+. Depletion of NAD^+ results in the inhibition of glycolysis, and stimulation of the nicotinamide adenine dinucleotide phosphate ($NADP^+$)-dependent hexose monophosphate shunt, ultimately resulting in the induction and secretion of proteases and subsequent cellular changes.

In the review by Papirmeister *et al.* (1991), it was noted that sulfur mustard-induced cytotoxicity is dose dependent and that DNA appeared to be more sensitive to mustard-induced alkylation than are other cellular constituents. The low-dose effects of sulfur mustard are characterized by genotoxicity and inhibition of mitosis. The loss of cellular reproduction may be due to bifunctional alkylation that ultimately prevents normal DNA replication. It was hypothesized that monofunctional DNA damage might be responsible for low-dose mutagenic and possibly carcinogenic effects.

Sulfur mustard-induced lipid peroxidation is a function of glutathione (GSH) depletion. For this mechanism, depletion of GSH results in an accumulation of reactive oxygen species via hydrogen peroxide-dependent processes (Miccadei *et al.*, 1988). The oxygen radicals react with membrane phospholipids forming lipid peroxides that alter membrane structure resulting in membrane breakdown.

Recent work has focused on the identification of possible biomarkers of sulfur mustard exposure and injury (Buxton *et al.*, 2000, 2001; Danne *et al.*, 2000). More recently, the role of metalloproteinases and collagen degradation (Gerecke *et al.*, 2005), platelet activating factor (Clark *et al.*, 2005, 2006), and interaction with cytochrome P450

processes (Brimfield and Hodgson, 2005; Brimfield *et al.*, 2006; Mancheco and Brimfield, 2006) are being investigated relative to the mechanism of action of sulfur mustard.

B. Nitrogen Mustards

A key component of nitrogen mustard toxicity is analogous to that of sulfur mustard: the formation of a cyclic onium cation. This occurs in the presence of polar solvents such as water (Somani, 1992). The immonium ion may react with nucleophiles such as nitrogen in the base components of nucleic acids and with sulfhydryl groups in proteins and peptides. The precise mechanism of nitrogen mustard activity is unclear but several have been proposed: DNA/RNA alkylation and resultant effects, effects on glutathione, membrane effects (protein cross-linking, ion transport effects), and cytoplasmic effects (release of lysosomal enzymes). The possible mechanisms of nitrogen mustard have been reviewed by Gray (1989). Results of preliminary work by Elsayed and Omaye (2006) in mice given HN2 intraperitoneally showed pulmonary alterations indicative of oxidative stress and impaired detoxification processes which are consistent with the aforementioned mechanisms.

C. Lewisite

Dermal or intravenous exposure to lewisite leads to local skin edema and pulmonary edema due to increased capillary permeability. The increased capillary permeability results in blood plasma loss and resultant physiological responses collectively referred to as ''lewisite shock''. Lewisite shock may be likened to shock observed in severe burn cases. It has been hypothesized that functional changes in the lungs, kidneys, respiratory tract, cardiovascular, and lymphatic systems may be the result of a disturbance of osmotic equilibrium (Goldman and Dacre, 1989).

Lewisite-induced vesicant and systemic toxicity are likely due, in part, to interactions with thiol groups (Goldman and Dacre, 1989). The interaction with enzyme sulfhydryl groups may cause inhibition of enzyme by the formation of stable cyclic structures with arsenic. These thiol interactions result in energy depletion leading to cell death (Young, 1999).

V. TOXICITY

A. Sulfur Mustard

The toxic effects of sulfur mustard in humans and animals have been extensively reviewed by ATSDR (2003), Sidell and Hurst (1992), Somani (1992), Watson and Griffin (1992), IOM (1993), NRC (2003), and Romano *et al.* (2008).

Sulfur mustard affects the skin, respiratory tract, and eyes. The acute effects include edema, ulceration, and necrosis of epithelial tissue. Systemic toxicity may also occur and is characterized by nausea and vomiting, fever, and malaise. There is evidence of systemic toxicity (gastrointestinal tract) following dermal exposure only (Dacre and Goldman, 1996). Delayed effects include conjunctivitis and blindness following ocular exposure and chronic bronchitis following inhalation exposure. Affected tissues may have an increased susceptibility to secondary infections, and possibility of carcinogenicity of the skin and respiratory tract.

Ambient temperature and humidity govern the degree of toxicity of sulfur mustard; in hot and humid conditions, lower mustard concentrations are required to produce debilitating effects. The severity of sulfur mustard effects is also greater in areas of the body with greater moisture (e.g. axilla, groin, eyes). Information regarding the toxic effects of long-term exposure to low levels of sulfur mustard that are not acutely toxic is limited.

Available data suggest that the location and severity of damage resulting from exposure to sulfur mustard are concentration dependent and a function of the highly reactive nature of sulfur mustard (Papirmeister *et al.*, 1991). The eyes are generally considered to be the most sensitive and rapidly responding target (Reed, 1918; Reed *et al.*, 1918; Anderson, 1942). For low exposures, sulfur mustard-induced injury appears to be limited to the upper respiratory tract (Eisenmenger *et al.*, 1991) and eyes (Reed, 1918; Reed *et al.*, 1918; Guild *et al.*, 1941; Anderson, 1942). In work with informed volunteer subjects, Anderson (1942) reported that Ct values of 60–75 mg-min/m^3 would result in conjunctivitis, photophobia, and ocular irritation, while Ct values of 75–90 mg-min/m^3 would cause a high proportion of casualties as determined by more severe ocular damage requiring several weeks of treatment. At higher concentrations, pulmonary effects would be expected (Eisenmenger *et al.*, 1991). Regardless of the target tissue, there is a latency period between initial exposure and development of effects. The eyes and respiratory tract appear to have the shortest latency period with effects appearing within hours depending on the exposure level.

In addition to the acute toxic effects on the eyes, skin, and respiratory tract, both acute and longer-term neuropsychiatric effects (e.g. depression, anxiety, neurasthenia, insomnia, post-traumatic stress syndrome) have been documented for individuals exposed to sulfur mustard (Romano *et al.*, 2008). Many of these effects have been documented for individuals exposed during noncombat (e.g. munitions plant workers) activities and are not always the result of high-level exposure that result in serious overt effects. Longer-term effects such as chronic bronchitis have been associated with occupational exposures that included episodes of acute toxicity, and delayed or recurrent keratitis may occur 8–40 years after a severe vapor exposure. Sulfur mustard-induced immunosuppression resulting in greater susceptibility to infections has also been reported.

TABLE 8.7. Acute lethality of sulfur mustard in laboratory species following inhalation exposure

Species	Lethality value	Concentration (mg/m^3) and exposure duration (min)	Reference
Rat	2 min LCt_{50}: 1512 mg-min/m^3 30 min LCt_{50}: 990 mg-min/m^3 60 min LCt_{50}: 840 mg-min/m^3	756 mg/m^3 (2 min) 33 mg/m^3 (30 min) 14 mg/m^3 (60 min)	Fuhr and Krakow (1945) (not verified)
Mouse	2 min LCt_{50}: 4140 mg-min/m^3 30 min LCt_{50}: 1320 mg-min/m^3 60 min LCt_{50}: 860 mg-min/m^3	2070 mg/m^3 (2 min) 44 mg/m^3 (30 min) 14.3 mg/m^3 (60 min)	Fuhr and Krakow (1945) (not verified)
Mouse	60 min LCt_{50}: 42.5 mg/m^3	42.5 mg/m^3 (60 min)	Vijayaraghavan (1997)
Monkey	10 min LCt_{50}: 800 mg-min/m^3	80 mg/m^3 (10 min)	Rosenblatt *et al.* (1975)
Dog	10 min LCt_{50}: 600 mg-min/m^3	60 mg/m^3 (10 min)	Rosenblatt *et a*l. (1975)
Cat	10 min LCt_{50}: 700 mg-min/m^3	70 mg/m^3 (10 min)	Rosenblatt *et al.* (1975)
Goat	10 min LCt_{50}: 1900 mg-min/m^3	190 mg/m^3 (10 min)	Rosenblatt *et al.* (1975)
Guinea pig	5 min LCt_{50}: 800 mg-min/m^3 10 min LCt_{50}: 1700 mg-min/m^3	160 mg/m^3 (5 min) 170 mg/m^3 (10 min)	Langenberg *et al.* (1998) Rosenblatt *et al.* (1975)

Acute lethality data in animals are summarized in Table 8.7. Based upon the animal data, interspecies variability in the lethal response to sulfur mustard vapor is less than an order of magnitude. For nonlethal effects, the animal data suggest that test species exhibit signs of toxicity that are qualitatively similar to humans when acutely exposed to sulfur mustard vapor. Ocular and respiratory tract irritations are clearly evident in studies using dogs, rats, mice, rabbits, and guinea pigs.

Effects of orally administered sulfur mustard in rats were studied by Sasser *et al.* (1996a). Repeated gavage administration of sulfur mustard in sesame oil produced epithelial hyperplasia of the forestomach at the highest dose tested but no deaths and no other treatment-related pathological lesions or changes in clinical chemistry or hematological parameters.

Results of a multigeneration study in rats given sulfur mustard by gavage showed no significant adverse effects on reproductive parameters at any dose level, but revealed dose-related lesions of the squamous epithelium of the forestomach (acanthosis and hyperplasia). It is likely that the forestomach lesions were a function of the treatment regimen whereby the bolus dose in an oil vehicle (sesame seed oil) would enhance the direct-contact effects of the sulfur mustard on the forestomach tissue. Studies by Hackett *et al.* (1987) in which rabbits were gavage dosed with sulfur mustard were equivocal regarding reproductive/developmental effects due in part to the dose regimen and overt maternal toxicity.

Studies in animals have shown that sulfur mustard may induce developmental and reproductive effects (reviewed in NRC, 1999, 2003). Acute exposures resulting in systemic uptake may have effects on reproductive organs, including inhibition of spermatogenesis. Fetal anomalies were observed in tests with rats given sulfur mustard during gestation but only at maternally toxic doses.

The genotoxicity of sulfur mustard is well documented. It is known to produce DNA cross-links, mutations following replication or repair errors, chromosomal breaks, and chromosomal aberrations. Occupational exposures have been associated with increased frequencies of somatic cell mutations, sister chromatid exchanges, and chromosome abnormalities. Studies with rats indicate that subchronic inhalation or oral exposures can produce dominant lethal effects.

The carcinogenicity of sulfur mustard in animals has been reviewed in IARC (1975), Watson *et al.* (1989), IOM (1993),

NRC (1999), and USACHPPM (2000). McNamara *et al.* (1975) studied the potential carcinogenicity of sulfur mustard in rats, mice, rabbits, guinea pigs, and dogs exposed via inhalation for up to one year. No tumors were detected in the mice, rabbits, guinea pigs, or dogs, but skin tumors (basal and squamous cell carcinomas, trichoepitheliomas, and keratoacanthomas) were associated with sulfur mustard exposure at the highest exposure tested (0.1 mg sulfur mustard/m^3 for 6.5 h followed by 0.0025 mg sulfur mustard/m^3 for 17.5 h/day, 5 days/week). An increased incidence of pulmonary tumors in Strain A mice was observed following intravenous injections (four doses over 2 days) of sulfur mustard (Heston, 1950), and an increase in injection site tumors in mice given subcutaneous injections of sulfur mustard over a 6-week period (Heston, 1953).

A study of the Iranian military veterans exposed to sulfur mustard under battlefield conditions during the Iran–Iraq conflict at levels sufficient to cause severe signs of toxicity indicated a potential increased incidence of chronic myelocytic leukemia (CML). In several earlier studies on WWI veterans who had been exposed to sulfur mustard, leukemia was not identified as a possible effect, although it is unclear if examination for CML had ever occurred in those populations. Confounders, such as exposure to benzene or radiation which complicate the analysis, have not yet been ruled out in the ongoing epidemiologic study of Iranian veterans. Two cases of CML were reported for Japanese workers exposed to sulfur mustard (Shakil *et al.*, 1993) but the incidences of CML in the entire population of sulfur mustard-exposed workers and in an unexposed control population were not reported. Studies in animals provide supporting evidence for the carcinogenicity of sulfur mustard although the results of some studies are compromised by insufficient exposure durations and injuries resulting from caging situations.

B. Nitrogen Mustards

Information regarding the toxicity of nitrogen mustards is not as extensive as that for sulfur mustard. Limited lethality data in animals are summarized in Table 8.8. Like sulfur mustard, exposure to nitrogen mustards may cause skin blistering as well as respiratory tract injury and ocular damage. Response data from tests with informed human volunteer subjects (NDRC, 1944) suggested a relative potency of HN3 > HN1 > HN2 for vesicant effects, although the differences were minor. Like sulfur mustard, dermal effects were enhanced by moisture (as from sweating). Estimated thresholds for skin blistering and ocular injury are summarized in Table 8.9. Ocular injury (irritation resulting in compromised operational effectiveness of military personnel) was detected at exposures much lower than those causing dermal effects. All of the toxic effects of nitrogen mustard appear to involve a latency period of several hours for ocular responses and several days for dermal blistering. Nitrogen mustards are alkylating agents with known mutagenicity, but there are no animal cancer bioassays and no human carcinogenicity data.

Nitrogen mustard and its hydrochloride salt have been shown to be teratogenic in mice and rats. Intraperitoneal administration of HN2-hydrochloride/g to mice during gestation resulted in serious teratogenic effects (Danforth and Center, 1954). Haskin (1948) and Murphy *et al.* (1958) reported similar findings in rats given HN2-hydrochloride/kg subcutaneously during gestation.

Nitrogen mustards are bifunctional alkylating agents that produce a carcinogenic response (primarily lung tumors and lymphomas) in mice following subcutaneous, intraperitoneal, and intravenous administration as well as by skin painting (IARC, 1987). Intravenous administration of nitrogen mustard to rats produced tumors in multiple organs (IARC,

TABLE 8.8. Lethality of nitrogen mustard (HN2)

Route	Species	Dose (mg/kg)	Exposure time	Effect	Reference
Oral	rat	10–85	–	LD_{50}	NDRC (1946)
	mouse	10–20	–	LD_{50}	Fox and Scott (1980)
Percutaneous	rat	14	–	LD_{50}	NDRC (1946)
		12	96 h	LD_{50}	Vojvodić *et al.* (1985)
	mouse	29–35	–	LD_{50}	NDRC (1946)
	monkey	<50	–	LD_{50}	NDRC (1946)
Subcutaneous	rat	6	–	LD_{50}	Vojvodić *et al.* (1985)
	mouse	1.4	–	LD_{50}	Fox and Scott (1980)
		2.6–4.5	–	LD_{50}	NDRC (1946)
Intraperitoneal	rat	1.8–2.5	–	LD_{50}	Fox and Scott (1980)
	mouse	4.4	–	LD_{50}	Fox and Scott (1980)
Intravenous	rat	1.1	–	LD_{50}	Fox and Scott (1980); NDRC (1946)
	mouse	~2	–	LD_{50}	NDRC (1946)

TABLE 8.9. Estimated effects thresholds in humans exposed to nitrogen mustard vapors

HN1	HN2	HN3	Effect
–	0.012 mg-min/m^3	–	No observable effect level during therapeutic use of HN2 (Van Vloten *et al.*, 1993)
90 mg-min/m^3	70 mg-min/m^3	42 mg-min/m^3	Moderate but reversible ocular effects (Porton Report, 1942a, b, 1943a, b, c, d; US Army Med. Div., 1945a, b; NDRC, 1946)
>21,170 mg-min/m^3	5,800 mg-min/m^3	1,800 mg-min/m^3	Median blistering Ct (10 min or 20 min exposure) for normal skin
		1,300 mg-min/m^3	Median blistering Ct (20 min exposure) for sweating skin (NDRC, 1944)

1987). Information in humans is limited to reports of squamous cell carcinomas of the skin following therapeutic application of nitrogen mustard in the treatment of mycosis, fungoides, and psoriasis (IARC, 1987).

C. Lewisite

The toxicology of lewisite has been reviewed by Goldman and Dacre (1989), Watson and Griffin (1992), Trammell (1992), and Hurst and Smith (2008). Its characteristic geranium-like odor is detectable at 14–23 mg/m^3 (Gates *et al.*, 1946). Lewisite may be lethal in humans following inhalation, dermal, or oral exposure. It is reportedly immediately highly irritating at estimated concentrations of 6–8 mg/m^3. Gates *et al.* (1946) estimated an LC_{50} of 3,300 mg/m^3 for 30 min for lewisite vapor absorption through the bare skin and an inhalation LC_{50} of 120 mg/m^3 for 10 min and 50 mg/m^3 for 30 min. Inhalation of 10 mg/m^3 lewisite for 30 min may result in severe intoxication and incapacitation lasting for several weeks, and inhalation of 10 mg/m^3 for 15 min caused inflammation of the eyes and swelling of the eyelids (Franke, 1977). Like sulfur mustard, moist tissues are particularly sensitive to lewisite. The eyes exhibit the greatest sensitivity (IOM, 1993).

The vesicant properties of lewisite result from direct contact with the skin. Signs of dermal toxicity (pain, inflammation) may be experienced within a minute after exposure. Acute lethality is usually the result of pulmonary injury. Ocular exposure may result in corneal necrosis. Due to its lipophilicity, percutaneous absorption of lewisite is rapid and, at a sufficient exposure, may be associated with systemic toxicity characterized by pulmonary edema, diarrhea, agitation, weakness, hypothermia, and hypotension (IOM, 1993). The threshold for severe systemic toxicity in humans following dermal exposure to lewisite has been estimated at 10 mg/kg (9.1–13.4 mg/kg) (Sollman, 1957).

Eldridge (1923) conducted tests on human volunteers to assess the effects of dermal exposure to lewisite vapor. The arms of men (one to seven men with previously determined average sensitivity to lewisite) were exposed to varying concentrations of lewisite vapor for periods ranging from 10 min to 3 h for the purpose of determining the concentration of lewisite required for blistering. The resulting dermal responses ranged from reddish discoloration to a clear watery blister over the entire burned area, accompanied by reddening, swelling, and hardening of the surrounding skin. The burns reached maximum severity in 36–48 h, and healing was complete in 6 days to 2 weeks. The men reported that the healed skin remained sensitive for several weeks after the healing was complete.

It has been hypothesized that fatalities following dermal exposure to lewisite may be due to blood plasma loss resulting from extensive capillary damage (lewisite shock) (Cameron *et al.*, 1946). Sollman (1957) estimated that an oral dose of as little as 2 ml in an adult human (equivalent to 37.6 mg/kg) may be fatal within several hours. The target tissues and organs for systemic toxicity of lewisite include the liver, gall bladder, urinary bladder, lung, and kidneys (Cameron *et al.*, 1946; Snider *et al.*, 1990). Generally, there is a data deficiency regarding definitive exposure–response data for lewisite.

In studies with rats, Silver and McGrath (1943) found little difference in the acute lethality of *cis*- or *trans*-lewisite exposed for 10 min. Ten-minute mouse LC_{50} values for the *cis*- and *trans*-isomers were 190 and 200 mg/m^3, respectively. All mice exposed to 240 mg/m^3 lewisite for 10 min died. Clinical signs in dogs exposed for 7.5 or 15 min included immediate continual eye blinking, followed by excessive nasal secretion, lacrimation, and sneezing (Armstrong, 1923). Ocular inflammation and vomiting also occurred in some dogs by the end of the 7.5- and 15-min exposures. Dogs exposed for 30 min or longer exhibited frequent retching, vomiting, extreme salivation, labored breathing, and inflammation of the entire respiratory tract. Necropsy revealed a thick membrane in the nostrils, larynx, and trachea, which was accompanied by purulent bronchitis, hemorrhage, pneumonia, edema, and congestion of the lungs.

Similar to the work on sulfur mustard, Sasser *et al.* (1989a) conducted experiments in rats given lewisite by gastric intubation (in sesame oil) at doses of 0.01, 0.1, 0.5, 1.0, or 2.0 mg/kg, 5 days/week for 13 weeks. A dose-related response was observed for lethality (deaths in the three

highest dose groups) and frequency and severity of forestomach lesions. The forestomach lesion incidence and severity were due, at least in part, to the bolus of dosing and the sesame oil vehicle.

Multigeneration reproductive studies in rats (Sasser *et al.*, 1989b) and teratology studies in rats and rabbits (Hackett *et al.*, 1987) given lewisite by gastric intubation were negative or compromised by concurrent maternal toxicity.

The carcinogenic potential of lewisite is not well understood. In a long-term follow-up study, Krause and Grussendorf (1978) reported the formation of a malignant lesion at the site of contact 8 years following a single, acute dermal exposure of a German soldier accidentally exposed to liquid lewisite on his lower right leg in 1940. Eight years later the lesion was diagnosed as malignant. Thirty-eight years after exposure, the area of contact remained ulcerated and diagnosed as Bowen's disease (an intradermal squamous cell carcinoma). Bowen's disease was also diagnosed in workers at a Japanese lewisite production facility (Inada *et al.*, 1978). Findings in these workers were not conclusive due to concurrent exposure to diphenylcyanoarsine and sulfur mustard. Furthermore, no quantitative estimates of dose or exposure rates were available (Inada *et al.*, 1978).

Increased incidences of cancer mortality (respiratory tract: 14%; digestive tract: 9.6%) in workers from the Okuno-Jima poison gas factory were reported by Wada *et al.* (1968). When cancer rates were correlated with job classification, the frequency of respiratory and gastrointestinal tract neoplasms was highest in the workers who were involved in the production of sulfur mustard or lewisite, followed by those who worked indirectly with sulfur mustard or lewisite. The lowest frequency occurred in the group having no direct contact with the vesicant agents (Yamakido *et al.*, 1985). Similar to the Bowen's disease findings, the cancer incidences were confounded by the fact that workers were also exposed to sulfur mustard, hydrocyanic acid, diphenylcyanoarsine, chloroacetophenone, and phosgene.

VI. RISK ASSESSMENT

A. Sulfur Mustards

1. Noncancer

Various standards and guidelines have been developed for sulfur mustard. These values are applicable to occupational exposures, emergency planning and response efforts, and remediation efforts. Airborne exposure limits (AELs) and health-based environmental screening levels (HBESLs) for sulfur mustard have been developed by the US Army (USACHPPM, 1999, 2000). Most health-based criteria for sulfur mustard vapor exposure are based upon protection of the eyes and respiratory tract which are the most sensitive targets.

Acute Exposure Guideline Levels (AEGLs) for sulfur mustard have been developed for emergency planning and emergency response applications (NRC, 2003). The AEGLs represent threshold exposure limits for the general public and are applicable to emergency exposure periods ranging from 10 min to 8 h.

Reference doses (RfDs), an estimate of a daily dose to humans that is likely to be without appreciable risk of deleterious health effects during a lifetime, have also been developed for sulfur mustard (NRC, 1999).

The various guidelines and standards for sulfur mustard have been summarized by ATSDR (2003).

2. Cancer

The International Agency for Research on Cancer (IARC) classified sulfur mustard as a Group 1 carcinogen (carcinogenic to humans) (IARC, 1987). The National Toxicology Program (NTP) considers ''mustard gas'' as a substance ''known to be a human carcinogen'' (DHHS, 1998). These assessments are based upon human and animal data.

Studies of occupational exposures to sulfur mustard indicate an elevated risk of respiratory tract and skin tumors following long-term exposure to acutely toxic concentrations. Overall, several factors are important regarding the assessment of the carcinogenicity of sulfur mustard. Increased cancer incidence in humans appears to be associated only with exposures that caused severe acute effects, and occupational exposures tended to involve repeated exposures and repeated injury of the same tissues. Because the therapeutic use of the sulfur mustard analog nitrogen mustard is associated with an increased incidence of CML, the reports of CML in HD-exposed individuals appear to be relevant to the carcinogenicity of sulfur mustard.

Cancer slope factors and unit risk values for sulfur mustard have been summarized by ATSDR (2003).

B. Nitrogen Mustards

1. Noncancer

Very few standards and guidelines are available for nitrogen mustards. AEGL values for the nitrogen mustards, HN1, HN2, and HN3, have been developed and are based upon ocular irritation in human volunteers (AEGL-2) and lethality in rodents (AEGL-3). Data were insufficient for derivation of level AEGL-1 values. The AEGL values are currently awaiting finalization. The US Army (USACHPPM, 1996, 2004) has developed Worker Population Limit (WPL) values and General Population Limit (GPL) values for nitrogen mustard (USACHPPM, 1996, 2004).

2. Cancer

Data are not available with which to quantitatively assess the cancer risk from nitrogen mustards, although IARC (1987) considers nitrogen mustard a Group 2A carcinogen based upon limited evidence in humans and sufficient evidence in animals.

C. Lewisite

1. Noncancer

The US Army has developed HBESLs for lewisite (USACHPPM, 1999). Additionally, a chronic oral reference dose (RfD) is available (NRC, 1999; USACHPPM, 1999), as are inhalation and dermal RfDs (USACHPPM, 1999). Interim Acute Exposure Guidelines values (AEGLs) have been developed for lewisite.

2. Cancer

Data regarding the potential carcinogenicity of lewisite are anecdotal and insufficient for a quantitative assessment. Although quantitative data are lacking, the position maintained by CDC (DHHS, 1988) is that some evidence suggests that lewisite may be a carcinogen. For environmental exposure and remediation concerns, the arsenic component and/or arsenic-containing degradation products are, however, relevant.

Although the carcinogenicity of lewisite is equivocal and a quantitative assessment not feasible, several lewisite degradation products are known carcinogens. Combustion products of lewisite include the inorganic arsenicals, arsenic trichloride, arsenic trioxide, and vinyl chloride. Inorganic arsenic is carcinogenic in humans and animals and is classified as a Group A carcinogen by the US EPA (2008). Arsenic trioxide and vinyl chloride are both considered Group A carcinogens by the US EPA (US EPA, 1984) and Group 1 carcinogens by IARC (IARC, 1987).

VII. TREATMENT

A. Sulfur Mustard

Medical management of sulfur mustard exposure begins with prevention of exposure. As previously noted in this chapter, the military use of sulfur mustard necessitated full-body protection. As a result, considerable effort has been expended in the development and evaluation of protective clothing and equipment (Schier and Hoffman, 2005). In general, these include respirators (air-purifying and atmosphere-supplying), and chemical-protective clothing (e.g. chemical and vapor impermeable coverings, clothing treated with adsorbing or neutralizing chemicals). Following exposure, rapid decontamination is essential and may include removal of contaminated clothing and removal/neutralization of the agent. Ocular exposure will necessitate rapid removal of the agent from the eyes by irrigating with water. Vapor exposure may necessitate respiratory support. Because there are no antidotes for sulfur mustard poisoning, medical management must rely on prevention, decontamination, and palliative treatment of signs and symptoms. The use of possible antidotes (e.g. antioxidants) has been reviewed by Smith *et al.* (2008) and polyurethane sponges containing detoxification additives are currently being developed and evaluated for decontamination/detoxification (Gordon *et al.*, 2006). The medical management of sulfur mustard (and other vesicant agents) has been reviewed by Munro *et al.* (1990) and Keyes *et al.* (2005).

B. Nitrogen Mustards

Medical management of nitrogen mustard exposure is similar to that for sulfur mustard and involves prevention of exposure and, where exposure has occurred, decontamination and support therapy. The use of antioxidants in the treatment of nitrogen mustard toxicity is currently under investigation (Hardej and Billack, 2006).

C. Lewisite

Similar to the mustard agents, exposure prevention is the first line of defense against lewisite. Rapid decontamination is especially relevant to lewisite exposure due to the rapid development of pain (1–2 min) associated with lewisite exposure. Unlike other vesicants, an effective antidote for lewisite toxicity exists in the form of British anti-lewisite (BAL; 2,3-dimercaptopropanol) which binds with arsenicals, thereby countering the lewisite-induced damage. Such chelation therapy is associated with notable side effects (e.g. renal effects) and requires careful medical management. More effective analogs of BAL have been developed with less significant side effects.

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

As chemical warfare agents, vesicants have received considerable attention over the last two decades due to concerns regarding destruction of agent stockpiles, remediation of contaminated sites, the documented and speculated use of these agents in regional conflicts, and possible use in subversive/terrorist activity. This elevated interest profile has resulted in summaries of older toxicological data, generation of new data, and a greater understanding of the effects of these agents on biological systems. Application of these data have been invaluable in the development of various health-based criteria, standards, and guidelines for use in remediation efforts, risk planning, and emergency response activities.

Future directions appear to focus on acquiring additional in-depth understanding of the mechanism of action of these agents through the development of experimental models for vesicant-induced injury, and an application of this information in the development of therapeutic measures for the prevention and treatment of vesicant-induced injury.

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CHAPTER 9

Arsenicals: Toxicity, their Use as Chemical Warfare Agents, and Possible Remedial Measures

SWARAN J.S. FLORA, GOVINDER FLORA, AND GEETU SAXENA

I. INTRODUCTION

Arsenic is a metalloid (semi-metal) member of group V elements of the periodic table having oxidation states of +3 (As III) and +5 (As V), in which the former (As III) is more toxic than the latter (As V) (Aposhian *et al.*, 2003). Both species occur in organic as well as inorganic compounds. Arsenic combines chemically with most nonmetals to form a variety of both inorganic and organic compounds. Organoarsenic compounds with trivalent arsenic were produced as pesticides as well as chemical warfare agents in the first half of the 20th century (Aposhian *et al.*, 2003; Li *et al.*, 2005). A number of organic arsenicals have been developed for use as chemical warfare agents. These arsenic-containing substances in the chemical weapon's program are of human and ecotoxicologic relevance. The story of arsine and lewisite encapsulates the key elements of the history of chemical weapons and their continuing power (Kunz, 1994; Stanek, 1991; Sugden, 2008). Although information about arsenic and its inorganic and organic derivatives is well documented, there is very little literature available on their role as chemical warfare agents. This chapter provides readers with updated information about the organic arsenicals as chemical warfare agents and also gives a comprehensive account of toxicity due to inorganic arsenicals. Arsenic poisoning has recently assumed an alarming proportion in some nine districts of West Bengal, India, so much so that it has been earmarked as ''the biggest arsenic calamity in the world''. In view of the rapid spread of various diseases arising out of arsenic contamination (e.g. arsenical dermatosis, melanosis, keratosis, edema, gangrene) in different areas of West Bengal and also in adjoining areas of Bangladesh, there is a need to provide the reader with information about the modes and sites of action following exposure to environmentally relevant levels of arsenicals and to determine the effects of arsenicals in susceptible populations.

II. BACKGROUND

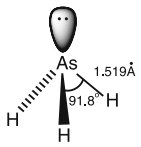
Arsenic is well known as an insecticide in the form of lead arsenate, arsenic acid, etc., and in pharmacy, especially in the form of salvarsan and neosalvarsan. Arsenic derivatives are also of value from the point of view of chemical warfare agents (Stanek, 1991; Wexler, 2004). World War I marked a new era in the modern warfare as persistent rumors were circulated in the early part of the war that the Germans were to use arsine (Szinicz, 2005; Thomas and Young, 2001). These rumors led to the use of sodium permanganate in the canister, but no arsine was actually used. Another suggestion which received considerable attention was the use of arsenide, which might decompose under the influence of atmospheric moisture with the liberation of arsine (Henriksson *et al.*, 1996; McManus and Huebner, 2005). However, calculation of the amount of arsenide required to establish a lethal concentration of arsine suggests no possibility of using the material in the field. Organic arsenic derivatives are the most important compounds from a military point of view. The first substance used was diphenylchloroarsine, a white solid, which readily penetrated the canister and caused sneezing. This was used alone, and in solution in diphenylchloroarsine. Later, methyl and ethyl dichloroarsines were introduced (Beckett, 2008).

Arsine, the most toxic form of arsenic, exhibits some characteristics that may make it useful as a chemical warfare (CW) agent. Arsine is a colorless, odorless, nonirritating gas and is 2.5 times denser than air (Henriksson *et al.*, 1996; Pullen-James and Woods, 2006; Thomas and Young, 2001). At concentrations above 0.5 ppm, a garlic-like odor may be noted, but arsine is toxic at much lower concentrations. Acute arsine poisoning due to inhalation of arsine gas (AsH_3) is rare but has no known antidote. It is the most acutely toxic form of arsenic causing rapid and severe hemolysis immediately on exposure. The mechanisms of hemolysis are not completely understood. Arsine has a short half-life (27–96 h) and is converted to various arsenic derivatives. Although it

has been investigated as a CW agent, arsine has no recorded battlefield use. During and prior to World War II, the British studied this agent and rejected its use in the field. They concluded it was more than ten times less toxic than phosgene (CG). In addition, it is difficult to manufacture and is highly flammable. Although it was determined that arsine was not a useful battlefield CW agent, it may still be useful as a small-scale weapon of assassination or terror. A number of other arsine-derived organoarsenic compounds have been developed and used as CW agents, including lewisite (L), methyldichloroarsine (MD), diphenylchloroarsine (DA), and ethyldichloroarsine (ED) (Ishizaki *et al.*, 2005).

Arsenicals are considered a threat, not so much from large nation states but from smaller, less developed nations and/or by terrorist organizations. The relative ease of production coupled with their effectiveness against an unprotected population make organic arsenicals a continued threat in the 21st century. This chapter describes the human health aspects of arsine, organic arsenicals, and inorganic arsenic, and the current status of development of suitable therapeutic measures.

III. ARSINE



Arsine (AsH_3) is a colorless, extremely flammable gas with a garlic-like odour. The most common synonyms for arsine are arsenic hydride, arsenic trihydride, hydrogen arsenide, and arsenous hydride. The relative molecular mass of arsine is 77.95. Its boiling point is −62°C and vapor pressure at 20°C is 1043 kPa. Arsine is a strong reducing agent, deposits arsenic on exposure to light and moisture, and is easily transformed into other oxidized arsenic forms [e.g. As (III) and As (V)]. The arsine gas is colorless, odourless, and 2.5 times denser than air (Table 9.1). Arsine is a class of organoarsenic compounds of the formula $AsH_{3-x}R_x$, where R = aryl or alkyl (Fowler and Weissber, 1974).

Possible sources of occupational exposure are many and include the semiconductor industry during microchip production and other industries in which workers are involved in galvanizing, soldering, etching, and lead plating (Landrigan *et al.*, 1983). It also can be produced inadvertently by mixing arsenic-containing insecticides and acids (Pullen-James and Woods, 2006). In humans and animals, arsine is metabolized to trivalent arsenic as well as pentavalent arsenic. Arsenic (III) is methylated to monomethylarsonate (MMA) and dimethylarsinate (DMA). Arsine metabolites are mainly excreted via urine (Apostoli *et al.*, 1997).

Arsine is supposed to be the most toxic form of arsenic. The acute toxicity of arsine is high in different species including humans. The target organ of arsine is the hematopoietic system and, in particular, the erythrocytes (Blair *et al.*, 1987). Inhaled arsine gas is distributed rapidly and causes massive red blood cell hemolysis that can potentially lead to cellular hypoxia (Apostoli *et al.*, 1997; Hatlelid et al., 1995, 1996; Peterson and Bhattacharyya, 1985). The mechanisms of action involved in hemolysis are not elucidated fully, but studies (Hong *et al.*, 1989; Winski *et al.*, 1997) reveal: (1) arsine causes a nonspecific disruption of ion gradients, leading to cell membrane instability, (2) sulfhydryl groups in cell membranes are probable targets of arsine toxicity, and (3) hemoglobin is an important subcellular target of arsine toxicity.

TABLE 9.1. Physical and chemical properties of arsine

Molecular formula	AsH_3
Molar mass	77.95 g/mol
Appearance	Colorless gas
Density	4.93 g/l, gas; 1.640 g/ml (−64°C)
Melting point	−117°C (157 K)
Boiling point	−62.5°C (210 K)
Solubility in water	0.07 g/100 ml (25°C)
Molecular weight	77.95

Arsine poisoning can lead to acute renal tubular necrosis and ultimately to oliguric/anuric renal failure (Rogge *et al.*, 1983). Renal failure can be attributed to (1) direct effect of arsine on renal tissue, (2) heme-pigment nephropathy, or (3) renal hypoxia secondary to massive hemolysis and decreased oxygen carrying capacity of the blood.

Effects of long-term exposure to low levels of arsine are not well documented; however, most of the reported deaths are believed to be secondary to acute renal failure. Exposure to other arsenic compounds to which arsine is metabolized can induce lung, bladder, kidney, and skin cancer in humans (Kleinfeld, 1980; Lenza, 2006).

A. Synthesis of Arsine

Arsine is formed whenever nascent hydrogen is released in the presence of arsenic or by the action of water on a metallic arsenide. The formation of arsine can be described with the help of the following reaction (Anthonis *et al.*, 1968a, b; Bellama and Macdiarm; 1968; Coles *et al.*, 1969; Jenkins *et al.*, 1965):

$$3\ H_2 + HAsO_2 \rightarrow AsH_3 + 2\ H_2O$$

AsH_3 is also prepared by the reaction of As^{3+} sources with H^- equivalents (Bellama and Macdiarm, 1968):

$$4\ AsCl_3 + 3\ NaBH_4 \rightarrow 4\ AsH_3 + 3\ NaCl + 3\ BCl_3$$

Alternatively, sources of As^{3-} react with protonic reagents to produce arsine:

$$Zn_3As_2 + 6\ H^+ \rightarrow 2\ AsH_3 + 3\ Zn^{2+}$$

B. Sources of Exposure

The main anthropogenic sources of arsine include its accidental formation, particularly in the chemical and nonferrous (like zinc, copper, and cadmium) metallurgical industries, production or use of the gas itself during manufacture of semiconductors as a doping agent (Aposhian, 1997; Winski *et al.*, 1997) and in battery production as an alloy with lead (Wald and Becker, 1986).

Arsine is extensively used in the semiconductor industry for epitaxial growth of gallium arsenide (GaAs) and as a dopant for silicon-based electronic devices (Sheehy and Jones, 1993). Arsine is also used in organic synthesis (Lewis, 1993), as an agent in the manufacture of light-emitting diodes, and for manufacturing certain glass dyes (HSDB, 1999).

C. Human Arsine Exposure

Occupational sources where exposures to arsine at levels sufficient to cause acute arsine intoxication have occurred include copper smelting and refinery (Pinto *et al.*, 1976), bronzing process (Clay *et al.*, 1977), a chemical company cleaning a clogged drain (Parish *et al.*, 1979), transistor industry (Kleinfeld, 1980), and burnishing of metals (Romeo *et al.*, 1997). Many processes including electrolyte refining, galvanizing, soldering, etching, lead plating, metal smelting, and extraction may expose workers to toxic concentrations of arsine. Workers in the electronics industry using GaAs to manufacture GaAs optoelectronic, microwave, and integrated circuit products are potentially exposed to arsine (Chein *et al.*, 2006; Sheehy and Jones, 1993). Unintentional formation of arsine can occur principally in the metallurgical industry as a result of arsenic contamination of many ores, such as zinc, lead, copper, cadmium, antimony, gold, silver, and tin (Braman, 1977; Fallenti *et al.*, 1968).

D. Metabolism of Arsine

1. Animal Studies

Levvy (1947) reported that an average of 64% of inhaled arsine was absorbed in mice exposed by inhalation route at concentrations of 25–2500 mg/m^3 for periods ranging from 0.40 min to 24 h. Blair *et al.* (1990b) measured the arsenic content in liver after exposure of rats to variable arsine concentrations (0.08, 1.6, and 8.1 mg/m^3 for 6 h/day for 90 days). Arsenic concentration in liver increased with airborne arsine concentration and was higher in females than in males. The arsenic level in 3–4 days after a 90 day exposure at a concentration of 8.1 mg/m^3 was 6–8 µg/g (compared with approximately 1.5 µg/g in controls).

The main route of arsine excretion is via urine after metabolism. Levvy (1947) studied the elimination of arsenic in arsine-exposed mice and compared it with the animals exposed to sodium arsenite. Arsenic was excreted exponentially in intravenously arsenite-administered mice and after 24 h, less than 10% of the dose remained. On the other hand, arsenic arising from inhalation exposure to 180 mg arsine/m^3 for 120 min was excreted more slowly and after 24 h about 45% of arsenic remained in the exposed mice. Buchet *et al.* (1998) exposed rats to 4–80 mg arsine/m^3 for 1 h; the major metabolites determined in urine were As (III) and As (V), MMA and DMA. When the exposure exceeded 60 mg/m^3, the proportional urinary excretion also showed an increase, indicating saturation of arsine/arsenic binding.

2. Human Studies

Arsine is rapidly absorbed into blood through the respiratory tract (Venugopal and Luckey, 1978). Arsenic can be detected in blood after a few days of exposure. The highest quantities of arsenic were found in liver, kidney, and spleen, and smaller amounts of arsenic were also found in the hair of workers occupationally exposed to arsine (Romeo *et al.*, 1997). Apostoli *et al.* (1997) detected the presence of arsenic in tissues, blood, and urine of workers in the petroleum industry who were poisoned with arsine. In a fatal case of arsine poisoning, arsenic was found in the liver at a concentration of 11.8 mg/g, spleen at 7.9 mg/g, kidneys at 3.2 mg/g, brain at 0.6 mg/g, and in the urine at 0.6 mg/ml. Trace amounts were also found in the blood (Apostoli *et al.*, 1997).

Inhaled arsine was rapidly dissolved in body fluids and oxidized to As (III) (Apostoli *et al.*, 1997). Part of As (III) is further oxidized to As (V), as indicated by the appearance of As (V) in urine of humans exposed to arsine 1–2 days following exposure. Trivalent arsenic is methylated to MMA and DMA (Romeo *et al.*, 1997).

E. Mechanism of Toxicity

After inhalation of arsine gas it causes rapid destruction of red blood cells leading to hypoxia and renal failure. The mechanism of toxicity and modes of action of arsine in humans and animals have not been fully studied. Two mechanisms have been proposed for arsine poisoning, (1) reaction with sulfhydryl groups (Blair *et al.*, 1990b; Waters *et al.*, 2004; Winski *et al.*, 1997) and (2) oxidative stress (Blair *et al.*, 1990a, b; Hatlelid *et al.*, 1996; Hatlelid and Carter, 1997). Besides these two mechanisms nonspecific disruption of ion gradient leading to cell membrane instability and lysis of red blood cells have also been proposed as mechanisms for the toxic effects of arsine (James and Woods, 2006). Hatlelid and Carter (1997) postulated that the hemolytic activity of arsine is related to oxidative stress through the formation of hydrogen peroxide and arsine adducts with hemoglobin, according to the following reaction:

$$H_2As - H + HbO_2 \rightarrow H_2As^* + 2\ Hb - O_2 - H$$

These products may then react to form methemoglobin and arsine peroxide, or, alternatively, the reaction may

produce hydrogen peroxide and arsenic adduct such as H_2As-H or H_2As-heme:

$$H_2As^* + 2\ Hb - O_2 - H \rightarrow adduct + H_2O_2$$

Such an adduct may probably damage hemoglobin molecules, leading to the rapid denaturation and precipitation of the proteins (Hatlelid and Carter, 1997).

Some studies have suggested that the sulfhydryl groups of glutathione (GSH) prevented hemoglobin oxidation and in this manner are essential for the maintenance of intact erythrocyte structure (Blair *et al.*, 1990b). In an *in vitro* study, a decrease in reduced GSH concentration in human RBCs was found to correlate with the hemolytic action of arsine (Pernis and Magistretti, 1960). Blair *et al.* (1990a) recorded a 60% decrease in reduced GSH level in erythrocytes exposed to arsine *in vitro*. However, later studies by Hatlelid *et al.* (1995) showed that the depletion of reduced GSH in RBCs in dogs neither preceded nor coincided with hemolysis.

According to the hypothesis of the sulfhydryl-dependent mechanism of arsine toxicity (Levinsky *et al.*, 1970), arsine reacts with the sulfhydryl group of Na^+/K^+-ATPase, causing an impairment in the sodium–potassium pump which subsequently causes red cell swelling and hemolysis. The affinity of trivalent arsenic for the sulfhydryl group is well known. Winski and co-workers reported profound abnormalities in membrane ultrastructure and in red blood cell volume (Winski et al., 1997), which were manifested by potassium leakage, sodium influx, and increases in hematocrit in arsine-exposed red cells, although no change in ATP and ATPase was observed following exposure to arsine. Hemolysis in arsine exposed RBCs was dependent on membrane disruption caused by arsine-hemoglobin metabolites, the ultimate toxic species (Winski *et al.*, 1997).

F. Effects on Humans

The data on arsine concentration in the workplace atmosphere are relatively scant. Toxicity of arsine to humans was first demonstrated in 1815 when a German chemist accidentally inhaled arsine vapor during an experiment. He became ill and soon died. A case report indicated that exposure to arsine by inhalation for a few hours at a concentration of 10–32 mg/m^3 might induce symptoms of poisoning, whereas exposure to 810 mg/m^3 for 30 min might be fatal (Romeo *et al.*, 1997). It has been reported that concentrations of 23–970 mg arsine/m^3 were associated with fatalities (Morse and Setterlind, 1950).

G. Acute Arsine Poisoning

Most patients report little or no discomfort at the time of exposure. Although a garlic-like odor may be noted with higher ambient arsine concentrations, serious toxicity may result from clinically nondetectable exposures. Following exposure, a dose-dependent latent period ensues, lasting up to 24 h (Apostoli *et al.*, 1997; Lenza, 2006; Levvy, 1947; Song *et al.*, 2006; Thomas and Young, 2001; Young *et al.*, 2003). Symptoms following sublethal arsine exposure may include abdominal pain, hematuria, and jaundice (Figure 9.1).

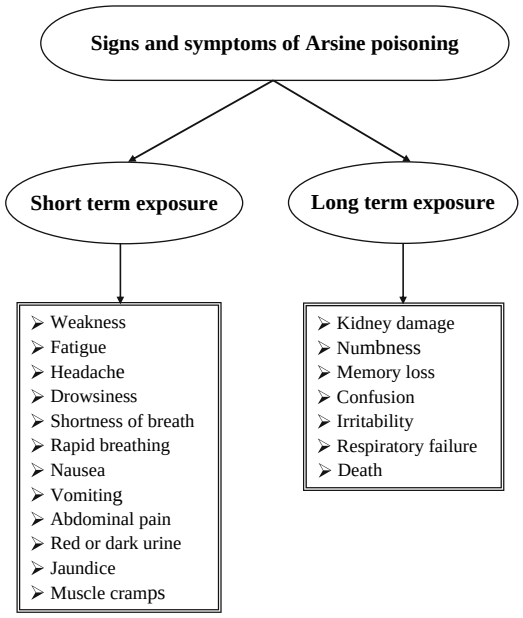


FIGURE 9.1. Signs and symptoms of arsine poisoning.

1. Physical Signs

Physical signs and their severity depend on the concentration of arsine gas and the duration of the exposure (Apostoli *et al.*, 1997; Lenza, 2006; Levvy, 1947; Song *et al.*, 2006; Thomas and Young, 2001; Young *et al.*, 2003).

- Vital signs – hyperthermia, tachypnea, tachycardia, hypotension.
- Head, ears, eyes, nose, and throat (HEENT) – discoloration of conjunctivae (red, orange, brown, or brassy; reportedly distinct from hyperbilirubinemia), scleral icterus, garlic odor to breath (possible).
- Pulmonary – rales from acute respiratory distress syndrome (ARDS) in severe exposure
- Gastrointestinal – abdominal tenderness, hepatomegaly.
- Genitourinary – costovertebral angle tenderness, colored urine (red, brown, or green from hemoglobinuria and/or methemoglobinuria).
- Extremities – possible paresthesias and Mees lines with chronic arsenic toxicity from arsine exposure.

2. Causes

- Arsine gas is used in the semiconductor industry when depositing arsenic on microchips. Exposure also may occur from producing, cleaning, or reclaiming GaAs wafers (Carter *et al.*, 2003; Chein *et al.*, 2006).

- Arsine is released during the production of hydrogen in an acid medium in contact with arsenic-contaminated metals (Braman, 1977).
- Arsine might be a potential chemical warfare agent (Lenza, 2006).

H. Immediate Effects

Clinical manifestation of arsine intoxication appears within 24 h of exposure (usually within a few hours). The period of latency depends on concentration and time of exposure. The initial symptoms include headache, malaise, weakness, dyspnoea, dizziness, abdominal pain, nausea, and vomiting. The urine might be dark red, usually 4–6 h following exposure, and jaundice of the skin and mucous membranes might appear usually 24–48 h after exposure. In some cases, hepatomegaly and splenomegaly with tenderness of costovertebral angle, fever, tachycardia, and tachypnea occur. Information on the concentration of arsine in the air or on the duration of the exposure in relation to the effects observed is mostly not available (Kleinfeld, 1980).

Hemolytic anemia is the most consistent clinical finding in humans. Massive hemoglobinuria may lead to anuria, which, if untreated, is often the cause of death. Both central and peripheral nervous systems may also be affected. Toxic pulmonary edema and acute circulatory failure have also been reported as the cause of death in arsine poisoning (Hatlelid *et al.*, 1996; Winski *et al.*, 1997).

I. Late Effects

Late consequences of acute arsine poisoning include chronic renal damage, hematological changes, polyneuritis, and neuropsychological symptoms (e.g. irritation, confusion, memory losses, agitation, and disorientation). Morphological changes in the kidneys of a truck driver with arsine-induced anuria have been reported earlier (Muehrcke and Pirani, 1968). Six months after arsine poisoning, the patient showed anemia and azotemia. Twenty-three months after recovery from acute renal failure, interstitial fibrosis was focal, and severe nephrosclerosis with renal insufficiency was present. Gosselin and co-workers described reversible polyneuritis of the upper and lower extremities that was observed 3 months after exposure. Peripheral neuropathy was still present 6 months after exposure (Gosselin *et al.*, 1982). Extreme restlessness, loss of memory, agitation, and disorientation occurred several days after exposure and lasted about 10 days in two patients heavily exposed to arsine. An increase in total cell count and macrophages in bronchoalveolar lavage was observed in an arsine-exposed worker. Progressive improvement in diffusing capacity of lungs was observed only after 2 months of treatment. Pinto and co-workers reported that electrocardiographic (ECG) changes in one case lasted for 10 months. Vertical white lines on the nails were observed in many cases 10 days to 3 weeks following arsine exposure (Pinto *et al.*, 1976). Hepatitis in an arsine-poisoned patient on the 20th day after the acute hemolysis has also been reported (Mora *et al.*, 1992).

J. Long-Term Exposure

Long-term exposure may cause symptoms similar to those observed in acutely poisoned individuals. The main differences from acute poisoning were in a delay in onset and development of peripheral neuritis, development of gastrointestinal tract involvement, and development of hemolysis and renal impairment (Risk and Fuortes, 1991; Watson and Griffin, 1992). Lowered hemoglobin levels were found in zinc ore smelting workers exposed to arsine for long periods and who had urinary arsenic concentration below 0.2 mg/liter. These urinary concentrations were estimated to correspond to air arsine concentrations below 0–16 mg/m^3. Once a special ventilation system had been installed, the hemoglobin levels in the workers gradually returned to their normal values (Risk and Fuortes, 1991; Watson and Griffin, 1992).

K. Diagnostic Tests

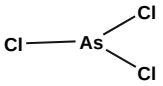
1. Laboratory Studies

- Complete blood count
  - o Hemolytic anemia – may be severe and rapidly developing, with pink serum resulting from free hemoglobin
  - o Elevated white blood cell count – may be seen early
- Methemoglobinemia
- Urinalysis
  - o Hemoglobinuria
  - o Proteinuria
  - o Serum chemistry panel
  - o Hemolysis can cause hyperkalemia, elevated lactate dehydrogenase, and hyperbilirubinemia
  - o Renal failure can cause elevated creatinine and blood urea nitrogen (BUN) levels
  - o Hepatic transaminases may be elevated
- Arsenic levels
  - o Blood and urine arsenic levels are elevated acutely
  - o A 24-h urine arsenic may help in monitoring chronic, low-level arsine exposures
- Electrocardiogram
  - o Peaked T waves from hyperkalemia may be seen
  - o Nonspecific ST segment and T wave abnormalities have been reported
  - o QT interval prolongation is possible from arsenic toxicity
- Imaging studies
  - o No routine imaging studies are indicated
  - o Chest radiography is indicated to detect acute respiratory distress syndrome (ARDS) in patients with pulmonary symptoms

2. Complications

- Hemolytic anemia
- Renal failure
- Hyperkalemia
- Death
  - o Overwhelming exposures cause rapid death from massive hemolysis
  - o Most deaths occur from renal failure in patients who survive acute exposure
- Chronic arsenic toxicity – patients surviving acute arsine exposure may develop chronic arsenic toxicity, including anemia and peripheral neuropathy.

IV. ORGANIC ARSENICALS



The arsenicals are a series of blister agents based around a chloroarsine ($AsCl_3$) molecule in which one of the chlorine atoms is replaced by an organic radical.

These chloroarsines are effective cytochrome oxidase destroyers, or blood agents. Arsenic seeks to replace calcium in the bones, thus causing bone marrow destruction as the endocrine system is concurrently attacked (Styblo and Thomas, 1997). Many organic radicals penetrate human skin, carrying their compounds with them (Cohen *et al.*, 2006).

A. Background

Interest in organic arsenicals dates back to the mid-19th century. Chemists discovered that an arsenic-chloride compound (chloroarsines) in which one of the chlorine atoms is replaced by an organic radical tends to be harmful both to insects and to human tissue (Bartelt-Hunt *et al.*, 2008; Beckett, 2008; Kunz, 1994; McManus and Huebner, 2005; Vilensky and Redman, 2003). The trench warfare stalemate during World War I created a tactical need for a chemical weapon that was both short acting (e.g. nonpersistent, volatile) and lethal (Stephenson, 2006). To fill this need, first weaponized organic arsenical, methyldichloroarsine (MD), was delivered. Two additional organic arsenicals, diphenylchloroarsine (DA) and ethyldichloroarsine (ED), soon augmented MD. A fourth organic arsenical named lewisite was discovered by US Army Medical Corps but was never deployed in World War I (Bartelt-Hunt *et al.*, 2008; Beckett, 2008; Kunz, 1994; McManus and Huebner, 2005; Vilensky and Redman, 2003).

B. Mechanism of Toxicity

The exact mechanism for the toxic effects of organic arsenicals is unknown. DNA alkylation and/or inhibition of glutathione-scavenging pathways are two postulated mechanisms (Nesnow *et al.*, 2002). On contact with arsenicals a blistering reaction occurs on skin, eye, or pulmonary tissues. The onset of symptoms after arsenical exposure occurs in seconds as compared to 4–8 h for mustard exposure. Either a liquid or vapor can cause toxicity. The organic arsenicals tend to have high volatility at room temperature and thus pose a significant vapor threat following exposure (Carter *et al.*, 2003; Cohen *et al.*, 2006; Devesa *et al.*, 2006; Gao and Burau, 1997; Kojima *et al.*, 2006) (Figure 9.2).

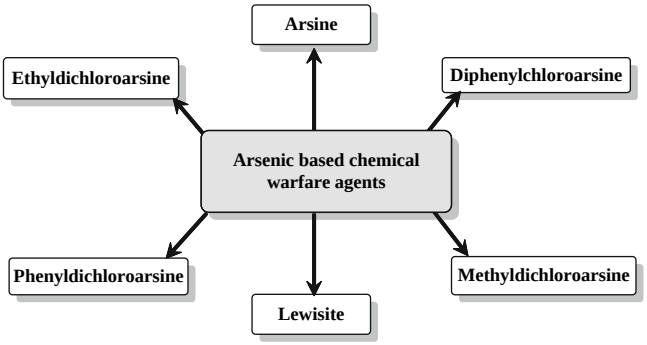


FIGURE 9.2. Arsenic-based chemical warfare agents.

Animal data and limited human trials suggested that organic arsenicals readily penetrate the skin. Within seconds of contact, the chemical fixes itself to the epidermis and dermis. Pain is immediate followed by destruction of subcutaneous tissue. The separation of dermis from epidermis together with capillary leakage causes fluid-filled vesicles (McManus and Huebner, 2005; Noort *et al.*, 2002).

C. Symptoms

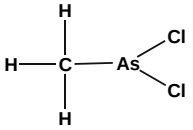
- Vapor contact with the conjunctiva may be the victim's first symptom. Severe conjunctival irritation and blepharospasm result upon eye contact leading to loosening of corneal epithelial cells and swelling and edema of the cornea.
- The respiratory tract's mucosa and submucosa are susceptible to vapor exposure. Mucosal damage starts in the nose and descends down the respiratory mucosa in a dose-dependent fashion. Immediate pain, lacrimation, and irritation accompany the damage. Damaged respiratory mucosa slough off, filling the airways with debris. Damage to the lung parenchyma causes the secretion of blood and mucus that, with the pseudomembranes, can cause asphyxiation.
- The gastrointestinal tract is also susceptible. DA vapor in particular produces a phenyl radical that causes vomiting. Vomiting usually develops within 1–2 min after exposure to DA.

The immediate onset of symptoms following exposure makes severe or systemic toxicity to organic arsenical unlikely. However, prolonged contact may lead to multi-organ involvement (Kinoshita *et al.*, 2007; Kojima *et al.*, 2006). Blood-borne arsenicals can trigger increased

permeability of capillaries throughout the body (Naranmandura and Suzuki, 2008). Leakage of proteins and plasma then can cause third space fluid shifts, hypovolemia, and shock. It may result in intravascular hemolysis of erythrocytes with subsequent hemolytic anemia (Wu *et al.*, 2003).

Organic arsenicals cause immediate signs, whereas signs of mustard exposure appear after a latent period of several hours. An erythematous rash appears within 15–30 min. This is followed by the development of fluid-filled vesicles. A lewisite skin lesion has more actual tissue destruction (but less surrounding erythema) than a mustard lesion. Compared to distilled mustard, lewiste is gram-for-gram more toxic. The LD_{50} (lethal dose for 50% of the population) of lewisite is 2.8 g on the skin.

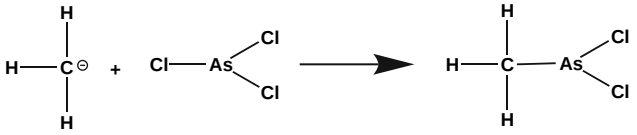
D. Methyldichloroarsine (MD)



Methyldichloroarsine was apparently used by the Germans in 1917. Methyldichloroarsine is a colorless liquid of powerful burning odor, which boils at 132°C. It is somewhat soluble in water and is soluble in organic solvents. The specific gravity is 1.838 at 20°C. The vapor pressure at 25° was found to be 10.83 mm Hg. Not only is the material toxic but it has remarkable vesicant properties, comparing favorably with mustard gas in this respect (Bennett and Dill, 1994).

1. Structure

The structure of MD consists of a trichloroarsine ($AsCl_3$) molecule combined through catalyzation with a methyl (CH_3) group.



2. Pathology

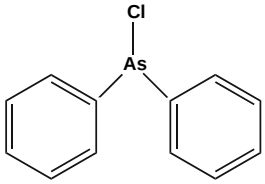
Chlorine bonds in MD give it its blistering qualities. The methyl group simply aids in its assimilation into the human body. Chlorine reactivity causes severe respiratory pain and damage to the membranes of the lungs. The methyl arsenic group readily penetrates the skin as a liquid and its prolonged exposure leads to systemic damage through bone calcium displacement and subsequent bone marrow destruction. Vapor concentration in open areas, given MD's high volatility, is not enough to cause Cl^--induced blistering although liquid contamination will give rise to blistering similar to that of distilled mustard within several hours. ''Dry-land drowning'' can occur as the lungs flood with water and mucus and the victim dies of a combination of blood poisoning and asphyxiation (Pitten *et al.*, 1999).

3. Physical and Chemical Properties

TABLE 9.2. Physical and chemical properties of methyldichloroarsine (MD)

Chemical formula	CH_3AsCl_2
Molecular weight	160.86
Freezing point	−55°C
Boiling point	133°C with decomposition
Flash point	No imminent hazard of explosion or fire
Liquid density	1.836 g/cc at 20°C
Median lethal dose (LCt_{50})	3,000 mg-min/m^3
Median incapacitating dosage (ICt_{50})	25 mg-min/m^3 (inhalation)
Vapor density	5.5 times as heavy as air
Vapor pressure	2.17 mm Hg at 0°C; 7.76 mm Hg at 20°C
Rate of detoxification	Sublethal dosages are rapidly detoxified, although arsenic poisoning may be accumulative if untreated
Persistency	Very short duration in humid climates due to high volatility and rapid hydrolysis. In dry, cold climates MD can persist up to several hours
Skin toxicity	Exposure of skin to liquid MD will cause blistering similar to that of HD within several hours. Vapor exposure in unlikely to cause blistering as sufficient concentration in unlikely in open areas
Eye toxicity	Exposure to even the slightest amount is highly irritating. At exposures above 30 mg-min/m^3 permanent corneal damage occurs
Latent heat of vaporization	49 calories/gm
Volatility	74,900 mg/m^3 at 20°C
Decontaminants	Bleaches and alkaline solutions such as sodium hydroxide (NaOH)
Rate of hydrolysis	Rapid
Hydrolysis products	Hydrochloric and methyl arsenic acids
Effect on metals and other materials	None on ferrous alloys. Slight oxidation to copper-based alloys

E. Diphenylchloroarsine

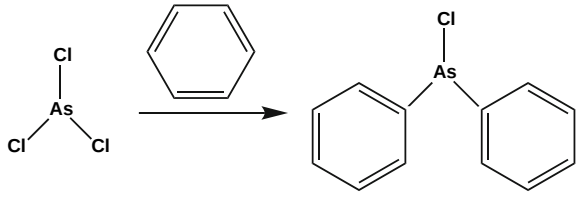


Diphenylchloroarsine (DA) is an irritating substance which was developed in 1918 for use in a smoke generator known as the M-device. The researchers described DA as having ''a very powerful irritant action on the mucous membranes of the eyes and nose, causes painful blistering of the skin, and is very dangerous for those working with it, since its vapor causes respiratory embarrassment, faintness, and long-lasting paralysis and anesthesia of the extremities''. However, after the entry of mustard (a far superior blister and casualty agent), DA proved to be less attractive as a chemical weapon (Ishii *et al.*, 2004; Kato *et al.*, 2007).

As DA and related compounds cause intense effects on the nasal and upper respiratory passages, they are referred to as ''sneeze gases'' (sternutators). Like other arsenicals, DA is a white solid, and was originally produced as both a casualty gas and a mask breaker during World War I. Military doctrine in World War I involving the use of DA counted on its being able to force soldiers to remove their protective masks, thus making them vulnerable to it or other chemical agents (Ishii *et al.*, 2004; Pitten *et al.*, 1999). Only very low concentrations of DA are needed to cause severe irritation of the nose and throat, approximately 0.0005 mg per liter of air (Hanaoka *et al.*, 2005). The median incapacitating dose (ICD_{50}) of DA is approximately 12 mg-min/m^3, while the median lethal dose (LD_{50}) is estimated at 15,000 mg-min/m^3. Due to the availability of other compounds with greater activity, however, less importance is attached to DA as a chemical weapon threat nowadays.

1. Structure

DA is prepared from trichloroarsine ($AsCl_3$):



Two chlorine ions are replaced by benzene groups, forming a stable compound which may be safely stored under all field conditions.

2. Effects

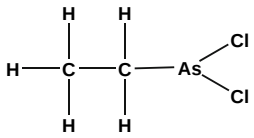
The immediate effects of DA are those associated with tear gas compounds: severe irritation to the eyes, nose, and throat. Severe headache and the feeling of tightness of the chest and bowels occur within a minute of inhalation of this compound. The headache rapidly develops into a general nausea which results in vomiting within 3 min. In closed or confined spaces DA can produce fatalities through first causing unconsciousness and then asphyxiation (Ochi *et al.*, 2004).

3. Physical and Chemical Properties

TABLE 9.3. Physical and chemical properties of diphenylchloroarsine (DA)

Chemical formula	$(C_6H_5)_2AsCl$
Molecular weight	264.5
Boiling point	333°C with decomposition
Decomposition point	300°C
Flash point	350°C
Median lethal dose (LCt_{50})	15,000 mg-min/m^3
Median incapacitating dosage (ICt_{50})	12 mg-min/m^3 (over 10 min)
Vapor density	Forms no significant vapor mass
Vapor pressure	0.0036 mm Hg at 45°C
Rate of detoxification	The human body will detoxify DA inhaled in brief exposures within 1 to 2 h
Persistency	Short duration
Skin toxicity	Irritating, nontoxic in open areas or in short exposures
Volatility	48 mg/m^3 at 45°C
Latent heat of vaporization	56.6 calories/gm
Eye toxicity	Irritating. Nontoxic in open areas or in short exposures
Decontaminants	Bleaches or caustic soda
Rate of hydrolysis	Slow in open container. Rapid when disseminated (in gaseous form)
Hydrolysis products	Hydrochloric and diphenylarsenous acids
Effect on metals and other materials	None when dry

F. Ethyldichloroarsine

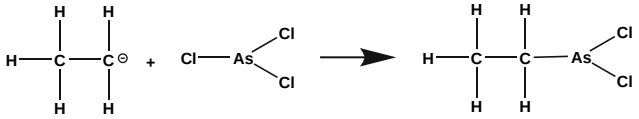


Ethyldichloroarsine (ED) was the third, and last, of the three blistering arsenicals developed in late 1917 to early 1918. Despite serious efforts made to weaponize this compound, little literature exists on the effectiveness and history of its use. Fast acting – compared to mustard or phosgene – ED is

a colorless liquid that smells like rotting fruit and has multiple effects on the body (Henriksson *et al.*, 1996). ED poses a significant vapor threat to exposed personnel. Within seconds of contact with the skin, the agent fixes itself to the epidermis and dermis causing immediate pain. The agent penetrates deeper into the skin layers causing the destruction of subcutaneous tissue. Fluid-filled blisters are formed only after prolonged exposure. Inhalation can cause pulmonary edema or ''dry-land drowning''. A lethal exposure, however, depends upon the period of exposure. A dose of 3,000 to 5,000 mg-min/m^3 is generally lethal.

1. Structure

The structure of ED consists of the trichloroarsine ($AsCl_3$) molecule combined through catalyzation with the ethyl (C_2H_5) group.



Production of ED is similar to that of MD, involving the ethylation of a chlorinated arsenite or arsenate salt, or reductions of arsenious oxide, As_4O_6, a naturally occurring compound (Bartelt-Hunt *et al.*, 2006).

2. Effects

Chlorine bonds in ED giving its blistering, lachrymatory, and harsh respiratory effects. Dosages as low as 5 mg-min/m^3 may cause severe discomfort to the eyes and throat. Sublethal dosages are detoxified by the body. ED's ethyl arsenic group may cause systemic damage to bone marrow and to the digestive and endocrine systems.

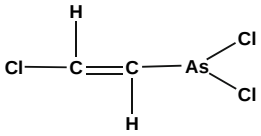
Blisters may appear within 2 to 4 h following skin redness or rash. However, like the mustards, ED actively attacks lung tissue. Damage to lung tissue is permanent to its survivors and presents a hazard area for future infections and tumors. ED is also highly toxic to the eyes. It may cause permanent corneal damage.

3. Physical and Chemical Properties

TABLE 9.4. Physical and chemical properties of ethyldichloroarsine (ED)

Chemical formula	$C_2H_5AsCl_2$
Molecular weight	174.88
Boiling point	156°C
Melting point	−65°C
Decomposition point	156°C
Flash point	No immediate hazard of fire or explosion
Median lethal dose (LCt_{50})	Between 3,000 and 5,000 mg-min/m^3
Median incapacitating dosage (ICt_{50})	5 to 10 mg-min/m^3
Vapor density	Six times as heavy as air
Vapor pressure	2.09 mm Hg at 20°C
Persistency	Short duration
Rate of action	Immediate effects on eyes and respiratory tract. Skin rash leads to blistering within 1 to 2 h
Rate of detoxification	Rapid for nonlethal dosages
Volatility	6,500 mg/m^3 at 0°C; 20,000 mg/m^3 at 20°C
Skin toxicity	Rapid advent of blistering (within 1 h of exposure to high concentrations of ED)
Latent heat of vaporization	52.5 calories/gm
Eye toxicity	Highly irritating. Splashed liquid may cause permanent corneal damage
Decontaminants	Bleaches and other oxidizers and strong alkaline such as NaOH
Rate of hydrolysis	Rapid
Hydrolysis products	Hydrochloric acid and ethylarsenous oxide
Effect on metals and other materials	Very destructive to rubber and plastics. No effect on ferrous alloys

G. Lewisite



The formulation of lewisite (chlorovinyldichloroarsine) as a war gas was achieved by W. Lee Lewis in 1918 (Goldman and Dacre, 1989). It is a colorless, oily liquid at room temperature with a faint ''geranium-like'' odor (Table 9.5). It is more volatile than sulfur mustard and, therefore, can be used as a vapor over greater distances. It was considered an alternative to sulfur mustard, which had become the main chemical warfare agent. Lewisite is a potent blistering agent. Like other blistering agents, it not only produces casualties but also restricts use of terrain, hampers troop movements, and requires cumbersome protective gear (Somani, 1992). To achieve greater effectiveness in combat, lewisite has been mixed with sulfur mustard. Because of its freezing point, lewisite is effective over a wider temperature range than sulfur mustard. Lewisite dissolves very slowly in water. The dissolved lewisite hydrolyzes rapidly to hydrochloric acid and lewisite oxide (Daniels *et al.*, 1990).

TABLE 9.5. Physical and chemical properties of lewisite

Boiling point	190°C
Freezing point	18 to 0.1°C (purity and isomer dependent)
Vapor pressure	0.394 mm Hg at 20°C
Vapor density	7.1 (compared to air)
Volatility	4,480 mg/m^3 at 20°C
Decomposition temperature	>100°C
Hydrolysis rates	Degrades under humid conditions
	Vapor – rapid
	Dissolved – rapid
Hydrolysis products	HCl and chlorovinyl arsenous oxide; alkaline hydrolysis destroys blister properties

In high concentrations, lewisite produces irritation and blistering of the skin and injury to the eyes and lungs promptly after exposure while, at lower levels, the effects resemble exposure to tear gas, with irritation of skin, eyes, and respiratory tract. Chronic exposure may lead to development of chronic bronchitis and predispose to Bowen's squamous cell intraepithelial cancer of the skin.

1. History and Background Information

Lewisite (also known as Agent L) is no longer considered a state-of-the-art chemical warfare agent (Franke, 1967; Goldman and Dacre, 1989). Lewisite is relatively simple and inexpensive to produce (Franke, 1967).

Lewisite acts promptly on exposure, persists with moderate potency, and is easily mixed with other chemical agents to augment toxic effects. Lewisite can be most effective when mixed with nerve agents. Once absorbed, lewisite induces vomiting, precluding the use of protective masks and making personnel vulnerable to other, more toxic chemicals. Lewisite is a significant threat to unprotected personnel and causes prompt incapacitation from eye injuries and respiratory irritation, coupled with long-term incapacitation from skin burns, pulmonary injury, and systemic illness (Sidell *et al.*, 1997; Wachtel, 1941). Large munitions expenditures were required to achieve effective concentrations in the field (Pechura and Rall, 1993).

Although the use of lewisite was suspected at times during the Iran–Iraq War, it was never proved present in the munitions studied (DIA, 1997; Dunn *et al.*, 1997; UN, 1984) and no elevated levels of arsenic were found in the blood and tissues of Iranian casualties treated in Europe (Heyndrickx, 1984). There is some human exposure experience from accidental exposure to lewisite (Pechura and Rall, 1993). The levels of exposure that resulted from accidents in occupational workers are not known.

Lewisite is easy to manufacture, and storage stability problems can be overcome. It can be dispersed by aerial spraying, shells, or bombs. Lewisite persists for 6 to 8 h on the ground in sunny weather. Its decomposition products are toxic, making decontamination difficult. Munitions containing lewisite may contain toxic stabilizers. Lewisite is effective as vapor, aerosol, or liquid (Boronin *et al.*, 1996; Goldman and Dacre, 1989).

Lewisite is reported to possess a characteristic (geranium-like) odor in the range of 0.8 mg/m^3 to more commonly cited 14–23 mg/m^3 median detection (Pechura and Rall, 1993). US forces have detectors for lewisite-paper and kits (M7 and M9A). Other forensic techniques for soil and material analysis already exists (e.g. gas chromatography). In biological tissues, increased arsenic levels are a surrogate for lewisite (Haddad and Wincester, 1983).

2. Toxicity and Mechanism of Action

Lewisite causes painful blistering of the skin and eyes. If decontamination does not occur within 1 min, lewisite produces severe damage to the cornea, and permanent loss of sight can result. Reddening of the skin begins within 30 min, and blistering appears about 13 h after exposure. As a vesicant, lewisite is about four times as fast acting as mustard and is much less persistent (Somani, 1992). Effects of lewisite are similar to those of sulfur mustard, except that it is absorbed through the skin and acts as a systemic poison. Exposure to lewisite leads to pulmonary edema, diarrhea, restlessness, weakness, subnormal temperature, and low blood pressure. Prolonged exposure leads to severe pain in the throat and chest (Bartelt-Hunt *et al.*, 2008; USDHHS, 1988).

Trivalent arsenic is considered the component of lewisite that is primarily responsible for its vesicant and systemic toxicity; thus, field drinking-water standards for lewisite are expressed in terms of the arsenic fraction (Aposhian *et al.*, 2003). Trivalent arsenic exerts its toxic effect by binding to sulfhydryl-containing proteins, especially enzymes, thus inhibiting pyruvate oxidation – a critical step in carbohydrate metabolism (Black, 2008). The lipid solubility of lewisite also contributes to its toxic effects; trivalent arsenic readily penetrates skin, exerting its toxic action systemically and causing painful localized blistering. As a systemic toxicant, lewisite produces pulmonary edema, diarrhea, restlessness, weakness, subnormal temperature, and low blood pressure. Vascular damage, induced by lewisite, is partly responsible for effects such as blistering, tissue perforation, and hemorrhaging. Edema and hemorrhaging associated with lewisite exposure can lead to shock and death (Flora *et al.*, 2007b, c).

Human data concerning the toxicity of lewisite via the oral route of exposure are not available. However, there are limited toxicity data on lewisite ingestion from animal studies. In animals, ingestion of lewisite can produce acute inflammation of the mucous membrane of the stomach or intestine, which is characterized by hemorrhage, necrosis of the epithelium, and submucous edema. Developmental effects have been reported in pregnant rats and rabbits

exposed to lewisite by intragastric intubation. A no-observed-adverse-effect-level (NOAEL) of 0.016 mg/kg/day in rabbits and 1.5 mg/kg/day in rats was identified (Daniels *et al.*, 1990; Goldman and Dacre, 1989). The NOAEL of 0.016 mg/kg/day in rabbits was selected for developing the proposed field drinking-water standards for lewisite. There are no data on chronic toxicity resulting from the ingestion of lewisite.

Lewisite, as an arsenical, might be carcinogenic, although no specific studies were found in which the carcinogenicity of lewisite was evaluated. There is evidence that arsenic might act as a co-carcinogen and promote the carcinogenic process. It is capable of producing DNA damage; however, direct tests of its mutagenic potential have been inconclusive (Datta *et al.*, 2007).

Given the limited epidemiological data, the proposed drinking-water standards for lewisite (Daniels *et al.*, 1990) were derived from three animal studies – a sparse database. The shortcomings of the animal studies for predicting the effects in humans exposed to lewisite in water, and in particular in military personnel exposed to field drinking water, have been clearly recognized (Daniels *et al.*, 1990). However, there are major data gaps on the toxicity of lewisite, and the usefulness of most of the studies is limited by the lack of a satisfactory animal model. Little information exists on the reaction of lewisite with biologically important molecules, although it is reasonable to assume that, as with sulfur mustard, DNA is a major target (IOM, 1996). There are no adequate data on the acute effects of lewisite following dermal exposure. Very little is known about its specific effects on skin, and data on its absorption, disposition, and excretion following dermal exposure are minimal. Microscopic examination of affected skin has not been pursued extensively. The proposed Army standards for exposure to lewisite in field drinking water were derived from a rabbit study, in which the NOAEL was estimated to be 0.016 mg/kg/day (Daniels *et al.*, 1990). Whether the rabbit is the species most sensitive to lewisite is not known, and the applicability of the rabbit data to the human situation requires further evaluation.

Neurological effects following acute exposure to lewisite have not been documented in animals. Acute exposure to high concentrations of lewisite leads to a shock syndrome that is thought to result from increased capillary permeability (Goldman and Dacre, 1989). No direct evidence exists that lewisite might cause neurological problems in humans, although arsenic is considered a neurotoxin and peripheral neuropathy has been reported in humans following a single arsenic exposure (Moore *et al.*, 1997).

3. Toxicokinetics

Lewisite is a local and pulmonary irritant, a vesicant, and a systemic poison. When ingested with food, it produces severe gastrointestinal irritation. The eyes, respiratory tract, and skin are the most likely sites of exposure when lewisite is used as a chemical warfare agent. The agent is lipophilic and readily penetrates intact skin. The approximate lethal dose (LD_{50}) is 35–40 mg/kg, an amount present in 2 ml of liquid agent (NATO, 1973). Lewisite toxicity resembles other trivalent arsenicals that produce peripheral and central neurotoxicity, hepatotoxicity, and epithelial damage. Death may result from fluid loss and hypovolemia secondary to capillary leakage – the so-called ''lewisite shock'' (Sidell *et al.*, 1997; Snider *et al.*, 1990; Watson and Griffin, 1992).

The cellular poisoning effects are attributed to the inhibition of cellular enzyme systems (Pechura and Rall, 1993; Watson and Griffin, 1992), especially as a result of arsenic complexing with sulfhydryl groups of proteins and enzymes. This agent affects many sulfur-containing enzymes, including amylase, lipase, cholinesterase, some adenosine triphosphate (ATP) enzymes, creatine phosphokinase, and of central importance (Snider *et al.*, 1990), the pyruvate oxidase system. Two mechanisms have been proposed for the above-mentioned effects (Sidell *et al.*, 1997): (1) reactions with glutathione leading to loss of protein thiol status, loss of calcium ion homeostasis, oxidative stress, lipid peroxidation, membrane damage, and cell death, and (2) reactions with sulfhydryl groups on enzymes leading to inhibition of pyruvate dehydrogenase complex, inhibition of glycolysis, loss of ATP, and cell death.

4. Clinical and Pathological Findings

There are few published case reports of human lewisite poisoning. Signs and symptoms of acute lewisite exposure include the rapid onset of irritation to the eyes and mucous membranes of the upper respiratory tract (lachrymation and rhinitis). In more serious cases of vapor intoxication, chest pain, nausea, vomiting, headache, weakness, convulsions, hypothermia, and hypotension occur (Katos *et al.*, 2007; NATO, 1973; Sidell *et al.*, 1997; USDHHS, 1988). The pathology literature is largely limited to serious acute exposures. Laboratory tests of the blood of persons exposed may show hemoconcentration; animal studies suggest elevated liver enzymes, including lactate dehydrogenase (LDH) (King *et al.*, 1994, 1992; Sasser *et al.*, 1996, 1999). The following subsections describe the effects on specific body sites.

a. Skin

Exposure of the skin to vapor causes immediate itching or stinging within 1 min, followed by erythema over 10 to 30 min. Mild exposures resemble sunburn with pain decreasing over 24 to 48 h. More intense exposures, including liquid contact, produce intense stinging and the formation of small vesicles over the next 24 h, with later enlargement of the vesicles with accumulation of a nontoxic fluid (McManus and Huebner, 2005). Systemic illness is more likely to occur if heavy exposures are to the liquid form, with later development of vomiting, pulmonary edema, or shock (Arroyo *et al.*, 2004; Kato *et al.*, 2007; King *et al.*, 1992).

b. Eye

Immediate eye pain and blepharospasm result from lewisite exposure, followed by conjunctival and lid edema. Severe exposures can produce necrotic injuries of the iris with depigmentation, hypopion, and synechia development. In contrast, very low levels may only involve the conjunctivae (McManus and Huebner, 2005). The eye lesions produced by lewisite are particularly serious: blindness will follow contamination of the eye with liquid lewisite unless decontamination is prompt.

c. Respiratory System

Mild respiratory exposures resemble upper respiratory infections, with sneezing, coughing, rhinitis, and mucous membrane erythema, possibly progressing to retrosternal pain, nausea, and malaise. More severe exposures cause lower respiratory effects, with continuous coughing, laryngitis, and aphonia (McManus and Huebner, 2005; Sasser *et al.*, 1996; Telolahy *et al.*, 1995).

d. Nervous System

Neurological findings are inconsistent despite reports of convulsions and coma with severe exposures (McManus and Huebner, 2005). Neurologic complications after mild exposures have not been described. Edema and hemorrhage in the brain are rare but no reports of degeneration of peripheral nerves were found.

e. Cardiovascular System

Bradycardia, dyspnea, hypotension, and hemoconcentration have been reported in severe intoxication. These effects are mediated by vasodilatation and increased capillary permeability (Watson and Griffin, 1992). Dilation of the right side of the heart in severe poisoning in animals has been reported.

f. Other Systems

Human ingestion experience is not documented (NAS, 1997), but would be expected to produce severe abdominal pain and bloody diarrhea. Nausea and vomiting occur from respiratory or dermal exposure (Fang, 1983). In human and animals vomiting is associated with retching (Sidell *et al.*, 1997). There is no documentation of liver effects from chronic lewisite exposure. There are no specific musculoskeletal findings, although weakness has been observed. There is little clinical information about effects on bone marrow and the immune and endocrine systems. Renal disorders, although theoretically possible, are not described. There is also no substantial evidence that lewisite is carcinogenic, teratogenic, or mutagenic (Sidell *et al.*, 1997).

V. INORGANIC ARSENIC

Arsenic is a metalloid belonging to group VA of the periodic table. It exists in three oxidation states: metalloid (0), trivalent (−3 or +3), and pentavalent (+5). The most common inorganic trivalent arsenic compounds are arsenic trioxide, sodium arsenite, and sodium trichloride. Pentavalent inorganic arsenic compounds are arsenic pentaoxide, arsenic acid, and arsenate, e.g. lead arsenate and calcium arsenate. In general, the toxicity of arsenic compounds is in the following order:

arsine > arsenites > arsenates > organic > elemental.

A. Sources and Uses

1. Uses

Current uses of arsenic are in pesticides, cotton desiccants, and wood preservatives. Arsenic is also used as a bronzing and decolorizing agent in the manufacture of glass, and in the production of semiconductors (Tanaka, 2004), as a desiccant and defoliant in agriculture, and as a byproduct of the smelting of nonferrous metals, particularly gold and copper, from coal residues (Hall, 2002).

2. Exposure

Arsenic exposure occurs from inhalation, absorption through the skin, and primarily by ingestion of arsenic contaminated drinking water. The exposure to arsenic may come from natural sources (groundwater, arsenic containing mineral ores), industrial processes (semiconductor manufacturing, wood preservatives, metallurgy, glass clarification, smelting, and refining of metals and ores), commercial products (pesticides, herbicides, fungicides, fire salts), food (sea food, kelp, wine, and tobacco), and medicines (antiparasitic drugs and folk medicines) (Kosnett, 1990). Acute arsenic poisoning is only limited to homicidal or suicidal attempts.

B. Absorption, Distribution, and Excretion

Respiratory absorption of arsenic is a two-stage process, involving deposition of the particles on to airway and lung surfaces, followed by absorption of arsenic from deposited particulates. Absorbance of inorganic arsenic from the gastrointestinal tract depends on the solubility and composition of arsenic compound. Both human and animal data show that over 90% of an ingested dose of dissolved inorganic arsenic is absorbed from the gastrointestinal tract to the bloodstream and distributed to organs/tissues after first passing through the liver. Trivalent and pentavalent inorganic arsenic have been reported to cross the placenta in laboratory animals and humans (Bollinger *et al.*, 1992). Following absorption by the lungs or the gastrointestinal tract, arsenic is transported via the blood to other parts of the body. Inorganic arsenic is rapidly cleared from the blood in both humans and common laboratory animals. In rats, absorbed arsenic is accumulated in the red blood cells due to its binding with hemoglobin. Recent studies indicate that the main form of arsenic bound to rat hemoglobin is

dimethylarsinic acid (DMA), the primary metabolite of inorganic arsenic (Lu *et al.*, 2004).

In humans as well as in most animal species, exposure to either arsenite or arsenate leads to an initial accumulation in the liver, kidneys, and lungs (Fujihara *et al.*, 2004). The clearance from these tissues is, however, rather rapid, and a long-term retention of arsenic is seen in hair, skin, squamous epithelium of the upper gastrointestinal tract, epidymis, thyroid, lens, and skeleton (Huges *et al.*, 2003). Two to four weeks after exposure ceases, most of the arsenic remaining in the body is found in keratin-rich tissues, such as skin, hair, and nails. Inorganic arsenic can cross the human placenta (Tabacova *et al.*, 1994). Arsenic metabolism is characterized by two main types of reaction: (1) reduction of the pentavalent arsenic to trivalent arsenic – this conversion of pentavalent arsenic species to trivalent arsenic species is catalyzed by arsenate reductase (Radabaugh and Aposhian, 2000; Radabaugh *et al.*, 2002), and (2) oxidative methylation reactions in which trivalent forms of arsenic are sequentially methylated to form mono-, di- and trimethylated products using *S*-adenosyl methionine (SAM) as the methyl donor and GSH as an essential co-factor (Vahter, 2002). Pentavalent arsenic has been reported to be less toxic than inorganic trivalent arsenic. The metabolic methylation had historically been considered as a detoxification process. Recently, it has been established that trimethylated arsenicals, particularly monomethylarsinous acid [MMA(III)] and dimethylarsinous acid [DMA(III)], exist as intermediates in the metabolic methylation process of inorganic arsenic in humans (Le *et al.*, 2000; Mandal *et al.*, 2001) and are more active than the parent inorganic arsenic for enzymatic inhibition (Styblo *et al.*, 1997), cytotoxicity (Petrick *et al.*, 2001), and genotoxicity (Nesnow *et al.*, 2002). The major route of excretion following exposure to inorganic arsenic is via the kidneys (Csanaky and Gregus, 2005). Only a few percent are excreted in feces (Mann *et al.*, 1996).

C. Biochemical and Toxic Effects

1. Hematopoietic

The hematopoietic system is affected by both short- and long-term arsenic exposure. Arsenic is known to cause a wide variety of hematological abnormalities like anemia, absolute neutropenia, leucopenia, thrombocytopenia, and relative eosinophilia – more common than absolute esinophilia, basophilic stippling, increased bone marrow vascularity, and rouleau formation (Rezuke *et al.*, 1991). These effects may be due to a direct hemolytic or cytotoxic effect on the blood cells and a suppression of erythropoiesis. The mechanism of hemolysis involves depletion of intracellular GSH, resulting in the oxidation of hemoglobin (Saha *et al.*, 1999). Arsenic exposure is also known to influence the activity of several enzymes of heme biosynthesis. Arsenic produces a decrease in ferrochelatase, and decrease in COPRO-OX and increase in hepatic 5-aminolevulinic acid synthetase activity (Woods and Southern, 1989). Subchronic exposure to arsenic has also been reported to inhibit δ-aminolevulinic acid synthetase (ALA-S) and ferrochelatase activities, leading to increased uroporphyrin (URO) and coproporphyrin (Woods and Fowler, 1978) and COPRO urinary excretion (Martinez *et al.*, 1983). In chronically exposed humans, arsenic alters heme metabolism as shown by an inversion of the urinary COPRO/URO ratio (Garcia Vargas *et al.*, 1996). A few recent studies also suggested a significant inhibition of blood δ-aminolevulinic acid dehydratase (ALAD) after subchronic and chronic arsenic exposure (Flora *et al.*, 2002; Flora, 1999; Kannan *et al.*, 2001). Martinez *et al.* (1983) reported that chronic exposure to arsenic alters human heme metabolism. Although anemia is often noted in humans exposed to arsenic, red and white blood cell counts are usually normal in workers exposed to inorganic arsenicals by inhalation (Morton and Caron, 1989). Anemia and leucopenia are common effects of poisoning and have been reported from acute, intermediate, and chronic exposure. These effects may be due to a direct effect of arsenic on the blood cells and a suppression of erythropoiesis. Keeping in view the above there was a proposal that the profile of urinary porphyrins could be used as early biomarkers for arsenic toxicity in humans chronically exposed to arsenic via drinking water.

2. Skin (Dermal)

Dermal changes most frequently reported in arsenic-exposed humans include hyperpigmentation, melanosis, hyperkeratosis, warts, and skin cancer. However, dermal effects appear to be highly dependent on time of exposure (Rossman, 2003). Arsenic-exposed skin cancer occurs mostly in unexposed areas such as trunk, palms, and soles. More than one type of skin cancer is reported and most common are Bowen's disease, squamous cell carcinomas, basal cell carcinomas, and combined forms. It appears that skin cancer lesions related to arsenic exposure act as early warning signals of the subsequent development of cancers of internal organs many years later (Tsuda *et al.*, 1995; Wong *et al.*, 1998). Brittle nails, the surface of which is marked by transverse bands (leukonychia striata arsenicalis transverses), have been associated with arsenic poisoning; the characteristic bands are known as Reynolds-Aldrich-Mees' lines.

3. Hepatic

Arsenic is one of the first chemical agents to which liver disease was attributed in humans (Clarkson, 1991). Early symptoms in patients with arsenic-induced hepatic injury include bleeding esophageal varices, ascites, jaundice, or simply an enlarged tender liver. Another important feature of chronic arsenic toxicity reported in West Bengal, India, is a form of hepatic fibrosis that causes portal hypertension, but does not progress to cirrhosis (Santra *et al.*, 1999, 2000; Rahman *et al.*, 1999). Recent animal studies have shown that hepatic enzyme changes occur following arsenic

exposure and these enzyme changes involved in the antioxidant defense system and membrane damage due to lipid peroxidation precede the pathomorphological lesions of arsenic-induced hepatic fibrosis in mice (Mishra *et al.*, 2008; Santra *et al.*, 2000).

4. Gastrointestinal

Gastrointestinal symptoms are common during acute poisoning. Gastrointestinal effects due to chronic arsenic poisoning are called arsenicosis. Workers exposed to high level of arsenic dust or fumes suffer from nausea, vomiting, and diarrhea (Morton and Caron, 1989). Patients complain of metallic taste and garlic odor. Clinical signs of gastrointestinal irritation due to acute arsenic poisoning include burning lips, painful swallowing, thirst, nausea, and severe abdominal colic (Goebel *et al.*, 1990). The toxic effects of arsenic on the gastrointestinal mucosal vasculature are vasodilatation, transduction of fluid into the bowel lumen, mucosal vesicle formation, and sloughing of tissue fragments. Rupture of the vesicle may cause bleeding, profuse watery stools (''rice-water stools''), and protein losing enteropathy. The most likely mechanism of gastrointestinal toxicity is damage of the epithelial cells, with resulting irritations.

5. Respiratory

Studies from West Bengal draw attention to both restrictive and obstructive lung disease (Mazumder *et al.*, 1998). Respiratory disease is more common in patients with the characteristic skin lesions due to chronic arsenic toxicity (Mazumder *et al.*, 2000). Humans exposed to arsenic dust or fumes inhalation are more likely to be encountered in mining and milling ores, or in industrial processing, such as the smelting industry which often produces irritation of mucous membrane, resulting in laryngitis, bronchitis, rhinitis, and tracheobronchitis, causing stuffy nose, sore throat, dyspnea, chest pain, and chronic cough (ATSDR, 2000). Pulmonary edema may occur, especially in cases of inhalation.

6. Cardiovascular

It has been suggested by several epidemiological studies that chronic inhalation of arsenic trioxide can increase the risk of death in humans from cardiovascular disease (Saha *et al.*, 1999). Both acute and chronic arsenic exposure cause altered myocardial depolarization and cardiac arrhythmias that may lead to heart failure. Arsenic causes direct myocardial injury, cardiac arrhythmias, and cardiomyopathy. Blackfoot disease, causing gangrene of the foot, is unique to a limited area on the southwestern coast of Taiwan, and is due to long-term exposure to high levels of inorganic arsenic in well water (range 0.01–1.82 mg/l) (TPHD, 1993; Tsai *et al.*, 1999). Epidemiological studies also indicate that excess intake of arsenic leads to a variety of vascular diseases such as Blackfoot disease, Reynaud's phenomenon, cardiovascular and cerebrovascular diseases, atherosclerosis, and hypertension (Engel *et al.*, 1994; Lewis *et al.*, 1999; Simeonova and Luster, 2004).

7. Reproductive and Developmental

Reproductive and developmental effects of inorganic arsenic on human and animal species have been reported (Concha *et al.*, 1998). Limited animal studies suggest that arsenic can produce malformation, intrauterine death, and growth retardation (Golub *et al.*, 1998). Arsenic readily crosses the placenta. Sarkar *et al.* (2003) also suggested that spermatogenesis and/or sperm function might be impaired by organic arsenicals. Ahmad *et al.* (2001) observed pregnancy outcome in women chronically exposed to arsenic through drinking water. The authors reported that arsenic contamination is also a threat to healthy and safe pregnancy outcome.

8. Neurological

After initial contradictory reports it is now established that arsenic can cross the blood–brain barrier and produces alternations in whole rat brain biogenic amines levels in animals chronically exposed to arsenite (Tripathi *et al.*, 1997). The neurological effects are many and varied. Usually, peripheral neuropathy, sensory neuropathy (Hafeman *et al.*, 2005), and encephalopathy are the initial complaints associated with acute arsenic poisoning. Acute exposure to arsenic in humans has been shown to result in problems of memory, difficulties in concentration, mental confusion, and anxiety (Hall, 2002; Rodriguez *et al.*, 2003). Other neurological symptoms arising due to arsenic are primarily those of a peripheral sensory neuritis, predominantly numbness, severe paresthesia of the distal portion of the extremities, diminished sense of touch, pain, heat and cold, and symmetrically reduced muscle power (Menkes, 1997).

9. Diabetes Mellitus

Noninsulin-dependent (type 2) diabetes is the prevalent form of diabetes mellitus found in populations chronically exposed to inorganic arsenic from the environment (Rahman *et al.*, 1998; Tseng, 2002). Type 2 diabetes is characterized by insulin resistance of internal organs and peripheral tissues that results in impaired glucose utilization, and, consequently, in abnormally high blood glucose levels between and especially after meals. Insulin resistance and β-cell dysfunction can be induced by chronic arsenic exposure and these defects may be responsible for arsenic-induced diabetes mellitus (Tseng, 2004).

D. Mechanisms of Toxicity

Trivalent inorganic arsenicals, such as arsenite, readily react with sulfhydryl groups, such as GSH and cysteine (Scott *et al.*, 1993; Delnomdedieu *et al.*, 1994). The complex between arsenic and the vicinal sulfhydryl group is particularly strong. Arsenite inhibits pyruvate dehydrogenase (PDH) activity (Hu *et al.*, 1998), perhaps by binding to the

lipoic acid moiety. Inhibition of PDH ultimately leads to decreased production of ATP. Inhibition of PDH may also explain in part the depletion of carbohydrates observed in rats administered arsenite (Reichl *et al.*, 1991). Methylated trivalent arsenicals such as MMA^{III} are potent inhibitors of GSH reductase (Styblo *et al.*, 1997) and thioredoxin reductase (Lin *et al.*, 1999). The inhibition may be due to the interaction of trivalent arsenic with critical thiol groups in these molecules. A mechanism of toxicity of pentavalent inorganic arsenic, such as arsenate, is its reduction to a trivalent form, such as arsenite. The reduction of arsenate to arsenite occurs *in vivo*. Another potential mechanism is the replacement of phosphate with arsenate.

1. Oxidative Stress

Oxidative stress has now been established as one of the major mechanisms involved in arsenic-induced carcinogenesis. A number of recent reports provided direct evidence of an inorganic arsenic-induced free radical formation or production of oxidative stress (Flora, 1999; Liu *et al.*, 2000; Sun *et al.*, 2005). From exposure to the free radical generations, DNA damage such as DNA single strand breaks can occur. It has been suggested in recent studies that arsenic exerts its toxicity through the generation of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion, singlet oxygen, and hydroxyl radical that can directly or indirectly damage cellular DNA, lipid, and protein. In addition, sodium arsenite has been shown to enhance heme oxygenase production, an indicator of oxidative stress (Applegate *et al.*, 1991). Reactive oxygen species that damage DNA *in vitro* are generated from iron released from ferritin. The results suggest that some clastogenic effects of arsenic are mediated via free radicals (e.g. peroxynitrite, superoxide, hydrogen peroxide, and possibly free iron). Vega *et al.* (2001) suggested that arsenic exposure could increase the production of ROS, activation of transcription factors (e.g. AP-1, *c-fos*, and NF-κB) and oversecretion of pro-inflammatory and growth promoting cytokines, resulting in increased cell proliferation and finally carcinogenesis. Increased ornithine decarboxylase (ODC) activity is often interpreted as a biomarker for cell proliferation (Kitchin, 2001). Arsenite appears to have an effect on the cell cycle, which may alter cell proliferation. The amplification of the gene which codes for the enzyme dihydrofolate reductase was enhanced by arsenic. Lee *et al.* (1988) suggested that the gene amplification induced by arsenic may have a role in its carcinogenic effects.

E. Diagnosis

1. Clinical Features

Clinical features like skin lesions and neuropathy are crude and imprecise indicators of the severity of poisoning. The early clinical symptoms of arsenic toxicity are headache, dizziness, insomnia, weakness, nightmare, numbness in the extremities, anemia, palpitations, and fatigue. White striae in the fingernails are also a useful clue in the diagnosis of arsenic toxicity; these white striae are also known as ''Mees' lines''. Other symptoms are anemia, leucopenia or pancytopenia, gangrene of the feet (blackfoot disease), hyperpigmentation, hypopigmentation, and hyperkeratosis. Arsenic accumulates in keratin-rich tissues such as skin, hair, and nails due to its high affinity for sulfhydryl groups; the arsenic level in hair and nails may be used as an indicator of past arsenic exposure. Elevated arsenic content in hair and nail segments, normally less than 1 part per million (ppm), may persist for months after urinary arsenic values have returned to baseline (Koons and Peters, 1994). The arsenic content of the fingernails and toenails has also been used as bioindicators of past arsenic exposure, and fingernail arsenic has been reported to be significantly correlated with hair arsenic content (Lin *et al.*, 1998).

Blood arsenic levels are highly variable. Blood arsenic, normally less than 1 μg/dl, may be elevated on acute intoxication. It is probably the most important diagnostic test for detecting arsenic exposure. Arsenic metabolites (inorganic arsenic + MMA + DMA) in urine have also been used as biomarkers of recent arsenic exposure (Yamauchi *et al.*, 1989).

2. Other Biomarkers

Essentially, all organ systems must be evaluated. Thus laboratory tests, should include complete blood count, liver and renal functional tests, and blood, nail and urine arsenic levels. Other biomarkers of arsenic exposure include non-erythrocyte porphyrin enzyme activities and urine transforming growth factor TNF-α, accompanied by induction of heme oxygenase, mitogen-activated protein kinases, the ubiquitin-dependent proteolytic pathway, and protein kinase C in various tissues. These tests are still being investigated in laboratories and their clinical usefulness remains to be proven (Chapell *et al.*, 2001).

VI. TREATMENT

Highly specific treatment is required for poisoning with arsenicals. There is no particular antidote for the treatment of arsine poisoning. Victims may be administered a high flow of oxygen. One of the earlier strategies for the treatment of arsine poisoning involved stopping the ongoing hemolysis which may lead to renal dysfunction (Kimmecki and Carter, 1995). Exchange transfusion is currently the treatment of choice (Pullen-James and Woods, 2006). Chelation therapy generally is not recommended to reduce hemolysis; however, chelating agents are shown to reduce arsenic in arsine-exposed subjects. Since World War II, dimercapol has been the standard treatment for poisoning by arsenicals. In the following paragraphs we discuss the efficacy of chelating agents for treating arsenicals, their drawbacks, and the current advancement in the area.

A. Chelating Agents and Chelation Therapy

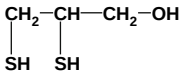
Chelating agents are organic compounds capable of linking together metal ions to form complex ring-like structures called chelates. Chelators act according to a general principle: the chelator forms a complex with the respective (toxic) ion and these complexes reveal a lower toxicity and are more easily eliminated from the body. This mechanism could be represented as:

Metal—*In vivo* site + Chelating agent → *In vivo* site + Metal—Chelating agent complex

Chelation may thus be defined as the incorporation of a metal ion into a heterocyclic ring structure (Flora and Sekhar, 2004).

Some of the chelating agents listed below have been reported to be useful in the treatment of arsenic.

1. 2,3-Dimercaprol (Dimercaprol; British Anti-Lewisite, BAL)



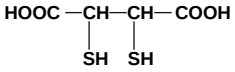
2,3-Dimercaprol (British Anti-Lewisite, BAL) was originally developed to treat the effects of lewisite, namely systemic poisoning and local vesication. The empirical formula of BAL is $C_6H_8OS_2$ and its molecular weight is 124.21. It is an oily, clear, colorless liquid with a pungent, unpleasant smell typical of mercaptans and having a short half-life. Because of its lipophilic nature it is distributed extracellulary and intracellularly. BAL is known to protect against the effects of lewisite and reverse the enzyme inhibition produced by it. Dimercaprol can be used parenterally against systemic toxicity of lewisite and also as an ointment for use against skin burn (Marrs *et al.*, 1996). Skin contamination may be treated with dimercaprol ointment.

a. Drawbacks

BAL is unstable and easily oxidized and therefore difficult to store, therefore it requires ready-to-use preparation. Beside rapid mobilization of arsenic from the body, it causes a significant increase in brain arsenic (Hoover and Aposhian, 1983). Due to its oily nature, administration of BAL requires deep intramuscular injection that is extremely painful and allergic (Flora and Tripathi, 1998). The chelating agent cannot be given intravenously thus denying the possibility of a loading dose. Other side effects include vomiting, headache, lachrymation, rhinorrhea and salivation, profuse sweating, intense pain in the chest and abdomen, and anxiety.

Two water-soluble analogs of dimercaprol have also been been studied as lewisite antidotes. They are meso 2, 3-dimercaptosuccinic acid (DMSA) and 2,3-dimercapto-1-propane sulfonic acid (DMPS). These two drugs circumvent two major disadvantages associated with treatment with BAL, i.e. the need for intramuscular injection and limitation of dose by toxicity.

2. Meso 2,3-Dimercaptosuccinic Acid (DMSA)



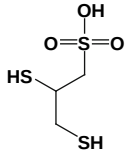
One of the chemical derivatives of dimercaprol (BAL) is DMSA. DMSA is an orally active chelating agent, much less toxic than BAL, and its therapeutic index is about 30 times higher (Angle and Kuntzelman, 1989). The empirical formula of DMSA is $C_4H_6O_4S_2$ and its molecular weight is 182.21. It is a weak acid soluble in water.

The LD_{50} value of sodium salt of DMSA in mice is: i.v. 2.4, i.m. 3.8, i.p. 4.4, and p.o. 8.5 gm/kg. The therapeutic index of DMSA was almost three times greater than that of DMPS because the LD_{50} of DMSA is about 3% greater than that of DMPS (Aposhian *et al.*, 1981). No significant loss of essential metals like zinc, iron, calcium, or magnesium was observed. Flora and Tripathi (1998) also reported a significant depletion of arsenic and a significant recovery in the altered biochemical variables of chronically arsenic-exposed rats. However, a double-blind, randomized controlled trial study conducted on a number of selected patients from arsenic-affected West Bengal (India) regions with oral administration of DMSA suggested that DMSA was not effective in producing any clinical and biochemical benefits or any histopathological improvements of skin lesions (Guha Mazumder *et al.*, 1998).

a. Drawbacks

DMSA distribution is predominantly extracellular, since it is unable to cross hepatic cell membranes. Hence, it is able to chelate arsenic from extracellular sites but not from intracellular sites. Adverse reaction of DMSA includes gastrointestinal discomfort, skin reaction, mild neutropenia, and elevated liver enzymes. Some evidence of embryo toxicity/fetal toxicity due to DMSA administration was also reported (Domingo, 1998).

3. Sodium 2,3-Dimercaptopropane-1-Sulfonate (DMPS)



DMPS was first introduced in the Soviet Union in the 1950s as ''Únithiol''. Its empirical formula is $C_3H_7O_3S_3Na$ and its

molecular weight is 210.3. DMPS, like DMSA, is another analog of BAL, and has been reported to be an effective drug for treating arsenic poisoning. A quantitative comparison has demonstrated that DMPS is 28 times more effective than BAL for arsenic therapy in mice (Hauser and Weger, 1989).

DMSA and DMPS are equally effective in providing significant depletion of body arsenic burden in chronically arsenite-exposed rats. Guha Mazumder *et al.* (1998) presented evidence on the efficacy of treatment of DMPS in a single-blind placebo-controlled trial of patients suffering with chronic arsenic poisoning in West Bengal, India. There was a significant decrease of clinical scores from pretreatment to post-treatment values among both DMPS and placebo groups. There was, however, no change in skin histology, hematology, and liver function test parameters in patients before and after the therapy with DMPS or placebo. No side effects were noticed in patients treated with DMPS.

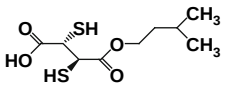
a. Drawbacks

No major adverse effects following DMPS administration in humans or animals have been reported (Hruby and Donner, 1987). However, a dose-dependent decrease in the copper contents was found in the serum, liver, kidneys, and spleen. Szincicz *et al.* (1983) observed that after 10 weeks of DMPS administration there was a decrease in copper concentration in serum and in various organs, an increase in iron contents of liver and spleen, and a decrease in hemoglobin, hematocrit, red blood cells, activity of alkaline phosphatase, and zinc content in the blood. Information regarding the developmental toxicity of DMPS is rather scarce. No abnormalities in the offspring with chronic oral DMPS treatment are reported. Oral administration of DMPS did not adversely affect late gestation, parturition, or lactation in mature mice and fetal and neonatal development does not appear to be adversely affected (Domingo, 1998).

4. Monoesters of DMSA

Recently, some mono- and diesters of DMSA, especially the higher analogs, have been developed and tried against cases of experimental heavy metal poisoning to address the shortcomings of DMSA, particularly depleting intracellular arsenic (Flora *et al.*, 2007a; Kalia and Flora, 2005; Mehta and Flora, 2001; Aposhian *et al.*, 1992). Due to its lipophilic nature it can easily cross the cell membrane and chelate arsenic from intracellular and extracellular sites. This is the best advantage of treatment with DMSA monoesters. It is also assumed that MiADMSA could be able to provide recovery in altered biochemical variables, particularly decreasing oxidative stress in tissues either by removing arsenic from the target organs and/or by directly scavenging ROS via its sulfhydryl group. These monoesters, like monoisoamyl DMSA (MiADMSA), mono n-amyl DMSA, mono n-butyl and mono iso-butyl DMSA, were significantly more efficient than DMSA in decreasing the arsenic content in most of the organs as soon as 1.5 h after administration. Among these monoesters, MiADMSA was the most effective in increasing the survival of mice and depleting tissue arsenic burden (Kreppel *et al.*, 1995; Flora *et al.*, 2004a, b). A few recent published studies clearly point to the fact that the analogs of DMSA were capable of crossing the membranes and were more effective in reducing the toxic metal burden in acute and subchronic arsenic intoxication (Flora *et al.*, 2004a, b, 2003; Flora and Sekhar, 2004; Saxena and Flora, 2004; Saxena *et al.*, 2005). Most of these conclusions are based on experimentally induced metal intoxication and no clinical data are available so far.

5. Monoisoamyl DMSA (MiADMSA)



Monoisoamyl dimercaptosuccinic acid (MiADMSA) is a C_5-branched chain alkyl monoester of DMSA. Kreppel *et al.* (1995) also reported the superior efficacy of MiADMSA and mono n-amyl DMSA in the protecting mice from the lethal effects of arsenic and in reducing body arsenic burden. These studies thus support that MiADMSA could be a potential drug to be used in the treatment of chronic arsenic poisoning. MiADMSA is a new drug and one of the most effective of the vicinal class of metal mobilizing agents (Jones *et al.*, 1992; Xu *et al.*, 1995). Although the compound is more toxic than the parent diacid DMSA (Mehta *et al.*, 2002; Flora and Mehta, 2003), its structural features and recent experimental evidences suggest that it might well be effective in chelating arsenic (Flora and Kumar, 1993; Flora *et al.*, 2002). More pronounced efficacy of MiADMSA could be attributed to its lipophilic characteristic. Lipophilicity and molecular size of this new drug might be important factors for the removal of arsenic from both intra- and extracellular sites possibly leading to better therapeutic efficacy. There could be a possibility of arsenic redistribution to the brain following treatment with this monoester. However, we observed no such effects in a recently conducted study (Flora *et al.*, 2005). It appears plausible that MiADMSA could be decreasing the oxidative stress in tissues either by removing arsenic from the target organs and/or by directly scavenging ROS via its sulfhydryl group (Mishra *et al.*, 2008).

No report is available for the therapeutic efficacy of MiADMSA against lewisite toxicity and other arsenicals. However, we reported the effect of MiADMSA on the reversal of gallium arsenide (GaAs)-induced changes in the hepatic tissue (Flora *et al.*, 2002). MiADMSA was found to be better than DMSA at mobilizing arsenic and in the turnover of the GaAs-sensitive biochemical variables.

Despite a few drawbacks/side effects associated with MiADMSA, the above results suggest that MiADMSA may be the future drug of choice owing to its lipophilic character

and the absence of any metal redistribution. However, significant copper loss requires further studies (Mehta and Flora, 2001; Mehta *et al.*, 2006). Moderate toxicity after repeated administration of MiADMSA may be reversible after the withdrawal of the chelating agent.

a. Drawbacks

It is reported that the toxicity of DMSA with an LD_{50} of 16 mmol/kg is much lower than the toxicity of MiADMSA with an LD_{50} of 3 mmol/kg but less than BAL (1.1 mmol/kg). Flora and Mehta (2003) reported that administration of MiADMSA led to no major alternations in heme synthesis pathway except for a slight rise in the zinc protoporphyrin levels suggesting mild anemia. MiADMSA was seen to be slightly more toxic in terms of copper and zinc loss and some biochemical alterations in the hepatic tissue in females as compared to male rats (Mehta *et al.*, 2006).

6. Role of Antioxidants

Oxidative stress can be partially implicated in arsenic toxicity and a therapeutic strategy to increase the antioxidant capacity of cells may fortify the long-term effective treatment of arsenic poisoning. This may be accomplished by either reducing the possibility of metal interacting with critical biomolecules and inducing oxidative damage, or bolstering the cells' antioxidant defenses through endogenous supplementation of antioxidant molecules (Flora *et al.*, 2007a). Although many investigators have confirmed arsenic-induced oxidative stress, the usefulness of antioxidants in conjunction with chelation therapy has not been extensively investigated yet. N-acetylcysteine (NAC) is a thiol, a mucolytic agent, and a precursor of L-cysteine and reduced glutathione. NAC is a source of sulfhydryl containing antioxidant that has been used to mitigate various conditions of oxidative stress. Combined administration of NAC and succimer post-arsenic exposure led to a significant recovery in biochemical variables indicative of oxidative stress and arsenic depletion from soft organs (Flora, 1999; Kannan and Flora, 2006). Various vitamins have been found to reduce the toxic manifestation of heavy metals. It was observed that vitamin E prevented the arsenite-induced killing of human fibroblasts (Lee and Ho, 1994). The protective mechanism of vitamin E could be attributed to its antioxidant property or its location in the cell membrane and its ability to stabilize the membrane by interacting with unsaturated fatty acid chains. Kannan and Flora (2004) reported that co-administration of vitamin C or vitamin E with DMSA or its monoisoamyl derivative (MiADMSA) produced profound recoveries in subchronically arsenic-exposed rats, although the results suggested that vitamin C was better in providing clinical recoveries and vitamin E was equally efficient in decreasing arsenic burden from the tissues. In an interesting study Wei *et al.* (2005) reported the involvement of oxidative stress in dimethylarsinic acid (DMA)-induced bladder toxicity and proliferation in rat, and inhibitory effects of vitamin C on these alterations.

7. Combination Treatment

As discussed above, metal chelators are given to increase the excretion of arsenic but unfortunately the use of these chelators are compromised by number of drawbacks (Mehta and Flora, 2001). These drawbacks open up the field for a search for new treatment which has no side effects. A number of strategies have been discussed in the recent past (Kalia and Flora, 2005). Among these strategies, combination therapy is a new and better approach to treat cases of metal poisoning (Flora *et al.*, 2007a; Mishra *et al.*, 2008). In a recent study, we investigated whether co-administration of thiol chelators like meso 2,3-dimercaptosuccinic acid (DMSA) or sodium 2,3-dimercaptopropane 1-sulfonate (DMPS) along with newly developed thiol chelators, e.g. monoisoamyl DMSA, is more beneficial than monotherapy with these chelators in counteracting chronic arsenic toxicity (Flora *et al.*, 2005; Bhadauria and Flora, 2007). It was concluded from these studies that concomitant administration of DMSA, a chelator known for its extracellular distribution with lipophilic chelators like MiADMSA, could play a significant and important role in abating a number of toxic effects of arsenic in animals compared to treatment with these chelators alone. We suggested that analogs having a long carbon chain (MiADMSA and MchDMSA) are better chelators than chelators with a shorter carbon chain (MmDMSA) or DMSA. It is assumed that analogs of DMSA eliminate arsenic simultaneously from the cell and provide assistance in bringing GSH homeostasis towards normalcy. Further combinational therapy with DMSA and MiADMSA or MchDMSA proved more beneficial than combined treatment with MmDMSA and DMSA (Mishra *et al.*, 2008).

As little experimental evidence is available there is a need for in-depth investigation in this area. It is thus proposed to investigate the effects of combination therapy particularly in the case of chronic arsenic poisoning, where a strong chelating agent is administered along with another structurally different chelating agent (Kalia and Flora, 2005) to evaluate whether combination treatment is able to promote the elimination of arsenic and restore arsenic-induced biochemical and clinical alterations.

VII. CONCLUDING REMARKS AND FUTURE DIRECTION

Arsenic is used in industry and agricultural production, and also appears in the food chain. Although information about arsenic and its inorganic and organic derivatives is now widely reported, there is very little information about their use as chemical warfare agents. The use of arsenicals remains a potential threat as they are relatively easy to manufacture and may cause significant morbidity and mortality. Knowledge about these arsenicals is very important to plan a response in an emergency. Lewisite is one of the arsenicals which has not been studied in detail. There is very little information available on the detailed

toxic effects of organic arsenicals particularly carcinogenicity, mutagenicity, and teratogenicity. Lewisite, an organic arsenical war gas which is a vesicant, has been reported for its ability to bind with thiol groups leading to the possibility of its undesirable effects on a variety of enzymes. Thus there is a very strong possibility that exposure to lewisite might also lead to carcinogenic effects, but this hypothesis requires experimental and epidemiological evidence. It is not known if lewisite is persistent. However, arsenic is an elemental poison and any residual hydrolysis, combustion, and decontamination product is likely to contain an arsenical compound. Some of the major thrust areas for future research include the possibility of delayed or latent effects arising after organic arsenical (particularly lewisite) exposure. These effects have been studied in detail recently after inorganic arsenic exposure; however, there is very little information available after organic arsenical exposure. Development of antidotes is another area which requires immediate attention. Although one approved antidote, BAL, is available for lewisite, further investigations are required with other derivatives of BAL like DMSA, DMPS, or monoesters of DMSA like MiADMSA. Chelators are generally not of any immediate benefit as far as toxicity of arsine is concerned and once hemolysis has begun. With some new chelating agents, like MiADMSA, at experimental stages the effectiveness and safety of these chelating against arsine poisoning need assurance. The toxicodynamics of arsine is also one area which requires exploration as this information will be of immense help in developing suitable antidotes, particularly for impending hemolysis by removing or displacing arsine.

Inorganic arsenic, particularly arsenic (III), is a well-documented potent carcinogen causing cancer of the bladder, lung, skin, and possibly liver and kidney. Because of failures in the attempt to study the carcinogenic effects of arsenic in animal models, the mechanism of arsenic-induced carcinogenic effects remains unclear. The newly discovered potency of trivalent methylated arsenic metabolites opens up new opportunities for mechanistic studies. No treatment of proven benefit is available to treat chronic exposure. Treatment options advocated are vitamin, mineral supplements, and antioxidant therapy. The benefits of these treatment measures need to be evidence based to receive endorsement and wider application. Further research work is also recommended in the areas of (1) molecular mechanisms of action of clinically important chelators, (2) intracellular and extracellular chelation in relation to mobilization of aged arsenic deposits and the possible redistribution of arsenic to sensitive organs like the brain, (3) effect of metal chelators on biokinetics during continued exposure to arsenicals, (4) combined chelation with lipophilic and hydrophilic chelators, (5) use of antioxidants, micronutrients, or vitamins as complementary agents or antagonists, (6) minimization of the mobilization of essential trace elements during long-term chelation, and (7) fetotoxic and teratogenic effects of chelators.

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CHAPTER 10

Psychotomimetic Agent BZ (3-Quinuclidinyl Benzilate)

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I. INTRODUCTION

Experience with the use of chemical warfare agents (CWAs) in WWI has led to an attempt to synthesize new chemicals for military use but without lethal effects (Ketchum and Sidell, 1997). The research was focused on contemporary incapacitants influencing behavior but without substantial effects on important vital functions. Incapacitating agents include the esters of glycolic acid, i.e. atropine-like anticholinergic compounds. BZ (3-quinuclidinyl benzilate QB, QBN, Ro 2-3308) is a prototype of the CNS depressants; and a prototype of CNS stimulants is LSD-25 (D-lysergic acid diethylamide) or cannabinol. LSD-25 was found as the first candidate for this group of CWAs. However, its physical and chemical properties as well as unpredictable behavior after exposure have led to its exclusion from military research. From many tested compounds, anticholinergic drugs were chosen for further research. The anticholinergics are generally ''glycolates'' (substituted glycolic and tropic acid esters) of which the representative and best-known member is atropine. Major symptoms of low-level atropinization include dry mouth, dilatation of pupils, and tachycardia. With toxic doses of atropine, central excitation becomes more prominent, leading to restlessness, irritability, disorientation, and hallucinations or delirium. With still larger doses of atropine, stimulation is followed by depression, coma, and medullary paralysis. A number of anticholinergic compounds influencing psychotic stage and higher nervous functions were tested (Table 10.1).

This group of compounds has both peripheral and central properties. It is known from ancient literature that atropine has psychotomimetic effects (extracts from *Atropa belladona* were used to induce hallucinations in ''wizards''). Central effects increase when the compounds are considered in the following rank: atropine, scopolamine, benactyzine, Ditrane, and, finally, BZ and other esters of glycolic acid (Albanus, 1970). BZ was originally studied for the therapy of gastrointestinal diseases. But even in small doses it produces side effects, such as confusion and hallucinations. Therefore, BZ was withdrawn from commercial studies and turned over to the US Army as a possible candidate for incapacitating agents (Sidell, 1982). BZ is known to neuropharmacologists as the standard drug for testing the central activity of muscarinic cholinergic receptors. At present, BZ is used in the research of cholinergic neurons in the peripheral and central nervous systems (Monica *et al.*, 2008; Yamada *et al.*, 2007), Parkinson's disease, Alzheimer's disease, and other types of dementia (Wyper *et al.*, 1993; Parkasi *et al.*, 2007). In the 1960s, a number of similar compounds including BZ were studied, in the framework of the military, through industrial liaison programs (Pearson, 2006); these compounds, by politico-military goals and requirements, must contain the following criteria:

- high potency for physiological action (acting in μg/kg or less)
- rapid onset (minutes)
- defined duration (optimally minutes–hours) and reversible effect
- stability at storage and delivery
- significant and predictable effect
- capability of rapid dissemination in defined conditions and
- high safety ratio.

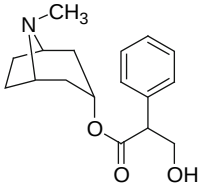
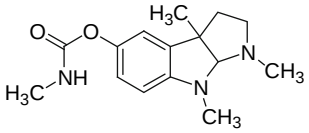
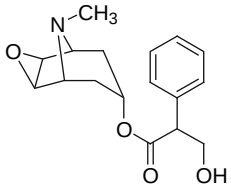
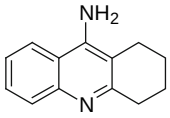
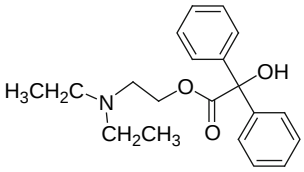
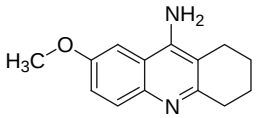
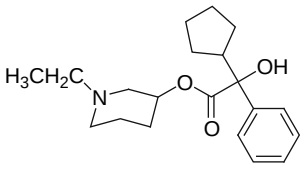
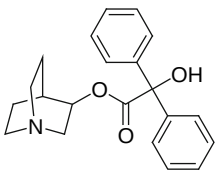
II. BACKGROUND

BZ is a white powder with a bitter taste. It has good solubility in water and organic solvents, and is stable at field conditions at a minimum of 1 or 2 days without loss of its incapacitating activity. Its physical and chemical properties are summarized in Table 10.2.

III. TOXICOKINETICS AND MECHANISM OF ACTION

BZ is absorbed by all the usual routes of administration (oral, parenteral, and inhalation). When administered by inhalation, the absorption into the transport system (blood stream) is more pronounced in comparison with oral administration. Experimental studies were performed with

TABLE 10.1. Structural formulae of some anticholinergic psychotomimetics and their antidotes

Compound		Compound	
Atropine		Physostigmine	
Scopolamine		Tacrine	
Benactyzine		7-Methoxytacrine	
Ditrane			
BZ, 3-quinuclidinyl benzilate			

parenteral administration, and in this case, i.v. administration is without any loss because of direct involvement of BZ in the transport system. In the transport blood, the compound is bound to the plasma proteins (preferably albumin) and it is transported to the sites of toxic effect – central and peripheral nervous systems. At these sites, BZ interferes with cholinergic nerve transmission at muscarinic sites, both in the peripheral autonomic nervous system and in the brain and spinal cord. Because of the wide distribution of these sites, measurable effects upon almost each phase of neural regulation may be observed. BZ readily crosses the blood–brain barrier, is distributed to all areas of the brain and spinal cord, and interacts with cholinergic receptors as a competitor with physiologically active transmitter acetylcholine. At the peripheral system, BZ binds to muscarinic acetylcholine receptors in smooth muscles, like atropine, and it has a very high affinity but without intrinsic activity (Fusek *et al.*, 1971).

At the central nervous system, BZ binds to all subtypes of muscarinic receptors, each of them having different functions in the brain (Lefkowitz, 2004). The particularly long duration of the central action of BZ may be related to its higher affinity

TABLE 10.2. Physical and chemical properties of 3-quinuclidinyl benzilate (BZ, QNB)

		Reference
Appearance	White crystals	
Moleculer weight	337.39	
Melting point	190°C (racemic 168°C)	Aleksandrov and Emelyanov (1990)
Boiling point	412°C	Aleksandrov and Emelyanov (1990)
Volatility	c_{max} = 0.0005 mg.l^{-1} (70°C)	Aleksandrov and Emelyanov (1990)
Solubility of hydrochloride	Very good	
Stability in substance	Compound is stable	
Stability in solution	Compound is stable in the water solution	
Persistence in terrain	Air oxidation proof (half-time 3–4 weeks) at 25°C and pH 7	Aleksandrov and Emelyanov (1990)
Method of determination of BZ in field conditions	In Czech army: (a) indicative tube type PT-51, (producer Oritest Ltd, Prague) (b) in field laboratory PPCHL-90	
Method of determination of BZ in biological agents in laboratory conditions	Determination: extract spectrophotometry of ion pairs Detection: TLC	Halamek and Kobliha (1993) Skalican *et al.* (1997)

for the nervous tissue, especially strong adsorption on mitochondria, the subcellular organelles concerned principally with the energy supply to the cell; this part of BZ has been found to reduce the oxygen consumption by nerve cells stimulated in various ways (Jovic and Zupanc, 1973).

IV. TOXICITY

The acute toxicity of BZ (expressed as LD_{50}) in different species following different routes of administration is shown in Table 10.4.

TABLE 10.3. Pharmacological activity of the compounds tested on isolated rat jejunum

Compound	i.a.	$pD_2 \pm P_{95}$	$pA_2 \pm P_{95}$
Acetylcholine	$\alpha = 1$	6.79 ± 0.06	
Muscarine	$\alpha = 1$	6.59 ± 0.05	
Atropine	$\beta = 0$		8.92 ± 0.05
Benactyzine	$\beta = 0$		7.64 ± 0.07
Scopolamine	$\beta = 0$		8.83 ± 0.06
Ditrane	$\beta = 0$		8.36 ± 0.07
BZ	$\beta = 0$		8.55 ± 0.09

i.a. = intrinsic activity
α = intrinsic activity of agonist
β = intrinsic activity of antagonist
pD_2 = negative decadic logarithm of agonist ED_{50} ($-\log ED_{50}$ agonist)
pA_2 = negative decadic logarithm of antagonist ED_{50} ($-\log ED_{50}$ antagonist)
P_{95} = 95% confidence limits

It is clear that acute toxicity is relatively low. However, the dose causing incapacitation is much lower. Therefore, the ratio between lethal and incapacitant dose is in the range of the logarithmic scale.

The effective doses (ED) of some psychotomimetic drugs for incapacitation in humans were assessed and are presented in Table 10.5.

The dose required to produce incapacitation is roughly 40 times lower than the lethal dose. For humans the effective dose (ED_{50}) for incapacitation by BZ under field conditions is about 60 mg.min/m^3 with a 75 kg body weight, volume of respiration of 15 l/min, and LD_{50} value about 200 μg/kg, p.o. administration.

A. Symptoms

BZ is active when it is given by the intravenous, intramuscular, inhalation, or oral route of administration. The fragmentary data available indicate that the higher dose caused the greatest effects and longest duration. From 1960 to 1969, field tests had been conducted with 3-quinuclidinyl benzilate by the US Army. Sidell's (1982) description of the effects induced by BZ (4.5–17.1 μg/kg) is as follows.

At low doses, the effects include dry mouth, decreased gastric motility, inhibition of sweating, increase in heart rate, papillary dilatation and loss of accommodation, mild sedation, and mental slowing.

At high doses these effects are severe. There are marked disturbances of function at all levels of the central nervous system: motor coordination, attentiveness, and control of thought and the learning process all decline. Confusion, restlessness, impairment of perception and interpretation, and memory span are observed. The first symptoms occur

TABLE 10.4. Lethal doses of 3-Quinuclidinyl benzilate and other anticholinergic drugs for different species

Animal	Route of administration	LD_{50} (mg/kg)		
		3-Quinuclidinyl benzilate (BZ)	Atropine	Scopolamine
Mouse	i.v.	22	74	163
	i.p.	110	256	119
	i.m.	42		
	p.o.	460	693	
Rat	i.v.	20	41	
	i.p.		256	
	i.m.	281		
	p.o.		733	1, 270
Guinea pig	i.v.	14	163	
	i.p.		277	
	p.o.		1,100	
Rabbit	i.v.	10		
	i.m.		588	
Cat	i.v.	12		
Dog	i.v.	12		
Pig	i.v.	5		

depending on the route of administration; judgment and deficient insight are all features of this syndrome. True hallucinations are present and if the dose is quite high the subject may become stuporous or even comatose for several hours.

After a single injection of BZ (5.0–6.4 μg/kg, i.m.), the following symptoms are observed at various time intervals:

- 10 min: light headedness and giggling.
- 30 min: dry mouth, blurred vision, nausea, chilly sensations, and twitching.
- 1 h: flushed skin, incoordination, fatigue, unsteadiness, sleepiness, and quivering legs.
- 2 h: many of the above, plus poor concentration, restlessness, hallucinations, slurred speech, and muscle fasciculation.
- 3 h: above, plus tremors.
- 4 h: above, plus difficulty in handling the subject, increased pulse to 130.
- 8 h: above, plus delirium and hallucinations.
- 24 h: persistent delirium, hallucinations, restlessness, unsteadiness, increased pulse in some but not all subjects.
- 48 h: persistent impairment of functions.

Mydriasis and disturbed accommodation remain unchanged depending on the dose after 2 or 3 days. BZ has a mean duration of incapacitation of about 70 h. In real situations, wide variations in dosage would occur and thus the results will also be varied over the large time interval.

V. RISK ASSESSMENT

BZ was stockpiled by the US military forces in 1980. However, in 1992, a US delegation to the Conference on Disarmament in Geneva declared that their stocks of BZ were destroyed. It is not known if stocks of BZ are held by any other armed forces. The military use of BZ was limited to

TABLE 10.5. The effective doses (ED) of some psychotomimetic drugs for incapacitation in man

Drug	Route of administration	ED (mg/kg)	Reference
LSD-25	i.m.	0.0005–0.001	Hoffman (1960)
	p.o.	0.001	Hollister (1968)
BZ	i.m.	0.006	Sidell *et al.* (1973)
	i.m.	0.01	Spivak and Milstejn (1973)
	i.v.	0.005	Spivak and Milstejn (1973)
Scopolamine	i.m.	0.024	Ketchum *et al.* (1973)
Atropine	i.m.	0.175	Ketchum *et al.* (1973)
Ditrane	i.m.	0.1–0.3	Gershon and Olariu (1960)
	i.m.	0.15	Ketchum *et al.* (1973)

special operations and, at present, BZ agent can be considered as a potential agent dedicated for special military operations. There exist a group of other incapacitants (not anticholinergic hallucinogens) known as nonlethal chemicals with very strong effects, e.g. fentanyl derivatives, as demonstrated in Moscow in 2002. Use of these incapacitants by terrorists and other small groups cannot be excluded either. Today, BZ is considered as one of a number of ''nonlethal'' incapacitating chemical weapons. These compounds are designed and primarily employed to incapacitate personnel or material while minimizing fatalities, permanent injury to personnel, and undesired damage to property and the environment (Pearson, 2006).

VI. TREATMENT

Based on the knowledge of mechanism of action (simply described as a lack of neuromediator acetylcholine), the therapeutic principle of BZ intoxication is to increase the acetylcholine level by inhibitors of cholinesterases. However, their use is limited to their ratio between therapeutic and toxic dose and therefore reversible inhibitors are preferred, although some experiments with interaction of highly toxic nerve agents (VX and sarin) and BZ on healthy volunteers were described (Sidell *et al.*, 1973). Physostigmine was the first antidote proposed against BZ intoxication. Its antidotal effect against intoxication with extract from *Atropa belladona* has been known for more than 150 years. It is known that physostigmine acts as a good antidote against scopolamine intoxication, as it is able to treat all kinds of symptoms, such as electrophysiological, psychiatric, and biochemical changes caused by this anticholinergic drug. Physostigmine was considered as one of the potential antidotes against anticholinergics, including esters of glycolic acid. Its therapeutic and toxic doses are very close, showing side effects and short time duration. There were some attempts to obtain new inhibitors having lower toxicity in comparison with physostigmine. Acridine derivatives were of great interest (Albert, 1966). From these compounds, 1,2,3,4-tetrahydro-9-aminoacridine (tacrine) was found to be very effective (Fusek *et al.*, 1974). It was found to be an antagonist against morphine and curare overdosage. It is an inhibitor of cholinesterases comparable with neostigmine or physostigmine and also having antihistaminic activity. Its effect is prolonged in comparison with physostigmine. During the studies of antidotes against anticholinergic hallucinogens, it was recognized as the most promising antidote at the time (Gershon and Angrist, 1973). The antidotal effect of Tacrine against Ditrane intoxication including improvement of the EEG changes was approved by Itil and Fink (1966) in 74 patients. Atropine, scopolamine, and Ditrane intoxications were successfully treated using physostigmine and Tacrine, but also sarin (Ketchum *et al.*, 1973). However, the toxicity of Tacrine was the reason for its limited use. Moreover, it has other limitations, for example it caused temporary changes in hepatic function tests and therefore was abandoned (Marx, 1987).

The 7-methoxy derivative of Tacrine (7-MEOTA) was synthesized and tested (Fusek *et al.*, 1986). 7-MEOTA inhibited *in vitro* rather more butyrylcholinesterase (BuChE) ($I_{50} = 3.5.10^{-7}$ M) than acetylcholinesterase (AChE) ($I_{50} = 3.5.10^{-6}$ M). The inhibition is a competitive–noncompetitive reversible type. The characteristics of AChE inhibition indicate that 7-MEOTA binds to the active surface of AChE in the gamma-anionic site, like galantamine or coumarine. Inhibition of blood AChE by *O*-ethyl-S-(2-dimethylaminoethyl) methylphosphonothioate (0.042 mg/kg = 1.5 × LD_{50}, i.m.) after premedication with 7-MEOTA (100 mg/kg, i.m.) was substantially smaller than in intoxicated animals pretreated with saline, which highlights its possible use for preventing intoxication by organophosphorus anticholinesterase agents. 7-MEOTA enhanced the contraction response of the guinea pig atria (ED_{50} positive inotropic effect = 1.7×10^{-6} M), reduced the frequency of contractions (by binding the compound to the effectors of the cholinergic system of the heart tissue), increased the contraction response of the isolated rat diaphragm, and antagonized the effect of *d*-tubocurarine. The dose of 3×10^{-6} M of 7-MEOTA entirely suppressed the effect of a dose of 2×10^{-6} M *d*-tubocurarine. 7-MEOTA elicited a contraction response (1×10^{-7} M) and intensified the response of the isolated rat jejunum to the applied concentration of cholinomimetics. The long-term increase of intestinal peristaltic after low concentrations of 7-MEOTA (1×10^{-7} M) bears witness to the inhibitory effect of the compound on tissue cholinesterases. In a dose-dependent manner, 7-MEOTA effectively antagonized symptoms of intoxication elicited by anticholinergics in dogs. A dose of 5 mg/kg, i.m. of 7-MEOTA shortened the time needed for a fall in the score of intensity of symptoms elicited by BZ (0.05 mg/kg, i.m.) from 210 min in untreated intoxication to 54 min, respectively (Fusek *et al.*, 1979).

Our results show that contributing to the effect of 7-MEOTA (besides the direct influence on effectors of the cholinergic system) can also be mediated through the action of the induced cholinesterase inhibition in the central and peripheral nervous systems. The more beneficial properties of 7-MEOTA in comparison with physostigmine suggests the possible therapeutic use of this compound in cases where hitherto physostigmine used to be applied. In contrast to physostigmine, the application of 7-MEOTA will not require repeated doses for maintaining the therapeutic effect at low dosage and has minimum side effects.

7-MEOTA is a potent, centrally active cholinesterase inhibitor. Therefore, this new medicament not only has use as an antidote against BZ intoxication but can be used in general as a drug for the treatment of cholinergic deficit neurological disorders, such as Alzheimer's disease. In isolated rat jejunum, 7-MEOTA increased muscle contractility and in isolated guinea pig ventricular myocytes this

compound was found to prolong transmembrane action potential and decrease the amplitude of the plateau. In isolated rat diaphragm, 7-MEOTA increased muscle contractility after electric stimulation of the phrenic nerve.

7-MEOTA was found to antagonize convulsive action of pentamethylenetetrazole and significantly decreased the number of surviving animals following administration of this drug. Results from behavioral studies indicated that 7-MEOTA antagonized anticholinergic syndrome evoked by scopolamine, Ditrane, and BZ.

TABLE 10.6. Lethal doses of Tacrine, 7-methoxytacrine and physostigmine in laboratory animals

Compound	Animal	Route of administration	LD_{50} (mg/kg)
Tacrine	Mouse	i.m.	28.9 (24.2–35.8)
	Rat	i.m.	33.8 (28.8–40.6)
	Rat	p.o.	103.7 (84.6–135.8)
	Rat	i.p.	20.2 (16.4–24.8)
	Rat	i.v.	12.0 (10.7–13.6)
	Rabbit	i.n.	13.3 (8.0–18.8)
	Dog	i.m.	12.6 (10.1–14.7)
7-Methoxytacrine	Mouse	i.m.	125 (110–143)
	Rat	i.m.	258 (224–313)
	Rat	p.o.	793 (662–950)
	Rat	i.p.	73.3 (61–90)
	Rat	i.v.	22.2 (19.9–25.2)
	Rabbit	i.m.	75.3 (60–89)
	Dog	i.m.	18.9 (15.2–22.1)
Physostigmine	Mouse	i.m.	0.86 (0.7–1.0)
	Rat	i.m.	2.2 (1.9–2.4)
	Dog	i.m.	0.83 (0.68–0.95)

The antidotal effect of 7-MEOTA on the anticholinergic syndrome was potentiated by nootropics, by diazepam, and especially by opioid peptides (Fusek, 1977).

7-MEOTA also antagonized side effects of tricyclic antidepressants and protected AChE against inhibition by some organophosphate anticholinesterase compounds.

Acute toxicity of 7-MEOTA was low, since LD_{50} (i.m.) was 125 mg/kg for mice and 258 mg/kg for rats. The oral LD_{50} in rats was found to be 793 mg/kg. Analogous values for THA at the same order were: 29, 34, and 104 mg/kg (Fusek, 1977). Also, 7-MEOTA was found to be markedly less toxic than THA in dogs and rabbits. A subacute toxicity study of 7-MEOTA (3 months' administration) demonstrated that the compound was well tolerated at doses of 25 mg/kg (i.m.) and 50 mg/kg (p.o.) in rats and 2.5 mg/kg (i.m. and i.v.) in beagle dogs. No pathological changes were observed in biochemical, haematological, and morphological investigations.

VII. ANALYTICAL METHODS

The fluorometric method for 7-MEOTA determination in biological material was developed, and blood concentration profiles of 7-MEOTA in rats and healthy volunteers were estimated (Filip *et al.*, 1991).

Maximal concentrations of 7-MEOTA in human blood were observed approximately 0.5–1 h after i.m. and 4 h after p.o. application of the drug. Half-life was 5 h, and an effective level was maintained for 12 h. Similar results in rats with radiolabeled [H^3]7-MEOTA were obtained. The main part of 7-MEOTA was eliminated by urine, and later by feces 6 h after administration. The majority of the compound was eliminated in unchanged form, and the minority was metabolized. Among the metabolites, 7-MEOTA and its conjugate with glucuronic acid were demonstrated; further metabolites were likely compounds with the OH group in position 1 or 2 (Patocka *et al.*, 1996).

In healthy volunteers, 7-MEOTA was well tolerated in a single dose of 2 mg/kg (p.o.) or 1 mg/kg (i.m.) following daily administration (7 days). The compound did not influence cognitive functions of healthy persons. The blood concentration profiles of volunteers corresponded well with those in rats.

On the basis of the results obtained, it was decided that 7-MEOTA would be studied in more detail including preclinical testing. The first and second phases of clinical testing on healthy volunteers were performed. It was found that 7-MEOTA is well tolerated after peroral or intramuscular administration (dose 2 mg/kg, p.o. and 1 mg/kg, i.m.) (Filip *et al.*, 1991). Clinical testing of this drug was performed on patients with tardive dyskinesias occurring after lengthy administration of neuroleptics with good therapeutic efficacy (Zapletalek *et al.*, 1989). As a result, it was introduced in the form of tablets (7-MEOTA, 100 mg)

and injections (50 mg in 2 ml) to the Czech Army as an antidote against psychotomimetic agents.

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

BZ was recognized as an incapacitant agent for military use. It was stockpiled and stored as CWA by the US Army but their stocks were destroyed in 1992. The use of this agent is not excluded. Its mechanism of action is based on the interaction with cholinergic receptors. The symptomatology is characterized by peripheral action (vegetative symptoms) and central symptomatology including hallucinations. The antidotes and effective antidotal therapy are of vital interest and are possible using commonly available physostigmine or 7-MEOTA, respectively. The use of BZ in the research of the cholinergic nervous system cannot be omitted.

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CHAPTER 11

Onchidal and Fasciculins

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I. INTRODUCTION

Onchidal and fasciculins are natural toxins, which produce their toxicity in mammalian systems by virtue of primarily acetylcholinesterase (AChE) inhibition. AChE hydrolyzes and inactivates acetylcholine, thereby regulating the concentration of the transmitter at the synapse. Termination of activation is normally dependent on dissociation of acetylcholine from the receptor and its subsequent diffusion and hydrolysis, except in diseases where acetylcholine levels are limiting or under AChE inhibition, conditions that increase the duration of receptor activation (Silver, 1963).

The toxins that inhibit the AChE are called anticholinesterase (anti-ChE) agents. They cause acetylcholine to accumulate in the vicinity of cholinergic nerve terminals, and thus are potentially capable of producing effects equivalent to excessive stimulation of cholinergic receptors throughout the central and peripheral nervous systems (Long, 1963). Nevertheless, several members of this class of compounds are widely used as therapeutics agents; others that cross the blood–brain barrier have been approved or are in clinical trial for the treatment of Alzheimer's disease.

The AChE can be classified in several ways. Mechanistically, it is a serine hydrolase. Its catalytic site contains a catalytic triad – serine, histidine, and an acidic residue – as do the catalytic sites of the serine proteases such as trypsin, several blood clotting factors, and others. However, the acidic group in AChE is a glutamate, whereas in most other cases it is an aspartate residue. The nucleophilic nature of the carboxylate is transferred through the imidazole ring of histidine to the hydroxyl group of serine, allowing it to displace the choline moiety from the substrate, forming an acetyl-enzyme intermediate. A subsequent hydrolysis step frees the acetate group. Understanding of the catalytic properties of the protein has assisted in our understanding of its inhibition by organophosphate and carbamate inhibitors. However, several questions remain to be answered regarding AChE catalysis; for example, the mechanism behind the extremely fast turnover rate of the enzyme (Fair *et al.*, 1994). Despite the fact that the substrate has to navigate a relatively long distance to reach the active site, AChE is one of the fastest enzymes (Nair *et al.*, 1994). One theory to explain this phenomenon has to do with the unusually strong electric field of AChE. It has been argued that this field assists catalysis by attracting the cationic substrate and expelling the anionic acetate product (Ripoll *et al.*, 1993). Site-directed mutagenesis, however, has indicated that reducing the electric field has no effect on catalysis. However, the same approach has indicated an effect on the rate of association of fasciculin, a peptide that can inhibit AChE (Schafferman *et al.*, 1994).

Naturally occurring irreversible inhibitors of AChE are toxins which are often selective inhibitors of protein function, and this property can often be exploited for a variety of purposes (Pita *et al.*, 2003). For instance, physostigmine, also called eserine, is a naturally occurring alkaloid inhibitor of AChE that has been used in understanding the kinetic mechanism of AChE.

II. BACKGROUND

This chapter covers natural agents that prolong the existence of acetylcholine after it is released from cholinergic nerve terminals. Natural toxins are chemical agents of biological origin including chemical agents and proteins, and can be produced by all types of organisms; such is the case of onchidal and fasciculins. Although a chemical and a protein, respectively, onchidal and fasciculins share the same toxic effect of inhibiting AChE, which is concentrated in synaptic regions and is responsible for the rapid catalysis of the hydrolysis of acetylcholine. As such, the natural toxins onchidal and fasciculins can produce disorders of neuromuscular transmission clinically categorized as either pre- or post-synaptic; some toxins simultaneously affect both sites. Chemical agents associated with neuromuscular transmission syndromes include the fasciculins, crotoxin, taipoxin, tubocurarine, organophosphorus compounds, and others (Anadón and Martínez-Larrañaga, 1985).

These aforementioned natural anti-ChE agents can be developed for a different utility including the extensive application as toxic agents (i.e. potential chemical warfare).

One of the first interesting structures with cholinergic properties isolated from a marine source is the chemical onchidal. This compound was first isolated from the mollusc *Onchidella binneyi* and has an acetate ester similar to acetylcholine. Upon isolation, onchidal was discovered to be an active site-directed irreversible inhibitor of AChE (Abramson *et al.*, 1989).

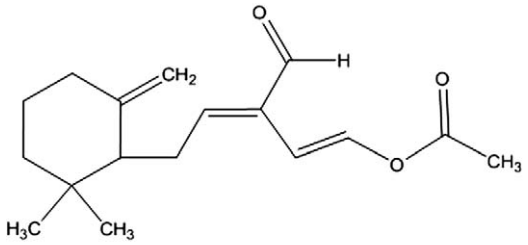
Neurotoxins from snake venoms have proved to be valuable tools for the understanding of synaptic transmission mechanisms. Likewise, the powerful inhibitory action of fasciculins against mammalian AChE makes them potentially useful tools for pharmacological and neurochemical research. Studies of their biochemical and electrophysiological effects on the central nervous system and biochemical characterization are now being carried out.

Natural toxins can be extremely potent and many of them are effective at far lower dosages compared to conventional chemical agents. Natural toxins, as compounds of biological origin, are often classed as biological agents, but they are not infectious and are more similar to chemicals with respect to their military potential for tactical use; therefore, they should be considered to be chemical agents. The Chemical Weapons Convention (2003) (available at: www.fas.harvard.edu/~hsp/cwc/cwcbyart.html) also includes natural toxins as chemical agents, and, specifically, includes the onchidal and fasciculins toxins in its control regime along with other highly toxic chemicals.

A. Onchidal

Onchidal is a toxic component of a certain poisonous marine opisthobranch mollusc. Like other opisthobranchs, the Onchidiacea family of molluscs does not have the protection of a hard external shell as do most molluscs. They rely instead on the production of a defensive secretion. When the animal is disturbed it secretes a viscous fluid from specialized glands. In two species of Onchidella (*Onchidella floridanun* and *Onchidella borealis*), this defensive secretion has been shown to act as a deterrent to potential predators, including fish and crabs. Chemically, it is a simple lipophilic acetate ester (see Figure 11.1).

Onchidal has been identified as the major lipid-soluble component of the defensive secretion of *Onchodella binneyi* and it has been proposed as the compound responsible for the chemical protection of *Onchidella*. *Onchodella binneyi* is an opisthobranch mollusc which inhabits the rocky intertidal zone near the area of Baja California, Mexico. The defensive secretion was obtained in the field by squeezing the mollusc and collecting the mucus discharge in capillary tubes. Large quantities of this material could be obtained after extraction of intact animals with acetone (Ireland and Faulkner, 1978). However, the distribution of onchidal in different species of *Onchidella* was not reported and, apart from inhibiting the growth of *Staphylococcus aureus* (MIC value was between 0.21 and 0.63 μg/ml), implying that onchidal is a potent inhibitor of Gram-positive



Onchidal

FIGURE 11.1. Chemical structure of onchidal.

microorganisms, no biological activity of onchidal was described. Additional studies demonstrated that onchidal is contained in several different species of *Onchidella* and that, once purified, is toxic to fish (Abramson *et al.*, 1989). Onchidal can be found in four of the eight known species of *Onchidella* collected from different countries (Table 11.1). In addition, the Abramson study found onchidal to be toxic to goldfish. Although goldfish are not potential predators of *Onchidella*, these results demonstrate that onchidal has a distribution and a biological activity consistent with its proposed role in the chemical defense of *Onchidella*.

B. Fasciculins

Mamba snake venom (*Dendroaspis angusticeps*, *polylepis*, *viridis*, *jamesoni*) contains a mixture of neurotoxic compounds including post-synaptic cholinoreceptor α-neurotoxins, dendrotoxins, fasciculins, and muscarinic toxins (Hawgood and Bon, 1991). Effects at the neuromuscular junction include AChE inhibition by fasciculins, and increased presynaptic release of acetylcholine by dendrotoxins (polypeptides which facilitate acetylcholine release in response to nerve stimulation); together with the high acetylcholine content of mamba toxin (6–24 mg/g), these effects are synergistic and enhance neurotoxicity and lethality. Moreover, the venom may contain other components having a synergistic action with dendrotoxin.

Toxins that facilitate neuromuscular transmission are a characteristic component of mamba venom. The four known fasciculins bind to a peripheral regulatory anionic site of AChE in a noncompetitive and irreversible manner

TABLE 11.1. Concentration of onchidal in different species of *Onchidella* (Abramson *et al.*, 1989)

Organism	Collection site	Onchidal concentration (μg/animal)
O. binneyi	Baja California, Mexico	230
O. borealis	Central California, USA	33
O. nigricans	New Zealand	18
O. patelloides	Australia	42

(Hawgood and Bon, 1991). The dendrotoxins comprise the second group of facilitatory neurotoxins and are present in most mamba venoms (with the exception of *Dendroaspis jamesoni*); they inhibit voltage-dependent K^+ channels in motor nerve terminals and facilitate acetylcholine release at the neuromuscular junction. Post-synaptic toxins present in mamba venom bind to and block nicotinic acetylcholine receptors. The muscarinic toxins present in mamba venom are small (7 kDa) proteins that selectively bind to muscarinic cholinergic receptors and may constitute up to 1% of the venom protein (Adem and Karlsson, 1985; Jerusalinsky and Harvey, 1994). About 12 muscarinic toxins have been isolated. M1 toxin binds noncompetitively and with high affinity to the M_1 muscarinic receptor sybtype. MTx1 and MTx2 show high affinity for both muscarinic M_1 and M_3 receptors; little is known about the receptor selectivity of MTx3 and MTx4. Dpα and Dpβ are also muscarinic agonists displaying similar affinity for both the M_1 and M_2 receptor subtypes. The last two agonists, DpMTx and DvMTX, are selective muscarinic agonists present in the venom of some mamba species; these agonists also show affinity for the M_1 muscarinic receptor subtype.

The fasciculins are a family of closely related ~6,750 Da peptides isolated from the venom of mambas (genus *Dendroaspis*), and are named after the long-lasting muscle fasciculations they produce in mice (Rodriguez-Ithurralde *et al.*, 1983). They are potent and selective inhibitors of AChE. Fasciculins (Fas) are 61 residue long polypeptides. They share a three-looped structural motif with other toxins, such as α-neurotoxins, cytotoxins, and muscarinic toxins, directed to diverse specific tagets. Four fasciculins are known (Table 11.2) which differ only by one to three residues and show selective and potent anti-AChE activity: Fas1 and Fas2 from the venom of *Dendroaspis angusticeps* (eastern green mamba) contains 61 amino acid residues including eight half-cystines (Rodriguez-Ithurralde *et al.*, 1983), ToxC from the venom of *D. polylepis polylepis* (black mamba) (Joubert and Taljaard, 1978), and Fas3, which was isolated from a particular batch of *D. viridis* (western green mamba) venom and found to have the same primary structure as ToxC (Marchot *et al.*, 1993). No fasciculin has been found in other *D. viridis* venoms (Marchot *et al.*, 1993), or in *D. jamesonii* (Jameson's mamba).

TABLE 11.2. Types of fasciculin identified

Fasciculins characterized	**From the mamba snake venoms (Elapidae family) (*Dendroaspis* genus)**
Fasciculin 1	*Dendroaspis angusticeps* (green mamba)
Fasciculin 2 (formally F_7 toxin)	*Dendroaspis angusticeps* (green mamba)
Fasciculin 3 (formally toxin C)	*Dendroaspis polylepis* (black mamba)
Fasciculin 4	*Dendroaspis viridis* (western green mamba)

Angusticeps-type toxins constitute a group of toxins typical for mamba venoms. They consist of 59–61 amino acid residues and four disulfides, and show sequence homology with short-chain post-synaptic toxins and cardiotoxins, but they are immunochemically distinct from these toxins. Eight toxins of this type have been sequenced and structurally classified into four subgroups (Joubert and Taljaard, 1978). Pharmacological studies on these *angusticeps*-type toxins have revealed that only toxins F_7 and C, which belong to subgroup I, exhibit a potent inhibition of cholinesterase of various tissues, except for that of chick skeletal muscle. Both F_7 and C stimulate mouse and toad skeletal muscles by their anticholinesterase activity, as well as possibly by facilitating acetylcholine release from nerve terminals (Lee *et al.*, 1985). Fasciculins account for 3–6% of the venom protein and the relative proportion between fasciculin 1 and 2 is about 1/3 (Cerveñanský *et al.*, 1994).

The structural similarity between onchidal (an acetate ester) and acetylcholine suggested that the toxicity of onchidal could result from inhibition of either nicotinic acetylcholine receptors or AChE. Although onchidal (1.0 mM) did not prevent the binding of ^{125}I-α-bungarotoxin to nicotine acetylcholine receptors, it inhibited AChE in a progressive, apparently irreversible, manner. The apparent affinity of onchidal for the initial reversible binding to AChE (K_d) was approximately 300 µM, and the apparent rate constant for the subsequent irreversible inhibition of enzyme activity (K_{inact}) was approximately 0.1 min^{-1}.

The fasciculins are a family of closely related peptides that are isolated from the venom of mambas and exert their toxic action by inhibiting AChE. The crystal structure of fasciculin 2 from green mamba (*Dendroaspis angusticeps*) snake venom was first resolved in 1992 (Le Du *et al.*, 1992). The three-dimensional (3D) structure of fasciculin 1 obtained from the US National Library of Medicine, National Center for Biotechnology Information, MMDB database is illustrated in Figure 11.2.

Fasciculins belong to the structural family of three-fingered toxins from Elapidae snake venoms, which include the α-neurotoxins that block the nicotinic acetylcholine receptor and the cardiotoxins that interact with cell membranes. The features unique to the known primary and tertiary structures of the fasciculin molecule were analyzed by Harald and associates (1995). Loop I contains an arginine at position 11, which is found only in the fasciculins and could form a pivotal anchoring point to AChE. Loop II contains five cationic residues near its tip, which are partly charge compensated by anionic side chains in loop III. By contrast, the other three fingered toxins show full charge compensation within loop II. The interaction of fasciculin with the recognition site on acetylcholinesterase was investigated by estimating a precollision orientation followed by determination of the buried surface area of the most probable complexes formed, the electrostatic field

FIGURE 11.2. The 3D protein structure of fasciculin 1 derived from green mamba (*Dendroaspis angusticeps*) snake venom. [Image obtained from the public domain at the US National Library of Medicine, National Center for Biotechnology Information, Molecular Modeling Database 3D Structure Database (MMDB)].

contours, and the detailed topography of the interaction surface. This approach has led to testable models for the orientation and site of bound fasciculin.

III. MECHANISM OF ACTION AND BIOLOGICAL EFFECTS

A. Onchidal

Onchidal is an irreversible inhibitor of enzyme AChE with a novel mechanism of action. It has been suggested, however, that its toxicity could be a consequence of the inhibition of either nicotinic acetylcholine receptor or AChE enzyme.

Incubation of AChE with onchidal resulted in the production of acetate, demonstrating that onchidal was a substrate for AChE, and approximately 3250 mol of onchidal was hydrolyzed/mol of enzyme irreversibly inhibited. Organophosphate and carbamate inhibitors of AChE have partition ratios (mol of toxin hydrolyzed/mol of enzyme irreversibly inhibited) that approach unity. Therefore, the relatively high partition ratio for onchidal suggests that the mechanism of inhibition utilized by onchidal may be distinctly different from other irreversible inhibitors (Walsh, 1984). The rate of hydrolysis of onchidal (K_{cat}) was 325 min^{-1}; this value is relatively slow suggesting that onchidal is not a very good substrate. The ability of AChE to hydrolyze onchidal raised the question of whether inhibition of enzyme activity resulted from onchidal itself or from a product of the enzymatic hydrolysis of onchidal. Enzyme kinetics revealed that onchidal was unable to completely inhibit higher concentrations of AChE. From the experiments performed by Abramson *et al.* (1989), onchidal was in molar excess and was completely hydrolyzed. Thus, irreversible inhibition of enzyme activity resulted either from onchidal itself or from a reactive intermediate produced during hydrolysis of onchidal (Walsh, 1984).

In another investigation, irreversible inhibition of enzyme activity was prevented by coincubation with reversible agents that either sterically block (edrophonium and decamethonium) or allosterically modify (propidium) the acetylcholine site (Barnett and Rosenberry, 1977). Enzyme activity was not regenerated by incubation with oxime reactivators; therefore, the mechanism of irreversible inhibition does not appear to involve acylation of the active site serine.

Because onchidal is an acetate ester similar to acetylcholine and because cholinergic neurotransmission is often the site of action of natural products involved in chemical defense, Abramson *et al.* (1989) investigated the ability of onchidal to inhibit AChE and the nicotinic acetylcholine receptor. Although onchidal did not prevent the binding of ^{125}I-α-bungarotoxin to nicotinic acetylcholine receptors, it was shown to be an active site-directed irreversible inhibitor of AChE. The structure and pharmacology of onchidal suggest that inhibition of AChE results from a novel covalent reaction between onchidal and an amino acid within the acetylcholine binding site. The onchidal could potentially be exploited in the design of a new class of natural anti-ChE agents and in the identification of amino acids that contribute to the binding and hydrolysis of acetylcholine.

B. Fasciculins

Various toxins in snake venoms exhibit a high degree of specificity in the cholinergic nervous system. The α-neurotoxins from the Elapidae family interact with the agonist binding site on the nicotinic receptor. α-Bungarotoxin is selective for the muscle receptor and interacts with only certain neuronal receptors, such as those containing α7 through α9 subunits. Neuronal bungarotoxin shows a wider range of inhibition of neuronal receptors. A second group of toxins, called the *fasciculins*, inhibits AChE. A third group of toxins, termed the muscarinic toxins (MT_1 through MT_4), are partial agonists and antagonists for the muscarinic receptor. Venoms from the Viperaridae family of snakes and the fish-hunting cone snails also have relatively selective toxins for nicotinic receptors.

Other reversible inhibitors, such as propidium and the peptide toxin fasciculin, bind to the peripheral anionic site on AChE. This site resides at the lip of the gorge and is defined by tryptophan 286 and tyrosines 72 and 124 (Taylor, 2001).

A large number of organic compounds reversibly or irreversibly inhibit AChE (Long, 1963), which bind either to the esteratic or the anionic subsite of AChE catalytic site or to the peripheral site of the enzyme. Most of them are synthetic substances, sometimes bearing insecticidal properties. Few natural inhibitors of AChE are known and, to date, fasciculins are the only known proteinic AChE

inhibitors and they have been shown to display a powerful inhibitory activity toward mammalian AChE. Iodination of fasciculin 3 provided a fully active specific probe of fasciculin binding sites on rat brain AChE (Marchot *et al.*, 1993). These authors demonstrate that fasciculins bind on a peripheral site of AChE, distinct from the catalytic site, and, at least partly, common with the sites on which some cationic inhibitors and the substrate in excess bind, since phosphorylation of the catalytic serine (esteratic subsite) by [1,3-^{3}H]diisopropyl fluorophosphate can still occur on the fasciculin 3. In the AChE complex, the structural modification induced by fasciculins may affect the anionic subsite of the AChE catalytic site.

Cholinesterases have a very different sensitivity towards fasciculins. AChEs from rat brain, human erythrocytes, and electroplax of electric eel (*Electrophorus electricus*) are inhibited by fasciculins with a K_i of about 10^{-11} M and pseudocholinesterases as human serum cholinesterase are inhibited by fasciculins with a K_i of about 0.5 μM. A second group of enzymes is partially (10–30%) inhibited by low concentrations (<0.5 nM) of fasciculin. Increasing the concentration of fasciculins to a toxic level of about 1 nM inactivates the enzymes to 90–110% of their initial activity. AChEs from guinea pig ileum, ventricle, and uterus behave similarly. A third group consists of enzymes insensitive to fasciculin; AChEs from chick biventer cervicis muscle and brain and from insects, heads of *Musca domestica* (common house fly), and cobra (*Naja naja*) venom. The biochemical mechanism of fasciculins involves displacement of propidium from its binding site on AChE. Since propidium is a probe from a peripheral anionic site, it is concluded that fasciculins also bind to the same site. The different sensitivity of cholinesterase to fasciculin should depend on the nature of their peripheral sites. Fasciculins are basic proteins of 61 amino acid residues and four disulfides, highly homologous to short α-neurotoxins and cardiotoxins. Indeed, a large number of AChE inhibitors are cations, e.g. neostigmine, physostigmine, and propidium.

The binding between fasciculin and AChE is strong, as indicated by a K_i of about 10^{-11} M. This should result from interaction of several amino acid residues in the toxin with the enzyme. A modification of one of these residues should not abolish but significantly decrease the activity. But the decrease in activity can also depend on structural perturbations caused by the modification. Chemical modification and structural data suggest that Lys 32 and 51 have a functional role (Cerveñanský *et al.*, 1994). This author acetylated the amino groups of fasciculin 2 with acetic anhydride. The monoacetyl derivatives of the ε-amino acids (Lys 25, 32, 51, and 58) retained between 28 and 43% of the initial activity and that of the α-amino group 72%. Acetylation of Lys 25 that has the most reactive amino group decreased the activity by 65% apparently without producing structural perturbation since the circular dichroism spectrum was not affected. The three-dimensional structure shows a cationic cluster formed by Lys 32, 51, Arg 24 and 28. A comparison of 175 sequences of homologous toxins shows that Lys 32 is unique for fasciculin. Acetylation of lysine residues in the cluster had a large effect and reduced the activity by 72% (Lys 32) and 57% (Lys 51).

Fasciculins inhibit AChE from mammals, electric fish, and some snake venoms with *K*i values in the pico- to nanomolar range; in contrast, avian, insect, and some other snake venom AChEs are relatively resistant, and high micromolar concentrations are required to inhibit mammalian butyrylcholinesterases (BuChE) (Marchot *et al.*, 1993). Dissociation constants of Fas1 and Fas3 are two-fold and 60-fold lower, respectively, than that of Fas2 for synaptosomal rat brain.

An examination of fasciculin association with several mutant forms of recombinant DNA-derivated AChE from mouse shows that it interacts with a cluster of residues near the rim of the gorge on the enzymes; the aromatic residues, Trp 286, Tyr 72, and Tyr 124, have the most marked influence on fasciculin binding, whereas Asp 74, a charged residue in the vicinity of the binding site that affects the binding of low molecular weight inhibitors, has little influence on fasciculin binding. The three aromatic residues are unique to the susceptible AChE and, along with Asp 74, constitute part of the peripheral anionic site. Fasciculin falls in the family of three-loop toxins that include the receptor blocking α-toxins and cardiotoxins. A binding site has evolved on fasciculin to be highly specific for the peripheral site on AChE. Acetylthiocholine affects rates of fasciculin binding at concentrations causing substrate inhibition. In the case of the mutant cholinesterase where rates of fasciculin dissociation are more rapid, steady state kinetic parameters also show ACh–fasciculin competition to be consistent with occupation at a peripheral or substrate inhibition site rather than the active center (Radic *et al.*, 1994).

Fasciculin inhibition of AChE is prevented by chemical modification of the enzyme at a peripheral site (Durán *et al.*, 1994). The specific interaction of fasciculin 2 with peripheral sites present in *Electrophorus electricus* AChE (K_i, 0.04 nM fasciculin) was investigated by chemical modification with *N*,*N*-dimethyl-2-phenylaziridium (DPA) in the presence of active or peripheral anionic site protective agents. An enzyme was obtained that compared to the native AChE and was 10^6 times less sensitive to fasciculin 2. This enzyme was fully inhibited by edrophonium and tacrine, and was 25–170 times less sensitive to several peripheral site ligands. It seems fasciculin 2 binding to an AChE peripheral site partially overlaps the site of other peripheral site ligands including acetylcholine.

IV. EXPERIMENTAL AND HUMAN TOXICITY

Administration of fasciculin 1 and fasciculin 2 to mice at doses of 1–3 mg/kg and 0.05–2.0 mg/kg, respectively, after

i.p. injection caused severe, generalized, and long-lasting fasciculations (5–7 h) followed by gradual recovery to normal behavior. *In vitro* preincubation with fasciculins at concentrations of 0.01 μg/ml inhibited brain and muscle AChE up to 80%. Histochemical assay for AChE showed an almost complete disappearance of the black–brown precipitate at the neuromuscular endplate after *in vitro* incubation with fasciculins. Fasciculins represent a new type of AChE inhibitors exerting muscle fasciculations through a powerful inhibition of enzyme activity at the neuromuscular endplate, interfering with the normal hydrolysis of ACh molecules. Fasciculins have also been demonstrated to be powerful inhibitors of brain AChE (Rodriguez-Ithurralde *et al.*, 1983).

The cause of death due to toxin F_7, an *angusticeps*-type toxin, isolated from the venom of *Dendroaspis angusticeps* was studied in anesthetized mice (Lee *et al.*, 1986). The carotid arterial blood pressure, ECG, and the respiratory movements were recorded. Within a few minutes after i.v. injection of toxin F_7 (1 mg/kg), both the rate and amplitude of the respiratory movements decreased and respiratory arrest took place within 15 min in most cases. Before respiratory arrest, marked bradycardia with various types of arrhythmia and oscillation of blood pressure were observed. Artificial ventilation could abolish these cardiovascular changes and maintain the blood pressure for a long period. Toxin F_7 caused a transient and slight increase of arterial blood pressure which could be prevented by hexamethonium. Intracisternal application of toxin F_7 (1 mg/kg) caused a long-lasting hypertension and bradycardia and the respiratory arrest time was significantly longer than after i.v. injection. A large dose (50 mg/kg, i.p.) of atropine, but not smaller doses (5–10 mg/kg), protected mice against respiratory failure induced by toxin F_7.

In rats, the phrenic nerve discharge was prolonged during respiratory depression. Since the toxin F_7 has a potent anti-ChE activity, it is concluded that the respiratory failure induced by toxin F_7 is peripheral in origin, chiefly, if not entirely, due to its anticholinesterase activity.

Strydom (1976) performed the purification of *Dendroaspis polylepis polylepis* (black mamba) venom, and found 12 low molecular weight proteins, of which 11 have subcutaneous LD_{50} values of less than 40 μg/g mouse.

Clinically, mamba bites may not provoke a major local reaction. If neurotoxins are injected by the bite, clinical symptoms appear within minutes to hours. Clinical signs of impairment of neuromuscular transmission (ptosis, ophthalmoplegia, bulbar symptoms, or generalized weakness) dictate administration of antivenom (Ludolfph, 2000). For Elapidae (coral snakes) venom is known that is a potential neurotoxin and may cause paresthesias, weakness, cranial nerve dysfunction, confusion, fasciculations, and lethargy. Often mild local findings, diplopia, ptosis, and dysarthria are common early symptoms. Patients die because of respiratory paralysis. In these cases, early and aggressive airway management is vital. Symptoms may be delayed by 8 to 12 h (Cameron, 2006).

V. RISK ASSESSMENT

Toxicity databases and computer-assisted computational predictive toxicology modeling are now becoming of increasing importance to risk assessors when risk assessment of a chemical using experimental animal toxicological studies is unclear because the data are equivocal or even absent (Benz, 2007). Furthermore, the European Community has passed a law called REACH (Registration, Evaluation, Authorization, and restriction of Chemicals), which entered into force on June 1, 2007, to identify and more effectively avoid the risks of the toxic properties of chemicals to humans and the environment (Lahl and Gundert-Remy, 2008). Under the REACH regulation, manufacturers or importers to the European Community with more than 1 ton of a chemical substance per year are required to assess the chemical's potential toxicological or environmental adverse effects, and to register this information in a central database (Saiakhov and Klopman, 2008). The REACH regulation has impact on the widespread use of computational predictive modeling because it rules that no animal test should be used if it can be replaced with other techniques such as reliable computer-based predictions (Lahl and Gundert-Remy, 2008; Saiakhov and Klopman, 2008). Therefore, the use of computer-assisted computational predictive toxicology software has arrived at a point of considerable attention worldwide because it may not only reduce, but eliminate the need for animal testing under certain circumstances. Since the acute and chronic toxic potential of onchidal in experimental animal studies is rather limited, strategies using *in silico* assessment of its toxic potential could be envisioned. Computational-based strategies with pharmaceuticals and natural product toxins have already been developed for use in safety evaluation and risk assessment practices at US government agencies (Benz, 2007; Valerio *et al.*, 2007; Arvidson *et al.*, 2008; Matthews *et al.*, 2008; Demchuk *et al.*, 2008; Bailey *et al.*, 2005; Contrera *et al.*, 2005). These strategies can be considered for an *in silico* risk assessment of onchidal. One approach is to combine different predictive models for the same toxicological endpoint to determine if a consensus prediction regarding the effect is possible (Matthews *et al.*, 2008). Another approach is to combine computational software that employs human rule-based approaches with software that utilize statistical algorithm-based predictions using QSAR models for the toxic potential of the compound of interest (Arvidson *et al.*, 2008). A variety of computational toxicology predictive software and data mining databases are available at no cost, commercially, or through non-profit organizations (Richard *et al.*, 2008; Marchant *et al.*, 2008; Yang *et al.*, 2008; Saiakhov and Klopman, 2008; Shi *et al.*, 2008). The information obtained from a predictive and data mining *in silico* assessment can be used judiciously as

a decision support tool in risk assessment and management regarding the safety of an organic chemical (Arvidson *et al.*, 2008; Matthews *et al.*, 2008).

In order to assess the potential toxicological effects of onchidal from a predictive standpoint, the author subjected the molecular structure of onchidal to an *in silico* computational analysis using a human rule-based software known as Derek for Windows (Deductive Estimation of Risk from Existing Knowledge; DfW), and a high-throughput *in silico* quantitative structure activity relationship (QSAR) screening software called MDL-QSAR that uses a statistical-based algorithm called discriminant analysis with validated models for genetic toxicity and rodent carcinogenicity (Contrera *et al.*, 2008). Details regarding the approaches of these software programs and the *in silico* screening procedures and methods have been well described previously (Marchant *et al.*, 2008; Matthews *et al.*, 2008; Contrera *et al.*, 2003, 2005, 2008). By employing these two different software programs with the molecular structure of onchidal, our *in silico* analysis produced predictive information pertinent to when conditions of potential acute and chronic human exposure are encountered (Table 11.3).

The results summarized in Table 11.3 show that the software DfW identified the α,β-unsaturated aldehyde moiety on onchidal as a structural alert and predicted this reactive center of the molecule to possess bacterial (*Salmonella typhimurium*) mutagenic potential at the reasoning level the software defines as ''plausible''. Plausible is an uncertainty term in the software's reasoning tree and is defined as ''The weight of evidence supports the proposition'' (Marchant *et al.*, 2008). The prediction of mutagenicity for the α,β-unsaturated aldehyde moiety on onchidal is consistent with a large body of scientific literature demonstrating the experimental mutagenicity of small organic molecules that have α,β-unsaturated aldehydic groups (Petersen and Doorn, 2004; Eckl, 2003; Burcham, 1998). In addition to the α,β-unsaturated aldehyde, DfW also identified the enol ether portion of onchidal's structure as a basis for a ''plausible'' prediction for dermal sensitization according to the rules of the software's computational-based reasoning. Both the mutagenicity and sensitization predictions arise as a consequence of the presence of alerts (alerts #302 for mutagenicity, #425 and #479 for dermal contact sensitization) in the onchidal query. In essence, these alerts describe the relationship between the chemical functionality and the occurrence of mutagenicity and dermal contact dermatitis. The software provides supporting evidence for the alert as well as a selected compound known to exhibit the toxic endpoint. An illustration of the DfW prediction for onchidal is shown in Figure 11.3. Given the potential for the chemical warfare application of onchidal, the information obtained from the DfW *in silico* predictive toxicological assessment seems to be useful if such an application of acute widespread human exposure would be encountered. Therefore, onchidal could be considered as a potential sensitizer for both Type I hypersensitivity and Type IV allergic contact dermatitis. Since many sensitizers are also dermal contact irritants, then another potential human health hazard that may be deduced for onchidal is irritant contact dermatitis. Clearly, under long-term exposure conditions the potential for mutagenic DNA damaging effects would be of concern. However, adverse effects from onchidal as a result of its ACE inhibition would likely supersede the period of time to the ensuing tissue injury produced as a result of DNA damaging effects.

MDL-QSAR *in silico* predictive screening for the mutagenic and carcinogenic potential of onchidal is presented in Table 11.3. The software classified onchidal to be of low mutagenic potential in bacteria, but classified the compound as high for potential to be a rodent carcinogen. MDL-QSAR utilizes a statistical algorithm-based approached called discriminant analysis that classifies a compound by determining the probability that the compound will fall into a membership class of either high or low for the toxic endpoint being predicted. In this case, it is predicted that onchidal be of low bacterial mutagenic potential in *Salmonella typhimurium* and of high rodent (rats and mice, male and female) carcinogenic potential with a high degree of confidence (probability for membership in class >85%). An illustration of MDL-QSAR's prediction for onchidal is given in Figure 11.4. Although the prediction of mutagenicity conflicts with DfW, the information is still useful from a risk assessment standpoint because a risk assessor will likely have additional information such as an estimate of exposure and route of exposure conditions as well as potential experimental studies that may ''weigh in'' during the overall analysis of human health risk.

TABLE 11.3. Summary of *in silico* predictive toxicological evaluations of onchidal by Derek for Windows and MDL-QSAR

Computational toxicology software	Prediction for onchidal
Derek for Windows	Mutagenicity – plausible Alert 302: α,β-unsaturated aldehyde Skin sensitization – plausible Alert 425: Enol ether Alert 479: α,β-unsaturated aldehyde
MDL-QSAR	Classified as low potential for rodent mutation *in vivo* Classified as high potential for rodent carcinogenicity High probability 0.8677 Low probability 0.1322

The computational toxicology software programs and models were obtained by FDA/CDER/ICSAS through cooperative research and development agreements with MDL Information Systems and Lhasa Ltd (Benz, 2007)

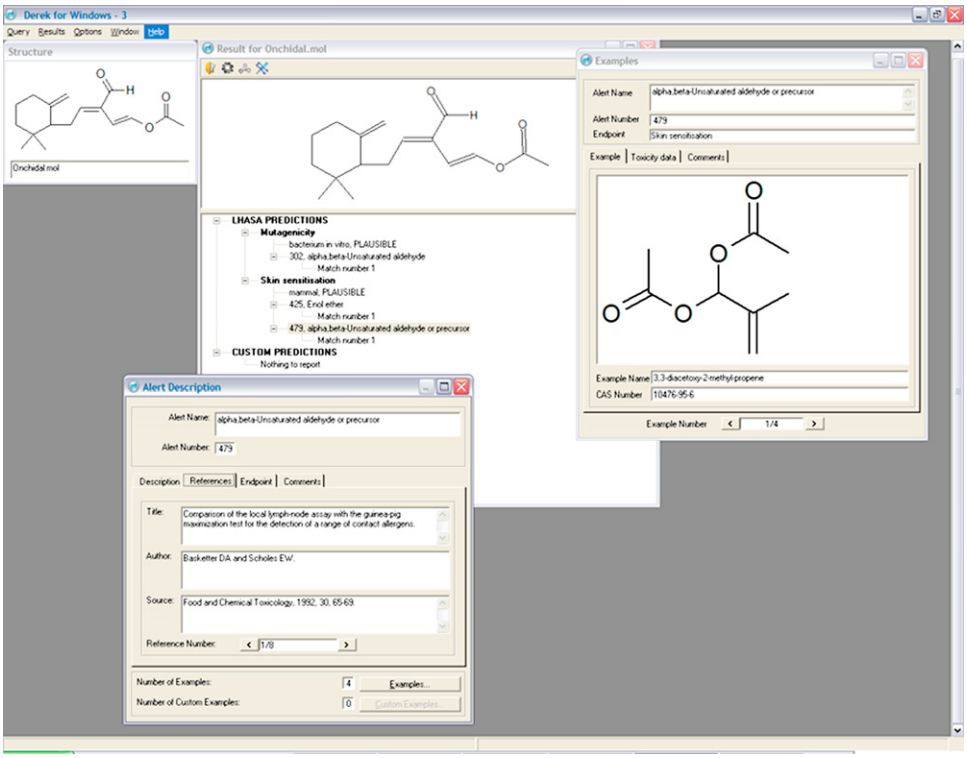


FIGURE 11.3. The Derek for Windows version 10.0.2 (DfW) prediction for onchidal under default processing contraints.

Overall, these computational toxicology models and many others developed at the US Food and Drug Administration, Center for Drug Evaluation and Research, Informatics and Computational Safety Analysis Staff (ICSAS) can predict with considerable precision the toxicological and adverse human effects of many classes of organic substances (Benz, 2007). Further information about ICSAS, including program activities, can be found at the FDA ICSAS internet website (http://www.fda.gov/cder/Offices/OPS_IO/default.htm).

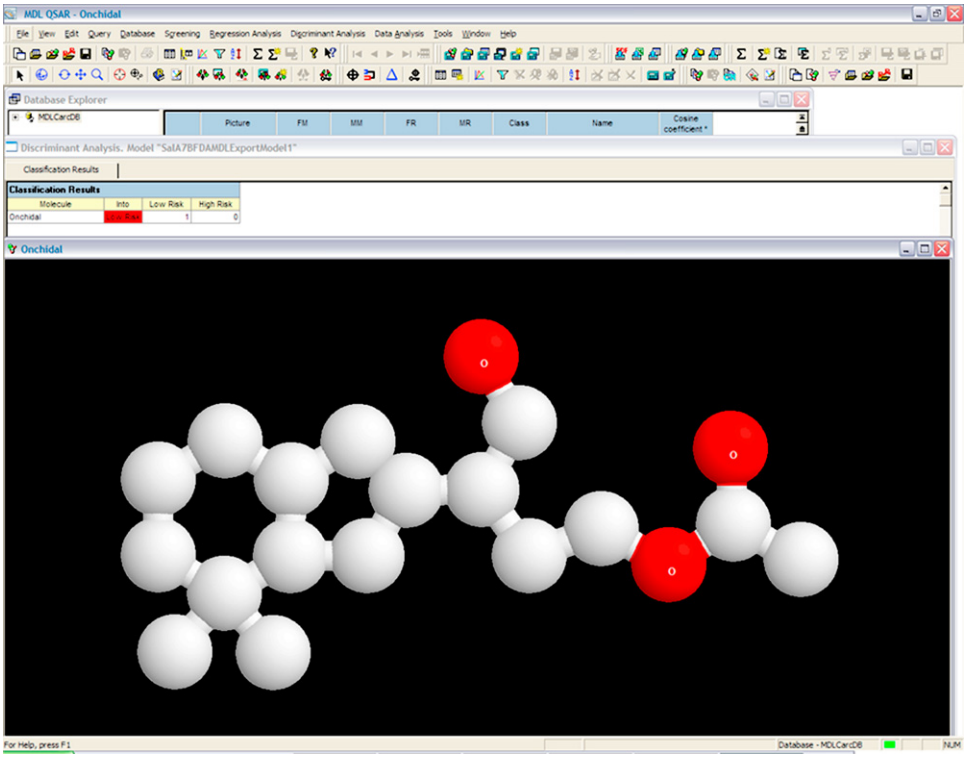


FIGURE 11.4. The MDL-QSAR version 2.2.2.0.7 prediction for mutagenic potential of onchidal in *Salmonella typhimurium.*

VI. TREATMENT

Antidotal therapy of the toxic effects of cholinesterase inhibitors such as onchidal and fasciculins used as potential chemical warfare agents is directed to blocking the effects of excessive acetylcholine stimulation and reactivating the inhibited enzyme. Atropine in sufficient dosage effectively antagonizes the actions at muscarinic receptor sites (Taylor, 2001). Atropine is used in adults at doses of 1–2 mg i.v. and in pediatrics at a dose of 0.05 mg/kg i.v.; doubling this dose every 5 min (DeLisle, 2006). Larger doses are required to get appreciable concentrations of atropine into the CNS. Atropine is virtually without effect against the peripheral neuromuscular compromise. The last-mentioned action of the anti-ChE agents as well as all other peripheral effects can be probably reversed by reactivators of cholinesterase such as pralidoxime (2-PAM) in the treatment of onchidal and fasciculins poisonings. Usual dosages are as follows: in adults 1–2 g i.v. over 15–30 min, may repeat in 1 h if necessary or start a drip at 500 mg/h; in pediatrics, 25 mg/kg i.v. over 15–30 min, follow with continuous infusion of 10–20 mg/kg i.v. (DeLisle, 2006). On the other hand, the antivenom is indicated for all confirmed eastern coral snake bites (western, no antivenom) (Cameron, 2006).

VII. CONCLUDING REMARKS AND FUTURE DIRECTION

The natural toxins onchidal and fasciculins behave as anti-ChE agents. Onchidal is an active site-directed irreversible inhibitor of AChE, and fasciculins are proteinic AChE inhibitors which bind to a peripheral regulatory anionic site of AChE in a noncompetitive and irreversible manner.

There is limited information about the toxicity, toxicokinetics, and toxicological properties of onchidal and additional data are needed to make a health effects-based risk assessment of the natural compound. Although fasciculins are much better known, data on toxicological properties and toxicokinetics will be of interest and useful for risk assessments despite it being generally accepted that the toxicity of this proteinic toxin occurs at very low doses.

Onchidal and fasciculins are interesting natural compounds and it is difficult to predict their toxicity. In the case of onchidal, *in silico* computational predictive modeling for toxic endpoints of interest may prove useful for risk assessment decision support. Likewise, it is a challenge to predict the military potential and human impact of these natural toxins since their affinity for enzyme inhibition depends upon the amount and duration of the human exposure.

Acknowledgment

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CHAPTER 12

Riot Control Agents

COREY J. HILMAS, MELISSA J. POOLE, ALEXANDRE M. KATOS, AND PATRICK T. WILLIAMS

I. INTRODUCTION

Nonlethal agents are a broad class of compounds intended to produce transient incapacitation of an individual or individuals. Both incapacitating agents and riot control agents (RCA) are separate classes of nonlethal agents. Although the two classes share the characteristic to incapacitate, a distinction must be drawn between these two types of agents. RCAs differ from incapacitating agents in several respects. RCAs possess a relatively short onset and limited duration of action. RCAs induce short-term toxic effects that subside within minutes following termination of the exposure. Additionally, modern RCAs have a very high safety ratio compared to incapacitating agents and first generation RCAs. Many incapacitating agents were developed during the Cold War which produced either limited lethality and/or prolonged morbidity. Consequently, incapacitating agents have been banned by international treaties recognized by the USA, including the Chemical Weapons Convention (CWC). Specifically, the CWC has placed a ban on the development, production, and possession of any chemical weapon intended to cause death or ''temporary incapacitation''. The USA considers these broad incapacitating agents as chemical warfare agents (CWAs). However, the USA does not recognize RCAs as CWAs, and therefore, US policy considers them to be legal for use by civilian police or the military. The CWC does prohibit their use in times of war. Thus, the USA has opted not to utilize RCAs in Iraq during the early 21st century against organized and armed insurgents.

While the field of nonlethal agents is diverse and interesting, we will limit our discussion to only those agents considered to be RCAs. The goal of RCAs is to temporarily incapacitate through irritating the skin and mucosal membranes of the eyes, airways, and digestive tract. As a result of their short-term toxicity, they are effective agents used by military and law enforcement personnel to disperse crowds, clear buildings, and quell riots. While RCAs are often thought of as ''tear gas'' or pulmonary irritants, they encompass more than this terminology would suggest. They are neither gases nor exclusively pulmonary irritants. Historically, RCAs were categorized as lacrimators, sternutators, and vomiting agents based upon their predominant toxicity on the eyes, lungs, or digestive tract. This nomenclature is outdated since modern RCAs affect a wide variety of organ systems. This fact will be clearly evident in the subsequent discussion concerning their mechanism of action and toxicity. Today, RCAs comprise a diverse array of chemical compounds with similar toxic effects since their introduction on the battlefield in the early part of the last century.

II. HISTORY

The Chinese were perhaps the first to employ pulmonary irritants with their stink bombs (Smart, 1996). The smoke emanating from them was a primitive sternutator designed to harass the enemy. RCAs were used during the 5th century BC Peloponnesian War when the Spartans used smoke from burning coal, sulfur, and pitch to temporarily incapacitate and confuse occupants of Athenian strongholds (Thoman, 2002). During antiquity, the Romans used irritant clouds to drive out their Spanish adversaries from hidden dwellings (Robinson, 1971). Almost all of these examples involved the use of incapacitating agents as an offensive tactical weapon as opposed to controlling crowds for defensive purposes.

World War I (WWI) marked the birth of RCAs as well as the modern age of CWAs (Figure 12.1). Both German and French forces used a wide variety of irritating agents, such as acrolein (papite), chloropicrin (PS), and diphenylaminearsine (DM; Adamsite); however, bromoacetone (BA) was the most widely used lacrimator agent at that time. At the end of WWI, the US military investigated the use of chloroacetophenone (CN) as a chemical irritant. First developed by Graebe in 1869 and formulated as Chemical Mace®, CN was the most widely used RCA up until World War II (Olajos and Stopford, 2004).

Two chemists, Carson and Stoughton (1928), synthesized 2-chlorobenzylidene malononitrile (CS); however, it was not adopted by the military as an official RCA until 1959. As a more chemically stable compound and having a greater potency with less toxicity than CN, it gradually replaced CN as the preferred RCA. CS was widely used during the Vietnam War to flush the Viet Cong out of the

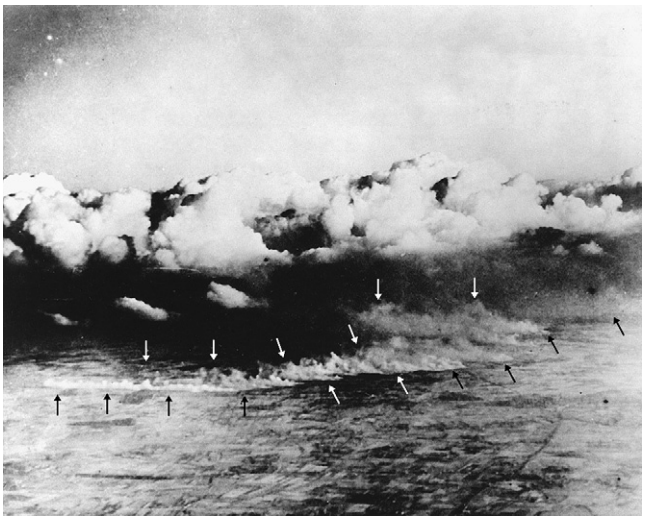

FIGURE 12.1. The birth of chemical warfare agents in World War I. The photograph depicts the initial chlorine gas attack by Germany at Ypres, Belgium, on April 22, 1915. The German Army released chlorine gas from cylinders to form a poisonous cloud (indicated by black and white arrows) directed toward the French lines by the prevailing winds. Photograph: courtesy of the US Army, Chemical Warfare Service, Edgewood, MD.

FIGURE 12.2. *Top*: US Army Engineers unpack and test a Mitey-Mite blower in the jungles of Vietnam. The Mighty-Mite aerosolized and dispersed smoke, CS powder, or other RCA as a means of tunnel denial. Photograph: courtesy of the US Army Engineer School, Fort Belvoir, VA. *Bottom*: American soldiers (''tunnel rats'') wearing M28 protective masks just prior to entry into underground tunnels previously saturated with CS. Photograph: courtesy of the US Army Research Development and Engineering Command, Historical Research and Response Team, Aberdeen Proving Ground, MD.

labyrinth of underground tunnels and bunkers throughout Southeast Asia (Figure 12.2). In the years following the Vietnam War, other militaries adopted CS. Saddam Hussein's forces used it against Iran during the Iran–Iraq War of the 1980s. Today, CS is commonly used by law enforcement agencies and militaries for riot control training, respirator training in boot camps, temporary incapacitation of an assailant, and civil disturbances. A famous case of RCA use by the US Federal government involved CS dissemination on the Branch Davidian cult members in 1993. Because of its high flammability rating, CS was believed to be a large contributor to the inferno that burned down the Waco, Texas, compound and its inhabitants. Even before fire broke out and destroyed the compound, it is believed that CS concentrations ranged from five to 60 times the amount required to deter individuals (Bryce, 2000).

During the 1980s and 1990s, the use of CS gas was rapidly on the decline and slowly being replaced by oleoresin capsicum (OC) spray. OC, an extracted resin from *Capsicum* pepper plants, was first developed in the 1970s as an alternative to CN and CS agents. Commercially available OC sprays used by the public are approximately 1% capsaicin, while formulations used by law enforcement agencies can contain up to 15% capsaicin. Most recently, a synthetic form of capsaicin called nonivamide, marketed as Captor, gained popularity as a defensive aerosol in the early 1990s (Olajos and Stopford, 2000).

Under the CWC of 1997, RCAs were banned from use as a method of warfare because in high concentrations RCAs are toxic chemicals with the potential to incapacitate individuals for prolonged periods, produce long-term sequelæ, and cause death. The CWC allows RCAs to be used in domestic riot control, as well as enforcement of domestic law and ''extraterritorial law enforcement activities undertaken by military forces'' (Rosenberg, 2003). These boundaries and definitions, while vague, were clarified in 2003 by President George W. Bush (Wade and Schmitt, 2003). Bush authorized the use of tear gas against Iraqi troops for defensive purposes as allowed in Executive Order 11850 of 1975. Many experts believed this would violate the CWC (which was not signed by Iraq) and give Saddam Hussein the power to use chemical agents against the US under the authority of the Geneva Protocol (Wade and Schmitt, 2003). In the end, RCAs were never used during that conflict.

III. BACKGROUND

A. The Agents and Their Physicochemical Properties

Unlike the majority of chemical agents which are liquid at room temperature, modern RCAs are crystalline solids with low vapor pressure (see Table 12.1). RCAs are typically administered as fine particles, aerosol sprays, or in solutions; therefore, they are not true gases. The inhalation toxicity of RCAs, as well as CWAs, is often indicated by the expression Ct. This term is defined as the product of concentration (C) in $mg \cdot m^{-3}$ multiplied by exposure time (t) in minutes ($mg \cdot min\ m^{-3}$). LCt_{50} and ICt_{50} are conventional terms used to describe airborne dosages that are lethal (L) or incapacitating (I) to 50% of the exposed population. The intolerable concentration (mg/m^3), ICt_{50} and minimal lethal concentration (mg/m^3) are provided in Table 12.1 for the most common RCAs. The ocular irritancy threshold (minimal irritant or minimal effective dose), estimated human LCt_{50}, and safety ratio are provided in Table 12.2 for these same RCAs. The modern RCAs are characterized by a high LCt_{50}, low effective Ct_{50}, low ICt_{50}, low minimal irritating concentration and large safety index ratio (LCt_{50}/irritancy threshold). As a rule of thumb, clinical signs and symptoms from RCA exposure generally subside within 30 min but may persist depending on dose and duration of exposure (Blain, 2003). Ortho-chloroacetophenone (CN) and chlorobenzylidene malononitrile (CS) are the classic representative agents of this class of compounds. The toxicity of CN and CS will be discussed in depth due to the vast volume of literature available for these compounds.

1. Chloroacetophenone (CN)

CN is a crystalline solid with a strong, pungent odor (see Figure 12.3). It is dispersed as a smoke, powder, or liquid formulation from grenades or other devices. It is perhaps better known under the trade name Chemical Mace® and was once used widely for self-protection. It was also the standard tear gas used by the military (Figure 12.4) and police personnel. It has been replaced in favor of the less toxic CS for riot control and capsaicin pepper spray for self-defense.

CN exhibits the greatest toxicity among RCAs in use today. Consequently, it has been replaced by compounds with higher safety ratios. CN is three- to ten-fold more toxic than CS in rats, rabbits, guinea pigs, and mice (Ballantyne and Swanston, 1978). Pathological findings in the lungs tend to be more severe and CN causes far greater edema. CN typically causes an acute, patchy, inflammatory cell infiltration of the trachea, bronchi, and bronchioles, in addition to early bronchopneumonia. CN not only demonstrates greater irritation to the skin than CS, it is also a more potent skin sensitizer (Chung and Giles, 1972). Patients frequently exposed to CN are at a high risk of developing allergic dermatitis (Penneys, 1971).

2. Ortho-chlorobenzylidene Malononitrile (CS)

The term CS was adopted after the two chemists, Carson and Stoughton, who synthesized the compound. CS is a white, crystalline powder with a pepper-like odor and low vapor pressure (see Figure 12.5). It is rapidly hydrolyzed following contact with water but minimally soluble in ethyl alcohol. CS is the most widely used RCA today, although many countries are switching to even less toxic compounds. CS is used by the US Armed Forces for gas discipline training exercises to help new recruits learn the importance of donning their protective masks quickly (Figure 12.6). It was also used by the USA during the Vietnam War for tunnel denial and crowd control (Figure 12.7) and by police forces for dispersing violent protests and incapacitating assailants.

3. Dibenz(*b,f*)-1:4-Oxazepine (CR)

Dibenz(*b,f*)-1:4-oxazepine (CR) (see Figure 12.8) is a potent sensory irritant with less toxicity than CS or CN (Ballantyne, 1977b). CR causes an immediate and effective irritation of the eyes, nose, and skin without persistent effects in these target organs. The irritation associated with CR is more transient compared to other RCAs. It is five to ten times greater in potency than CS; therefore, a smaller concentration is needed to cause irritation (low minimal irritant concentration or dose) and incapacitation (low ICt_{50}) (see Tables 12.1 and 12.2). CR has a favorable safety ratio; it is safer than other RCAs based on its higher LCt_{50} (Table 12.1) and greater LCt_{50}/irritancy threshold (safety ratio). In humans, the effects caused by CR are identical to CS. The LCt_{50} for humans is estimated at $>$100,000 $mg.min/m^3$. Despite its reduced toxicity in man, CR is not entirely without risk. CR is fairly stable, resists weathering, and persists in the environment (Sidell, 1997); therefore, enhanced toxicity may occur with prolonged exposure.

4. Diphenylaminechlorarsine (DM)

Diphenylaminechlorarsine (DM) (see Figure 12.9) or Adamsite are pro-emetic agents used in WWI. DM has greater toxicity than other RCAs and has been abandoned in favor of compounds with less toxicity and greater safety ratios. While toxicity is typically delayed with DM exposure, toxic signs and symptoms can occur within minutes after exposure. Systemic toxicity may also be more pronounced and prolonged. Symptoms often subside hours after exposure. Because DM is an antiquated RCA, this compound is irrelevant today and will not be discussed further.

5. Oleoresin Capsicum (OC)

Oleoresin capsicum (OC) is an oily resin derivative from capsicums and composed of several related compounds. Capsicums are solanaceous (nightshade species) plants from the genus *Capsicum*. More than 20 species fall within the genus. Capsaicinoids are considered the active ingredients of OC. These active compounds are endocrine products of glands found in the plant placenta and are a mixture of two unsaturated and three saturated homologs

TABLE 12.1. Physical characteristics and toxicity data for the common RCAs

		Physical characteristics			Toxicity data			
Agent	**Discovered in**	**Solubility**	**Vapor pressure (mm Hg @ 20°C)**	**Vapor density**	**Onset**	**Intolerable concentration (mg/m^3)**	**ICt_{50} ($mg \cdot min/m^3$)**	**Minimal lethal concentration[g] (mg/m^3)**
CS	1928 (Carson and Stoughton)[a]	Insoluble in water Soluble in organic solvents	0.00034	6.5	Immediate	5	3–10	2,500
CN	1871 (Graebe)[b]	Poorly soluble in water	0.0054	5.3	Immediate	35	20–40	850–2,250
DM	1915 (Wieland)[c] and 1918 (R Adams)[d]	Insoluble in water Poorly soluble in organic solvents except acetone	2×10^{-13}	9.6	Delayed with long recovery period	5	22–150	1,100–4,400
CR	1962 (Higginbottom and Suschitzkey)[e]	Sparingly soluble in water Stable in organic solvents	0.00059	6.7	Immediate	1	1	10,000
Bromobenzyl cyanide (CA)	1881 (Riener)[f]	Insoluble in water Soluble in organic solutions	0.12	4.0	Immediate	0.8	30	1,100

References: Maynard (1999); Sidell (1997); Smith and Stopford (1999); Olajos and Salem (2001).
[a]Carson and Stoughton (1928)
[b]Graebe (1871)
[c]Wiegand (1915); Wieland and Rheinheimer (1921)
[d]Sartori (1939)
[e]Higginbottom and Suschitzky (1962)
[f]Prentiss (1937)
[g]Estimate for minimal lethal concentration (10 min exposure)

TABLE 12.2. Health risk considerations for the common RCAs

Agent	Irritancy threshold[a] (mg/m^3)	Estimated human LCt_{50}[c] ($mg \cdot min/m^3$)	Safety ratio[d]	Adverse effects
CN	0.3[a]	8,500–22,500	28,000	Danger of permanent eye injury, vesiculation, bronchopneumonia, reactive airways, documented fatality cases
CS	0.004[a]	25,000–150,000	60,000	Same as CN, but fatality cases not authenticated, enhanced persistence compared to CN and CS
CR	0.002[a]	100,000	100,000	No significant respiratory toxicity
OC	0.0003[b]	not available	>60,000	Eye, skin, respiratory toxicity, significant morbidity in neonate, fatality involving case of in-custody use
DM	~1 [a]	11,000–44,000	11,000	No longer used
CA	0.15[a]	11,000	11,000	Predominantly a lacrimatory agent, no longer used

[a]Ocular irritancy thresholds unless indicated otherwise
[b]Threshold for respiratory complaints by capsaicinoids: Stopford and Sidell (2006); Lankatilake and Uragoda (1993)
[c]Values obtained from references: Maynard (1999); Sidell (1997); Smith and Stopford (1999); Olajos and Salem (2001)
[d]Values derived from estimate of the human LCt_{50} (lower bound)/irritancy threshold (minimal effective dose). Therefore, ranges are not provided for the safety ratios

(see Figure 12.10). Capsaicinoids are isolated through a volatile solvent extraction of the dried, ripened fruit of chili peppers. The capsaicinoids are distilled, dried, and compounded together. The final oleoresin contains several branched-chain alkyl vanillylamides, in addition to capsaicin, the major component in OC. The predominant capsaicinoid components of OC are capsaicin (70%), dihydrocapsaicin (20%), norhydrocapsaicin (7%), homocapsaicin (1%), and homodihydrocapsaicin (1%) (Salem *et al.*, 2006; see Figure 12.10).

Capsaicinoids cause dermatitis as well as nasal, ocular, pulmonary, and gastrointestinal effects in humans. OC gained popularity in the 1990s as a defensive weapon for civilians and law enforcement agencies because they produce an immediate, temporary immobilization and incapacitation when sprayed directly into the face or eyes. It is important to note that hand-held pepper spray formulations can contain OC by themselves or a mixture of OC and CS.

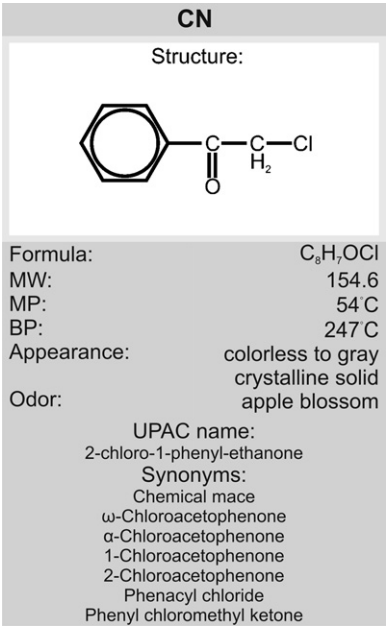

FIGURE 12.3. Chemical structure and physicochemical properties of chloroacetophenone (CN).

FIGURE 12.4. US soldier in protective clothing disseminating CN aerosol using the M33A1 disperser. Photograph: courtesy of the US Army Research Development and Engineering Command, Historical Research and Response Team, Aberdeen Proving Ground, MD.

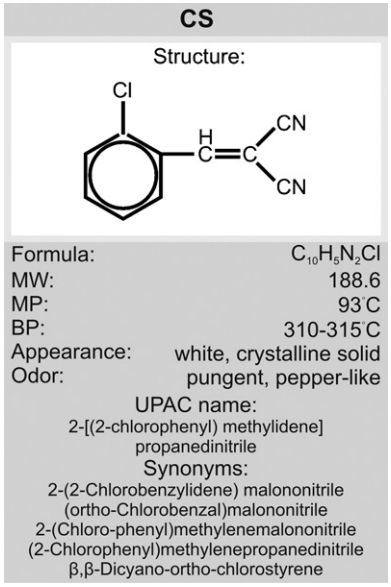

FIGURE 12.5. Chemical structure and physicochemical properties of ortho-chlorobenzylidene malononitrile (CS).

6. Pelargonic Acid Vanillylamide (PAVA)

Other capsaicinoids are available. Pelargonic acid vanillylamide (PAVA or nonivamide), shown in Figure 12.10, is a ''synthetic'' form of capsaicin. Nonivamide was first synthesized by Nelson (1919). Nonivamide was originally found to be a minor component in *Capsicum annum* peppers (Constant and Cordell, 1996); however, the majority of PAVA is derived from synthesis rather than extraction from natural plant sources. As a result, the composition and concentration of PAVA can remain consistent (Haber *et al.*, 2007).

FIGURE 12.6. Aerial spraying of a Chemical Warfare School class with CS tear gas during a training event. Photograph: courtesy of the US Army Research Development and Engineering Command, Historical Research and Response Team, Aberdeen Proving Ground, MD.

FIGURE 12.7. US Army soldiers using CS tear gas in South Vietnam. Photograph: courtesy of the US Army Research Development and Engineering Command, Historical Research and Response Team, Aberdeen Proving Ground, MD.

In order for PAVA to work, it must be directed at the subject's eyes. The pain to the eyes is reported to be higher than that caused by CS tear gas (Smith *et al.*, 2004; ACPO, 2006). The effects are immediate but will subside 15–20 min after exposure to fresh air. PAVA does display disadvantages. While PAVA has a high rate of effectiveness, it has proven to be ineffective against those under the influence of alcohol (ACPO, 2006). Additionally, the Smith *et al.* (2004) study mentions a number of cases where PAVA was used without effect. The effect of PAVA was also reported to be almost instantaneous, with the undesirable effect that recovery was also immediate. PAVA is commercially available in two forms, Captor I and Captor II. Captor I contains 0.3% PAVA with a solvent of equal parts ethanol and water. Captor II contains 0.3% PAVA with propylene glycol, water, and ethanol (COT, 2007).

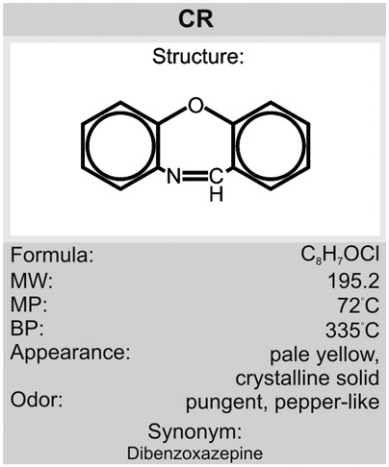

FIGURE 12.8. Chemical structure and physicochemical properties of dibenz(b,f)-1:4-oxazepine (CR).

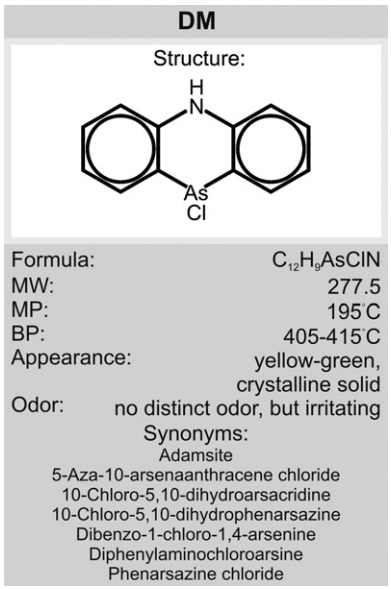

FIGURE 12.9. Chemical structure and physicochemical properties of diphenylaminechlorarsine (DM).

IV. MECHANISM OF ACTION

A. CS, CN, and CR

The mechanisms of action through which RCAs act are not completely understood. One explanation for the toxic effects of RCAs is the production of hydrochloric acid through reduction of chloride ions on mucosal membranes (Worthington and Nee, 1999). This may help explain the marked, focal irritation and burns on skin resulting from exposure to CS (Anderson *et al.*, 1996). In addition, CS and CN are SN_2 alkylating agents (Cucinell *et al.*, 1971; Ballantyne and Swanston, 1978); in contrast, the vesicant mustard is an SN_1 alkylating agent. The SN_2 moniker describes direct reaction of the agent with nucleophilic compounds in a bimolecular fashion. In particular, they react with intracellular thiol or SH-containing enzymes, thereby inactivating them (Ballantyne, 1977a). Mackworth (1948) first showed that CN and other first generation lacrimators used during WWI (bromoacetophenone, ethyl iodoacetate, chloropicrin, bromobenzyl cyanide) strongly inhibited thiol-containing succinic dehydrogenase and pyruvic oxidase, major players of crucial metabolic pathways. Some suggest that lactic dehydrogenase is completely insensitive to lacrimators (Mackworth, 1948), but only lacrimators from the iodoacetate family were ever studied by this group. Another group reported that lactic dehydrogenase is in fact strongly inhibited by CS (Cucinell *et al.*, 1971). Chloropicrin also interferes with oxygen transport to the tissues by reacting with SH groups on hemoglobin.

In addition, CS reacts with the disulfhydryl form of lipoic acid, a coenzyme in the pyruvate decarboxylase system (Olajos and Salem, 2001). Alteration in dihydrolipoic acid biochemistry can lead to decreased acetyl CoA levels, resulting in cellular injury. Therefore, tissue injury seems to be related to inactivation of these metabolic enzyme systems. The damage is transient because the enzymes can be rapidly reactivated if exposure is terminated (Beswick, 1983).

Based on these studies, it has been suggested that alkylation of nucleophilic sites, including SH-containing enzymes, is the underlying biochemical lesion responsible

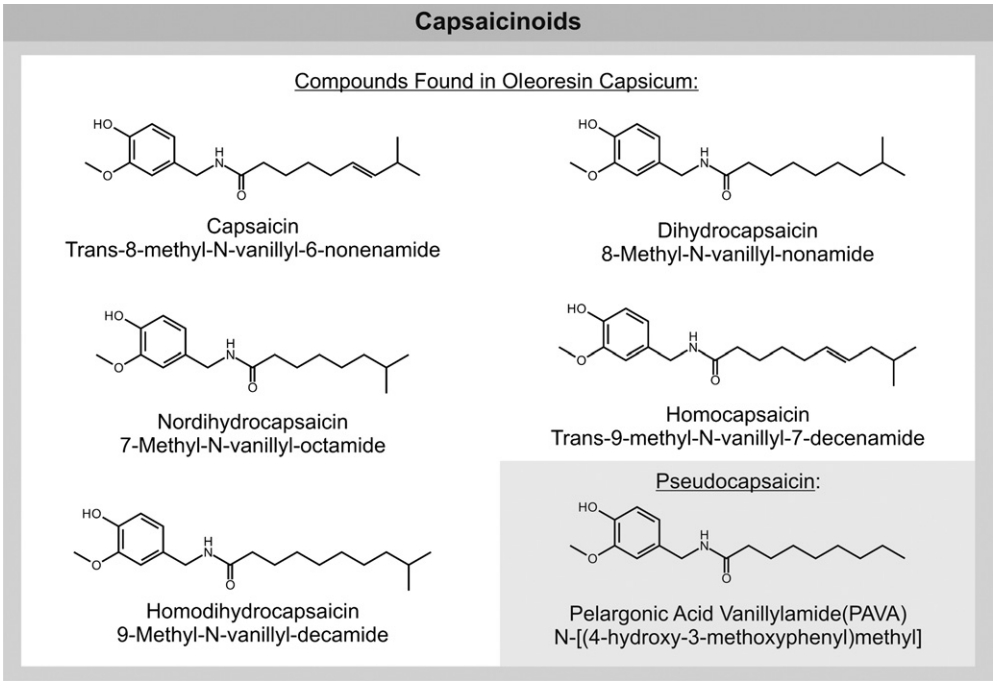

FIGURE 12.10. Chemical structures of the most common capsaicinoids found in oleoresin capsicum.

for lacrimator-induced toxicity. However, pain from RCA exposure can occur without tissue injury. It has been suggested that the irritant and painful effect of CS may be bradykin mediated (McNamara *et al.*, 1969; Cucinell *et al.*, 1971; Olajos and Salem, 2001). CS causes bradykinin release *in vivo* in humans (Cucinell *et al.*, 1971) and *in vitro* (Blain, 2003). Elimination of bradykininogen *in vivo* abolishes the systemic response to CS (USAMRICD, 2000; Salem *et al.*, 2006).

The metabolism of CS to cyanide (see section V) was once thought to be responsible for agent-induced lethality in animals (Cucinell *et al.*, 1971; Jones and Israel, 1970). Despite reports on alleged fatality cases, mortality in humans following CS administration has not been authenticated (Ballantyne, 1977a; Hill *et al.*, 2000; Olajos and Salem, 2001). CS has been demonstrated to cause death in dogs (Cucinell *et al.*, 1971). CS is hydrolyzed to malononitrile and 2-chlorobenzaldehyde (Brewster *et al.*, 1987). Further metabolism of malononitrile yields two potential cyanides, which could interact with sulfur thiols to yield thiocyanate. Cyanide typically causes death immediately, but animals administered CS by inhalation far above the lethal C*t* do not die immediately; death occurs 12 to 24 h after exposure. In fact, death seems to be due to airway and lung damage (Ballantyne and Swanston, 1978; Ballantyne and Callaway, 1972). Studies to ascertain cyanide production after CS exposure in humans showed negligible levels of plasma thiocyanate (Swentzel *et al.*, 1970; Leadbeater, 1973). Another study revealed low levels of cyanide production in mice administered carbon 14-labeled CS (Brewster *et al.*, 1987). In short, cyanide is not liberated in sufficient quantity from CS metabolism to become toxic enough to cause death.

While cyanide inhibition of cytochrome *c* oxidase may not account for the full spectrum of toxicity in CS exposure, cyanide toxicity may include an array of biochemical interactions (Way, 1984). These include lipid peroxidation (Johnson *et al.*, 1987), cyanide release of endogenous opioids to cause respiratory paralysis (Leung *et al.*, 1986), disruption of neuronal calcium homeostasis (Johnson *et al.*, 1986), and phospholipids hydrolysis (Sakaida and Farber, 1990). The mechanism of action for CN follows very closely that of CS as they are both alkylating agents. The effect of both agents on SH-dependent enzyme systems has been studied (Lovre and Cucinell, 1970; Cucinell *et al.*, 1971). Less is known regarding the mechanism of action for CR intoxication.

B. Capsaicinoids

Capsaicinoids interact with a population of neuropeptide-containing afferent neurons and activate a ''vanilloid'' receptor (Szallasi and Blumberg, 1990, 1992; Szallasi *et al.*, 1991). There seems to be a requirement by the receptor for a vanilloid ring and an acyl chain moiety for activity (Szallasi and Blumberg, 1999; Caterina and Julius, 2001). Vanilloid receptors are part of a superfamily of transient receptor potential (TRP) cation channels (Montell *et al.*, 2002). Binding of a vanilloid-containing ligand to the receptor causes channel opening, influx of Ca^{2+} and Na^{+}, depolarization of the neuron, and release of neuropeptides (Lundblad and Lundberg, 1984; Martling, 1987). In addition to transitory excitation of primary afferents, activation of these receptors leads to a prolonged refractory period, indicative of an apparent nonconducting, desensitized state of the receptor. In this refractory period, primary afferents become unresponsive to further application of capsaicinoids. Furthermore, it has been suggested that influx of Ca^{2+} and Na^{+} may lead to rapid cellular damage and eventual cell death (Jancso *et al.*, 1984), possibly by Ca^{2+}-dependent protease activity. Administration of capsaicin in neonatal rats causes destruction of the dorsal root ganglion neurons (Jancso *et al.*, 1977).

The biological actions of capsaicin are primarily due to release of the neuropeptide substance P, calcitonin gene-related peptide (CGRP), and neurokinin A from sensory neurons. These transmitters from primary sensory neurons communicate with other cell types. They produce alterations in the airway mucosa and neurogenic inflammation of the respiratory epithelium, airway blood vessels, glands, and smooth muscle. Alterations in multiple effector organs lead to bronchoconstriction, increased vascular permeability, edema of the tracheobronchial mucosa, elevated mucosal secretion, and neutrophil chemotaxis (Lundberg and Saria, 1982; Lundberg *et al.*, 1983, 1984; Blanc *et al.*, 1991; Tominack and Spyker, 1987). Capsaicin-induced effects of bronchoconstriction, vasodilation, and plasma protein extravasation are mediated by substance P. In addition, substance P can cause bronchoconstriction through stimulation of c-fibers in pulmonary and bronchial circulation.

V. TOXICOKINETICS

The uptake, distribution, and metabolism of CS, CR, and capsaicins (but not CN) have been well characterized.

A. Uptake, Distribution, and Metabolism of CS

CS is rapidly absorbed and distributed throughout the body after inhalation exposure. Pharmacokinetic studies show that CS is removed from circulation quickly with first-order kinetics, following inhalation exposure. CS half-life is just under 30 s (Olajos, 2004). Short half-lives in the circulatory system are also demonstrated for the major CS metabolites (2-chlorobenzyl malononitrile and 2-chlorobenzaldehyde) (Leadbeater, 1973). Currently, it is thought that significant amounts of CS, near the tolerable concentration around 10 mg/m^3, would not be absorbed following CS inhalation. The absorption of CS from the digestive tract in cases of exposure by ingestion is unknown at this time. Systemic toxicity

has been noted after ingestion of CS pellets (Solomon *et al.*, 2003).

In mammalian species, CS rapidly hydrolyzes to form 2-chlorobenzaldehyde and malononitrile (Leadbeater, 1973; Paradowski, 1979; Rietveld *et al.*, 1986). The malononitrile intermediate is further metabolized from two cyanide moieties, which are converted to thiocyanate (Cucinell *et al.*, 1971). The aldehyde intermediate undergoes oxidation to 2-chlorobenzoic acid or reduction to 2-chlorobenzyl alcohol. These metabolites are conjugated and excreted in the urine.

B. Uptake, Distribution, and Metabolism of CR

Absorption of CR after aerosol inhalation is rapid with a plasma half-life of 5 min; this is consistent with half-life estimates following intravenous administration (Upshall, 1977) and gastrointestinal uptake (French *et al.*, 1983). Corneal tissue has been demonstrated to take up CR and metabolize it to the lactam derivative (Balfour, 1978; King and Holmes, 1997).

A number of studies have investigated the bioconversion, fate, and elimination of CR in various animal species (French *et al.*, 1983; Furnival *et al.*, 1983; Balfour, 1978; Harrison *et al.*, 1978). Human metabolic studies on CR have not been performed due to the high degree of sensitivity of human tissues to CR. The maximum tolerated dosage is far too low to allow for detection in metabolic studies (Olajos, 2004). The lactam derivative dibenz[*b*,*f*]1:4-oxazepin-11-(10H)-one is a primary metabolic product of metabolism and a direct precursor of the urinary hydroxylated metabolites. In rats, the lactam, a dihydro-CR metabolite, an amino alcohol of CR, and an arene oxide are metabolites in CR degradation. In the rat, the major mechanism for elimination is sulfate conjugation and biliary excretion to a limited extent. Phase I metabolism by microsomal mixed function oxidases involves reduction of CR to the amino alcohol, oxidation to form the lactam ring, and hydroxylation to form the hydroxylactams. Phase II conjugation reactions sulfate the hydroxylactam intermediates for renal elimination. Amino alcohol intermediates are conjugated with glucuronide for biliary secretion.

C. Uptake, Distribution, and Metabolism of CN

The uptake, distribution, and fate of CN have been poorly characterized despite numerous investigations reporting its toxicity. Inhalation of lethal CN, which does not metabolize to liberate cyanide, also causes death secondary to effects on the pulmonary system (pulmonary congestion, edema, bronchopneumonia, cellular degeneration in the bronchiole epithelium, and alveolar thickening) in mice, rats, guinea pigs, and dogs (Olajos and Salem, 2001). CN presumably reacts irreversibly with the free sulfhydryl groups of proteins and enzymes. It is thought that CN metabolically converts to an alkylating agent with this affinity for SH groups and nucleophilic sites in tissues (Mackworth, 1948; Olajos, 2004).

D. Uptake, Distribution, and Metabolism of Capsaicins

Capsaicin and capsaicinoids undergo Phase I metabolic bioconversion to catechol metabolites via hydroxylation of the vanillyl ring moiety (Lee and Kumar, 1980; Miller *et al.*, 1983). Metabolism involves oxidative, in addition to nonoxidative, mechanisms. An example of oxidative conversion involves the liver mixed-function oxidase system to convert capsaicin to an electrophilic epoxide, a reactive metabolite (Olajos, 2004). Surh and Lee (1995) have also demonstrated the formation of a phenoxy radical and quinine product; the quinine pathway leads to formation of a highly reactive methyl radical (Reilly *et al.*, 2003). The alkyl side chain of capsaicin also undergoes rapid oxidative deamination (Wehmeyer *et al.*, 1990) or hydroxylation (Surh *et al.*, 1995; Reilly *et al.*, 2003) to hydroxycapsaicin as a detoxification pathway. An example of nonoxidative metabolism of capsaicin is hydrolysis of the acid–amide bond to yield vanillylamide and fatty acyl groups (Kawada *et al.*, 1984; Oi *et al.*, 1992).

VI. TOXICITY

RCAs produce a wide variety of physiological effects in man. Figure 12.11 illustrates these generalized toxic signs and symptoms of exposure. The clinical effects in the figure are representative of those encountered after CN or CS exposure. CR causes qualitatively similar effects to those caused by CS, except it has greater potency. The predominant anatomical regions affected include eye, lung, and skin. RCAs also cause nasal, oral, neuronal, and gastrointestinal effects.

A. Ophthalmological Effects

1. CN and CS

The eyes are a major target for the short-lived toxic effects of RCAs. Eye findings from RCA toxicity can range in severity from conjunctival erythema to ocular necrosis. Lacrimation, conjunctival erythema/edema, blepharitis, and erythema are the most typical findings after exposure to all RCAs. Toxic signs may further include periorbital edema (Vaca *et al.*, 1996; Yih 1995), blepharospasm or spasms during eyelid closure (Grant, 1986; Blain, 2003), apraxia of eyelid opening, ophthalmodynia, corneal injury, and ocular necrosis (Grant, 1986). Figure 12.12 illustrates and summarizes the common toxic ophthalmological signs and symptoms associated with RCA aerosol exposure. It is important to note that eye findings tend to be more severe in

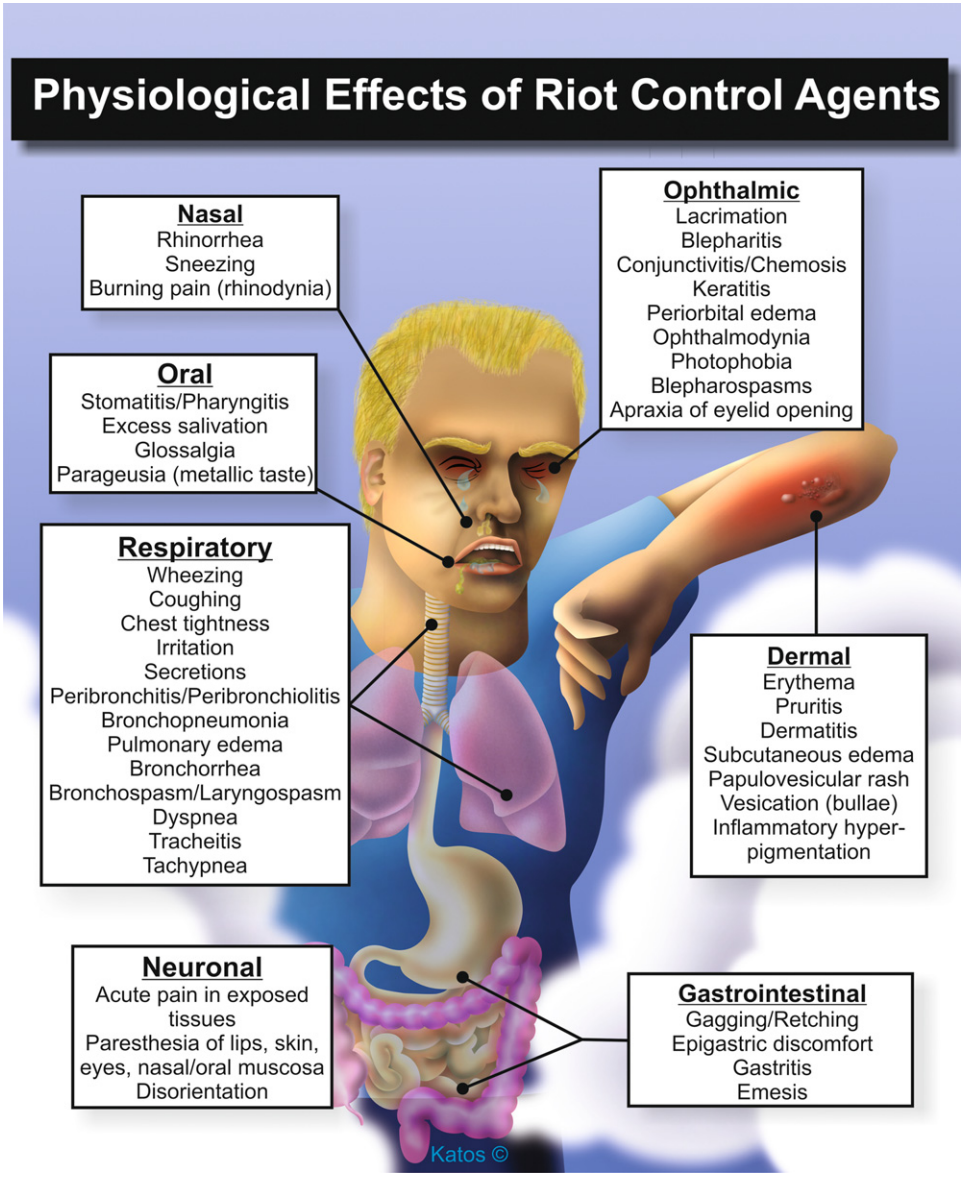

FIGURE 12.11. Physiological effects of riot control agents. Illustrated, copyright protected, and printed with permission by Alexandre M. Katos.

RCA exposure victims if they are wearing contact lenses (Solomon *et al.*, 2003).

Erythema and edema may last up to 48 h and vascularizing keratitis is not uncommon (Ballantyne *et al.*, 1974), but symptoms generally subside after 30 min (Beswick, 1983) depending on the concentration and duration of exposure (Blain, 2003). Recovery is typically complete within 15 to 30 min after exposure, but a few signs such as erythema of the lid margins and photophobia may persist slightly longer. The conjunctivae may appear injected or even progress to fulminant conjunctivitis and blurred vision following some RCAs including CS (Euripidou *et al.*, 2004). Toxic signs in the conjunctivae from CN Mace exposure can include conjunctivitis, sloughing, limbal ischemia, and symblepharon formation (adhesion of the eyelids to the eyeball) (Scott, 1995). Permanent eye injury is unlikely except after exposure to high concentrations of CN Mace (Grant, 1986). While permanent eye damage is uncommon, raised intraocular pressure from edema may precipitate acute angle closure glaucoma if left untreated. Long-term sequelæ may include cataracts, vitreous hemorrhage, and traumatic optic neuropathy (Gray and Murray, 1995).

In studies involving human exposure (Rengstorff and Mershon, 1969a, b), CS (0.1% or 0.25% in water; 1.0% in triocyl phosphate) sprayed or administered as ophthalmic drops onto the eyes, caused apraxia of eyelid opening with blepharospasm upon eyelid closure for 10 to 135 s. It also caused a transient conjunctivitis but no corneal damage upon further inspection with a slit lamp. Rabbit eyes contaminated with CS as a solution (0.5–10% in polyethylene glycol), as a solid, or thermally dispersed as a smoke (15 min at 6,000 mg/m^3) showed a greater toxicity with solution. CS in solution caused profuse lacrimation, conjunctivitis, iritis, chemosis, keratitis, and corneal vascularization at concentrations at or above 1%.

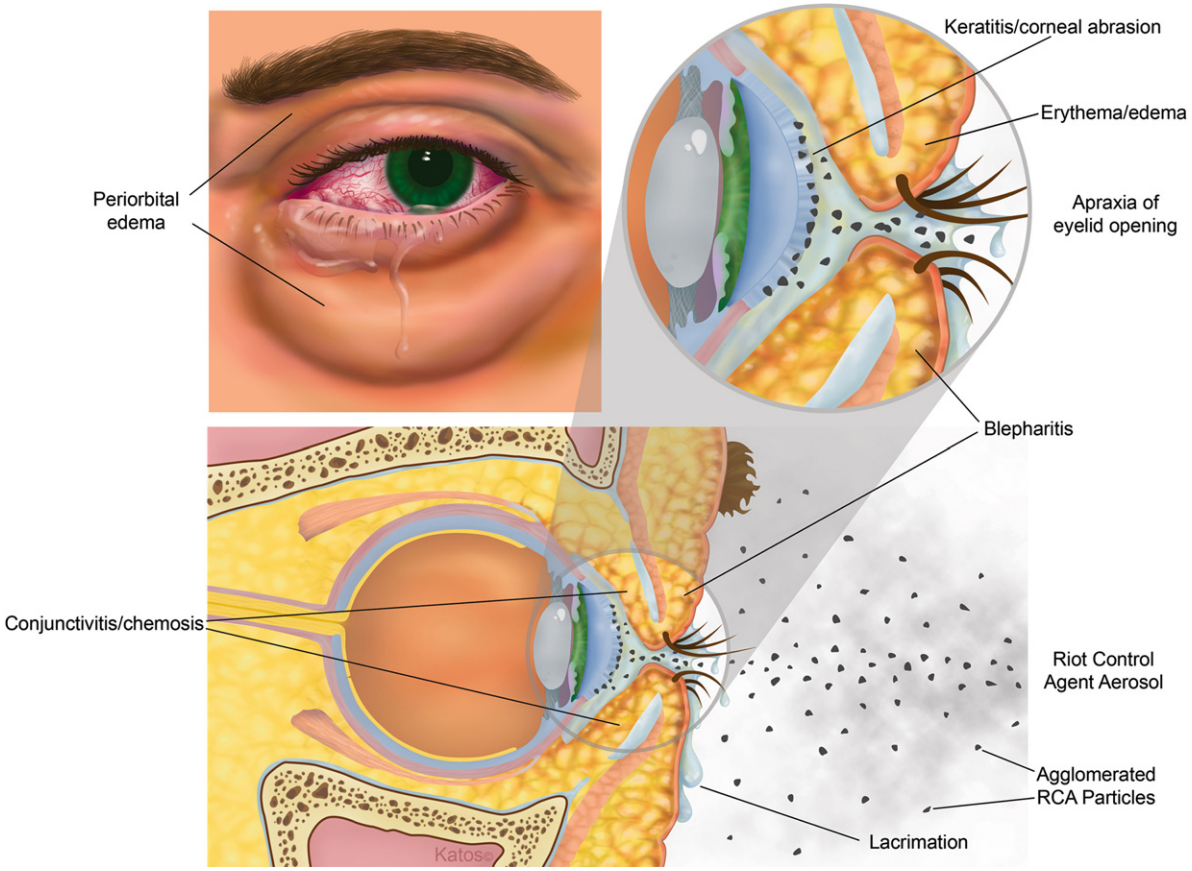

FIGURE 12.12. Exposure of the eye to CS aerosol. *Top left panel*: external view of left eye immediately after exposure to CS aerosol, showing scleral injection, periorbital edema, and lacrimation. *Bottom panel*: Penetration of CS aerosol into the eye, sagittal view. Following exposure to CS, the eye responds with inflammation, edema (chemosis), lacrimation, erythema, eye pain, and eyelid closure. *Top right panel*: Close-up of the eye and eyelids, sagittal view. Inflammation of the eyelids (blepharitis), conjunctivae (conjunctivitis), and cornea (keratitis) are apparent. The eye, in turn, responds with spasms of eyelid closure (blepharospasms) followed by an inability to open the eyelids (apraxia of eyelid opening). Agglomerated CS particles can penetrate the eye upon initial contact and cause corneal abrasions. Illustrated, copyright protected, and printed with permission by Alexandre M. Katos.

The lesions tended to be more severe and have a greater duration at higher doses. Histologically, the cornea appeared with patchy denudation of the epithelium and infiltration of neutrophils to the site of injury (Ballantyne *et al.*, 1974). In general, more severe eye exposures have resulted from CN compared to CS agent.

CN causes a similar constellation of ocular signs and symptoms as CS, but CN toxicity is likely to be more severe in the eyes and skin. CN sprayed into the eyes from a distance causes lacrimation, edema of the corneal epithelium and conjunctivae, and reversible epithelial defects of the cornea (Leopold and Lieberman, 1971). At close range, CN can cause long lasting and permanent damage to the eye. Because RCAs are solids, it is possible for a particle to clump or agglomerate, causing penetration into corneal or conjunctival tissues (see Figure 12.12). Agglomerated CN particles can penetrate eye tissue as a result of tear gas cartridge discharge (Levine and Stahl, 1968). In addition to large powder CN agglomerates, traumatic effects from the propellant charge, wadding, or foreign pieces from the cartridge should also be suspected when evaluating eye damage from CN.

While RCAs produce short-lived effects, rabbits exposed to 10% CN solution caused iritis and conjunctivitis >7 days and corneal opacity (Grant, 1986) lasting longer than 2 months (Gaskins *et al.*, 1972). In comparison, CS at the same concentration produced moderate conjunctivitis without iritis or corneal opacity, and eyes returned to normal by the end of one week. Another difference between the two agents is that CN produces a more severe reaction than CS when applied to the eye in powder form or as a spray at close range (McNamara *et al.*, 1968).

In addition to opacification, additional corneal effects from particulate CN exposure include possible penetration of the corneal stroma, severe scarring and ulceration, and deficits in the corneal reflex (Blain, 2003; Scott, 1995). Penetration of the corneal stroma may lead to stromal edema and later vascularization, resulting in further ocular complications. These may include pseudopterygium, infective keratitis, symblepharon, trophic keratopathy, cataracts,

hyphema, posterior synechia, secondary glaucoma, vitreous hemorrhage, and traumatic optic neuropathy (Hoffman, 1967). Furthermore, a 4% CN formulation produced permanent corneal injury while a 10% CS product did not (Gaskins *et al.*, 1972). In animal studies, high concentrations of CN produce ocular necrosis (Grant, 1986).

The eyes are also affected from CS agent without direct contact of the agent with the eye. In one report, seven patients were exposed to oral ingestion of a juice drink contaminated by CS pellets (Solomon *et al.*, 2003). In addition to mild headache and gastrointestinal irritation, patients complained of ocular irritation and lacrimation. The majority of symptoms resolved within 24 h of exposure.

2. CR

Higginbottom and Suschitzky (1962), the chemists who discovered CR, first noted the intense lacrimal response to this compound. A splash of CR (0.01 to 0.1% range solution) causes immediate ophthalmodynia, lacrimation, and blepharospasm, similar to CS and CN (Sidell, 1997). These effects can last 15 to 30 min before subsiding. Blepharitis (edema of the eyelids), periorbital edema, and injected conjunctivae can last for up to 6 h. In rabbits and monkeys, CR (0.1% solution) causes mild, transient erythema, chemosis, and keratitis in the eye. Moderate conjunctivitis has been demonstrated with higher CR concentrations (5% solution) applied directly to the rabbit eye (Rengstorff *et al.*, 1975). Ballantyne *et al.* (1975) showed that increasing CR concentrations as a solution caused dose-dependent corneal thickening but minor eye effects (mild conjunctivitis and lacrimation) as an aerosol. In animal studies, the effects of CR on the eye are very transient as they are cleared in 1 h, and it produces far less toxicity to the eye than CN (Salem *et al.*, 2006).

3. Capsaicin

Capsaicin causes conjunctivitis, periorbital edema/erythema, ophthalmodynia, blepharospasm, blepharitis, corneal abrasions, and lacrimation. In a retrospective study of 81 patients who presented to the emergency department following aerosol exposure from law enforcement use of OC, 56% of individuals developed ophthalmodynia, 44% conjunctivitis, 40% conjunctival erythema, 13% lacrimation, and 9% corneal abrasions (Watson *et al.*, 1996). Another study involved exposure of 47 human volunteers to OC for evaluating effects on the cornea and conjunctivae (Zollman *et al.*, 2000). All subjects reported significant eye pain, blurred vision, and lacrimation 10 min after exposure to OC pepper spray, but symptoms improved by 1 h later. Corneal abrasions were not apparent, but 21% of subjects showed evidence of punctate epithelial erosions and reduced corneal sensitivity. Corneal abnormalities were absent 1 week after exposure. Another human study identified 23% of subjects (7 of 30) with corneal abrasions following aerosol exposure to OC spray (Watson *et al.*, 1996). In mice, a single subcutaneous injection of 12.5, 25, or 50 mg/kg capsaicin causes corneal changes characterized by neuronal axon degeneration in the corneal epithelium (Fujita *et al.*, 1984).

B. Nasal/Pharyngeal Toxicity

RCAs produce oral and nasal symptoms immediately after exposure. Inhalation exposure to CN and CS causes rhinorrhea, sneezing, and burning pain within seconds (Beswick, 1983); a similar burning sensation with increased salivation occurs after oral contact with aerosolized powder or solution. The salivation, pharyngitis, and glossalgia occur within minutes after exposure (Thorburn, 1982; Beswick, 1983). A CR solution (0.01 to 0.1% range) splashed in the mouth causes salivation and burning of the tongue (Ballantyne and Swanston, 1974) and palate for several minutes. Splashes of the solution can cause nasal irritation and rhinorrhea (Sidell, 1997). Fumes from burned *Capsicum* plants or capsaicin-containing pepper sprays are highly irritating to the nasal mucosa and cause immediate rhinorrhea (Morton, 1971; Collier and Fuller, 1984; Geppetti *et al.*, 1988).

C. Cardiovascular Toxicity

While the evidence is not overwhelmingly impressive, RCAs have apparent effects on the cardiovascular system. Tachycardia and mild hypertension have been noted after exposure (Beswick, 1983). This response may result from anxiety or a response to the pain as opposed to any toxicological effect. The initial response to aerosolized CS is hypertension and irregular respiration, suggestive of the Sherrington pseudoaffective response. Bypassing the pain receptors of the nose and upper airway by endotracheal administration of CS leads to a decrease in blood pressure and bradypnea, effects also seen after intravenous injection. This suggests the initial pressor effect and irregular respiration are generalized responses to noxious stimuli rather than pharmacological effects of CS. Splash contamination of the face or whole-body drenching with dilute CR solution (0.0010 and 0.0025%) causes an immediate increase in blood pressure and bradycardia (Ballantyne *et al.*, 1973, 1976). Intravenous administration of CR in cats causes a transient but dose-dependent tachycardia. These pressor effects are postulated to be secondary to CR effects on sympathetic tone to the cardiovascular system (Lundy and McKay, 1975) or the result of stress and discomfort from the irritation (Ballantyne, 1977a, b).

RCAs have been shown to have a direct effect on the heart. One report linked exposure of high CS concentrations to the development of congestive heart failure (Hu *et al.*, 1989). Furthermore, underlying cardiac disease has been shown to exacerbate toxicity from CS (Worthington and Nee, 1999).

D. Respiratory Toxicity

CS and CN are disseminated as an aerosol powder or solution. Therefore, the most common route of CN or CS absorption is by inhalation. Inhalation of RCAs causes burning and irritation of the airways leading to cough, chest tightness, dyspnea (Beswick, 1983, Hu *et al.*, 1989; Blain, 2003), shortness of breath (Euripidou, 2004), bronchospasm (Hu and Christiani, 1992), and bronchorrhea (Folb and Talmud, 1989). Estimates of the minimal irritant exposure and ICt_{50} are 0.004 and 5 mg·min/m^3, respectively, for CS (Olajos and Salem, 2001). Similar estimates have been made for CN (0.3–1 and 20–50 mg·min/m^3; Olajos and Salem, 2001). Other estimates report that 31 mg/m^3 CN vapor is intolerable to humans after 3 min (Punte *et al.*, 1962).

Laryngospasm can occur either immediately or delayed for 1 to 2 days after CN or CS exposure. Delayed onset laryngotracheobronchitis 1–2 days post-exposure, characterized by wheezing, dyspnea, tachypnea, hoarseness, fever, and purulent sputum, was reported in three of eight patients exposed to high concentrations of CN (Thorburn, 1982). Long-term bronchodilator therapy was required in one patient with pre-existing pulmonary disease. Reactive airways are associated with high-level exposure to CS and CR (Blain, 2003). Paroxysmal cough, shortness of breath, and chest tightness, characteristic of reactive airway disease, have been demonstrated to last up to several weeks. Pulmonary effects typically resolve by 12 weeks post-exposure.

Pulmonary edema may occur up to 24 h post-exposure (Stein and Kirwan, 1964; Gonmori *et al.*, 1987). Gonmori *et al.* (1987) reported a fatality from chloropicrin spray intoxication. The patient, an 18-year-old female, developed pulmonary edema 3 h after exposure. Furthermore, a 43-year-old man developed pulmonary edema complicated by pneumonia, heart failure, and hepatocellular damage after CS intoxication (Krapf and Thalmann, 1981). Delayed onset bronchopneumonia may occur from prolonged exposure to some RCAs in enclosed spaces (Beswick, 1983).

There is no evidence that CS causes permanent lung damage after one or several exposures to field concentrations (Blain, 2003). Inhalation of an irritant might be expected to exacerbate underlying pulmonary disease such as asthma, emphysema, or bronchitis. Histories of asthma and chronic obstructive pulmonary disease may exacerbate effects from CS (Worthington and Nee, 1999) or CN (Thorburn, 1982). CS may exacerbate chronic bronchitis or precipitate an attack in known asthmatics (Anonymous, 1971).

1. CN and CS Toxicity in Animals

In animal studies, the cause of death from CN inhalation is the result of toxicity in the pulmonary system. Post-mortem examination from acute toxicity lethality studies in animals exposed to CN aerosols reveal pulmonary congestion, edema, emphysema, tracheitis, bronchitis, and bronchopneumonia in dogs and pulmonary congestion, edema, and bronchopneumonia in mice, rats, and guinea pigs (Olajos and Salem, 2001). Sublethal CN aerosol exposure (62.6 mg/m^3, 0.1 LC_{50}) for 60 min causes cellular degeneration in the bronchiole epithelium and alveolar septal wall thickening due to infiltration of mononucleocytes (Kumar *et al.*, 1995).

Exposure to aerosol CS (unreported concentration) in male Wistar rats for 20 min can cause decreased minute ventilation and induce histological lesions of the trachea (cytoplasmic vacuoles in epithelial cells) and lung (emphysema) (Debarre *et al.*, 1999). Lower respiratory tract injury, including fibrosing peribronchitis and peribronchiolitis, can be produced by chloropicrin (Buckley *et al.*, 1984).

2. CR

CR does not produce any significant respiratory toxicity (Sidell, 1997). CR causes tachypnea and labored breathing in multiple animal species. In humans, aerosol exposure to CR causes respiratory irritation, choking, and dyspnea. One human study involving aerosol exposure to CR (0.25 mg/m^3) in volunteers for 60 min noted decreased expiratory flow rate minutes after exposure. CR was thought to stimulate irritant receptors in the conducting portion of the pulmonary system, causing bronchoconstriction (Ashton *et al.*, 1978). Additionally, CR increased blood volume in the lungs by driving sympathetic tone. Two animal studies evaluated the effect of CR aerosol exposure on the physical and ultrastructural changes in rat lungs (Pattle *et al.*, 1974; Colgrave *et al.*, 1983). Even high CR aerosol doses did not produce significant pulmonary damage. Gross examination of the lungs was normal in both studies. Microscopic examination showed mild congestion, lobar hyperinflation characteristic of emphysema and hemorrhage. Further pulmonary damage was evident on electron microscopy. CR exposed lungs showed capillary damage of the endothelium and a thickened, swollen epithelial layer (Colgrave *et al.*, 1983).

3. Capsaicin

In children, capsaicin spray was demonstrated to cause a severe bronchospasm and pulmonary edema (Winograd, 1977; Billmire *et al.*, 1996). In the Billmire study, a 4-week-old infant was exposed to 5% pepper spray after discharge from a self-defense device. The infant suffered respiratory failure and hypoxemia, requiring immediate extracorporeal membrane oxygenation. Inhaled capsaicin causes an immediate increase in airway resistance (Fuller, 1991). This dose-dependent bronchoconstriction after capsaicin inhalation in humans is the same as that demonstrated in asthmatics and smokers (Fuller *et al.*, 1985). The capsaicin-induced bronchoconstriction and release of substance P is due to stimulation of nonmyelinated afferent C-fibers.

E. Neurologic Toxicity

RCAs are irritants to the peripheral nervous system (Anonymous, 1999). CN and CS interact with receptors on sensory nerves in the eyes, other mucous membranes, and skin, resulting in discomfort and burning pain. Their neurologic toxicity can range from paresthesias of the lips to burning pain of the eyes (ophthalmodynia), tongue (glossalgia), nose (rhinodynia), throat (pharyngodynia), and skin (dermatalgia). The reaction of CN with sulfhydryl (SH)-containing proteins and enzymes is the cause of denaturation associated with sensory nerve activity (Chung and Giles, 1972). As RCAs affect the senses, the feeling can become disorienting after exposure, which may explain why some experience temporary loss of balance and orientation after exposure (Thorburn, 1982).

Agitation and panic may develop in those not previously exposed to CN (Beswick, 1983; Stein and Kirwan, 1964). Syncope has also been reported (Athanaselis *et al.*, 1990; Thorburn, 1982), but this is likely attributed to panic. Headaches have been reported in 48% of symptomatic persons exposed to chloropicrin (Goldman *et al.*, 1987). When CN was released into 44 prisoner cells, eight inmates experienced malaise and lethargy and among those hospitalized, one experienced syncope and a severe systemic illness (Thorburn, 1982).

A clinical case report of hand injuries caused by accidental discharges from tear gas pens (Adams *et al.*, 1966) revealed specific neuronal toxicological findings. In each case, CN penetrated into the skin to cause a wound. Neurological examination indicated hyperesthesia of select digits in all cases. Biopsies of digital neurons taken for pathology showed thickened epineurium and tendon sheaths. Some of the patients complained of paresthesias months after exposure. The study suggests a possible link between direct chemical injury and nerve damage. The same investigators exposed the sciatic nerves of rabbits to agent by discharge of a CN pen or by dusting the exposed nerve with 0.2 g CN powder. These animal studies suggested CN can cause inflammation and necrosis in skeletal muscle, loss of axon cylinders, and replacement of neural elements with granulation tissue and fibroblasts (Adams *et al.*, 1966). Animals exposed to CR exhibit fasciculations, tremors, convulsions, and ataxia; intraperitoneal administration of CR can also cause muscle weakness (Salem *et al.*, 2006).

Capsaicin activates receptors in trigeminal (cranial nerve V) and intestinal neurons. These include pain receptors located in the mouth, nose, stomach, and mucous membranes. Trigeminal neurons utilize substance P as their primary pain neurotransmitter. Capsaicin first induces the release of substance P from the neuron and then blocks the synthesis and transport of substance P to the effector side (Bernstein *et al.*, 1981; Tominack and Spyker, 1987). Substance P depolarizes neurons to produce dilation of blood vessels, stimulation of smooth muscle, and activation of sensory nerve endings (Helme *et al.*, 1987; Tominack and Spyker, 1987). Jancso characterized the effects of capsaicin as an initial intense excitation of sensory neurons followed by a lengthy period of insensitivity to physicochemical stimuli (Jancso *et al.*, 1968, 1987; Buck and Burks, 1986). Substance P is associated with sensory (pain) or skin inflammation afferents. It is also a peripheral mediator of neurogenic inflammation and smooth muscle contraction (Lembeck and Holzer, 1979; Pernow, 1983). It contributes to contraction of the esophagus, trachea, respiratory tract, and levator palpebrae muscles (blepharospasm and apraxia of eyelid opening). Capsaicin directly applied to the eye causes a neurogenic inflammation, involving vasodilatation and extravasation of fluid, and unresponsiveness to chemical stimuli. Capsaicin renders the skin of humans and animals insensitive to various types of painful chemical stimuli (Bernstein *et al.*, 1981). In humans, OC exposure eventually causes loss of the corneal blink reflex (Olajos and Salem, 2001), which is mediated by sensory input from cranial nerve V and motor output via cranial nerve VII.

F. Gastrointestinal Toxicity

Many reviews state that gastrointestinal effects do not occur upon inhalational exposure to RCAs with the exception of DM; however, nausea, vomiting, and alterations in taste are commonplace in clinical case reports of exposure to CS (Solomon *et al.*, 2003; Athanaselis, 1990) and CN (Thorburn, 1982; Blain, 2003). The involvement of retching and emesis tends to occur if the individual is sensitive, the concentration is sufficiently high, the exposure prolonged, the range is close, or the event occurs in a confined space. Vomiting was reported in 25% of patients with severe reactions to CN in a confined area (Thorburn, 1982). Emesis did not resolve until the following week in one patient. Inhalation of RCAs often leads to parageusias or altered taste of the tongue. In particular, a metallic or burning sensation is often reported (Folb and Talmud, 1989).

Ingestion of CS can also produce episodes of nausea, vomiting, crampy abdominal pain (Blain, 2003), and diarrhea (Blain, 2003; Solomon, 2003). Seven patients in the Solomon study drank juice contaminated with CS pellets and primarily developed gastrointestinal symptoms. Two of the seven patients reported emesis and diarrhea; all patients reported abdominal pain, epigastric discomfort, and burning gastroesophageal reflux. Symptoms resolved 24 h later. Surprisingly, they did not develop parageusia or burning of the tongue after CS ingestion which is often the case after inhalational CS exposure. Another study designed for patients to taste an admixture of sugar and CS (5–10 pellets, 500 mg each and dissolved in 10 liters of water) indicated that patients experienced a 30 s delay in onset of altered taste (Kemp and Willder, 1972); this was most likely due to a masking effect from the sugar. In animal studies, rabbits and rats develop gastroenteritis upon CN or CS exposure by ingestion (Gaskins *et al.*, 1972).

Biting and ingesting *Capsicum* plants can cause nausea and vomiting (Morton, 1971; Tominack and Spyker, 1987; Snyman *et al.*, 2001). Nausea has also been reported in individuals exposed to pepperball tactical powder containing capsaicin (Hay *et al.*, 2006). Capsaicin causes effects on gastric mucosa including mild erythema, edema, epithelial cell damage (Desai *et al.*, 1976), and gastric hemorrhage (Viranuvatti *et al.*, 1972; Desai *et al.*, 1977; Kumar *et al.*, 1984).

G. Dermatological Toxicity

CN and CS are primary irritants of the integumentary system able to cause first and second degree burns (Stein and Kirwan, 1964; Weigand, 1969; Hu *et al.*, 1989). Low concentrations of either agent cause erythema, pruritis, subcutaneous edema, paresthesias, and/or burning sensations in exposed areas of the skin within minutes. Erythema is often the first sign of contact dermatitis, occurring minutes after exposure and subsiding about an hour after exposure. These agents follow a time course of skin damage similar to mustard agent. Further, if the skin is wet or abraded, the toxic effects on the skin are more prominent (Holland and White, 1972; Thorburn, 1982; Sidell, 1997). Exposure to higher doses leads to worsening erythema, edema, vesication with bullae (observed hours later), and fever. The extent of toxic effects also depends on thickness of the stratum corneum and time of exposure. Furthermore, contact with water up to 48 h after exposure can exacerbate the painful symptoms (Pinkus, 1978; Blain, 2003). High humidity, diaphoretic subjects, and warm temperatures can all exacerbate the contact dermatitis from RCAs (Hellreich *et al.*, 1969). Areas of occlusive dress over the skin may also result in worse reactions.

Higher concentrations of CS or longer exposures will result in more than erythema, pruritis, and burning pain. Papulovesicular rashes are not uncommon with high concentrations of RCAs. Typically, edema and vesiculation (bullae dermatitis) follow 24 h after CS or CN exposure (Sidell, 1997). Common sites of bullae are areas under the cuff of a shirt or glove and just under the collar. One study examined the effect of high CS concentrations (300 mg/m^3), tested on the arms of volunteer study patients, at times ranging between 15 and 60 min exposure (Hellreich *et al.*, 1967). All participants experienced burning pain approximately 5 min after exposure onset. A C*t* range of 4,440 and 9,480 $mg.min/m^3$ caused an immediate patchy erythema, which subsided after 30 min. A C*t* range of 14,040 and 17,700 $mg.min/m^3$ led to greater dermal toxicity and required several hours to subside. Bullous dermatitis occurred in 50% of subjects as a delayed reaction. These bullous lesions resolved in 2 weeks, but an inflammatory hyperpigmentation of the skin remained by 6 weeks post-exposure. Differences in individual sensitivities are due to skin pigmentation, complexion, and susceptibility to sunburns (Hellreich *et al.*, 1969).

Exposure to other RCAs causes similar dermal effects. CN is a more potent irritant than CS. In a human study involving dermal application, CN (0.5 mg) powder caused irritation and erythema when on the skin for 60 min (Holland and White, 1972). It took 20 mg CS to cause similar effects for the same duration of exposure. Exposure to 5% capsaicin pepper spray causes immediate and severe erythema and edema in the skin (Herman *et al.*, 1998). Similarly, pepper ball pellets fired at individuals will cause erythema, pain, and edema at the site of impact. The initial point of contact may become infected, scar, or heal with hyperpigmentation (Hay *et al.*, 2006).

Dermal exposure to CN or CS may lead to an allergic contact dermatitis (Madden, 1951; Penneys, 1971), a delayed hypersensitivity reaction developed from a previous exposure to RCAs. CS and CN are both skin sensitizers, but CN is more potent (Chung and Giles, 1972). Initial exposure to either may not cause significant toxic signs or symptoms. Exposure to small amounts of the same agent years later, however, may produce a severe allergic erythematous, patchy rash with edema, bullae, purpura, and necrosis. Sensitization is likely to occur after dermal exposure to high concentrations of RCAs (Penneys *et al.*, 1969; Holland and White, 1972; Leenutaphong and Goerz, 1989). Hypersensitivity reactions can persist for up to 4 weeks (Leenutaphong and Goerz, 1989). This phenomenon has been demonstrated so far by CN (Ingram, 1942; Kissin and Mazer, 1944; Steffen, 1968; Frazier, 1976) and CS (Ro and Lee, 1991) but not CR.

Dermal exposure to CR causes a burning sensation and erythema several minutes later. Burning pain goes away after 15 to 30 min, but the erythema lasts up to 2 h (Holland, 1974). CR does not induce inflammatory cell migration to the site of injury, bullous dermatitis, or contact sensitization (Ballantyne, 1977a). Repeated application of CR to the skin (applied 5 days/week for 12 weeks) has little effect (Marrs *et al.*, 1982). Similar to the eye and lungs, CR does not demonstrate significant toxicity to the skin.

CS, CN, or CR can pose a toxic danger hours after dissemination as they are persistent in the environment. During the riots of the late 1960s, CS was frequently used to control crowds. Inadvertently, firefighters in those metropolitan areas sometimes were exposed as they entered buildings where CS had been disseminated. The force of water from firehose and movement within the buildings reaerosolized enough agent toxic enough to cause erythema and edema around their eyes and other areas of exposed skin (Rengstorff and Mershon, 1969a).

While capsaicinoids may have a vesicant effect, depending on length of exposure, in most cases it produces a burning sensation and mild erythema. Capsaicins cause erythema and burning pain without vesiculation when applied topically to human skin (Smith *et al.*, 1970; Burnett, 1989; Watson *et al.*, 1996; Herman *et al.*, 1998). Skin blistering and rash may occur after chronic or prolonged capsaicin exposures (Morton, 1971).

H. Other Toxicity

One report noted renal tubular nephritis in a worker killed after an explosion inside a plant manufacturing CS agent (Cookson and Nottingham, 1969). Hepatocellular injury has been linked to serious CS inhalation (Krapf and Thalmann, 1981). To date, animal studies have not documented any relationship between RCA exposure and teratogenicity (Himsworth *et al.*, 1971; Upshall, 1973; Folb and Talmud, 1989). CS did not demonstrate mutagenic potential with the Ames assay (Rietveld *et al.*, 1983). Similarly, CR did not have carcinogenic effects in mice or hamsters (Blain, 2003); CS lacks mutagenicity in several test systems (Daniken *et al.*, 1981; Wild *et al.*, 1983).

I. Lethality

Human deaths have been reported from RCA exposure (Thorburn, 1982; Ferslew *et al.*, 1986; Danto, 1987). Death is usually the result of excessive concentrations used, confined spaces, and prolonged exposures. Death occurs hours after initial exposure, and post-mortem findings are consistent with severe airway damage seen in animals. Deichmann and Gerarde (1969) reported a fatality following exposure to high CN vapor concentrations (5.4 gm in a 34 m^3 room) for less than 20 min, which equates to approximately 160 mg/m^3. Estimates of the human LCt_{50} range between 25,000 and 150,000 $mg \cdot min/m^3$ for CS and between 8,500 and 22,500 $mg \cdot min/m^3$ for CN (Olajos and Salem, 2001). High doses of CR aerosol do not produce lethality in animals; CR aerosols of 68,000 $mg \cdot min/m^3$ are not lethal in mice, guinea pigs, or rabbits. The large safety ratio for CR is clearly evident as compared to the other agents.

While OC is widely regarded as a safe substance with low toxicity (Clede, 1993), more research should be conducted in light of recent deaths involving pepper spray use by law enforcement agencies. One case involving an inmate who died in custody implicated pepper spray as a direct contributor to death (Steffe *et al.*, 1995). Billmire *et al.* (1996) reported the life-threatening effects in a 4-week-old infant exposed to OC spray as a result of an accidental discharge.

VII. RISK ASSESSMENT

The ideal process in RCA risk assessment is to characterize the effectiveness and risk from exposures to situations where RCAs may be used (NAS, 1983, 1984; NAS/NRC, 1994; TERA, 2001; Patterson *et al.*, 2004). In order to do that, one must identify all pertinent effects of the RCA in question, develop a dose–response assessment, consider an exposure assessment, and finally characterize the risk. When used as intended, RCAs are thought to be safe and of sufficient low toxicity. They are designed with the purpose of disabling a targeted individual through sensory irritation of the eyes, respiratory tract, and skin. As discussed previously, they are not without additional, unwanted effects especially in circumstances where high concentrations are used or exposure is prolonged. The previous sections have provided sufficient discussion regarding the potential toxicity to humans as a result of exposure to RCAs, including case reports.

A. Identification of Intended and Unintended Effects

By providing a minimal force alternative for controlling and managing individual(s), RCAs are a desired public health and safety tool for military, domestic law enforcement, and civilian use. As with any chemical intended to benefit the public, it is important first to identify the compounds, their potential adverse impact (unintended effects), and their beneficial impact (intended effects). There are a number of chemicals designed and used as RCAs. In general, they are compounds with low vapor pressures and dispersed as fine particles or in solution from a variety of devices. These dispersal methods can include the gamut from aerial spray (Figure 12.6) to large spray tanks (see Figures 12.2 and 12.4) and small, hand-held devices for self-protection. The modern RCAs used today include CN, CS, CR, OC, and PAVA. Their major adverse effects are summarized in Table 12.2. The intended effect for all RCAs is a change in behavioral response of the target. A better measure of this intended effect would be the actual physiological effects produced by RCAs on the eyes, skin, and respiratory tract (Patterson *et al.*, 2004). These are the target organs designed for harassment by RCAs (see Figure 12.11 for review).

Each physiological effect can be evaluated and categorized on a broad spectral continuum from mild to severe. At lower aerosol dosages, the effects from RCAs will be reversible with no serious injury. For instance, typical mild ophthalmic effects include lacrimation (tearing) and transient burning pain (ophthalmodynia). When used at higher levels, in confined spaces and/or for prolonged duration, there is a greater potential for the toxicity to escalate. Moderate effects would include conjunctivitis, keratitis, blepharitis, chemosis, and periorbital edema. Severe effects result from significantly prolonged duration or high concentrations leading to irreversible damage in the tissues (i.e. loss of vision). These include corneal abrasions, scarring, or opacification. Very serious effects on the eye include symblepharon, pseudopterygium, cataracts, hyphema, posterior synechia, secondary glaucoma, vitreous hemorrhage, and traumatic optic neuropathy. The same analysis can be applied to effects on the skin, respiratory tracts, and additional organ systems affected for each agent.

B. Dose Response

Dose–response assessment involves evaluating the dose required to produce a particular effect of interest. Ideally,

quantitative data on specific doses and their corresponding responses are desired. In reality, threshold data for a particular target organ or effect in a target organ are often available as a substitute. The ophthalmic threshold levels and toxicity estimate for human responses to CN, CS, and CR are shown in Table 12.2. If empirical dose–response data are available, a dose–response evaluation for a given RCA might include plotting the percent of individuals responding as a function of dose for each toxicological sign or symptom and target organ of interest. Dose–response curves can then be used in modeling studies to estimate the probabilities of intended and unintended effects for a particular risk assessment scenario (Patterson *et al.*, 2004).

C. Exposure Assessment

The crux of exposure assessment is creating a scenario for human exposure to a given RCA and identifying the exposure factors. This would involve describing the intended target(s), environmental conditions (windy, rainy weather, etc.), crowd size and characteristics, delivery device (tear gas canisters or grenades, powder or aerosol), hazards associated with the delivery system like blunt trauma, as well as the nature of the agent selected (physicochemical properties, solvents, concentration/dose), and duration of exposure. An exposure assessment might include estimation of the amount of systemic exposure through RCA inhalation, absorption through the skin from dermal contact, or intestinal uptake after ingestion. Availability of quality data for each of the aforementioned exposure factors will estimate exposure with high confidence and minimal uncertainty level. Unavailability of data is a major limitation if models are used to estimate exposure.

D. Characterization of the Risk and Risk Management

Estimating or developing probabilities of toxic effects within a population is at the heart of risk characterization. It integrates dose–response and exposure assessments. It is designed to provide the probability of occurrence for effects induced by a given RCA given a particular exposure scenario. For example, a decision-maker will use risk characterization to estimate the probability of a group of effects occurring as a result of clearing a confined space with CS. The probability can be derived as a function of the number of tear gas grenades employed. Unfortunately, there is a dearth of specific Federal risk assessment and risk management guidance or mandates on RCAs. Therefore, the potential for risk management or mitigation of concerns is not optimized for the health and benefit of the public good (Hauschild, 2004). This is partly due to the fact that the process for assessing risk of toxic chemicals has yet to be standardized among Federal programs (Burke *et al.*, 1993; Rhomberg, 1997). Computer modeling to aid risk assessment without empirical data to feed the model can be an academic exercise. The two in combination can be a powerful predictor for risk assessment of any toxic chemical.

VIII. TREATMENT

Exposure to RCAs leads to a generalized stress reaction, causing leukocytosis (Thorburn, 1982; Park and Giammona, 1972), hypokalemia, elevated total protein, increased globulin, and high bicarbonate levels (Beswick *et al.*, 1972). Treatment for RCA toxicity is not often required since the course of intoxication is self-limiting for the most part. Serum toxicological testing is not available to detect RCAs (Sidell, 1997). Clinical signs and symptoms from RCA exposure subside in less than an hour. Initial care involves removing the victim from a potentially crowded area of dispersal immediately to minimize exposure time. It is important to note that these victims may require additional assistance during evacuation because of their reduced vision and disorientation. In circumstances where the concentration of agent is substantially elevated or the area of release is confined, increased complications and risks of morbidity may arise in the eyes, skin, airways, and lungs.

A. Eyes

If the eyes are involved to any degree, a protective mechanism to close the eyelids will be initiated as a result of conjunctivitis, iritis, or keratitis. Photophobia, blepharospasm, and apraxia of eyelid opening prevent the clinician from evaluating the damage. However, a local anesthetic applied to the eye will help with eye pain and allow for further evaluation of the eye by slit lamp. Contact lenses should be immediately removed and the eyes flushed of any dusting or agglomerated solid particles (see Figure 12.12). Eyes should be irrigated with copious volumes of water or saline for at least 15 min to adequately flush the irritant. Diphoterine has also been used to decontaminate eyes and skin after CS tear gas exposure (Viala *et al.*, 2005). If symptoms or signs of eye toxicity persist, consultation with an ophthalmologist is critical. Elderly patients should be monitored for evidence of possible acute glaucoma (Yih, 1995).

B. Skin

Early signs of skin toxicity at the time of clinical presentation will often be contact or allergic dermatitis since blisters form hours later. Removal of clothing should be the first step in decontamination. Placement of contaminated clothes in sealed plastic bags by first responders will prevent secondary contamination as a result of reaerosolized agent (Horton *et al.*, 2005). Early studies of CS indicated that mixing CS with sodium hypochlorite (or household bleach) produced a greater reaction than CS alone in patch testing

(Punte *et al.*, 1963). Despite its usefulness as a decontaminant for many chemical agents, hypochlorite should never be used to decontaminate RCAs on skin. Use of water for decontamination of skin may result in an initial worsening of the burning sensation (described previously). A solution of 6% sodium bicarbonate, 3% sodium carbonate, and 1% benzalkonium chloride has been shown to provide immediate relief from CS dermatitis as the alkaline solution hydrolyzes the agent (Weigand, 1969; Sidell, 1997). Consultation with a burn unit should be considered when large areas of skin are involved or when children are affected. Medical treatment for dermatitis may include topical steroids such as triamcinolone acetonide (Hellreich, 1967; Sidell, 1997), oral antihistamines for pruritis, and topical antibiotics such as silver sulfadiazine (Hellreich, 1967; Roberts, 1988). Systemic antibiotics can be given for secondary infection. Oozing lesions from bullae dermatitis should be treated with wet dressings, changed daily. Deroofing closed vesicles is controversial (Carvajal and Stewart, 1987; Roberts, 1988). Tetanus prophylaxis should be considered.

C. Respiratory

Removing an exposed patient from the source of intoxication to fresh air will provide immediate improvement. Patients should be evaluated for hypoxia with pulse oximetry and arterial blood gases. Pulmonary function tests may be helpful in patients with prolonged pulmonary complaints and followed until symptoms resolve. Chest radiography might be useful if concentration was sufficiently high, exposure was prolonged, or dispersal occurred in a confined space. Pulmonary edema may be delayed for 12 to 24 h after exposure, suggesting a need for follow-up radiographs (Stein and Kirwan, 1964; Solomon, 2003). Laryngospasm is a serious complication that may require tracheal intubation to secure a patent airway. Bronchospasm may be treated with inhaled beta-2 agonists, steroids (methylprednisolone), and aminophilline (Ballantyne and Swanston, 1978; Folb and Talmud, 1989). Arterial blood gas (Vaca *et al.*, 1996) and pulse oximetry should be continued if patients are symptomatic hours after exposure.

IX. CONCLUDING REMARKS AND FUTURE DIRECTION

The goal of RCAs is to harass or produce temporary incapacitation. Use of irritants to harass enemies dates back several thousand years. Today, law enforcement agencies and military personnel use RCAs for quelling protestors, controlling crowds, subduing combatants, clearing buildings, training in chemical warfare, and area denial. Individuals use hand-held devices for self-protection against an assailant. RCAs are dispersed as aerosols or sprays, causing irritation of mucous membranes of the eyes, respiratory tract, and skin. Symptoms and signs of toxicity typically subside by 30–60 min.

Several lines of evidence suggest that RCAs are safe if used as they were originally intended. Even though RCAs are considered safe, nonlethal, temporary incapacitating agents, they are not without risk. Some of the adverse clinical effects from RCA exposure reported in the literature have involved indiscriminate use (excessive concentrations), prolonged exposure, and dissemination of compound in a confined space. In short, these nonlethal agents can pose a serious health hazard in their intended targets. Some RCAs have such a poor safety profile that they have been abandoned long ago (DM and CA). CN and CS have a large body of literature from which to compare and contrast their safety, toxicity, and potency. As the data clearly suggest, CS is a safer compound to use compared to CN. The latest newcomers to the RCA scene are the inflammatory capsaicinoids. OC and PAVA are highly effective irritants that cause similar symptoms to CN and CS. Capsaicinoids gained considerable attention in the 1990s from police departments and the public at large for safe, effective chemical incapacitation of individuals. These compounds are primarily used as defensive sprays by law enforcement to subdue a combative suspect or by individuals for self-protection. While OC, PAVA, and related capsaicinoids produce a similar constellation of toxic signs and symptoms, they are not currently used to control crowds at the level of a riot. If OC-containing pepper spray is preferred for riot control, more research will be required to determine whether it is indeed safe for humans. Finally, risk assessment is a process which can identify gaps in the literature and therefore serves to highlight research needs.

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CHAPTER 13

Fluoroacetate

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I. INTRODUCTION

Fluoroorganic compounds attracted the attention of researchers nearly 70 years ago when among a large class of biologically inert chemicals a group of very toxic compounds was revealed, having the general formula CH_2FCOOR and the common name ''fluoroacetate'' (FA). The toxicological effects of FA do not become apparent immediately even after exposure to lethal doses, but after a latent period of half an hour to several hours for animals and humans. The level of FA in some Australian plants can reach up to 5 g/kg dry weight (Hall, 1972) and can cause death of domestic animals, sometimes with appreciable economic damage (McCosker, 1989; Minnaar *et al.*, 2000a). FA can be found in fog and rain drops in some industrial regions (Rompp *et al.*, 2001). The best known representative of FA is its sodium salt (SFA, compound 1080). This substance is used in several countries for controlling populations of some vertebrates. There are also series of fluorocompounds whose metabolism is connected with the formation of FA, these are: antineoplastic drugs (5-fluorouracil and isomers of fluoronitrosourea); N-(2-fluoroethyl) derivatives of the narcotic analgetics normeperidin and normethazocin; pesticides, 1,3-difluoro-propanol and fluoroacetamide (FAA, compound 1081); and 1-(di)halo-2-fluoroethans and fluoroethanol (Reifenrath *et al.*, 1980; Tisdale and Brennan, 1985; Feldwick *et al.*, 1998). The urgency of the problems associated with FA toxicity; and therapy for acute intoxication has greatly increased in connection with a new threat – international terrorism (Holstege *et al.*, 2007). Physicochemical features of FA, the lack of taste and odor, delayed manifestation of toxicity, and similarity of clinical signs of intoxication to some natural indispositions are all characteristics that necessitate comprehensive studies of mechanisms of action of FA and a search for effective therapeutic means for treatment of acute intoxication.

II. BACKGROUND

FA was initially synthesized in 1896 and only decades after that was found in *Dichapetalum*, *Gastrolobium*, *Oxylobium*, *Acacia* and *Palicourea* plants growing in Australia, South Africa, and South America (Oerlichs and McEwan, 1961; De Oliveira, 1963; McEwan, 1964; Aplin, 1971; Vickery *et al.*, 1973). Chemically pure FA is a very stable compound, and the energy of dissociation of the fluorocarbon bond in the molecule is regarded as one of the highest among the natural compounds (Ichiyama *et al.*, 2004). However, FA is broken down in biological preparations from plants (Minnaar *et al.*, 2000a). After inhalation or ingestion, FA is easily absorbed by tissues and its high toxicity is independent of its route of entry into organisms (Chenoweth, 1949). The mechanism of toxic action of FA is widely known as ''lethal synthesis'' (Peters, 1952; Peters and Wakelin, 1953), the essence of which is conversion of nontoxic FA to toxic fluorocitrate (FC) within the cells of an organism. The main reasons for death are considered to be disbalance of intracellular ions, osmotic disbalance, and deficit of ATP as a consequence of aconitase blockade (Buffa *et al.*, 1973). The latent period from the moment of poisoning with FA to manifestation of clinical signs is 0.5–3 h (in warm-blooded animals). This period reflects penetration of FA into blood and cells and conversion of FA to FC, with the consequent uncoupling of intracellular metabolism. Death usually occurs within 24–48 h, but can be later. At autopsy there are no specific signs of intoxication (Peters *et al.*, 1981). For warm-blooded animals unadapted to FA the lethal dose is less than 2 mg/kg (Atzert, 1971). But there is a considerable species-specific difference in clinical signs of intoxication and differences in sensitivity to the poison (Chenoweth, 1949). The mean lethal dose varies within the range from 0.05 mg/kg for dogs to 150 mg/kg for possums. The most common criterion for tolerance, or sensitivity, of animals to FA is intensity of metabolism. Thus, in the lizard *Tiliqua rugosa* the level of metabolism of FA is ten times lower in comparison with that of rat (*Rattus norvegicus*), and the lethal dose for lizard is 100 times higher than that of rat (Twigg *et al.*, 1986). Low intensity of metabolism means low conversion of FA to FC, which makes more effective excretion and detoxication possible. In the absence of specific clinical, physiological, and morphological signs of intoxication, determination of FA in tissues together with

citrate and fluoride ions can be a diagnostic confirmation of FA intoxication (Harrison *et al.*, 1952; Schultz *et al.*, 1982; Koryagina *et al.*, 2006).

III. TOXICOKINETICS

A. Detoxification

The main pathway of detoxification of FA is its defluorination via a glutathione-dependent mechanism involving nucleophilic attack on the β-carbon atom and formation of fluoride and S-carboximethylglutathion, with subsequent cleavage of the latter into amino acids and S-(carboxymethyl) excreted in the urine as a conjugate complex (Mead *et al.*, 1979, 1985; Tecle and Casida, 1989). The highest defluorinating activity was found in liver, followed by kidney, lung, heart, and testicles in a descending line. No defluorinating activity was found in brain. The activity of enzymes responsible for defluorination depends on glutathione (GHS) concentration with a maximum above 5 mmol/l, the apparent K_m being 7 mmol/l at saturating concentrations of GHS (Soiefer and Kostyniak, 1983). Defluorination is mainly carried out by anionic proteins having glutathione transferase activity, though the anionic fraction contains nearly 10% of proteins without this activity but is also capable of defluorination of FA. Moreover, cationic enzymes were shown to be responsible for about 20% of cytosolic defluorination of FA (Wang *et al.*, 1986). The GHS-dependent enzyme defluorinating FA is not identical to GHS-dependent S-transferases; it is an FA-specific defluorinase having an acidic isoelectric point (pH = 6.4) and a molecular weight of 41 kD (27 kD for the main subunit) (Soiefer and Kostyniak, 1984). Activity of defluorinase isoenzymes varies markedly and has been the subject of recent research (Tu *et al.*, 2006).

B. Analytical Procedure

Analysis of biological samples of FA is problematic because of the high polarity of the fluorine–carbon bond in the molecule. Liquid chromatography (LC) has been applied for analysis of FA in different media (Livanos and Milham, 1984; Allender, 1990), and analysis of FA in plants and gastric contents by HPLC with UV detection has also been described (Ray *et al.*, 1981; Minnaar *et al.*, 2000b). Being a nonvolatile substance, FA was commonly analyzed by gas chromatography (GC), as a methyl derivative (Stevens *et al.*, 1976), ethyl or *n*-propyl derivatives (Peterson, 1975) and as pentafluorobenzyl esters (Okuno *et al.*, 1982; Vartiainen and Kauranen, 1984). Derivatization with 2,4-dichloroaniline in the presence of N,N-dicyclohexylcarbodiimide was used for GC analysis of SFA in water (Ozawa and Tsukioka, 1987) and blood serum (Demarchi *et al.*, 2001). A modified procedure by Eason *et al.* (1994) achieved low detection limits for FA at the level of 0.01 μg/g in plasma and urine and 0.002 μg/g in tissue and feces of sheep and goats. However, this procedure is labor and time consuming, and the GC-electron capture detection procedure applied is considered to be unreliable at this level of sensitivity.

The main problem for GC analysis of FA in biological samples is coelution of the matrix components. This can be overcome by sampling the analyte from an equilibrium vapor phase. Static head-space analysis of SFA as ethyl fluoroacetate, with a linear range for SFA in water of 5–200 μg/ml and a detection limit of 0.5 μg/ml has been reported (Mori *et al*, 1996). Solid-phase microextraction (SPME) from an equilibrium vapor phase has all the advantages of head-space analysis, while being a much more sensitive technique. We reported on a novel procedure for determination of FA in water and biological samples, involving ethylation of FA with ethanol in the presence of sulfuric acid, SPME of the ethyl fluoroacetate formed with subsequent analysis by GC-MS (Koryagina *et al.*, 2006). To overcome the problem of the presence of the components coeluting with FA derivatives we made use of GC-MS in the SIM (selective ion monitoring) mode. To avoid a partial overlapping of the internal standard's peak with the sample matrices' components, quantification was performed with the use of two internal standards, carbon tetrachloride and toluene. GC-MS was performed on a Shimadzu QP5000 GC-MS system, using a Supelco SPB-5 capillary column. A stable PDMS-Carboxen-Stable-Flex microfiber (75 μm) was used. The GC-FID, combined with SPME under the optimal conditions, achieved reliable determination of FA in water in the concentration range 0.001–10 μg/ml. The calibration plot for the determination of SFA in biological samples was linear in the SFA concentration range 0.01–5.0 μg/ml for both internal standards, and a linear relationship in blood plasma was observed in the range 0.01–5.0 μg/ml ($r = 0.95$). With toluene as internal standard, the linear regression equation was $Y = 0.014\,X$ [Y was a ratio S(EthylFA)/S(toluene); X was the concentration of SFA, μg/ml]. The RSD (relative standard deviation) for fluoroacetate quantification at 0.1 μg/ml was 12% ($n = 5$). With carbon tetrachloride as internal standard, a linear relationship in plasma was observed in the range 0.01–5.0 μg/ml ($r = 0.98$). The linear regression equation was $Y = 0.1656\,X$ [Y was the ratio S(EthylFA)/S(CCl4); X was the concentration of FA, μg/ml]. The RSD for FA quantification at 0.1 μg/ml was 6% ($n = 5$), and the detection limit was 0.01 μg/ml ($S/N = 3$). The calibration characteristics of rat organ homogenates were identical to those of plasma.

C. Tissue Distribution and Elimination

The data on toxicokinetics of FA are rather contradictory, apparently depending on analytical procedures and dose of the poison; also, there is evidence for animal species specificity. The first data on toxicokinetics of FA demonstrated its rather uniform distribution between organs, with some

predominance in heart, brain, and kidneys (Hagan *et al.*, 1950; Gal *et al.*, 1961). The half-life was calculated to be not less than 2 days, and this could cause secondary toxicity arising from ingestion of meat from the poisoned animals (Aulerich *et al.*, 1987). For the purposes of risk assessment for humans in case of secondary poisoning with meat, sheep and goats were given FA (0.1 mg/kg) and their tissues were analyzed for FA content. The half-life of FA was shown to be 10.8 h for sheep and 5.4 h for goats; maximal concentration of FA 2.5 h after the poisoning was revealed in blood plasma (0.098 μg/ml), followed by kidneys (0.057 μg/g), skeletal muscles (0.042 μg/g), and liver (0.021 μg/g). Only traces of FA were found in all the tissues examined 96 h after the poisoning (Eason *et al.*, 1994). At 1 and 12 h after introduction of SFA (0.2 μg/kg) to rats a similar ratio of FA was found in rat plasma (0.26 and 0.076 μg/ml, correspondingly) (Eason and Turck, 2002), the half-life period being 2.9 h. On the other hand, for rabbits under subacute intoxication with FA the half-life was found to be 1.1 h, and the level of FA in rabbit muscles, kidneys, and liver was much higher than in blood plasma (Gooneratne *et al.*, 1995).

In our laboratory, the use of the above-mentioned SPME method in combination with GC-MS produced the following results (Figure 13.1): maximal concentrations were found in rats 1 h after the poisoning, 2.2 μg/ml in blood plasma and 1.89 μg/g in brain; there was 3–4 times less FA in rat kidneys, liver, and heart (from 0.64 to 0.50 μg/g). After a further 2 h the distribution between the tissues was more equal, resulting from a prominent decrease of FA in plasma and brain and a small decrease or even elevation of FA in other organs. A further decrease of FA was found in all the tissues, except for heart, 24 h after the poisoning. After 72 h, no FA was detected in plasma; we did not measure FA in rat organs at this point. The half-life was calculated to be 3.6 h.

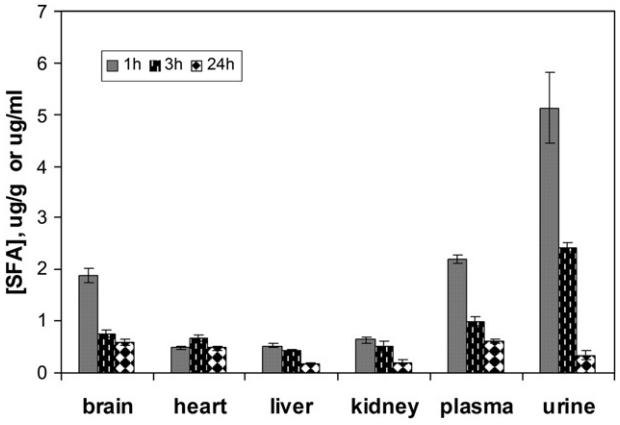

FIGURE 13.1. Data on determination of FA (recounted as SFA) in rat organ homogenates and body fluids, at times following poisoning with SFA at a peroral dose of 2 mg/kg ($1/2LD_{50}$). Standard deviations (shown) were based on 4–6 replicate analyses.

IV. MECHANISM OF ACTION

A. Molecular Mechanism of Aconitase Inhibition

The mechanism of the inhibitory effect of FA on aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3] is one of the most interesting in biochemistry. Upon entering an organism, nontoxic FA undergoes a series of metabolic conversions the result of which is synthesis of highly toxic fluorocitrate (FC); this process was termed ''lethal synthesis'' (Peters, 1952). FC is formed by the enzymatic condensation of fluoroacetyl-CoA with oxaloacetate, catalyzed by citrate (*si*)-synthase (EC 4.1.3.7) (Eanes and Kun, 1974; Kirsten *et al.*, 1978). FC was initially considered to be a competitive aconitase inhibitor, but in the early 1990s it was suggested that FC acts as a ''suicide substrate'', because it has a high affinity for aconitase at any concentration of the competitive citrate (Clarke, 1991). Aconitase effects conversion of citrate to isocitrate through an intermediate, *cis*-aconitate, which binds with aconitase in two different ways, swung 180° to the C^{α}—C^{β} bond (Gawron and Mahajan, 1966). Aconitase includes a [4Fe-4S] cluster and the catalytic conversion involves substrate coordination to a specific iron atom in this cluster, Fe_a (Lauble *et al.*, 1992). The single inhibitory isomer was shown to be (–)-*erythro*-2-fluorocitrate (2R, 3R) (Carrell *et al.*, 1970), from which aconitase removes fluoride ion with a stoichiometry of 1 F^- per enzyme molecule (Kent *et al.*, 1985; Tecle and Casida, 1989). The defluorination results in generation of an actual aconitase inhibitor, 4-hydroxy-*trans*-aconitate (HTA), which binds very tightly – though not covalently – with aconitase (Kent *et al.*, 1985; Lauble *et al.*, 1996). The natural aconitase substrate isocitrate should be at a 10^6-fold excess in order to slowly displace HTA from its complex with aconitase. The HTA–aconitase complex involves four hydrogen bonds, which hold together HTA, a water molecule, Asp165, and His167 (Lauble *et al.*, 1994, 1996). In contrast, isocitrate has only one such bond.

B. Physiological and Biochemical Effects of FA

1. Effects of FA and FC on Mitochondria and Other Intracellular Organelles

Functional disturbances of mitochondria (MCh) precede the appearance of structural anomalies (Buffa and Pasquali-Ronchetti, 1977) and consist of their decreased capacity to oxidize the substrates introduced. Within the mitochondrial matrix, FA induces changes which develop in several minutes resulting in its swelling and loss of electronic density. These changes are explained by accumulation of citrate, rise of osmotic pressure, and decrease of energy production (Corsi and Granata, 1967; Buffa and Pasquali-Ronchetti, 1977). Change in the level of ATP is not caused by uncoupling of respiration and phosphorylation (Fairhurst *et al.*, 1958; Corsi and Granata, 1967). Mitochondrial

volume changes are accompanied by their conformational reorganizations: these are displacement of granules and disintegration of cristae, and extension and rupture of their membranes. Axonal cylinders stretch in 3–4 h after small doses of the poison and in 1–2 h after lethal doses. The cylinders are filled with MCh (most of which are being swelled and degenerated), multilamellar lisosome-like bodies, vesicules, and neurofibrils. In the Golgi complex, a condensation of cisternae takes place (McDowell, 1972). Concurrently, a disruption of endoplasmic reticulum, swelling of nucleus, and reduction of aggregated chromatin can be seen.

Having studied *in vitro* effects of FC on rat liver MCh we revealed that maximal inhibition of respiration was registered when MCh were uncoupled (Figure 13.2). The level of alkalinization of the medium at addition of ADP was much lower in the presence of FC, thus evidencing an inhibition of ATP synthesis. The amplitude of alkalinization was also decreased, which could be caused by incomplete ATP synthesis, an additional transmembrane redistribution of protons, and/or change of the binding constant of ADP. FC induced a leak of Ca^{2+} from MCh, which was consistent with the observed inhibition of oxygen consumption in respiratory state 1. Addition of the substrates caused re-entry of Ca^{2+} into MCh. In the presence of FC, the MCh only partially took up the Ca^{2+} ions added to the medium, followed by their spontaneous efflux through an electroneutral $2H^+/Ca^{2+}$ exchanger with $K_{1/2} = 10$ µmol/l (Teplova *et al.*, 1992).

The effects observed under exposure of MCh to SFA developed at much higher concentrations (from 4 mmol/l), as compared to FC, and greatly depended on respiratory substrates. With pyruvate as substrate, the time period of oxidative phosphorylation (OP) and the level of NADH oxidation increased linearly at increasing SFA concentration in the medium (Zinchenko *et al.*, 2007). However, with

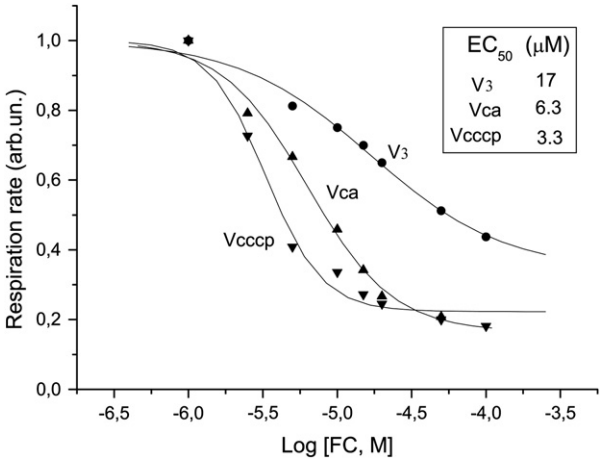

FIGURE 13.2. Effects of FC on respiration of rat liver MCh. Dependence of respiration rate activated by ADP (V_3), calcium transport (V_{Ca}), and protonophore CCCP (Vcccp) upon concentration of FC. Substrates: pyruvate *plus* malate.

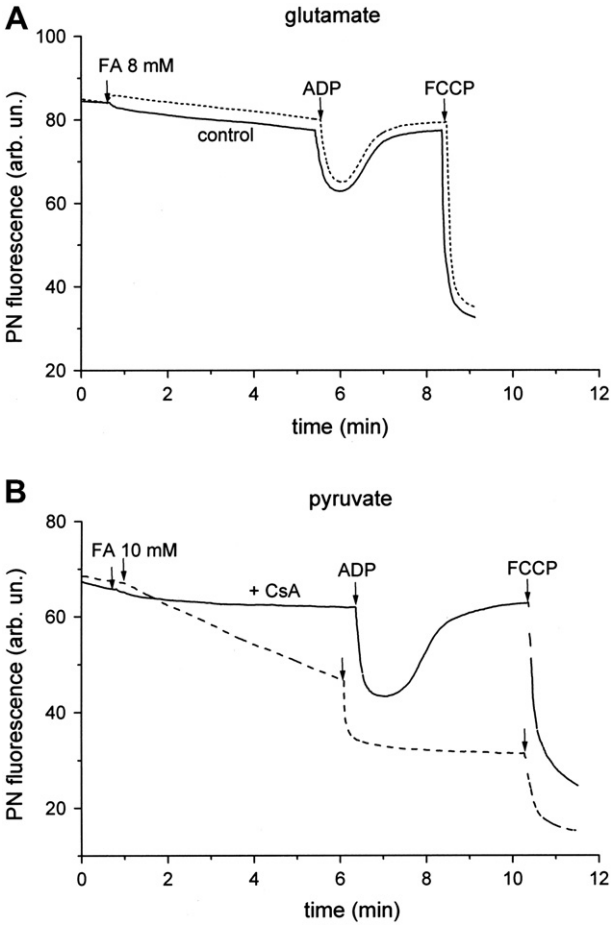

FIGURE 13.3. Effects of FA on redox state of pyridine nucleotides (PN) of rat liver MCh. (A) Glutamate as respiratory substrate. (B) Prevention of PN oxidation and/or leakage by cyclosporin A (CsA) when pyruvate used as respiratory substrate. Additions: (A) SFA 8 mmol/l (dots) or sodium acetate 8 mmol/l (control line), ADP 120 µmol/l, FCCP 1 µmol/l; (B) SFA 10 mmol/l (dots) or SFA 10 mmol/l plus CsA 1 µmol/l (line).

utilization of succinate and especially glutamate, SFA had no effect on OP in concentrations as high as 8 mmol/l (Figure 13.3A) and even 16 mmol/l (not shown here). Moreover, the effect of SFA with pyruvate as respiratory substrate can be prevented by incubation of MCh with cyclosporine A, a known inhibitor of the mitochondrial transition pore (Figure 13.3B). This means that under exposure to FA development of mitoptosis and apoptosis is possible, but opening of the pore is reversible in nature and preventing oxidation or leak of NADH from MCh can turn them back to normal functional state.

2. Effects of Fluoroacetate on Isolated Cells

The effects of FA on the physiological and biochemical status of cells and tissues are tightly dependent upon the level of their oxidative metabolism. Thus, FA does not inhibit phagocytosis because of the low level of TCA cycle

activity within macrophages (Cifarelli *et al.*, 1979). We investigated a series of cell types, transformed lines and those obtained from animals, under exposure to FA or FC. The level of NAD(P)H in Ehrlich ascite tumor (EAT) cells slowly decreased and the level of Ca^{2+} increased when the cells were incubated with SFA (Zinchenko *et al.*, 2007). SFA could induce depletion of intracellular calcium stores and activation of influx of extracellular Ca^{2+} ions through the store-operated calcium (SOC) channels. Discovery of other calcium channels such as TRPV5 and TRPV6 (Hoenderop *et al.*, 2003; van de Graaf *et al.*, 2006), which remain inactivated when $[Ca^{2+}]_i$ is increased and become activated when $[Ca^{2+}]_i$ is decreased, stimulated the investigation of the level of calcium ions in endoplasmic reticulum (ER) with chlortetracycline (CTC). Under exposure to ATP, FC does not affect the velocity of calcium efflux from ER, so the signal transmission from P2Y receptor via G-protein is not inhibited in EAT cells (Zinchenko *et al.*, 2007). However, FC induced a growth in both amplitude of Ca^{2+} leakage and velocity of its influx into ER. A rather long period (8–10 min) of Ca^{2+} influx into ER was observed, which indicated efflux of intracellular Ca^{2+} from cells by plasma membrane Ca-ATPase immediately after mobilization and leaving ER. This greatly reduces $[Ca^{2+}]_i$ for transport back to ER. It was demonstrated earlier (Zinchenko *et al.*, 2001) that the velocity of return transport of Ca^{2+} into ER depends upon activity of plasma membrane SOC channels. Therefore, we suggest that FA (or FC) can induce entry of calcium ions into cells through SOC channels.

The toxic effects of FC on endothelial cells have been shown to be similar to its effects on other energy-dependent tissues: a reduction of ATP level and oxygen consumption but accumulation of lactate and considerable decrease of protein synthesis (Rist *et al.*, 1996). We have demonstrated a gradual decrease of the mitochondrial membrane potential and elevation of $[Ca^{2+}]_i$ under exposure to SFA (unpublished data). Conversely, in cardiomyocytes SFA induced a slow enhancement of the mitochondrial membrane potential together with a rise of basal $[Ca^{2+}]_i$; propagation of calcium waves along the surface of sarcoplasmic reticulum, or visible elevation and velocity of spreading of the pre-existing waves, was also observed (Zinchenko *et al.*, 2007). Probably the increased level of $[Ca^{2+}]_i$ is the reason for its transport into MCh with a subsequent inhibition of the proton ATPase and rise of the membrane potential. Mechanistically, this phenomenon could be explained by the existence of a Ca^{2+}-dependent protein inhibiting H^+-ATPase (Hubbard and McHugh, 1996).

We have also studied the kinetic parameters of platelet aggregation in experiments with rats *in vitro* and *ex vivo* (Mindukshev *et al.*, 2006). Aggregation of platelets was induced with ADP over the concentration range 10–100 nmol/l. The median effective concentrations (EC_{50}) of ADP for the cells exposed to SFA, 10 and 5 mmol/l, were calculated to be 25 nmol/l and 35 nmol/l, correspondingly, and these platelets can be characterized as hypersensitive to ADP. Studying the kinetic parameters of platelet aggregation under intoxication of rats with SFA, we encountered a problem of spontaneous aggregation of the platelet-rich plasma, which was in agreement with the data on primary transition of the platelets to the hypersensitive state. However, the cells that avoided aggregation demonstrated an extremely high level of desensitization. In some experiments, ADP could not induce platelet aggregation at very high (nonphysiological) concentrations near 10 μmol/l.

Under intoxication with SFA a significantly reduced thymus, as well as a prominent quantity reduction of freshly obtained thymocytes and elevation of apoptosis, were revealed (Figure 13.4). SFA also caused an acceleration of apoptosis of control and dexamethazone-treated human lymphocytes *in vitro*, although spontaneous apoptosis of

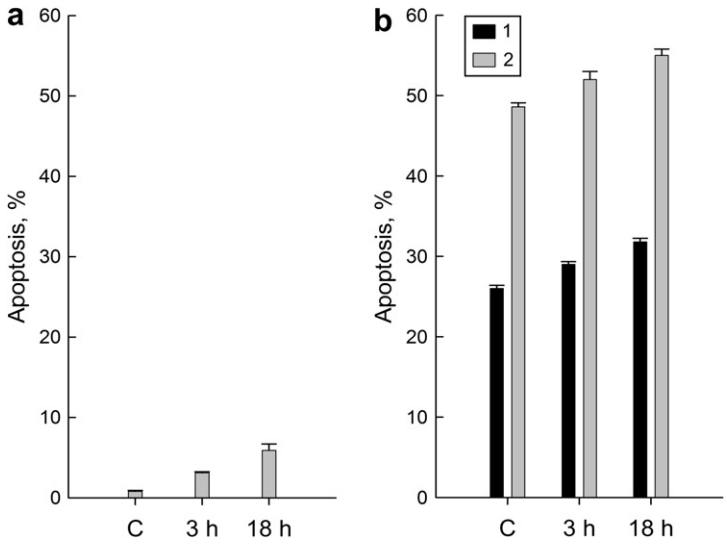

FIGURE 13.4. Effect of SFA on development of apoptosis of rat thymocytes 3 and 18 h after administration of SFA at $1/2LD_{50}$. Registration of apoptosis with Hoechst-33258. (A) Apoptosis in freshly isolated thymocytes; (B) Apoptosis in thymocytes cultivating for 20 h after isolation in the absence (1, black) and presence (2, gray) of dexamethasone.

human neutrophils was inhibited (not shown here). Moreover, SFA practically had no effect on reactive oxygen species (ROS) production by peritoneal macrophages of mice. One can suggest that the inhibitory effect of FA on neutrophil apoptosis is realized through ROS, whereas the enhanced apoptosis and depression of the cells responsible for adaptive immunity is a nonspecific reaction under SFA intoxication, reflecting a general decline and redistribution of energy resources of the organism.

3. Biochemical Parameters Under Intoxication with Fluoroacetate

Among the biochemical effects caused by FA are: accumulation of citrate and disturbance of its transport from MCh; elevation of lactate and disturbances in carbohydrate metabolism; decrease of free fatty acids (FFA) concentration; elevation of adenosine and ammonia; disbalance of bivalent cations and acid–base equilibrium; changes in GABA balance in brain; and rise in phosphates and various enzymes in blood plasma (Buffa and Peters, 1950; Engel *et al.*, 1954; Elliott and Phillips, 1954; Maytnert and Kaji, 1962; Williamson, 1967; Stewart *et al.*, 1970; Bgin *et al.*, 1972; Eanes *et al.*, 1972; Buffa *et al.*, 1973; Liang, 1977; Kirsten *et al.*, 1978; Taitelman *et al.*, 1983a; Bobyleva-Guarriero *et al.*, 1984; Bosakowski and Levin, 1986; Szerb and Redondo, 1993). However, among such a variety of biochemical changes citrate seems to be the only parameter whose qualitative (but not quantitative) trends are not a matter of controversy. In rat hearts under acute intoxication with FA, concentration of citrate can exceed control values 8–15 times (Bosakowski and Levin, 1986). Elevation of citrate concentration is in direct proportion to respiratory activity of a tissue: metabolically active tissues – such as heart, kidneys, and spleen – maximally accumulate citrate, though in liver, which is also characterized by high respiratory level and metabolic activity, a small accumulation of citrate has been observed (Cole *et al.*, 1955; Twigg *et al.*, 1986). In our experiments with rats poisoned with SFA at a dose of $1/2LD_{50}$, concentration of citrate in blood plasma and organs increased within 1 h (Figure 13.5). The most prominent elevation of citrate was revealed 6 h after the poisoning in heart (5×), kidneys (3×), and brain (2.5×). There was doubling of the level of citrate in blood plasma after 1 h, though it was the only biochemical parameter of plasma that remained elevated for 3 days.

Transfer of citrate through the inner membrane of MCh is provided by a tricarboxylate transporter (m.w. 32.5 kD), which also catalyzes transport of *treo*-D_s-isocitrate, *cis*-aconitate and other tricarboxylates (LaNoue and Schoolwerth, 1979; Kaplan *et al.*, 1990). This is electroneutral exchange for either another tricarboxylate or dicarboxylate (e.g. malate or succinate), or for phosphoenolpyruvate. Formation of glutathione-citryl thioester is irreversibly inhibited by (–)erythrofluorocitrate ($IC_{50} = 25$ pmol FC/mg protein), which makes a stable adduct with the synthase (Kun *et al.*, 1977). However, the block of citrate transport

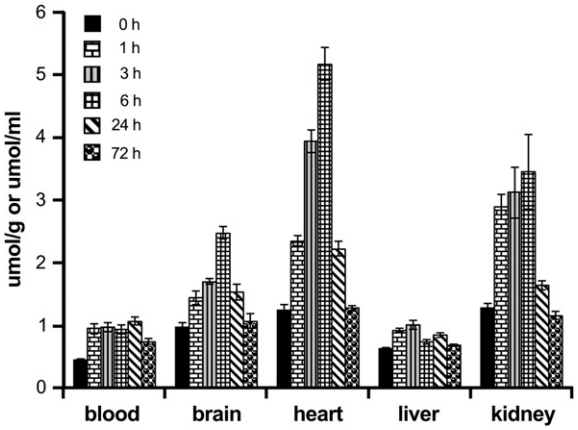

FIGURE 13.5. Concentration of citrate in blood and organs of rats under intoxication with SFA at $1/2LD_{50}$.

is not absolute and universal for all the organs and tissues. There are data on citrate transfer from MCh to cytosol with its subsequent utilization by cytoplasmic aconitase (c-aconitase), which is virtually not affected under FA intoxication, and then by cytoplasmic NADP-dependent isocitrate dehydrogenase (cICDH) (Max and Purvis, 1965). Around 32% of citrate produced in MCh can be transported to cytosol (Buffa *et al.*, 1972). These processes should be regarded as being adaptive and positive, they lead to reduced oxygen consumption because the NADPH generated does not require further oxidation in the respiratory chain and can be utilized in other metabolic pathways.

Among the negative consequences of citrate accumulation is a change of electrolyte composition and acid–base disbalance in the organism. Moreover, elevation of citrate level in cells leads to disturbance of glucose metabolism due to inhibition of the key glycolytic enzyme phosphofructokinase (Bowman, 1964; Peters, 1972). Hyperglycemia during intoxication with FA can be very prominent, in spite of inactivation of gluconeogenesis (Godoy *et al.*, 1968; Bobyleva-Guarriero *et al.*, 1983, 1984). Nevertheless, we could not find significant changes in rat blood glucose level throughout the periods of intoxication with FAA or SFA at a dose of $1/2LD_{50}$; at the same time there was a significant increase of glucose level in liver, heart, and brain (unpublished data). This may signify a utilization of glucose by other tissues and first of all by skeletal muscles, as a result of which the local increase of glucose in organs is not reflected by the level of glucose in blood. Thus, glucose cannot serve as a reliable criterion of intoxication.

Some researchers considered the elevated glucose level to be a result of decreased insulin secretion by pancreatic β-cells due to their damage by FA (Cole *et al.*, 1955; Karam and Grodsky, 1962). Along with hyperglycemia there was hyperketonemia, observed characteristically for the diabetic state, caused by inhibition of TCA cycle and depletion of oxaloacetate (Williamson, 1967; Buffa *et al.*, 1973; Taitelman *et al.*, 1983a). Also consistent with diabetes is

inhibition of hormone-induced lipolysis in adipose tissue (Taylor *et al.*, 1977). Moreover, FA increased glucose conversion to fatty acids, and such coincidence of antilipolytic and lipogenetic effects of FA provides a basis for suggesting a relation in effects of FA and insulin. However, injection of insulin does not alleviate FA intoxication in general and ''FA diabetes'' in particular (Reichelt, 1979). During FA intoxication, the initial hyperglycemia can even be reversed into hypoglycemia (Boquist *et al.*, 1988), so this effect of FA was considered to be an insulin-like phenomenon (Zieve *et al.*, 1983). The principal distinction, however, should be depletion of glycogen stores in different tissues under intoxication with FA (Godoy *et al.*, 1968; Boquist *et al.*, 1988). After poisoning with FA, glycogen levels in animal tissues may decrease by 75% in 1 h and by 90% in 2 h (Buffa *et al.*, 1973; Zhou *et al.*, 1984). According to our data, during SFA intoxication ($1/2LD_{50}$) glycogen levels are maximally decreased after 6 h in both liver (by 55%) and brain (by 40%), and the dynamics of the glycogen levels was similar in these organs. Such a decrease could result from the indirect action of adrenalin or sympathetic regulation (Buffa *et al.*, 1973). In addition, inhibition of *de novo* glycogen synthesis has been reported (Zhou *et al.*, 1984).

Nevertheless, disturbances in hormonal regulation during the FA intoxication can also take place: reduction of calcium concentration in blood plasma could be caused by a poor reabsorption of calcium ions in kidneys due to a decrease of parathyroid hormone level; an excess of Ca^{2+} excretion up to 0.173 mg/min (the control rate being 0.06 mg/ml) has been registered (Perez and Prindt, 1977). Decrease of calcium level could be the reason for the so-called ''hypocalcemic tetanus'' (Roy *et al.*, 1980), manifesting itself as typical convulsions, disturbances of blood clotting, and hypotension leading to vascular attacks. The level of decrease of calcium correlates with extension of the Q-T interval on ECG, which is a consequence of broad spectrum of cardiac arrhythmia (Buffa and Peters, 1950; Arena, 1970).

ATP level is usually reduced, though ADP and AMP levels can be elevated in the first hours of the FA intoxication, with subsequent decrease (Bowman, 1964; Stewart *et al.*, 1970). There are other reports of a constant level of ATP in some organs and tissues. For example, FA did not affect ATP and GTP, as well as cyclic nucleotides and levels in hepatocytes *in vitro* (Dohi and Murad, 1981). When dogs were intoxicated with sublethal doses of FA there was no observable decrease in oxygen consumption and ATP level; this was explained by utilization of glutamate and aspartate which can enter the TCA cycle distally of aconitase (Liang, 1977). The inversion of reactions at glutamate dehydrogenase (GDH) is a simple and effective compensative mechanism during blockade of the TCA cycle in kidney cells: instead of glutamine synthesis, glutamate is deaminated to form 2-oxoglutarate to support the flow of reducing equivalents in the TCA cycle and ATP synthesis, while the ammonia produced neutralizes local tissue acidosis (Yu *et al.*, 1976). Such utilization of glutamate may account for the significant reduction of glutamate level in rat organs, beginning at the first hour after intoxication with FA. The data obtained according to the GC method of Matsumura *et al.* (1996) have shown a decrease of glutamate, aspartate, and some other amino acids in rat brain (Figure 13.6), as well as a decrease of glutamate and nearly complete absence of glutamine in blood plasma of rats and rabbits (not shown here) 3 h after poisoning with SFA. The levels of amino acids in blood plasma of animals indicate the extent of protein breakdown in muscles, on the one hand, and the level of their utilization by other organs and tissues, on the other hand. Under intoxication with FA, glutamate and its precursor glutamine are probably the main nutrients. Elevation of amino acid levels in blood plasma of rats within 3 h after poisoning signifies an elevation of protein breakdown. Furthermore, this indicates that other amino acids – because of their transport, catabolism, etc. – are not nutrients of primary importance under energetic deficit conditions.

Elevation of lactate level in the blood of animals poisoned with FA has been reported (Engel *et al.*, 1954; Taitelman *et al.*, 1983a). In agreement with these workers, we observed a prominent rise in lactate levels in blood just after convulsions (unpublished data). In rat heart and brain, lactate levels decreased under intoxication with SFA or FAA, irrespective of convulsions. During SFA intoxication the decrease in lactate level (and increase of glucose level) in rat heart takes place earlier and to a greater extent than for FAA intoxication: 38% decrease in 3 h for lactate, as compared with 25% in 6 h in the case of FAA; 100% increase in 3 h for glucose, as compared with 67% in 6 h in the case of FAA. Also, the maximal increase of citrate was registered at 24 h after poisoning with FAA, but at 6 h after poisoning with SFA. These and other biochemical data are

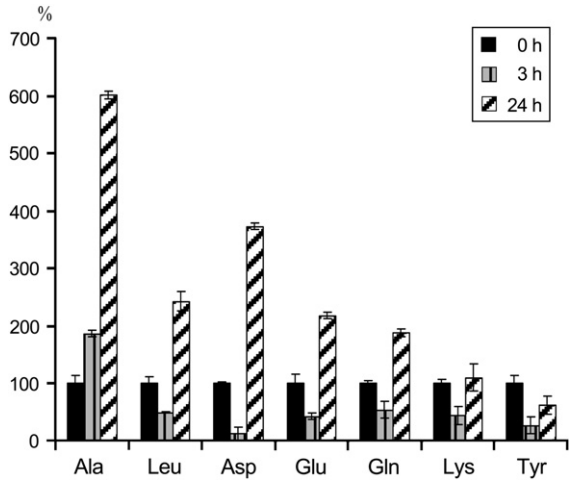

FIGURE 13.6. Changes of some amino acids in rat brain 3 and 24 h after administration of SFA at $1/2LD_{50}$.

consistent with clinical pictures of intoxication with equipotential doses of SFA and FAA: intoxication with SFA is generally more violent and takes a shorter period of time.

4. Effects of FA on the Cells of the Nervous System: Interaction of Glia and Neurons

Acetate is metabolized in astrocytes nearly 18 times faster than in cortical synaptosomes, though activity of acetyl-CoA synthase in synaptosomes is almost double that in astrocytes (5.0 and 2.9 nmol/min per mg of protein, respectively). The principal difference in the acetate metabolism rates is explained by differences in the kinetics of its transport, which is mediated by a monocarboxylate carrier (Hosoi *et al.*, 2004); acetate uptake by astrocytes, unlike synaptosomes, rapidly increases and follows saturation kinetics (V_{max} = 498 nmol/mg protein/min, K_M = 9.3 mmol/l) (Waniewski and Martin, 1998). Having penetrated into astrocytes at one site, FA can diffuse into other cells through gap junctions (Ransom, 1995). Citrate accumulating in astrocytes is readily released from cells and effectively penetrates other astrocytes (Westergaard *et al.*, 1994). The TCA cycle in nerve tissues is blocked by FA but not completely, only by 35–55% (Patel and Koenig, 1968). This leads to decreased consumption of glucose and increased consumption of glutamine (if the latter is available); no reduction of ATP was observed (Hassel *et al.*, 1994). The natural metabolic pathway is switched over to utilization of glutamine, glutamate, and 2-oxoglutarate in the TCA cycle. GDH of astroglia plays a big role in this switching over, promoting the ATP-independent utilization of glutamate (Plaitakis and Zaganas, 2001). The absence of an aspartate/glutamate mitochondrial exchanger (the key component of the malate/aspartate cycle) in astrocytes also plays in support of this (Xu *et al.*, 2007). There is little GDH in neurons as compared to astrocytes, with activity of GDH depending not only upon proximity to glutamatergic fibers and terminals, but also upon activity of neighboring neurons regardless of their functional specialization; a deficiency of GDH activity in astroglia may be a cause of cytotoxic effects of glutamate and aspartate (Aoki *et al.*, 1987).

The taking of glutamate by astrocytes is an electrogenic process in which one molecule of glutamate is cotransported with three sodium ions (or $2Na^+$ and $1H^+$), being exchanged for $1K^+$ and $1OH^-$ or $1HCO_3^-$ (Bouvier *et al.*, 1992). To re-establish the ionic balance, Na^+/K^+-ATPase would work with ATP provided by phosphoglycerate kinase bound to plasma membrane. This stimulates glycolysis and lactate production in astrocytes. Lactate is released from astrocytes and then taken by neurons to be further oxidized. Pyruvate, which is also produced in astrocytes, can be utilized in the TCA cycle to form 2-oxoglutarate or transaminated to form alanine; the latter can also enter neurons (Tsacopoulos and Magistretti, 1996; Tsacopoulos, 2002). However, the rate of alanine metabolism through alanine transaminase (ALT) in synaptosomes is much less than the rate of its uptake; moreover, neuronal ALT and AST work mainly to synthesize alanine and aspartate (Erecinska and Silver, 1990), hence a stable elevation of alanine level, in contrast to that of other amino acids, in brain of rats poisoned with SFA (Figure 13.6). The role of alanine as a source of glutamate is increased during the restoration period after ischemia/hypoxia, when alanine concentration is elevated and glutamate concentration is reduced.

Inhibition of glutamate uptake by astroglial cells can be one of the causes of convulsions observed under intoxication with FA (Szerb and Issekutz, 1987). The toxic effect is governed mainly by citrate, which chelates calcium ions (Fonnum *et al.*, 1997). Intrathecal injection of FC in mice caused convulsions in about 15 s, while in about 37 min by intracerebroventricular injection (Hornfeldt and Larson, 1990). Moreover, intrathecal injection of sodium citrate caused the same effect. This means that the main target of FC and citrate, and the area for generation of convulsions, should be the spinal cord. Convulsions could also be generated by other compounds having the common property of chelating calcium ions; these are EDTA, EGTA, glutamate, and lactate (Hornfeldt and Larson, 1990). Thus, along with elevation of citrate level, activation of anaerobic oxidation of glucose in neurons followed by accumulation of lactate in cerebrospinal fluid could also lead to coma and convulsions (Stewart *et al.*, 1970). Chelation of zinc and other divalent cations by citrate enhances the signaling activity of NMDA receptors (Westergaard *et al.*, 1995). In addition, disturbances of GABA metabolism were revealed as a result of the TCA cycle blockade: after injection of FA, there was initially an elevated level of GABA registered in different regions of the brain, followed by its reduction concurring with the beginning of clonicotonic convulsions (Maytnert and Kaji, 1962; Stewart *et al.*, 1970).

The convulsive state is aggravated by increasing the concentration of ammonia ions (Raable, 1981), an excess of which can lead to redistribution of K^+ and Cl^- ions, disturbances of neuronal depolarization and hyperpolarization, and impairment of post-synaptic inhibition. The neuron dysfunctions observed result in encephalopathy, ataxia, convulsions, and coma (Iles and Jack, 1980; Raable and Lin, 1983, 1984; Xiong and Stringer, 1999). On the other hand, FC affecting astroglia cause a drop in membrane potential and depolarization, and decrease of $[K^+]_i$ (Largo *et al.*, 1997); this should lead to compensatory transport of bicarbonate ions into astrocytes and acidification of the extracellular medium. Together with the natural carbonate acidification of the medium close to chemoceptors of the retrotrapezoid nucleus, this activates the diaphragmal nerve and increases the expired minute ventilation (Erlichman *et al.*, 1998; Holleran *et al.*, 2001): maximum ventilation is attained at 4% CO_2 against 8–10% in control hypercapnic trials. Control of extracellular pH in nervous tissue is coupled with functioning of the Na^+/HCO_3^- cotransporter, existing in plasma membrane of astrocytes but lacking in that of neurons (Deitmer, 1992; Romero and Boron, 1999). This transport has an electrogenic character, because two or

even three bicarbonate ions are transferred per one sodium ion. Again, however, a continuous supply of glutamine to the glutamatergic nerve terminals is the necessary condition of respiratory rhythm generation; blockade of the TCA cycle in astroglial cells with FA can impair the respiratory activity (Hulsmann *et al.*, 2000).

5. Physiology of Blood Vessels Under Intoxication with FA

FA does not affect circulation in resting organs, but a significant increase of blood flow can be seen in working respiratory muscles (Johnson and Reid, 1988). Conversely, a reduction of blood was registered in hepatic artery, and contractive activity of isolated portal veins was suppressed after introduction of FA into the medium (Liang, 1977). These data, along with data on the effects of FA on endothelial cells *in vitro*, suggested that endothelium of blood vessels could be one of the primary targets for FA. If so, the endothelium-dependent relaxation of blood vessels would be affected. To test the hypothesis, we administered SFA to rats subcutaneously at a dose of 2–3 mg/kg (LD_{50}–LD_{84}), and investigated endothelium-dependent relaxation of rat aorta 3 and 24 h after the poisoning. Norepinephrine in saturating concentrations induced a rapid constriction of aorta followed by a smooth transition to plateau; in contrast, vasoconstricting hormones angiotensin II, vasopressin and 5-hydroxytryptamine induced a bell-shaped vasoconstricting response of aorta. To assess the functional state of endothelium, carbocholine was introduced at 10^{-5}mol/l. Acting on muscarinic receptors of endothelial cells, it induced generation of nitric oxide and release of endothelium-derived hyperpolarizing factor (McCulloch *et al.*, 1997). All the agonists applied had similar effects on contraction of aortas obtained from control and poisoned animals (not shown here). The experiment clearly demonstrated that FA has no influence upon the contractile properties of isolated rat aorta at 3 and 24 h after poisoning. This endothelial function is not affected, at least directly, under intoxication with FA.

6. Body Temperature of Rats and Rabbits Under Intoxication with FA

One of the main pathophysiological features of intoxication with FA is decrease of the body core temperature of endotherms, which indicates a disturbance of heat production and/or regulation (Brockmann *et al.*, 1955; Taitelman *et al.*, 1983b; Misustova *et al.*, 1980). It is interesting to note that the effects of FC are comparable with those of selective inhibitors of p38 MAP-kinases (activation of which precedes production of pyrogens) and antagonists of cytokines TNF, IL-1, and IL-6 (Milligan *et al.*, 2001, 2003). In our experiments, following administration of lethal doses of SFA to male rats, a marked decrease of rectal temperature was registered beginning from 1 h after the poisoning and gaining minimal levels in 6 or 24 h depending on doses. Then a gradual increase in temperature took place in surviving rats, returning to normal in 2–7 days. For example, under intoxication with SFA at a dose LD_{50}, a minimal rectal temperature of the surviving male rats (31.5°C as compared to 38.5°C in control animals) was registered in 6 h, and 7 days after the poisoning the temperature was 1°C below the control level. Under intoxication with FAA at equipotential doses, a minimal rectal temperature of the surviving male rats (32.6°C as compared to 38.9°C in control animals) was registered in 2 days, and even 7 days after the poisoning the temperature was 3°C below control level. But we observed fewer changes of the rectal temperature in rabbits after s.c. administration of SFA at a dose LD_{50}: maximal decrease was only 1°C (38°C as compared to 39.1°C) in 6 h. According to our observations, decrease of the temperature below 38°C in 3 h can serve as a reliable sign forecasting the lethal outcome of the poisoned rabbits.

7. Electrophysiological Studies of FA Intoxication

Clinical analysis of ECG of rats poisoned with SFA or FAA (Kuznetsov *et al.*, 2007) revealed a similar dynamic of the temporal parameters of ECG, with slowing down and delay of the repolarization processes being the most important (Table 13.1). A drop in amplitudes of the atrial and ventricular ECG complexes can be observed within an hour after poisoning, followed by decrease of the systolic index in 24 h thus indicating an impairment of the contractile capacity of myocardium. Registration of ECG of rats which died in 2 days of intoxication with SFA revealed a sharp drop of heart rate (down to 120–180 per min) 24 h after poisoning, together with complete absence of the P wave which reflects atrial depolarization (Figure 13.7A). Simultaneous reduction of both amplitude and duration of the T wave can be seen. An upward shift of the ST segment, though not accompanied by growth of the T wave amplitude, was registered in 70% of rats (Figure 13.7B). The cumulative evidence of the shape and amplitude changes of the ECG waves indicates a development of acute myocardial ischemia, though a transient one and maximally expressed 24 h after poisoning. Reduction of the S wave amplitude could be caused by disturbances of excitation processes in basal ventricular regions and in some areas of the right vetricle. Taking into account an increase in duration and shape distortions of the ventricular complex, one cannot exclude an incomplete right bundle-branch block. A significant extension of the T wave during the course of examination is indicative of deceleration of the fast repolarization of myocardium, though the process of slow repolarization (the QT interval in ECG, corresponding to the systole of ventricles) is accelerated within 3–24 h after poisoning.

Respiratory rhythm was gradually increased in rats under intoxication with SFA, and there were additional respiratory components in 50% of animals 24 h after administration of the poison (Figure 13.8) that may indicate disturbances of innervation of respiratory muscles. Spectral analysis of the

TABLE 13.1. Parameters of ECG (averaged cardiocycle) of adult rats in normal state and different terms after introduction of SFA at 1/2LD$_{50}$

	Parameters										
	Amplitude (mV)				Duration (s)						
Terms	**P**	**R**	**S**	**T**	**P**	**T**	**PQ**	**QRS**	**QT**	**RR**	**SI**
Background	0.297 ± 0.019	0.973 ± 0.131	0.723 ± 0.137	0.747 ± 0.071	0.018 ± 0.001	0.036 ± 0.001	0.050 ± 0.001	0.019 ± 0.001	0.057 ± 0.001	0.140 ± 0.003	40.7
1 h	0.144 ± 0.016***	0.669 ± 0.083	0.235 ± 0.088*	0.469 ± 0.089*	0.018 ± 0.001	0.049 ± 0.002***	0.050 ± 0.001	0.022 ± 0.002	0.070 ± 0.002***	0.139 ± 0,005	50.4
3 h	0.170 ± 0.017***	0.826 ± 0.066	0.161 ± 0.059**	0.518 ± 0.083	0.021 ± 0.001*	0.044 ± 0.002**	0.057 ± 0.004	0.022 ± 0.001*	0.067 ± 0.002***	0.182 ± 0.011**	36.8
1 day	0.124 ± 0.030***	1.153 ± 0.171	0.089 ± 0.050**	0.538 ± 0.143	0.019 ± 0.001	0.051 ± 0.002***	0.048 ± 0.002	0.024 ± 0.002*	0.075 ± 0.004***	0.237 ± 0.036*	31.7
3 days	0.195 ± 0.023**	1.309 ± 0.122	0.102 ± 0.052**	0.403 ± 0.102**	0.019 ± 0.004	0.046 ± 0.003**	0.052 ± 0.004	0.025 ± 0.001**	0.071 ± 0.003***	0.158 ± 0.005**	44.9
7 days	0.167 ± 0.012***	1.106 ± 0.113	0.450 ± 0.088	0.458 ± 0.041**	0.018 ± 0.001	0.062 ± 0.003***	0.052 ± 0.002	0.025 ± 0.001**	0.088 ± 0.003***	0.171 ± 0.007***	51.5

*$p < 0.05$
**$p < 0.01$
***$p < 0.001$
SI – systolic index, calculated after formula SI = (QRST*100)/RR

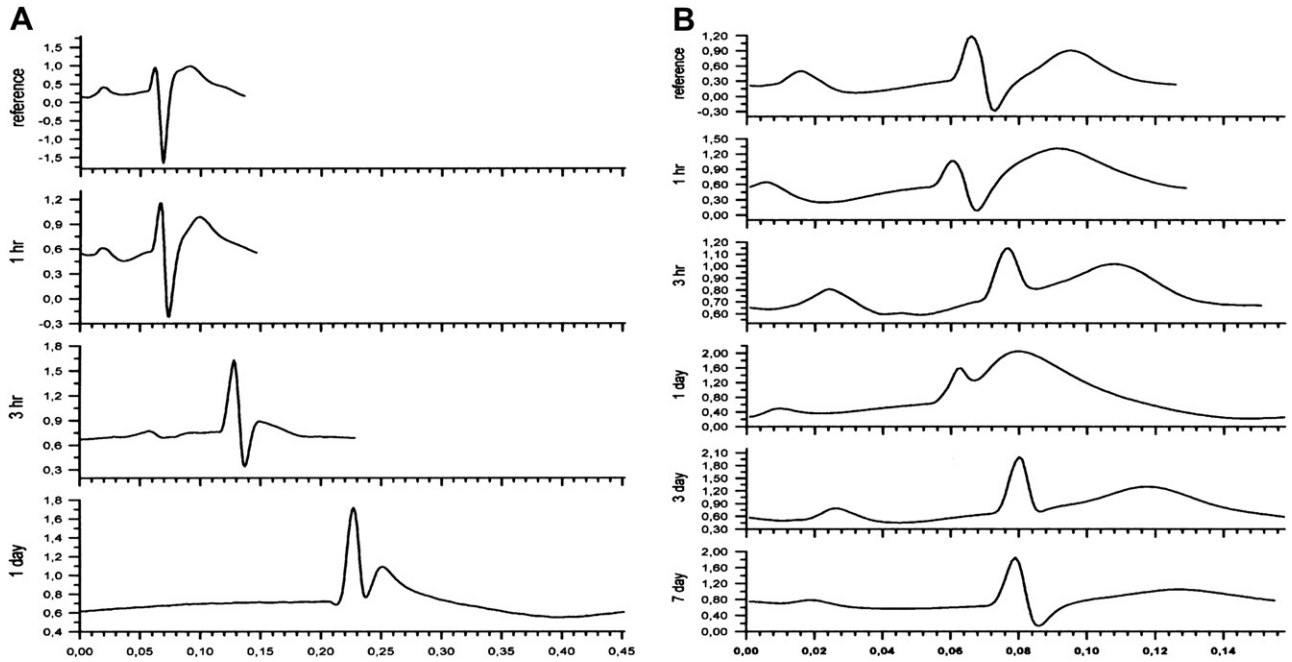

FIGURE 13.7. (A) ECG (averaged cardiocycle) of rat that died nearly 2 days after introduction of SFA at LD_{50}. (B) ECG (averaged cardiocycle) of rat that survived after introduction of SFA at LD_{50}. Along the *x*-axis – time (s), along the *y*-axis – amplitude (mV).

respiratory curve demonstrated that there was an enhanced synchronization of the respiratory rhythm observed within 3 h after poisoning. Simultaneously, the amplitude of respiration increased followed by a gradual decrease to the third day. Over the same period, a certain reduction of lability of respiratory rhythm was noted, accompanied by the appearance of two distinct peaks corresponding with frequencies of 90 and 120 cycles of respiration per minute. By the seventh day, the respiratory spectrogram was similar to the initial one, though the frequency of respiration was not completely restored. Comparison of spectrograms of respiration and ECG demonstrates disturbances of control mechanisms underlying generation of the second-order waves (respiratory arrhythmia visible at the spectrogram as a peak in the high-frequency region 0.8–2.5 Hz). One day after administration of SFA, there was a marked frequency

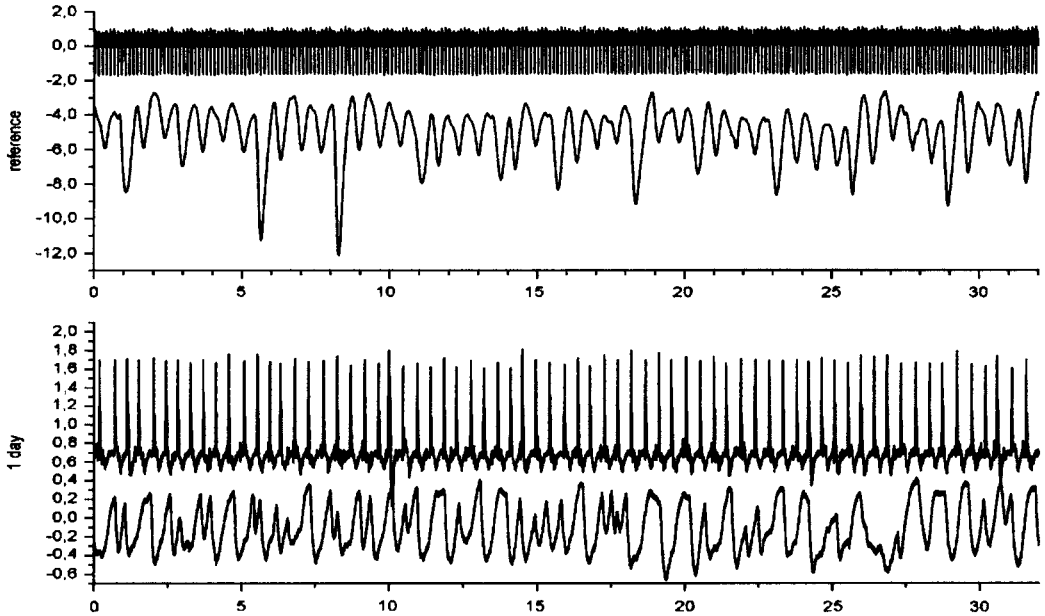

FIGURE 13.8. Records of ECG (upper) and respiratory rhythm (lower) from a narcotized rat before and 24 h after introduction of SFA.

maximum at the respiratory spectrum, in contrast to that of the ECG spectrogram.

Analysis of the heart rate variability (HRV) demonstrates that 1 h after poisoning an enhancement of parasympathetic influence took place, and this was accompanied by insignificant and paradoxical enhancement of heart and respiratory rates (Table 13.2). Then against a background of enhancement of humoral (metabolic) and sympathetic influences and simultaneous decline of parasympathetic influence, a stable decrease of heart and respiratory rates took place indicating a prominent divergence between vagosympathetic balance and resulting physiological parameters. Previously it was shown in experiments with dogs that systemic, pulmonary, and coronary hemodynamic parameters during the first hours after introduction of FA were not mediated by the autonomic nervous system and adrenergic neuromediators (Liang, 1977). This is in partial agreement with our results obtained with rats, though this cannot be extrapolated to all the periods of intoxication and all animal species.

V. TOXICITY AND RISK ASSESSMENT

A characteristic feature of the clinical picture of intoxication with FA is a latent period of 0.5 to 6 h (Egekeze and Oehme, 1979). The duration of the latent period depends on animal species' metabolism and dose administered (Chenoweth, 1949; Goncharov *et al.*, 2006). A broad variability of clinical manifestations of FA effects in different animal species is one of its characteristic features. There is a correlation between food specificity and toxic effect of FA; the cardiovascular system is mainly affected in herbivores, while the CNS is mainly affected in carnivores. According to this, four groups were recognized in terms of clinical signs of intoxication (Chenoweth and Gilman, 1946). The first comprised herbivores (rabbits, goats, sheep, cattle, and horses), in which FA induced ventricular fibrillation without notable CNS disorders (Marais, 1944; Chenoweth, 1949; Egekeze and Oehme, 1979). The second group comprised dogs and guinea pigs, in which the CNS was primarily affected. In dogs, a species highly sensitive to FA, symptoms of secondary intoxication appear after a latent period of 1 to 10 h (Chenoweth and Gilman, 1946; Egyed and Shupe, 1971). For animals of the third group the clinical pattern of intoxication is similar to that of the second group of animals, but slightly less pronounced. This group comprised rats and hamsters relatively tolerant to FA. After a latent period lasting 1–2 h, tremor and elevated excitability were common symptoms. Death usually occurred within 4–6 h as a result of respiratory depression, after exposure to high FA doses (Chenoweth and Gilman, 1946; Pattison, 1959). The surviving animals demonstrated depression, weakness, ataxia, and strongly pronounced bradycardia down to 30 heartbeats per minute. At sublethal doses of FA, a full recovery can occur in 72 h after poisoning (Chenoweth and Gilman, 1946; Pattison, 1959). A mixed response to FA exposure was described in animals of the fourth group – cats, pigs, and rhesus monkeys; it included disturbances of both CNS and cardiovascular system. On acute poisoning, adynamia, salivation, vomiting, frequent defecation, pupil dilatation, nystagmus, accelerated respiration, enhanced excitability, tremor, and clonicotonic convulsions were observed in these animals (Chenoweth and Gilman, 1946; Gammie, 1980).

This classification has been revised recently (Sherley 2004). The division of animals into cardiac and neurological symptomatic groups is considered to be unnatural as it ignores common neurological signs manifested in all the groups: among these are tremor, ataxia, hypersensitivity, myotonic convulsions, weakness, and partial paralysis. The cardiac response in a pure form was not a common event and was described just for a limited number of animals, though CNS involvement is obviously widespread.

As for humans, exposure to stock solution during formulation and dermal or respiratory exposure during application of baits, as well as accidental or intentional acute intoxications, are the main human health concerns. Formulators and pest control workers are the largest occupational risk group (Norris, 2001). The clinical picture of acute intoxication of humans is similar to that of rhesus monkeys, and among the symptoms are nausea, vomiting, abdominal pains, salivation, irrational fear, weakness, tachypnoe, cyanosis, and sometimes sweating and increased temperature (Brockmann *et al.*, 1955; Pattison, 1959; Arena, 1970; Taitelman *et al.*, 1983b). Psychomotor agitation and sometimes a loss of spatiotemporal feeling can occur. In addition, tremor, nystagmus, involuntary dejection and urination, muscle spasms, hypertonus of the extremities, and even alalia, have been reported (Gajdusek and Lutheer, 1950; Harrison *et al.*, 1952; Robinson *et al.*, 2002). The most characteristic signs of intoxication involve generalized recurrent convulsions alternating with deep depression. Sudden loss of consciousness and coma may occur. These symptoms were associated with metabolic acidosis and hypotension (Pattison, 1959; Chi *et al.*, 1996, 1999), as well as cardiac rhythm disturbances, such as tachycardia, bradycardia, asystolia, and sustained ventricular fibrillations (Gajdusek and Lutheer, 1950; Reigart *et al.*, 1975; Trabes *et al.*, 1983). Death usually occurs in 3 h to 5 days of heart block, arrhythmia, or respiratory failure (Reigart *et al.*, 1975; Montoya and Lopez, 1983). Important diagnostic symptoms registered with ECG are arrhythmia, the QT and ST intervals, and the T wave (Pattison, 1959; Taitelman *et al.*, 1983b; Chi *et al.*, 1996). Kidneys are among the most sensitive organs: acute renal failure associated with uremia and increased level of creatinine in serum can be observed under acute FA poisoning (Chung, 1984; Chi *et al.*, 1996). Pathomorphological abnormalities of humans poisoned with FA are also nonspecific and similar to those of animals. In the case of lethal outcome, petechial hemorrhages and excess blood filling of internal organs (Hayes, 1982), edema

TABLE 13.2. Analysis of heart rate variability of adult rats in time and frequency domains under intoxication with SFA at $1/2LD_{50}$

	Period of examination											
	Control		1 h		3 h		24 h		3 days		7 days	
Parameters	**Value**	**Shift %**	**Value**	**Shift %**	**Value**	**Shift %**	**Value**	**Shift %**	**Value**	**Shift %**	**Value**	**Shift %**
Heart rate, contr/min	424.6 ± 6.1	–	441.6 ± 9.4	+4.0	340.1 ± 11.4***	−20.0	291.8 ± 18.5***	−31.3	370.6 ± 6.0***	−12.7	341.0 ± 6.0***	−19.7
Coefficient of arrhythmia, rel.un.	0.049 ± 0.003	–	0.058 ± 0.005	+18.4	0.156 ± 0.042*	+218.4	0.245 ± 0.073*	+400	0.053 ± 0.003	+8.2	0.051 ± 0.006	+4.0
Value of VLF (ms^2)	0.041	8.8	0.053	7.6	0.087	11.8	0.060	9.7	0.043	10.1	0.044	10.9
Value of LF (ms^2)	0.075	16.1	0.107	15.2	0.128	17.3	0.114	18.4	0.073	17.1	0.079	19.6
Value of HF (ms^2)	0.350	75.1	0.542	77.2	0.523	70.9	0.445	71.9	0.311	72.8	0.280	69.5
Value of To (ms^2)	0.466	–	0.702	+50.6	0.738	+58.4	0.619	+32.8	0.427	−8.4	0.403	−13.5
LF/HF, rel.un.	0.214	–	0.197	−7.9	0.245	+14.5	0.256	+19.6	0.235	+9.8	0.282	+31.8
HF/To, rel.un.	0.751	–	0.772	+3.8	0.709	−5.6	0.719	−4.3	0.728	−3.1	0.695	−7.5
Respiration rate/min	94.1 ± 3.0	–	101.9 ± 3.1	+8.3	94.4 ± 3.2	+0.3	98.8 ± 3.7	+5.0	111.9 ± 6.1*	+18.9	120.2 ± 8.1**	+27.7

Coefficient of arrhythmia = $(RR_{max}-RR_{min})/RR_{mean}$

Values of shifts (%) for **VLF**, **LF**, and **HF** indices are given against **To** index of corresponding period of examination. For other parameters the shift was calculated against the initial control value

of lungs and brain, and sometimes mediastenal emphysema and acute inflammatory reaction with coagulating necrosis in esophagus were registered in humans (Brockmann *et al.*, 1955). The morphological basis of cardiotoxic effects is acute myocardial dystrophy, a characteristic of which is diffuse lesions of cardiac muscle (Pattison, 1959; Taitelman *et al.*, 1983b). Acute renal failure develops due to the influence of FA on subcellular structures of kidneys. Metabolic acidosis aggravates the clinical course of renal failure. Diffuse degeneration of renal tubules was observed (Hayes, 1982). For cases that lack clinical and morphological specificity, biochemical data and primarily citrate and fluoride levels can be used for diagnostic purposes (Pattison, 1959; Schultz *et al.*, 1982). Thus under acute intoxication with FAA, citrate (108 μg/g in heart and 23.9 μg/g in kidney) and fluoride (6.3 mg/g dry weight of heart and kidney) were found in human corpse; the dose of FAA was estimated to be near 23 mg/kg (Hayes, 1975). In addition, the indubitable diagnostic confirmation of the intoxication should be based on determination of the poison in tissues. Under acute SFA poisoning with lethal outcome, FA was found in urine (368 μg/ml), liver (58 μg/g), and brain (76 μg/g) (Harrison *et al.*, 1952).

Among the after-effects that develop after acute intoxication with FA are various neurological disturbances: impaired muscular tonus and reflex activity, and transient spasmodic and meningeal syndromes. Long after an acute poisoning (from 1.5 to 9 years) a tendency for epileptoid seizures, ataxia, extremity muscular hypertension, spastic tetraplegia, blindness of cortical origin, diffuse brain atrophy, and psychic disorders were observed (Pridmore, 1978; Trabes *et al.*, 1983). A case of chronic intoxication with FA of a farm worker has been described (Parkin *et al.*, 1977): the clinical signs were renal insufficiency and less pronounced injuries of other organs.

VI. TREATMENT

Decades of studies on the toxicology of FA have led scientists to the conclusion that treatment of intoxications can be successful only if timely general and symptomatic therapy is applied, but not specific antidotes (Dorman, 1990; Norris, 2001). Much experimental work over an extensive period has been undertaken in an effort to find effective donors of acetate groups, because of their ability to inhibit conversion of FA to FC. Ethanol, monoacetin (glycerol monoacetate), acetamide, and cortisone acetate were tested for their potency to serve as antidotes (Hutchens *et al.*, 1949; Chenoweth, 1949; Cole *et al.*, 1955; Giller, 1956; Egyed, 1971; Egyed and Shlosberg, 1977). Therapeutic effect was revealed for simultaneous introduction of ethanol and acetate (Hutchens *et al.*, 1949; Tourtelotte and Coon, 1949). Negative effects of monoacetin and acetamide were enhancement of hyperglycemia and metabolic acidosis, damage to capillaries and hemolysis of red blood cells, and increase of citrate concentration in different organs (Engel *et al.*, 1954; Egyed and Shlosberg, 1973). Administration of cortisone acetate inhibited the FC synthesis and prevented development of ketosis, though increased hyperglycemia (Cole *et al.*, 1955).

Several antidotes were tested for their capacity to activate transport of the TCA cycle intermediates through mitochondrial membranes. For this purpose fluoromalate was proposed, though any positive result was negligible (Peters *et al.*, 1972). Malate was also tested, but proved to be effective only in *in vitro* experiments (Buffa *et al.*, 1972). Also *in vitro*, glutathione and a series of SH-containing compounds (cysteamine and *N*-acetylcysteine) were tested (Mead *et al.*, 1985). However, they were incapable of replacing glutathione in enzymatic defluorination of FA and have not found practical application. TCA cycle intermediates (succinate, malate, citrate, and glutamate) were tested, but did not exhibit protective effect (Hutchens *et al.*, 1949). A positive result was observed in experiments with mice, which were administered calcium gluconate and succinate (Omara and Sisodia, 1990). This therapy was hardly more effective than ethanol. Some 16 years later another research group tested the therapy with cats, which are known to be much more sensitive to FA. Again, differences in survival between treated and nontreated animals were not significant ($p > 0.05$) (Collicchio-Zuanaze *et al.*, 2006). Administration of calcium chloride to cats under acute intoxication with FA made it possible to postpone their death by up to 166 min combination of calcium chloride with monoacetin gave a similar effect (Taitelman *et al.*, 1983a). Nevertheless, calcium chloride caused reduction of the QT interval and favored survival of humans in case of their intoxication with FAA (Taitelman *et al.*, 1983b).

Our strategy for development of therapeutic means of treating acute FA intoxication was based on a deep analysis of the biochemical literature, together with our own experimental data. Thus, a high sensitivity of aconitase to inhibition by superoxide anion and nitric oxide (Gardner *et al.*, 1994; Andersson *et al.*, 1998; Castro *et al.*, 1998) means that ROS and NO could be competitive antagonists of FC to avert its effect on aconitase. Also, as considered earlier, during FA intoxication glutamate could be utilized in the TCA cycle through GDH or transaminases (Yu *et al.*, 1976; Liang, 1977; Hassel *et al.*, 1994). Moreover, the effects of FC could be prevented by prior introduction of isocitrate (bypass of inhibited aconitase) and fructose-1,6-bisphosphate (energy substrate for neurons) (Lian and Stringer, 2004).

We have demonstrated that FA can adversely affect mitochondrial functions only if pyruvate was available as respiratory substrate, and that changes of redox-state of pyridine nucleotides (PN) or their leakage from MCh could be critical factors that impair mitochondrial respiration and lead to cell death (Zinchenko *et al.*, 2007). Opening of the mitochondrial pore is a reversible phenomenon: prevention of oxidation and/or leakage of NADPH from MCh can

restore the normal functional state of MCh. For example, when succinate or glutamate was used as a respiratory substrate, mitochondrial functions were not affected by FA (Figure 13.3A).

As for other alternative substrates, we suggest that the accumulating intracellular citrate could be one of them. As discussed earlier, blockade of citrate transport from MCh under FA intoxication is not an obligatory event, and citrate can enter cytosol to be further utilized by cICDH (Max and Purvis, 1965; Buffa *et al.*, 1972). The cICDH activity is almost equally distributed between cytosol and MCh of astroglia and microglia, whereas cICDH accounts for about 75% of activity in neurons and oligodendrocytes (Minich *et al.*, 2003). We have not found data on the ratio of mitochondrial and cytoplasmic aconitases in cells of the nervous system, but it is interesting to note that a similar ratio of m- and cICDH exists in hepatocytes (Rakhmanova and Popova, 2006), and that c-aconitase accounts for 65% of the aconitase in these cells (Konstantinova and Russanov, 1996). In rat heart a similar ratio of m- and c-aconitases has been revealed: 35 and 65%, correspondingly (Medvedeva *et al.*, 2002). Based on these data, one may suggest that an effective pathway for citrate utilization and NADPH synthesis exists in these (and other) cells in case of inhibition of m-aconitase. This alternative pathway could play a positive physiological role because NADPH might be used for anabolic reactions and heat generation, glutathione reduction and NO synthesis, and regulation of blood vessel tone by means of ROS generation (Winkler *et al.*, 1986; Bobyleva *et al.*, 1993; Lee and Yu, 2002; Gupte and Wolin, 2006). As was pointed out earlier, studies focused on the pentose cycle as the main source of NADPH need to be re-evaluated taking into consideration the metabolic activity and substrate specificity of a tissue (Winkler *et al.*, 1986). cICDH along with malic enzyme and transhydrogenase participates in NADPH regeneration to further reduce glutathione in brain mitochondria (Vogel *et al.*, 1999), but cICDH can provide a seven-fold greater generation of NADPH as compared to malic enzyme (Winkler *et al.*, 1986). The level of cytoplasmic NADPH can influence potassium channels and calcium balance (Wolin *et al.*, 2005; Gupte and Wolin, 2006). In our *in vitro* studies, FA induced a slow elevation of $[Ca^{2+}]_i$ in different cells (Zinchenko *et al.*, 2007). This could indicate an activation of the SOC channels; the process is not affected by FA and does need ATP to be implemented, at least in glial cells (Lian and Stringer, 2004). We suppose this mechanism to be common for many types of cells, and this could explain a primary hypersensitivity of platelets exposed to FA (Mindukshev *et al.*, 2006). In cardiomyocytes, elevated $[Ca^{2+}]_i$ can stimulate their functional activity observed in our experiments *in vitro* and also supported *in vivo* by a primary increase of systolic index (Table 13.1). As for modulating effects of Ca^{2+} on bioenergetics of MCh, it is pertinent to recall ''classic'' activation of the TCA cycle dehydrogenases followed by increase of mitochondrial potential and NADH generation: 2-oxoglutarate dehydrogenase (OGDH) and mICDH can be activated by calcium ions through allosteric mechanisms and pyruvate dehydrogenase is activated due to dephosphorylation by the Ca^{2+}-dependent phosphatase (McCormack *et al.*, 1990; Hansford, 1994). The exact role of these dehydrogenases in the bioenergetic status of MCh affected by FA needs to be clarified, though one can suppose that OGDH could derive a special benefit from such an activation if it is provided with exogenic or endogenic 2-oxoglutarate.

TABLE 13.3. Assessment of therapeutic effectiveness of METIS preparations under acute intoxication of rats with SFA

Therapy	Index of therapeutic efficiency: Ratio LD_{50} treated/LD_{50} nontreated
Ethanol, $n = 42$	**1.6**
METIS-1, $n = 48$	**2.5**
METIS-2, $n = 92$	**3.3**
METIS-4, $n = 39$	**4.3**

n – number of animals used in experiment to calculate the index

According to the above discussion, we have defined several directions for biochemical correction under acute intoxication with FA and suggested suitable preparations for therapeutic complexes: (1) competitive inhibition of FA and CoA interaction; (2) competitive inhibition of FC and aconitase interaction; (3) replenishment of the TCA cycle distally of aconitase; (4) utilization of accumulating citrate. In a previous publication we presented the first data on effectiveness of a therapeutic complex named METIS (Goncharov *et al.*, 2006). Further experiments proved the validitity of the therapeutic approach, and we have now appreciably enhanced the efficacy of the complex (Table 13.3). In addition to these data on the index of therapeutic effect, a spectrum of physiological and biochemical data was obtained. Animals treated with METIS complex had little changes of body weight, temperature, and oxygen consumption. Dynamics of citrate in brain, kidneys, and blood was also improved, and kinetic parameters of platelet aggregation were corrected. Comparative analysis of the FA level in tissue homogenates, blood plasma, and urea of rats revealed that the METIS complexes reduced the level of FA in brain almost two-fold, thus indicating inhibition of FA utilization first of all in the cells of the nervous system.

VII. CONCLUDING REMARKS AND FUTURE DIRECTION

The extreme toxicity of FA is determined by its similarity to acetate, which has a central role in cell metabolism. FA enzymatically condenses with CoA-SH to produce

fluoroacetyl-CoA, which replaces acetyl-CoA entering the TCA cycle and produces FC. The latter reacts with aconitase and blocks the TCA cycle. Energy production is reduced, as well as concentration of metabolites generated distally to aconitase. 2-Oxoglutarate is the most important of them, being a precursor of glutamate, which is a neuromediator in the CNS and participates in neutralizing ammonia either directly through glutamine synthase or indirectly through the urea cycle. Accumulation of citrate is one of the causes of metabolic acidosis. Chelating of Ca^{2+} is apparently one of the central events in pathogenesis of intoxication.

The first papers on toxicology of FA were published in the 1940s. The long history of investigations was fruitful, with several important discoveries: biochemical mechanism of ''lethal synthesis''; structure of aconitase; functional relations of glia and neurons; and switching of metabolic pathways. However, the main problem of toxicology (for any poison) was not solved – development of an effective therapy. Analysis of the scientific literature has demonstrated that reciprocal relations of signaling and metabolic pathways under intoxication with FA are unclear. Inhibition of m-aconitase causes blockade of TCA cycle, reduction of pyridine nucleotides, accumulation of citrate, disturbances of intracellular signaling, deenergization, and cell death. However, the dynamics and significance of these events are different depending of the type of cells and tissues, which is why it is very difficult to predict the primary reaction of different cells and more so the whole organism.

Biochemical pathways underlie the basis of physiological rhythms; they should have a certain space–time structure and presuppose coordinated interactions of different cells. Thus, one of the causes of disturbances of normal respiration under FA intoxication could be disturbances in rhythmic activity of respiratory neurons; but suppression of these neurons is a consequence of the inhibiting effect of FA on astrocytes, not neurons (Hulsmann *et al.*, 2000). We described development of cardiac and respiratory tachyarrhythmias reflecting reproduction of decasecond rhythms characteristic for immature or abnormal excitatory structures (Kuznetsov *et al.*, 2007). Previously, it was suggested that such endogenic rhythmic activity could be determined by the level of the pentose cycle activity (Kuznetsov, 1999, 2002). This cycle indeed plays an important role in neurons, protecting them from oxidative or traumatic stress (Ben-Yoseph *et al.*, 1994; García-Nogales *et al.*, 2003; Bartnik *et al.*, 2005). However, it should be noted that although the activity of NADPH-generating enzymes of the pentose cycle in astrocytes (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) is 2–3 times higher than in brain as a whole, the activity of cICDH is also very high in these cells (Rust *et al.*, 1991). This metabolic pathway is interesting primarily because under FA intoxication citrate is accumulated, and the pathway may be regarded as a form of biochemical adaptation that facilitates utilization of the central metabolite. Modern data suggest that spatial and temporal division of m- and c-aconitases not only provides regulation of iron balance in cells, but actually provides regulation of balance between catabolic and anabolic processes (Tong and Rouault, 2007).

Providing the cells have utilized citrate entering cytosol, another problem should be utilization of generating NADPH. One possible and very important mechanism of PN oxidation is heat generation through shiver and nonshivering thermogenesis. Rise in activity of NADPH-generating enzymes and pathways, including c-aconitase and cICDH, is accompanied by enhanced thermogenesis (Bobyleva *et al.*, 1993). It was shown that NADPH could be used together with or even instead of NADH as a reducing cofactor for cytoplasmic glycerophosphate dehydrogenase (Bobyleva *et al.*, 1993; Fahien *et al.*, 1999). But if the role of this pathway for transferring electrons from cytoplasma to MCh in skeletal muscles is rather clear, then the level of activity and functional state of glycerophosphate shuttle in brain cells are contradictory and serve as a subject for discussion. Activity of glycerophosphate shuttle in brain is explained by the need for glycerol-3-phosphate as a substrate for phospholipid synthesis in oligodendroglia (Adler and Klucznik, 1982; Nguyen *et al.*, 2003). In neurons and astrocytes, the activity of glycerophosphate dehydrogenases is much lower than in oligodendrocytes (Rust *et al.*, 1991; Nguyen *et al.*, 2003). There are data, however, that indicate an important role of this shuttle in astrocytes, taking into consideration (1) the absence of malate–aspartate shuttle in these cells (Waagepetersen *et al.*, 2001; McKenna *et al.*, 2006), and (2) the elevated level of mRNA of cICDH in astrocytes after convulsions, under exposure to morphine, indometacine, and some other preparations (Link *et al.*, 2000). In conclusion, we suggest that future progress in toxicological studies of FA and development of effective therapy will depend on comprehensive consideration of these and other modern data, together with reevaluation of old and forgotten data.

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CHAPTER 14

Strychnine

JIRI PATOCKA

I. INTRODUCTION

Strychnine is a poisonous alkaloid of indole type found in the genus *Strychnos*. It is a basic compound which forms colorless or white rhombic crystals. The compound has a bitter taste and melts at around 290°C. Strychnine was the first alkaloid to be identified in plants of the genus *Strychnos*, Family Loganiaceae. *Strychnos*, created by Linnaeus in 1753, is a genus of trees and climbing shrubs of the gentian order. The genus contains 196 various species and is distributed throughout the warm regions of Asia (58 species), America (64 species), and Africa (75 species). Plants of the genus *Strychnos* have opposite leaves and bear cymes of white or yellowish flowers that have a four-lobed or five-lobed calyx, a four-parted or five-parted corolla, five stamens, a solitary pistil and bear fruit in the form of a berry. The seeds and bark of many plants in this genus contain the powerful poison strychnine. Strychnine is obtained commercially from the seeds of the Saint-Ignatius' bean (*Strychnos ignatii*) and from the nux-vomica tree (*Strychnos nux-vomica*) (Volfova and Patocka, 2003). Strychnine was first discovered by French chemists Joseph-Bienaime Caenoiu and Pierre-Joseph Pelletier in 1818 in the Saint-Ignatius' bean. In some *Strychnos* plants a 9,10-dimethoxy derivative of strychnine, alkaloid brucine, is also present (Li *et al.*, 2006). Brucine is not as poisonous as strychnine.

II. BACKGROUND

A. Chemistry and Physicochemical Properties

Strychnine has a molecular formula of $C_{21}H_{22}N_2O_2$ and its structural formula is shown in Figure 14.1. CAS No: 57-24-9 (base), 60-41-3 (sulfate). It occurs as white crystals or powder, odorless, with a melting point of 286°C, boiling point 270°C at 5 mm Hg, density 1.36 g/cm^3, vapor density 11.0 (air = 1) and vapor pressure 0 torr at 20°C (Mackison *et al.*, 1981). Strychnine is a stable compound and incompatible only with strong oxidizing agents.

Water solubility of strychnine is negligible, only 160 mg/l at 25°C; the pH of saturated solution is 9.5. The octanol/water partition coefficient (logK_{ow}) is 1.93 (Hansch *et al.*, 1995). Strychnine is very slightly soluble in ether, slightly soluble in benzene, ethanol (6.7 g/cm^3), and acetone, and soluble in chloroform and pyridine (Budavari, 1996).

B. History

The toxic and medicinal effects of strychnine have been well known from the times of ancient China and India. The inhabitants of these countries had ancestral knowledge of the species nux vomica and Saint-Ignatius' bean. The species *Strychnos nux-vomica* is a tree native to Indonesia which attains a height of 12 m. The tree has a crooked, short, thick trunk and the wood is close grained and very durable. The fruit is an orange color about the size of a large apple with a hard rind and contains five seeds, which are covered with a soft wool-like substance. The ripe seeds look like flattened disks, which are very hard. These seeds are the chief commercial source of strychnine and were first imported to and marketed in Europe as a poison to kill rodents and small predators. *Strychnos ignatii* is a woody climbing shrub of the Philippines. The fruit of the plant contains as many as 25 seeds embedded in the pulp. The seeds contain more strychnine than other commercial alkaloids. The properties of nux-vomica and Saint-Ignatius seeds are substantially those of the alkaloid strychnine.

Strychnine was discovered and identified as the main toxic principle of *Strychnos* in 1818, although *nux vomica*, the unpurified plant extract in which it is the active component, had been known and used for both medicinal and criminal purposes for some time. Historic records indicate that the strychnine alkaloid had been used to kill dogs, cats, and birds in Europe as far back as 1640. The structure of strychnine was first determined in 1946 by Sir Robert Robinson and in 1954 this alkaloid was synthesized in a laboratory by Robert W. Woodward. This is one of the most famous syntheses in the history of organic chemistry. Both chemists won the Nobel prize (Robinson in 1947 and Woodward in 1965).

C. Therapeutic Purpose

Strychnine does have a history of use for therapeutic purposes, although in most cases this was entirely misguided, and not a little dangerous. It has a very bitter taste and so

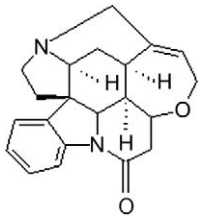

FIGURE 14.1. Chemical structure of strychnine.

stimulates salivary and gastric secretion. This increases appetite, and was used to counteract the loss of appetite associated with illness, giving the impression that strychnine had restorative properties. In fact, any constitutional improvement resulting from the increased appetite would probably be outweighed by the harmful effects of strychnine, and the underlying illness would be more or less unaffected. This is the same mechanism that gives tonic water its apparent ''tonic activity'', although in that case the bitter agent is quinine, which is much less dangerous.

Strychnine has an undeserved reputation as a useful therapeutic agent. There is no current justification for its presence in any medication. However, preliminary experimental reports suggest that judicious treatment with strychnine may modify the neurological deterioration in some infants with nonketonic hyperglycinemia, a rare metabolic disorder characterized by abnormally high concentrations of glycine in the brain and cerebrospinal fluid. Strychnine was used as a tonic stimulant for atonic constipation and as a stomachic and bitter. It produces no selective gastrointestinal effects and has no place in the therapy of any gastrointestinal disorder. At present, strychnine has no demonstrated therapeutic value in human medicine, despite a long history of unwarranted popularity. It was first employed in medicine in 1540, but did not gain wide usage until 200 years later (Gilman *et al.*, 1985).

Although strychnine was earlier used extensively in animals, it no longer has a rational place in the therapeutic armamentarium of the veterinarian. Its only interest is from a veterinary toxicology standpoint because animals are sometimes poisoned inadvertently or intentionally (Booth and McDonald, 1982).

III. PHARMACOKINETICS AND TOXICOKINETICS

A. Absorption, Distribution, Metabolism and Excretion

Strychnine is rapidly absorbed from the gastrointestinal tract, mucous membranes, and parenteral sites of injection (Thienes and Haley, 1972) and also from the oral cavity (LaDu *et al.*, 1971). A nonfatal case of strychnine poisoning through dermal exposure is also described (Greene and Meatherall, 2001). Strychnine is transported by plasma and erythrocytes, but protein binding is slight and distribution to the tissues rapidly occurs. Within a few minutes of ingestion, strychnine can be detected in the urine, and excretion by that route accounts for about 15% of sublethal (4 mg) dose over 6 h. Little difference was noted between oral and intramuscular administration of strychnine with a 4 mg dose. Blood levels in human poisoning were less than 0.5 μg/ml from 1 to 48 h following ingestion a sublethal dose (700 mg), 2.7 μg/ml in a patient who survived the acute episode, and 40 μg/ml in a patient who died following a massive ingestion (Haddad and Winchester, 1983). In persons killed by strychnine, the highest concentrations are found in the blood, liver, and kidney (Hayes and Laws, 1991).

The half-life of absorption is about 15 min and the half-life of metabolism is about 10 h. The apparent volume of distribution in one patient was 13 l/kg (Ellenhoorn *et al.*, 1997).

Strychnine is rapidly metabolized by the liver microsomal enzyme system requiring NADPH and O_2. Five metabolites formed *in vitro* by rabbit liver were isolated and identified as 2-hydroxystrychnine, 11,12-dehydrostrychnine, strychnine-21, 22-epoxide, 21,22-dihydroxy-22-hydrostrychnine, and strychnine-N-oxide which was the major metabolite and accounted for approximately 15% of the metabolized strychnine. All other metabolites accounted for less than 1% (Mishima *et al.*, 1985). Similar metabolites were identified in rat urine where the major metabolite was strychnine-21,22-epoxide (Oguri *et al.*, 1989). The metabolic fate of strychnine in humans is unknown.

B. Toxicokinetics

Strychnine competes with the inhibitory neurotransmitter glycine producing an excitatory state characterized clinically by hyperreflexia, severe muscle spasms, and convulsions. However, the toxicokinetics after overdose have not been well described. In most severe cases of strychnine poisoning, the patient dies before reaching the hospital (Shadnia *et al.*, 2004).

Palatnick *et al.* (1997) described a case of a 34-year-old male presented to the emergency department 20 min after ingesting 125 ml of 2% strychnine sulfate (2.25 g). He was alert and oriented and experiencing muscle spasms. His condition deteriorated prompting sedation, muscle paralysis, and tracheal intubation. He was given activated charcoal 100 g per nasogastric tube. He was admitted to intensive care where he was managed with diazepam, pentobarbital, and pancuronium. Despite mild rhabdomyolysis, he recovered and was extubated on day three. Although receiving prophylactic heparin therapy, a massive fatal pulmonary embolus ensued. Eighteen blood specimens for strychnine analysis were obtained from 20 min to 51 h after ingestion. Serum concentrations were determined with gas chromatography–mass spectroscopy. Disappearance followed a first-order process with a $t_{1/2}$ of 16 h. Results confirmed the findings of an earlier case report of

19 strychnine levels obtained between 4 and 19 h which described first-order kinetics with a $t_{1/2}$ of 10–16 h (Palatnick *et al.*, 1997).

In a case report of Wood *et al.* (2002) a 42-year-old man ingested an unknown quantity of strychnine powder. Eight serum samples were taken over the first 5 days and analyzed subsequently for strychnine concentrations. The initial concentration at 1.5 h after ingestion was 4.73 mg/l, falling to 0.38 mg/l at 74 h post-ingestion. Serum concentrations followed a monoexponential elimination curve with a calculated elimination half-life of 12 h. The initial serum concentration of 4.73 mg/l is the highest reported concentration in a patient who has survived.

IV. MECHANISM OF ACTION

Strychnine acts as a blocker or antagonist at the inhibitory or strychnine-sensitive glycine receptor, a ligand-gated chloride channel in the spinal cord and the brain (Song *et al.*, 2006). The glycine receptor is the receptor for the amino acid neurotransmitter glycine (Rajendra *et al.*, 1997). It is one of the most widely distributed inhibitory receptors in the central nervous system. Strychnine-sensitive glycine receptor is a member of a family of ligand-gated ion channels. This ionotropic receptor can be activated by a range of simple amino acids, except glycine, β-alanine and taurine, and can be selectively blocked by the high-affinity competitive antagonist strychnine. The receptor is arranged as five subunits surrounding a central pore, with each subunit composed of four α helical transmembrane segments. There are presently four known isoforms of the α subunit (α_{1-4}) of GlyR that are essential to bind ligands and a single β subunit. The adult form of the glycine receptor is the heteromeric $\alpha_1\beta$ receptor, which is believed to have a stoichiometry of three α_1 subunits and two β subunits or four α_1 subunits and one β subunit (Kuhse *et al.*, 1993, 1995). The strychnine-binding subunit of the glycine receptor shows certain homology with nicotinic acetylcholine receptors (Grenningloh *et al.*, 1987). The glycine receptor is highly enriched in microdomains of the post-synaptic neuronal surface apposed to glycinergic afferent endings. There is substantial evidence suggesting that the selective clustering of glycine receptors at these sites is mediated by the cytoplasmic protein gephyrin (Meier *et al.*, 2000).

V. TOXICITY

Strychnine has been placed in Toxicity Category I, indicating a high degree of acute toxicity, for oral and ocular effects. Inhalation toxicity is also presumed to be high. Acute toxicity of strychnine to birds is very high. Mammalian studies indicate that strychnine is very highly toxic to small mammals on both an acute oral basis and a dietary basis. The extent of poisoning caused by strychnine depends on the amount and route of strychnine exposure, time of exposure, and in humans the person's condition of health. The signs of toxicity, including death, usually occur within 1 h.

A. Animal Toxicity

Reported toxic doses of strychnine by different routes of adinistration in some animals and humans are summarized in Table 14.1.

Strychnine toxicity in rats is dependent on sex. It is more toxic to females than to males when administered s.c.or i.p. and differences are due to higher rates of metabolism by male rat liver microsomes (Parke, 1968). Dogs and cats are more susceptible among the domestic animals, pigs are believed to be as susceptible as dogs, and horses are able to tolerate relatively larger amounts of strychnine (Humphreys, 1988). Birds affected by strychnine poisoning exhibit feathers fluffed or held tightly against the body, ataxia, wing droop, salivation, tremors, muscle tenseness, and convulsions. Death occurs as a result of respiratory arrest.

The clinical signs of strychnine poisoning relate to its effects on the central nervous system. The first clinical signs of poisoning include nervousness, restlessness, twitching of the muscles, and stiffness of the neck. As the poisoning progresses, the muscular twitching becomes more pronounced and convulsions suddenly appear in all the skeletal muscles. The limbs are extended and the neck is curved to opisthotonus. The pupils are widely dilated. As death approaches, the convulsions follow one another with increased rapidity, severity, and duration. Death results from asphyxia due to prolonged paralysis of the respiratory muscles (Humphreys, 1988). Following the ingestion (swallowing) of strychnine, symptoms of poisoning usually appear within 15 to 60 min.

B. Human Toxicity

People exposed to low or moderate doses of strychnine by any route will have the following signs or symptoms: agitation, aprehension or fear, ability to be easily startled, restlessness, painful muscle spasms possibly leading to fever and to kidney and liver injury, uncontrollable arching of the neck and back, rigid arms and legs, jaw tightness, muscle pain and soreness, difficult breathing, dark urine, and initial consciousness and awareness of symptoms. The reported medium lethal doses of strychnine in humans range from 5 to 120 mg/kg.

There are some cases of human poisoning. A 46-year-old man presented 2 h after ingestion of about 250 mg strychnine eperienced severe violent, generalized convulsions, triggered by external stimuli. During the convulsion-free periods, there were no abnormal signs in the physical examination (Scheffold *et al.*, 2004). A 28-year-old man was admitted 2 h after ingestion of 1 to 1.5 g of strychnine. He was severely agitated and in mild respiratory distress; his blood pressure was 90/60 mm Hg, pulse 110/min, and

TABLE 14.1. Toxicity values (LD_{50}) of strychnine in humans and animals

Organism	Route	LD_{50} (mg/kg)	Source
Bird – wild	Oral	16	Tucker and Haegele (1971)
Cat	Intravenous	0.33	RTECS (1935)
Cat	Oral	0.5	Moraillon and Pinoult (1978)
Dog	Intravenous	0.8	Longo *et al.* (1959)
Dog	Subcutaneous	0.35	RTECS (1935)
Dog	Oral	0.5	Moraillon and Pinoult (1978)
Duck	Oral	3.0	Tucker and Haegele (1971)
Human	Oral	100–120	Zenz *et al.* (1994)
Human (adult)	Oral	30–100	Gossel and Bricker (1994)
Human (children)	Oral	15	Gossel and Bricker (1994)
Human	Oral	30–60	Lewis (1996)
Human	Oral	5–10	Ellenhorn *et al.* (1997)
Human (adult)	Oral	50–100	Migliaccio *et al.* (1990)
Human	Oral	100–120	Palatnick *et al.* (1997)
Mouse	Intraperitoneal	0.98	Setnikar *et al.* (1960)
Mouse	Intravenous	0.41	Haas (1960)
Mouse	Oral	2.0	Prasad *et al.* (1981)
Mouse	Parenteral	1.06	Zapata-Ortiz *et al.* (1961)
Mouse	Subcutaneous	0.47	Sandberg and Kristianson (1970)
Pigeon	Oral	21.0	Tucker and Haegele (1971)
Quail	Oral	23.0	Tucker and Haegele (1971)
Rabbit	Intravenous	0.4	Longo *et al.* (1959)
Rabbit	Oral	0.6	RTECS (1935)
Rat	Oral	16.0	Spector (1956)
Rat	Oral	2.35	Ward and Crabtree (1942)

RTECS – Registry of Toxic Effects of Chemical Substances

peripheral pulses weak. He had generalized hyperactive reflexes and had several generalized tonic–clonic convulsions in the emergency department. Treatment consisted of gastric lavage with water, oral administration of activated charcoal and sorbitol solution, continuous intravenous administration of midazolam, and then sodium thiopental, furosemide, sodium bicarbonate and hemodialysis for acute renal failure. His clinical course included respiratory distress, agitation, generalized tonic–clonic convulsions, hyperactivity, oliguria, and acute tubular necrosis prior to recovery in 23 days. This patient ingested what would normally be a fatal amount of strychnine. He had signs and symptoms of severe toxicity but recovered, suggesting that with aggressive supportive care patients may have favorable outcomes (Shadnia *et al.*, 2004). In another case report described by Wood *et al.* (2002), a 42-year-old man presented shortly after ingestion of an unknown substance had in fact taken a lethal quantity of strychnine powder. After a respiratory arrest, with intensive supportive management requiring admission to an intensive care unit, he survived.

People exposed to high doses of strychnine may have the following signs and symptoms within the first 15 to 30 min of exposure: respiratory failure (inability to breathe), possibly leading to death or brain death; no postmortem lesions are observed with the exception of small pinpoint hemorrhages in the lungs resulting from death due to asphyxia; and rigor mortis occurs shortly after death and persists for days.

Toxicity of strychnine in humans, expressed as LDLo (lethal dose low), is approximately 30 mg/kg. It is evident that strychnine is less toxic in humans than in most other animals. If the person survives the toxic effects of strychnine poisoning, long-term health effects are unlikely. However, long-term effects may result from damage caused by the poisoning, for example brain damage from low oxygen or kidney failure. People severely affected by strychnine poisoning are not likely to survive.

1. Clinical Symptomatology

The symptomatology of human intoxication beginning 15 to 30 min after ingestion of strychnine, usually without any warning, may cause the subject to fall into violent convulsions. Convulsions lead to severe lactic acidosis which secondarily result in visceral (lung, heart, kidney,

liver, and brain) collapse and death (Gordon and Richards, 1979). Prodromal symptoms are described such as apprehension, restlessness, heightened acuity of hearing, vision and feeling, hyperreflexia, abrupt movement, and muscular stiffness of face and legs. Generalized convulsions last from 30 s to 2 min. Ten to 20 minutes after exposure, the body's muscles begin to spasm, starting with the head and neck. At first, convulsions are clonic, but a tetanic phase quickly intervenes. The body typically arches in hyperextension, the legs are adducted and extended, the arms are flexed over the chest, and the fists are tightly clenched. The jaw is rigidly clamped, the face has a fixed grin and the eyes protrude in a fixed stare (Philippe *et al.*, 2004).

The convulsions progress, increasing in intensity and frequency until the backbone arches continually. Death comes from asphyxiation caused by paralysis of the neural pathways that control breathing, or by exhaustion from the convulsions. The subject usually dies within 2–3 h after exposure. At the point of death, the body ''freezes'' immediately, even in the middle of a convulsion, resulting in instantaneous rigor mortis.

C. Diagnosis

A tentative diagnosis can be made based on clinical signs and history. However, a positive diagnosis can only be made by identifying strychnine in the stomach contents, viscera, or blood. The drug can be identified by chemical tests and microscopic identification of typical strychnine crystals.

VI. RISK ASSESSMENT

A. Human Health Hazard

The human health assessment for strychnine is based on the acute toxicity. Strychnine has been placed in Toxicity Category I, indicating the greatest degree of acute toxicity, for oral and ocular effects. It has been reported that the probable lethal oral dose is 1.5 to 2 mg/kg (Gosselin *et al.*, 1984). Inhalation toxicity is also presumed to be high. An oral dose of 1.5 to 2 mg/kg is equivalent to 70 to 93 mg/m^3 exposure for 30 min for a 70 kg human being.

Strychnine was first registered as a pesticide in the USA in 1947; however, this natural toxin had been used in many countries to control vertebrate animals for many years prior to 1947. Currently, strychnine is registered for use only below ground as a bait application to control pocket gophers. The end-use products are formulated as a grain-based bait or a paste. Baiting can be done manually, or with the use of application equipment.

The European Union (EU) withdrawal of strychnine marks its end as a method of mole control. The EU Directive 91/414/EEC is midway through an ambitious program to review all pesticides used within Member States. This requires manufacturers to provide health and safety data to support the continued registration of their products. Strychnine was to be reviewed in the fourth part of this program but manufacturers have failed to provide such data. Despite last ditch appeals by users, from September 1, 2006 it was no longer legal to use.

B. Safety Data

The strychnine oral reference dose (RfD) of 0.0003 mg/kg/day or 0.02 mg/day for a 70 kg person is derived from the Seidl and Zbinden (1982) short-term to subchronic study by applying an uncertainty factor of 10,000. This factor accounts for extrapolation from a less than chronic to a chronic exposure study, extrapolation from animals to humans, and differences in sensitivity among the human population. An additional factor of 10 is used because an LOAEL/FEL (2.5 mg/kg/day) was utilized in the estimation of the RfD instead of an NOAEL. The immediately dangerous to life and health (IDLH) dose for strychnine by NIOSH REL is 0.15 mg/m^3 and the current OSHA PEL is 0.15 mg/m^3.

The work of Seidl and Zbinden (1982) is the only oral short-term or subchronic study reported, in which rats received daily doses of 0–10 mg/kg of strychnine by gavage for 28 days. Data recorded for the surviving animals included blood cell counts, electrocardiograms, eye examinations, urine chemistry, weight gain, tissue histology, organ weights, behavioral tests, and food and water consumption. Mortality was observed in five out of 12 male rats receiving 10 mg/kg, one in 12 in each of the 5 mg and 2.5 mg/kg groups. All deaths occurred 0.5–6 h after oral doses.

Additional studies (Gitzelmann *et al.*, 1978) reported that a 6-month-old human patient received strychnine doses of 0.3–1.1 mg/kg/day over an 18-month period without any adverse effects. However, the patient may have had a higher strychnine tolerance as a result of nonketotic hyperglycinemia.

The risk phrases of strychnine are R26, R27, and R28.

VII. TREATMENT

There is no specific antidote for strychnine but recovery from strychnine exposure is possible with early hospital treatment. Treatment consists of removing the drug from the body (decontamination) and getting supportive medical care in a hospital setting. Supportive care includes intravenous fluids, medications for convulsions and spasms, and cooling measures for high temperature.

Treatment of strychnine poisoning involves an oral administration of an activated charcoal which absorbs any unabsorbed poison within the digestive tract. Unabsorbed strychnine can be removed from the stomach by gastric lavage with tannic acid (strong tea) or potassium permanganate solutions to oxidize strychnine. Seizures are controlled by anticonvulsants, such as phenobarbital or diazepam, along

with muscle relaxants such as dantrolene to combat muscle rigidity. Because diazepam, as the anticonvulsant of choice, is not effective in all cases, a combination with midazolam, fentanyl, or pancuronium is recommended in controlling the convulsions (Scheffold *et al.*, 2004). The fatal outcome of strychnine poisoning demands an aggressive management with early intubation, control of muscle tremors, and prevention of rhabdomyolysis and renal failure. If the patient survives past 24 h, recovery is probable.

Small doses of strychnine were once used in medications as a stimulant, a laxative, and as a treatment for other stomach ailments. Strychnine has stimulant effects at low doses but because of its high toxicity and tendency to cause convulsions the use of strychnine in medicine was eventually abandoned once safer alternatives became available.

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

Strychnine is a highly poisonous natural substance that is used in some countries for the control of wild animals. Today, strychnine is used primarily as a pesticide, particularly to kill rats. Its use is restricted by law. Because strychnine is highly toxic and can be rapidly absorbed through the mucous membranes of the mouth, stomach, and small intestines, theoretically it may be used as a military toxicant or terroristic agent. There are three main ways that strychnine can enter the body: inhalation, ingestion, and through broken skin.

Goal-directed misuse of strychnine against humans is unlikely; its misuse against domestic animals, however, is realistic and more likely.

Uncommonly, strychnine is found mixed with ''street'' drugs such as LSD, heroin, and cocaine. It is very likely that seizures observed occasionally after cocaine application may be caused by admixed strychnine (Haddad and Winchester, 1983; Wijesekera *et al.*, 1988). As a result of analysis of heroin samples seized in the Florence area between 1975 and the first half of 1981 no dangerous substances were found and strychnine, if present, was found only in very low concentrations (Mari *et al.*, 1982).

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CHAPTER 15

Superwarfarins

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I. INTRODUCTION

Chemical warfare agents may be manufactured from a wide range of commercially manufactured household industrial products such as bleach, antifreeze, fertilizers containing anhydrous ammonia, pesticides, or anticoagulant rodenticides particularly superwarfarins, to name a few (WHO, 2008). Some chemical warfare agents, e.g. nerve agents, are capable of harming or killing a large number of people when dispersed in air. On the other hand, superwarfarins may be used to harm or terrorize people through the ingestion of contaminated food or water.

Superwarfarins are the ''second-generation anticoagulant rodenticides''. They are referred to as ''superwarfarins'' in the modern medical literature (Pavlu *et al.*, 2005; Sharma and Bentley, 2005; Dolin *et al.*, 2006, POISINDEX, 2007). Superwarfarins are a group of commercially available long-acting anticoagulant rodenticides that are structurally similar to warfarin but are many times more potent and many have the capacity to cause severe bleeding problems that may last for 2 to 8 months in humans (Ellenhorn *et al.*, 1997; Goldfrank *et al.*, 2002).

This group of long-acting anticoagulants may be used as chemical warfare agents because of their high potency and duration of action. The capability of terrorists to use these commercially available poisons is dependent upon the availability of large amounts of high concentration product whether bought or stolen, the target population and its vulnerability, and a method of effective delivery and dissemination (EPA, 2005; WHO, 2008). Although these anticoagulants may be absorbed through the skin and lungs, the main route of exposure is ingestion of food or water containing the product (Jones *et al.*, 1984; Katona and Wason, 1989; Swigar *et al.*, 1990; Wallace *et al.*, 1990; Exner *et al.*, 1992; Rauch *et al.*, 1994; Gallo, 1998; Corke, 1997). The United Nations report from 1969 defines chemical warfare agents as ''chemical substances, whether gaseous, liquid or solid, which might be employed because of their direct toxic effects on man, animals and plants …''. Superwarfarin poisoning may result in a number of casualties if these substances are ingested (Baker *et al.*, 2002; EPA, 2005; Palmer *et al.*, 1999; POISINDEX, 2007; HSDB, 2008).

Superwarfarin rodenticides are used to kill urban and agricultural rodent pests. They are readily available to the general public and pest controllers, are easy to obtain and conceal, so may pose a risk of being used as chemical warfare agents (EPA, 2003, 2005; WHO, 2003, 2008).

These rodenticides are available as meal bait packs, pellets, mini pellets, blocks, mini blocks, wax blocks, liquid bait formulations, tracking powder, and concentrate formulations (POISINDEX, 2007; WHO, 2008; Wilton, 1991).

II. BACKGROUND

Anticoagulants were discovered in the early 20th century after livestock had eaten moldy sweet clover contaminated with bis-hydroxycoumarin and died of hemorrhagic disease. Newer long-acting warfarin derivatives such as brodifacoum, bromadiolone, diphenadione, chlorophacinone, and a few more can produce profound and prolonged anticoagulation and bleeding after a latency period that is generally 24 to 72 h (Chong *et al.*, 1986; Greeff *et al.*, 1987; Swigar *et al.*, 1990; Wallace *et al.*, 1990; Routh *et al.*, 1991; Wilton, 1991; Exner *et al.*, 1992; Rauch *et al.*, 1994; Hui *et al.*, 1996; Tecimer and Yam, 1997; Gallo, 1998; Chua and Friedenberg, 1998; Gill and Redfern, 1980; FDA, 1985; Smolinske *et al.*, 1989; IPCS, 1995a–e; EPA, 2003, 2005).

In the 1940s, a small British pharmaceutical company suggested that dicoumarol might have rodenticidal properties. Trials carried out by Armour and Barnett (1950) confirmed the idea and started the era of anticoagulant rodenticides. Warfarin was the first anticoagulant rodenticide introduced into the market shortly after World War II and became widely used in many countries. Other anticoagulant compounds with potency similar to that of warfarin were also synthesized. These early anticoagulant rodenticides have often been called ''first-generation anticoagulant rodenticides''. These first-generation compounds generally have moderate toxicity, with acute LD_{50} values ranging from 10 to 50 mg/kg body weight (Table 15.2).

The first-generation compounds often needed continuous bait exposure for rodent control. Many rodent species developed a resistance to warfarin (Jackson *et al.*, 1975)

TABLE 15.1. Common commercial products containing superwarfarins

Name	Molecular formula	Commercial names
Brodifacoum CAS: 56073-10-0	C_{31}-H_{23}-Br-O_3	D-Con Mouse-Prufe I & II®, Havoc®, Klerat®, Ratak Plus®, Talon G®, Void® Finale, Folgorat, Matikus, Mouser, Rodend, Volak, Volid
Difenacoum CAS: 56073-07-5	C_{31}-H_{24}-O_3	Compo®, Diphenacoum®, Frunax DS®, Matrak®, Neosorexa®, Rastop®, Ratak®, Ratrick®, Silo®
Bromadiolone CAS: 28772-56-7	C_{30}-H_{23}-Br-O_4	Apobas®; Bromard®; Bromone®, Bromatrol®; Bromorat®; Contrac®; Deadline®; Hurex®; Lanirat®; Maki®; Morfaron®; Musal®; Maki®, Ramortal®; Ratimon®; Rodine-c®; Slaymore®; Super-caid®; Toidon®
Diphacinone CAS: 82-66-6	C_{23}-H_{16}-O_3	Diphacine®, Ditrac®, Gold Crest®, Kill-Ko®, P.C.Q.®, Promar®, Ramik®, Rat Killer®, Rodent Cake®, and Tomcat®
Chlorophacinone CAS: 3691-35-8	C_{23}-H_{15}-Cl-O_3	Caid®, Liphadione®, Microsul®, Ramucide®, Ratomet®, Raviac®, Rozol®, Topidox®
Difethialone[a] CAS: 104653-34-1	C_{31}-H_{23}-Br-O_2-S	None to report
Pindone[a] CAS: 83-26-1	C_{14}-H_{14}-O_3	Pestanal®, Pindone®, Pival®, Pivalyn®, Pivalyl Valone®, Tri-ban®
Coumatetralyl[a] CAS: 5836-29-3	C_{19}-H_{16}-O_3	Racumin®
Coumafuryl[a] CAS: 117-52-2	C_{17}-H_{14}-O_5	Fumarin®, Tomarin®
Valone CAS: 83-28-3	C_{14}-H_{14}-O_3	None to report
Flocoumafen[a] CAS: 90035-08-8	C_{33}-H_{25}-F_3-O_4	None to report

Available forms include: meal bait packs, pellets, mini pellets, blocks, mini blocks, wax blocks, liquid bait formulations, and tracking powder
[a]No longer produced or used in the USA

presumably due to continued exposure and widespread use. Consequently, new chemical structures were synthesized and used as anticoagulant rodenticides. These newer compounds are generally more toxic than warfarin with acute LD_{50}s of 0.2–3.9 mg/kg body weight. For example, a bait concentration of only 50 ppm of brodifacoum is adequate to give control in a single feeding for most rodents and noncommensal species (Matolesy *et al.*, 1988). These newer compounds were called ''second-generation anticoagulant rodenticides'' and are often now referred to as ''superwarfarins'' in the contemporary medical literature (Chong *et al.*,1986; Greeff *et al.*, 1987; Swigar *et al.*, 1990; Wallace *et al.*, 1990; Routh *et al.*, 1991; Wilton, 1991; Exner, 1992; Rauch *et al.*, 1994; Hui *et al.*, 1996; Tecimer and Yam, 1997; Gallo, 1998; Chua and Friedenberg, 1998; Pavlu *et al.*, 2005; Sharma and Bentley, 2005; Dolin *et al.*, 2006).

During the past 30 years there have been more than 600 articles published in the medical literature relating to the clinical assessment, laboratory testing, and treatment of patients exposed to superwarfarins. A great number of these articles are related to children under 6 years old, who accidentally ingested small amounts of these products and, in most cases, did not experience adverse effects (AAP, 2003, Brands *et al.*, 1995, Ingels *et al.*, 2002; Osterhoudt and Henretig, 2003). Also, there are a few cases describing severe bleeding or bleeding-related complications from patients who intentionally ingested large amounts, as well as a few fatal ingestion cases (AAPCC, 2006; Casner, 1998; Walker and Beach, 2002; Wallace *et al.*, 1990). Most of the

TABLE 15.2. The oral LD_{50} values (mg/kg body wt) of some anticoagulant rodenticides

Animals	Bromadiolone	Brodifacoum	Difenacoum
Rat (acute)	0.65	0.27	1.8
Rat (chronic)	(0.06–0.14) × 5	(0.05–0.08)	0.15 × 5
Mouse	0.99	0.4	0.8
Rabbit	1.0	0.2	2.0
Pig	3.0	10.0	80.0
Dog	10.0	3.5	50.0
Cat	25.0	25.0	100.0
Chicken	5.0	10.0–20.0	50.0
Guinea pig	2.8	–	–
Opossum	–	0.17	–
Sheep	–	10.0	100.0

health hazards are associated with accidental ingestion of superwarfarins, and the risk for dermal and inhalation exposure is minimal (Bruno *et al.*, 2000, POISINDEX, 2007). One-third of these publications are related to domestic animals and a few to nontarget wild animals (Newton *et al.*, 1990; Stone *et al.*, 1999), with a small number of reported deaths. All animal exposures are due to accidental direct and indirect ingestion of these rodenticides. The great majority of animal exposures include dogs, which may need gastric decontamination, and in some cases referral to a veterinarian for further clinical evaluation and treatment (Borst and Counotte, 2002; DuVall *et al.*, 1989; Hornfeldt and Phearman, 1996; McConnico *et al.*, 1997; Robben *et al.*, 1997). In most cases gastric decontamination will be recommended and treatment with vitamin K_1 may be needed. There are few data of the incidence or mortality rates from animal exposures to rodenticides.

Warfarin and dicoumarol found application as both oral anticoagulants and as rodenticides. Sweet clover requires the action of molds to form dicoumarol; giant fennel does not. Giant fennel (*Ferula communis*) grows in Mediterranean countries. It has a naturally occurring anticoagulant effect. An association between the plant and anticoagulation was first reported in the 1950s (Costa, 1950a, b; Carta, 1951). It was further investigated in Italy (Mazzetti and Cappelletti, 1957; Corticelli and Deiana, 1957; Corticelli *et al.*, 1957; Cannava, 1958) then in Israel (Shlosberg and Egyed, 1983). The anticoagulant activity of the plant in Morocco has recently been reviewed (Lamnaouer, 1999).

Warfarin and its congeners are still used as therapeutic agents. Oral anticoagulants available therapeutically in Europe include warfarin, phenprocoumaron, and nicoumalone – also called acenocoumarol (Shetty *et al.*, 1993). Oral anticoagulants are used therapeutically to reduce thromboembolic events. Warfarin examples include a reduction in catheter-related thrombosis (Guidry *et al.*, 1991; Magagnoli *et al.*, 2006), early venous thrombosis after operations (Calnan and Allenby, 1975; Pan *et al.*, 2005), including hip surgery, atrial fibrillation (Middlekauff *et al.*, 1995; Reiffel, 2000), and myocardial infarction (Asperger and Jursic, 1970). A number of adverse events have been recognized, and most are related to drug interactions (Dayton and Perel, 1971).

An association between vitamin K and coagulopathies was made in the mid-1930s (Dam, 1935; Fieser *et al.*, 1939). Soon, thereafter Prof. Link reported the discovery of dicoumarol in the moldy hay (Last, 2002). Naturally occurring coumarin in the sweet-clover hay is converted by fungi to dicoumarol. Dicoumarol was found to be the causative agent of the disease, so the elements needed for the disease were coumarin-containing plant material plus mold growth. Subsequently, a range of molecules were synthesized. One named warfarin became the most popular (Duxbury and Poller, 2001). Warfarin takes its name, in part, from the Wisconsin Alumni Research Foundation.

A. American Association of Poison Control Centers Data on Superwarfarins

Every year there are tens of thousands of accidental ingestions of long-acting anticoagulant rodenticides reported worldwide in the medical literature. These include the annual report from the American Association of Poison Control Centers (AAPCC). During the past 23 years the AAPCC reported 209,047 exposures to long-acting anticoagulant rodenticides (LAAR), which included 24 deaths due to LAAR ingestion. All fatalities were in adults who intentionally committed suicide.

III. CLASSIFICATION OF SUPERWARFARINS

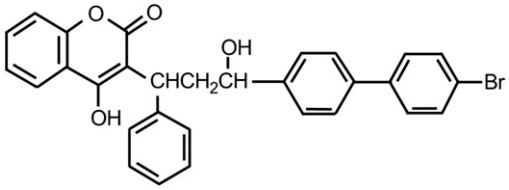
Anticoagulant rodenticides are also categorized by chemical structure. The chemical structure of the currently marketed products fits in one of two chemical classes: 4-hydroxycoumarins and indanediones.

A. 4-Hydroxycoumarins

This group of compounds have a 4-hydroxycoumarin ring with different side-chain substituents at the 3-position. Commonly used superwarfarin anticoagulant rodenticides in this group are bromadiolone, brodifacoum, coumatetralyl, coumafuryl, and difenacoum. Brodifacoum, difenacoum and bromadiolone are three of the most commonly used rodenticides around the world. Brodifacoum is the most frequently used rodenticide in the USA. These rodenticides share most of their physical and chemical characteristics, as well as their toxicokinetics, toxicodynamics, and mechanism of toxicity, and the medical toxicological management is the same for all superwarfarins.

1. Bromadiolone

Chemical formula: $C_{30}H_{23}BrO_4$

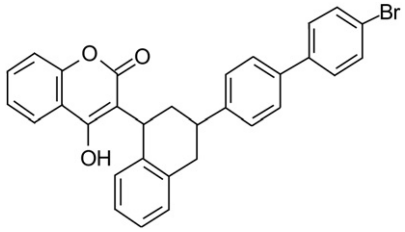


Bromadiolone [3-(3-(4′-bromobiphenyl-4-yl)-3-hydroxy-1-phenyl propyl)-4-hydroxycoumarin] was synthesized and marketed by the French company Lipha SA during the mid-1970s. It is used widely for control of commensal and field rodents in many countries. Technical grade bromadiolone is 97% pure. It is a yellowish powder and stable up to 200°C (Chalermchaikit *et al.*, 1993). It is very soluble in dimethylformamide (730 g/l), but less soluble in ethyl acetate

(25 g/l), and ethanol (8.2 g/l), and sparingly soluble in water (0.019 g/l). Bromadiolone is considered more palatable to rodents than most other anticoagulants. Its concentration in baits is usually 50 ppm (Chalermchaikit *et al.*, 1993). Although bromadiolone is considered a ''second-generation anticoagulant rodenticide'', some resistance problems have been reported with *Rattus norvegicus* and *Mus musculus* in the UK and Denmark (IPCS, 1995d; Lund, 1984; Rowe *et al.*, 1981).

2. Brodifacoum

Chemical formula: $C_{31}H_{23}BrO_3$



Brodifacoum [3-(3-(4′-bromobiphenyl-4-yl)-1,2,3,4-tetrahydro naphth-1-yl)-4-hydroxycoumarin] is one of the newer and more potent second-generation anticoagulant rodenticides. It was first introduced in 1977 by Sorex Ltd of London, and then developed by the Imperial Chemicals Incorporated (ICI) Plant Protection Division (Chalermchaikit *et al.*, 1993).

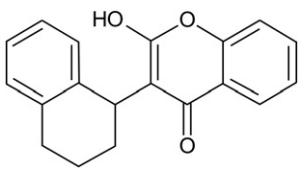
Pure brodifacoum is an off-white to fawn colored powder with a solubility of 6–20 g/l in acetone, 3 g/l in chloroform, 0.6–6 g/l in benzene, and less than 10 mg/l water. It is very stable in the environment with no loss after 30 days' exposure to direct sunlight (Chalermchaikit *et al.*, 1993).

Brodifacoum has been marketed in several countries for the control of a wide range of rodent pest species. It is available as a 0.005% pellet for rat and mouse control, a smaller 0.001% pellet for field rodent control, and as 29 g wax blocks for sewer rat control. It is the only anticoagulant rodenticide found to produce 100% mortality in most rodent species after only a 24 h dose (Chalermchaikit *et al.*, 1993). Brodifacoum was effective against warfarin-resistant rats and mice in 1984, but the possibility of resistance has been raised (Lund, 1984).

There is variation in the susceptibility of species to brodifacoum. Dogs are susceptible and are commonly exposed to potentially toxic quantities of brodifacoum (Chalermchaikit *et al.*, 1993).

3. Coumatetralyl

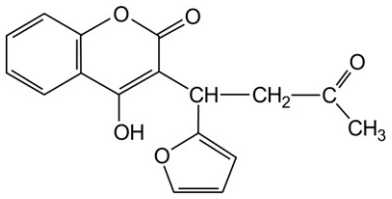
Chemical formula: $C_{19}H_{16}O_3$



Coumatetralyl [3-(alpha-tetralyl)-4-hydroxycoumarin] was introduced by Bayer AG with the trademark name of Racumin. It has been used for commensal rodent control in many countries. It is formulated as a dry bait (0.0375%), a liquid bait of its sodium salt, and a 0.75% tracking dust (Chalermchaikit *et al.*, 1993). Pure coumatetralyl is a colorless powder which is stable at temperatures below 150°C. Its solubility is 20–50 g/l in propan-2-ol, 50–100 g/l in methylene dichloride, and 4 mg/l in water. The acute and chronic LD_{50}s to *R. norvegicus* are 16.5 and 0.3 mg/kg for five consecutive doses, respectively. Chickens are somewhat resistant to coumatetralyl, with a chronic LD_{50} of 50 mg/kg for eight consecutive doses. Signs did not appear in fish until the concentration of coumatetralyl reached 1,000 mg/l in water (Chalermchaikit *et al.*, 1993). In spite of its low toxicity, it is reported to be a little more effective than warfarin against *R. norvegicus*, apparently due to a higher palatability. Coumatetralyl was introduced after the detection of warfarin-resistant rat populations and showed considerable success for a number of years, but resistant pests have been reported in the UK and Denmark (Rowe and Redfern, 1968; Lund, 1984).

4. Coumafuryl

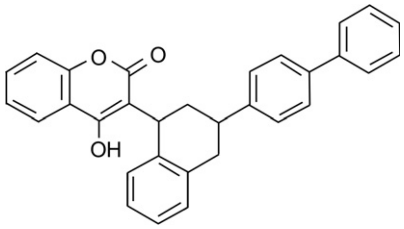
Chemical formula: $C_{17}H_{14}O_5$



Coumafuryl [3-(alpha-acetonylfurfuryl)-4-hydroxycoumarin] is a German anticoagulant, introduced in 1952, and is used at 0.025–0.05% in baits. Its toxicity is considered equal to warfarin for *R. norvegicus* but slightly less efficient against *M. musculus*. The chronic LD_{50} in *R. norvegicus* is 1.4 mg/kg for five repeated doses. Cats and dogs seem to be almost as susceptible as rats, with dogs being killed by 2 mg/kg for five repeated doses and cats by 10 mg/kg for four repeated doses (Chalermchaikit *et al.*, 1993).

5. Difenacoum

Chemical formula: $C_{31}H_{24}O_3$

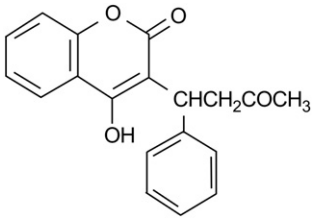


Difenacoum [3-(3-p-diphenyl-1,2,3,4-hydronaphth-1-yl)-4-hydroxycoumarin] was synthesized in the UK and marketed in 1975 by Sorex Ltd under the trademark

''Neosorexa'', and by ICI Plant Protection Division under the trademark ''Ratak'' as a 0.005% pelleted bait, and as a wax block. Pure difenacoum is an off-white powder with a solubility of greater than 50 g/l in acetone, 600 mg/l in benzene, and less than 10 mg/l in water. It is more toxic than warfarin, but less palatable (IPCS, 1995c). Difenacoum is still effective against many populations of warfarin-resistant rats (Desideri *et al.*, 1979), but resistance may be developing in the UK (Greaves *et al.*, 1982).

6. Warfarin

Chemical formula: $C_{19}H_{16}O_4$



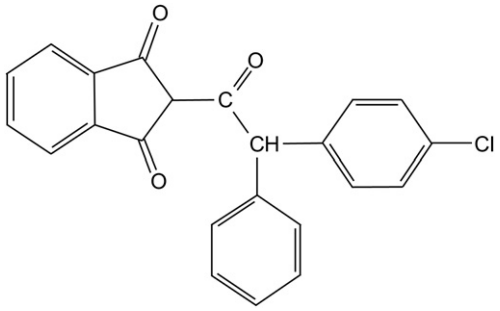
Warfarin [3-(a-acetonylbenzyl)-4-hydroxycoumarin] was the first anticoagulant rodenticide introduced shortly after World War II after development by the Wisconsin Alumni Research Foundation. Warfarin is still used widely, especially for the control of *R. norvegicus* in areas where resistance has not developed. In its racemic form, warfarin is colorless and crystalline, insoluble in water, but readily soluble in acetone, dioxane, and moderately soluble in alcohols. Warfarin is formulated as dry bait (0.005–0.05%) as well as a liquid bait, based on the sodium salt, and a tracking dust (0.5–1.0%). It is generally applied as the S-isomer, which has a toxicity ten times greater than the R-isomer. The acute and chronic LD_{50}s for *R. norvegicus* are around 10–12 and 0.75 mg/kg for five repeated doses, respectively (Colvin and Wang, 1974). Warfarin is sometimes combined with an antibacterial agent, sulfaquinoxaline, in order to reduce the bacterial production of vitamin K in the rat intestine, but the effectiveness of this combination has not been proven. Warfarin is considered one of the safest anticoagulants, as far as domestic and other nontarget animals are concerned. Serious resistance problems have been reported in Europe. It has recently been evaluated against sewer rats in London (Channon *et al.*, 2000).

B. Indanediones

This group of compounds has a 1,3 indanedione structure with different side-chain substituents at the 2-position. The most common superwarfarins in this group are chlorophacinone and diphacinone.

1. Chlorophacinone

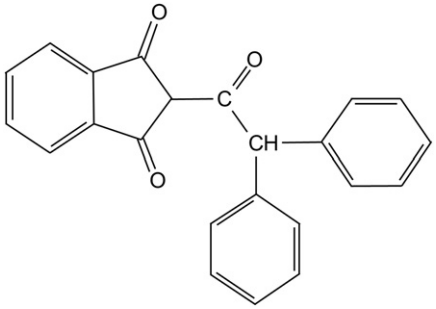
Molecular formula: $C_{23}H_{15}ClO_3$



Chlorophacinone [2-(alpha-4-chlorophenyl-a-phenylacetyl)-1,3-indandione] was first introduced during the mid-1960s by Lipha SA of France, at concentrations of 0.05% in baits and 0.2% in tracking dust. Pure chlorophacinone is a yellow crystalline solid which is very soluble in acetone, ethanol, ethyl acetate, but is sparingly soluble in water. It is quite stable and resistant to weathering. Chlorophacinone does not induce bait-shyness and is compatible with cereals, fruits, roots, and other potential bait substances. Its acute LD_{50} in *R. norvegicus* is about 20.5 mg/kg which is less toxic than warfarin, but it has a stronger initial effect on rats and mice. For control of house mice populations, a prolonged feeding period is needed. Chlorophacinone may not be effective against warfarin-resistant rodents (Chalermchaikit *et al.*, 1993).

2. Diphacinone

Molecular formula: $C_{23}H_{16}O_3$



Diphacinone (2-diphenylacetyl-1,3-indandione) is an old anticoagulant rodenticide, introduced by Vesicol Chemical Corp. and the Upjohn Co. It has been produced and used primarily in the USA as a 0.005% dry or liquid bait. Pure diphacinone is a yellow powder which is very soluble in chloroform (204 g/kg), toluene (73 g/kg), xylene (50 g/kg), and acetone (29 g/kg), but sparingly soluble in water (0.30 g/l). It will decompose in water due to sunlight. The acute LD_{50}s in *R. norvegicus* are 22.7 mg/kg in females and 43.3 mg/kg in males. It is more toxic than warfarin to rats, mice, and dogs, but its palatability is somewhat lower. Diphacinone may not be effective against some warfarin-resistant rodents (Chalermchaikit *et al.*, 1993). The anticoagulant rodenticides are marketed to have efficacy against a number of target pest species.

IV. TOXICOKINETICS

A. Absorption, Metabolism, and Excretion in Laboratory Animals and Humans

Superwarfarins are primarily well absorbed from the gastrointestinal tract. Almost 90% is absorbed with peak plasma concentrations often occurring within 12 h of ingestion. Binding to plasma proteins may prolong distribution and half-life. Toxicity after dermal or respiratory exposure is rare (Berry *et al.*, 2000; Boermans *et al.*, 1991) but not unreported (Spiller *et al.*, 2003).

The metabolism and elimination of the trans-isomer was more rapid than those of the cis-isomer. The elimination from the liver is biphasic with an initial rapid phase of 3 days and a slower phase with a half-life of 120 to 130 days. The liver is the major organ for the accumulation and storage, which has been found mainly as the unchanged parent compounds. The major route of elimination in different species after oral administration is via the feces. The urine is a very minor route of elimination (Watt *et al.*, 2005).

V. MECHANISM OF ACTION

The mechanism of action of all anticoagulant rodenticides is similar to that of warfarin, specifically inhibition of vitamin K_1 epoxide reductase (Park *et al.*, 1979; Leck and Park, 1981; Breckenridge *et al.*, 1985). In the coagulation cascade, the clotting factors II, VII, IX, and X must bind calcium ions to be active in clot formation. The Ca^{2+}-binding ability requires converting glutamyl residues on these clotting factors to carboxyl glutamyl residues by the process of carboxylation. This carboxylation uses vitamin K_1 hydroquinone as a cofactor. This vitamin K-dependent carboxylase reaction converts vitamin K_1 hydroquinone to its epoxide form, vitamin K_1 2,3-epoxide. In the normal cycle, vitamin K_1 2,3-epoxide is reduced to the original vitamin K_1 (phylloquinone) by epoxide reductase, and thus ''recycled''. The anticoagulant rodenticides produce their effect by interfering with vitamin K_1 epoxide reductase, resulting in the depletion of vitamin K_1 and subsequently impairing the synthesis of normal clotting factors II, VII, IX, and X (Craciun, 1997, 1998). Clinical coagulopathy soon follows the depletion of vitamin K_1 in the liver. These clotting factors in the dog have plasma half-lives of 41, 6.2, 13.9, and 16.5 h, respectively. The coagulation system continues to function well until about 3 to 5 days after ingestion when the natural decay of clotting factors occurs. It has been suggested that they bind more strongly to the liver than warfarin resulting in more persistent effects (Babcock *et al.*, 1993; Barnett *et al.*, 1992; James *et al.*, 1998; Jackson and Suttie, 1977; Suttie, 1986; Murphy and Gerken, 1989). The interrelationship of vitamin K, prothrombin, and gamma-carboxyglutamic acid is reviewed in Stenflo (1978). The interaction of warfarin and vitamin K is reviewed in Suttie (1990).

VI. TOXICITY

A. Clinical Effects: Signs and Symptoms

Clinical signs and symptoms of acute intoxication by superwarfarins range from a mild tendency to bleed in less severe poisoning cases to severe coagulopathy. Mild bleeding tendencies are often recognized clinically as nose or gum bleeding, hemoptysis, ecchymosis, bloody or melenotic stools, hematuria, abdominal or flank pain, enhanced bruising, or ventral hematomas. Severe bleeding may lead to shock and death. Internal and external bleeding are the most frequent clinical signs followed by tachycardia and hypotension, then multiple organ failure due to substantial blood loss. The onset of the signs of poisoning may not be evident until a few days after absorption (Baker *et al.*, 2002; Casner, 1998; Chua and Friedenberg, 1998; Corke, 1997; Nighoghossian *et al.*, 1990; Ross *et al.*, 1992; Swigar *et al.*, 1990; Tsutaoka *et al.*, 2003; Vogel *et al.*, 1988; Weitzel *et al.*, 1990; Wilton, 1991).

1. Animal Toxicology

Clinical signs are usually delayed until 24–36 h post-ingestion. The most common signs include vomiting, diarrhea, dyspnea, weakness, depression, anorexia, hematuria, and melena. Other signs such as pale mucous membranes, bleeding from nose and gums, and generalized bruising may be noticeable. Internal bleeding also causes generalized pain, fever, and lameness from bleeding into a joint, bleeding into the thorax or abdomen, brain, pericardium, or sudden death (Berny *et al.*, 1995; Braithwaite, 1982; Munday and Thompson, 2003).

2. Pediatric Exposures

The great majority of human exposures are children under the age of 6 due to accidental or unintentional ingestion. They usually do not require any medical intervention or routine follow-up laboratory studies and can be adequately managed by Poison Control Centers with home observation and parent education (Ingels *et al.*, 2002; Kanabar and Volans, 2002; Mullins *et al.*, 2000; Shepherd *et al.*, 2002). Children with acute unintentional superwarfarin ingestions may often be managed without gastric decontamination or prophylactic vitamin K. Laboratory testing for coagulopathy should be reserved for cases involving clinically evident bleeding abnormalities (Ingels *et al.*, 2002).

A small number of reported cases of children have presented with mild to moderate hematological effects, requiring minimal to nonmedical intervention (Babcock *et al.*, 1993, Golej *et al.*, 2001; Osterhoudt and Henretig, 2003; Smolinske *et al.*, 1989; Travis *et al.*, 1993; Watts *et al.*, 1990).

Intentional suicidal ingestion of large amounts of product conveys a greater risk for severe toxicity and increased mortality and should be referred to a healthcare facility for examination and treatment if needed (Ingels *et al.*, 2002).

3. Adult Exposures

The great majority of adult exposures to rodenticides are due to deliberate acute and chronic surreptitious ingestion. Bleeding disorders may persist for 6 weeks to many months. Serious poisoning had been reported in adults with massive overdoses. These long-acting anticoagulants have produced rapid and persistent bleeding due to hypoprothrombinemia (Barnett *et al.*, 1992; Berry *et al.*, 2000; Chong *et al.*, 1986; Exner *et al.*, 1992; Gallo, 1998; Hoffman *et al.*, 1988; Katona and Wason, 1989; Mack, 1994; McCarthy *et al.*, 1997; Morgan *et al.*, 1996; Routh *et al.*, 1991; Wallace *et al.*, 1990). There is a risk of spontaneous abortion with long-acting anticoagulants (Lipton and Klass, 1984; Nelson *et al.*, 2006; Zurawski and Kelly, 1997).

The severity of the intoxication depends on the amount of rodenticide ingested, preexisting co-morbidity, and co-ingestion of other toxic substances (Palmer *et al.*, 1999; Stanziale *et al.*, 1997; Tecimer and Yam, 1997; Seidelmann *et al.*, 1995; Walker and Beach, 2002). Fatalities are usually due to intentional suicidal ingestion of large amounts. Bleeding disorders and organ failure have been described in adults due to deliberate acute and chronic surreptitious ingestion. Twenty four deaths have been reported by the American Association of Poison Control Centers in the past 20 years (see Table 15.3; AAPCC-TESS annual reports from 1983–2006).

4. Household Pets and Farm Animal Exposures

Household pets and farm animals may be accidentally exposed to rodenticides. The possible effects on nontarget organisms can be considered in two categories, specifically direct poisoning and secondary poisoning. Secondary poisoning is generally considered to be after consumption of anticoagulant rodenticide poisoned rodents. The most common type of exposure is direct exposure of eating a cereal-based bait containing the rodenticide. The most commonly affected household pets are dogs, followed by cats, hamsters, rabbits, and pet birds (Boermans *et al.*, 1991; Hornfeldt and Phearman, 1996; McConnico *et al.*, 1997; Munday and Thompson, 2003; Park and Leck, 1982; Peterson and Streeter 1996; Radi and Thompson, 2004; Redfern and Gill, 1980; Robben *et al.*, 1997, 1998; Woody *et al.*, 1992).

Cats may be more resistant to the toxic effects of brodifacoum and difenacoum than dogs. Cases of abortion and hemorrhage in sheep and goats after misuse of brodifacoum have been reported (Jones, 1996; Watt, 2005).

5. Nontarget Wildlife Exposures

Nontarget wildlife may be exposed to rodenticides. The possible effects on nontarget organisms can also be considered in two categories, specifically direct poisoning and secondary poisoning. The potential for secondary poisoning is more likely in carnivorous wildlife (Borst and Counette, 2002; DuVall *et al.*, 1989; Eason *et al.*, 2002; Greaves *et al.*, 1982; James *et al.*, 1998; Mahmoud and Redfern, 1981; Mathur and Prakash, 1980; Newton *et al.*, 1990; Stone *et al.*, 1994). The most commonly affected species are birds such as great horned owls, barn owls, eastern screech-owls, golden eagles, red-tailed hawks, Cooper's hawks, and crows. Bird species varied in their susceptibility. Other wild animals may be exposed such as polecats, wild cats, foxes, etc. Brodifacoum was highly toxic for fish when tested as a technical material.

6. Laboratory/Monitoring and General Recommendations

Superwarfarins lower the blood concentrations of the vitamin K-dependent clotting factors II, VII, IX and X; this results in prolongation of prothrombin time (PT) and partial thromboplastin time (PTT). PT and PTT should be repeated at least twice daily until a normal PT and PTT are established. Also, the blood clotting time and the bleeding time should be measured. Blood is often demonstrable in the excreta. Secondary hypochromic or microcytic anemia may be marked (Goldfrank *et al.*, 2002; Nelson *et al.*, 2006).

A PT 24 to 48 h after exposure in asymptomatic children with accidental ingestions of large or unknown amounts should be obtained. In adults with deliberate ingestions and children with clinical evidence of bleeding, an initial PT and PTT should be obtained and then repeated at 24 and 48 h post-ingestion (Manoguerra and Cobaugh, 2005).

7. Analytical Methods

A number of analytical methods have been reported for detecting anticoagulant rodenticides in various matrices. Early fluorimetric methods were used to detect warfarin in serum (Corn and Berberich, 1967; Fasco *et al.*, 1977; Hanna *et al.*, 1978; Keiser and Martin, 1974; Lee *et al.*, 1981; Lewis *et al.*, 1970; O'Reilly *et al.*, 1962; Vesell and Shively, 1974; Welling *et al.*, 1970) and GLC for warfarin (Mildha *et al.*, 1974).

Warfarin-specific methods were generally not adequate for the anticoagulant rodenticides, so a number of other methods were developed. These include thin-layer chromatography (TLC), high-pressure liquid chromatography (HPLC), mass spectroscopy (MS), and antibody-mediated tests. Coumarin anticoagulant rodenticides were initially detected using TLC (Lau-Cam and Chu-Fong, 1972; Mallet *et al.*, 1973). A high-performance TLC method with an estimated detection limit of 200 ppb and 87% recovery from liver has recently been reported (Berny *et al.*, 1995).

Early HPLC methods focused on an individual chemical. For example, methods to detect chlorophacinone in formulations (Vigh *et al.*, 1981; Grant and Pike, 1979), brodifacoum in serum (Murphy and Gerken, 1989), brodifacoum (Koubek *et al.*, 1979; Hoogenboom and Rammell, 1983; Keiboom and Rammel, 1981; Ray *et al.*, 1989), bromadiolone (Subbiah *et al.*, 2005; Hunter, 1983), chlorophacionone (Hunter, 1985), difethiolone (Goldade *et al.*,

TABLE 15.3. Number of exposures to long-acting anticoagulant rodenticides and deaths reported by the American Association of Poison Control Centers – Toxic Exposure Surveillance System (AAPCC-TESS) Annual Reports: 24-year data

Year	No. of participating centers	Population served (millions)	No. of human exposures reported (all substances)	No. of exposures/ thousand population	No. of exposures to long-acting anticoagulant rodenticides (LAAR)	No. of deaths due to anticoagulants (alone and combined with other substances)	Reason: intentional suicidal (IS)	% of deaths from exposure to (LAAR)
1983	16	43.1	251,012	5.8	1,724	0	No deaths	0.00%
1984	47	99.8	730,224	7.3	3,703	2	IS	0.05%
1985	56	113.6	900,513	7.9	5.098	2	IS	0.04%
1986	57	132.2	1,098,894	8.3	7,723	1	IS	0.01%
1987	63	137.5	1,166,940	8.5	4,382	0	No deaths	0.00%
1988	64	155.7	1,368,748	8.8	5,133	1	IS	0.02%
1989	70	182.4	1,581,540	8.7	6,116	0	No deaths	0.00%
1990	72	191.7	1,713,462	8.9	8,215	0	No deaths	0.00%
1991	73	200.7	1,837,939	9.2	10,826	1	IS	0.009%
1992	68	196.7	1,864,188	9.5	10,864	0	No deaths	0.00%
1993	64	181.3	1,751,476	9.7	10,692	1	IS	0.009%
1994	65	215.9	1,926,438	8.9	12,868	1	IS	0.008%
1995	67	218.5	2,023,089	9.3	13,423	1	IS	0.007%
1996	67	232.3	2,155,952	9.3	13,345	1	IS	0.007%
1997	66	250.1	2,192,088	8.8	13,405	3	IS	0.02%
1998	65	257.5	2,241,082	8.7	16,019	0	No deaths	0.00%
1999	64	260.9	2,201,156	8.4	15,982	2	IS	0.01%
2000	63	270.6	2,168,248	8.0	16,006	0	No deaths	0.00%
2001	64	281.3	2,267,979	8.1	16,423	2	IS	0.01%
2002	64	291.6	2,380,028	8.2	17,100	3	IS	0.02%
2003	64	294.7	2,395,582	8.1	16,891	2	IS	0.001
2004	62	293.7	2,438,644	8.3	16,054	0	IS	0.00
2005	61	296.4	2,424,180	8.2	14,740	1	IS	0.006
2006	61	299.4	2,403,539	8.0	13,118	0	No deaths	0.00
Total			43,482,940		222,165	24	IS	0.01%

Source: Extracted from reviewing the AAPCC-TESS annual reports from 1983 to 2006. http://www.aapcc.org/annual.htm

1998), and difenacoum (Mundy and Machin, 1977) in tissue have been reported.

Then a method was developed to look for all the anticoagulant rodenticides on the market at the time. It succeeded in extracting and detecting eight anticoagulant rodenticides in serum and liver using fluorescence and UV detection. Samples were extracted with acetonitrile then cleaned up on solid phase columns. Four hydroxycoumarins were detected by fluorescence with excitation at 318 and emission at 390 nm. The indandiones were detected at 285 nm. An extraction recovery of 75% from serum and 69% from liver was reported. Hydroxycoumarins may be detected down to about 1 ng/ml of serum and 1 ng/g of liver, and indandiones down to 10 ng/ml of serum and 10 ng/g of liver (Chalermchaikit *et al.*, 1993; Felice *et al.*, 1991; Felice and Murphy, 1989). Another HPLC method for detecting brodifacoum in serum and liver using difenacoum as the internal standard has been reported (O'Bryan and Constable, 1991). There is also a method for the simultaneous detection of five superwarfarin rodenticides in human serum (Kuijpers *et al.*, 1995).

Other serum methods have been reported, for example, detection limits of 3 to 12 ng/ml for fluorescence and 20 to 75 ng/ml for UV detection (Kuijpers *et al.*, 1995; McCarthy, 1997; Mura *et al.*, 1992; Feng *et al.*, 1999).

Tissue methods include a solid phase cartridge extraction from liver with recoveries ranging from 52% for difenacoum to 78% for warfarin. The limit of detection is 10 ppb for warfarin and difenacoum and 110 ppb for chlorophacinone (Fauconnet *et al.*, 1997; Jones, 1996; Addison, 1982).

HPLC methods have also been published to distinguish cis- and trans-isomers of difenacoum with detection limits of 5 ng/ml (Kelly *et al.*, 1993). An early interesting approach was use of a post-column pH shift to enhance fluorescence detection (Hunter, 1985, 1983). Several earlier HPLC methods have also been reported (Hunter 1983; Mundy and Machin, 1982; AOAC, 1976a, b): diphacinone (Bullard *et al.*, 1975, 1976), fluorescence for bromadiolone (Deepa and Mishra, 2005), brodifacoum (Fu *et al.*, 2006), brodifacoum in tissues (Hoogenboom and Rammell, 1983), difenacoum (Hadler and Shadbolt, 1975), determinaiton of Rozol in parafinized formualtions (Kawano and Chang, 1980), and bromadiolone in tissues (Nahas, 1986).

A recent method uses DAD detection (Yang *et al.*, 2001). An interesting new method uses HPLC to detect anticoagulant rodenticides in soft drinks (Dimuccio *et al.*, 1991). An ion pair liquid chromatography method has been reported to detect chlorophacionone and diphacinone in oats (Primus *et al.*, 1998).

Contemporary confirmatory methods use mass spectroscopy. Most recently, liquid chromatography-electrospray ionization-mass spectroscopy (LC-EIS-MS) has been reported for the analysis of ten anticoagulant rodenticides with a limit of quantitation of about 5 µg/l (Grobosch *et al.*, 2006). Other recent methods use LC-MS-MS for unknown drugs including warfarin (Marquet *et al.*, 2003) and LC-ESI-MS and HPLC UV to detect anticoagulant rodenticides as low as 20 ng on column (Mesmer and Flurer, 2000). One of the earlier MS methods used a direct probe technique to detect indandione residues in food animals (Braselton *et al.*, 1992).

A cell culture/ELISA assay has recently been developed to detect anticoagulant rodenticides in treated grain (Lawley *et al.*, 2006). A prior immunoassay was developed to detect diphacinone and chlorophacionone (Mount *et al.*, 1988). Enantiomers of warfarin, coumachlor, and coumafuryl can be separated chromatographically (Armstrong *et al.*, 1993).

Serum concentration of dogs with anticoagulant rodenticide poisoning ranged from less than 10 ng/l to 851 ng/l for brodifacoum, difethialone, and difenacoum (Robben *et al.*, 1998).

Animal samples are routinely analyzed in veterinary diagnostic laboratories. For example, the Texas Veterinary Medical Diagnostic Laboratory, in College Station, Texas (http://tvmdlweb.tamu.edu), performs such analyses. Other laboratories performing anticoagulant rodenticide analyses on animal samples can be obtained from www.aavld.org Human samples are generally analyzed at either the National Medical Services Laboratory in Willow Grove, PA (800-522-6671) or the Medtox Scientific Laboratories in St Paul, MN (800-832-3244).

VII. GENERAL TREATMENT RECOMMENDATIONS

A. Referral to Healthcare Facility

In case of suspected terrorist act, misuse, intentional criminal, or any deliberate intentional suicidal ingestion, or when the amount ingested is either a large amount, or cannot be determined, the patient should be referred to a healthcare facility for clinical and laboratory assessment, and treatment if necessary (POISINDEX, 2007; Manoguerra and Cobaugh, 2005).

B. Home Observation Criteria

Accidental ingestion of a small piece or less than a few pellets can be adequately managed at home by Poison Control Centers, or by a healthcare professional with home observation and parent education. Usually, these types of exposures do not require any medical intervention or routine follow-up laboratory studies. Gastric decontamination has no effect on the clinical outcome after ''taste'' amounts are ingested by children (Kanabar and Volans, 2002; Mullins *et al.*, 2000; Shepherd *et al.*, 2002).

If the amount ingested by a child is a ''moderate amount'' (more than a handful, or a mouthful), or is questionably ''high'', then it is recommended that the parent contact their physician or call a local Poison Control Center

for instructions on how to induce emesis with syrup of ipecac (AAP, 2003; Tenenbein *et al.*, 1987).

There is no risk of poisoning for animals ingesting taste amounts, but if the amount ingested is unknown, or cannot be estimated, then a local Poison Control Center or veterinarian should be contacted for instructions on how to induce emesis and obtain a 24 to 48 h blood test (PT) to determine the need for treatment with vitamin K_1 (Munday and Thompson, 2003; Murphy and Gerken, 1989; Woody *et al.*, 2003).

C. Treatment at a Healthcare Facility

A PT should be obtained 48 h after exposure in asymptomatic children with accidental ingestion of a suspected large amount of rodenticide (Babcock *et al.*, 1993; Berry *et al.*, 2000). Adults with intentional ingestion and children with clinical evidence of bleeding, should obtain an initial PT and PTT, repeated at 24 and 48 h post-ingestion (Goldfrank *et al.*, 2002; Greeff *et al.*, 1987; Hoffman *et al.*, 1988). If any significant prolongation or evidence of bleeding is observed, PT should be repeated every 6 to 12 h. Determination of factors II, VII, IX, and X may be abnormal in patients with a normal PT and PTT and may provide earlier evidence of significant ingestion (Brands *et al.*, 1995; Corke, 1997; Pavlu *et al.*, 2005; Spahr *et al.*, 2007). Hemoglobin and hematocrit should be monitored in patients with clinical evidence of bleeding or significant coagulopathy. Determination of ABO blood type may be necessary in cases of toxic ingestions and bleeding. Patients may require red blood cell transfusions, or the administration of fresh frozen plasma (Bruno *et al.*, 2000; Ellenhorn *et al.*, 1997; Laposata *et al.*, 2007; Olmos and Lopez, 2007).

1. Emesis

Currently, there is a controversy regarding the use of syrup of ipecac; the American Academy of Pediatrics reversed its policy position about using syrup of ipecac to help with poisoning emergencies in children (AAP, 2003). Simultaneously, the American Association of Poison Control Centers (AAPCC) is still indicating that syrup of ipecac does have a place in therapy, and ''concluded that individual practitioners and poison control centers are best able to determine the particular patient population, geographic and other variables that might influence the decision to recommend having ipecac on hand'' (Manoguerra and Cobaugh, 2005). We recommend that the first action for a caregiver of a child who may have ingested one of these rodenticides is to consult with their local Poison Control Center (AAP, 2003).

Emesis with syrup of ipecac has been recommended for children with a history of accidental ingestion of small amounts, more than a ''grain or two'', if it can be administered within 1 h from the time of ingestion (Katona and Wason, 1989).

TABLE 15.4. Dosing of ipecac

Adult[a]	15–30 ml
Adolescent[a]	15–30 ml
Child 1–12 years old	15 ml
Child 6–12 months	Dose: 5–10 ml (Position child in left lateral decubitus position to reduce risk of aspiration)
Child under 6 months of age	NOT recommended for pre-hospital use

Katona and Wason (1989)

Emesis is contraindicated in patients with a prolonged PT or a bleeding disorder due to the risk of bleeding following ipecac-induced increased intracranial pressure (POISINDEX, 2007). Taste amounts, or a few pellets, or a bite on one block bait, do not require emesis. More than two mouthfuls or one block bait, or an unknown amount ingested, then emesis is most effective if initiated within 30 min to 1 h from the time of ingestion. The decision to induce or not to induce emesis is often controversial, and must be carefully considered. It could be most appropriate in the prehospital setting and is not recommended once the patient is in the emergency room (Krenzelok *et al.*, 1997; Chyka and Seger, 1997).

Contraindications: Patients with a bleeding disorder, particularly those under treatment with anticoagulants, or with history of chronic long-acting anticoagulant ingestion, are at risk from gastrointestinal and central nervous system bleeding from ipecac-induced emesis. The administration of activated charcoal is preferred when large amounts or chronic ingestion have occurred. Also, it is contraindicated if there is a risk for choking or aspiration, central nervous system excitation or depression, coma, seizures, signs of oral, pharyngeal, or esophageal irritation (Chyka and Seger, 1997; Golej *et al.*, 2001; Goldfrank *et al.*, 2002).

Before or after ipecac is administered, patients should be encouraged to drink water. Adults are given approximately 8 ounces (240 ml), and children 4 to 8 ounces (120 to 240 ml) (POISINDEX, 2007; Goldfrank *et al.*, 2002).

2. Activated Charcoal

For patients with a potentially toxic ingestion who are awake and able to protect their airway, activated charcoal diluted in water may be administered before going to the

TABLE 15.5. Dosing of activated charcoal

Charcoal dose Recommended to dilute 240 ml of water per 30 g charcoal (FDA, 1985)	
Adults and adolescents	50 to100 g
Children aged 1 to 12 years	25 to 50 g
Infants up to 1 year old	1 g/kg of body weight

Chyka and Seger (1997)

hospital. It is more effective when administered within 1 h post-ingestion. It is recommended to dilute 240 ml of water per 30 g charcoal (Chyka and Seger, 1997).

In patients who are at risk from the abrupt onset of seizures or mental status depression, activated charcoal should be administered by medical or paramedical personnel capable of airway management to prevent aspiration in the event of spontaneous emesis (POISINDEX, 2007; Ellenhorn *et al.*, 2002).

Use of a cathartic with activated charcoal is not routinely recommended as there is no evidence that cathartics reduce drug absorption and because cathartics can cause adverse effects such as nausea, vomiting, abdominal cramps, electrolyte imbalances, and occasionally hypotension. Complications include emesis and aspiration (Chyka and Seger, 1997; Golej *et al.*, 2001).

3. Gastric Lavage

Gastric lavage is recommended within 1 to 2 h post-ingestion. In chronic ingestions, it is not recommended as it may induce bleeding in adults with significant coagulopathy and is not necessary in children after accidental ingestion (Brands *et al.*, 1995; Ellenhorn *et al.*, 2002).

4. Laboratory Monitoring

A PT and PTT should be obtained 24 and 48 h post-ingestion in asymptomatic children with nonintentional ingestion of a ''large amount''. Adults with intentional ingestions and children with clinical confirmation of bleeding should obtain an initial PT and PTT, repeated at 24 and 48 h post-ingestion, followed by blood type verification (Barnett *et al.*, 1992; Ellenhorn *et al.*, 1997; Robben *et al.*, 1998). If any significant prolongation or evidence of bleeding is observed, PT should be repeated every 6 to 12 h. Determination of factors II, VII, IX, and X may be abnormal in patients with a normal PT and PTT, which may provide earlier proof of significant ingestion. Serial hemoglobin and hematocrit in patients should be followed with clinical evidence of bleeding or significant coagulopathy (Babcock *et al.*, 1993; Robben *et al.*, 1998). Hematocrit should be monitored closely at least every 4 h until the patient is stable (Brands *et al.*, 1995).

Hematest should be performed in stools and vomit for occult blood, and prothrombin time (PT) and partial time of thromboplastin (PTT) monitored routinely. A PT and PTT obtained within 48 h post-ingestion may not be predictive of subsequent coagulopathy (Greeff *et al.*, 1987). A 24 h and 48 h PT and PTT are therefore recommended every 6 to 12 h to assess efficacy of therapy. If prolongation is observed, then PT or INR should be repeated (Hoffman *et al.*, 1988; Smolinske *et al.*, 1989).

Antidote: Vitamin K_1 (Phytonadione: AquaMephyton®, Mephyton®) is the specific antidote and should be administered to any patient with a prolonged PT (Braithwaite, 1982; Bruno *et al.*, 2000; Tsutaoka *et al.*, 2003).

Blood and fresh or frozen plasma are recommended if the anticoagulation is severe.

Administration of vitamin K_1 is recommended if anticoagulation is excessive. A small intravenous dose of 1 to 5 mg, titrated to return PT to the therapeutic range, has been recommended. In anemic patients, the hematocrit should be monitored about every 4 h until it is stable. Stools and vomit may also be tested using Hematest (Jackson and Suttie, 1997; Hornfeldt and Phearman, 1996).

Oral vitamin K_1 may be administered in small ingestions and after the patient has been stabilized. Recommended doses are 15 to 25 mg p.o. in adults, 5 to 10 mg in children (Greeff *et al.*, 1987), and 2.5 to 5 mg/kg body weight in animals. A large daily maintenance dose of vitamin K_1 may be required for prolonged therapy in severe overdose, particularly in patients in whom vitamin K_1 absorption is variable (Lipton and Klass, 1984; Hoffman *et al.*, 1988; Ross *et al.*, 1992).

Intravenous phytonadione may be instituted in severe cases where rapid correction is needed. The adult dose is a minimum of 10 mg diluted in saline or glucose, injected i.v. at a rate not exceeding 5% of the total dose per minute. Doses should be repeated each at 6 to 8 h. Initial i.v. doses of 25, 100, 150, 160, and 400 mg have been required in patients actively bleeding (Hoffman *et al.*, 1988; Vogel *et al.*, 1988). Anaphylaxis may occur if vitamin K_1 is injected too rapidly.

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

Today there is a greater risk than ever before that extremist or terrorist groups may use industrial or household chemical substances to harm, kill, or terrorize our society because these substances are very easy to obtain and conceal. Also, numerous highly toxic chemicals can be stolen and released or detonated from storage tanks or from transportation and manufacturing facilities. These commercial and industrial chemicals stored in large quantities are considered ''Agents of Opportunity'' because they are readily commercially available. A large number of industrial and household chemicals have the potential to be used as chemical warfare or terrorist agents. These include the superwarfarins. However, the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) classifies these rodenticides in the ''Low Toxicity Group''. Nevertheless, they may be used to harm and terrorize people through the ingestion of contaminated food or water. Superwarfarins are available to consumers as meal bait packs, pellets, mini pellets, blocks, mini blocks, wax blocks, liquid bait formulations, as well as in tracking powder, in diluted to concentrated formulations. The ingestion of small amounts may not cause any bleeding problems. Ingestion of greater amounts provides increased risk of severe bleeding in 36 to 48 h. The coagulopathy may last several weeks to months despite vitamin K_1 treatment.

Inhibition of synthesis of vitamin K_1-dependent clotting factors may also occur following repeated ingestion of small amounts making these agents insidious.

The use of chemical weapons by terrorists still remains a big threat. Until recently, United Nations resolutions required a complete disclosure of chemical warfare agents and their destruction. More action is needed to prevent radical groups from accessing or accumulating these agents. Participation of the entire community, particularly the civilian population, is needed. Participation may increase due to educational programs that boost awareness, improved surveillance, and reporting to the authorities of any suspicious activity, or purchase/stockpile of large quantities of these commercial products.

What to do? Be wary if people from a community that attended the same event, meal, party, or restaurant present with similar signs of bleeding disorders. Ingestion of superwarfarins may go unnoticed when these are mixed with food, and signs or symptoms are delayed 36 to 48 h. Consequently, the victims may not associate the ingestion with the coagulopathy.

Identification of superwarfarins is easy today. Analytical methods for the detection of these products in serum and tissues are readily available. Routine laboratory tests for coagulopathy may help support the need for such analytical chemistry testing. Stockpiles of vitamin K_1 may be useful to consider, particularly in places where large stocks of superwarfarin rodenticides are used such as livestock areas.

Although viral infections are manifest with different signs and symptoms there is a possibility that members from a community who are victims of superwarfarins may begin to panic thinking that they were victims of a ''virus'', such as Ebola, but the signs and symptoms are different. Consequently, good differential diagnostic and laboratory work-up algorithms are needed for both humans and animals. Teams of subject matter experts for both humans and animals may be useful in this regard.

Superwarfarin intoxication may have no signs or symptoms other than the appearance of bleeding in the stools, urine, mucous membranes, thoracic or abdominal cavity. Ebola virus causes sudden hemorrhagic fever, weakness, muscle pain, headache, and sore throat, followed by vomiting, diarrhea, rash, limited kidney and liver functions, and both internal and external bleeding.

Community education programs should be developed to inform residents about superwarfarin rodenticides and other household commercial chemicals with the potential to be used as chemical warfare or terror agents. Fact sheets may be one method of informing the public. Identifying those at risk and when they should be informed may be a useful part of this education campaign – this may also improve security in industrial plants, and limit any access from outsiders in all industrial and storage facilities where flammable or highly toxic chemicals are stored, particularly livestock facilities.

Finally, surveillance of food sources, particularly those derived from livestock, should be assessed to determine whether these agents are available to consumers in low concentrations which may lead to long-term accumulation and eventual coagulopathies.

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CHAPTER 16

Thallium

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I. INTRODUCTION

Thallium (Tl) is a soft, bluish-white metal that occurs naturally in the earth's crust. It was discovered by Sir William Crookes in 1861 while making spectroscopic determinations of tellurium in residue material from a sulfuric acid plant. The name comes from the Greek word ''thallos'' which means a green shoot or twig, a reference to the green spectral emission lines originally used to identify the element. It is a heavy metal (density 11.83 g/cm^3, atomic number 81) whose use is mainly in the electronics industry (e.g. infrared detectors, semiconductor materials) with smaller quantities used in glass manufacturing and pharmaceutical industries, including the radioactive isotope Tl-201. Thallium can be released into the environment by cement manufacture, the burning of certain coal deposits and the production of nonferrous metals (Kazantzis, 2000; Peter and Viraraghavan, 2005). Thallium is a highly toxic element and salts of Tl are colorless, odorless, and tasteless. Thallium has no known biological function and has been the least studied of the toxic metals such as lead, mercury, and cadmium. Thallium salts were introduced as pesticides in Germany in 1920. The sulfate salt is most common and has been widely used as a rodenticide and ant killer. Tl has been associated with intentional and accidental poisonings since that time, although problems decreased greatly after its use was banned in major parts of the world (Saddique and Peterson, 1983).

II. BACKGROUND

Thallium has two important oxidation states, Tl (+1) and Tl (+3). The trivalent form more closely resembles aluminum and the monovalent form more resembles alkali metals such as potassium. The toxic nature of the monovalent Tl is due to its similarity to potassium in ionic radius and electrical charge. Thallium sulfate use as a pesticide was restricted in 1965 in the USA and the World Health Organization (WHO) recommended in 1973 against its use as a rodenticide due to its toxicity (WHO, 1973). From 1912 to 1930, thallium compounds were used extensively for medicinal purposes; for example in the treatment of ringworm (because of the depilatory effects), dysentery, and tuberculosis. The narrow margin between toxicity and therapeutic benefit, however, eventually eliminated the practical use of these compounds. Due to their highly toxic nature, delayed symptoms, and lack of taste or odor, Tl salts have been used for suicide attempts and in the intentional poisoning of individuals or small groups of people. Although the reported symptoms of Tl poisoning are diverse, the classic syndrome involves gastroenteritis, polyneuropathy, and alopecia. Fictional accounts of Tl as the agent of an intentional poisoning include Agatha Christie's book *The Pale Horse*. More recent accounts or suspicions of Tl use include medical case reports as well as lay press reports. Chronic Tl exposure has been reported in the industrial setting and exposure limits have been established (Peter and Viraraghavan, 2005). The radioactive isotope Tl-201 is a gamma emitter and is used in cardiac imaging, similar to technetium-99, and has a half-life of approximately 3 days. Although Tl-201 is the most common isotope in use, Tl-204 decays by beta particle emission and has a half-life of 3.8 years.

III. TOXICOKINETICS

Thallium is rapidly absorbed from the gastrointestinal tract and is well absorbed through the skin. There is little information concerning absorption from the respiratory tract. Once absorbed, Tl is rapidly distributed throughout the body to all organs, with the highest concentrations occurring in the kidney following an acute exposure. Both monovalent and trivalent Tl appear to distribute in similar manners, and it is not known if metabolic processes can change the valence state. Thallium can pass the placental barrier as well as the blood–brain barrier (Sullivan, 1992).

Elimination of Tl is mainly through the gastrointestinal tract but elimination also occurs through the kidneys, saliva, hair, skin, sweat, and breast milk. Relative amounts excreted by each route vary by species. Thallium is likely excreted through intestinal and gastric secretions associated with potassium loss or excretion. Likewise, reabsorption of Tl also occurs, mainly from the colon. The estimated biological half-life of Tl is 10 days but values up to 1 month have been reported (WHO, 1996).

IV. MECHANISM OF ACTION

Although the precise mechanism of action of Tl is unknown, its similarity to potassium has been shown to play a significant role. Thallium has an atomic radius similar to potassium and has shown a 10-fold affinity over potassium in Na^+/K^+-ATPase, resulting in lower activity of the enzyme. Tl will inhibit the influx and efflux of potassium in mitochondria, without affecting the movement of sodium. In addition to disturbing mitochondrial function, Tl has been shown to increase the levels of hydrogen peroxide and increase lipid peroxidation and oxidative stress (Hanzel and Verstraeten, 2005). Thallium can also inactivate sulfhydryl groups including those affecting the permeability of the outer mitochondrial membrane. Thallium can act as a Lewis acid, having an affinity for organosulfur compounds, which may account for its action to cause hair loss. The binding of cysteine by Tl may inhibit keratinization of hair by preventing the crosslinking of proteins (Mulkey and Oehme, 1993).

V. TOXICITY

Available human literature on Tl is mainly case reports from the results of acute poisonings, accidental ingestions, or suicide attempts. Although the acute classic syndrome of Tl poisoning involves gastroenteritis, polyneuropathy, and alopecia, not all these effects are observed in every case. The onset and sequence of symptoms will vary with the dose and duration of exposure. The lowest known toxic dose in the human is 0.31 g, which was reported to cause symptoms but did not cause death (Cavanagh *et al.*, 1974). Oral doses of 6–40 mg/kg body wt have been lethal within 10–12 days. Other oral human toxic doses are given as 10–15 mg/kg body wt (WHO, 1996; Moore *et al.*, 1993). Children have been poisoned with Tl at 4–8 mg/kg body wt.

Several hours following an acute exposure, initial symptoms may include gastroenteritis including nausea, vomiting, and diarrhea. With a relatively small dose, these symptoms may be relatively mild and diffuse, with little progression for 2–5 days. Gastrointestinal bleeding or constipation may then develop along with central and peripheral nervous system effects. These include paraesthesia with reports of the feeling of ''burning feet''. Additional neurological symptoms can include lethargy, delirium, seizures, and coma (Tsai *et al.*, 2006). An initial presentation simulating Guillain-Barré syndrome has been reported (Misra *et al.*, 2003). Nonspecific kidney and liver damage can develop. In severe exposures, circulatory symptoms may include hypertension, tachycardia, and cardiac failure. Initial dermatological involvement may include anhydrosis (which can cause fever) and this can be followed some time later by diaphoresis. In the second week following exposure additional dermatologic symptoms appear including an increased darkening of the hair papillae followed in several days by a developing alopecia. By 3 weeks following exposure there may be almost complete alopecia. At this time in the syndrome there may be ataxia and tremors with a painful neuritis in the lower extremities which may be severe. Following a lethal dose, death commonly occurs within 10–12 days caused by renal or cardiac failure. Recovery from Tl poisoning can require several months and residual neurological problems may remain including weakness, memory impairment, and psychological disturbances (Pau, 2000; Tsai *et al.*, 2006).

VI. RISK ASSESSMENT

Although reported as an agent of intentional poisoning for an individual or small group of people, the broad use of Tl as an agent in chemical warfare or terrorism has not been reported (Salem *et al.*, 2008). The most commonly available radioisotope of Tl-201 is a gamma emitter with a short half-life, making it a poor candidate for a radiological dispersion device (Burnham and Franco, 2005; Chin, 2007). Although the Tl-204 isotope is a beta emitter with a half-life of 3.8 years, its commercial use is limited. The chelating agent used for treatment of Tl exposure (Prussian blue) is also used in the treatment of radiocesium exposure, and thus is included in many antidote stockpiles (Ansari, 2004).

VII. TREATMENT

Diagnosis of Tl poisoning is based upon exposure history if available, compatible clinical time course and symptoms, along with the finding of above-background levels of Tl in urine, serum, or other clinical specimens. Appropriate methodology for urine or blood Tl includes atomic absorption spectroscopy (flame or flameless) and evolving methodology such as inductively coupled plasma emission spectroscopy (ICP). Use of colorimetric analyses of these specimens can lead to false positives (CDC, 1987). Normal or background concentration of Tl in urine is given as <0.5 μg/l up to a level of <10 μg/l, depending upon the laboratory reference, with concentrations elevating several hundred- to several thousand-fold following an acute exposure. Treatment should be initiated when 24-h urinary Tl excretion exceeds 0.5 mg. Additionally, a toxic level of Tl in the urine of >300 μg/l has been suggested (Sullivan, 1992). Blood Tl levels in exposure situations are less well characterized and values above 5 μg/l are considered to be evidence of excess Tl exposure (CDC, 1987). Prussian blue [ferric-hexacyanoferrate (II)] is the treatment of choice for Tl exposure in that it acts by binding Tl in the gastrointestinal tract, making it unavailable for reabsorption. This will increase the fecal excretion of Tl and decrease the half-life. Prussian blue is also used in the treatment of radiocesium and acts by the same mechanism [Yang *et al.*, 2008]. Suggested dosage regime is 3 g given orally three times a day for adults and adolescents. Children between

the ages of 2 and 12 years can be given 1 g orally three times a day. The clinician should insure proper gut functioning because constipation is a common finding in Tl intoxication. Fluid diuresis and other symptomatic and supportive care should also be provided.

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

Thallium remains a toxic metal of concern for both accidental and intentional exposure. Its historical use as an intentional poison against individuals or small groups is based on its delayed onset of symptoms and the nature of its salts being tasteless and odorless. Although the incidence of intoxication with Tl has been decreasing with its decreasing availability, clinicians should remain familiar with clinical features, diagnostic considerations, and treatment regimens. History has shown Tl not to be an agent of choice for chemical warfare or terrorism, and the use of radiological Tl for these purposes is also unlikely. However, preparations for such an unlikely event are relatively straightforward with major considerations being the stockpiling of Prussian blue, also needed for radiocesium treatment, and the retention of analytical capabilities for Tl in biological and other samples. Future directions for Tl should include the further characterization of environmental contamination with Tl, the characterization of its chronic effects in humans and basic research to better explain its mechanism of action at the cellular and molecular level.

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CHAPTER 17

Polycyclic Aromatic Hydrocarbons: Exposure from Emission Products and from Terrorist Attacks on US Targets – Implications for Developmental Central Nervous System Toxicity

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I. INTRODUCTION

The goal of this chapter is to stimulate research activity to develop both novel intervention strategies and therapeutic approaches to mitigate the neurotoxicity associated with prenatal exposure to polycyclic aromatic hydrocarbons (PAHs). The focus of this chapter is PAH exposure of vulnerable populations as a result of terrorist attacks on US targets. We will attempt to define the signature of prenatal PAH exposure-induced neurotoxicity within the context of human epidemiological data and translate this to appropriate animals models.

A critical review of the post-September 11, 2001 literature has resulted in a specific focus on studies that have revealed critical neural signaling/activity pathways and the identification of primary targets of PAH toxicity, as well as toxicity modifiers. It is hoped that as a result of this review, the interdisciplinary conduct of simultaneous temporal measurements of relevant markers and integrative analysis of critical signaling processes during development will increase two-fold; such that a better understanding of the mechanism of PAH exposure-induced neurotoxicity results.

II. BACKGROUND

A. Particulate PAH Release in the World Trade Center Disaster

The attack on the World Trade Center (WTC) on September 11, 2001 caused the largest acute environmental disaster in the history of New York City (Lioy *et al.*, 2002). As a result of this terrorist attack, combustion of more than 90,000 liters of jet fuel at temperatures above 1,000°C released a very dense and toxic atmospheric plume which contained thousands of tons of particulate matter (PM) composed, in part, of polycyclic aromatic hydrocarbons (PAHs). The plume dispersed over lower Manhattan, Brooklyn, and for miles beyond. The toxic materials contained in this plume entered nearby offices, schools, and residential buildings (Clark *et al.*, 2003; Lioy *et al.*, 2002; McGee *et al.*, 2003).

The populations that were faced with the greatest risk of exposure to this toxic plume included firefighters, police, paramedics, other first responders (Prezant *et al.*, 2002; Centers for Disease Control and Prevention [CDC], 2002), construction workers and volunteers who worked initially in rescue and recovery and then for many months cleared rubble at Ground Zero. Other vulnerable populations and individuals at a potentially elevated risk included women who were pregnant on September 11 and succeeding weeks in lower Manhattan and adjacent areas of Brooklyn and workers who cleaned WTC dust from nearby buildings as well as community residents including the 3,000 children who resided within 1 km of the towers and the 5,500 who attended school there. A consequence of the intense fire and the subsequent complete collapse of the two main towers was the development of a large plume of dust and smoke that released both particles and gases into the atmosphere.

An assessment of the potential exposure to dust and smoke among the residential and commuter populations was conducted and samples were taken from three undisturbed protected locations to the east of the WTC site (on Cortlandt, Cherry, and Market Streets). Analysis of samples provided for the determination of (a) chemical and physical characteristics of the material present in the dust and smoke

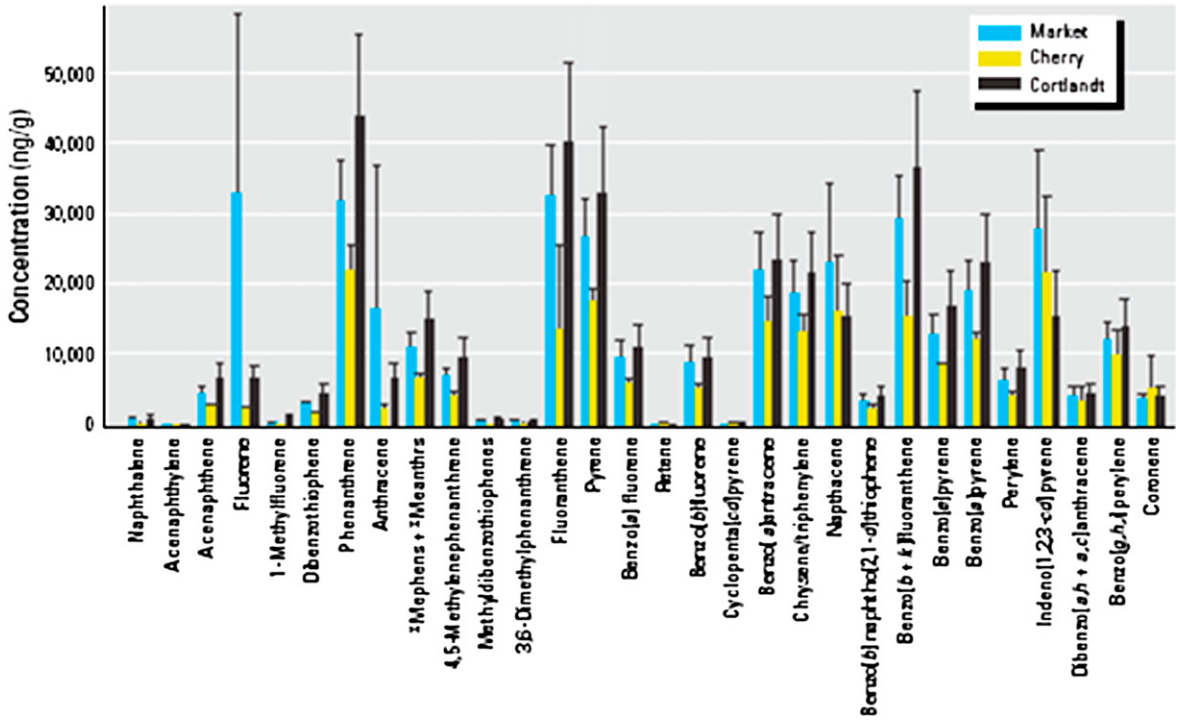

FIGURE 17.1. Airborne $PM_{2.5}$ mass concentrations at NYU Downtown Hospital (five blocks east of Ground Zero) and other sites in Manhattan, September through mid-October 2001. PS, public school. Data from Thurston *et al.* (2003) and adapted from Lioy *et al.* (2002). Adapted with permission from *Environ. Health Perspect.*

that settled from the initial plume, and (b) information on contaminants that could potentially affect acute or long-term human health by inhalation or ingestion. The highest concentrations of the 40 specific PAHs reported in Figure 17.1 were found in the Cortlandt Street sample. This is logical because this site was deemed to be the site closest to the fire after the collapse; however, a larger variety of other PAHs at concentrations >10 μg/g were found in the Market Street samples. The intense and uncontrolled fire(s) would be expected to burn at different temperatures, and the homogeneity of the material that burned would lead to a variety of unburned or partially burned hydrocarbons. These PAHs were found to be derived from burning plastics, metals, woods, synthetic products, and other materials, using morphologic analyses. It was anticipated and subsequently verified that the actual compounds and materials, that were present in the plume might be similar to those found in building fires. The morphologic analyses of the large mass of material present in the toxic plume found that the total concentrations of the above-mentioned 40 typical PAHs with higher molecular weights were in excess of 200–300 μg/g. The distribution of the 40 PAH compound levels ranged from hundreds of nanograms per gram to >40 μg/g. The concentration of benzo(a)pyrene ranged from 12 to 24 μg/g, and the highest values were detected at the Cortlandt Street site.

Data collected to date clearly document that PAHs were present in the samples at levels of 5 μg/g to hundreds of micrograms per gram as a result of the incomplete combustion of jet fuel and building materials that were produced by the intense fire. Concentrations of the individual compounds, e.g. benzo(a)pyrene, [B(a)P] were >20 μg/g, and the total mass of PAHs present was in excess of 0.1% of the mass. When placed in the context of the vast amounts of other materials present in the air during the first day after the collapse and fires, these levels were high and could lead to significant short-term inhalation exposure. Based on the PAH results from air samples collected after September 25, 2001 the types of PAHs released into the atmosphere at that time were similar to the PAHs detected in the settled dust and smoke samples collected during the first week after the collapse and fires (ATSDR, 1995, 2002).

B. Exposure of Pregnant Women to PAHs as a Result of the WTC Terrorist Attack

Pregnant women who were either working in the WTC or residing in the communities of lower Manhattan on September 11, 2001 were successfully recruited to a Mount Sinai cohort population for the purpose of assessing pregnancy outcomes and impacts on their infants (Berkowitz *et al.*, 2003). Of the 187 recruited pregnant women, 12 were inside the WTC towers at the time of attack and an additional 122 (65%) were within ten blocks. A comparison group ($n = 2{,}367$) consisted of all private patients not known to have been near the WTC who delivered at Mount Sinai Hospital during the same time period.

TABLE 17.1. Unadjusted birth outcomes by place of residence and employment (within 2 miles of the WTC)

Birth outcomes	Group 1: resided	Group 2: worked	Group 3: neither resided nor worked	*p*-Value
Length of gestation (days)	277.7 [*n*=80]	275.5 [*n*=51]	279.0 [*n*=169]	0.026
Birth weight (g)	3339.6 [*n*=80]	3442.7 [*n*=51]	3511.8 [*n*=169]	0.019
Birth length (cm)	50.06 [*n*=80]	51.44 [*n*=48]	51.15 [*n*=165]	0.008
Head circumference (cm)	34.10 [*n*=80]	34.18 [*n*=49]	34.51 [*n*=164]	0.097
Ponderal index	2.75 [*n*=80]	2.54 [*n*=48]	2.65 [*n*=165]	0.286
Percent SGA (<10th percentile)	8.75 [*n*=80]	5.88 [*n*=51]	5.33 [*n*=169]	0.581
Percent SGA (<20th percentile)	23.8 [*n*=80]	15.7 [*n*=51]	18.3 [*n*=169]	0.465

C. Birth Outcomes of Pregnant Women in or Near the WTC During Terrorist Attack

Term infants born to women who were pregnant on September 11, 2001 and who were living within a 2-mile radius of the WTC during the month after the event showed significant decrements in term birth weight (−149 g) and birth length (−0.82 cm), compared with infants born to the other pregnant women studied, after controlling for socio-demographic and biomedical risk factors. The decrements remained significant with adjustment for gestational duration (−122 g and −0.74 cm, respectively). Women in the first trimester of pregnancy at the time of the WTC attack delivered infants with significantly shorter gestation (−3.6 days) and a smaller head circumference (−0.48 cm), compared with women at later stages of pregnancy, regardless of the distance of their residence or work sites from the WTC. The observed adverse effects suggest an impact of pollutants and/or stress related to the WTC disaster and have implications for the health and development of exposed children (Lederman *et al.*, 2004).

D. Conclusion from WTC Prospective Epidemiology Cohort Studies

An increase in incidence in small for gestational age (SGA) was the major adverse health effect seen in infants born to women who were inside the towers or within approximately ten blocks of the WTC on September 11 (Berkowitz *et al.*, 2003). The incidence of SGA infants was two-fold greater among the WTC mothers than in a demographically similar comparison population not known to have been in lower Manhattan on September 11, 2001 ($p < 0.01$). SGA is an index of intrauterine growth restriction (IUGR). Biologically plausible causes of IUGR in these babies include exposures to fine PM and PAHs. Previous studies have found associations between particulate air pollution and IUGR (Bobak *et al.*, 2001; Dejmek *et al.*, 1999). Other investigations have linked air pollution to preterm births (Ritz *et al.*, 2000). Additionally, high levels of PAH-DNA adducts in umbilical cord leukocytes have been associated with reduced birth size (Perera *et al.*, 1998). Prenatal exposure to cigarette smoke, which contains PAHs among other toxins, is a well-established risk factor for IUGR. Maternal stress is another possible cause of the observed increase in SGA, but the authors in the prospective cohort studies did not detect any correlation between reported levels of stress and SGA incidence. An important question that arose as a result of the studies discussed above was: Will the increased frequency of SGA observed in babies born to women who were within or near the WTC on September 11 result in long-term adverse effects on growth and cognitive development?

E. The Human Health Relevance of Prenatal Exposure to PAHs

Exposure to benzo(a)pyrene, the prototypical PAH, occurred through the inhalation of particulates in the ambient air that was produced as a result of the terrorist attack on the WTC as described above. Subsequent evidence from human epidemiological studies has shown that unintended prenatal exposure of the fetus to PAHs adversely affects fetal development resulting in low birth weight and reduced head circumference that can manifest as neurobehavioral deficits in the early years of childhood. Neurobehavioral deficits in offspring from PAH-exposed mothers have been quantified as low scores on selective types of cognitive and neuromotor functioning (Hack *et al.*, 1991; Perrera *et al.*, 2003; Landrigan *et al.*, 2004). Recently published studies on PAH-exposed populations provide further evidence that exposure to environmentally relevant levels of PAH adversely affects childhood development as assessed by the mental development index on the Bailey Scales of Infant Development (BSID-II) (Perera *et al.*, 2006).

While the brain levels of PAH in the children who scored low on the mental development index component of the BSID-II cannot be known, studies such as the aforementioned are valuable from a translational standpoint as they can assist in the design of molecular neurotoxicity studies. Mechanistic studies that utilize transgenic mouse models will facilitate the understanding of the molecular signaling events that give rise to the observed neurotoxic effects resulting from exposure to PAHs during development. The

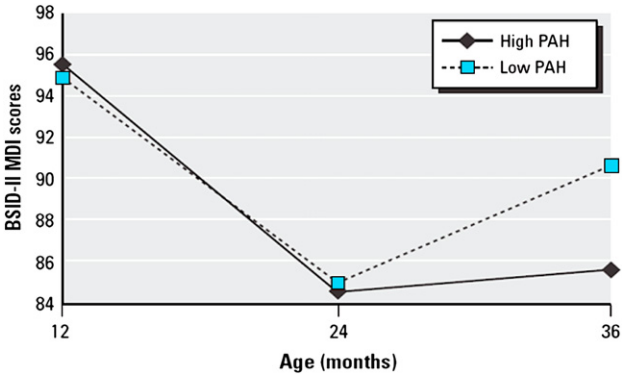

FIGURE 17.2. Estimated effects of prenatal PAH exposure on cognitive development in children 12 months through 36 months of age by GEE. The model was adjusted for the child's exact age at test administration, child's sex, ethnicity, gestational age at birth, quality of the (caretaking) home environment, and prenatal exposure to ETS and CPF. Adapted with permission from *Environ. Health Perspect.* and Perera *et al.* (2006).

gaps with respect to several reports in the literature illustrate the need for such basic mechanistic studies.

III. PAH-INDUCED PHYSIOLOGICAL AND BEHAVIORAL TOXIC MANIFESTATIONS IN ANIMAL MODELS: EARLY PAH STUDIES WHICH IMPLICATED THE CNS AS A TARGET

In the 1970s it was shown that pregnant mice treated with PAHs produced offspring with a high incidence of brain tumors (Rice *et al.*, 1978). Acute B(a)P exposures in laboratory animals were shown to cause malignant brain tumors (Markovits *et al.*, 1976). Additionally, malignant transformations of fetal mouse brain cells were observed subsequent to *in vitro* B(a)P exposure (Markovits *et al.*, 1976). High levels of several PAHs including B(a)P were found in soil, sludge, and water samples along with other solvents and metals that were used for processing motor oil and chemical wastes during the mid-1970s and early 1980s (Kilburn and Warshaw, 1995). Later in the 1980s, microinjections of diesel exhaust fraction (rich in PAHs) into the rat hippocampus and striatum were shown to cause neuronal lesions in a study by Andersson *et al.* (1998). Plant workers in Poland employed to process coke were reported to develop neurotic syndromes with vegetative dysregulation, and loss of short-term memory; their prevalence depended on the level of exposure to B(a)P (Majachrzak *et al.*, 1990). Children born in years of maximal air pollution in the Czech Republic were shown to have learning disorders that were attributed to elevated levels of PAHs in the atmosphere from the mining and combustion of coal (Otto *et al.*, 1997). In the USA, neurological symptoms were reported from a community that was chronically exposed to B(a)P, benz(a)anthracene, chrysene, naphthalene, fluorine, and pyrene dumped at a site in Texas from the 1960s until the 1970s (Dayal *et al.*, 1995). Similarly, residents in close proximity to a combustion Superfund site in Louisiana were reported to have displayed neurophysiological and neuropsychological impairments. Paternal occupational exposure to PAHs was shown to be associated with an increased risk of neuroectodermal tumors in children from Italy, France, and Spain (Cordier *et al.*, 1997a, b). Similarly, an association between paternal exposure to creosote (rich in PAHs) and diagnosed cases of neuroblastoma in children was seen (Kerr *et al.*, 2000). As can be seen from the early work, *in utero* exposure to this toxic chemical produces functional alterations in nervous system functioning resulting in various forms of developmental disorders or behavioral impairments in higher mammals (Srám, 1999).

In the late 1990s a mounting body of literature suggested that a likely long-term effect of prenatal exposure to PAHs was to interfere with the development and function of normal CNS development. Although the mechanism of PAH-induced impairment of CNS function was not well understood at the time, it was clear that additional research on PAH-induced neurobehavioral alterations and the underlying cellular and molecular mechanisms was warranted. At the time, we commenced studies [at environmentally relevant B(a)P concentrations] to assess the developmental neurotoxicological effects subsequent to exposure to B(a)P aerosol (Hood *et al.*, 2000; Ramesh *et al.*, 2001a, b, 2002; Wormley *et al.*, 2004; Wu *et al.*, 2003). Additionally, PAHs have been implicated as causative agents of lung, breast, esophageal, pancreatic, gastric, colorectal, bladder, skin, prostate, and cervical cancers in humans and animal models. Other than carcinogenicity, PAHs have also been reported to cause hemato-, cardio-, renal, neuro-, immuno-, reproductive, and developmental toxicities in humans and laboratory animals. Details of the toxicities and cancers caused by PAHs are beyond the scope of this chapter. Interested readers may refer to the reviews of ATSDR (1995), WHO (1999), Pickering (1999), and Ramesh *et al.* (2004).

A. Development of a Susceptibility Exposure Paradigm to Access the Effects of Prenatal Exposure to PAHs on CNS Development

Over the years, work from our laboratory focused on the refinement of a susceptibility exposure paradigm to better assess the effects of prenatal exposure to environmental toxicants on certain aspects of CNS development (Hood *et al.*, 2000; Ramesh *et al.*, 2001a, b; Wormley *et al.*, 2004). Because CNS events have ''windows of susceptibility'' during development, it was reasoned that there should be a time frame when the lowest dose and shortest duration of exposure to an environmental contaminant would be

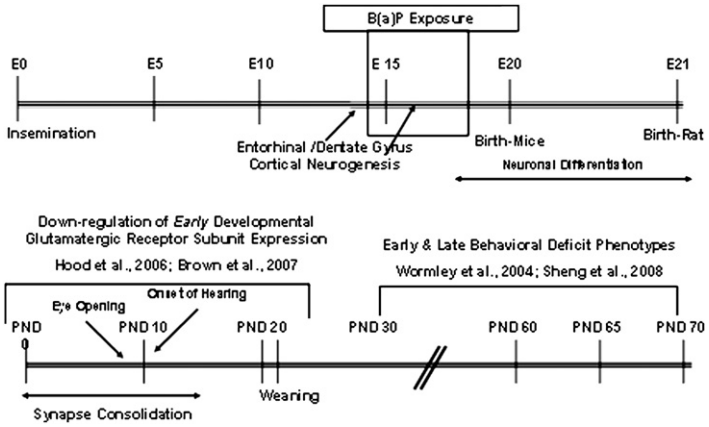

FIGURE 17.3. A Modified Barker Hypothesis: the susceptibility exposure paradigm used as a model to assess the effects of prenatal exposure to polycyclic aromatic hydrocarbons. Adapted with permission from *Neurotoxicology* and Brown *et al.* (2007).

expected to have a significant negative impact on brain development.

The susceptibility exposure paradigm was developed based on the peak periods of neuroepithelial cell proliferation (neurogenesis) for specific brain structures. Gestation days 14–17 represent the peak period of neurogenesis for the cerebral cortex in the mouse. Subsequent to this peak period of neurogenesis, each neuronal cell continues to mature through a process of migration, settling to a specific location and extending projections to a designated target site. In many cases such as for the external germinal layer, this process of migration continues well after birth and in the human can continue for 7 months to 2 years after birth. Specifically, cortical synapses at birth are still immature and in the human the morphological characteristics of maturity are reached between 6 and 24 months after birth. The use of our susceptibility exposure paradigm has allowed us to ask relevant questions pertaining to nervous system dysfunction during development that arise as a result of modulation of the process of neurogenesis.

B. Benzo(a)pyrene Aerosol CNS Disposition Studies as a Model of Prenatal Environmental Contaminant Exposure

We routinely use nose-only inhalation exposure of B(a)P aerosol to evaluate the consequence of prenatal exposure to this toxicant on physiological and behavioral endpoints. The properties of this B(a)P aerosol are shown in Figure 17.4. The aerosol typically exhibits a trimodal distribution with a 93% cumulative mass less than 5.85 μm, 89% cumulative mass less than 10 μm, 55.3% cumulative mass less than 2.5 μm, and 38% less than 1 μm. Fifty-five percent of the aerosol generally has a cumulative mass less than $PM_{2.5}$ and the mass median aerodynamic diameter (MMAD) + geometric standard deviation for this mode is consistently 1.7 ± 0.085 μm. For several years we employed a rat model exposing timed pregnant dams to inhalation concentrations of 25, 75, and 100 $\mu g/m^3$.

C. Benzo(a)pyrene Metabolite CNS Disposition in Offsprings

Bioavailability and B(a)P disposition studies conducted in timed pregnant rat dams have demonstrated a progressive increase in plasma and cortex metabolite concentrations in offspring pups as a function of B(a)P aerosol exposure concentration. Additionally, differences in metabolite disposition from plasma to cortex within individual exposure concentration cohorts were noted. No detectable levels of B(a)P metabolites are ever in the control (carbon-black exposed; Hood *et al.*, 2000) rats as reported in Wu *et al.*

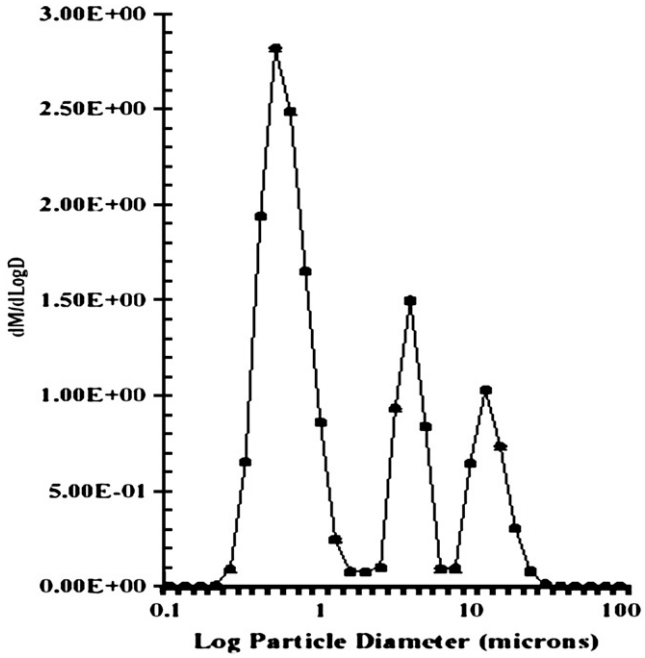

FIGURE 17.4. Differential particle size distribution of B(a)P: carbon black aerosol (100 $\mu g/m^3$). Subsequent to exposure, substrate post-weights were recorded and entered into the Win-CIDRS (Windows–Cascade Impactor Data Reduction Program) to generate the particle size distribution for the particulate aerosol. From Hood *et al.* (2000).

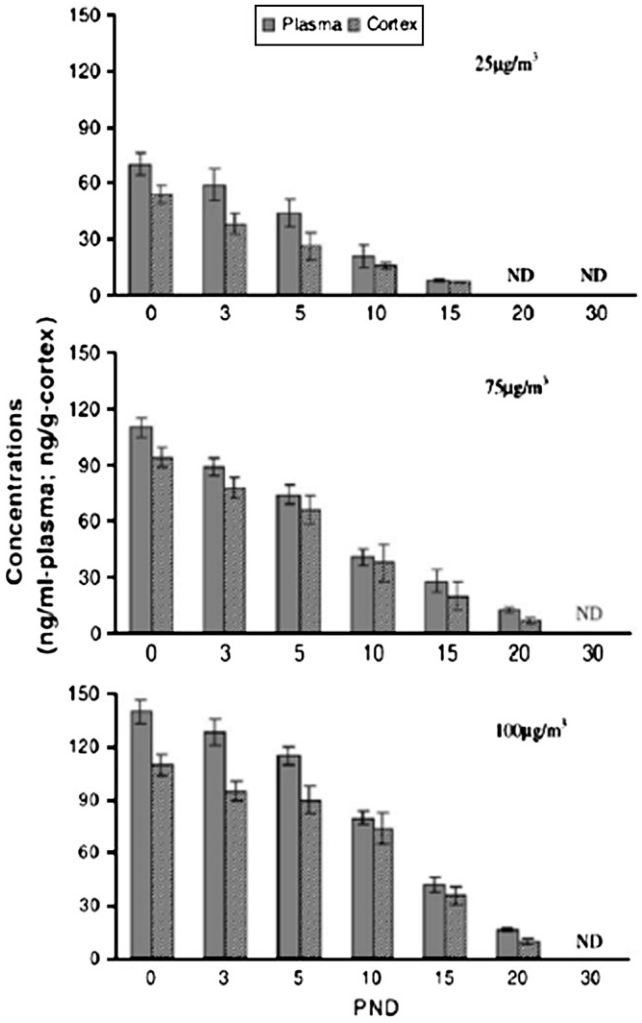

FIGURE 17.5. The time course of mean plasma and cerebrocortical total B(a)P metabolite concentrations in offspring pups. Adapted from Hood *et al.* (2000).

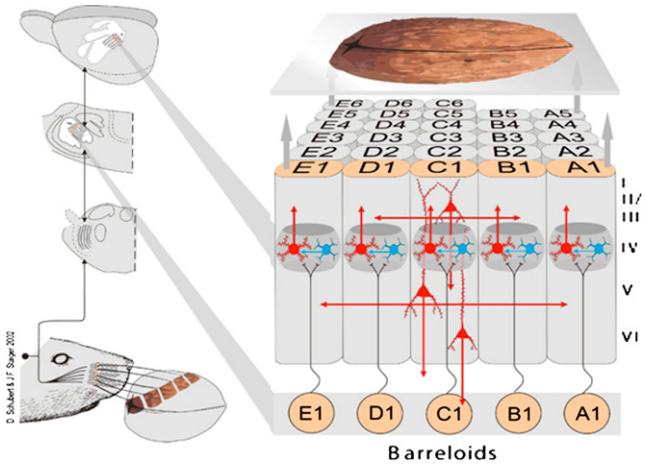

FIGURE 17.6. Main elements in the neural pathway from the axons innervating the whisker follicles on a rat's face (lower left) to the primary somatic sensory cortex (upper right). The diagram shows the rat palpating a walnut with its whiskers and perceiving the walnut through activity transmitted in the sensory pathway to cortex. The pathway contains a first synapse in the brainstem trigeminal nuclei (''Trig''), a second synapse in the contralateral thalamus (''Thal'') and a third relay from thalamus to barrel cortex [called barrels (Cortex)]. There is a separate channel for each whisker on the rat's face up to and including primary sensory cortex as illustrated on the right, each labeled by a row (A, dorsal to E, ventral) and a number starting from posterior and counting anteriorly. The roman numerals to the right of barrel cortex identify the six cortical layers. The results in the present paper were generated by analyzing neurons in the barrel cortex using single cell electrophysiology and biochemical techniques (adapted from Woolsey and Van der Loos, 1970).

(2003). Importantly, total metabolite distribution at an exposure concentration of 100 μg/m^3 was reported and the B(a)P 4,5; 7,8; 9,10-dihydrodiols, B(a)P 3,6-dione, 3- and 9-hydroxy B(a)P metabolites were detected and the B(a)P 7,8; 9,10-diols, and 3-hydroxy B(a)p were found to be predominant in cerebrocortical extracts.

D. Identification of Prenatal PAH Exposure-Induced Deficits in Offspring Cortical Neuronal Activity and Behavior

Recent B(a)P studies have established reductions in learning and memory correlates both in rodent and humans (Grova *et al.*, 2007; Wormley *et al.*, 2004; Widholm *et al.*, 2003; Gilbert *et al.*, 2000; Hack *et al.*, 1991; Perrera *et al.*, 2003, 2006; Landrigan *et al.*, 2004). We decided to use the rat cortex as a model of primary somatosensory (S1) cortex (Figure 17.6). Because of the unique organization of the rodent whisker to cortex pathway, and the already demonstrated effects of other toxins, such as alcohol, lead, and TCDD on this cortex as the system was ideal for deciphering the effects of B(a)P on a well-characterized model (Rema *et al.*, 1998; Rema and Ebner, 1999; Wilson *et al.*, 2000; Benusková *et al.*, 2001; Hood *et al.*, 2006). Whiskers are arranged in vertical and horizontal arrays on both sides of the face. Each whisker projects to a cellular aggregate called a barrel in layer IV of the S1 cortex (Woolsey and Van der Loos, 1970). The arrangement of this whisker-to-barrel circuit makes the S1 cortex in offspring animals that received a prenatal insult an *ideal* system to quantify alterations in stimulus–response relationships which manifest as a result of *in utero* exposure. The results from McCallister *et al.* (2008) shown in Figure 17.7 are consistent with earlier studies (Hood *et al.*, 2006) of the neurotoxic effects in offspring cortex subsequent to prenatal TCDD exposure. B(a)P-exposed offspring also exhibit diminished, stimulus-evoked activity in the barrel field cortex. In this work, responsive neurons were identified in most penetrations by advancing the electrode at 20 μm intervals, monitoring spontaneous activity and then manually stimulating the whiskers to test for evoked responses from neurons near the electrode. When post-stimulus time histogram (PSTH) time bins are subdivided into 10 ms time domains, the data

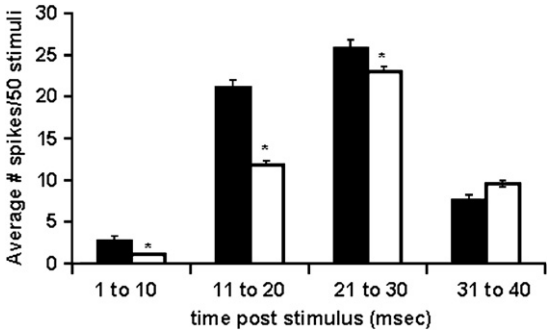

FIGURE 17.7. Bar graph of the mean ± SE stimulus evoked activity from control and PAH-exposed (300 µg/kg BW) offspring. White bars represent vehicle control offspring and black bars represent B(a)P-exposed offspring. Data are derived from a suprathreshold intensity (50 V stimulus applied to piezoelectric element) with whiskers deflected approximately 600 µm from the face. Response magnitudes (+SEM) were assessed for statistical differences within each group (* $p < 0.05$, ANOVA). This epoch analysis shows that prenatal B(a)P exposure significantly impacts the shorter latency components (from McCallister *et al.*, 2008).

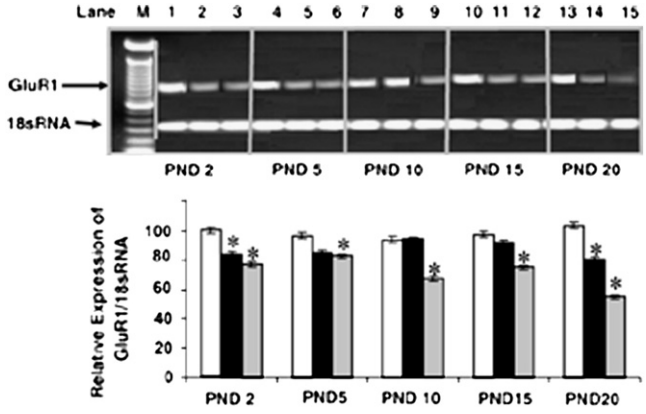

FIGURE 17.8. Offspring pre-weaning hippocampal developmental expression profile of GluR1 mRNA on PND 2, 5, 10, 15, and 20 following gestational exposure to B(a)P on GD14–17. Upper panel shows the electrophoretic agarose gel results from semi-quantitative RT-PCR. The upper band is the GluR1 (791 bp) subunit and the lower band is the internal control, 18sRNA (324 bp). Lower panel displays a histogram of the densitometric quantitation of the relative expression of target to internal 18sRNA control. M represents the DNA marker. Lanes 1–3: offspring GluR1 mRNA levels for PND 2; (lane 1) white bar – control, (lane 2) black bar – 50 mg/kg B(a)P, and (lane 3) gray bar – 150 mg/kg B(a)P. Lanes 4–6: offspring GluR1 mRNA levels for PND 5; (lane 4) white bar – control, (lane 5) black bar – 50 mg/kg B(a)P, and (lane 6) gray bar – 150 mg/kg B(a)P. Lanes 7–9: offspring GluR1 mRNA levels for PND 10; (lane 7) white bar – control, (lane 8) black bar – 50 mg/kg B(a)P, and (lane 9) gray bar – 150 mg/kg B(a)P. Lanes 10–12: offspring GluR1 mRNA levels for PND 15; (lane 10) white bar – control, (lane 11) black bar – 50 mg/kg B(a)P, and (lane 12) gray bar – 150 mg/kg B(a)P. Lanes 13–15: offspring GluR1 mRNA levels for PND 20; (lane 13) white bar – control, (lane 14) black bar – 50 mg/kg B(a)P, and (lane 15) gray bar – 150 mg/kg B(a)P. * $p < 0.05$ relative to control on the respective PND.

are very similar to what was observed in TCDD-exposed offspring. Prenatal B(a)P exposure clearly induces suppression of evoked cortical neuronal activity and is greatest in the early latency components of the response after stimulation of the best whisker (Figure 17.7). The overall reduction in B(a)P-induced suppression is clearly apparent in the average responses. Further, the most robust suppression after prenatal B(a)P is found at the two shortest post-stimulus epochs of 1–10 ms and 11–20 ms. The short latency response (3–10 ms) of cortical neurons is thought to reflect activity generated at the thalamocortical synapses that depend heavily on alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors (Armstrong-James *et al.*, 1993). This is the component of the cortical response that seems to be most strongly diminished in B(a)P-exposed offspring. The longer latency responses (11–40 ms) have been shown to be strongly dependent on N-methyl-D-aspartate (NMDA) receptor activation (Armstrong-James et al., 1993). Therefore, the data from our laboratory suggest that prenatal B(a)P-exposure reduces evoked cortical neuronal activity and indicates that these reductions are due to environmental contaminant-AhR agonist effects on the normal development of both AMPA and NMDA subunits.

Interestingly and consistent with previously reported results for prenatal TCDD exposure (another environmental contaminant found to be released as a result of the terrorist attack on the WTC) the effects on the aforementioned AMPA-mediated single cell responses were validated by RT-PCR. Glutamate receptor, AMPA-type subunit (GluR1) temporal developmental mRNA expression was determined in control and B(a)P-exposed offspring. The results shown in Figure 17.8 were normalized to 18sRNA expression. The results presented in this pre-weaning developmental expression profile figure from Brown *et al.* (2007) reveal a significant 50% down-regulation in GluR1 developmental expression in the 150 mg/kg body wt B(a)P-exposed offspring by PND 20 as compared to offspring controls.

In order to ascertain a behavioral phenotype for PAH-exposed offspring, an operant behavioral paradigm was used. The results from Wormley et al. (2004) are shown in Figure 17.9. On PND 100, offspring male and female littermates were prepared for initiation of learning behavior analysis (Weiss and Laties, 1964). Initially, animals were trained to press a lever in the operant chamber. This initial session is referred to as magazine training. In this session, animals are trained to retrieve pellets from the delivery trough that are dispensed every 120 s (variable time, VT 120) on average independently of the animal's behavior. Random lever pressing also initiates delivery of a food pellet. If ten lever presses occurred during a 20-min magazine training period, the schedule was automatically switched to a fixed-ratio of 1 (FR1; specifying that one lever press would produce one food pellet). If no responses occurred, the VT 120 s schedule automatically shifted to an

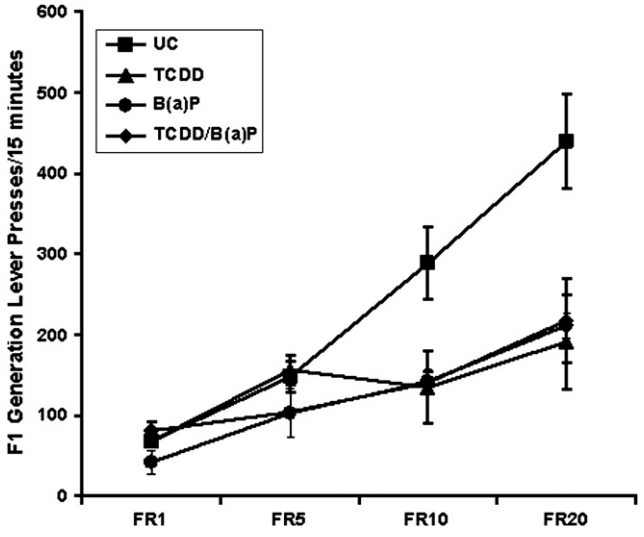

FIGURE 17.9. Gestational exposure to environmental contaminants (including PAH aerosol) produced as a result of the terrorist attack on the WTC; results in significant deficits in learning behavior in offspring animals. Control ($n = 8$) and B(a)P-exposed ($n = 8$) F1 generation animals were magazine trained where they learn to press a lever n number of times (FR) in order to receive a food reinforcement. Automated overnight shaping of this learning behavior proceeded at FR(n) until the animal met the criteria for inclusion in the study. Acquisition of data at an FR(n) for 15 min each day for 5 days was followed by data averaging and statistical analysis (* $p < 0.05$).

FR1 after 30 min. This FR1 component then proceeded for an additional 100 responses or 60 min, whichever occurred first. Further training proceeded overnight in a 12-h session and is referred to as automated overnight shaping. During this overnight, automated session the FR1 schedule was in effect. All animals responded well by the end of the overnight session as revealed by analysis of the cumulative record for each animal per overnight session. At the end of each testing session, the animals were returned to their cages. Analysis of fixed-ratio performance learning behavior was initiated on the following day by analyzing behavior for 15 min/day for 5 days (equals 5 session block; $n = 5$ F1 generation animals/group). The analysis was conducted for an FR1, FR5, FR10, and FR20 (Weiss and Laties, 2000). Data for five session blocks/FR were analyzed and the mean ± SEM was plotted for each FR versus lever presses/15-min acquisition session. These findings emphasize that as the complexity of the task increased from FR1 to FR5 to FR10 to FR20, the exposed offspring animals reveal significant deficits in lever pressing learning behavior which does not occur in the controls (Figure 17.9). The B(a)P exposed offspring animals simply were not able to learn the lever pressing task beyond a complexity of FR5. These results demonstrate that there is clearly a reduced capacity for behavioral learning in offspring progeny subsequent to prenatal PAH exposure. The results from these *in vivo* experiments correlate well with behavioral studies in animals and humans, which have reported deficits in learning and memory tasks produced by prenatal exposure to polychlorinated biphenyl compounds (Schantz *et al.*, 2003).

The translational relevance of our work utilizing this susceptibility exposure paradigm to assess the effects of PAH exposure during gestation is based on the fact that the earliest synapses develop during the early postnatal (PND13-15) in the mouse model. Unintended prenatal exposure of the fetus to environmental contaminants has been shown to result in alterations in neurodevelopment such as reduced head circumference and neurobehavioral deficits such as poorer outcome on selective aspects of cognitive and neuromotor functioning in both rodent and human offspring. Previous studies from our group utilized this susceptibility exposure paradigm to uncover deficits in behavioral learning in parallel with reduced expression of glutamate receptor subunits (Wormley *et al.*, 2004). As mentioned above, findings presented in a recent report from the Perera group have demonstrated for the first time that prenatal exposure to polycyclic aromatic hydrocarbons during development results in a significant reduction in mental development index at 36 months in children born to mothers in the urban cohort that was studied (Figure 17.2 above; Perera *et al.*, 2006). The phenotype of the 3-year old children born to these women who were prenatally exposed to PAHs resembled the observed phenotype of rodents from our studies where the effect of B(a)P-induced dysregulation of developmental signaling processes was to degrade neuronal function to result in significant deficits in neuronal activity, plasticity, and behavioral learning (Wormley *et al.*, 2004; Brown *et al.*, 2007).

Overall, the results from these electrophysiology and molecular studies quantify deficits in early postnatal glutamatergic receptor subunit neuronal activity, developmental expression, and behavioral learning suggesting dysregulation of temporal developmental expression of glutamate receptor subunit expression as a result of prenatal B(a)P exposure when synapses are developing for the first time.

IV. UNDERLYING MECHANISMS OF PAH NEUROTOXICITY

Polycyclic aromatic hydrocarbons exert their toxicity in the central nervous system through various mechanisms. The following is a brief overview of the pathways involved.

A. Biotransformation

The relatively high affinity of these chemicals for lipid-rich tissues (Lipniak and Brandys, 1993) leads to disposition of B(a)P in rodent brain regions (Das *et al.*, 1985). The intraregional distribution of cytochrome P450 in brain tissues contributes to quantitative differences in PAH metabolite levels (Saunders *et al.*, 2002; Brown *et al.*, 2007). Several

cytochrome P450 enzymes, such as CYP1A1, CYP1A2, CYP1B1, CYP2B, CYP2C, and CYP3A, are involved in the metabolism of PAH compounds in both animal models and humans (Shimada *et al.*, 1989; Guengerich and Shimada, 1991). Using C57BL/6J mice, Shimada *et al.* (2003) studied the induction of PAHs in brain tissue. The CYP1A1 was constitutively expressed in brain tissue at very low levels, but highly induced by B(a)P. On the other hand, CYP1B1 was significantly expressed in brain tissues. This isoform was the most highly inducible one in brain tissues. Recent studies by Kommaddi *et al.* (2007) have demonstrated the existence of a unique P4501A1 variant in human brain that failed to bioactivate PAHs. The existence of such variants provides clues why some people are vulnerable to toxicity while others are not. To what extent these novel site-specific biotransformation pathways prevail over the established epoxide and quinone pathways in causing/altering the PAH toxicity is a potential area for exploration. Besides metabolites produced in the brain by the cytochrome P450 (Ravindranath and Boyd, 1995), metabolites sequestered in the plasma lipoproteins and transported to the brain through vascular circulation (Polyakov *et al.*, 1996) also contribute to the brain PAH metabolite pool.

Though the disposition of B(a)P in rodent brain regions has long been established, studies linking neurotoxicity to the spatial and temporal distribution of PAHs and their metabolites are limited (Saunders *et al.*, 2002; Wu *et al.*, 2003; Brown *et al.*, 2007). In studies conducted by our research group, when B(a)P was administered to rats either through oral (Saunders *et al.*, 2002, 2006) or inhalation (Wu *et al.*, 2003; Brown *et al.*, 2007) routes, a concordance between temporal and spatial distribution of B(a)P metabolites and behavioral activity was noticed. Adult F-344 rats were exposed to 0**,** 25, 50, 100, and 200 mg/kg of B(a)P through oral gavage, and their motor activity was monitored at 0, 2, 4, 6, 12, 24, 48, 72, and 96 h time points. The accumulation of both B(a)P parent compound and metabolites in the striatum and hippocampus 2 h post-oral treatment of B(a)P corresponded with the onset of behavioral effects and their peak concentrations at 6 h also coincided with the behavioral deficits (Figure 17.10). These observations document that metabolism plays an important role in modulating B(a)P-induced neurotoxic effects in rats.

In addition to the behavioral changes produced by oral exposure to B(a)P in adult rats (Saunders *et al.*, 2002), we also documented behavioral deficits produced as a result of gestational exposure to B(a)P. Timed pregnant Long-Evans rats were exposed in an oral subacute exposure regimen to 0, 25, and 150 µg/kg body wt B(a)P on gestation days 14–17. On PND 2, 5, 10, 15, and 20, offspring pups were randomly selected and analyzed for B(a)P disposition and glutamate receptor subunit developmental expression. A dose-dependent increase and post-exposure time-dependent increase in B(a)P metabolite concentrations and a significant interaction effect of dose and time ($p < 0.005$) were also observed in plasma and

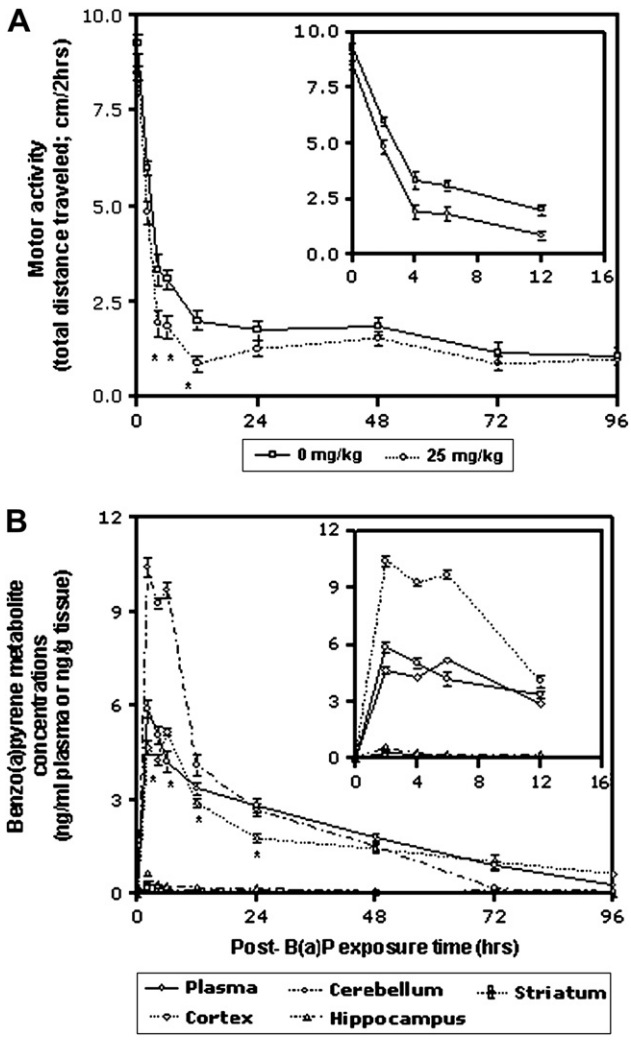

FIGURE 17.10. Relationship between motor activity (A) and disposition (B) of benzo(a)pyrene [B(a)P] metabolites in plasma, cortex, cerebellum, hippocampus, and striatum of male F-344 rats orally dosed with 25 mg/kg body weight (Saunders *et al.*, 2006). Values represent mean + SE for six rats/treatment group at each time point. * $p < 0.05$ between control and B(a)P treatment group in the case of motor activity (A), and between the time points showing peak metabolite concentrations and other time points post-B(a)P exposure (B). Data in the inset depict motor activity (A) and disposition (B) of B(a)P from 0 to 16 h time points.

cerebrocortical extracts (Figure 17.5) in offspring pups. Gestational B(a)P-exposure has also been found to reduce both hippocampal plasticity (Wormley *et al.*, 2004) and cortical neuronal activity (McCallister *et al.*, 2008; Figure 17.7). Glutamatergic receptor subunit expression ratios have also been shown to be inversely proportional to time and have paralleled with metabolite levels (Brown *et al.*, 2007).

Our studies have also revealed that the total B(a)P metabolite load in brain regions as well as the qualitative distribution of metabolites in the aforementioned brain

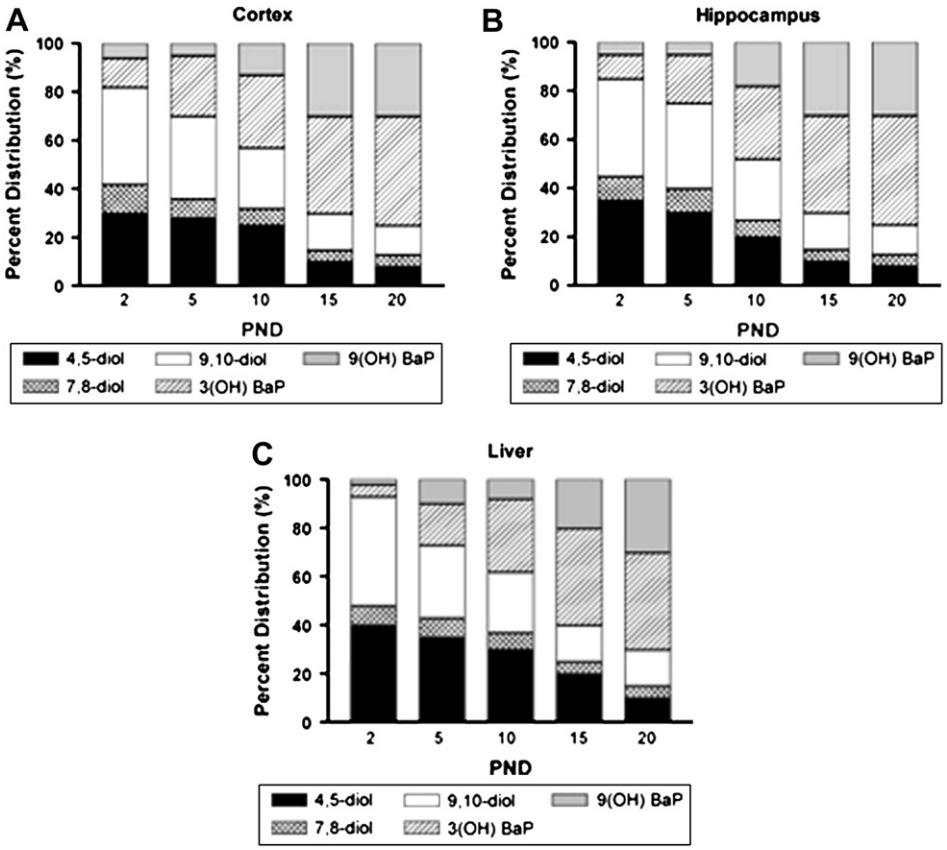

FIGURE 17.11. Percentage distribution of B(a)P metabolites in offspring pups. (A) Cerebral cortex, (B) hippocampus, and (C) liver (from Brown *et al.*, 2007). Timed pregnant dams were dosed with 25 μg B(a)P/kg BW. Offspring pups were sacrificed on PND 2, 5, 10, 15, and 20 and metabolite concentrations were determined and authenticated using standards obtained from the National Cancer Institute.

regions are important determining factors of toxicity. The concentrations of B(a)P diol metabolites (4, 5; 7, 8, and 9, 10 diols) were high up to PND 10, whereas the hydroxy metabolites (3 and 9-OH) constituted higher percentages at PND 15 and 20 (Figure 7.11). The formation of diols during this early period of development is interesting in that the diols can be converted further into dihydrodiol epoxides. From a toxicity standpoint, the dihydrodiol epoxides are important as they are very reactive to nucleophilic attack by nucleophilic sites in cellular macromolecules. The predominance of hydroxy metabolites at PND 15 and 20 indicates that the mechanism of detoxification may be more prominent at later stages of development. The total metabolite concentrations and the specific type in plasma and brain support the contention that B(a)P metabolism plays an important role in modulating neurobiological effects during early developmental expression of key synaptic proteins (McCallister *et al.*, 2008).

B. Oxidative Stress

During PAH metabolism, the uncoupling of electron transfer and oxygen reduction from monooxygenation by CYP1A1 and CYP1A2 results in the release of O_2-, H_2O_2, and OH- (Dutton *et al.*, 1989). These reactive oxygen species (ROS) or oxyradicals react with DNA, proteins, and membrane lipids in the intracellular milieu (Nebert *et al.*, 2000; Zangar *et al.*, 2004). In addition to the CYP family of enzymes, dihydrodiol dehydrogenase (Bolton *et al.*, 2000) and peroxidases (Mulder *et al.*, 2003) were reported to be involved in metabolic conversion of B(a)P to reactive and redox active *o*-quinones, resulting in the formation of ROS such as superoxide anion, H_2O_2, and hydroxyl radicals. Several laboratories have hypothesized that many cellular effects of B(a)P may be attributed to its metabolism to BaP quinones and their subsequent ability to generate intracellular ROS and lipid peroxidation (Lemiere *et al.*, 2005; Casetta *et al.*, 2005). The increase in lipid peroxidation and subsequent decrease in antioxidant defense systems may contribute to an increased susceptibility to oxidative stress (Badary *et al.*, 2003).

Since ROS are generated during metabolism, Saunders *et al.* (2006) conducted studies on antioxidant enzymes during peak periods of B(a)P-induced neurobehavioral activity to see whether neurobehavioral toxicity is driven by oxidative stress as a result of B(a)P metabolism. A correlation between time-dependent changes in motor activity and oxidative stress has been noted by these authors. For example, B(a)P caused inhibition in activity of SOD and glutathione peroxidase and an increase in catalase and lipid peroxidation at 6 and 96 h post B(a)P treatment [time periods where motor activity peaked and diminished post-B(a)P exposure], respectively. These findings reiterate that B(a)P-induced acute neurobehavioral toxicity may occur through oxidative stress due to inhibition of brain antioxidant scavenger systems.

C. Cholinesterase Inhibition

Inhibition of acetylcholinesterase (AChE) is one of the mechanisms of neurotoxicity caused by many commonly used chemicals. Acetylcholine is responsible for neurotransmission at all neuromuscular junctions as well as many synapses within the central nervous system (IPCS, 2001). Published reports demonstrate potent acetylcholinesterase inhibition *in vitro* following exposure to B(a)P (Jett *et al.*, 1999; Tzekova *et al.*, 2004). To study the PAHs increased chlorpyrifos inhibition of cholinesterase, Jett *et al.* (1999) added specific concentrations (2 to 28 μM) of pyrene, B(a)P, fluoranthene, and anthracene to an incubation mixture containing chlorpyrifos (1 to 180 nM concentrations) and purified cholinesterase. They then measured cholinesterase inhibition in the presence or absence of different concentrations of the four PAHs, noting the concentrations that caused cholinesterase to be inhibited by 50%, as well as the maximal activity of the enzyme. The data suggested that the combined effects of the PAHs were additive, but not synergistic with the effects of low concentrations of chlorpyrifos. Although all four PAHs inhibited cholinesterase activity by themselves, their potencies differed, with B(a)P having the greatest relative effect on cholinesterase in combination with chlorpyrifos, and fluoranthene having the least.

D. Tyrosine Hydroxylase Inhibition

Andersson *et al.* (1998) show marked inhibition of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine biosynthesis in the striatum and hippocampus after intracranial injections of PAH mixtures. Inhibition of TH can lead to reductions in striatal dopamine (Stephanou *et al.*, 1998; Andersson *et al.*, 1998), which may also contribute to the suppression in motor activity (Saunders *et al.*, 2006).

These results provide additional evidence that enzyme repression is an important mechanism in B(a)P-induced neurotoxicity and likely results from oxidative stress in the nervous system. Inhibition of Na^+/K^+-ATPase, an important enzyme in muscle contraction and nerve excitability, in addition to decreased motor conduction velocities may explain the suppression of motor activity observed in B(a)P intoxicated rats (Kim *et al.*, 2000; Saunders *et al.*, 2001). Furthermore, there is also strong experimental evidence showing that oxidative stress and lipid peroxidative products can cause decreases in dopamine and inhibit Na^+/K^+-ATPase activity as well (Madrigal *et al.*, 2003).

E. Monitoring and Treatment of Individuals Exposed to PAHs (First Responders, Pregnant Women)

Adverse health symptoms resulting from acute exposure to PAHs were rarely reported in humans. So far, no therapeutic agents have been developed to counteract PAH toxicity. Law enforcement personnel and deployed defense personnel injured in explosions and suspected to be exposed to PAHs can be treated in the same way as civilians and firefighters are treated after smoke inhalation. These cohorts must be followed for several years for behavioral perturbations, reproductive failure, and cancerous signs as PAHs have been reported to cause toxicity to several organ systems (Ramesh *et al.*, 2004). The serum immunoglobulin levels of exposed service personnel must be regularly monitored because immunosuppression is the first adverse effect in people occupationally exposed to PAHs (Szczeklik *et al.*, 1994). Also chromosomal damage including chromosomal aberrations (sister chromatid exchanges) in peripheral blood lymphocytes of these individuals (Cheng *et al.*, 2007) should be evaluated for several years as latency periods between occupational PAH exposure and development of cancer can last several years. Police, army and emergency management teams involved in rescue and cleanup operations (first responders) must be advised to carry portable personal monitors to keep an eye on their exposure to PAHs, similar to those used by policemen and bus drivers in epidemiology studies involving PAHs (Farmer *et al.*, 2003).

V. CONCLUDING REMARKS AND FUTURE DIRECTION

Incendiary and explosive devices are used in most terrorist attacks. As a result of combustion of fuel and hazardous materials, PAHs are released in high volumes. Exposure of civilians or deployed personnel to fumes containing PAHs constitutes an acute exposure scenario. Additionally, defense forces involved in extinguishing oil well fires, and cleanup tasks are exposed to low levels of PAHs over a more protracted time period. In addition, over 1.3 million civilian and military personnel are occupationally exposed to hydrocarbon fuels, particularly gasoline, jet fuel, diesel fuel, or kerosene on a near daily basis. Studies have reported acute or persisting neurotoxic effects from acute, subchronic, or chronic exposure of humans or animals to hydrocarbon fuels (Ritchie *et al.*, 2001), specifically burning of jet fuels, which release PAHs in considerable proportions.

The toxicity to the nervous system depends on the delivered dose and exposure duration. In the case of pregnant women, pharmacokinetic processes (absorption, distribution, metabolism, and excretion) govern PAH disposition within the mother and the nervous system of children. Moreover, unique physiological features, such as the presence of a placental barrier and the gradual development of the blood–brain barrier influence PAH disposition and thus modulate developmental neurotoxicity. Because CNS effects are dependent upon ‘‘windows of susceptibility’’ when the lowest dose and shortest duration of exposure to environmental PAHs will have the greatest negative impact on brain development, a susceptibility exposure paradigm has proven to be the most reliable model in which to study developmental insult. The intent of this chapter was to review

the relevant available literature regarding characterizing the neurotoxic effects of prenatal exposure to PAHs (a toxicant that was released as a result of the terrorist attack on the WTC) in various models. Characterizing the prenatal neurotoxic effects of PAH exposure will lead to a better understanding of the results of PAH exposures. It remains a significant challenge to continue to develop and refine appropriate investigational models that are capable of reliably assessing the consequences and underlying mechanisms by which environmental toxicants alter CNS function and behavior.

Exposures of newborns to PAHs depend on pharmacokinetic processes operating in the mother, and transfer through breast milk. Since it is difficult to characterize these pathways in humans, physiologically based pharmacokinetic (PBPK) and pharmacodynamic (PD) models need to be developed using appropriate animal models, and incorporating key parameters such as dose, exposure duration, and developmental stage (Dorman *et al.*, 2001). Thus, development of PBPK and PBPD models for PAHs is an immediate need that will help in not only characterizing the dose–response relationship, but also extrapolation of results from animal studies to humans.

Exposure to a single PAH compound seldom occurs in situations of relevance to local law enforcement agencies or defense forces. Often, people are exposed to PAHs in combination with other toxicants such as aliphatic hydrocarbons and/or metals (NRC, 2005). Information on toxicity of chemical mixtures, of which PAHs are a constituent, is lacking. Until such studies are undertaken in animal models, it is difficult to establish whether PAHs have an additive or a synergistic effect in determining neurotoxicity.

Studies are also warranted on developing biomarkers that complement a battery of neurotoxicity tests in field situations. One of the enzymes that can be used as an environmental biomarker is lactate dehydrogenase (LDH; an indicator of cell injury). It is a key enzyme in the anaerobic pathway of energy production. LDH is important for neuromuscular physiology in conditions of chemical stress when high levels of energy may be needed within a short period of time (often faced by troops deployed for a special operation). One such example of civilian and service personnel exposure is the first Persian Gulf War when departing Iraqi troops set fire to more than 600 oil wells in Kuwait. As the oil burned, the magnitude of hydrocarbons released through smoke plumes contributed to adverse health effects (Hobbs and Radke, 1992). In smoke inhalation studies involving rats, LDH activity was found to increase more than once and did not return to control values 4 weeks after exposure (cited in NRC, 1999). These findings suggest that LDH levels serve as a robust biomarker, worthy of consideration.

Since PAHs disrupt the antioxidant defense system thereby inducing oxidative stress, markers of oxidative stress such as glutathione levels, catalase, and superoxide dismutase activities could be measured in plasma along with the total blood burden of PAHs for personnel exposed to PAHs (Singh *et al.*, 2007). To determine if there is any correlation between blood PAH levels and oxidative damage, measuring the concentrations of 8-oxodG adducts in blood cells serves as a useful metric (Singh *et al.*, 2008). Recent studies also discuss the quantification of a group of compounds called F(2)-isoprostanes (which are prostaglandin F_2-like compounds derived from the nonenzymatic oxidation of arachidonic acid) as the most reliable biomarkers of oxidative stress (Milne *et al.*, 2007). A subclass of these compounds called neuroprostanes (F_4-neuroprostanes) are markers of brain lipid peroxidation and are potential therapeutic targets of neurodegenerative diseases (Montine *et al.*, 2004).

The research described in this chapter represents the first step leading to the development of novel methodology to systematically discover and develop therapeutic glutamate receptor/aryl hydrocarbon receptor antagonists that will prevent neurotoxicity associated with PAH exposure in first responders and/or pregnant women. A fundamental premise of future research takes into account the need for simultaneous temporal measurements and integrative physiological analysis of critical signaling processes to better understand the mechanisms of PAH-induced neurotoxicity. Future experiments should also define the signatures of PAH exposure-induced neurotoxicity and novel therapeutics/protectants in animal models.

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CHAPTER 18

PCBs, Dioxins, and Furans: Human Exposure and Health Effects

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I. INTRODUCTION

Polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) are members of the group of halogenated aromatic hydrocarbons (HAHs). This group of chemicals has been identified by the national and international agencies as priority environmental pollutants posing significant effects on aquatic and terrestrial animals including humans (Loganathan and Kannan, 1994; Jacobson, 1994; Van den Berg *et al.*, 1998; Kodavanti *et al.*, 2008). PCBs were produced in large quantities (millions of pounds) for a variety of industrial uses during the 1940s to the late 1970s especially by the developed nations. However, PCDDs (dioxins) and PCDFs (furans) were never produced commercially, but are formed in small quantities as byproducts of combustion of various industrial as well as natural processes. Due to their persistent, bioaccumulative and toxic properties, residues of these compounds are found in every component of the global ecosystem (Lipnick *et al.*, 2001; Kodavanti *et al.*, 2008). Some PCBs (coplanar PCBs), dioxins, and furans are three structurally and toxicologically related families of compounds that are classified as the most toxic synthetic chemical substances to a variety of animal species including humans (Tucker *et al.* 1983; Loganathan *et al.*, 1995; ATSDR, 1998). Production of PCBs was banned during the 1970s; however, already produced PCBs are still causing environmental and health problems (Loganathan *et al.*, 2008; Sajwan *et al.*, 2008). Fortunately, these compounds were never used as chemical warfare agents, weapons of mass destruction, or agents of threat or terror by contamination of air, water, food/feed, etc. However, inadvertent poisonings of these chemicals have caused significant environmental and health problems. In this chapter, historical background, chemical characteristics, analysis, pathways of human exposure to these compounds, and toxic effects associated with the exposures are presented.

II. BACKGROUND

PCBs were first synthesized in the early 1880s (Schmidt and Schultz, 1881) and commercial production began in 1929. Biphenyls were reacted with Cl_2 in the presence of ferric chloride catalyst, where some of the hydrogen atoms are replaced by chlorine atoms. PCBs were produced as complex mixtures potentially containing 209 congeners formed by chlorinating biphenyl with 1 to 10 chlorines (Figure 18.1). The amount of chlorination of biphenyls corresponded to the duration of the chlorination process. For example, Aroclor 1221, 1242, 1248, 1254, 1260, and 1268 are commercial preparations that were formerly produced by the Monsanto Chemical Company in the USA (St Louis, MO) that contain 21, 42, 48, 54, 60, and 68% chlorine by weight, respectively, as indicated by the last two digits in the numerical designation (Giesy and Kannan, 1998). The PCB mixture formulations were different depending on the country of origin, and were produced in Germany (Clofen), France (Phenoclor and Pyralene), Japan (Kanechlor), Italy (Fenclor), Russia (Sovol), and Czechoslavakia (Delor). PCB mixtures were produced for a variety of uses such as fluids in electrical transformers, capacitors, heat transfer fluids, hydraulic fluids, lubricating and cutting oils, and as additives in plastics, paints, copying paper, printing inks, adhesives, and sealants (Loganathan *et al.*, 1989; Safe, 1990).

Dioxins and furans are not produced deliberately, but are produced unintentionally as byproducts of combustions of organic matter in the presence of chlorine. Dioxins and furans consists of 135 possible chlorinated dibenzofuran and 75 chlorinated dibenzo-p-dioxins with from 1 to 8 chlorine substituents (Figure 18.2). PCDDs/DFs are found as byproducts during the manufacture of some industrial chemicals such as PCBs, polychlorinated naphthalenes, chlorinated phenols, chlorinated phenoxyacids, polychlorinated diphenyl ethers, polyvinyl chlorides, and chlorinated phenoxy-2-phenols (Hutzinger *et al.*, 1985; Hryhorczuk *et al.*, 1986; ATSDR, 2001; Masunaga *et al.*,

Generalized structures of highly toxic chlorinated organic compounds

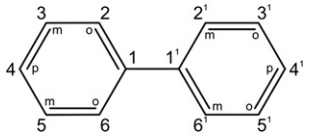
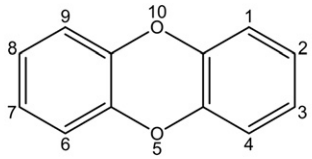
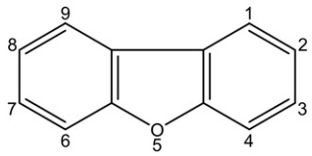
Compound	Molecular mass	Chemical structure
PCBs	188-498	
PCDDs	218-460	
PCDFs	202-444	

FIGURE 18.1. Generalized structures of PCBs, dioxins, and furans.

2001a). These compounds are also formed during incineration of industrial and municipal waste, forest fires, fireplaces, and combustion engines (Loganathan *et al.*, 1997; Feil and Larsen, 2001). Due to anthropogenic as well as natural processes, PCBs, dioxins, and furans are widely dispersed in the global environment and their presence was reported in air, water, soil, sediment, aquatic and terrestrial organisms including human tissues (Loganathan and Kannan, 1994; Safe, 1994; Giesy and Kannan, 1998; Masunaga *et al.*, 2001b; Ogura *et al.*, 2001).

III. HUMAN EXPOSURE TO PCBs, PCDDs, AND PCDFs

Direct human exposure to PCBs/PCDF occurred due to inadvertent poisoning by consumption of PCB contaminated food: Yusho and Yucheng poisoning (oil disease) in Japan and Tailwan during 1968 and 1979, respectively are examples of such food poisoning. The Yusho Support Center Report (2007) states that ''39 years have passed since the outbreak of YUSHO, the PCB/dioxin tragedy – the most unprecedented incident in the history of mankind whereby people ingested toxic chemicals unknowingly, directly through food''. The outbreak of a strange disease ''Yusho'' (Kanemi Oil Poisoning) occurred in the western part of Japan in 1968. The major symptoms and signs of the disease consisted of acne form eruptions, pigmentation of the skin, nails, and conjunctivae, increased discharge from the eyes, and numbness in the limbs (Yao *et al.*, 2002; Yusho Support Center Report, 2007). The epidemic was identified later (1969) to be an unprecedented mass food poisoning caused by the ingestion of commercial brand rice oil that had been contaminated by PCBs and their related compounds. The number of people who were reported to have ingested the rice oil was about 14,000 and 1,867 persons were designated as Yusho victims. A similar outbreak ''Oil Disease'' occurred in Taiwan in 1979. Toxicological studies revealed that PCDF congeners, including 2,3,4,7,8-pentachlorodibenzofuran, played an important role in the manifestation of the above mentioned diseases. Research conducted on Yusho victims revealed harmful effects of exposure which continued for two generations (Yusho Support Center Report, 2007).

Direct human exposure to dioxin occurred in southern Vietnam and also in Seveso, Italy. It was estimated that southern Vietnam has been contaminated by 160 to 600 kg of dioxin as a result of 80 million liters of defoliant herbicides (Agent Orange, a 50:50 mixture of 2,4,5-T and 2,4-D) being sprayed by the US military over a large area of forests and crops of southern Vietnam from 1962 to 1971 (Westing, 1984; Schecter, 2006; Le Hong Thorn, 2007). The defoliant was contaminated with a very toxic form of dioxin (TCDD) known to have caused adverse effects on human health. Dioxin may cause harmful effects on whole body and can affect separately the functioning of systems such as the nervous system, immune responses, carcinogenicity, hepatotoxicity, metabolic and enzyme toxicity (Le Hong Thorn, 2007). Another example of direct human poisoning of dioxin was the food poisoning of Mr Viktor Yushchenko (See Figure 18.3), the Ukrainian presidential candidate, in 2004. Dioxin poisoning caused a mysterious illness that resulted in his face becoming pockmarked and ashen (www.Mindfully.org). Manahan (1989) classified dioxin (2,3,7,8-TCDD) as super toxic in comparison with other known toxic substances (Table 18.1).

FIGURE 18.3. Dioxin poisoning – Ukrainian former Prime Minister and presidential candidate Mr Viktor Yushchenko, with his face disfigured by illness due to dioxin poisoning. Photo source: www.mindfully.org; date: November 19, 2004 (accessed on July 13, 2008).

TABLE 18.1. Toxicity level of various chemical compounds (prepared from Manahan, 1989)

Toxicity level	Compounds	LD_{50} estimated from laboratory animals[a]
Slightly toxic	Ethyl alcohol	10,000 mg/kg
	Sodium chloride	5,000 mg/kg
Moderately toxic	Malathion (organophosphorus pesticide)	1,000 mg/kg
	Chlordane (termite exterminator)	500 mg/kg
Very toxic	Heptachlor (pesticide)	100 mg/kg
	Parathion (pesticide)	10 mg/kg
Extremely toxic	Dioxin (2,3,7,8-TCDD)	5 mg/kg (hamster)
	Tetraethyl pyrophosphate (pesticide, raticide)	1 mg/kg
	Tetrodotoxin (toxin of blowfish)	0.1 mg/kg
Super toxic	Dioxin (2,3,7,8-TCDD)	0.0006 mg/kg (guinea pig)
	Botulin (toxin of botulinum)	0.00001 mg/kg

[a] LD_{50} are rough values estimated form oral-dose experiment on laboratory animals (usually rats). Unit: mg/kg food

IV. PHYSICOCHEMICAL PROPERTIES AND GLOBAL DISTRIBUTION

The unusual industrial versatility of PCBs was directly related to physical and chemical properties which include resistance to acids and bases, compatibility with organic materials, resistance to oxidation and reduction, excellent insulating properties, nonflammability, and thermal stability (Hutzinger, 1985). Physical and chemical stability of PCBs is vital to the industrial applications and the same properties have been responsible for global environment contamination. In addition, multimedia releases and volatility lead to long-range environmental transport, both via water and atmosphere, resulting in widespread environmental contamination of humans and wildlife at sites distant from their use (Lipnick and Muir, 2001; Loganathan *et al.*, 2008). In PCBs, dioxins, and furans, the properties vary widely and depend on the number and position of chlorine atoms attached to the molecule. In general, vapor pressure, water solubility, and biodegradability decrease with the increasing number of chlorine atoms. Lipophilicity adsorption capacity shows a reverse trend (Loganathan and Kannan, 1994). Because of these unique properties, PCBs, dioxins, and furans have been detected in air and water (rivers, lake ecosystems) (Pearson *et al.*, 1997; Loganathan *et al.*, 1998a, b, 2001). Apart from this, these compounds were recorded in fish, birds, and marine mammals of several other ecosystems such as Atlantic, Baltic, and Pacific ocean (Kawano *et al.*, 1988; Loganathan *et al.*, 1999). They have been identified in processed fish and other food products (Kannan *et al.*, 1997; Patandin *et al.*, 1999). Further, the residues of these contaminants were found in human adipose tissue, blood, and milk and also in numerous other matrices (Loganathan *et al.*, 1993, 1998a, b, 1999; Petreas *et al.*, 2001; Czaja *et al.*, 2001). Because of the large production and indiscriminate use of PCBs in industries, these contaminants extended their boundaries of distribution over the global environment and this was evidenced by their detection even in pristine environmental media and biota such as the Arctic and Antarctic atmosphere, hydrosphere, and biosphere (Muir *et al.*, 1988; Corsolini *et al.*, 2002; Kumar *et al.*, 2002). The discovery of the widespread environmental occurrence, the increased general environmental concern, and the apparent link to carcinogenesis and other health disorders prompted public outcry which resulted in prohibition of PCBs as well as chlorinated pesticides in several developed nations during the early 1970s. The following section deals with the chemical analysis of PCBs, dioxins, and furans in environmental and biological samples.

V. ANALYTICAL METHODS

PCBs and PCDDs/DFs consist of a total of 419 individual congeners. These congeners have quite a variety of toxicity and some, especially planar dioxins, furans, and dioxin-like PCBs (non-*ortho* chlorine substituted coplanar PCBs), are extremely toxic even at very low concentrations (Table 18.1). Therefore, determination of some toxic congeners to a very low concentration (parts per trillion) has become important.

Congener-specific determination was required for those congeners that have toxic equivalency factors (TEFs), namely 2,3,7,8-chlorine substituted dioxins and dioxin-like PCBs (Table 18.2). Thus, they are analyzed using high-resolution gas chromatography–high-resolution mass spectrometry (HRGC-HRMS). A schematic flow chart of the representative analysis procedure for environmental and biological samples is shown in Figure 18.4 based on standard analytical methods such as US EPA Method 1613, 1668, 1668a and JIS K0312 (US EPA, 1994, 1997, 1999; JISC, 2008).

Biological samples are either freeze dried or dewatered (homogenized) with anhydrous sodium sulfate salt and spiked with internal standards (cleanup spike) and then extracted using Soxhlet apparatus. Obtained extracts are concentrated and their solvents are changed to an appropriate solvent such as hexane. Then, the sample extracts are taken through a series of cleanup procedures to remove lipid and other interfering chemicals. Then eluates are concentrated again and spiked with internal standards. The prepared sample extracts were injected into HRGC-HRMS and monitored by multiple ion monitoring mode. Concentrations of target analytes are calculated by the isotope dilution method. Stable isotope labeled target compounds are used as internal standards and spiked into samples. Calibration with internal standards and determination by isotope dilution are necessary to obtain reliable data under very low concentration and after repeated pretreatment and cleanup procedures.

Solid samples such as soil and sediments are air dried and extracted using Soxhlet/Dean-Stark apparatus. Aqueous samples are separated into solids and filtrates by filters. Solids are Soxhlet extracted similar to biological samples and filtrates are liquid–liquid extracted. These extracts are then cleaned up and injected into HRGC-HRMS, similar to biological samples.

In cases when congener-specific information is not necessary, other simpler methods can be used. EPA Methods 608 and 8082a use GC/ECD to determine the concentration of PCBs in terms of Aroclor (Federal Register, 1984; US EPA, 2007). EPA Method 680 (US EPA, 1985) uses GC/MS (low resolution mass spectrometry) to determine the homolog concentration of PCBs.

VI. MECHANISM OF ACTION AND TOXICITY

The toxicity of PCBs and PCDDs/DFs is complicated by the presence of large numbers of congeners, each with its own toxicity. Commercial PCB mixtures elicit a broad spectrum of toxic responses that are dependent on several

factors including chlorine content, purity, dose, species, age and sex, and duration of exposure. Immunotoxicity, carcinogenicity, and developmental toxicity as well as biochemical effects of commercial PCB mixtures have been studied extensively in various laboratory animals, fish, and wildlife (Giesy and Kannan, 1998). Several studies have confirmed the common receptor mediated mechanism of action of toxic halogenated aromatics and have resulted in the development of a structure–activity relationship for this class of chemicals (Safe, 1990). The most toxic halogenated aromatic compound is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) which is assigned the maximum toxicity factor of 1, and the relative toxicities of individual halogenated aromatics have been determined relative to TCDD (i.e. toxic equivalents). The 17 congeners of PCDD/DFs and the 12 congeners of dioxin-like PCBs are assigned TEFs lower than TCDD (Table 18.2). In June 1997, the World Health Organization established the levels of toxicity factors (WHO-TEFs) to be applied to evaluating the risks for humans and animals (WHO-TEF, 1998). The WHO consultation set the tolerable daily intake (TDI) between 1 and 4 pg TEQ/kg body weight, emphasizing that the aim was to lower the TDI to a level under 1 pg TEQ/kg body weight (Guerzoni and Raccanelli, 2004). The most toxic PCB congeners are those that have chlorine substitution in most of the non-*ortho* positions such as 3, 4,

TABLE 18.2. Toxic equivalency factors (TEFs) for dioxins and dioxin-like PCBs

TEF SYSTEM	I-TEF	WHO-TEF (1998)			WHO-TEF (2006)
Congener	Human	Human & mammals	Fish	Bird	Human & mammals
PCDDs					
2,3,7,8-TCDD	1	1	1	1	1
1,2,3,7,8-PeCDD	0.5	1	1	1	1
1,2,3,4,7,8-HxCDD	0.1	0.1	0.5	0.05	0.1
1,2,3,6,7,8-HxCDD	0.1	0.1	0.01	0.01	0.1
1,2,3,7,8,9-HxCDD	0.1	0.1	0.01	0.1	0.1
1,2,3,4,6,7,8-HpCDD	0.1	0.01	0.001	<0.001	0.01
OctaCDD	0.001	0.0001	–	–	0.0003
PCDFs					
2,3,7,8-TCDF	0.1	0.1	0.05	1	0.1
1,2,3,7,8-PeCDF	0.05	0.05	0.05	0.1	0.03
2,3,4,7,8-PeCDF	0.5	0.5	0.5	1	0.3
1,2,3,4,7,8-HxCDF	0.1	0.1	0.1	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1	0.1	0.1	0.1
1,2,3,7,8,9-HxCDF	0.1	0.1	0.1	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1	0.1	0.1	0.1
1,2,3,4,6,7,8-HpCDF	0.01	0.01	0.01	0.01	0.01
1,2,3,4,7,8,9-HpCDF	0.01	0.01	0.01	0.01	0.01
OctaCDF	0.001	0.0001	0.0001	0.0001	0.0003
Non-ortho-PCBs					
3,4,4′,5—TeCB (#81)		0.0001	0.0005	0.1	0.0003
3,3′,4,4′—TeCB (#77)		0.0001	0.0001	0.05	0.0001
3,3′,4,4′,5—PeCB (#126)		0.1	0.005	0.1	0.1
3,3′,4,4′,5,5′—HxCB (#169)		0.01	0.00005	0.001	0.03
Mono-ortho-PCBs					
2,3,3′,4,4′—PeCB (#105)		0.0001	<0.000005	0.0001	0.00003
2,3,4,4′,5—PeCB (#114)		0.0005	<0.000005	0.0001	0.00003
2,3′,4,4′,5—PeCB (#118)		0.0001	<0.000005	0.00001	0.00003
2′,3,4,4′,5—PeCB (#123)		0.0001	<0.000005	0.00001	0.00003
2,3,3′,4,4′,5—HxCB (#156)		0.0005	<0.000005	0.0001	0.00003
2,3,3′,4,4′,5′—HxCB (#157)		0.0005	<0.000005	0.0001	0.00003
2,3′,4,4′,5,5′—HxCB (#167)		0.00001	<0.000005	0.00001	0.00003
2,3,3′,4,4′,5,5′—HpCB (#189)		0.0001	<0.000005	0.00001	0.00003

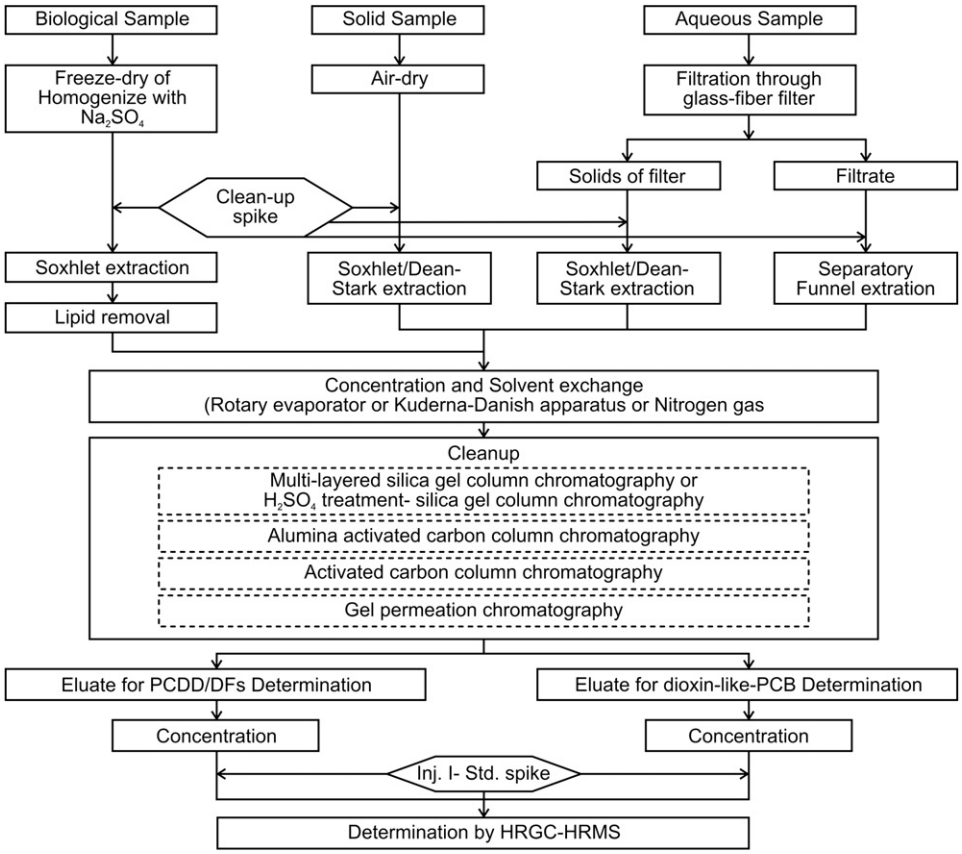


FIGURE 18.4. Analytical procedures of dioxins and ''dioxin-like PCBs''.

and 5 in each ring. These coplanar PCB congeners (Figure 18.2) are structurally similar to highly toxic 2,3,7,8-TCDD and exhibit similar toxic responses (Ah-receptor mediated toxicity) (Figure 18.5). 2,3,7,8-TCDD and structurally related halogenated aromatic compounds induce a variety of microsomal enzymes primarily in the liver. 2,3,7,8-TCDD evokes dose-related induction of cytochrome-P-450-associated AHH (aryl hydrocarbon

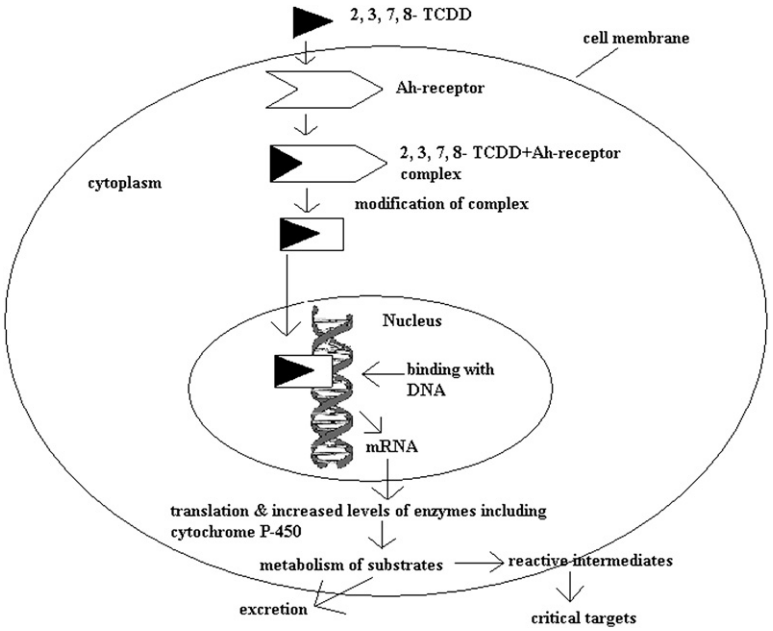


FIGURE 18.5. Possible mechanism of toxic action of 2,3,7,8-TCDD.

hydroxylase) activity. The most widely studied of these responses is the induction of AHH and EROD (markers of CYP1A activity) in mammalian cell cultures and in laboratory rodents (Goldstein and Safe, 1989).

Ah-receptor mediated toxicity resulted in wide range of biological responses, including alterations in metabolic pathways, body weight loss, thymic atrophy, impaired immune responses, hepatotoxicity, chloracne and related skin lesions, developmental and reproductive effects, and neoplasia.

VII. CONCLUDING REMARKS AND FUTURE DIRECTION

PCBs, dioxins, and furans are persistent organic pollutants (POPs) which have negative effects on the environment and health of humans including skin toxicity, immunotoxicity, neurotoxicity, negative effects on reproduction, teratogenicity, endocrine disruption, and a predisposition to cancer. A major pathway of exposure to these chemicals is through consumption of food contaminated by these chemicals. The Committee of Experts on Food of the European Commission proposed a dose called ''Tolerable Weekly Intake'' (TWI) given by the total of dioxins and PCBs of 14 pg TEQ per kg of body weight, that is an average of 2 pg TEQ/day/kg of body weight (Guerzoni and Raccanelli, 2004). By reducing the environmental contamination, we can diminish the food chain accumulation and ultimately we can reduce the intake levels of PCBs and dioxins and their toxic effects.

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CHAPTER 19

Cyanide Toxicity and its Treatment

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I. INTRODUCTION

Cyanide refers to a highly toxic chemical compound containing one atom of carbon and nitrogen each. Deaths due to cyanide poisoning are relatively rare largely due to its limited availability (Musshoff *et al.*, 2002). However, the name cyanide and its toxic characteristic have long been a source of dread for humans. The first reported isolation of hydrocyanic acid from Prussian blue was by the Swedish chemist C.W. Scheele in 1782 and he was reported to be the first victim of this poison in 1786 when he accidentally broke a vial of the chemical in the laboratory and died from vapor poisoning (Ballantyne, 1987).

Since the days of ancient Rome, cyanide and its derivatives were used in weapons (Sykes, 1981). Nero used cherry laurel water, which contained cyanide as its main toxic component, to poison members of his family and others who displeased him. Napolean III proposed the lacing of soldiers' bayonets with cyanides during the Franco-Prussian War. Hydrogen cyanide (HCN) was not very successful as a chemical warfare agent during World War I because it was not persistent. During the same period, the French army employed artillery shells under the name Vincennite (50% HCN, 30% arsenic trichloride, 15% stannic chloride, and 5% chloroform) but due to high volatility of HCN, these munitions could not be used effectively. Also, German soldiers were adequately equipped to protect themselves from HCN exposure. In 1916, the French experimented with cyanogen chloride which was heavier and less volatile than HCN. The effect of cyanogen chloride was similar to HCN. Additionally, it caused marked lacrimation, rhinorrhea, and bronchial secretions similar to phosgene. However, these effects were considered to be of little military importance compared to its tissue effects. During World War II, the Nazis used Zyklon B (briquettes of calcium sulfate impregnated with 40% HCN) made by IG Farben, Germany, to exterminate millions of civilians and enemy soldiers in the death camps (Robinson, 1971; Baskin, 1998). It is also reported that in the late 1980s, there was the possible use of cyanide against the inhabitants of the Syrian city of Hama, the inhabitants of the Kurdish city of Halabja, Iraq, and in Shahabad, Iran, during the Iran–Iraq war (Baskin and Rockwood, 2002). Cyanide gas precursor compounds were found in several subway restrooms in Tokyo following the release of sarin in Tokyo in 1995 (Sidell, 1996). Allegedly, cyanide was added to the explosives used in the first attack on the World Trade Center in New York (Brennan *et al.*, 1999).

The use of cyanides for murder, suicide, and accidentally has been passably reviewed by Gee (1987). In 1973, 109 passengers and in 1980, 303 pilgrims were feared killed in Paris and Riyadh, respectively, due to inhalation of HCN from smoldering plastic inside aircraft which accidentally caught fire (Mohler, 1975; Weger, 1983). In 1985, the explosion of a Boeing 737 during take-off at Manchester, England, revealed that 20% of the 137 victims who escaped had dangerously high levels of carbon monoxide, while 90% had significantly elevated levels of cyanide. Similarly, short circuits in electrical wires also caused fire on ships which led to the combustion of plastic materials releasing HCN, which resulted in severe poisoning (Levine *et al.*, 1978). The murders of two Ukrainians, Mr Rebet (1957) and Mr Bandera (1959), were committed by a Soviet agent in Munich using a gas pistol containing cyanide (Anders, 1963). In September 1982, seven people in Chicago died when cyanide (acetatminophen, manufactured by McNeill Consumer Products Co., Fort Washington, PA), was illicitly placed in bottles of Tylenol (Wolnick *et al.*, 1984). In 1978, 912 members of a religious sect in Port Kaituma, Guyana, committed mass suicide after consuming a grape-flavored drink laced with cyanide (Thompson *et al.*, 1987; Sidell *et al.*, 1997). The first case of illegal euthanasia in Spain was reported when a tetraplegic patient, whose repeated legal requests for euthanasia were refused, consumed potassium cyanide (KCN) (Blanco and Garcia, 2004).

The toxicology of cyanide and its treatment modalities have been extensively discussed by many authors (Vennesland *et al.*, 1981; Way, 1984; Ballantyne and Marrs, 1987; Gonzales and Sabatini, 1989; Borowitz *et al.*, 1992; Salkowski and Penney, 1994; Marrs *et al.*, 1996; Baskin and Brewer, 1997). Additionally, over the years thousands of research articles on cyanide have appeared. Cyanide and its effects, being such an old poison, seem to be known universally. But most of the crucial molecular mechanisms underlying cyanide toxicity have been elucidated only in the recent past (Gunasekar *et al.*, 1996; Sun *et al.*, 1997;

Borowitz *et al.*, 2001). Cyanide is a very interesting molecule; it has always fascinated researchers, and is the reason for further study. This chapter attempts to enlighten the reader with the known facts about cyanide and some of the recent advances made in its toxicology and antagonism.

II. SOURCES OF EXPOSURE

The use of cyanide for military purposes has been very insignificant and most of the cases of poisoning have involved civilians, for example in the case of fires and industrial accidents. The human to cyanide interaction is very common due to its wide industrial applications. Worldwide industrial consumption of cyanide is estimated to be 1.5 million tons per year, and occupational exposures account for a significant number of cyanide poisonings (Cummings, 2004). Cyanide poisoning may occur from a broad range of exposures and is summarized in Table 19.1. Some of the major sources of poisoning are as follows.

A. Fire Smoke

Residential fires may result in life-threatening poisoning (Megarbane *et al.*, 2003). In a closed space, fire induces a combination of oxygen deprivation directly related to combustion, and simultaneous intoxication by asphyxiant and irritant gases (Baud, 2007). Cyanide can be liberated during combustion of products containing carbon and nitrogen. These products include wool, silk, polyurethane, polyacrylonitriles, melamine resins, and synthetic rubber (Vogel *et al.*, 1981; Bismuth *et al.*, 1987; Homan, 1987). It is estimated that significant levels of cyanide are present in almost 35% of all fire victims (Clark *et al.*, 1981; Barillo *et al.*, 1994; Sauer and Keim, 2001). Carbon monoxide is also an important component of fire smoke and its concomitant exposure with HCN is known to exacerbate the toxicity as both cause tissue hypoxia by different mechanisms (Barillo *et al.*, 1994).

TABLE 19.1. Various possible sources of cyanide poisoning

Fire smoke	Smoke generated after combustion of silk, polyurethanes, polyacrylonitriles, nylon, melamine resins, plastics, etc., in accidents including industrial, residential, car, aircraft, ship fire
Industrial exposure	Plastics production, dyeing, printing and photography, fumigation of pesticides/ rodenticides, synthetic rubber production, fertilizer production, metal polish, tanning in leather industry, electroplating, metallurgy, paper and textile manufacture
Drugs	Sodium nitroprusside, Laetrile, Succinonitrile
Dietary	Cassava, lima beans, linseed, bamboo sprout, macadamia nuts, hydrangea, Rosaceae family (plum, peach, pear, apple, bitter almond, cherry), *Sorghum* species (Johnson grass, sorghum, Sudan grass, arrow grass), *Linum* species (flax, yellow pine flax)
Others	Cigarette smoking, phencyclidine synthesis, ingestion of nail polish remover, suicide, homicide, terrorist attack, chemical warfare, capital punishment

B. Industrial Exposure

Occupational exposures to cyanide in industry have caused serious problems (Blanc *et al.*, 1985; Peden *et al.*, 1986). Cyanide is used in many chemical syntheses, plastics and rubber processing, gold and silver extraction, tanning, metallurgy, photography, and fumigation of pesticides/ rodenticides (ATSDR, 1997). Cyanide poisoning can also occur following contamination of the skin with cyanide solution or inhalation of released HCN during electroplating in jewelry polishing (Sullivan and Krieger, 2001; Megarbane *et al.*, 2003). Exposure to silver brightener, which contains cyanide, has also caused death. Subsequent to initial lethal inhalation of cyanide, an extensive post-mortem transcutaneous diffusion of cyanide was also shown to occur (Seidl *et al.*, 2003). Cyanogens are complex nitrile-containing materials which can generate free cyanide of toxicological significance. Many of the cyanogens occur naturally or are produced synthetically. The synthetic cyanogens have a variety of industrial, domestic, and therapeutic uses (Ballantyne, 1987). Some of the synthetic cyanogens include acetonitrile, acrylonitrile, adiponitrile, malononitrile, propionitrile, succinonitrile, sodium nitroprusside, tetremethyl succinonitrile, etc. Fires involving nitrogen-containing polymers, often found in fibers used in fabrics, upholstery covers, and padding, produce HCN (Tsuchiya and Sumi, 1977).

C. Drugs

Cyanide is a metabolic product of amygdalin (Laetrile®) which was introduced as an antineoplastic agent in the 1950s, and was responsible for several cyanide poisoning cases (Hall *et al.*, 1986; Bromley *et al.*, 2005). Intestinal beta-d-glucosidase digests the amygdalin, releasing HCN. Also, iatrogenic exposure to cyanide may result following the use of sodium nitroprusside, an anti-hypertensive agent (Vesey and Cole, 1985) and succinonitrile, an antidepressant (Ryan, 1998). Sodium nitroprusside is used medicinally as Nipride® and its intravenous infusion is used to lower blood pressure in hypertensive emergencies. This application of sodium nitroprusside occasionally causes classical cyanide toxicity (Kurt, 1983). Death due to mercuric

cyanide or mercuric oxycyanide poisoning was reported due to possible ingestion of an antiseptic or a hair lotion commercialized in France (Labat *et al.*, 2004).

D. Dietary

Cyanide occurs naturally in cassava (*Manihot esculenta* Crantz) as linimarin, a cyanogenic glycoside. Cassava roots are a major source of energy for millions of people in the tropics. However, chronic ingestion of cassava-based foods accounts for several toxicological effects, including various neuropathies, amblyopia, etc. (Rosling, 1989; Kamalu, 1995). Several common plants also contain cyanogenic glycosides and their ingestion can result in death due to improper processing (Conn, 1988). The family Rosaceae, which includes plum, peach, pear, apple, bitter almond, and cherry, is responsible for many cases of cyanide poisoning (Ryan, 1998).

E. Other Sources

There are numerous other instances of cyanide poisoning which include illicit synthesis of phencyclidine, ingestion of nail polish remover (acetonitrile), in terrorist attacks, as suicidal, homicidal, and chemical warfare agents, and in capital punishment. HCN was considered to be a notorious chemical warfare agent because of its rapid lethal actions. It was used as a war gas in World War I (Gee, 1987), World War II (Williams and Wallace, 1989), and in the Iran–Iraq War (Lang *et al.*, 1986; Heylin, 1988). Due to poor persistence of HCN in the environment and lack of delivery system, cyanides were not optimistically considered for future use in wars. However, its possible use in local terrorist actions cannot be overlooked. Because cyanide can be released from both synthetic and natural materials, terrorist acts with explosives or incendiaries, which could cause fire in enclosed spaces, make cyanide and cyanogenic compounds potential threats for terrorism (Rotenberg, 2003). During the last decade, Japan has experienced the largest burden of chemical terrorism-related events in the world, which included the Nagano cyanide incident in 1998, and two intentional cyanide releases in Tokyo subway and railway station restrooms, which were thwarted in 1995 (Okumura *et al.*, 2003). Because of the natural cyanide found in tobacco, cigarette smokers show significant increase in blood cyanide levels as compared to nonsmokers. Visual abnormalities of tobacco amblyopia are usually associated with heavy smoking and vitamin B_{12} deficiency (Wilson, 1983; Homan, 1987).

III. TOXIC LEVELS OF CYANIDE

It is not easy to determine the lethal doses of cyanide to man. Morbidity or mortality depends upon the magnitude of poisoning, which varies with the dose and form of cyanide and the route of poisoning. The maximum permissible concentration for HCN in human is 11 mg/m^3 (Ballantyne, 1974). Taken orally the fatal dose of HCN to adults is estimated at 50–100 mg, and for KCN about 150–250 mg (DuBois and Geiling, 1959). Figures estimated in terms of body weight for HCN vary from 0.7 to 3.5 mg/kg (Hallstrom and Moller, 1945). However, victims ingesting as much as 3.0 g of KCN have been saved with immediate therapy (Van Heijst *et al.*, 1987). Table 19.2 shows that inhalation of HCN at a concentration of 300 mg/m^3 (approximately 270 ppm) will be immediately fatal while at 20–40 mg/m^3 mild symptoms will appear after several hours of exposure (Rumack, 1983; FOA, 1992). Victims having a blood cyanide level of 2.5–3.0 μg/ml frequently succumb to respiratory cessation within 20–30 min of exposure or may survive even up to 3 h (Ballantyne, 1974; Van Heijst *et al.*, 1987). Oral ingestion of cyanide may also produce rapid onset of symptoms because many human doses far exceed the minimal lethal dose. Studies on experimental animals have shown that absorption of cyanide decreases with a more alkaline stomach and that normally most cyanide is absorbed within 2–3 h of ingestion (Ryan, 1998). However, substances such as nitrile compounds and amygdalin from plants require enough time for conversion to cyanide before they can produce symptoms of toxicity (Ryan, 1998). Laeterile is synthesized from amygdalin. One gram of laterile contains the equivalent of 60 mg of cyanide, and each laeterile tablet may contain up to 100 mg of laeterile. A 12- to 18-tablet laeterile overdose is sufficient to produce severe metabolic acidosis and convulsions (Ellenhorn *et al.*, 1997). The lethal toxicity of HCN and its alkali salts by different routes for different species of animals and sexes has been largely discussed elsewhere (Ballantyne, 1984, 1987). A study carried out in rabbits revealed the following order of decreasing toxicity of KCN administered by different routes: intravenous > intramuscular > intraperitoneal > per oral > instillation into conjunctival sac > percutaneous (Ballantyne, 1984). The comparative per oral LD_{50} values of KCN for different species of animals are given in Table 19.3. Rabbit was found to be more susceptible than rat and mouse.

TABLE 19.2. Toxicity of hydrogen cyanide by inhalation

Concentration		
mg/m^3	**ppm**	**Effects**
20–40	18–36	Slight symptoms after several hours
50–60	45–54	Endurable for 20–60 min without effect
120–150	110–135	Very dangerous (fatal) after 30–60 min
150	135	Lethal after 30 min
200	181	Lethal after 10 min
300	270	Immediately lethal

TABLE 19.3. Acute lethal toxicity of KCN by oral route for different species of animals

Species	Sex	LD_{50} (mg/kg)	Reference
Mouse	Male	8.50	Sheehy and Way, 1968
	Male	12.5	Bhattacharya *et al.*, 2002
Rat	Male	10.0	Hayes, 1967
	Female	7.49	Ballantyne, 1984
	Female	14.1	Bhattacharya and Vijayaraghavan, 2002
Rabbit	Female	5.82	Ballantyne, 1984

IV. DETECTION AND ESTIMATION OF CYANIDE

Determination of cyanide or its metabolites in biological fluids is necessary for forensic, clinical, military, research, and veterinary purposes. The choice of analytical methods depends on a variety of factors like sensitivity, specificity, rapidity, convenience, facilities, and expertise. The selection of biological sample, time of sampling, time to analyze and storage conditions, and interfering substances are other factors which influence the choice of analytical methods (Troup and Ballantyne, 1987). There are several convenient and sensitive methods for measuring cyanide in biological fluids but many of them have limitations. Some of the methods are summarized below.

One of the most common procedures includes diffusion and trapping of cyanide in the alkaline media prior to colorimetric analysis in pyridine–pyrazolone mixture (Epstein, 1947). This method was subsequently modified for microdiffusion analysis of cyanide (Feldstein and Klendshoj, 1954). This procedure is widely used during the treatment of cyanide intoxication and thiosulfate is known to interfere in the colorimetric estimation, which was subsequently resolved (Morgan *et al.*, 1979). A rapid (about 20 min), specific, and sensitive spectrophotometric method for whole blood cyanide assay has also been developed (LaForge *et al.*, 1994). All these procedures are based on the König reaction, which starts with the production of cyanogen chloride. A spectrofluorometric determination of cyanide and thiocyanate based on modified König reaction in a flow-injection system was also reported with detection limits of 30 nM for both anions (Tanaka *et al.*, 1992). Spectrophotofluorometry is also a convenient, sensitive method provided prior microdiffusion is carried out to isolate and concentrate the cyanide. The fluorometric methods using pyridoxal are more sensitive and require fewer and more stable reagents than the colorimetric method (Takanashi and Tamura, 1970). However, sodium thiosulfate is known to interfere with the chemical conversion of the fluorophore and it is possible to circumvent the interference by using acetate buffer (pH 5.2) as the acidifying agent. Another fluorescent method with an advantage over pyridoxal uses para-benzoquinone and this method is not known to have any extraneous interference (Guilbault, 1976).

The potentiometric determination of cyanide using ion selective electrodes has become yet another very popular technique, because it is convenient, rapid, and sensitive method of analysis (Frant *et al.*, 1972). Microdiffusion of biological samples containing cyanide is recommended prior to potentiometric determination. The use of a cyanide ion-selective electrode in combination with the Conway microdiffusion method for the measurement of cyanide concentrations in human red blood cells and plasma was reported with remarkable recovery of cyanide (Yagi *et al.*, 1990). Ion chromatographic determination of sulfide and cyanide in real matrices by using pulsed amperometric detection on a silver electrode was reported by Giuriati *et al.* (2004).

The measurement of HCN directly by gas chromatography has also been reported but this method lacks sensitivity with most detectors (Valentour *et al.*, 1974). Gas chromatographic techniques are not widely used for measuring cyanide because other methods are more convenient. A simple and sensitive method was devised for determining cyanide and its major metabolite, thiocyanate, in blood using an extractive alkylating agent (pentafluorobenzyl bromide). The detection limits of cyanide and thiocyanate were 0.01 and 0.003 μmol/ml, respectively (Kage *et al.*, 1996). Rapid quantitation of cyanide in whole blood by automated headspace gas chromatography was performed on clinical samples from fire victims. This method could detect a wide concentration of blood cyanide (30–6,000 μg/l) in about 17 min (Calafat and Stanfill, 2002).

A direct and sensitive isotope dilution-mass spectrometry determination of blood cyanide by headspace gas chromatography was developed with the detection limit of 0.3 μmol/l (Dumas *et al.*, 2005). This method was also compared with other techniques in a round robin exercise. Cyanide can also be measured by indirect atomic absorption spectrometry where a metal–cyanide complex is formed which is then extracted in organic solvent to determine the metal content (Manahan and Kunkel, 1973). An original high-performance liquid chromatographic-mass spectrometric (HPLC-MS) procedure was developed for the determination of cyanide in whole blood. The limits of detection and quantitation were 5 and 15 ng/ml, respectively (Tracqui *et al.*, 2002). Also, several other methods including HPLC, using post-column derivatization with *o*-phthalaldehyde (Sumiyoshi *et al.*, 1995), capillary electrophoresis with fluorescence detection (Chinaka *et al.*, 2001), polyphenol oxidase/clay biosensors (Shan *et al.*, 2004), capillary electrophoresis microchip (Lu *et al.*, 2004), ICT-based probes (Badugu *et al.*, 2005), and micro-chemiluminiscence (Lv *et al.*, 2005) have been reported for different environmental or biological samples. However, their utility to detect cyanide in blood samples is yet to be ascertained.

A technique to detect cyanide currently utilized by water treatment facilities was employed to rapidly detect concentrations of cyanide in the clinically important range (Rella *et al.*, 2004). The CYANTESMO test strips accurately and rapidly detect cyanide greater than 1 μg/ml. A paper test for cyanide (CYANTOSNO) in whole blood is now commercially available in the USA (Ellenhorn *et al.*, 1997).

V. TOXICOKINETICS OF CYANIDE

A. Absorption

Oral absorption of cyanide is rapid, and toxic effects can occur within minutes. When salts of cyanide are ingested, hydrochloric acid in the stomach causes the release of HCN, which is readily absorbed as cyanide ion (CN^-). Cyanide (hydrocyanic acid, HCN) is a small molecule with good lipid and water solubility. It is rapidly absorbed irrespective of route of exposure owing to its low molecular weight (Borowitz *et al.*, 2001). It is mainly absorbed by the respiratory and gastrointestinal mucosa and through skin and eyes on prolonged contact (Ballantyne, 1974; Ellenhorn *et al.*, 1997; Ryan, 1998). In experimental rabbits, cyanide introduced into the conjunctival sacs was quickly absorbed in significant quantities to produce systemic toxicity (Ballantyne, 1983). Dermal exposure is rare but large surface area of the skin facilitates absorption of toxic consequences. Nitriles are more readily absorbed through the skin but the onset of toxicity is delayed.

B. Distribution

After absorption, cyanide distributes to a volume of approximately 40% total body weight. The distribution is rapid and is completed within 5 min after a single intravenous dose (Sylvester *et al.*, 1983). Cyanide is rapidly transported in the body by blood and about 60% is bound to plasma proteins, a small amount is present in the red blood cells (RBC), and the remainder present as free cyanide (Ryan, 1998). The concentration of cyanide in RBC is estimated to be at a RBC/plasma ratio of 100/1 (Ellenhorn *et al.*, 1997). After acute exposures, the plasma elimination half-life of cyanide was observed to be 14.1 min (Egekeze and Oehme, 1979). After oral poisoning, a significant amount of cyanide was traced in the brain, blood, kidney, stomach wall, liver, and urine (Ansell and Lewis, 1970). This indicates that after absorption, cyanide is widely distributed in all the tissues.

C. Elimination

Cyanide is rapidly detoxified in mammals. The detoxification rates of cyanide following intravenous administration in humans, dogs, guinea pigs, and rabbits are 0.017, 0.020, 0.04, and 0.008 mg/kg body weight/min, respectively (Hinwich and Saunders, 1948; NIOSH, 1975). The major pathway of cyanide detoxification (approximately 80%) is through enzymatic transulfuration to thiocyanate (SCN^-), which is nontoxic and excreted in the urine (Lang, 1933). This reaction is catalyzed by an enzyme, rhodanese (thiosulfate cyanide sulfur transferase; EC.2.8.1.1). Rhodanese uses a precursor like thiosulfate which is a source of sulfane sulfur (divalent ionized sulfur bound to another sulfur atom). The endogenous supply of this substance is very limited. Therefore, detoxification of cyanide largely depends on an exogenous supply of thiosulfate (Westley *et al.*, 1983). However, cyanide detoxification by this pathway is often debated because rhodanese is located principally in the mitochondria and penetration of the cell wall and mitochondrial membrane by thiosulfates is very slow (Bhat and Linell, 1983). It is presumed that the sulfane sulfur binds first to the serum albumin to yield a sulfane sulfur albumin complex which eventually reacts with cyanide to form thiocyanate (Way, 1984; Westley *et al.*, 1983). In normal metabolism of cyanide, the serum albumin–sulfane complex may be the primary detoxification mechanism (Sylvester *et al.*, 1983). Unlike other chemical warfare agents, cyanide appears biologically in blood, urine, and expired breath (Lundquist *et al.*, 1988). Cyanide is eliminated from the body by several mechanisms. After 3 h, approximately 90% of injected cyanide has been shown to be eliminated in the dog model (Sylvester *et al.*, 1983). A small amount of cyanide is excreted in the urine and via the lungs after being incorporated into cyanocobalamin (vitamin B_{12}), oxidated to formate and carbon dioxide, and incorporated with cystine (Ballantyne, 1987). Other minor pathways of detoxification include enzymes like mercaptopyruvate sulfurtransferase, thiosulfate reductase, and cystathionase γ-lyase or disulfide cystine, 2-iminothiazolidine-4-carboxylic acid (2-ICA) or its tautomer, 2-aminothiazolidine-4-carboxylic acid (2-ACA) (Wood and Cooley, 1956; Baskin *et al.*, 2004). Cyanide reacts with cystine to produce β-thiocyanoalanine, which spontaneously undergoes ring closure to form 2-ICA and 2-ACA, depending on the pH in the cells (Borowitz *et al.*, 2001). To determine the effect of species on cyanide metabolism, toxicokinetics of cyanide was studied in rats, pigs, and goats after oral dosing of KCN (Sousa *et al.*, 2003). The study showed that metabolism of cyanide and its main metabolite, thiocyanate, is species linked, with goat being the most sensitive to the toxic effects of cyanide.

VI. MECHANISM OF ACTION

The toxic effect of cyanide is attributed predominantly to the production of anoxia following inhibition of the metal-containing enzymes. The critical interaction appears to be the inhibition of the terminal respiratory chain enzyme, cytochrome oxidase a_3 (containing iron) within the mitochondria. The enzyme is essential for the production of adenosine triphosphate (ATP). As a result, aerobic oxidative

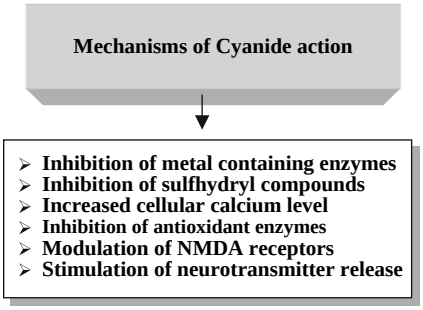

FIGURE 19.1. Mechanisms for the toxic manifestations of cyanide exposure.

metabolism and phosphorylation are impaired leading to cellular hypoxia. The pyruvate that is produced can no longer be used and is now reduced to lactate. The shunt from aerobic to anaerobic metabolism leads to profound lactic acidosis (Solomonson, 1981). Cyanide toxicity may not be ascribed solely to a single biochemical lesion but a complex phenomenon. Cyanide reacts with several metalloenzymes, carbonyl groups of different enzymes, coenzymes, and substrates, resulting in inhibition of normal activity. Cyanide also interacts with sulfhydryl compounds like cystine, mercaptopyruvate, reduced glutathione, and oxidized glutathione to form different complexes (Way, 1984; Borowitz *et al.*, 1992). Cyanide also strongly interacts with iron in protein molecules, inhibiting enzymes including carbonic anhydrase and succinic dehydrogenase (Ballantyne, 1987). Formation of cyanhemoglobin by interaction of cyanide with ferric iron abolishes the ability of hemoglobin to carry oxygen (Way, 1984). The mechanism of cyanide toxicity is summarized in Figure 19.1.

Cyanide is regarded as a selective neurotoxin and its toxicity has frequently been associated with elevated levels of cellular calcium (Johnson *et al.*, 1986), inhibition of antioxidant defense enzymes in the brain (Ardelt *et al.*, 1989), and generation of reactive oxygen species, leading to lipid peroxidation (Ardelt *et al.*, 1994; Kanthasamy *et al.*, 1997). Cyanide raises intracellular calcium through activation of voltage-sensitive calcium channels (Johnson *et al.*, 1987), direct redox modulation and enhancement of *N*-methyl-D-aspartate (NMDA) receptor function (Patel *et al.*, 1992; Sun *et al.*, 1997), and mobilization of intracellular calcium stores (Yang *et al.*, 1997). It was proposed that elevated cytosolic calcium activates proteases, which in turn converts xanthine dehydrogenase to xanthine oxidase. In the presence of oxygen, xanthine oxidase can catalyze the formation of superoxide radicals that initiate lipid peroxidation (Figure 19.2) (Chan and Fishman, 1985). Modulation of NMDA receptor has been widely implicated in cyanide-induced neurotoxicity. Nitric oxide and reactive oxygen species generation after NMDA receptor activation was found to mediate cyanide-induced neurotoxicity (Gunasekar *et al.*, 1996). Several other studies also showed activation of NMDA receptors during cyanide toxicity (Patel *et al.*, 1992; Sun *et al.*, 1997). Cyanide can stimulate release of glutamate from intracellular stores resulting in elevation of cytosolic Ca^{2+} through NMDA receptor activation. There is also evidence that cyanide may interact directly with NMDA receptor to enhance NMDA receptor-mediated Ca^{2+} influx (Patel *et al.*, 1994; Sun *et al.*, 1995). It was further shown that cyanide selectively interacted with NMDA subunits, possibly by formation of thiocyanate adduct with a cysteine residue located in NR1 receptor subtype (Arden *et al.*, 1998). Cyanide is also known to

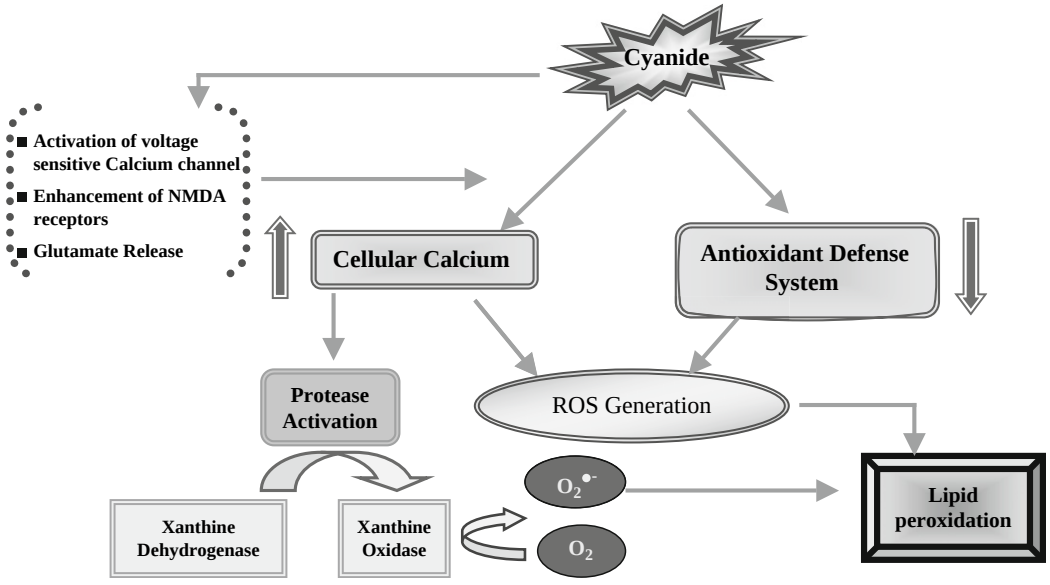

FIGURE 19.2. Pathway of cyanide-induced oxidative stress. Abbreviations used: ROS – reactive oxygen species; O_2 – Oxygen; $O_2^{\bullet-}$ – superoxide anion.

interact with cystine to produce 2-ICA and 2-ACA, and the former is responsible for memory loss, convulsions, and loss of consciousness (Bitner *et al.*, 1991). Cyanide can produce dopaminergic toxicity characterized by loss of dopaminergic neurons in the basal ganglia that is accompanied by impaired motor function (Kanthasamy *et al.*, 1994). Cyanide is also known to stimulate neurotransmitter release in both the central and peripheral nervous systems (Kanthasamy *et al.*, 1991). It is suggested that the convulsive effects of cyanide are due to changes in the levels of dopamine (Cassel, 1995). It has also been opined that protein kinase C, calmodulin, and nitric oxide (NO)-dependent cyclic guanosine monophosphate (GMP)-dependent enzymes may contribute to the induction of convulsions. ATP depletion may also contribute in part to the development of cyanide-induced convulsions (Yamamoto, 1995). Other biochemical processes that may mediate or at least influence cyanide toxicity include lactic acidosis, mitochondrial ADP ribosylation, and hyperammonemia (Borowitz *et al.*, 1992). It is possible that some of these mechanisms are independent of cellular energy deficit but produce severe effects on the cells. Overall, it appears that oxidative stress plays a crucial role in cyanide-induced neurotoxicity (Ardelt *et al.*, 1989; Kanthasamy *et al.*, 1994). Another important aspect of cyanide toxicity is its cardiotoxic manifestations. However, it has been excluded from the present chapter as it has been adequately discussed by others (Baskin, 1991; Borowitz *et al.*, 1992).

VII. DIAGNOSIS AND CLINICAL FEATURES OF CYANIDE POISONING

Signs and symptoms of acute cyanide poisoning are often nonspecific and vary in both time and intensity depending upon the scale of exposure (Ballantyne, 1987; Ellenhorn *et al.*, 1997). Low concentrations may produce delayed and various nonspecific symptoms like headache, dizziness, nausea, vomiting, confusion, coma, and incontinence of feces and urine, but exposure to high concentrations may lead to a series of events like dyspnea, incoordination of movements, convulsive seizures, coma, and cardiac and/or respiratory failure culminating in death (Ballantyne, 1974; Way, 1984). Pathologically, no particular lesions can define cyanide toxicity, although animal experiments indicate that the lesions are principally in the central nervous system (CNS), particularly necrosis in the white matter (Ballantyne, 1974). Probably the most widespread pathologic condition attributed to chronic cyanide poisoning is tropic ataxic neuropathy following cassava consumption (Rosling, 1989; Kamalu, 1995). Other effects associated with cassava consumption include tropical pancreatitis (Gee Varghese, 1985), tropical neuropathies (Banea *et al.*, 1997), and endemic goiter (Abuye *et al.*, 1998). Repeated or prolonged skin contact to low concentrations of KCN dust may lead to dermatitic effects (cyanide rash) characterized by itching and skin eruptions (NOHSC, 1993).

TABLE 19.4. Whole blood cyanide levels and clinical signs and symptoms

Whole blood cyanide		
µg/ml	**µmol/l**	**Signs and symptoms**
0–0.5	8–20	No symptoms
0.5–1.0	20–38	Tachycardia, flushing, headache, hyperpnea, dizziness
1.0–2.5	48–95	CNS depression including giddiness, tachypnea, nausea, vomiting feeling, suffocation, confusion
2.5–3.0	95–114	Respiratory depression, convulsion, coma, cyanosis, apnea, circulatory collapse, fixed dilated pupils
≥3.0	114	Death

While the signs and symptoms of cyanide poisoning are well documented, more than 55 signs and six biochemical idiosyncrasies are known to possibly occur during cyanide poisoning (Hall *et al.*, 1987). Signs and symptoms of acute cyanide poisoning reflect cellular hypoxia and are often nonspecific. Onset of symptoms depends on dose, route, and duration of exposure. In general, the signs and symptoms correlate with the whole blood cyanide level, and are usually tachycardia, flushing, headache, hyperpnea, and dizziness (Table 19.4). Laboratory tests suggestive of cyanide intoxication include: arterial blood gases (metabolic acidosis with normal PO_2), serum electrolytes (elevated anion gap), central venous percent O_2 saturation (elevated), calculated arterial percent O_2 saturation (normal), and measured arterial percent O_2 saturation (decreased). Quantitative determination of cyanide in whole blood, urine, gastric contents and tissues, and plasma thiocyanate levels are also important (Ballantyne, 1983). Unfortunately, these tests take several hours and the results may not be available to the clinician during the acute phase of poison management. Usually the ''Lee-Jones test'' is a quick bedside test that can qualitatively detect cyanide in gastric aspirate but may give false positive cases with many drugs (Hall *et al.*, 1987).

VIII. TREATMENT OF CYANIDE POISONING

The onset of toxicity after cyanide poisoning is very fast. The prognosis of the victim depends on termination of further exposure, supportive care, and institution of immediate and aggressive specific treatment. Early diagnosis and clinical information would enhance the chances of recovery. The first principle of therapy is termination of further exposure, which can be facilitated by the following: (a) remove the victim from the contaminated atmosphere,

(b) apply a protective mask for the patient as soon as possible to prevent further inhalation, (c) remove off any liquid on skin or clothing as soon as possible, (d) remove all contaminated clothing; rinse skin with soap and copious amounts of water or water alone if there is liquid on the skin, and (e) gavage and administer activated charcoal if cyanide was ingested (Baskin and Brewer, 1997).

A. Supportive Therapy

The details of supportive therapy are presented in Table 19.5. Briefly, prior to specific therapy, the patient is given supportive aid. This includes mechanical airway support, artificial ventilation with 100% oxygen, possibly delivered via an Ambu bag containing the contents of two ampoules of amyl nitrite (0.6 ml), and cardiac monitoring (Van Heijst *et al.*, 1987). Anecdotal evidence suggests that hyperbaric oxygen augments the protective efficacy of nitrite–thiosulfate therapy (Goodhart, 1994). Lactic acidosis resulting from anaerobic metabolism and convulsions should be treated with intravenous administration of sodium bicarbonate and diazepam, respectively (Van Heijst and Meredith, 1990; Baskin and Brewer, 1997). The use of antidotes should be restricted to patients in deep coma with respiratory insufficiency.

B. Specific Antidotal Therapy

Patients who are critical and do not satisfactorily respond to supportive therapy should be administered specific cyanide antidotes as outlined in Table 19.5. Cyanide antidotes have been classified into three main groups based on their mechanism of action: (1) methemoglobin inducers, (2) sulfur donors, and (3) cobalt compounds. The definitive treatment of cyanide poisoning differs in various countries due to different medical practices and guidelines. The safety and efficacy of all the antidotes are still debated upon. There is no worldwide consensus for treatment of cyanide intoxication.

1. Methemoglobin Inducers

The basic aim of rapid detoxification of cyanide is prevention or reversal of inhibition of cytochrome oxidase by cyanide. This is usually facilitated by providing a large pool of ferric iron in the form of methemoglobin to complex cyanide. Cyanide preferentially competes with the Fe^{3+} of methemoglobin as compared to that of cytochrome oxidase, and eventually binds with the former to form cyanmethemoglobin. Thereby, the activity of inhibited cytochrome oxidase is restored (Baskin *et al.*, 1992). The various methemoglobin inducers employed as cyanide antidotes are discussed below.

a. Amyl Nitrite

Although inhalation of amyl nitrite as a first aid measure to cyanide poisoning has been known for many years (Pedigo, 1888), its efficacy as a methemoglobin inducer is often disputed due to its inability to generate methemoglobin greater than 6% (Jandorf and Bodansky, 1946). About 15% of methemoglobin is required to challenge one LD_{50} of cyanide (Van Heijst *et al.*, 1987). The protective effect of amyl nitrite is attributed to its vasodilatory effect which can reverse the early cyanide-induced vasoconstriction (Van Heijst and Meredith, 1990). Artificial ventilation with amyl nitrite broken into Ambu bags has been reported as a life-saving therapy in cyanide poisoned dogs, prior to induction of significant levels of methemoglobinemia (Vick and Froehlich, 1985).

b. Sodium Nitrite

Sodium nitrite is the most prevalent drug for cyanide poisoning. It takes about 12 min to generate approximately

TABLE 19.5. Supportive therapy and specific antidotal therapy for acute cyanide poisoning

	Agents	Drug category and action
Supportive therapy	100% oxygen or hyperbaric oxygen	Oxygen: Potentiates the efficacy of nitrite–thiosulfate therapy
	Sodium bicarbonate	Alkalizing agent: Corrects lactic acidosis
	Diazepam	Anticonvulsant: Depresses CNS activities
	Epinephrine	Sympathomimetic: Improves coronary and cerebral blood flow, corrects anaphylactoid reactions
Specific antidotal therapy	Amyl nitrite Sodium nitrite 4-Dimethylaminophenol	Methemoglobin inducers: Converts hemoglobin to methemoglobin which binds with cyanide to form cyanmethemoglobin. In the presence of sodium thiosulfate cyanide is excreted as thiocyanate
	Sodium thiosulfate	Sulfur donors: Facilitates enzymatic conversion of cyanide to thiocyanate
	Dicobalt edetate Hydroxocobalamin	Cobalt compounds: Forms stable metal complexes with cyanide

40% of methemoglobin after intravenous administration of the recommended dose (Van Heijst *et al.*, 1987). In spite of this delay in inducing a significant level of methemoglobinemia, a reasonable protection offered by sodium nitrite can be attributed to its vasodilatory effects (Van Heijst and Meredith, 1990). A major drawback with sodium nitrite is that it causes serious cardiovascular embarrassment, particularly in children (Berlin, 1970). Since methemoglobinemia impairs oxygen transport, sodium nitrite cannot be recommended for fire victims where concomitant exposure of HCN and carbon monoxide usually occurs. Since carbon monoxide also reduces the oxygen carrying capacity of blood, administration of sodium nitrite would further aggravate the underlying hypoxic condition. Sodium nitrite is also contraindicated for individuals with glucose-6-phosphate dehydrogenase (G6PD) deficient red cells because of the possibility of serious hemolytic reactions (Way, 1984). Excessive levels of methemoglobin are known to be reverted by intravenous administration of 30 ml of 1% methylene blue solution (Van Heijst *et al.*, 1987). In the USA, the Lilly Cyanide Antidote Kit (manufactured by Eli Lilly and Company, Indianapolis, IN) includes 10 ml ampoules of 3% sodium nitrite solution and 50 ml of 25% sodium thiosulfate solution. The kit also contains amyl nitrite encased in glass ''pearls'' which are meant to be broken so the drug could be inhaled. In India, a similar kit is manufactured by Troikaa Pharmaceuticals Ltd, Thiol.

c. 4-Dimethylaminophenol

The relatively slow rate of methemoglobin formation by sodium nitrite prompted the development of rapid methemoglobin formers like aminophenols. 4-Dimethylaminophenol (DMAP) is the treatment of choice for cyanide poisoning in Germany. Given intravenously, a dose of 3.25 mg/kg DMAP was reported to produce methemoglobin levels of 30% within 10 min and 15% methemoglobinemia was attained within 1 min without any immediate effect on the cardiovascular system (Kiese and Weger, 1969). However, there are differences in individual susceptibility to DMAP which may result in undesirable levels of methemoglobin even after normal therapeutic doses (Van Dijk *et al.*, 1987). Intramuscular injection of DMAP results in local abscess and fever. Its clinical utility remains limited on account of its other toxicological implications like nephrotoxicity (Weger, 1983).

2. Sulfur Donors

After the initial therapy of methemoglobin inducers, the cyanide has to be converted to thiocyanate which is eliminated in urine. This enzymatic detoxification of cyanide is facilitated by a sulfur donor like sodium thiosulfate. The mechanism of this reaction was discussed earlier under elimination of cyanide. High tissue oxygen markedly potentiates the effects of this reaction. In cases where methemoglobin formation is not desirable, sodium thiosulfate together with oxygen alone is sufficient. The utility of thiosulfate alone is limited because of its short biological half-life and its small volume of distribution (Sylvester *et al.*, 1983). Also, thiosulfate is contraindicated in patients with renal insufficiency as the thiocyanate formed may cause toxicity (Van Heijst and Meredith, 1990).

3. Cobalt Compounds

The cobalt ion, which forms a stable metal complex with cyanide, is an effective therapeutic agent against cyanide poisoning (Evans, 1964; Hillman *et al.*, 1974; Linell, 1987). Various cobalt-containing compounds known to antagonize cyanide poisoning are discussed below.

a. Dicobalt Edetate (Kelocyanor)

This compound chelates cyanide as cobalticyanide. This drug is known to antagonize cyanide more quickly than the nitrites but its clear superiority has not been established. Intravenous administration of 300 mg of dicobalt edetate in glucose solution is the current treatment of choice in France and the United Kingdom. Serious side effects like vomiting, urticaria, anaphylactoid shock, hypotension, and ventricular arrythmias have been reported in patients receiving Kelocyanor (Van Heijst and Meredith, 1990).

b. Hydroxocobalamin (Cyanokit)

With the exchange of the hydroxy group of hydroxocobalamin (vitamin B_{12a}) for cyanide, nontoxic cyanocobalamin (vitamin B_{12}) is formed (Hall and Rumack, 1987). An injectable solution of hydroxocobalamin (5 g in water) has been used in France and Germany. In France a 4 g hydroxocobalamin solution in 80 ml of sodium thiosulfate has also been used (Van Heijst and Meredith, 1990). EMD Pharmeceutical Company has produced a lyophilized packaging of 2.5 g of hydroxocobalamin that can be readily reconstituted in a 100 ml sodium chloride solution. This product is pending FDA approval. It has been in use in Europe since 1996 as the Cyanokit™. Hydroxocobalamin is also used in other countries, including Sweden, Denmark, Spain, Japan, and Hong Kong. Now, sodium thiosulfate or hydroxocobalamin has also been recommended for empiric treatment of cyanide poisoning (Hall *et al.*, 2007). There are several disadvantages in the clinical use of this drug. It has a relatively short half-life as it decomposes in light, and the dose required to counter cyanide poisoning is quite large. Also, recorded side effects include anaphylactoid reactions and acne (Van Heijst and Meredith, 1990). However, in a case study, clinical laboratory data did not show any evidence of toxicity (Borron *et al.*, 2007).

C. Investigational Drugs

Numerous agents analogous to the specific antidotes or compounds belonging to different pharmacological classes have also been evaluated against experimental cyanide poisoning. The cardiovascular implications and poor pharmacokinetics of nitrite led to evaluation of yet another group

of methemoglobin formers like aminophenones and derivatives. Out of all these compounds, *p*-aminopropiophenone (PAPP) was considered to be the most suitable agent for prophylaxis (Marrs and Bright, 1986; Bright, 1987). Hydroxylamine was examined as a rapid methemoglobin former with anticonvulsive properties, which conferred significant protection against acute cyanide poisoning (Kruszyna, *et al.*, 1982). Combinations of sodium nitrite with DMAP or hydroxylamine were found to provide sustained prophylaxis against acute cyanide poisoning in rats by virtue of rapid, protracted, and desired levels of methemoglobinemia (Bhattacharya *et al.*, 1991, 1993). However, toxicity of these regimens prevented their further use (Bhattacharya *et al.*, 1995). A number of 8-aminoqunoline analogs of primaquine (e.g. WR242511) were also studied as potential prophylactic drugs because they induced elevated levels of methemoglobin for a longer duration (Steinhaus *et al.*, 1990). Stroma-free methemoglobin solution (SFMS) was yet another alternative treatment proposed for cyanide poisoning (Ten Eyck *et al.*, 1985). SFMS was formed by oxidizing outdated human blood and intravenous administration of this solution did not impair the oxygen carrying capacity of blood as caused by most other methemoglobin formers. It was shown to directly sequester cyanide to protect a 4 × LD_{90} dose of sodium cyanide in rats. Among sulfur donors, several compounds containing sulfane sulfur, like polythionates, thiosulfonates, persulfides, etc., were examined as cyanide antidotes (Isom and Johnson, 1987). Compounds containing more lipophilic sulfane sulfur (e.g. ICD1021) or those which can be actively transported into the cells to mediate enzymatic detoxification of cyanide were synthesized as new anti-cyanide drugs (Baskin *et al.*, 1999). Antagonism of cyanide intoxication with murine carrier erythrocytes containing bovine rhodanese and sodium thiosulfate has been demonstrated (Cannon *et al.*, 1994; Petrikovics *et al.*, 1995). Exogenous supplementation of the enzyme has been reported to accelerate the transulfuration of cyanide to thiocyanate. However, stability and sensitivity of the enzyme remain to be addressed. Several cobalt compounds were also evaluated against experimental cyanide poisoning but none of them except cobalt edetate and hydroxocobalamin could be put to human use (Way, 1984; Linnell, 1987; McGuinn *et al.*, 1994).

Cyanide is a nucleophile known to react with various carbonyl moieties like ketones and aldehydes to yield cyanohydrin derivatives (Morrison and Boyd, 1976). Sodium pyruvate (Schwartz *et al.*, 1979), α-ketoglutarate (Moore *et al.*, 1986), pyridoxal-5′-phosphate (Keniston *et al.*, 1987), and many other carbonyl compounds and their metabolites or nutrients which interact with cyanide to form cyanohydrin complexes (Way, 1984; Niknahad *et al.*, 1994; Bhattacharya and Tulsawani, 2008) have been reported to afford significant protection against acute cyanide poisoning *in vitro* or *in vivo*. Out of all these agents, α-ketoglutarate, either alone or with sodium thiosulfate, has been considered to be a promising antidote for cyanide (Borowitz *et al.*, 1992). In a recent study, α-ketoglutarate and *N*-acetyl cysteine were found to protect rat pheochromocytoma (PC12) cells from cyanide-induced cytotoxicity and altered energy metabolism (Satpute *et al.*, 2008). *N*-acetyl cysteine, a free radical scavenger, was earlier found to attenuate cyanide-induced cytotoxicity and DNA damage in isolated rat thymocytes (Bhattacharya and Lakshmana Rao, 1997). Also, in several other acute (Tulswani and Bhattacharya, 2007) and subacute studies (Tulsawani *et al.*, 2005), α-ketoglutarate was found to antagonize cyanide poisoning in experimental animals. This agent is being actively pursued as an oral treatment for cyanide poisoning, particularly in instances where nitrites are not recommended (Bhattacharya, 2004). At present, phase-I clinical trials with α-ketoglutarate are being conducted in India.

There are several other compounds which are regarded as nonspecific cyanide antidotes, and their mechanisms have been only tentatively explained. These compounds are usually not very effective *per se* but as adjuncts significantly augment the efficacy of conventional antidotes. Chlorpromazine (phenothiazine), phenoxybenzamine (α-adrenergic blocker), centrophenoxine (neuroregulator), etomidate (nonbarbiturate hypnotic and anticonvulsant), naloxone (morphine antagonist), and flunarizine, verapamil, and diltiazem (calcium-channel blockers) are some of the compounds (Way, 2004; Marrs, 1988; Bhattacharya, 2000). Additionally, a few more compounds have been found to antagonize cyanide poisoning in the recent past. They include protein kinase C inhibitor (Maduh *et al.*, 1995), nitric oxide generator (Sun *et al.*, 1995), endothelium-derived relaxing factor/nitric oxide releasers or calcium ionophore A23187 (Baskin *et al.*, 1996), local anesthetic, procaine hydrochloride (Jiang *et al.*, 1998), and antioxidants like melatonin, a pineal hormone (Yamamoto and Tang, 1996) and its metabolite, 6-hydroxymelatonin (Maharaj *et al.*, 2003). In general, antioxidants are found to be good adjuncts to nitrite–thiosulfate therapy. Another study has shown that ATP, encapsulated in unilamellar fusogenic lipid vesicle when injected intraperitoneally in mice prior to cyanide, significantly increased the survival time and the fatal dose of cyanide (Chiang *et al.*, 2004). Above all, extracorporeal filtering or hemodialysis has also been found to supplement specific cyanide antidotes. This would hasten the elimination of cyanide and its metabolites. Hemodialysis achieved dramatic response in patients with severe acidosis (Wesson *et al.*, 1985). This is only an anecdotal case report and has not been substantiated further.

IX. CONCLUDING REMARKS AND FUTURE DIRECTION

In today's scenario, cyanide is considered to be neither an important CW agent nor an agent of choice for suicidal or homicidal purposes. However, threat from its possible use in local terrorism cannot be overlooked. Also, the occupational

hazard of cyanide is likely to increase with its escalating industrial use. Serious epidemiological studies and reporting in poison control centers can be observed in many developed nations. But many cases of fatal cyanide poisoning from fire smoke, drug side effects, dietary sources, and industrial exposures may go undetected due to poor surveillance in many countries. Cyanide toxicity is mainly ascribed to its ability to inhibit cytochrome oxidase, an end-chain enzyme of cellular respiration. Numerous new mechanisms of action of cyanide unfold the complex toxic phenomena occurring at cellular and molecular levels. Many of these mechanisms have been the target for pharmacological interventions. Although very primitive, the combination of sodium nitrite and sodium thiosulfate still continues to be the most favored treatment for cyanide poisoning. Hydroxocobalamin has also been used as a cyanide antidote with considerable success in the recent past. If we could circumvent the side effects of this drug, possibly it would be the most successful second-generation antidote for cyanide. Cyanide antagonism by scavengers like carbonyl compounds, particularly α-ketoglutarate, has also been very encouraging in animal models. Further clinical trials would determine its safety for human use. Also, the stability of this molecule needs to be addressed. Perhaps these kinds of molecules could be of use for occupational exposures, chronic dietary cyanide poisoning or as prophylaxis for fire-fighters/personnel engaged in evacuation operations in cyanide-contaminated areas. Another area of interest would be the development of field-based detection systems for rapid diagnosis of cyanide poisoning or for retrospective detection of cyanide in biological samples. This is a prerequisite for effective therapeutic measures.

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CHAPTER 20

Carbon Monoxide: From Public Health Risk to Painless Killer

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Carbon monoxide causes more health problems and death every year than any other poison or many together.
Lewin (1920) – Translation from German, Pankow (2000)

I. INTRODUCTION

The colorless carbon monoxide (CO) is everywhere. Wherever there is combustion there is CO; it is the predominant product above 800°C. The concentration of CO might vary from 0.1 ppm in clean atmosphere to 5,000 ppm in the proximity of domestic wood fire chimneys (Fawcett *et al.*, 1992) and is present in significant quantities in cigarette smoke (Hartridge, 1920; Hoffman *et al.*, 2001). The atmospheric lifetime of CO is 1 to 2 months, which allows its intercontinental transport (Akimoto, 2003).

The universal presence and unsuspecting inhalation of CO make it the most common source of poisoning in the modern world and the leading cause of unnatural death in the USA. However, CO is not on the list of chemicals used as chemical weapons in wars (Elsayed and Salem, 2006) although there are reports that the Nazis used CO gas against Jewish people in and around 1941 since CO was cheaper than bullets.

Mysterious illnesses, strange visions, and inexplicable deaths of entire households, generally attributed to haunted houses, are most likely due to malfunctioning chimneys resulting in carbon monoxide poisoning. Even nonlethal concentrations of CO can exert dramatic effects in the presence of pre-existing cardiac diseases. Continued exposure to relatively high, yet nonlethal, concentrations of CO can adversely affect a pregnant woman and her fetus. CO is a contributory factor in the adverse effects of smoking (Akrawi and Benumof, 1997; United Nations Environment Program and WHO, 1979); moreover smokers occasionally set the entire home afire producing excessive quantities of CO (Sacks and Nelson, 1994).

CO is also endogenously generated. Indeed, CO is a bit like other endogenous gases of great physiological and pathological significance like O_2, NO, and hydrogen sulfide (H_2S). CO also shares some properties with hydrogen cyanide (HCN) in so far as both produce hypoxia.

Given the toxicological and epidemiological importance of CO, it has been the subject of several books and monographs since 1920 (Bouletreau, 1970; Bour and Ledingham, 1967; Buchwald *et al.*, 1979; Coburn *et al.*, 1977; Cooper, 1966; Drinker, 1938; Flandin and Guillemin, 1942; Gras, 1967; Grut, 1949; Hamilton, 1921; IPCS, 1999; Jain, 1990; Lewin, 1920; Mannaioni *et al.*, 2006; Nicloux, 1925; Pankow, 1981; Penny, 1996; Putz *et al.*, 1976; Raymond and Vallaud, 1950; Shephard, 1983; Tiunov and Kustov, 1980; Von Oettingen, 1944). CO has also been a subject of numerous reviews some of which are referred to here (Beck, 1927; Blumenthal, 2001; Ernst and Zibrak, 1998; Fenn, 1970; Goldsmith and Landow, 1968; Gorman *et al.*, 2003; Kao and Nanagas, 2006; Killick, 1940; Lawther, 1975; Lilienthal, 1950; McGrath, 2006; Morandi and Eisenbud, 1980; Penny, 1990; Piantadosi, 2002; Prockop and Chichkova, 2007; Pugh, 1959; Raub *et al.*, 2006; Root, 1965; Ryter and Otterbein, 2004; Thom and Keim, 1989; Von Berg, 1999; Weaver, 1999; Winter and Miller, 1976).

During the last 20 years, however, the attention of researchers has shifted from the toxicity of CO to its physiological role (Snyder and Ferris, 2000; Wu and Wang, 2005). As a result the toxicity of CO is sometimes not mentioned as much as it should be. For example, the first edition of the textbook *The Pharmacological Basis of Therapeutics* by Louis Goodman and Alfred Gilman published in 1941 (MacMillan, New York) devoted five and one-quarter pages to carbon monoxide; the 11th edition of the same book edited by L.L. Brunton, J.H. Lazo, and K.L. Parker and published in 2006 (McGraw-Hill) mentions nothing under the heading of carbon monoxide; the word carbon monoxide appears only in relation to the therapeutic uses of oxygen. Similarly, the *Pharmacological Reviews* had the subject reviewed twice – first in 1950 (Lilienthal, 1950), when CO was known for its toxicity, and the second 55 years later (Wu and Wang, 2005), which only deals with its physiological role.

The pharmacology of CO spans a broad spectrum, from public health risk to endogenous modulator of important biochemical events to lethal poison. Given the theme of this

book, this chapter will mainly focus on the toxicology of CO. However, for the sake of completeness, physiological roles of endogenous CO are also presented. CO relates to all forms of life; however, its roles in microorganisms (Frankland, 1927) and plants (Han *et al.*, 2008; IPCS, 1999; K. Liu *et al*., 2007; Muramoto *et al.*, 2002; Wilks, 1959) are not covered in this chapter.

II. HISTORICAL BACKGROUND

An adequate amount was known about carbon monoxide at the beginning of the 20th century; Haldane (1922) devoted 14 pages on the history of carbon monoxide in his book titled *Respiration*; it occupied almost the same number of pages in the second edition 13 years later (Haldane and Priestley, 1935). An excellent description of the history of CO is contained in an article by Pankow (2000). Since most of the pioneering work on CO was done in Germany, and Pankow is based in Halle, Germany, he has been able to do justice to the celebrated first book on CO by Lewin (1920) and other related literature. There is another book called *Carbon Monoxide: The Silent Killer* by the University of Toronto Professor Roy J. Shephard. The title contains every ingredient of the history and toxicology of this mysterious poison – not invented by humans but made by nature itself like arsenic or cobra venom.

CO has always been a part of the universe. However, atmospheric CO has increased over time. When volcanoes erupted, continents collided, and winds embraced the trees sparking fires millions of years ago, all this contributed to the stock of CO. However, when CO first made a significant presence in the air we breathe, humans lived in the open. A very long time must have passed by before humans inhabited caves or built enclosures for protection from the effects of the weather or the tyranny of predators. In the process, however, humankind invited the unwanted guest – carbon monoxide, the silent killer. So where there is smoke, there is not only fire but also CO; in terms of human cost, the latter is more dangerous than the former. Yet it must have taken several thousands of years to tame the fire, and over those years CO has claimed many innocent victims who went to sleep after a hearty meal never to wake up. The knowledge of these mysterious events has been unraveled over time.

Just as concoctions of cinchona, bark of willow, and leaves of the common foxglove plant were used during ancient times without knowing that they contained, respectively, quinine, aspirin, or digoxin, so is the story of CO. The toxicity of CO was recorded by Aristotle (384–322 BC) in the third century BC; by the first century BC, charcoal fumes were used for suicide and executions without any knowledge of the exact nature of the killer (Lewin, 1920; Shephard, 1983). Byzantine emperor Julian the Apostate and his successor, Jovian, were poisoned in AD 363 and 364, respectively, because coal was used to heat their braziers (Lascaratos and Marketos, 1998).

The industrial revolution in Britain and Europe, and the discovery of the New World introduced coal for domestic heating and cooking. In 1700, Bernardino Ramazzini (1633–1714), a physician and professor at the University of Modena, published the book *De Morbis Arteficium Diatriba* (*Diseases of Workers*) which was translated from the original Latin text into Italian, French, German, English, and Dutch. Ramazzini recognized that fumes from burning coal by confectioners caused headache and dyspnea, and miners encountered ''noxious vapors''; burning coals, unless vented out, could ''Kill a man on the instant'' (Pankow, 2000; Shephard, 1983).

In their review, Prockop and Chichkoa (2007) write ''CO was first prepared by the French chemist de Lassone in 1716. Because it burned with a blue flame, de Lassone thought it to be hydrogen.'' In his book *Carbon Monoxide: The Silent Killer* (page 7), Shephard (1983) attributes this episode to the illustrious British chemist Joseph Priestley (1733–1804) who mistook this ''noxious vapor'' for hydrogen for the same reason – it burned with a bright blue flame. However, both Prockop and Chichkoa (2007) and Shephard (1983) agree that the identity of this noxious vapor was evaded until Cruikshank demonstrated that the gas which burned with the blue flame was indeed an oxide of carbon (CO) that could be converted into a dioxide of carbon (CO_2) by exploding it with oxygen.

Because the cause of CO poisoning which often resulted in death was unknown, myths emerged. Friedrich Hoffmann (1660–1742) of Halle, Germany, recounts that two farmers and a student used charcoal fire on a Christmas night; the farmers died and the student was found very sick; theologians, who claim to possess great knowledge, opined that it was a divine curse (Pankow, 2000).

French physiologist Claude Bernard (1857) was perhaps the first to describe the toxicity of carbon monoxide. In 1846, he forced a dog to inhale CO; of course the dog died and on autopsy he noted that the blood was crimson color in all the heart chambers as well as in the veins (Bernard, 1865). Claude Bernard correctly assumed that the crimson color was due to excess of oxygen in the blood. It was left to the celebrated British physiologist J.S. Haldane (1860–1936), to whom this chapter is dedicated, to identify carboxyhemoglobin (COHb) and determine its chemical nature (Haldane, 1895a).

By the end of the 19th century, Haldane had not only described the effects of exposure to various concentrations of CO on himself and mice but also discovered that the symptoms of CO poisoning could be alleviated by oxygen (Haldane, 1895a; Haldane and Priestley, 1935), which remains the most effective antidote against CO toxicity to date (Sluijter, 1967; Tibbles and Edelsberg, 1996). Haldane (1895a) also noted that ''symptoms of poisoning occur far more rapidly in mice than in man [himself]'' because the relationship of the body mass to surface area is

such that the mouse ''produces [CO] in an hour 10 grammes per kilo body weight, while man produces only about half a gramme''. Haldane was the first to use mice for the detection of CO in mines until canaries took over this service in 1921. By 1987 chemical methods for the detection of CO replaced the fate of the canaries.

Haldane worked out the physical chemistry of the CO–hemoglobin interaction; his First Law states: ''When a solution containing hemoglobin is saturated with a gas mixture containing O_2 and CO the relative proportions of the haemoglobin which enters into combination with the two gases are proportional to the relative partial pressures of the two gases, allowing for the fact that the affinity of CO for hemoglobin is about 3 times greater than that of O_2.'' This law is valid today; however, Haldane underestimated the affinity of CO for hemoglobin.

III. EPIDEMIOLOGICAL CONSIDERATIONS

Morbidity and mortality from CO poisoning vary from country to country because of the different standards of surveillance, extent of urbanization, housing conditions, source of energy, and so on. The major source of CO in the modern era is from the burning of petrol gas. Naturally, megacities with a population of over 10 million have a much higher level of atmospheric CO than smaller cities and rural areas.

Demography is changing rapidly in many countries especially in the two countries with populations of over one billion each – China and India; with surging economies in both countries the number of automobiles is fast increasing. In China, for example, the number of automobiles has been increasing at a rate of 13% per year since the 1980s; there were 18 to 21 million automobiles in China in 2000 compared with 11 million barely three years earlier in 1997; it is estimated that 3,000 kg of CO is discharged from every 1,000 automobiles each day (Chen and Wang, 2000).

In most countries, CO accounts for the maximum number of unintentional deaths in peace time. The estimated annual number of emergency admissions due to unintentional CO poisoning is approximately 40,000 in the USA (Weaver *et al.*, 1999), 5,000–8,000 in France (Annane *et al.*, 2001), 6,000 in Italy (Gandini *et al.*, 2005), and 523 in Poland (Sokal and Pach, 2000). Annual unintentional deaths from CO poisoning are approximately 600 in the USA (Cobb and Etzel, 1991) and 1,500 in the UK (Hamilton-Farrel and Henry, 2000).

Atmospheric CO is related to human civilization; consequently, its concentration varies markedly from east to west and from the south to north. Also, CO in the atmosphere varies greatly within different regions of the same country denoting uneven industrialization. In order to highlight these variables, we deal with this subject under two separate headings – the external source and the endogenous source of CO; details can be found in an excellent article by Vreman *et al.* (2000).

It is common knowledge that death due to CO poisoning is painless. The generation of CO in an enclosed space is also well known. Consequently, CO poisoning has emerged as the method of choice for suicides in favor of hanging and firearms.

IV. TOXICOKINETICS AND TOXICODYNAMICS

A. Sources of CO

There are two main sources of CO, exogenous and endogenous. While the atmospheric CO is the principal cause of CO toxicity, the endogenous source is physiologically very important (Maines, 1997; Marks *et al.*, 1991; Wu and Wang, 2005), and under certain conditions may even become pathological (Nezhat *et al.*, 1996).

1. External Sources of CO

Carbon monoxide is a product of incomplete combustion as encountered in the operation of vehicles, heating, coal power generation, and biomass burning (Godish, 2003). Natural geographical events such as volcanic eruptions, emission of natural gases, degradation of vegetation and animals, and forest fires all contribute to atmospheric CO. Approximately 40% of global CO comes from these natural sources. Human intervention such as fossil fuel consumption, garbage disposal, tobacco smoke, charcoal fires, etc., contribute to the remaining 60% of global CO (Jain, 1990; Vreman *et al.*, 2000). Because human activity and density differ from place to place due to socioeconomic factors, atmospheric CO varies greatly from place to place. CO emission in the USA in 2001 was 120.8 million short tons of which 74.8 million came from on-road vehicles (McGrath, 2006).

Apart from various other changes, the developing countries are characterized by increasing migration of rural population to slums and shanty towns on the outskirts of cities like São Paulo, Mexico, Johannesburg, Mumbai, Shanghai, etc.; this is associated, among other things, with an increase in atmospheric CO. Fortunately, atmospheric CO has not exceeded safety levels globally, or in any specific areas including, for example, Mexico City and Los Angeles, but it can. It is reassuring that many efforts are being made by government agencies to reduce CO emissions.

2. Endogenous sources of CO

The knowledge that CO is normally present in the body dates back to 1894 when Grehant (1894) detected a combustible gas in blood. By 1898, this combustible gas was suspected to be carbon monoxide (De Saint Martin, 1898; Nicloux, 1898). At the time, methods did not exist to ascertain if the CO in the blood was generated inside the

body or was derived from the air. It was not until 1949 that the evidence for endogenous production of CO was firmly established (Sjorstrand, 1949). Tenhunen *et al.* (1968, 1969, 1970) elaborated on the role of heme oxygenase in the generation of CO.

The major source of endogenous CO in a healthy individual is from the degradation of heme by heme oxygenase (HO) – HO-1 and HO-2. The enzyme HO-1 is inducible and HO-2 is constitutive; heme oxygenase degrades heme into CO and biliverdin and the latter is rapidly converted into bilirubin (Coburn, 1979; Coburn *et al.*, 1963, 1967; Mores and Sethi, 2002). Current literature emphasizes only a physiological role for endogenously generated CO (Boehning and Snyder, 2003; Choi and Otterbein, 2002; Mannaioni *et al.*, 2006; Ryter and Otterben, 2004; Wu and Wang, 2005) although it is quite likely that it could add to the toxicity of inhaled CO.

Heme oxygenase is the rate-limiting step in the production of CO and its activities account for 86% of endogenous CO production; the remaining 14% is derived from nonheme sources. The lifespan of red blood cells is approximately 120 days; the older the erythrocyte the greater is its CO output. In neonates, red blood cells have a shorter lifespan and relative to erythrocytes of adults, they produce two to three times more CO (Fallstrom, 1968). HO-2 is activated during neuronal stimulation by phosphorylation by the enzyme CK2 (Boehning *et al.*, 2003).

The rate of COHb formation is a function of inhaled CO concentration and duration (Figure 20.1). The rate of CO production and excretion parallels the rate of bilirubin production; hence a measurement of COHb serves as a measure of heme degradation and bilirubin production.

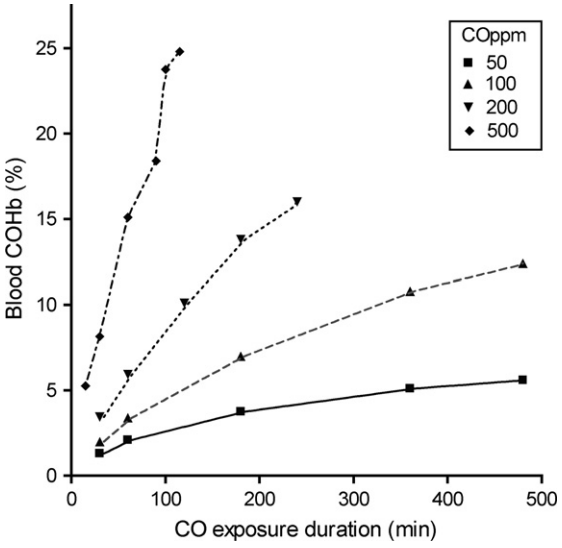

FIGURE 20.1. Relationship between the duration of exposure to different concentrations of carbon monoxide and blood carboxyhemoglobin (COHb) in healthy volunteers. The plot is based on data of Stewart and Peterson (1970).

Indeed, a measurement of end-tidal CO in breath corrected for inhaled CO is used as a measure of assessing infants at risk of severe hyperbilirubinemia because CO and bilirubin are produced in equimolar amounts (Bartoletti *et al.*, 1979).

A small fraction (20%) of endogenous CO is derived from other hemoproteins such as myoglobin and many other iron-containing enzymes (Coburn, 1970; Vreman *et al.*, 2000). This serves as an example of the use of endogenous CO monitoring for diagnostic purposes.

In addition to the major exogenous and endogenous sources of CO described above, certain medical procedures within the body can also generate CO. For example, laser and bipolar electrocautery during laparoscopy can generate over 200 ppm CO in the body cavity, which finds its way into the circulation increasing COHb levels (Wu *et al.*, 1998), sufficient to induce toxicity (Nezhat *et al.*, 1996). Anesthetic machines equipped with drying material like soda lime or barium hydroxide were found to generate CO (Baum *et al.*, 1995; Moon *et al.*, 1992) from several anesthetic agents (Fang *et al.*, 1995). Likewise pulmonary function tests based on determining the CO diffusion capacity as a means of determining the alveolar-capillary diffusion capacity for gases can also elevate CO and in turn COHb (Vreman *et al.*, 2000).

B. Physicochemical Properties of CO

CO is a colorless, odorless, tasteless, noncorrosive stable diatomic molecule in the atmosphere. Unlike hydrogen peroxide (H_2O_2) or superoxide ($O^{\bullet}$), CO is not a radical. CO has low water solubility and negligible quantities dissolve in blood at normally encountered pressure (Shephard, 1983). CO has a shorter inter-atomic distance ($\sim$1.13 Å) than would be anticipated for a single bond. It has a high heat of formation from the constituent atoms (bond strength 2.07 MJ/mol) and the electric charge is distributed rather uniformly between the carbon and oxygen atoms. Pauling (1960) postulated that under normal circumstances CO existed as a hybrid containing roughly equal proportions of three structures. According to the modern atomic theory, carbon atoms have the first shell filled with two electrons in S orbitals, while the second shell contains two electrons in the S orbital and two in the P orbital (Shephard, 1983). The general properties of CO are presented in Table 20.1.

C. Methods for CO Measurement

Appropriate techniques exist for the quantitation of CO both in gaseous and tissue samples. In either case caution needs to be exercised to ensure that CO concentration is not altered by interaction with the sample vessel during storage and transport. For example, Vacutainer tubes were found to greatly alter CO levels when used for storage of blood samples (Vreman *et al.*, 1984). Furthermore, routine monitoring of air for public health requires a simple and yet reliable sample collecting system. Ambient CO levels at the

TABLE 20.1. Physicochemical properties of carbon monoxide

Property	Description
Chemical structure	
Molecular weight	28.01
Critical point	−140°C at 3495 kPa
Melting point	−199 °C
Boiling point	−191.5 °C
Fundamental vibration transition	2143.3 cm^{-1} (4.67 μm)
Density at 25°C, 101.3 kPa	1.145 g/l
Specific gravity relative to air	0.967
Solubility in water at 0°C	35.4 ml/l
Solubility in water at 25°C	21.4 ml/l
Explosive limit in air	12.5–74.2%

Source: UN Environment Program and WHO (1979); Pryor *et al.* (2006); Shephard (1983)

workplace can be very low requiring sensitive analytical techniques. With the recognition of the hazardous nature of CO and its almost universal presence, several sophisticated methods for quantifying CO have been developed (IPCS, 1999; Vreman *et al.*, 2000).

1. Measurement of Blood CO

Haldane (1895b) wrote: ''In view of the very poisonous nature of carbonic oxide, and the comparative frequency with which cases of poisoning by this gas occur, much attention has been given to its detection and estimation … The method now to be described is very simple, and depends on the fact that when a hemoglobin solution is well shaken with air containing carbonic oxide and the proportion of hemoglobin, which finally combines with carbonic oxide, varies with the percentage of carbonic oxide in the air. By determining colorimetrically the proportion of the hemoglobin which has combined with the carbonic oxide it is thus possible to infer the percentage of carbonic acid in the air.''

''The bottle containing the blood solution and sample air must be covered with a cloth during the process of shaking, and (although this is by no means so important) very bright light should be avoided during the process of titration with the carmine solution'' (Haldane, 1896).

The currently employed method of quantifying blood CO and the precaution that is needed for accuracy are simply refinements of the method described by Haldane (1895b, 1896) more than a century ago; colorimetric detection was soon replaced by the spectroscopic detection method (Hartridge, 1912) and has undergone further sophistication since then. Both gas chromatography and spectrophotometry are considered appropriate although the former is favored (Coburn *et al.*, 1964; Collison *et al.*, 1968; Constantino *et al.*, 1986; Kane, 1985; Vreman *et al.*, 1984, 1998). However, blood CO is still measured by determining COHb levels, which are normally undetectable but might be involved in the transfer of CO between cells. The measurement of COHb is useful not only in the quantification of CO but also to detect neonatal hemolysis (Necheles *et al.*, 1976; Ostrander *et al.*, 1982).

COHb is quite stable and its concentration does not change over a long period (up to 6 months) if the blood sample is stored in the dark and under sterile conditions. Blood levels of COHb are not expected to exceed 5% at ambient levels of CO. IPCS (1999) focuses on methods which can accurately measure COHb below 10%. A method which simply requires finger-prick blood is convenient for mass screening and is described in detail by Commins and Lawther (1965); in this method, the sample is diluted in ammonia solution, which is divided into two parts; from one of these, CO is displaced by oxygen and the COHb containing part is placed in the sample beam of a spectrophotometer so that the instrument records the difference between the absorbance of COHb and oxyhemoglobin.

Several techniques for measuring COHb exist (IPCS, 1999). The sensitive techniques require the release of CO from hemoglobin into a gas phase; CO can then be detected directly by a number of methods such as infrared absorption, difference in thermal conductivity between CO and the carrier gas, amount of ionization following conversion of CO to methane, or the release of mercury vapor resulting from interaction of CO with mercuric oxide.

The conventional method of expressing CO in blood samples is as percent COHb and it is determined by the formula:

$$\%\text{COHb} = [\text{CO content}/(\text{HB} \times 1.389)] \times 100$$

where CO content is the CO concentration in ml/100 ml blood, Hb is hemoglobin in g/100 ml blood and 1.389 is the combining capacity of CO for Hb in ml of CO per gram Hb.

The blood level of COHb as a percent of total hemoglobin is directly related to the exposure duration at any concentration of CO (Figure 20.1).

Blood COHb measurements have been used to monitor exposure of populations to atmospheric CO; which also functions as a measure of air pollution; using this approach, New Hampshire, Vermont, and the city of St Louis, Missouri were found to have blood COHb levels in the range 1 to 1.5% in the early 1970s (Davis and Ganter, 1974; Kahn *et al.*, 1974).

a. Ambient Air CO

Because CO concentration in ambient air and at workplaces is usually quite low, reliable methods for sample collection and transport as well as highly sensitive methods for its measurement are needed (IPCS, 1999; Smith and Nelson, 1973). The sampling method recommended by WHO (IPCS, 1999) comprises a sample introduction system

consisting of a sampling probe, an intake manifold, tubing, and air remover. Known gas concentration is periodically collected to verify the method.

According to IPCS (1999), the analyzer system consists of an analyzer as well as sample preconditioning components fitted with a moisture control system such as the Non-Dispersive-Infrared (NDIR) analyzer. The infrared absorption near 4.6 μm, characteristic of CO, is used to measure its concentration. The most sensitive analyzers can detect CO concentrations as low as 0.05 mg/m^3 (0.044 ppm). The NDIR analyzer designed by Luft (1962) is considered appropriate because it is little affected by flow rate, requires no wet chemicals, has a short response time, and is sensitive over wide concentration ranges.

Other analyzers include gas chromatography, which is a sensitive, automated, and semi-continuous technique in which CO is separated from water, CO_2, and hydrocarbons (other than methane) by a stripper column and CO is passed through a catalytic reduction tube where it is converted to methane. The converted CO is passed through a flame ionization detector; its sensitivity range is 0.026 to 43.7 ppm (IPCS, 1999). Other methods such as small personal exposure monitors (PEMs) can measure CO concentrations on a continuous basis and store data on internal digital memories (Ott *et al.*, 1986).

b. Home Detectors

Residential CO detectors are designed like smoke detectors and provide protection from excessive CO concentrations inside homes by sounding alarms. They are based on an interactive-type sensor, such as tin oxide, artificial Hb that relies on an interaction between CO, and the sensitive element to generate an alarm. The alternate current powered home detectors have a metallic sensor that reacts with CO; the battery-powered ones have a chemically treated gel that darkens upon exposure to CO. They are designed to sound the alarm within 90 min at CO concentrations of 100 ppm; within 35 min at 200 ppm or within 15 min at 400 ppm (IPCS, 1999). CO concentration of 400 ppm can increase COHb to 10%, above which CO toxicity is observed.

c. CO in Expired Breath

Measurement of CO in the expired breath is based on the assumption that CO in alveolar air is in equilibrium with the partial pressure of CO in blood which in turn is in equilibrium with CO bound to Hb, that is COHb (Douglas *et al.*, 1912).

D. Absorption, Distribution, and Elimination of CO

Atmospheric carbon monoxide can travel thousands of kilometers from its source and has a half-life of 1–2 months (Akimoto, 2003). Although the biological effects of CO differ depending upon whether it is inhaled or endogenously produced, the ultimate fate of CO is the same regardless of its source.

Exogenous CO reaches the body solely by pulmonary absorption. Once inhaled, CO combines reversibly with hemoglobin and to a smaller extent with myoglobin and other iron-containing macromolecules. Because most of the CO in the body is bound to hemoglobin, the relative affinities of CO and oxygen for hemoglobin are of critical significance in terms of both its toxicity and excretion. This relationship was first described by Haldane (1912), in what is known as Haldane's first law, represented by the following equation:

$$(PO_2 \times \mathrm{COHb})/(P\mathrm{CO} \times \mathrm{O_2Hb}) = \mathrm{M}$$

where M is the affinity constant, thought to be between 208 and 245 at 37°C, and PCO and PO_2 are the partial pressures of CO and oxygen, respectively.

Haldane had conducted the experiments on himself and found that he needed to inhale 0.07% CO in the air containing 20.9% oxygen in order for Hb to achieve 50% oxygen and 50% CO saturation. On this basis he calculated the relative affinities of CO and oxygen for Hb as 1.299. While more refined techniques have estimated the affinity of CO for Hb to be much higher (Ernst and Zibrak, 1998; Roughton and Darling, 1944; Sendroy and Liu, 1930), the fundamental relationship proposed by Haldane (1912) remains valid and has been discussed in detail by others (Forster, 1970; Roughton, 1970); this relationship between CO, oxygen, and hemoglobin explains both the high toxic potential of CO and the need of hyperbaric oxygen to treat CO overdose (Jay and McKindley, 1997; Pace *et al.*, 1950; Peterson and Stewart, 1970).

Under normal physiological states there is almost always a certain amount of COHb even when breathing CO-free air, because the breakdown of Hb results in endogenous production of CO, which can result in 0.5 to 0.8% COHb in normal blood (Lawther, 1975). At any CO concentration in the air, COHb will attain an equilibrium (16% at 100 ppm ambient CO and 1.7% at 10 ppm CO); it follows that if CO in the air is lower than that required for a given COHb% at equilibrium, CO will leave COHb and be exhaled until a new equilibrium is established. This has practical implications. For example, a smoker with a relatively high COHb at equilibrium may exhale CO. Also, if the basal COHb is high an equilibrium at a high CO concentration in the air will be achieved more quickly than it would be at an initial low COHb.

The reaction of CO with ferrous iron is relevant to its pharmacology. Shephard (1983) provides the following description of this reaction. When reacting with ferrous iron, carbon atoms of CO form a sigma bond, involving also a D-orbital electron from the third shell of Fe^{2+}. In the body, Fe^{2+} also has four nitrogen linkages that contribute a substantial electron density; the effective valency is less than Fe^{2+}, enabling a more readily reversible reaction with both oxygen and carbon monoxide.

The solubility of CO in water is about 20% less than that of O_2 such that it is practically absent from the solution in blood.

Reaction of CO with hydroxyl radicals ($OH^{\cdot}$) is the major method of removing CO from the atmosphere (IPCS, 1999). The cycle of hydroxyl radicals is coupled to cycles of CO, methane, water, and ozone; they are produced by the photolysis of ozone followed by the reaction of the excited oxygen atoms with water vapor to produce two hydroxyl radicals: ($O(^1D) + H_2O \rightarrow OH^{\cdot} + OH^{\cdot}$). A small proportion of atmospheric CO is removed by the soil.

Carbon monoxide is eliminated from the body almost exclusively via the lungs. When respiring room air, the elimination half-life of CO in a healthy adult is approximately 4 h, which can be reduced to 1 h when breathing oxygen at normal atmospheric pressure (Bartlett, 1968; Pace *et al.*, 1950; Roughton and Root, 1945); high concentration of COHb can shorten the elimination half-life of CO (Haldane, 1985a; Henderson and Haggard, 1921) and low concentration can increase it (Petersen and Stewart, 1970). On the other hand, half-life for a drug is a meaningful feature only if it is not dose independent; which is not the case with CO (Jay and McKindley, 1997).

V. MECHANISM OF TOXICITY

Wu and Wang (2005) chose to name endogenous CO as ''Dr Jekyll'' and not ''Mr Hyde'' because it is good rather than bad; if they are correct, then this mysterious gas proves more than any other agent the correctness of the dictum of Paracelsus (1493–1541) that: ''All substances are poisonous. Only the dose differentiates a poison from a remedy.'' If Paracelsus had known about the views of Wu and Wang (2005) regarding CO, he might well have added – not only the ''dose'' of the substance but also its source.

There are very few agents like the anticoagulant warfarin whose toxicity and therapeutic effects are produced by the same mechanism. In most cases beneficial and harmful effects recruit different mechanisms of action. As far as CO is concerned not only does its mechanism of toxic and physiological actions differ, but the source from where it is derived matters too. So the mechanisms of action of inhaled- and heme-generated CO also seem to differ. Since the focus of this chapter is CO toxicity, we will attempt to elaborate the mechanism of its toxicity more than the mechanism of its physiologic actions.

The French physiologist Claude Bernard (1857) and the British physiologist Haldane (1895a) inferred that the toxicity of CO was caused by its interaction with hemoglobin. In his review of carbon monoxide, Lilienthal (1950) writes: ''It has been said many times that the effect of CO on man may be attributed to two actions and, in essence, to these two actions alone: (a) occupation of the Hb molecule by CO, with a resultant decrease in the O_2 transport capacity of the blood; and (b) alteration of O_2Hb dissociation

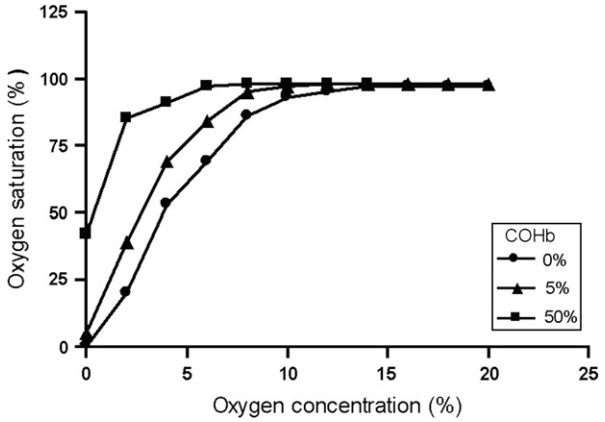

FIGURE 20.2. Effect of carboxyhemoglobin (COHb) upon the shape of the oxyhemoglobin dissociation curve in healthy males. Based on data of Haldane and Priestley (1935) as presented by Shephard (1983).

characteristic produced by COHb, with a resultant impaired unloading of O_2 at the tissues.'' The O_2Hb dissociation curve is shown in Figure 20.2.

Both features described by Lilienthal (1950) boil down to hypoxia as the principal mechanism of CO toxicity thereby confirming the basic observations of Claude Bernard and J.S. Haldane made 150 years earlier and working out details of CO–hemoglobin interaction. Haldane (1894a, 1896) knew that the affinity of CO for hemoglobin was greater than that of oxygen, even if he underestimated it. The color of COHb is red, which explains the cherry-like discoloration of CO victims; this is generally but erroneously believed to be a specific characteristic of cyanide poisoning. Obviously, any time oxygen is not fully extracted from the arterial side, the venous blood will become cherry-red.

More sophisticated techniques than the ones used by Haldane have established that CO binds to hemoglobin with an affinity 200 times greater than that of oxygen (Ernst and Zibrak, 1998; Roughton and Darling, 1944; Sendroy *et al.*, 1930). Carbon monoxide diffuses from the alveoli to the blood in pulmonary capillaries across the alveoli–capillary membrane, which is composed of pulmonary epithelium, the capillary epithelium, and the fused basement membranes of the two. The uptake of CO by Hb is very rapid and the transfer of CO is diffusion limited (Prockop and Chichkoa, 2007).

The greater the duration of the exposure to CO, the concentration of CO in the inhaled air, and the alveolar ventilation, the greater the total amount of COHb. While the uptake of CO by Hb is very rapid, the release of CO from COHb complex is extremely slow. As the concentration of COHb increases the formation of oxyhemoglobin at any concentration of oxygen in the inhaled air decreases. There is a strong case for using COHb concentration as a predictor of CO toxicity (Renwick and Walton, 2001).

There is yet another mechanism by which CO produces hypoxia. COHb shifts the dissociation curve of the

remaining oxyhemoglobin to the left, which further decreases the release of oxygen (Douglas *et al.*, 1912; Roughton and Darling, 1944), which causes headaches when mild and death when it is extreme. Douglas *et al.* (1912) also observed that ''the presence of a small proportion of oxygen may greatly increase the formation of CO–haemoglobin, and *vice versa*'' and this ''paradoxical effect explains the favorable physiological effect sometimes produced by carbon monoxide in conditions of great anoxhaemia''. The studies of Pace *et al.* (1950) on human volunteers demonstrated that the release of CO bound to hemoglobin and its expiration can be increased by inhalation of hyperbaric oxygen (Figure 20.3).

However, the data of Goldbaum *et al.* (1975, 1976) imply that formation of excess of COHb might not account for the lethal effects of CO; these researchers found that dogs breathing 13% CO died within 1 h after achieving COHb levels between 54 and 90% but transfusion of blood containing 80% COHb, which resulted in 57 to 64% COHb in the recipient dog, did not cause marked toxicity. It would thus appear that the toxicity of CO results from a combination of tissue hypoxia due to COHb plus other effects of CO, which would explain why COHb levels do not always correlate with the severity of clinical effects (Brown and Piantadosi, 1992; Myers, 1984; Norkool and Kirkpatrick, 1985; Rottman, 1991).

Besides hemoglobin, CO binds to many heme-containing proteins such as myoglobin, guanylyl cyclase, and cytochrome oxidase (Chance *et al.*, 1970; Hill, 1994; Kao and Nanagas, 2006; Omaye, 2002). However, the affinity of CO for cytochrome oxidase is very low and this interaction would require a dose far exceeding the lethal dose of CO (Prockop and Chichkoa, 2007). On the other hand, even at sublethal concentrations binding of CO to cytochrome may

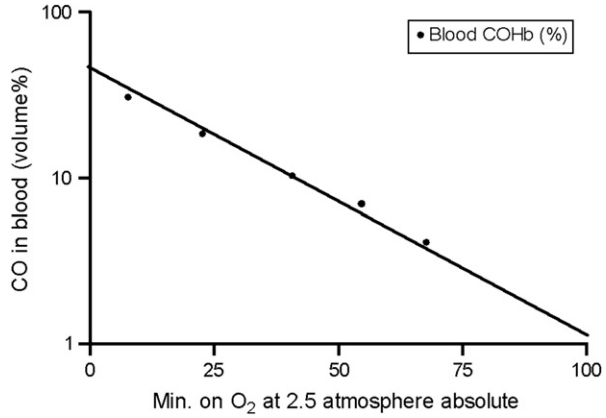

FIGURE 20.3. Data are from a healthy male volunteer, who breathed a low concentration of carbon monoxide and then oxygen at 2.5 atmospheric pressure, absolute. Blood was collected at different times after starting oxygen. Data points are taken from Pace *et al.* (1950) and may not be exactly the same as in their figure. Each point is the mean of triplicate determination and intercept indicates CO level at time zero prior to oxygen therapy.

lead to the generation of superoxides (Hardy and Thom, 1994; Zhang and Piantadosi, 1992) and interfere with cellular respiration. Cellular metabolism remains inhibited even after COHb levels decline to normal range (Brown and Piantadosi, 1992; Olson, 1984; Piantadosi *et al.*, 1995).

The interaction of CO with myoglobin can explain some of its toxic effects; it might impair oxygen supply to the mitochondria, which can result in myocardial ischemia. Patients with underlying cardiac conditions are therefore at a high risk of cardiac arrhythmias following exposure to CO (DeBias *et al.*, 1976; Olson, 1984; Sangalli and Bidanset, 1990) as well as chest pain and even death. A study of Swiss soldiers accidentally exposed to CO noted a substantial number had developed chest pain some time after exposure to CO (Henz and Maeder, 2005). The interaction of CO with skeletal muscle myoglobin can cause muscle weakness (Herman *et al.*, 1988; Richardson *et al.*, 2002; Wolf, 1994).

Nitric oxide (NO) and NO donors as well as phosphodiesterase inhibitors increase gyanylyl cyclase and hence cGMP causing marked vasodilatation; patients on nitrates for angina are well aware that the drug may cause dizziness and loss of consciousness. CO also activates guanylyl cyclase and there is always a possibility that the resulting hypotension and cerebral vasodilatation may lead to a loss of consciousness; it is therefore prudent also to treat an increase in cGMP as a potential toxic effect and not only as a physiological activity of CO or proof of a neurotransmitter role for CO as suggested by many (Verma *et al.*, 1993; Snyder and Ferris, 2000; Wu and Wang, 2005).

Any event that leads to hypoxia can lead to reperfusion and secondary hyperoxia especially if vascular autoregulation is not fully developed such as in premature births and certain animal models (Chemtob *et al.*, 1991; Hardy *et al.*, 1997, 2000). Hyperoxia leads to generation of superoxides and other radicals, which are involved in a number of pathologies (Brault *et al.*, 2007; Kowluru *et al.*, 2001; Pryor *et al.*, 2006). Recent studies suggest that hypoxia caused by the formation of COHb may also be followed by reperfusion injury on the top of that produced by the initial hypoxia. The oxidants generated during reperfusion can oxidize essential proteins and nucleic acid and can cause lipid peroxidation leading to demyelination of CNS lipids and other effects (Ernst and Zibrak, 1998; Prockop and Chichkoa, 2007).

In addition, CO can promote neutrophil adhesion to the microvasculature resulting in the activation of xanthine oxidase and generation of oxidative radicals which ultimately culminate in brain lipid peroxidation, which is the likely cause of delayed neurological sequelae (Gilmer *et al.*, 2002; Hardy and Thom, 1994; Ischiropolous *et al.*, 1996; Thom, 1990, 1993; Thom *et al.*, 1994, 1997, 2001). The observed brain lipid peroxidation in CO poisoning is likely to be caused by changes in cerebral blood flow plus oxidative damage (Hardy and Thom, 1994; Ischirpolous *et al.*, 1996; Thom, 1990, 1992; Thom *et al.*, 1997; Zhang and Piantadosi, 1992).

CO might also alter myelin basic protein, which may react with lipid peroxidation products to produce immunologic cascade (Thom *et al.*, 2004). Other events that could contribute to CO toxicity include glutamate-induced neuronal injury (Ishimaru *et al.*, 1992; Penny and Chen, 1996; Piantadosi *et al.*, 1997), atherogenesis (Lightfoot, 1972; Thom *et al.*, 1999), and apoptosis (Piantadosi *et al.*, 1997).

The model of carbon monoxide toxicity proposed by Kao and Nanagas (2006) combines the cascade of changes resulting from three primary events – binding to HB, direct cellular injury, and increased NO activity. CO is not a radical but many of the injuries produced by it are those that are caused by oxidative stress, which is secondary to hypoxia. In the model of Kao and Nanagas (2006), the oxidant is NO, which contributes to oxidative damage to the brain and produces the clinical syndrome of delayed neurologic sequelae (Thom *et al.*, 1997).

VI. TOXICITY OF CARBON MONOXIDE

A. Acute Toxicity

All chemicals produce toxicity; however, only a few of them cause death every time if taken in overdose; carbon monoxide is one of them. The toxicity of CO has been extensively reviewed (Drinker, 1938; Gorman *et al.*, 2003; Hamilton, 1921; Kao and Nanagas, 2006; Lilienthal, 1950; Mannaioni and Vannacci, 2006; McGrath, 2006; Prockop and Chichkova, 2007; Putz *et al.*, 1976; Robkin, 1997; Ryter and Otterbein, 2004; Samoli *et al.*, 2007; Thom and Kleim, 1989) or contained in monographs (IPCS, 1999; Penny, 2000; Shephard, 1983).

The acute toxicity of CO spans from dizziness to death. Symptoms of CO poisoning begin at approximately 20% COHb and death occurs between 50 and 80% COHb (Ryter and Otterbein, 2004); The relationship between blood COHb and toxic signs and symptoms is presented in Table 20.2.

Haldane (1895a) provided the first description of the relationship of CO in inhaled air to its toxic effect on humans. In the first experiment on himself and a mouse, Haldane inhaled 0.5% CO for 11.5 min with no obvious symptoms except ''hyperapnea after running upstairs''. In the second experiment, Haldane and his mouse inhaled 0.39% CO; at 15 min his COHb was 23% but he had no symptoms while the mouse started panting after 1.5 min and lay helplessly on its side by 15 min; at 29 min his pulse was 120 beats/min and he felt distinctly ''abnormal'' while the mouse lay on its back with hyperapnea; at 30.5 min the COHb was 39% and he stopped inhaling CO but felt giddy and had palpitations after running up 24 steps and could not see well; upon resting for 2–3 min, Haldane felt better and hyperapnea disappeared; 45 min after stopping the inhalation of CO, COHb was 23%, at 105 min 18% and at 186 min 5%.

Not satisfied with the data Haldane had so far collected he did nine more experiments (total of 11) with his companion mouse; aside from all other facts, Haldane came to the conclusion that mice are more sensitive than humans to CO toxicity and COHb remains elevated for a long time.

The higher sensitivity of mice than of humans led to the introduction of canaries in the coalmines. To our knowledge, CO toxicity on humans was again experimentally determined 75 years after Haldane's experiments (Hosko, 1970; Peterson and Stewart, 1970; Stewart *et al.*, 1970). Stewart *et al.* (1970) exposed 25 human volunteers to CO concentrations from <1 to 1,000 ppm for variable time periods and determined COHb as well as subjective symptoms and objective signs (EEG recording, EECG, hand and foot reaction); the main conclusions of these researchers was that even exposure up to 100 ppm CO for up to 24 h did not produce detectable subjective or objective changes; exposure to 200 ppm CO caused mild frontal headache in 4 h and headache was experienced within 1 h at 500 ppm. Full recovery took place in all cases when subjects were transferred to a hyperbaric oxygen chamber; COHb strongly correlated with inhaled CO concentration reaching approximately 20% at 100 ppm and 30% at 200 ppm CO (Peterson, 1970; Peterson and Stewart, 1970). Hosko (1970) found that at COHb greater than 20%, visually evoked responses were altered in human volunteers; at high altitudes even a much smaller increase in COHb (5–10%) can exert similar effects (Lilienthal and Fugit, 1945).

The data cited above and the relationship of COHb to CO toxicity presented in Table 20.2 clearly show that the acute toxicity of CO is concentration dependent; this is of great

TABLE 20.2. Relationship of carboxyhemoglobin (COHb) to toxicity in humans following exposure to carbon monoxide

COHb %	Signs and symptoms
<10	No effects in healthy individuals[a]
10–20	Mild headache, exertional dyspnea, cutaneous vasodilation
20–30	Throbbing headaches, nausea
30–40	Severe headaches, dizziness, visual disturbance, fatigue
40–50	Tachypnea, tachycardia, collapse, syncope
50–60	Coma, convulsions, Cheyne-Stoke's respiration
60–70	Cardiorespiratory depression, possible death
>70	Respiratory failure and death

[a]Symptoms may appear in subjects with cardiovascular disease. Concentration of COHb depends upon the duration of exposure to any concentration of CO in the air and therefore not included in the table. Exposure to approximately 200 ppm for 2 h results in 10% COHb; ~12,000 ppm CO would produce lethal concentrations of COHb within 2–3 breaths. Based on data from Goodman and Gilman (1941); Renwick and Walton (2001)

relevance in setting permissible or desirable atmospheric CO concentration standards. However, the binding affinity of CO for Hb is 200 to 300 times greater than that of oxygen to Hb; the effects of CO therefore must be considered cumulative. Prolonged exposure to CO would increase COHb more than can be accounted for by its ambient concentration; likewise a long period of CO-free atmosphere would be needed for COHb to return to normal levels as also revealed by the data of Haldane (1895a). As a caution it should be noted that toxic symptoms of CO poisoning do not necessarily correlate with COHb levels; for example, seizures in a four-year-old child were noted at COHb levels of approximately 25%, while 40% is the usual level for such symptoms (Herman, 1998). Putz *et al.* (1976) exposed 30 volunteers for 4 h to 5–70 ppm CO and found that 5% COHb (achieved following exposure to 70 ppm CO) impaired coordination, response time to light, and auditory acuity. However, exposure to CO during sleep, which increased COHb to 12.5, did not impair performance after awakening although it had some effect on sleep pattern (O'Donnell *et al.*, 1971).

Air pollution, which consists of particulate matter, CO, sulfur oxide, nitrogen oxide, hydrogen sulfide, acid gases like HF and HCl, volatile organic solvents, radiation, etc., is estimated to be responsible for 800,000 annual deaths worldwide (Cohen *et al.*, 2005); a review of the health effects of these pollutants found that respiratory and cardiovascular mortality was consistently associated with particulate matter and CO (Curtis *et al.*, 2006). A large study of the effect of air pollution on mortality was investigated in 19 European cities under the APHEA (Air Pollution and Health: A European Project) project. Samoli *et al.* (2007) found a significant association of CO both with total and cardiovascular mortality. However, because CO is not the only noxious air pollutant, such epidemiological studies fail to categorically establish a role of CO because it is also possible that CO toxicity is aided by the presence of other pollutants. Purser (1996) analyzed scenarios in the event of fire in aircraft which release a host of toxic gases including CO and concluded potentially serious consequences in terms of performance of the crew and the survival of passengers.

It is of interest that big cities like Mexico City and Los Angeles harbor not only high concentrations of CO in the air but also an excess of noise and there are both clinical and experimental data (Cary *et al.*, 1997; Fechter *et al.*, 1988) to show that noise increases the toxicity of CO as well as of other noxious chemicals.

Edmund Hillary and Sherpa Tenzing conquered Mount Everest on May 29, 1953; this sparked great enthusiasm about mountain climbing. However, at high altitudes the partial pressure of oxygen is low and even a small amount of CO can be fatal. For example, two healthy men died at a height of about 6,000 m in Alaska from exposure to CO generated just by a cooking stove in their tent (Foutch and Henrichs, 1988). Even a 5–10% increase in COHb can cause appreciable deterioration in flicker fusion frequency at high altitudes (Lilienthal and Fugit, 1945). Also, altitude exaggerates CO toxicity in subjects with coronary artery disease (McGrath, 2006).

Atmospheric CO is the main source of exposure to the population; for this reason WHO has set the following limits for CO exposure (IPCS, 1999):

100 mg/m^3 (87 ppm) for 15 min
60 mg/m^3 (52 ppm) for 30 min
60 mg/m^3 (26 ppm) for 60 min
10 mg/m^3 (9 ppm) for 8 h (the usual working period per day).

B. Delayed Toxicity

The delayed CO toxicity is also referred to as the interval form of toxicity (Mannaioni *et al.*, 2006) with significant effects on the central nervous system (Raub and Benignus, 2002). The interval form refers to neuropsychiatric symptoms within several days or even weeks after asymptomatic existence following recovery from unconsciousness caused by exposure to CO. The most common symptoms of the ''interval form'' of delayed CO toxicity are lethargy, behavior changes, forgetfulness, memory loss, and Parkinsonian symptoms (Yoshii *et al.*, 1998). These symptoms are attributed to diffuse demyelinization of cerebral white matter in specific regions of the brain (Hsiao *et al.*, 2004; Mannaioni *et al.*, 2006; Okeda et al., 1981; Yoshii, *et al.*, 1998). Neurological symptoms were found to accompany lesions in the brain as detected by magnetic resonance imaging in several other studies (Hsiao *et al.*, 2004; Parkinson *et al.*, 2002).

Daily exposure of dogs for 11 weeks to 100 ppm CO resulted in persistent ECG changes as early as 2 weeks and cardiac muscle degeneration at the end of the study; observed disturbances in gait and posture were attributed to lesions in the cerebral cortex (Hamilton and Hardy, 1974). Visual disturbances following recovery from a period of CO-induced unconsciousness have also been reported (Kelly and Sophocleus, 1978). Moderate to severe CO poisoning can shorten lifespan because of myocardial injury (Henry *et al.*, 2006).

A lack of close correlation between COHb levels and the severity of CO toxicity is well recognized (Mannaioni *et al.*, 2006); this clearly suggests the involvement of other factors. These other factors most probably include xanthine oxidase-derived reactive oxygen species, which are produced due to hyperperfusion following the initial hypoxia. This inference is supported by several animal studies (Sokal and Kralkowska, 1985; Thom, 1992; Zhang and Piantadosi, 1992).

CO and cyanide have certain similar toxicities, which are a consequence of hypoxia such as disruption of cochlear functions (Tawackoli *et al.*, 2001). CO has been found to cause hearing loss both in humans (Goto *et al.*, 1972; Makishima, 1977; Morris, 1969; Sato, 1966) and in animal

models (Douglas *et al.*, 1912; Fechter *et al.*, 1988; Liu and Fechter, 1995).

C. Cardiovascular Toxicity

CO can cause as well as exacerbate underlying cardiovascular diseases (Atkins and Baker, 1985; Thom and Kleim, 1989). CO seems to affect each step in the course of ischemic heart disease (Goldsmith and Aronow, 1975; Turino, 1981). CO has been found to exacerbate the atherogenic potential of cholesterol in monkeys (Webster *et al.*, 1970). Inhalation of CO during treadmill exercise in patients with coronary artery disease hastens the onset of anginal symptoms and causes a prolongation of ECG changes (Anderson *et al.*, 1973; Aronow and Cassidy, 1975). A number of deaths from myocardial infarction have been recorded at COHb levels much lower than the levels required to produce neurological effects (Atkins and Baker, 1985; Scharf *et al.*, 1974). The potential of CO to aggravate myocardial pathology is understandable since the disease is associated with relative decrease in oxygen supply to the myocardium; at any concentration CO can only further increase hypoxia as well as interfere with any compensatory mechanism. Given that cardiac disease is common in the developed world, which also has more than the usual level of CO in the atmosphere, important public health measures would be required to deal with this issue.

Anemia will exert a profound effect on CO saturation and its toxicity because most, if not all, of CO in the blood is bound to hemoglobin. This subject assumes special importance because anemia is quite common in the general population, particularly in slum dwellings in developing countries. Anemic subjects approach equilibrium levels of COHb more rapidly than those with normal hemoglobin at any level of exposure to CO. For example, a 4 h exposure of subjects with hemoglobin levels of 7 g/100 ml could attain COHb concentrations of 4–5% compared with an anticipated level of 2.5% for normal individuals; this fact coupled with greater production of endogenous CO would pose a greater threat of toxicity in anemic than in normal subjects (United Nations Environmental Program and WHO, 1979).

D. Maternal, Fetal, and Infant Toxicity of CO

Carbon monoxide crosses the placenta freely. Blockage of maternal and fetal hemoglobin by CO places the fetus at serious risk especially in the final weeks of gestation (Sheppard, 1983). Maternal and fetal hemoglobins are not identical. Fetal hemoglobin has a higher affinity for CO than the maternal hemoglobin. Almost 2 days are needed to achieve equilibrium in maternal and fetal hemoglobin and at equilibrium fetal COHb is slightly higher than the maternal COHb (Longo, 1970; Hill *et al.*, 1977).

Smoking is a well-recognized risk factor during pregnancy (Rogers, 2008). Norman and Halton (1990) analyzed 60 cases of exposure of pregnant women to CO and concluded that severe acute exposure to CO can lead to fetal deaths as well as anatomical malformation and functional alterations. Cigarette smoke has also been found to decrease fetal weight in rats (Carmines and Rajendran, 2008). Cigarette smoke contains 4,800 identified chemicals including CO, HCN, and of course nicotine; there is no difference in the toxicities of different brands of cigarettes (Hoffmann *et al.*, 2001). The well-established adverse effects of smoking on the fetus may be contributed by CO. While effects of chronic exposure to CO at levels that can be delivered by smoking CO have not been clearly documented, several animal studies and studies with embryos reviewed by Robkin (1997) suggest that the adverse effects of smoking on fetal development are due to factors other than CO.

CO poisoning of pregnant women can cause fetal death and malformation (Norman and Halton, 1990; Rogers, 2008). Several reports document that exposure to CO from ambient air pollution during pregnancy can decrease fetal weight and lead to preterm births (Gilboa *et al.*, 2005; Leem *et al.*, 2006; S. Liu *et al.*, 2007; Mannes *et al.*, 2005; Ritz *et al.*, 2002; Wilhelm and Ritz, 2005; Ziaei *et al.*, 2005). However, a large study conducted in 96 counties in the USA between 1999 and 2002 examined the effect of air pollution on pregnancy outcome; the study analyzed 3,538,495 births including 6,639 infant deaths and air composition of particulate pollutants >2.5 μm as well as CO, SO_2, and O_3; the study concluded that infant mortality was contributed by particulate matters and not by CO (Woodruff *et al.*, 2008).

In animal models, exposure to low levels of CO during pregnancy, which does not reveal obvious signs of fetal toxicity at birth, results in neurological and other abnormalities in later life (Annau and Fetcher, 1994; Benagiano *et al.*, 2005; Carratu *et al.*, 1997; Giustino *et al.*, 1999). The commonly encountered behavioral abnormalities in children could be due to prenatal exposure to low concentrations of CO; this possibility is very much real, and, if so, the prophylaxis is a monumental task and a major public health challenge since people have no choice but to breathe the air around them.

E. Tolerance to CO Toxicity

Hypoxia is the principal mechanism of CO toxicity. Hypoxia can also be produced by the low partial pressure of oxygen at high altitudes. The fact that people living at high altitudes do not exhibit signs of hypoxia implies adaptation to low oxygen pressure. It is thus no surprise that continuous exposure to CO also leads to adaptation (Killick, 1948). In one of the earliest studies on adaptation to CO, mice were exposed to successively higher concentrations of CO for 6–15 weeks; by the end CO concentration was nearly 3,000 ppm and COHb 60–70%; symptoms of this very high COHb were far less in the adapted than in the nonadapted mice (Killick, 1937); the underlying mechanism of this adaptation seems increased synthesis of hemoglobin (IPCS,

1999). These data have been confirmed by other researchers (Clark and Otis, 1952; Wilks *et al.*, 1959).

Adaptation to CO toxicity also seems to occur in humans. Doing experiments on herself, Killick (1940) found diminished symptoms and lower COHb on chronic exposure to CO than in the beginning, which is in accord with the data of Haldane and Priestley (1935). Adaptation to hypoxia is the reason why people living at high altitudes feel perfectly normal while a visitor from the plains may feel quite unwell. Indian and Pakistani soldiers are facing one another in Siachen of Kashmir, the highest place for any military confrontation in the world. Unless the soldiers are acclimatized before they go to Siachen, many develop fatal pulmonary edema; if they are acclimatized, the incidence of pulmonary toxicity is considerably reduced.

VII. PHYSIOLOGICAL ROLES OF CARBON MONOXIDE

The primitive earth atmosphere was composed of CO_2, CO, nitrogen, and water, and somehow radiation, heat, and sparks contributed to the generation of amino acid from these basic materials (Kobayashi *et al.*, 2002; Miyakawa *et al.*, 2002). If this is true, it should be possible to experimentally prove this; we are not aware whether this has been done. However, if it is true, nothing could be physiologically more important than CO, nitrogen, and H_2O.

Furchgott and Zawadzki (1980) made the seminal observation that vascular endothelium was essential for the vasorelaxant effect of acetylcholine; soon after, this vasorelaxant substance was identified as nitric oxide (Palmer *et al.*, 1987). It was subsequently found that NO increases guanylyl cyclase activity and cGMP and performs numerous physiological functions (Moncada *et al.*, 1991; Culotta and Koshland, 1992). The study of the physiological role of CO is relatively recent but it would not be surprising that in years to come CO may be on a par with NO. The excellent and extensive review of the physiological role of CO by Wu and Wang (2005) most certainly paints a rosy picture of CO; one can interpret the review to imply that the infamous CO is about to become a cure for many maladies.

NO and CO have several common properties; both are gases; both are putative neurotransmitters; both cause vasorelaxation; both increase cGMP; both inhibit platelet aggregation; both increase cGMP albeit from different pools; and both are synthesized endogenously although CO also had an exogenous source (Hartsfield, 2002; Snyder and Ferris, 2000). Being gases, neither CO nor NO directly depends upon plasma membrane transporter or channels for its entry into cells. Also, there exists a certain interdependence between CO and NO. NO augments HO-1 gene expression and consequently CO formation (Hartsfield, *et al.*, 1997); in its turn heme oxygenase can decrease NO synthesis (Maines, 1997). On the basis of data from our laboratory, we have proposed that *trans*-arachidonic acid increases the activity of heme oxygenase-2 leading to the generation of CO, which increases cGMP and thereby causes relaxation of cerebral microvasculature of rats as depicted in Figure 20.4 (Kooli *et al.*, 2008).

At the same time there are differences between CO and NO. For example, NO is an unstable gas and radical while CO is a stable gas and not a radical. CO binds only ferrous heme but NO binds to both ferrous and ferric hemoprotein (Hartsfield, 2002). The combination rate of NO with hemoglobin is faster and dissociation slower than that of CO (Sharma and Ranney, 1978) so that the affinity of NO for hemoglobin is ~1,500 times that of CO (Foresti and Motterlini, 1999). Formation of endogenous CO in a variety of tissues has been demonstrated (Marks *et al.*, 2002). Given the complexity of colocalization and activities of heme oxygenase and nitric oxide synthase, it has been speculated that ''CO and NO could function in a synergistic, compensatory, and/or counterregulatory way'' (Hartsfield, 2002).

The source of endogenous CO and NO and the enzymatic mechanisms in their synthesis differ (Hibbs *et al.*, 1987; Marletta *et al.*, 1988; Moncada *et al.*, 1991; Wu and Wang, 2005). There is no common mechanism of coordinating the release and activities of these two molecules; it is therefore difficult to conceptualize what would be the ultimate physiological effect of any interaction between the two molecules. Usually, if a substance has physiological effect, its excess or deficiency manifests in some physical disorder. While there is strong evidence that decreased formation of NO can affect blood pressure homeostasis, no condition has yet been identified which can be attributed to an excess or deficiency of endogenous CO. In contrast the toxic effects of exogenous CO are well characterized and there is no reason to assume that the source of this molecule makes fundamental difference in its pharmacology. In any case, this chapter focuses on CO toxicity. Putative physiological functions of endogenous CO have been extensively reviewed (Baringa, 1993; Choi and Otterbein, 2002;

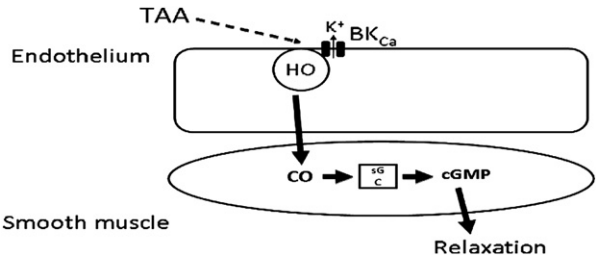

FIGURE 20.4. A model depicting the involvement of CO in mediating relaxation of brain pial microvessels of rats by trans-arachidonic acid (TAA). In this model TAA acts on calcium-dependent potassium cannels (BKca) leading to activation of hemeoxygenase-2 (HO-2), which releases carbon monoxide (CO). CO activates guanylyl cyclase (sGC) activity; the resulting increase in cGMP causes dilation of rat brain pial microvessels. Reproduced from Kooli *et al.* (2008) with permission from *Free Radical Biology & Medicine*.

Mannaioni and Vannacci, 2006; Marks *et al.*, 1991; Snyder and Ferris, 2000; Wu and Wang, 2005). A brief account of the postulated physiological roles of CO is presented below.

A. CO as a Putative Neurotransmitter

The colocalization of heme oxygenase-2 (HO-2), the enzyme responsible for the release of CO from COHb, and guanylyl cyclase in discrete brain areas lacking NOS coupled with changes in cGMP by agents and procedures that alter CO, has been advanced as evidence for a putative neurotransmitter role for CO (Verma *et al.*, 1993). However, these workers (Verma *et al.*, 1993) did not test whether or not changes in cGMP were associated with any changes in neuronal activity, nor did Baringa (1993) feel any need for such details in their commentary in the same issue of *Science*. In further support for neurotransmitter role for CO, Boehning *et al.* (2003) demonstrated that HO-2 is activated by phosphorylation of CK2 (casein kinase 2) during neuronal depolarization and that CK2 activation is essential during CO-mediated nonadrenergic noncholinergic relaxation of the smooth muscle. Also, a role of heme oxygenase and CO has been demonstrated in olfactory neurons in culture (Ingi and Ronnett, 1995; Ingi *et al.*, 1996), myenteric plexus (Zakhary *et al.*, 1996), and vas deferens (Burnett *et al.*, 1998).

The researchers cited above have provided strong evidence that CO is released during heme breakdown and that liberated CO can increase cGMP. However, according to the established criteria, a neurotransmitter, among other things, must transmit a message across a synapse; unless clear evidence is presented for an effect of CO on neuronal transmission, CO ought to be treated only as a putative but not a proven neurotransmitter.

B. Other Physiologic Effects of CO

Brune and Ullrich (1987) demonstrated that CO increases the activity of guanylyl cyclase like NO and inhibits platelet aggregation triggered by a variety of agents. CO was found to dilate coronary arteries and increase coronary blood flow in rats (McFaul and McGrath, 1987; McGrath and Smith, 1984); these researchers did not examine if this was associated with an increase in cGMP but they excluded involvement of an adrenergic system. Similar vasodilator effects of CO have been found on porcine coronary artery (Graser *et al.*, 1990), rabbit cerebral arteries (Brian *et al.*, 1994), rabbit aorta (Furchgott and Jothianandin, 1991), and rat aorta (Lin and McGrath, 1988). CO produces similar effects on cerebral blood flow; this subject has been reviewed by Koehler and Traystman (2002). CO has been shown to relax cerebral microvasculature (Leffler *et al.*, 1997) and pulmonary as well as systemic vessels (Villamor *et al.*, 2007) of newborn pigs. The vasorelaxant effect of CO is endothelium independent (Vedernikov *et al.*, 1989; Wang *et al.*, 1997) and exerted via K^+ channels (Graser *et al.*, 1990; Wang and Wu, 1997), in a manner similar to the oxidant OCl^- (Varma *et al.*, 2006), which is also endogenously produced (Weiss *et al.*, 1985, 1986). Also, CO can increase cerebral blood flow in rats (MacMillan, 1975), rabbits (Meyer-Witting *et al.*, 1991), and fetal sheep (Rosenberg *et al.*, 1986).

Heme oxygenase activation as well as CO induce the synthesis of vascular endothelial growth factor (Dulak *et al.*, 2002) but suppress its induction by hypoxia (Liu *et al.*, 1998). Heme oxygenase-1 plays a protective role against vasoconstriction and proliferation (Duckers *et al.*, 2001; Lee *et al.*, 1996; Sammut *et al.*, 1998), regulates vascular cGMP (Morita *et al.*, 1995), suppresses endothelial cell apoptosis (Brouard *et al.*, 2000), and activates mitogen-dependent protein kinase pathway (Otterbein *et al.*, 2000). Paradoxically, CO can prevent ischemic lung injury by suppressing fibrinolysis (Fujita *et al.*, 2001).

A number of studies have examined the anti-inflammatory activities of CO (Abraham *et al.*, 1988; Mannaioni *et al.*, 2006). An activation of the heme oxygenase system and exposure to CO *in vitro* (Chauveau *et al.*, 2005; Gibbons and Farrugia, 2004; Otterbein *et al.*, 1999, 2000; Pae *et al.*, 2004; Sethi *et al.*, 2002) and *in vivo* (Dolinay *et al*, 2004; Nakao *et al.*, 2003; Neto *et al.*, 2004; Otterbein *et al.*, 1999) revealed anti-inflammatory activity in animal models but not in humans (Mayr *et al.*, 2005). CO can inhibit apoptosis of endothelial cells (Soares *et al.*, 2002). There is some evidence that upregulation of HO-1 might be beneficial in inflammatory diseases (Willis *et al.*, 1996). In any case, the ability of CO to inhibit platelet aggregation and inflammation has important therapeutic implications and needs to be explored.

Liver is a site for cytochrome P450 synthesis as well as degradation of senescent erythrocytes and consequently catabolism of hemoglobin and heme oxygenase, which mediates the generation of endogenous CO. Since oxyhemoglobin can capture CO, it would reduce available CO, which could and has been shown to increase hepatic vascular resistance (Suematsu *et al.*, 2000). There is some evidence that endogenous CO formation in the hypothalamus might modify hypothalmo-pituitary-adrenal axis (Mancuso *et al.*, 1997).

In conclusion, there is definite evidence that endogenously produced CO exerts biological effects. If these are important physiological effects, one can assume that a deficiency in endogenous CO generation might manifest as a disease state or worsen existing diseases. Vascular NO is thought to be of relevance in maintaining vascular tone and its deficiency can cause hypertension or platelet aggregation. Diseases caused by a deficiency of hormones or neurotransmitters are ameliorated by substitution therapy. There is no reason to think that exogenous CO cannot substitute for a deficiency of endogenous CO. For example, endogenous CO deficiency increases acid-dependent bile flow; this can be corrected by exogenous CO (Shinoda *et al.*, 1998; Suematsu *et al.*, 2000). A lower

incidence of Parkinson's disease among smokers than in nonsmokers is attributed to nicotine in cigarettes (Quick, 2004). Fortunately, the beneficial effects of nicotine in cigarettes have not led to promoting smoking. Hopefully, any useful role for CO would also not lead to promoting the therapeutic value of cigarettes, which is a rich source of CO.

VIII. TREATMENT OF CARBON MONOXIDE OVERDOSE

A. Oxygen

When Humphrey Bogart rescued Audrey Hepburn from a garage where she had turned on the ignition keys of several cars in the film *Sabrina*, she knew that exhaust fumes could kill and he knew that fresh air could save life. The treatment of CO intoxication by oxygen was elaborated by Haldane (Haldane, 1895a, 1922; Haldane and Priestley, 1935). Perhaps the general public is aware of this therapy.

It was implicit in Haldane's studies that oxygen at high pressure would be more effective than at atmospheric pressure. However, there was a reluctance to resort to this practice because of the fear of oxygen toxicity. End and Long (1942) successfully demonstrated the value of oxygen at high pressure to treat CO overdose in laboratory animals. To our knowledge the first controlled study on the value of hyperbaric oxygen against CO poisoning was done by Pace *et al.* (1950) on ten human volunteers, five men and five women; data of these workers show that changing ambient pO_2 from 0.2 to 2.5 atmospheric pressure absolute increased the rate constant (k) of elimination of CO from 0.0028 to 0.0315 min, which would alter the half-life of CO elimination from 246 to 21 min.

B. Other Therapeutic Measures

CO poisoning leads to several abnormalities such as generation of oxidants, lipid peroxidation, disturbance in glucose metabolism, and so on, which compound the toxic effect of CO caused by hypoxia; measures other than oxygen are intended to deal with these secondary changes.

1. Allopurinol and *N*-Acetylcysteine

There is strong evidence that some of the toxic effects of CO poisoning are produced by release of reactive oxygen metabolites and xanthine oxidase plays a major role in these processes (Sokal and Kralkowska, 1985; Thom, 1992; Zhang and Piantadosi, 1992). Xanthine oxidase is a nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase, which is converted to oxidase under ischemic conditions utilizing molecular oxygen in place of NAD and in the process generates superoxide radicals and hydrogen peroxide. Allopurinol is a xanthine oxidase inhibitor and *N*-acetylcysteine is a sulfhydryl donor. The efficacy of these agents against neuronal injury caused by CO has been documented (Thom, 1992; Howard *et al.*, 1987).

2. Insulin

Hyperglycemia, which follows stroke or myocardial infarction, worsens the resulting neurological insult. Acute CO poisoning is also characterized by hyperglycemia, which has been found to worsen brain dysfunction in rats (Penny *et al.*, 1990). Similar observations have also been made in CO-poisoned patients (Pulsinelli *et al.*, 1980). Moreover, neurological complications of CO poisoning in diabetic patients seem worse than in healthy individuals. There is evidence that insulin treatment is effective in reducing neuronal damage caused by stroke or cardiac arrest, and similar encouraging results have been found after CO poisoning in humans (White and Penny, 1994).

3. Other Measures

Due to the role of excitatory amino acids in ischemic neurodegeneration, Ishimura *et al.* (1991) studied the effect of several agents including NMDA receptor antagonists against CO poisoning in mice with encouraging results. We are not aware of any clinical studies on the efficacy of NMDA receptor antagonists in reducing neurological toxicity in humans after CO poisoning. Cerebrolysin, produced by enzymatic breakdown of lipid-free proteins of porcine brain, have been found to offer protection against certain neurological changes after CO poisoning in animal models. Hypothermia has also been found of benefit in CO poisoning (Peirce *et al.*, 1972).

IX. CONCLUDING REMARKS AND FUTURE DIRECTION

We have attempted to deal with most though not all aspects of the pharmacology, toxicology, and physiology of carbon monoxide. The literature on CO is immense and dates back to 1857 or even earlier. The omnipresence of this colorless, odorless, tasteless, and nonirritating gas, generated by incomplete combustion and ever present in megacities, has traveled a long way from being a leading cause of unintentional death and an agent of choice for suicide to finding a physiological role as big as a neurotransmitter like nitric oxide.

Many currently used drugs like acetyl salicylic acid, digitalis, nitrates, and quinine have existed for a long time, and so have many poisons like cyanide and neurotoxins. However, their mechanism of action was not known until the middle of 20th century or even later. CO is unique. The details of its mechanism of toxicity were published by Haldane as early as 1895 and subsequent work has only elaborated these details while affirming its correctness. The generation of COHb is the principal cause of CO toxicity; however, it is not the only one. Much research has been done to elaborate these additional mechanisms of CO

toxicity but we have not discussed them in detail, just as we have not gone into detail about the physiological role of endogenous CO as one of the many warfare chemicals.

If detected in time, CO poisoning can be treated with hyperbaric oxygen plus other supplementary measures. However, safeguarding against chronic toxicity, if it results from chronic exposure to low levels of CO, is a difficult challenge. Many megacities are highly polluted with CO. Burning petroleum in automobiles as the chief source of CO in the modern world would most probability increase rather than decrease atmospheric CO, ozone, nitrogen, and hydrogen sulfide, etc.; this poses one of the great challenges of modern times to public health care. It is the responsibility of the environmental toxicologists, other scientists, and a socially conscious population to do whatever they can to ensure that measures are taken to protect the citizenry from hazardous chemicals in the air we breathe.

Acknowledgments

We thank Ms Emily Gibson for compiling and checking references. This article is dedicated to the memory of Professor John Sanderson Haldane, FRS (1861–1936), who conducted experiments on himself with poisonous carbon monoxide to save the life of miners, and whose laws on the exchange of gases remain valid even today.

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CHAPTER 21

Methyl Isocyanate: The Bhopal Gas

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I. INTRODUCTION

Methyl isocyanate (MIC) is the smallest member of the isocyanate family and the most reactive and toxic of all. MIC was almost unheard of until the fateful night December 3, 1984, when nearly 30 metric tons of this poisonous chemical spewed out of the Union Carbide India Ltd (UCIL) pesticide plant within a period of 45–60 min (Jayaraman, 1984). Bhopal turned into a ''city of death'', wrote the fortnightly *India Today* (December 30, 1984). The journal *Nature* (Opinion, 1984) vented its anger thus: ''… the anguish vividly carried round the world by the television cameras seems not to have matured into the anger, even hysteria, there would have been had the [Bhopal] accident occurred on the edge of a European city – or in Connecticut'' (the headquarters of Union Carbide was in Danbury, Connecticut, USA).

There was only one scientific report on MIC toxicity (Kimmerle and Eben, 1964) until the Bhopal disaster; this led *Lancet* (Editorial, 1984) to comment: ''In a year's time we will have learnt a lot more about methyl isocyanate – at an appalling price.'' As is to be expected, the Bhopal disaster evoked immense interest among journalists, scientists, the corporate world, lawyers, social activists, and the Indian government. A discussion of the toxicology of MIC necessitates a narrative of different events specific to the Bhopal disaster. However, this chapter mainly focuses on four aspects. One, why did MIC escape in such huge amounts and can this reoccur at other places where MIC is manufactured and stored? Two, what are the physicochemical characteristics of MIC that make it the most toxic of all isocyanates and deadlier than cyanide? Three, what is the toxicity profile of MIC? Four, what was done and not done to deal with the tragedy? Other aspects such as social cost, legal implications, and issues of rehabilitation (Budiansky, 1985; Dhara and Dhara, 1995; Varma, 1986) are equally important; however, these aspects are not being discussed.

II. BACKGROUND

At the time of the disaster the population of Bhopal was approximately 800,000; more than one-quarter of the population was exposed to toxic gases. The toxicity of a chemical, barely tested in animal models, was suddenly being observed on unsuspecting thousands of children, women, and men. The magnitude of the disaster was so enormous that some called it the Nagasaki and Hiroshima of peace time. ''This may be how the world will end – not with a bang but with an ecological whimper'', wrote Abu Abraham in the *Sunday Observer*, Bombay, December 23, 1984. ''India's disaster – The night of death'' was displayed on the front cover of the *Time* magazine (December 17, 1984, New York). *Nature* (Opinion, 1984) expressed its dismay at the lack of international concern for this tragedy. *Chemical and Engineering News* (1985) published a special issue ''Bhopal the continuing story''. A British medical student, who had arrived just one day earlier in Bhopal to start her elective, had this to say (Sutcliffe, 1985): ''The dead and dying arrived by the truckload, others came by rickshaw or were carried by relatives. For some the effort of the journey itself proved too much, and they died soon after arrival.''

Bhopal is known as the city of the world's worst industrial disaster. That might be why the 20th anniversary of the Bhopal episode in 2004 was covered by news media all over the world. Commentaries appeared in *Nature Medicine* (Padma, 2005), *Science* (Crabb, 2004), *The Lancet* (Sharma, 2005) and other journals. By this time the story of MIC and Bhopal had been the subject of several books and monographs (Eckerman, 2005; Everest, 1985; Lapierre and Moro, 2001; Morehouse and Subramaniam, 1986; Sinha, 2007; Sufrin, 1985) and reviews (Bucher, 1987; Dhara and Dhara, 1995, 2002; Dhara *et al.*, 2002; Dhara and Gassert, 2002; Dhara and Kriebel, 1993; ICMR, 1985, 1986, 2004; Lepkowski, 1985; Marwick, 1985; Mehta *et al.*, 1990; Sriramachari, 2004; Sriramachari and Chandra, 1997; Varma, 1986; Varma and Guest, 1993; Varma and Varma, 2005; Varma and Mulay, 2006).

Given the social dimensions of the tragedy, a documentary ''Bhopal beyond genocide'', directed by Tapan Bose and Suhasini Mulay, was produced in 1986 by Cinemart Foundation, New Delhi. ''Bhopal: The Search for Justice'', directed by Peter Raymond and Lindalee Tracey, was produced by White Pine Pictures and the National Film Board of Canada in 2004. The play ''Bhopal'' by Rahul Varma was staged in Canada and its Hindi version titled ''Zahrili Hawa (poisonous gas)'' was staged in Indian cities under the direction of the renowned theater personality, Habib Tanweer.

Soon after the Bhopal accident, the government of India intervened in two important areas; first, it assumed all rights for negotiations about the liability of the Union Carbide Corporation and subsequently with its new owners Dow Chemical Corporation and, second, assured an investigation into the causes of the disaster as well as a comprehensive study of the acute and long-term effects of exposure of the Bhopal population to MIC or other gases. While the details of the negotiations are not integral to this chapter, the promised toxicological studies were scarcely done. Although the Indian Council of Medical Research (ICMR) released approximately $2 million for research on MIC toxicity, there is no indication that scientists were assigned to undertake a systematic study of different areas to unravel the complete picture of MIC toxicity.

As time passed, three important developments made the study of MIC toxicology problematic. First, there has been a significant movement of the population, which usually is not a characteristic of Indian cities. For example, out of 317 children identified in 1985 to have been exposed to the gas *in utero*, fewer than 100 could be traced 20 years later. Second, there is evidence of contamination of groundwater, which makes it difficult to distinguish between effects due to exposure to toxic gases and those due to ingestion of groundwater toxins. Third, people in Bhopal are looking for relief from their suffering and are not keen on being subjects of a researcher's inquisitiveness; therefore honest scientific inquiry necessitates a rapport with the population, which the government-commissioned scientists have not been able to establish.

III. THE MAKING OF A DISASTER

The specific details of how MIC escaped from Tank E-610 at the UCIL plant in Bhopal have been described in detail elsewhere (Varadarajan *et al.*, 1985; Varma, 1986; Varma and Varma, 2005). It is highly unlikely that an accident will repeat itself in exactly the same way it did in Bhopal on December 2–3, 1984. On the other hand, a brief description of the Bhopal episode is relevant to speculate how such accidents can occur and what needs be done to ensure that they do not happen elsewhere.

In the case of Bhopal, water entered into pipes on the floor of the factory during routine cleaning but safety slips had not been placed at the joints. Water reached Tank E-610. The exothermic reaction between water and MIC increased the temperature of the tank converting liquid MIC into gas. The increase in pressure forced open the vent valve letting most of the MIC escape to the outside as gas. Various safety measures to neutralize MIC, such as caustic soda scrubbers, were not functional or unserviceable. Even if safety measures had been in perfect workig order, they could not have handled such a big leak. Fortunately, MIC stored in the other two tanks (E-611 and E-619) was not affected; it was later converted to pesticide in what was termed ''Operation Faith'' during December 16–22, 1984. The disaster had frightened the people of Bhopal to such an extent that despite all assurances by the Indian government, almost one-half of the population left the town, some with their entire belongings, during this exercise.

An examination of the causes of the Bhopal disaster clearly indicates that the accident would not have occurred if all necessary precautions had been taken in the maintenance and operation of the Union Carbide Pesticide Plant. This required continued and apt maintenance, regular inspection by independent authorities, sufficiently well-trained staff, and location of the plant far away from residential areas and not just within 1 km of the railway station and within 3 km of two major hospitals, as was the case in Bhopal.

Around the time of the Bhopal disaster, MIC was used in the USA (West Virginia), Germany, and Japan; at none of these places was MIC stored in large quantities. One can assume that other cautionary measures were followed more rigorously in these places than in Bhopal, suggesting that developing countries with very poor regulatory processes require more stringent safety measures than in developed countries.

Whether or not the operation of hazardous industries can be both safe and profitable is debatable. What is not debatable is that safety must remain the top consideration. There is a strong case to demand state control of hazardous corporate operations (Varma and Varma, 2005) and global monitoring of potentially toxic materials (Baxter, 1986; Sriramachari and Chandra, 1997). Following the cyclohexane explosion in Flixborough in 1974, an Advisory Committee on Major Hazards was set up in the UK. The European Economic Council Directive (1982) was triggered by the Seveso accident of 1976 in Italy. However, the Bhopal disaster of 1984 was far worse than the Flixborough or Seveso accidents or for that matter any other in history which has not led to additional regulations outlining corporate and state responsibilities. MIC is dangerous and is to be treated on a par with nuclear establishments, requiring the utmost care in maintenance.

IV. TOXICOKINETICS OF ISOCYANATES

MIC is a member of the isocyanate family of chemicals. The high chemical reactivity of isocyanates is central to their toxicity as well as commercial uses. No clinical use of isocyanates has so far been demonstrated. In view of these considerations, this section will elaborate in some detail the relationship between the structure of MIC and other isocyanates, and between their physicochemical properties and toxicities.

A. Chemistry of Isocyanates

Organic isocyanates were first synthesized in 1849. Isocyanates (Table 21.1) are highly reactive heterocumulene

TABLE 21.1. Commonly used isocyanates

Isocyanates	MW	LC_{50} ppm[a]	Ceiling ppm[b]
Methyl isocyanate (MIC)	57	5.1	0.02
Hexamethylene diisocyanate (HDI)	168	55.9	0.02
Toluene diisocyanate (TDI)	174	49.0	0.02
Isophorone diisocyanate (IPDI)	222	28.5	0.02
Diphenylmethane diisocyanate (MDI)	250	36.0	0.02
Dicyclohexylmethane diisocyanate (SMDI)	262	–	0.01
1,5-Naphthalene diisocyanate (NDI)	210	–	0.02

[a] LC_{50} (lethal concentration killing 50 experimental animals) values are after 4 h exposure

[b] To convert ppm to mg/m^3, divide it by (24.4/MW); see review by Varma (1986)

chemicals. The general structure of isocyanates is R–N=C=O which is distinct from cyanate N≡C–O–H. The reactivity of organic isocyanates is due to the strain in the cumulative double bonds (–N=C=O) of isocyanates (D'Silva *et al.*, 1986; Varadarajan *et al.*, 1985; Westcott, 1985).

Most of the commercially used isocyanates are diisocyanates and R is an aromatic ring. MIC is an exception; its structure is H_3C–N=C=O. The physicochemical properties of MIC differ from those of other isocyanates (Lowe, 1970; Tse and Pesce, 1978; Westcott, 1985; Worthy, 1985). Because of high chemical reactivity of MIC with alcohols, it serves as an intermediate in the production of the pesticide carbaryl. Diisocyanates are primarily used for the manufacture of polyurethanes.

1. Synthesis of MIC

MIC (CH_3N=C=O) can be synthesized using different reactions. The commercial synthesis of MIC by the Union Carbide Corporation, Bayer, and Dupont is described below:

At Union Carbide Corporation, Bhopal, India:

$$CH_3NH_2 \text{ (monomethyl amine)} + COCl_2 \text{ (phosgene)} \rightarrow CH_3NCO + 2HCl$$

At Bayer, Germany:

$$CH_3NH\text{–}CO\text{–}NHCH_3 \text{ (dimethyl urea)} + (C_6H_5O)_2CO \text{ (diphenyl carbamate)} \rightarrow CH_3NCO + \text{byproducts}$$

At Dupont, USA:

$$CH_3\text{–}NH\text{–}CHO \text{ (methyl formamide)} + O_2 \rightarrow CH3NCO + H_2O$$

MIC was produced and stored in Bhopal. Other chemicals used for the production of carbaryl, such as chlorine, methylamine, caustic lye, and chloroform, were brought to Bhopal and stored in tanks. CO and phosgene were not stored but produced and utilized.

Various steps in the production of carbamate pesticide at Bhopal were (Varadarajan *et al.*, 1985):

1. Petroleum coke (2C) was reacted with oxygen to produce 2CO.
2. CO and chlorine were reacted to produce phosgene $(COCl_2)$–.
3. Phosgene and methylamine (CH_3NH_2) were reacted to form methylcarbamoyl chloride ($CH_3NHCOCl$) plus HCl.
4. Methylcarbamoyl was then pyrolyzed to yield methyl isocyanate (CH_3NCO) and HCl.
5. Finally, MIC was reacted with a slight excess of α-naphthol in the presence of a catalyst in carbon tetrachloride solvent to produce the desired pesticide carbaryl.

In the system used in Bhopal, the stored liquid MIC was transferred through pipes to one metric ton charged pots under nitrogen pressure of 16 psi. These charged pots were connected to two reactors where MIC and α-naphthol reacted to produce carbaryl (see below). The charging of α-naphthol was done by dissolving it in carbon tetrachloride at approximately 50°C in the presence of a catalyst trimethylamine. The reaction between MIC and α-naphthol is exothermic. The temperature was maintained at 70°C for efficient production of carbaryl.

$$CH_3N{=}C{=}O + \alpha\text{-Naphthol} \rightarrow 1\text{-Naphthyl-}N\text{-methyl carbamate (carbaryl)}$$

The alternate route of production of carbaryl involves reaction of α-naphthol with phosgene to generate α-naphthol chloroformate which upon reaction with methylamine leads to the formation of carbaryl.

2. Physicochemical Reactions with MIC

MIC can interact with a large number of molecules as well as with itself. Indeed, 21 identified (Varadarajan *et al.*, 1985) and almost ten unidentified chemicals (Sriramachari, 2004) were detected in the culprit MIC Tank E-610. An MIC trimer as well as other metabolites of MIC such as dimethyl isocyanurate and 2,4-dione of methyl isocyanate were identified in autopsy samples from Bhopal victims (Chandra *et al.*, 1991, 1994; Saraf *et al.*, 1995). Reaction of MIC with water is important because this will occur whenever MIC comes into contact with a body or environment, as happened in Bhopal. It is important to note that while excess water can neutralize MIC, a small quantity of water is sufficient to generate heat during the reaction, which would lead to vaporization of MIC, as actually happened in Bhopal. Some important interactions of MIC are enumerated below:

1. Polymerization (self-addition of many MIC molecules).
2. Trimerization:

$$3(CH_3N{=}C{=}O) \rightarrow \text{Trimethyl isocyanurate}$$

3. Dimerization is common with aromatic isocyanates but is rare with aliphatic isocyanates like MIC.
4. Additive reactions with molecules containing reactive hydrogen species, which migrate to the nitrogen of MIC.
5. General reaction of MIC with molecules containing hydroxylic groups:

$$CH_3N{=}C{=}O + ROH \rightarrow CH_3NHCOOR \ (\text{urethane})$$

6. Reactions with water:

$$CH_3NCO + H_2O \rightarrow CH_3NH_2 \ (\text{MMA}) + CO_2$$

$$2(CH_3NCO) + H_2O \ (\text{excess}) \rightarrow CH_3NHCONHCH_3 \ (\text{DMU}) + CO_2$$

$$3(CH_3NCO) \text{ in excess} + H_2O \rightarrow CH_3NHCON\ (CH_3)\ CONHCH_3\ (\text{TMB}) + CO_2$$

$$4(CH_3NCO) + H_2O \rightarrow \text{DMI} + (CH_3)2NH\ (\text{DMA}) + CO_2$$

7. Reaction with DMA:

$$CH_3NCO + \text{DMA} \rightarrow (CH_3)2N{-}CO{-}NH{-}CH_3\,(\text{TMU})$$

8. Reaction with alcohols and phenols (used by Union Carbide Corporation, India, to produce carbaryl pesticide):

$$CH_3NCO + \alpha\text{-Naphthol} \rightarrow \text{1-Naphthyl-}N\text{-methyl carbamate (carbaryl)}$$

9. Reaction with primary and secondary amines:

$$CH_3NCO + R{-}NH_2 \rightarrow CH_3{-}NH{-}CO{-}NH{-}R$$

10. Reaction with nitrates and nitrites: the reaction of MIC with nitrates and nitrites, which are normally present in water, can yield carcinogenic nitrosamines.
11. Decomposition into hydrogen cyanide (HCN): when MIC pyrolyzes into the gaseous phase in the range 427–548°C at 55–300 torr, the major decomposition product is HCN (Blake and Ijadi-Maghsoodi, 1982). This reaction is of relevance in view of the conjecture that the culprit gas in Bhopal was HCN. However, MIC does not degrade to HCN at low temperatures and pressures. The temperature in Tank 610 was estimated to have reached approximately 200°C, far below that required for conversion of MIC into HCN.
12. Reaction with HCN at normal temperatures leading to the formation of other cyanides (Slotta and Tschesche, 1927).
13. Reactions with body constituents.

In general, interactions between isocyanates and endogenous molecules are reversible (Axness and Fleeker, 1979; Gibson and Hickman, 1982; Newman and Farquhar, 1987; Tse and Pesce, 1978; Twu and Wold, 1973). MIC has been shown to cause greater interaction with macromolecules than aryl isocyanates because of the slower hydrolysis of the former than of the latter in biological medium (Brown *et al.*, 1987); indeed, this has been suggested to be the cause of sensory and pulmonary irritation with MIC and not with toluene diisocyanate (Ferguson *et al.*, 1986). Reversible conjugation of isocyanates with glutathione (Pearson *et al.*, 1990; Slatter *et al.*, 1991), which occurs both spontaneously and enzymatically (Brusewitz *et al*, 1977; Mennicke *et al.*, 1983), may have been the mechanism of distributing MIC molecules to different parts of the body and for its diffuse toxicity profile (Baillie and Slatter, 1991; Bruggeman *et al.*, 1986; Pearson *et al.*, 1991).

Kinetic analysis of hydrolysis revealed a half-life of 2 min for MIC in aqueous solution, which is much slower than that for aryl isocyanates (Brown *et al.*, 1987). Interaction of isocyanates with cholinesterases is reversible and MIC is far less potent than aryl isocyanates in this respect (Brown *et al.*, 1987). At the same time MIC can act as a hapten leading to generation of antibodies in both animals and humans, although it results in low titers (Karol and Kamat, 1988; Karol *et al.*, 1987).

A reactive *S*-(*N*-methylcarbamoyl) glutathione (SMG) has also been isolated from the bile of rats administered methyl isocyanate (Pearson *et al.*, 1990, 1991); the corresponding mercapturic acid has been isolated from the urine (Slatter *et al.*, 1991). Since MIC-SMG conjugate is reversible, it is likely that it serves as a method of transport of MIC to different organs. MIC can carbamylate macromolecules (Bhattacharya *et al.*, 1988; Ramachandran *et al.*, 1988; Segal *et al.*, 1989; Sriramachari *et al.*, 1991). MIC has been shown to be an effective anti-sickling agent *in vitro*, combining with α-amino groups of hemoglobin and thus increasing its oxygen binding affinity (Lee, 1976).

3. Quantification of MIC

The method of collection of MIC samples in the workplace, and under controlled experimental conditions, differs because its concentrations are in the range of parts per billion (ppb) in the former case and several orders of magnitude higher in the latter case.

The methods of sampling MIC in air at the workplace are mainly by pumped sampling in combination with impingers, reagent-coated or impregnated with sorbent tubes for collection of the analytes. The isocyanate thus collected is reacted with an amino-based reagent such as 1-(2-methoxyphenyl) piperazine (2MP) or other similar substances. Details of sampling and measurement of MIC are described in detail by others elsewhere (Henneken *et al.*, 2003; Von Zweibergk *et al.*, 2002). Sampling under controlled experimental conditions can be done using gas-tight syringes.

Several techniques have been used for the quantitative analysis of MIC. Kimmerle and Eben (1964) used the spectrophotometric method at 352–353 nm. Most studies following the Bhopal disaster used GC-MS equipped with appropriate detectors, while Adkins *et al.* (1987) used infrared spectroscopy between 3.3 and 3.6 nm. The method employed in Alarie's lab (Ferguson *et al.*, 1986) and by us (Varma *et al.*, 1987, 1988, 1990) used a Perkin-Elmer Model 3920 gas chromatograph equipped with a nitrogen–phosphorus detector; the column used for the chromatographic analysis of MIC was made of glass, packed with 3% OV-210 on Chromosorb WHP (SO/l00 mesh), run isothermally at 60°C, and permitted on-column injection of samples. Helium was passed through the column at 20 ml/min (inlet pressure: 60 psi). The injector and interface temperatures were both set at 200°C. Air and hydrogen passed into the nitrogen–phosphorus detector at 30 and 1 to 3 ml/min, respectively. An MIC calibration curve was prepared by injecting known volumes of pure MIC gas into glass vessels of 1,000 ml thus producing MIC of different dilutions. These vessels contained glass beads for mixing and were equipped with Teflon-coated septa, enabling the withdrawal of desired volumes for subsequent injection onto the chromatography column by gas-tight (Unimetrics) syringes. The absolute retention time for MIC under these conditions was approximately 1 min and sensitivity of the method was 0.8 ng MIC. Following calibration, samples were withdrawn from the animal exposure chamber with the same syringes. Samples were taken approximately every 3–5 min during the exposure period and the mean concentration was determined for each exposure. Coefficients of variation for each mean were under 20%, indicating low variation between samples analyzed during exposure.

V. MECHANISM OF ACTION

Physicochemical properties of MIC relevant to its toxicity are presented in Table 21.2. MIC exerts a wide spectrum of toxic effects, including sensory and pulmonary irritation (Alarie *et al.*, 1987; Ferguson *et al.*, 1986; Kamat *et al.*, 1985, 1992; Kimmerle and Eben, 1964), reproductive toxicity (Varma, 1987), ocular toxicity (Andersson *et al.*, 1984; Salmon *et al.*, 1985) and neurological toxicity (Bharucha and Bharucha, 1987; Sethi *et al.*, 1987). There is some evidence indicating teratogenic and carcinogenic effects of MIC (Goswami, 1986; Saxena *et al.*, 1988; Ghosh *et al.*, 1990; Dikshit and Kanhere, 1999). However, unlike the toxicity of pesticides or cyanide, which can be clearly attributed to inhibition of cholinesterase (Taylor, 1980) or cytochrome oxidase (Goldstein *et al.*, 1968), respectively, specific biochemical mechanisms, which can explain these diverse toxic effects of MIC, have so far not been identified. The paucity of data on MIC toxicity at the time of the Bhopal disaster was because of a lack of systematic study of the clinical toxicity of MIC and the incompleteness of documents of Union Carbide (1978).

TABLE 21.2. Physicochemical properties of methyl isocyanate (MIC) and toluene diisocyanate (TDI)

Properties	MIC	TDI
Appearance	Colorless liquid	Colorless to yellow liquid
Flammability	Flammable	Not flammable
Specific gravity (water = 1) at 20°C	0.96	1.22
Boiling point (°C)	39.1	251
Autoignition temperature (°C)	535	–
Vapor density (air = 1)	1.97	6.0
Vapor pressure at 20°C (mbar)	464	0.06
Weight (mg/m^3) = 1 ppm	2.5	7.12

The immediate effect of exposure to MIC in Bhopal was lacrimation, choking sensation, and difficulty in breathing followed in many cases by death. There was a lag period of 4–6 h between exposure to MIC and death in Bhopal (Varma, 1986). Delayed death was also observed in experimental animals (Varma *et al.*, 1988; Dodd *et al.*, 1986, 1987; Fowler *et al.*, 1987a; Bucher *et al.*, 1987a, b; Boorman *et al.*, 1987; Stevens *et al.*, 1987; Fowler and Dodd, 1987a, b; Alarie *et al.*, 1987). It would thus appear that the lethal effects of MIC were caused by pulmonary complications; these complications could be attributed to pulmonary irritation, which could ultimately lead to fluid exudation and pulmonary edema (Alarie *et al.*, 1987), tissue hypoxia (Fowler *et al.*, 1987b), or acidosis (Fedde *et al.*, 1987). Hypoxia does not seem to result from inhibition of oxygen utilization as is the case with cyanide (Alarie *et al.*, 1987). There is some evidence that at high concentrations MIC can reversibly inhibit cholinesterase (Brown *et al.*, 1987; Troup *et al.*, 1987); this could cause pulmonary edema. However, other characteristic signs of excess acetylcholine such as papillary constriction or skeletal muscle paralysis were not reported in Bhopal victims.

1. There are reports of biphasic death in Bhopal. The first phase – within 2 days of exposure – was followed by a second phase of deaths during subsequent months and years (Varma, 1986; Varma and Guest, 1993; Varma and Mulay, 2006). Interestingly, the pattern of death in experimental animals (rats, mice, and guinea pigs) following exposure to MIC was similar to that observed in Bhopal; even excessively high concentrations (3,506 ppm for 15 min) of MIC were not lethal to rats within 10 min but 1,000 ppm killed 69% of animals between 4 and 6 h (Dodd *et al.*, 1987). Qualitatively

similar data have been reported by others at MIC concentrations that could have been achieved in Bhopal (Bucher *et al.*, 1987b; Varma *et al.*, 1988; Ferguson and Alarie, 1991). Exposures to MIC have consistently led to a significant decline in body weight within 24–48 h (Dodd *et al.*, 1986; Bucher *et al.*, 1987b; Varma, 1987; Varma *et al.*, 1988), suggesting significant loss of body fluid. Overall, both clinical observations in Bhopal and experimental data suggest that initial deaths were caused by hypoxemia resulting from pulmonary edema plus alveolar obstruction, and delayed deaths were the result of pulmonary complications.

2. It is possible that high chemical reactivity of MIC leads to its interaction with and disorganization of tissue macromolecules; this could also explain numerous other biological effects of MIC. There is evidence that MIC can deplete key components, which can contribute to death (Kolb *et al.*, 1987).

In the experiments conducted by Kimmerle and Eben (1964), human volunteers were exposed to unadulterated MIC; the symptoms observed in these volunteers are identical to those noted in Bhopal, albeit far less severe because as the concentration of MIC was increased to 21 ppm, volunteers could not tolerate it even for seconds. We are not aware of any studies examining whether MIC and HCN toxicities are additive or synergistic; on purely theoretical grounds at least an additive effect can be predicted.

In summary, while MIC can cause specific effects, its overall toxicity seems to result from its ability to physically damage any cell or organ it comes in contact with and the ultimate effects, including death, are secondary to the physical insult inflicted by MIC.

VI. THE CYANIDE CONTROVERSY: MIC OR HCN?

There was strong contention that the lethal as well as persistent maladies in Bhopal were caused by hydrogen cyanide and not MIC. Even though the proponents of the cyanide theory do not say so, their thesis does imply that MIC by itself is neither lethal nor capable of exerting long-term effects. Nothing could be further from truth. The mechanism of toxicities of MIC and HCN differ in another important way. Because the toxicity of HCN depends upon interaction with ferric ion, conversion of ferrous to ferric form in the hemoglobin by nitrites or supplying molecules with greater affinity for the CN^- ion than provided by F^{3+} such as by thiosulfate acts as an antidote against cyanide. Neither sodium nitrite nor sodium thiosulfate was found to reduce the toxicity of MIC (Bucher *et al.*, 1987a; Varma *et al.*, 1988) nor did exposure to MIC cause any decrease in oxygen utilization (Alarie *et al.*, 1987).

The lethal effects of MIC were due to hypoxemia primarily because of pulmonary effects. HCN also causes death by hypoxemia by a well-known mechanism and the brain is the most susceptible organ. In this sense there is a superficial similarity between MIC- and HCN-induced fatalities. Indeed deaths due to many toxins and disease states result from pulmonary failure. However, data taken together with extensive information in literature (Ballantyne and Marrs, 1987; Curry, 1963; Goldstein *et al.*, 1968; Way, 1984) strongly contradict the HCN hypothesis (Varma, 1989).

At a temperature range of 427–548°C and a pressure range of 55–300 torr, MIC has been shown to decompose into HCN (Blake and Ijadi-Maghsoodi, 1982). However, the temperature inside the culprit MIC-containing tank was estimated to be only 250°C (Varadarajan *et al.*, 1985) at which temperature MIC does not degrade into HCN. There is evidence that MIC can combine with HCN even at low temperatures (Slotta and Tschesche, 1927); however, this reaction can only reduce toxicities of both HCN and MIC and not make more ''lethal cyanogens'' as speculated by Sriramachari (2004) because the toxic potency of cyanides depends upon the dissociation of the –CN ion (Goldstein *et al.*, 1968).

The possible decomposition of MIC into HCN has been treated as evidence that toxicity in Bhopal was caused by HCN (Mangla, 1989). However, a meticulous study by D'Silva *et al.* (1986) did not detect any HCN in the residue of Tank 610 (from which MIC escaped) nor did they observe the formation of HCN from MIC by experimentally duplicating the conditions that led to the disaster.

Notwithstanding the negative data about the presence of any HCN, if some MIC did decompose into HCN, the quantity of HCN generated as well as of unconverted MIC would be lower than it would be if no breakdown of MIC took place; in either case it would reduce the toxicity of both MIC and HCN. There is yet another point that needs to be considered. Once the vent valve of the tank containing MIC was forced open by the pressure, the escaping vapor was ejected upwards. Even if it is assumed that most of the MIC was converted into HCN, it is highly unlikely that significant amounts will descend onto the ground since the relative vapor density of HCN is 0.9 relative to 1.0 of air. Even if somehow HCN did descend to the ground, it is unlikely that it will achieve a fatal concentration in an area of approximately 40 km^2 in which the population was affected. Moreover, profuse lacrimation, which was witnessed in Bhopal residents, is not a characteristic of HCN poisoning.

Contrary to popular belief, cyanide is not a potent killer although it is an extremely fast killer if the dose is high enough (Goldstein *et al.*, 1968). HCN, the most potent of all cyanides, is lethal only at concentrations well over 200 ppm (Table 21.3). Alarie *et al.* (1987) exposed guinea pigs, which are more sensitive to MIC than rats or mice (Fowler and Dodd, 1987a), to as high as 340 ppm for a short period and all animals recovered. In contrast, MIC could be fatal at as low as 3 ppm and kill a majority of animals at 30–40 ppm for three to four exposures. Because of the relatively low

TABLE 21.3. Properties of methyl isocyanate (MIC) and hydrogen cyanide (HCN)

Property	MIC	HCN
Molecular weight	57	27
Appearance	Liquid	Liquid/gas
Boiling point (°C)	39.1	25.7
Vapor density (air = 1)	1.97	0.95
Threshold limit value (ppm)	0.02	10.0
Lethal level 1 h exposure (ppm)	3.0	100
Concentration immediately fatal (ppm)	Undetermined	>270
Antidote	None	Sodium thiosulfate
Long-term effects	Many	None identified

toxic potency of HCN, its threshold limit for an 8 h exposure is set at 10 ppm for HCN compared with 0.02 ppm for MIC (Table 21.3). It would be impossible to work in an atmosphere of 10 ppm MIC.

Almost no deaths occurred in Bhopal until 4 h after the MIC leak; indeed, most deaths occurred between 24 and 48 h after the discharge of the poisonous gas (Varma, 1986). This was followed by delayed deaths months and years after the accident (Varma and Mulay, 2006). This pattern of fatalities characterized by delayed and not immediate deaths in Bhopal (Varma, 1986) is mimicked by exposure of experimental animals to MIC (Boorman *et al.*, 1987; Bucher *et al.*, 1987a, b; Dodd *et al.*, 1987; Fowler and Dodd, 1987a, b; Stevens *et al.*, 1987; Tepper *et al.*, 1987; Uraih *et al*, 1987; Ferguson and Alarie, 1991). In contrast, at lethal concentrations, HCN would cause deaths almost immediately; if deaths do not occur within 4 h, it is highly unlikely that they would occur at all.

Also, there is definite evidence of long-term effects in survivors of the Bhopal disaster (Varma and Guest, 1993; Dhara and Dhara, 2002; Ranjan *et al.*, 2003; Sriramachari, 2004; Varma and Varma, 2005; Varma and Mulay, 2006) and in animals exposed to MIC (Boorman *et al.*, 1987; Fowler and Dodd, 1987; Karol *et al.*, 1987; Stevens *et al.*, 1987; Bucher *et al.*, 1987a, b; Varma *et al.*, 1988; Ferguson and Alarie, 1991). On the other hand, a single dose of cyanide is not known to produce long-term effects in survivors (Goldstein *et al.*, 1968), although continued ingestion, as a result of cyanogenic foods, can cause chronic toxicity (Cliff *et al.*, 1984).

The cyanide theory was triggered by the following events. First, in response to an enquiry from Bhopal immediately after the effects of the gas leak were felt in Bhopal, a senior official at the Union Carbide Plant in West Virginia is reported to have advised administration of sodium thiosulfate; although the instruction was soon withdrawn, the impact of the initial misinformation was not lost. Second, Dr Chandra of the Gandhi Memorial Hospital at Bhopal reported observing cherry-red venous blood at autopsy, characteristics of cyanide poisoning; although the characteristic almond odor of the breath and stomach contents was not reported, his suggestion that poisoning was caused by cyanide gained further currency. Third, the cyanide theory gained momentum in the Indian and international press after the release of the finding of ICMR's double-blind study that sodium thiosulfate exerted beneficial effects and increased urinary excretion of thiocyanate; indeed the Supreme Court of India intervened to instruct in August 1985, that is 7 months later, the ''mass detoxification'' of Bhopal victims with sodium thiosulfate. The cyanide theory acquired further credence because in an exemplary humanitarian gesture, the German toxicologist Dr Daunderer brought a generous supply of sodium thiosulfate ampoules and supervised its use in Bhopal victims (Sriramachari, 2004). Finally, in the midst of chaos and confusion caused by a disaster of the magnitude of Bhopal, rumors are bound to find their way. Even during the Tokyo subway episode when miscreants released organophosphate sarin, cyanide poisoning was suspected until the identity of the incriminating chemical was quickly established.

There are two aspects to the cyanide controversy. One, was the poisoning in Bhopal caused or significantly contributed by HCN? Two, did sodium thiosulfate provide relief? The overall evidence clearly indicated that the major culprit in the Bhopal disaster was MIC and not HCN. If the findings of cherry-red venous blood led to a suspicion of cyanide poisoning, autopsy blood (not liver or lungs) samples could have been tested for cyanide, even days or weeks after the accident (Ballantyne *et al.*, 1974), which was not done. The possibility that MIC itself can lead to underutilization of oxygen and thus give rise to cherry-red venous blood was critically examined by Salmon (1986) using a UV spectrometer following addition of MIC to rat blood; they found a role for methylamine, a hydrolysis product of MIC in color change, which can be misinterpreted when examined by the naked eye as was done in Bhopal.

As to the second question concerning the beneficial effects of sodium thiosulfate, the answer is not simple, but neither does it indicate that the disaster in Bhopal was caused by MIC being converted into HCN.

Sodium thiosulfate is well known as an effective antidote against cyanide poisoning, for which purpose it should be administered almost immediately and not several days and months later (Chen and Rose, 1956; Goldstein *et al.*, 1968); at the same time experimental data show that it is not an antidote against MIC (Alarie *et al.*, 1987; Nemery *et al.*, 1985b; Varma *et al.*, 1988). It is reasonable to assume that administration of sodium thiosulfate provided some relief to Bhopal residents but this does not prove that the poisoning in Bhopal was caused by HCN.

Cyanide has been part of the environment of living organisms during the evolutionary process and the body is endowed with a specific enzyme, thiosulfate-cyanide sulfurtransferase ''rhodanese'' (EC 2.8.1.1), which can convert cyanide into relatively nontoxic thiocyanate. Cyanide in the body can arise from several sources; its levels in normal human blood may be as high as 0.15 mg/l (~6 µmol/l) and might be even higher in patients with renal and respiratory disease (Cailleux *et al.*, 1988). Cyanide can be sequestered in the body from certain foods, ''cyanogenic glucosides'', as well as smoking, and other environmental factors. The cyanide content in 200 g of moist peach seed is 88 mg, greater than a fatal dose of HCN. Chronic toxicity of HCN can result from chronic (and not single) ingestion of the poison especially in workers in many industries where HCN can be a normal pollutant. Bhopal residents who were most severely affected by the disaster lived in shanty towns in highly polluted areas and cooked with charcoal or firewood, and many were smokers. It is therefore very likely that their normal cyanide blood concentration was significant; this would explain the relief experienced by them following injection of sodium thiosulfate at any time – days and months after the disaster. The double-blind study by ICMR, which recommended the routine use of sodium thiosulfate in Bhopal victims, is of dubious merit; of course the drug will relieve some symptoms better than the placebo. For the same reason administration of sodium thiosulfate would increase urinary thiocyanate. What ICMR should have done is to perform the same study on a population in shanty towns in other cities of India to determine if sodium thiosulfate is more beneficial in Bhopal than, say, in Mumbai or Kolkata.

No drug, including sodium thiosulfate, should be administered without clear indication. Like all other drugs, sodium thiosulfate is also not without its own toxicity. Sodium thiosulfate can even be converted into cyanide by the enzyme thiosulfate oxidase (Goldstein and Reiders, 1951), which is the reason why its overzealous use in cases of cyanide poisoning can lead to recurrence of symptoms.

VII. TOXICITY OF ISOCYANATES

MIC is less frequently used than toluene diisocyanate (TDI), diphenylmethane diisocyanate (MDI), naphthalene diisocyanate (NDI), hexamethylene diisocyanate (HDI), and other isocyanates. In order to highlight the distinctiveness of MIC toxicity, the toxicity of diisocyanates is described briefly.

Although organic isocyanates were first synthesized in 1849, their use, mainly for the manufacture of polyurethanes, increased after World War II. The toxicity of isocyanates can be due to direct irritant effect on lungs, eyes, skin, and exposed mucosa as well as secondary to pulmonary toxicity, allergic response, and nonpulmonary direct effects. The direct effects can be instantaneous as is the case with MIC, or ensue after a lag period of hours.

By the year 1956, more than 100 cases of illnesses and four cases of death due to poisoning by TDI were reported (Baader, 1956). In view of the increased use of isocyanates, the National Institute of Occupational Safety and Health, USA, projected as early as 1978 that approximately 50,000 to 100,000 workers would be exposed to these chemicals within 2 years (NIOSH, 1978); this estimate was based on exposure at the workplace in polyurethane production, upholstery work, wire coating, and spray painting in the absence of any accidental spill (Axford *et al.*, 1976; Editorial, 1966; Fuchs and Valade, 1951; Hill, 1970; Williamson, 1965).

The routine exposure of workers to isocyanates is by inhalation rather than oral ingestion; their toxicity is greater following inhalation than following oral ingestion as well as if they produce both pulmonary and sensory irritation than if they only cause sensory irritation (Weyel *et al.*, 1982). MIC is both a sensory and pulmonary irritant (Ferguson *et al.*, 1986). The smaller the isocyanate molecule, the greater its toxicity (Carney, 1980; Rye, 1973), which makes MIC the most toxic of all isocyanates (Varma, 1986). Also, the toxicity of isocyanates is directly related to their volatility and vapor pressure (Rye, 1973).

TDI seems to be the most toxic of diisocyanates. Eosinophilia, pulmonary emphysema, and right-sided heart failure were observed in some cases of TDI poisoning (Brugsch and Elkins, 1963). TDI can cause inflammatory changes at exposed mucous membranes in experimental animals (Sangha and Alarie, 1979; Sangha *et al*, 1982; Schmidt-Nowara *et al.*, 1973; Stevens *et al.*, 1987; Weyel *et al.*, 1982; Weyel and Schaffer, 1985; Wilson and Wilson, 1959; Wong *et al.*, 1985; Zapp, 1957). Repeated exposure to a dose of TDI as low as 5–10 ppm can be lethal in animals (Henschler *et al.*, 1962). Human toxicity of TDI has been documented by several workers (Adams, 1970; Axford *et al.*, 1976; Baader, 1956; Brugsch and Elkins, 1963; Bruckner *et al.*, 1968; Charles *et al.*, 1976; Editorial, 1966; Fuchs and Valade, 1951; Le Quesne *et al.*, 1976; Wilson and Wilson, 1959). Specific antibodies against TDI have been demonstrated in rabbits (Scheel *et al.*, 1964), guinea pigs (Karol, 1983), and humans (Karol, 1981), which would explain precipitation of symptoms following a second exposure to TDI in subjects who had recovered from previous exposure (Editorial, 1966).

In summary, all isocyanates are toxic and lungs are the main but not the only targets of their toxicity. The toxicity of isocyanates may range from transient reversible to long-term irreversible damage and may prove fatal.

A. Toxicity of Methyl Isocyanate

Experimental study on the toxicity of MIC vapor on rats, mouse, rabbits, and guinea pigs as well as on human volunteers was first reported in 1964 (Kimmerle and Eben,

1964). For the next 20 years from 1964 to 1984 no follow-up of these studies was done. The world had to have its worst industrial disaster in the last month of 1984 to prompt a renewed interest in the toxicology of MIC; this provoked *The Lancet* (Editorial, 1984) to comment: ''In a year's time we will have learned a lot more about methyl isocyanate – at an appalling price.'' It is to the credit of Kimmerle and Eben (1964) that their stringent observations were confirmed by all the studies since 1984. On the other hand, acute toxicity studies are not predictors of long-term effects (Salmon, 1985).

It is tragic that the toxicity of poisons is tested on humans during wars, and far too often by the most developed countries. It is unfortunate that human toxicity of chemicals is observed during industrial accidents or environmental neglect. In this sense, Bhopal offered the most expansive opportunity to observe and investigate the toxicity of MIC on such a large scale on a human population as well as on livestock and vegetation. Surprisingly, however, most of the obvious questions raised in the aftermath of the Bhopal disaster have not been investigated in a rigorous manner and the answers to many questions remain at best anecdotal.

Whereas inhalation is the commonest mode of entry of MIC into the body, its toxicities have been demonstrated following injections (Jeevarathinam *et al.*, 1988; Meshram and Rao, 1988; Pearson *et al.*, 1990; Varma, 1987); there is evidence that the toxicity of MIC is not contributed by its metabolites (Jeevaratnam *et al.*, 1992a).

For the sake of simplicity, data on the toxicity of MIC on humans and animals are presented separately. It is worth mentioning, however, that almost all the data derived from animal studies seem to confirm what has been observed in humans in Bhopal.

1. Toxicity of MIC In Animal Models

a. Mortality

Barely 4 h after the disaster, Bhopal, on the morning of December 3, 1984, was littered with dead animals – 790 buffaloes, 18 bullocks, 84 calves, 270 cows, 483 goats, 90 dogs, and 23 horses (Varma, 1986). According to autopsy reports, dead animals showed swollen livers and lymph nodes, bloated digestive tracts, engorged blood vessels, edema, necrosis in lungs with blood clots, and congested heart and kidney (Varma, 1986). House flies survived probably because they stood still and MIC did not enter into the body in sufficient concentrations through the wings and skin.

The lethal effect of MIC was first reported by Kimmerle and Eben (1964) who estimated an LC_{50} value of 5 ppm in rats following a 4-h exposure, and 21 ppm following a 2-h exposure. Unlike cyanide, deaths followed several hours after exposure and continued up to 18 days. In later studies it was found that a 10 min exposure to as high as 3,506 ppm was not immediately lethal (Dodd *et al.*, 1987) although guinea pigs died during exposure to high concentrations of greater than 500 ppm. In general, deaths following exposure to MIC occur 1 to 2 days later and a second phase of mortality follows after a week or longer (Alarie *et al.*, 1987; Boorman *et al.*, 1987; Bucher *et al.*, 1987a, b; Dodd *et al.*, 1987; Fowler *et al.*, 1987; Fowler and Dodd, 1987a, b; Stevens *et al.*, 1987; Varma *et al.*, 1988). There seem to be species differences in lethal toxicity of MIC; guinea pigs being more sensitive than rats (Dodd *et al.*, 1987). Experimental studies during the first 2 years have been reviewed by Nemery *et al.* (1987).

b. Pulmonary Toxicity

Kimmerle and Eben (1964) reported that MIC caused lacrimation, mucosal irritation, and pulmonary edema in rats, mouse, rabbits, and guinea pigs; death followed acutely after a few hours as well as after several days (up to 18 days). The LC_{50} in rats following a 4 h exposure was 5 ppm; it was 21 ppm following a 2 h exposure. Later studies found that MIC causes both sensory and pulmonary irritation; if death did not ensue, the recovery from these pulmonary effects was very slow (Alarie *et al.*, 1987; Ferguson *et al.*, 1986). Exposure to MIC caused concentration-dependent degenerative changes in bronchiolar and alveolar epithelium in rats and guinea pigs resulting in plugging of major airways and atelectasis (Nemery *et al.*, 1985a; Fowler *et al.*, 1987), increase in lung weight (Bucher *et al.*, 1987a; Stevens *et al.*, 1987), pulmonary (Boorman *et al.*, 1987; Bucher *et al.*, 1987a; Dutta *et al.*, 1988; Fowler and Dodd, 1987a, b) as well as olfactory epithelial necrosis (Uraih *et al.*, 1987), airway obstruction (Stevens *et al.*, 1987; Tepper *et al.*, 1987), and a compromised cardiopulmonary function (Tepper *et al.*, 1987) in surviving animals.

c. Ocular Toxicity

As was the case in humans, lacrimation has also been found to be one of the earliest effects of MIC vapor in experimental animals (Bucher *et al.*, 1987; Dodd *et al.*, 1986, 1987; Ferguson and Alarie, 1991; Gupta *et al.*, 1987; Kimmerle and Eben, 1964; Varma *et al.*, 1988). However, exposure of rats to 3, 10, or 30 ppm MIC for 2 h, which approximates the situation in Bhopal, was not found to cause any damage to cornea although copious lacrimation was observed up to 3 months. Similar findings have been reported in mice (Boorman *et al.*, 1987). On the other hand, exposure of lens explants to MIC *in vitro* has been shown to cause opacity (Harding and Rixon, 1985). It is very likely that profuse lacrimation acted as a protective mechanism by both chemically inactivating MIC and offering physical protection.

d. Reproductive Toxicity

Exposure of mice on day 8 of gestation (gestation period 19 days) to 2, 6, 9, and 15 ppm MIC for 3 h or 1–3 ppm for 6 h on days 14–17 of gestation caused concentration-dependent fetal loss and maternal mortality (Varma, 1987; Varma *et al.*, 1987, 1990); lengths of different fetal bones

were significantly reduced following exposure of mice to 9 and 15 ppm MIC (Varma, 1987). MIC also caused maternal and fetal toxicity in rats; pregnancy loss accompanied sudden decrease in progesterone although it could not be determined which of the two events occurred first (Varma *et al.*, 1990). Given the extensive nature of MIC toxicity, it is difficult if not impossible to determine if MIC-induced reproductive toxicity is a direct effect on the conceptus or a consequence of general toxicity; however, several observations indicate the possibility of a direct effect.

Radio-labeled MIC rapidly reaches the fetus (Ferguson *et al.*, 1988). Intraperitoneal injection of MIC also caused reproductive toxicity of a similar magnitude as following inhalation (Varma *et al.*, 1990). Moreover, MIC metabolites methylamines also produced reproductive toxicity without other obvious effects on pregnant mice (Guest and Varma, 1991); of the three amines tested, monomethylamine, dimethylamine, and trimethylamine, the last one was most toxic *in vivo* as well as in mouse embryos in culture (Guest and Varma, 1991, 1992; Guest *et al.*, 1994). Interestingly, administration of trimethylamine during mouse pregnancy resulted in stunting of male but not female progeny (Guest and Varma, 1993), similar to that reported years later in Bhopal victims (Ranjan *et al.*, 2003). Another metabolite of MIC, *S*-(*N*-methylcarbamoyl) GSH, exerted marked toxicity on cultured mouse embryos (Guest and Varma, 1994; Guest *et al.*, 1992) as well as yolk sac and limb bud (Guest and Varma, 1994). However, exposure of male and female mice to low concentrations (1–3 ppm) of MIC did not seem to affect their reproductive performance one week post-exposure (Schwetz *et al.*, 1987).

e. Genetic and Immunotoxicity

MIC has been found to generate specific antibodies in guinea pigs following both inhalation and subcutaneous injections (Karol *et al.*, 1987). However, immunologic response to 3 ppm MIC in mice was considered minimal by one group (Tucker *et al.*, 1987) but not by another group of investigators (Dwivedi *et al.*, 1988). In consideration of the socioeconomic status of the victims, Saxena *et al.* (1991) studied B lymphocyte cell activity in protein-deficient rats and found no evidence of significant effects on immune response; likewise, Luster *et al.* (1986) did not observe appreciable compromise in immune function in mice exposed up to 3 ppm MIC for 4 consecutive days.

Conner *et al.* (1987) did not observe any effect on sister chromatid exchange in bone marrow, alveolar macrophages, and lymphocytes of mice exposed to MIC; however, cell cycling of lymphocytes and bone marrow cells from mice exposed to >15 ppm MIC was almost completely suppressed. On the other hand, MIC was found to be genotoxic in rats (Dutta *et al.*, 1988) and caused dose-related increases in sister chromatid exchange as well as chromosomal aberrations in hamster ovary cells in addition to cell cycle delay in mice (Shelby *et al.*, 1987). MIC has also been reported to be mutagenic in mammalian and bacterial cell cultures (Caspary and Myhr, 1986; Meshram and Rao, 1988); MIC has been estimated to have a 76.6% probability of being a genotoxic carcinogen but only in tests with low specificity (Ennever and Rosenkranz, 1987).

f. Other Toxic Effects

MIC caused dose-dependent necrosis of brain cells and muscle cells (Anderson *et al.*, 1988) of rats in culture; these findings could explain neuromuscular complaints in Bhopal victims. Exposure of mice to 1–3 ppm MIC was found to inhibit erythroid precursors, pluripotent stem cells and granulocyte-macrophage progenitor; recovery from this inhibitory effect was found within 3 weeks after 1 ppm but not after 3 ppm (Hong *et al.*, 1987). At higher concentrations of 6–15 ppm, MIC inhibited cell cycling in bone marrow, alveolar cells, and T lymphocytes (Conner *et al.*, 1987; Shelby *et al.*, 1987); similar data were reported by others (Tice *et al.*, 1987; Mason *et al.*, 1987). MIC can inhibit bone marrow cell proliferation in mice (Meshram and Rao, 1988). MIC can cause necrosis in whole-brain cell cultures (Anderson *et al.*, 1990) and inhibit differentiation in muscle cell cultures (Anderson *et al.*, 1988).

Exposure of rats, mice, and guinea pigs to MIC vapor caused dramatic body weight decrease in the first 2 days, which was followed by incomplete to complete recovery (Bucher *et al.*, 1987a; Dodd *et al.*, 1987; Fowler and Dodd, 1987a, b; Varma 1987). The most likely cause of the rapid decrease in body weight is fluid loss, which may also explain the increase in hematocrit. A decrease in food intake may contribute to the persistence of decreased body weight.

MIC also caused an increase in creatinine kinase, hemoglobin, hematocrit, reticulocytes, neutrophils, and blood PCO_2 in rats and guinea pigs (Bucher *et al.*, 1987b; Dodd *et al.*, 1987; Troup *et al.*, 1987) and a decrease in blood pH and PO_2 (Fedde *et al.*, 1987; Jeevaratnam *et al.*, 1991; Maginniss *et al.*, 1987; Troup *et al.*, 1987). MIC can cause hyperglycemia, lactic acidosis, and hypothermia in rats (Jeevaratnam *et al.*, 1992c) as well as inhibit mitochondrial respiration (Jeevaratnam *et al.*, 1992b) and disrupt erythrocyte membranes (Jeevaratnam and Vaidyanathan, 1992). MIC was found to be a weak reversible inhibitor of cholinesterase (ChE) *in vitro* (Brown *et al.*, 1987; Troup *et al.*, 1987) but not *in vivo* (Troup *et al.*, 1987); both groups of workers concluded that ChE inhibition could not have contributed to fatalities in Bhopal. Mishra *et al.* (1991) exposed rats to different concentrations of MIC vapor for 8 min and measured drug metabolizing enzymes in lungs and found that aminopyrene demethylase and aniline hydroxylase activities were inhibited but glutathione-*S*-transferase activity was increased.

2. Toxicity in Humans

The human toxicity of the noxious gases that engulfed Bhopal on the night of December 2–3, 1984 has been reviewed by several investigators (Dhara and Dhara, 2002;

Mehta *et al.*, 1990; Sriramachari, 2004; Varma and Guest, 1993; Varma and Mulay, 2006). Dureja and Saxena (1987), two of the earliest physicians who were involved in the rescue, graded symptoms into four categories: (1) minor eye ailments, throat irritation and cough; (2) severe conjunctivitis, keratitis, acute bronchitis and drowsiness; (3) severe pulmonary edema; and (4) convulsions followed by cardiorespiratory arrest. Similar findings were reported by others (Kamat *et al.*, 1985; Misra *et al.*, 1987).

The human cost is vividly narrated in the various books mentioned in Section II of this chapter. While there is no doubt that the major toxic chemical responsible for acute and chronic toxicity in Bhopal was MIC, there is also ample evidence that the toxic fumes contained other noxious substances. Whether or not these additional chemicals exerted additive or synergistic effects on MIC toxicity cannot be stated categorically. It is reasonable to assume that major effects, both acute and chronic, were due to MIC.

a. Acute Toxicity

i. Nonlethal Effects Eye irritation, lacrimation, choking sensation, and difficulty in breathing were first reported by Kimmerle and Eben (1964) who exposed human volunteers to MIC vapor; the observation of these workers was confirmed on thousands of people following the Bhopal disaster. Also, many of the victims lost consciousness; some but not all regained it (Varma, 1986).

ii. Fatal Effects Although the precise number of people who died after being exposed to MIC is still not known, our estimate based on fatalities in 3,270 households surveyed to determine effects on pregnancy (Varma, 1987) would suggest 6,000–8,000 deaths within 24–72 h after the gas leak in Bhopal. Deaths can be attributed to pulmonary edema. As mentioned earlier, deaths did not occur as quickly as is characteristic of cyanide poisoning but after a delay of several hours.

b. Subacute and Chronic Toxicity

i. Mortality Anecdotal reports suggest that the Bhopal disaster resulted in approximately 20,000 deaths over approximately a 2-year period. Since late deaths have been observed by several workers in animal models (Alarie *et al.*, 1987; Boorman *et al.*, 1987; Bucher *et al.*, 1987a, b; Dodd *et al.*, 1987; Fowler *et al.*, 1987; Fowler and Dodd, 1987a, b; Stevens *et al.*, 1987; Varma *et al.*, 1988), the reports of late deaths in humans are a reasonable assumption. It is very likely that severe lung damage accounted for most of the late deaths although a contributory role of dehydration, internal hemorrhage, and other complications cannot be ruled out.

c. Pulmonary Complications

Examination of 500 exposed people within 3 days of the Bhopal disaster identified alveolar edema in 40% and destructive lesions in 8% (Sharma and Gaur, 1987); similar data have been reported by others (Bhargava *et al.*, 1987). A retrospective study of 978 patients found 7.14% mortality, breathlessness, and cough in 95%, irritation and choking in the throat in 46% and chest pain in 25% (Misra *et al.*, 1987). Evidence of necrotizing lesions in the respiratory tract (ICMR, 1985) as well as radiological changes and compromise in lung function have been documented (Gupta *et al.*, 1988; Misra and Nag, 1988). Since the prevalence of compromise in lung function was higher in the population closer to the pesticide plant than in the population further away, it is very likely that it was a result of exposure to the toxic gases rather than preexisting bronchitis, tuberculosis or emphysema. A follow-up of 113 exposed patients revealed worsening of pulmonary symptoms at 2 years relative to those at 1 year; forced expiratory flow (FEF) between 25 and 75% declined progressively over a 2-year period (Kamat *et al.*, 1985, 1987, 1992; Patel *et al.*, 1987), a 1–7 year period (Vijayan and Kuppurao, 1993; Vijayan and Sankaran, 1996; Vijayan *et al.*, 1989, 1995), and a 10-year period (Acquilla *et al.*, 1996; Dhara *et al.*, 2002). Likewise, other workers found direct relationship between pulmonary function compromise and inflammatory alveolitis and the severity of exposure (Vijayan *et al.*, 1989, 1995). A causative relationship between the intensity of exposure to toxic gases and a decrease in $FEF_{25\%–75\%}$ is also suggested by another follow-up study of 454 adults conducted 10 years after the disaster (Cullinan *et al.*, 1996, 1997).

Persistent airway hyperreactivity after a single exposure to chemical irritant has been termed Reactive Airways Dysfunction Syndrome (RADS) (Brooks et *al*., 1985). There is a strong likelihood of RADS among the victims of the gas exposure at Bhopal (Nemery, 1996); however, whether or not the exposed Bhopal population suffers from RADS has not been carefully studied.

A study by Avashia *et al.* (1996) (the medical director of the Union Carbide Institute at West Virginia Plant in 1984) concluded that prolonged low exposure of workers to MIC did not cause any pulmonary complications; the level of exposure was not quantified. Even in Bhopal people who lived far away from the pesticide plant and were exposed to very low concentrations of MIC did not develop pulmonary complications because no chemical produces detectable toxicity at all doses.

d. Ocular Toxicity

There are reports that eye irritation and some level of lacrimation was a common experience of workers of the pesticide plant. Indeed, because of these frequent episodes of eye irritation, workers initially believed that they did not initially suspect something unusual was happening in the early morning of December 3, 1984. It would seem that eyes are most sensitive to MIC toxicity since eye irritation was experienced even by people who lived quite far from the pesticide plant and seemed not to have experienced pulmonary and other symptoms (Varma, 1986). Exposure to

MIC produced ocular burning, watering, pain, and photophobia (Andersson *et al.*, 1984, 1985, 1988; Dwivedi *et al.*, 1985), conjunctivitis, and corneal opacity (Maskati, 1986). Within the first 2 weeks of disaster Anderson *et al.* (1988) found no case of blindness in a community-based survey; surprisingly, the incidences of photophobia and interpalpebral erosion were highest in areas where the death rates were lowest.

Two-year follow-up studies revealed persistent eye watering, itching, redness, photophobia, burning, Bitot spots, and even corneal opacity (Andersson *et al.*, 1986, 1990; Khurrum and Ahmad, 1987; Raizada and Dwivedi, 1987). It is noteworthy that in a gas-exposed cohort of 232 children admitted to the Pediatric and Eye Ward of the Hamidia Hospital, Bhopal, extensive evidence of respiratory and cardiac complications was not accompanied by equally serious eye injuries (Dwivedi *et al.*, 1985). It is very likely that poor living conditions, which favor infection, especially in children, further worsened ocular toxicity (Crabb, 2004; Dhara and Dhara, 2002). On the other hand, it was feared at the time of the accident that a large number of survivors might be left with severe visual impairment; fortunately this does not seem to be the case, which does indicate that profuse watering, a toxic effect of MIC, also had the effect of minimizing ocular toxicity.

e. Reproductive Toxicity

A follow-up of 865 pregnant women living close to the pesticide plant at the time of the Bhopal disaster found that 379 (43.8%) did not give birth to live babies (Varma, 1987). Another follow-up study of 2,566 pregnant women from 18,978 households also found pregnancy loss in 23.6% women as compared to 5.6% in 1,218 control cohorts (Bhandari *et al.*, 1990). Kanhere *et al.* (1987) found that exposure to toxic gases resulted in decreased placental and fetal weights, in addition to the increased loss of pregnancy; infant death within 1 month, 2 years, and 5 years after birth was approximately 14% compared with 2.6–3% within the pre-accident period (Varma, 1987, 1991). Other effects of exposure to MIC in women include leucorrhea, suppression of lactation, pelvic inflammatory diseases, and irregular menstruation (Varma, 1986). No effect on spermatogenesis was detected within 6 months after the Bhopal disaster (Daniel *et al.*, 1987).

f. Genotoxicity

In a study involving 43 gas-exposed females and 40 gas-exposed males 3 years after the disaster, a significant increase in chromosomal aberration was reported; these aberrations included breaks, gaps, and dicentric rings, which were more marked in females than in males (Ghosh *et al.*, 1990). Chromosomal aberrations (Goswami *et al.*, 1985, 1990; Ghosh *et al.*, 1990) and cell cycle abnormalities have been identified in Bhopal victims (Deo *et al.*, 1987).

g. Carcinogenicity

A cancer registry was initiated by the Indian Council of Medical Research (ICMR) in 1986. However, no conclusive evidence of an increase in cancer in the exposed population has been documented. Dikshit and Kanhere (1999) analyzed the incidence of cancer in gas-exposed males during1987–1992 but found no significant increase in cancer. On the other hand, these researchers (Dikshit and Kanhere, 1999) predicted that a true estimate of any increase in the incidence of cancer can only be made ‘‘15–20 years’’ after the accident, but no such study has since been published.

h. Immunotoxicity

Saxena *et al.* (1988) studied 31 exposed adults and found a significant increase in abnormal lymphocytes; however, there was no middle ground in humoral and cellular immunity in the exposed population in Bhopal. Anti-MIC antibodies were detected in blood samples from gas-exposed subjects but the clinical implications are not clear (Karol *et al.*, 1987).

i. Neurotoxicity and Psychological Effects

Soon after the Bhopal disaster there were displays of bizarre and starkly different drawings by the surviving children almost all of them depicting gusts of flames going upwards. Many of these drawings reflected loss of parents or other family members or friends. While these drawings are unlikely to be specific to MIC, they most probably reflected the children’s response to unanticipated horror. Psychological trauma was experienced by adults similar to that experienced by soldiers returning from combat missions. One study categorized the post-disaster psychological impact into four categories – post-traumatic stress disorder characterized by anxiety, restlessness, and sleep disorder; pathological grief reactions expressed as suicidal tendencies, and helplessness at not being able to save family members; emotional reaction to physical problems imposed upon them; and exacerbation of preexisting problems (Murthy and Isaac, 1987). A survey of 164 gas-exposed children 105 days after the disaster found them apprehensive and jittery (Irani and Mahashur, 1986).

The authors of this chapter encountered a volunteer at Sambhavana Trust Clinic located near the now decommissioned pesticide plant. This young man was perfectly normal on the many occasions we saw him but other members of the clinic told us that from time to time he would become very depressed; his parents had died during the disaster. One day in 2007 we received a message from Bhopal that he had committed suicide. There are reports of other such cases although it is never possible to causally link such tragic events with exposure to MIC.

Sethi *et al.* (1987) reported that a large number of survivors suffered from organic neurological problems including neuroses, anxiety states, and accentuation of previous psychological problems. Bharucha and Bharucha

(1987) also observed neurological and neuromuscular abnormalities in both adults and children and concluded that the incidence was lower than to be expected following a disaster of such magnitude; 24 of the 47 children examined by these authors experienced coma lasting for a maximum duration of 24 h. Neurological problems have also been reported by others (Gupta *et al.*, 1988; Kamat *et al.*, 1985; Misra and Kalita, 1997). Raphael and Middleton (1988) have suggested that 30–59% of those exposed to a disaster may suffer from traumatic neuroses.

j. Other Toxic Effects

Soon after the disaster, Bhopal was flooded with vendors selling all kinds of pills and buyers who hoped for a cure. Some did not know what to expect in the days following the accident and hoped that the pills would safeguard against existing ailments such as loss of appetite, weakness, breathlessness, and so on, and prevent complications. This scenario is typical of developing countries.

Twenty five years later, victims of the disaster still occupy the hospital beds and clinics and visit private practitioners of all branches of medicine complaining of vague to specific symptoms. In general, however, it is difficult to relate many of the symptoms, such as weakness, loss of appetite, and menstrual problems, to exposure to MIC. However, many subjects continue to suffer from pulmonary and visual problems.

As mentioned before, most of the victims of the disaster belonged to economically disadvantaged groups and lived in poor housing with nonexistent sanitary facilities. They thus suffered from many chronic diseases including bronchitis, tuberculosis, malaria, and so on. The Bhopal disaster certainly worsened these maladies.

VIII. TREATMENT

By their very nature disasters involving chemicals pose special problems because they involve a large number of people in a state of panic. Most places are not equipped to deal with such a situation especially if there is confusion about the nature of the chemical. If the chemical is a pulmonary irritant, as is the case with MIC, there is a good likelihood of cyanide poisoning as happened in Bhopal and for a short period in Japan when miscreants exploded organophosphates in the subway. The other reason for mass confusion is the erroneous belief that there exist antidotes for every poison.

People are usually unaware that supportive therapy rather than antidotes is the cornerstone to managing drug overdose or poisoning; a few exceptions include cyanide, narcotic analgesics, acetaminophen, methanol, organophosphates, digitalis, and CO. It was legitimate for the media and people in Bhopal to demand an antidote. Therefore conveying accurate information to the panicking population was vital. In the case of Bhopal, nondisclosure of the nature of, and lack of proper information about, the chemical involved, leading to improper cautionary measures proved as harmful as the poison itself. For example, if the community had been warned not to run away and encouraged to cover their faces with a wet cloth, the benefits would have been significant. In the midst of all this confusion, the doctors in Bhopal worked out as rational a treatment as possible, which comprised atropine, antibiotic eye drops, and antispasmodics. Treatment of pulmonary edema requires hospitalization and positive pressure respiration; Bhopal had neither enough beds nor equipment to provide this.

Long-term treatment is also supportive and is unrelated to the initiating factor. For example, the treatment of pulmonary, ophthalmic, or neurological complications has nothing to do with whether these are the late effects of poisoning by MIC or phosgene. Cyanide, unless ingested on a regular basis from the environment or food, does not produce long-term disability if death has not occurred.

A redeeming feature of the Bhopal tragedy was the overwhelming response of the citizens. Hundreds had flocked into Bhopal on the morning of December 3 from nearby villages and some came from further away. Doctors performed a commendable job working for long hours without a break. In contrast, the medical team dispatched by Union Carbide arrived 10 days later and tried to assure everyone that MIC is rapidly destroyed and no long-term effects are to be expected. The government of India failed both to summon a high level medical team to deal with the disaster and to provide any sort of ongoing aid. Voluntary groups and nongovernmental organizations shared the major burden of assuring people and helping in both the treatment and rehabilitation.

IX. TOXIC POTENTIAL OF MIC BEYOND THE BHOPAL DISASTER

Bhopal was the first case of mass exposure of humans, animals, and vegetation to MIC. Several factors influenced the toxicity of MIC such as the living conditions of the victims. Under identical conditions in a developed country the consequences would most likely be different. At the same time, the exposure of the Bhopal population was to a specific concentration of MIC and for a specific duration.

Accidental release of MIC can happen wherever the chemical is stored. A minor leak occurred from the Union Carbide plant in West Virginia only a few months after the Bhopal disaster and a nearby school had to be evacuated. Exposure to MIC at higher concentrations and for a longer duration than happened in Bhopal can also occur and can be fatal to a substantially greater percentage of population regardless of where it happens. The fact that MIC is heavier

than air makes it a potentially highly hazardous chemical both in enclosed and open spaces.

If animal experiments were solely performed to answer questions relating to the Bhopal disaster, the use of excessively high concentrations of MIC (Dodd *et al.*, 1987; Troup *et al.*, 1987; Fedde *et al.*, 1987; Fowler *et al.*, 1987) and repeated exposures would not have much relevance. However, workers are likely to encounter repeated exposure to MIC; indeed, anecdotal reports suggest that the impending disaster was not expected because workers were used to minor leaks and consequently eye irritation in the Union Carbide plant. Although no worker died inside the plant in Bhopal, because MIC spewed outside the factory, an accident worse than Bhopal cannot be ruled out, especially in an enclosed space.

Confusing instructions and the lack of transparency about the identity of the poison by the authorities at the Union Carbide headquarters only accentuated the tragedy. It can be assumed that whenever a full-scale disaster occurs, rumors are likely to spread. Even in the Japan subway tragedy where the incriminating agent was the organophosphate nerve agent sarin, rumors had it that the substance was cyanide.

X. CONCLUDING REMARKS AND FUTURE DIRECTION

We have described the chemistry, pharmacology, and toxicology of methyl isocyanate in the context of more commonly used diisocyanates. Combatants and civilians have been subjected to lethal and debilitating chemical agents during war. However, no poisonous chemical other than MIC has the notoriety of killing nearly 8,000 people within 72 hours and many more in the subsequent years on a scale as large as happened in Bhopal. Unlike the 9/11 tragedy where total casualties are accurately known, no one is still certain about the exact death toll immediately after and in the subsequent months and years in Bhopal. While acute deaths were most likely caused by pulmonary edema, only a well-planned epidemiological study coordinated by official agencies could have determined the nature and the magnitude of long-term effects. Unfortunately, this was not the case and many of the long-term effects cannot be identified retroactively. Carcinogenicity and genotoxicity require long-term follow-up of a large population. So far the results have been disappointing.

MIC toxicity amply demonstrates that the full dimension of the toxicity of a chemical (also its therapeutic potential) cannot be predicted from its chemical structure but can be approximated by careful and painstaking studies. Such an enquiry into MIC would be well deserved.

India may be the 12th largest economy in the world but the victims of Bhopal do not benefit from it. In February to May 2008, children, women, and men marched a distance of 800 km to the Indian capital Delhi with meager demands for clean water, appropriate therapy, and rehabilitation. The last time this was done was 2006; some promises were made but not fulfilled. What will happen this time, only time will tell.

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CHAPTER 22

Chlorine

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I. INTRODUCTION

Chlorine is the most abundant naturally occurring halogen. When formed experimentally, chlorine is a greenish-yellow, diatomic gas (Cl_2) with a pungent, irritating, or suffocating odor. The vapor is 2.5 times heavier than air and will form a cloud in the vicinity of a release. Solubility in water is low, approximately 4.4 g/l. Chlorine is extremely reactive and enters into substitution or addition reactions with both inorganic and organic substances. Other relevant chemical and physical properties are listed in Table 22.1. Because chlorine is so reactive, it is normally detected in the environment at only very low levels. Moist chlorine unites directly with most elements. Chlorine is a strong oxidizer; reaction with water produces hydrochloric (HCl) and hypochlorous acid (HOCl) as follows (ATSDR, 2007; O'Neil *et al.*, 2001; Teitelbaum, 2001):

$$Cl_2 + H_2O \leftrightarrow HCl + HOCl$$

Chlorine is used in the manufacture of a variety of nonagricultural chemicals. The major use of chlorine is in the manufacture of ethylene dichloride, which in turn is used to make vinyl chloride and subsequently polyvinyl chloride. Other uses include a bleaching agent in the paper industry [along with chlorine dioxide (ClO_2)], commercial and household bleaching agents [in the form of chlorates (ClO_3) and hypochlorites (OCl)], and a biocide in water purification and waste treatment systems (CEH, 2005; Teitelbaum, 2001).

As of July 2005, more than 500 companies produced chlor-alkali (chlorine and sodium hydroxide from brine) at over 650 sites worldwide with a total annual capacity of about 55.6 million metric tons of chlorine. About half of all plants are located in Asia. Global consumption of chlorine in 2004 was nearly 50 million metric tons. Consumption is greatest in the USA followed by Western Europe, China, and Japan (CEH, 2005).

The odor of chlorine is pungent and irritating (ATSDR, 2007). According to Amoore and Hautala (1983), the odor threshold is 0.31 ppm with a range of 0.2–0.4 ppm in other studies. There is considerable variation in detecting the odor among subjects; for many individuals, the ability to perceive the odor decreases over exposure time (NIOSH, 1976).

This chapter summarizes the known human health effects of chlorine, including information from studies with laboratory animals. Where quantitative data were available, the relationship of exposure concentration and exposure duration to toxicity is explained. Emphasis is on acute exposures.

II. HISTORICAL BACKGROUND

Chlorine gas was used as a chemical warfare agent during World War I (Withers and Lees, 1985; Haber, 1986; Salem *et al.*, 2008). During the early years of the war, both the Germans and the Allies used irritant gases as chemical weapons. By early 1915, Fritz Haber, a German chemist, proposed using chlorine as a chemical weapon. By this time, the German army had advanced into Belgium and France. During February and March 1915, trenches were dug and gas cylinders containing chlorine were installed to the north and northeast of Ypres, Belgium. Allied shelling resulted in some breached cylinders and a few German gas casualties during this time. By early April 1915, over 5,000 chlorine-containing cylinders containing about 168 tons of chlorine had been placed along a four-mile frontline near Ypres. On April 22, 1915, as a strong wind blew in the direction of the Allies, the valves were opened, and the released chlorine drifted as a cloud toward the French and Canadian lines. Allied protective gear was rudimentary, and estimated casualties for the battle ranged from 3,000 to 15,000 killed or wounded. Following this attack, the Germans led repeated chlorine gas attacks near Ypres but failed to capture the town. The concentrations that caused casualties are unknown.

III. TOXICOKINETICS

Chlorine is considered a direct-acting irritant to the target tissues, the eyes and respiratory tract. Death is due to acute respiratory failure. Chlorine gas reacts at the site of contact and very little of the chemical is absorbed into the bloodstream (Eaton and Klaassen, 2001). Any chlorine that is absorbed becomes part of the body pool of chlorine, and toxicokinetics is not involved in the mechanism of action.

TABLE 22.1. Chemical and physical data

Parameter	Value	Reference
Synonyms	Bertholite; hypochlorite; hypochlorous acid	ATSDR (2007)
Molecular formula	Cl_2	O'Neil *et al.* (2001)
Molecular weight	70.9	O'Neil *et al.* (2001)
CAS registry number	7782-50-5	O'Neil *et al.* (2001)
Physical state	Gas	O'Neil *et al.* (2001)
Color	Greenish-yellow	O'Neil *et al.* (2001)
Solubility in water	4.4 g/l @ 25°C	O'Neil *et al.* (2001)
Vapor pressure	5025 mm Hg @ 20°C	Matheson Gas Co. (1980)
Vapor density (air = 1)	2.48 @ 20°C	ATSDR (2007)
Density (water = 1)	1.56 at boiling point	Teitelbaum (2001)
Melting point	−101°C	O'Neil *et al.* (2001)
Boiling point	−34.05°C	O'Neil *et al.* (2001)
Flammability	Nonflammable	Matheson Gas Co. (1980)
Conversion factors	1 ppm = 2.9 mg/m^3 1 mg/m^3 = 0.34 ppm	ATSDR (2007)

IV. MECHANISM OF TOXICITY

The toxicity of chlorine is related to its oxidizing capacity (ATSDR, 2007). As noted, chlorine gas combines with water in the epithelial lining of the upper respiratory airways forming hydrochloric and hypochlorous acids. Hydrochloric acid is 33 times less potent as a sensory irritant than chlorine (Barrow *et al.*, 1977; Barrow and Steinhagen, 1982); whereas an aerosol of sodium hypochlorite and chlorine at equivalent air concentrations induces similar decreases in respiratory rate and changes in lung function parameters in mice (Morris *et al.*, 2005). This suggests that the oxidant properties of chlorine alone are sufficient to account for the observed responses. The precise mechanism of the interaction of chlorine or its reaction products with cellular components resulting in pulmonary edema is not known.

At low concentrations, e.g. 9 ppm, only sensory receptors are affected, triggering changes in respiratory dynamics such as a decreased respiratory rate. At irritant concentrations, the mechanistic response to chlorine is due to stimulation of the trigeminal nerve endings in the respiratory mucosa which results in a decrease in respiratory rate (Alarie, 1981). In studies with the mouse, this response is usually measured as the RD_{50}, the concentration of a chemical that decreases the respiratory rate by 50%. Another reaction, reflex bronchoconstriction, is a local reaction in which cholinergic-like agents bind to respiratory tract cell surface receptors and trigger an increase in the intracellular concentration of cyclic guanosine monophosphate, which in turn facilitates contraction of the smooth muscles that surround the trachea and bronchi. Contraction of these muscles causes a decrease in airway diameter and a corresponding increase in resistance to airflow which may result in wheezing, coughing, a sensation of chest tightness, and dyspnea (Witschi and Last, 2001).

HOCl, the reaction product of chlorine gas in the respiratory tract, reacts with thiol groups present in enzymes (Smith *et al.*, 2008). Results of *in vitro* studies show that enzymes containing thiol groups (glutamylcysteine synthetase, cysteine, and methionine) were 100 times more reactive to HOCl than amino acids that did not contain thiol groups. Inactivation of enzymes involved in antioxidant defenses would render the cell vulnerable to oxidative stress. Cellular glutathione, a cysteine-containing tripeptide, is also reduced by exposure to HOCl. Decreased glutathione may impair the ability of tissues to suppress lipid peroxidation reactions.

V. TOXICITY

Chlorine is an eye and respiratory tract irritant and, at high doses, has direct toxic effects on the lungs. Although only moderately soluble in water, inhaled chlorine is soluble in the epithelial lining of the respiratory tract by five orders of magnitude over its physical solubility (Nodelman and Ultman, 1999). At high concentrations the scrubbing capacity of the upper airways is saturated and chlorine reaches the lungs where it causes edema and hemorrhage. The irritant properties of chlorine have been studied in human volunteers and the acute, repeat-exposure, and chronic inhalation toxicity has been studied in several laboratory animal species.

A. Human Exposures

The irritant properties of chlorine have been documented in studies with healthy human volunteers as well as in individuals with airway hypersensitivity (Table 22.2).

TABLE 22.2. Summary of irritant effects in humans[a]

Concentration (ppm)	Exposure duration	Effect	Reference
0.5	8 h[b]	Perception of odor, no discomfort, no effects, no changes in pulmonary function measurements for healthy individuals, some changes for an atopic individual	Anglen (1981); Rotman *et al.* (1983)
1.0	8 h	Irritation (itchy eyes, runny nose, mild burning in throat); transient changes in pulmonary function measurements; asthmatic-like response in an atopic individual	
2.0	15 min to 4 h	No perceptible irritation at 30 min; throat irritation at 1 h; no significant changes in pulmonary function at 2 h; at 4 h irritation characterized as nuisance	
1.0	2 h	No noticeable effect	Joosting and Verberk (1974)
2.0	2 h	Very slight irritation of eyes, nose, and throat; no changes in pulmonary function tests	
4.0	2 h	Nuisance level of throat irritation, perceptible to nuisance level of nose irritation	
0.4	1 h	No changes in pulmonary function parameters in subjects with airway hyperreactivity/asthma	D'Alessandro *et al.* (1996)
1.0	1 h	Modest changes in pulmonary function parameters (FEV_1 and R_{aw}) for both healthy and asthmatic subjects	
0.5	15 min	NOAEL for nasal airway resistance and pulmonary peak flow in healthy subjects; change in nasal airway resistance for rhinitic subjects	Shusterman *et al.* (1998)
1.0	15 min	Increase in nasal air resistance for subjects with allergic rhinitis subjects greater (49% from baseline) than controls (10%); older subjects responded with greater increase than younger subjects; no gender difference	Shusterman *et al.* (2003)
0.5	6 h/day, 3 days	NOAEL for pulmonary function changes	Schins *et al.* (2000)

[a]The Anglen (1981) and Joosting and Verberk (1974) studies were performed with healthy adults. Atopic individuals were included in the Shusterman *et al.* (1998, 2003) and Rotman *et al.* (1983) studies, and healthy subjects as well as asthmatics were included in the D'Alessandro *et al.* (1996) study

[b]Eight-hour studies were composed of two segments with a 30-min or 1-h break after 4 h

FEV_1 = forced expiratory volume at 1 second

R_{aw} = airway resistance

A concentration of 0.4–0.5 ppm for up to 8 h is generally considered a no-adverse-effect level for nose and eye irritation (Anglen, 1981; Rotman *et al.*, 1983; D'Alessandro *et al.*, 1996). Nasal airway resistance and pulmonary peak flow are not affected at 0.5 ppm (Shusterman *et al.*, 1998; Schins *et al.*, 2000). At 1 ppm, some subjects report slight nose, throat, and eye irritation, and transient alterations in pulmonary function tests may occur. A concentration of 4 ppm was considered a ''nuisance'' level of nose and throat irritation (Joosting and Verberk, 1974).

Volunteers showed differing sensitivity to chlorine exposure. Following a 15-min exposure to 0.5 ppm chlorine, unmedicated subjects with seasonal allergic rhinitis had increased nasal air resistance, but no change in allergy symptoms or pulmonary peak air flow (Shusterman *et al.*, 1998). In the Rotman *et al.* (1983) study, a subject with obstructive airway disease, who was defined by the authors as ''atopic'' suffered an asthmatic-like attack following the exposure to 1.0 ppm for 4 h. Healthy subjects as well as subjects with airway hyperreactivity (defined by response to a methacholine challenge) showed no change in pulmonary function parameters following a 1-h exposure to 0.4 ppm chlorine (D'Alessandro *et al.*, 1996). Following a 1-h exposure to 1.0 ppm, there were statistically significant changes in FEV_1 (forced expiratory volume in 1 second) and R_{aw} (airway resistance) for both normal and hyperreactive subjects compared to baseline values, with hyperreactive subjects showing a greater relative decrease in FEV_1 (16%) than healthy subjects (4%) and a greater relative increase in R_{aw} (108%) than normal subjects (39%). For all subjects, return to baseline occurred by 24 h post-exposure.

The lethal effects of chlorine in humans are well documented, but lethal concentrations can only be estimated. Death is due to pulmonary edema. Probit analysis of available information on the lethality of chlorine to animals and man was used by Withers and Lees (1985) to estimate a concentration lethal to 50% of the population (LC_{50}). Their model incorporated the effects of physical activity, inhalation rate, the effectiveness of medical treatment, and the lethal toxic load function. The estimated 30-min LC_{50} at a standard level of activity (inhalation rate of 12 l/min) for the regular, vulnerable, and average (regular + vulnerable) populations, as described by the authors, were 250, 100, and 210 ppm, respectively. The LC_{10}s for the three populations were 125, 50, and 80 ppm, respectively.

B. Laboratory Animal Studies

Acute lethality studies in rodents are listed in Table 22.3. Thirty-min LC_{50} values range from 504 ppm in the mouse to 700 ppm in the rat (Zwart and Woutersen, 1988). The 60-min LC_{50} and LC_{01} values for the rat were 455 ppm and 288 ppm, respectively (Zwart and Woutersen, 1988).

TABLE 22.3. Summary of acute lethal inhalation studies with laboratory animals

Species	Concentration (ppm)	Exposure duration	Effect	Reference
Rat	5500	5 min	LC_{50}	Zwart and Woutersen (1988)
	2841		No deaths	
	1946	10 min	LC_{50}	
	700	30 min	LC_{50}	
	547		No deaths	
	455	60 min	LC_{50}	
	288		LC_{01}	
	322		No deaths	
Rat	293	60 min	LC_{50}	MacEwen and Vernot (1972)
	213		No deaths	
Mouse	290	6 min	No deaths	Bitron and Aharonson (1978)
		11 min	LC_{50}	
		15 min	80% mortality	
	170	55 min	LC_{50}	
		2 h	80% mortality	
Mouse	302	10 min	LC_{50}	Alarie (1980)
Mouse	1057	10 min	LC_{50}	Zwart and Woutersen (1988)
	754		No deaths	
	504	30 min	LC_{50}	
Mouse	137	60 min	LC_{50}	MacEwen and Vernot (1972)
Mouse	200	60 min	LC_{01}	O'Neil (1991)
	150		No deaths	

LC_{50} = the concentration that results in 50% mortality
LC_{01} = the concentration that results in 1% mortality

TABLE 22.4. Summary of acute nonlethal inhalation studies with laboratory animals

Species	Concentration (ppm)	Exposure duration	Effect	Reference
Rat	1,500	2, 10 min	Edema, metaplasia of lung	Demnati *et al.* (1995)
	500	5 min	Slight perivascular edema of lung	
	100	2 min	No effect	
Rat	25	10 min	RD_{50}	Barrow and Steinhagen (1982)
Rat	10.9	6 h	RD_{50}	Chang and Barrow (1984)
Rat	9.1	6 h	Lesions of the nasal passages, less severe lesions of the lower respiratory tract	Jiang *et al.* (1983)
Mouse	9.3	10 min	RD_{50}	Barrow *et al.* (1977)
Mouse	3.5	60 min	RD_{50}	Gagnaire *et al.* (1994)
Mouse	9.1	6 h	Lesions of the nasal passages; less severe lesions of the lower respiratory tract	Jiang *et al.* (1983)

RD_{50} = concentration that lowers the respiratory rate by 50% during a 10-min exposure

The 30-min LC_{50} for the mouse was much lower than for the rat, 137 ppm (MacEwen and Vernot, 1972).

Exposure to extremely high concentrations for short periods of time is tolerable in laboratory rodents (Table 22.4). In the rat, exposure to 1500 ppm for 2 min produced only slight effects including mild perivascular edema and occasional small clusters of polymorphonuclear leukocytes in the mucosa of large airways (Demnati *et al.*, 1995). The concentration that lowers the respiratory rate by 50% (RD_{50}), a measure of extreme irritation, is 9.3 ppm (Barrow *et al.*, 1977). The RD_{50} is a standard 10-min test conducted with male Swiss-Webster mice (ASTM, 1991). When this approximate concentration (9.1 ppm) was extended to a 6-h duration, moderate to severe lesions of the respiratory tract and peribronchiolitis were observed in rats and mice (Jiang *et al.*, 1983). Lesions of the lower respiratory tract were less severe. The lower mouse RD_{50} value of Gagnaire *et al.* (1994) may be due to the use of a different strain of mice.

In chronic studies, male and female rats and mice tolerated up to 2.5 ppm chlorine gas for 6 h/day, 5 days/week for 2 years (CIIT, 1993; Wolf *et al.*, 1995). Concentration-dependent lesions confined to the nasal passages were observed in all animals. These lesions were most severe in the anterior nasal cavity and included respiratory and olfactory epithelial degeneration, septal fenestration, mucosal inflammation, respiratory epithelial hyperplasia, squamous metaplasia and goblet cell hypertrophy and hyperplasia, and secretory metaplasia of the transitional epithelium of the lateral meatus. Body weight was depressed compared to controls but no early deaths occurred. A similar lack of lower respiratory tract effects was seen in monkeys exposed to 2.3 ppm chlorine for 6 h/day, 5 days/week, for 1 year (Klonne *et al.*, 1987). At these concentrations, chlorine is effectively scrubbed in the anterior nasal passages as indicated by the absence of lesions in the lung.

VI. RISK ASSESSMENT

Young children, especially children with asthma, are of concern in that they may experience increased sensitivity to irritants than asthmatic adults. However, children with asthma, ages 1–17, reacted in a similar manner to methacholine challenge (Avital *et al.*, 1991) as asthmatic adults challenged with histamine (Crockcroft *et al.*, 1977). The methacholine challenge is a medical test used to assist in the diagnosis of asthma. Breathing nebulized methacholine provokes narrowing of the airways, detected during a spirometry test. Reaction to histamine is similar. Although children were not tested, Shusterman *et al.* (2003) reported that airway resistance in the youngest group of tested subjects, ages 18–34 years, responded in a less pronounced manner to chlorine exposure than older subjects, ages 52–69 years.

Clinical studies with volunteers have shown that asthmatics or persons with airway hypersensitivity have increased sensitivity to the irritant effects of chlorine. The clinical studies of Anglen (1981), Rotman *et al.* (1983), D'Alessandro *et al.* (1996), and Shusterman *et al.* (1998, 2003) show that individuals with airway hyperreactivity or asthma react with increased sensitivity to the presence of chlorine. Taken together the studies show that human subjects exposed to 1.0 ppm chlorine for up to 8 h/day show some evidence of sensory irritation and transient respiratory tract changes. No such changes were reported in volunteers exposed to 0.5 ppm chlorine. These studies have been used by various agencies to develop standards and guidelines for chlorine exposure in emergency as well as occupational settings (Table 22.5).

Of interest in risk assessment is the relationship between concentration (C) and exposure duration (t) for a set endpoint such as irritation or death. This relationship can be described by $C^n \times t = k$, where k is a constant (ten Berge *et al.*, 1986). ten Berge and Vis van Heemst (1986) analyzed

TABLE 22.5. Standards and guidelines for chlorine

	Exposure duration				
Guideline	**10 min**	**30 min**	**1 h**	**4 h**	**8 h**
AEGL-1[a]	0.5 ppm	0.5 ppm	0.5 ppm	0.5 ppm	0.5 ppm
AEGL-2	2.8 ppm	2.8 ppm	2.0 ppm	1.0 ppm	0.71 ppm
AEGL-3	50 ppm	28 ppm	20 ppm	10 ppm	7.1 ppm
ERPG-1[b]			1 ppm		
ERPG-2			3 ppm		
ERPG-3			20 ppm		
EEGL[c]					3 ppm
IDLH[d]		10 ppm			
PEL – Ceiling[d]					1 ppm
REL – Ceiling[d]					0.5 ppm
TLV-TWA[e]					0.5 ppm
TLV-STEL					1.0 ppm
MAK[f]					0.5 ppm
MAK – Peak Limit					1.0 ppm
MAC – Ceiling[g]					1.0 ppm

[a]AEGLs (Acute Exposure Guideline Levels) (NRC, 2004) represent threshold exposure limits for the general public and are applicable to emergency exposure periods ranging from 10 min to 8 h. Three levels – AEGL-1, AEGL-2, and AEGL-3 – are developed for each of five exposure periods (10 and 30 min, 1 h, 4 h, and 8 h) and are distinguished by varying degrees of severity of toxic effects. The three AEGLs are defined as follows:

AEGL-1 is the airborne concentration [expressed as parts per million or milligrams per cubic meter (ppm or mg/m^3)] of a substance above which it is predicted that the general population, including susceptible individuals, could experience notable discomfort, irritation, or certain asymptomatic, nonsensory effects. However, the effects are not disabling and are transient and reversible upon cessation of exposure.

AEGL-2 is the airborne concentration (expressed as ppm or mg/m^3) of a substance above which it is predicted that the general population, including susceptible individuals, could experience irreversible or other serious, long-lasting adverse health effects or an impaired ability to escape.

AEGL-3 is the airborne concentration (expressed as ppm or mg/m^3) of a substance above which it is predicted that the general population, including susceptible individuals, could experience life-threatening health effects or death.

[b]ERPG (Emergency Response Planning Guidelines) (AIHA, 1997) also represent guidelines for the general public. Three ERPG levels are defined, each for a period of 1 h:

The ERPG-1 is the maximum airborne concentration below which it is believed nearly all individuals could be exposed for up to 1 h without experiencing other than mild, transient adverse health effects or without perceiving a clearly defined objectionable odor.

The ERPG-2 is the maximum airborne concentration below which it is believed nearly all individuals could be exposed for up to 1 h without experiencing or developing irreversible or other serious health effects or symptoms that could impair an individual's ability to take protection action.

The ERPG-3 is the maximum airborne concentration below which it is believed nearly all individuals could be exposed for up to 1 h without experiencing or developing life-threatening health effects.

[c]EEGL (Emergency Exposure Guidance Level; NRC, 1984) is the concentration of contaminant that can cause discomfort or other evidence of irritation or intoxication in or around the workplace, but avoids death, other severe acute effects and long-term or chronic injury. EEGLs were developed for healthy military personnel. The EEL (Emergency Exposure Limit) for chlorine was set in 1966 and 1971 on the basis of nasal and eye irritation. After a review of the Rotman *et al.* (1984) data, the 8-h EEL was kept at 3 ppm but the 24-h EEGL was lowered to 0.5 ppm.

[d]IDLH (Immediately Dangerous to Life and Health; NIOSH, 2005) represents the maximum concentration from which one could escape within 30 min without any escape-impairing symptoms, or any irreversible health effects.

NIOSH REL-Ceiling (Recommended Exposure Limits – Ceiling; NIOSH 2005) is a 15-min ceiling.

OSHA PEL-TWA (Occupational Safety and Health Administration, Permissible Exposure Limits – Time Weighted Average; NIOSH, 2005) is defined analogous to the ACGIH-TLV-TWA, but is for exposures of no more than 10 h/day, 40 h/week.

[e]ACGIH TLV-TWA (American Conference of Governmental Industrial Hygienists, Threshold Limit Value – Time Weighted Average; ACGIH, 2001) is the time-weighted average concentration for a normal 8-h workday and a 40-h workweek, to which nearly all workers may be repeatedly exposed, day after day, without adverse effect.

The ACGIH TLV-STEL (Threshold Limit Value – Short Term Exposure Limit; ACGIH, 2001) is defined as a 15-min TWA exposure which should not be exceeded at any time during the workday even if the 8-h TWA is within the TLV-TWA. Exposures above the TLV-TWA up to the STEL should not be longer than 15 min and should not occur more than four times per day. There should be at least 60 min between successive exposures in this range.

[f]MAK [Maximale Arbeitsplatzkonzentration (Maximum Workplace Concentration)] (Deutsche Forschungsgemeinschaft (German Research Association), 2007) is defined analogous to the ACGIH-TLV-TWA. Two exposure peaks/work shift above the MAK are allowed, but the TWA must be maintained.

MAK Spitzenbegrenzung (Peak Limit) (German Research Association, 2007) constitutes the maximum average concentration to which workers can be exposed for a period up to 30 min with no more than two exposure periods per work shift; total exposure may not exceed the 8-h MAK.

[g]MAC [Maximaal Aanvaarde Concentratie (Maximal Accepted Concentration)] (SDU Uitgevers (under the auspices of the Ministry of Social Affairs and Employment), The Hague, The Netherlands, 2000) is defined analogous to the ACGIH-TLV-TWA.

the data of Anglen (1981) on irritation response in humans. Regression analysis of the percent of subjects reporting a nuisance irritation response to concentrations of 1 and 2 ppm over exposure durations of 30 and 120 min resulted in an *n* value of 1.9. Thus, the nuisance irritation response for longer and shorter exposure durations can be calculated. Probit analysis of the 5-, 10-, 30-, and 60-min LC_{50} values for the rat in the study by Zwart and Woutersen (1988) yields an *n* value of approximately 1.

VII. TREATMENT

According to IPCS (2008), patients without immediate symptoms may require no treatment, but a full physical examination and a record of respiratory peak flow may be of use in assessing any subsequent respiratory effects. Patients with mild effects require a full physical examination including peak flow before discharge and are advised to return if symptoms recur or develop over the following 24 to 36 h. Patients showing immediate moderate or severe effects should be checked for lung function and the chest should be x-rayed. Oxygen and bronchodilators such as salbutamol, orally or inhaled, may be used for bronchospasm. Pulmonary edema should be treated with positive end expiratory pressure, or constant positive airway pressure. Corticosteroids may inhibit the inflammatory response and should be considered in severe cases. Arterial blood gases should be monitored and hyperchloremic acidosis should be treated. Patients with preexisting respiratory disease should be assessed and considered for hospital admission for at least 24 h.

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

The database on chlorine is robust. The irritant properties and toxicity of chlorine have been studied with controlled human exposures as well as with multiple species of laboratory animals. Exposure durations range from acute to chronic. Subjects with rhinitis or asthma appear to be more sensitive to the irritant effects of chlorine than healthy individuals. Thus, risk assessment addresses the potential for greater effects in these sensitive populations.

Chlorine is a direct-acting irritant to the eyes and respiratory tract. The mechanisms of action for both irritation and lethal effects are described. Toxicokinetics is not involved in the mechanism of action. The relationship between concentration and exposure duration for the set endpoints of irritancy and mortality can be described mathematically. There are no recommendations for further research.

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CHAPTER 23

Phosgene

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I. INTRODUCTION

Phosgene is a colorless gas at ambient temperature and pressure. Its odor has been described as similar to newly mown hay (Leonardos *et al.*, 1968). This mild odor and the weak acute irritant properties, however, provide little warning of its presence (Lipsett *et al.*, 1994).

Phosgene is manufactured from a reaction of carbon monoxide and chlorine gas in the presence of activated charcoal. Manufacture of phosgene is approximately 1 million tons per year in the USA; it is used in the manufacture of isocyanates, polyurethanes, polycarbonates, and pharmaceuticals and involves more than 10,000 workers (Currie *et al.*, 1987a). Manufacture of phosgene in the USA is almost entirely captive, in that >99% is used in the manufacture of other chemicals within a plant boundary (US EPA, 2003). The odor threshold is between 0.5 and 1.5 ppm (NIOSH, 1976); unfortunately, the odor threshold is inadequate to protect against toxic inhalant exposure as damage to the deep respiratory tract can take place at lower concentrations (Sidell *et al.*, 1997). Phosgene has an odor safety classification rating of ''E'' which indicates that fewer than 10% of attentive persons can detect the TLV (Amoore and Hautala, 1983).

Inhalation is the most important route of exposure for phosgene. Because of its mild upper respiratory, eye, and skin irritancy, and a mildly pleasant odor, an exposed victim may not actively seek an avenue of escape before lower respiratory damage has occurred (Currie *et al.*, 1987a; Lipsett *et al.*, 1994). Small amounts of phosgene can be irritating to the eyes (lacrimation) and throat (coughing) as phosgene undergoes hydrolysis to create hydrochloric acid which acts as an irritant. Far more dangerous, however, is the pulmonary edema that can develop after a latent period of 1–24 h post-exposure. Exercise can increase the extent of pulmonary edema (Marrs *et al.*, 1996). If severe clinical signs are not present after 48 h, then chances for full recovery are great. Physical and chemical properties of phosgene are summarized in Table 23.1.

II. BACKGROUND

Phosgene was first made in 1812 and was eventually used as a chemical warfare agent in WWI. Because of its higher density compared to air, phosgene gas can accumulate in low-lying areas; thus concentrated pockets within war trenches caused significant exposure in WWI. German troops in WWI used phosgene gas as a chemical warfare agent against the British troops. Possibly up to 85% of the deaths in WWI were a result of phosgene gas exposure (Ministry of Defence, 1987). Sandall (1922) examined 83 British soldiers 3 years after phosgene exposure. Shortness of breath upon exertion (70%), cough with expectoration (54%), tight feeling in chest (25%), sporadic giddiness (14%), and nausea (12%) were the most frequently reported complaints. No physical lung abnormalities were noted in 53% of the men.

The concept of a ''death product'' was introduced by Haber to explain the relationship between the extent of exposure to phosgene and death (Haber, 1924). According to ''Haber's law'', the biological effect of phosgene is directly proportional to the exposure expressed as the product of the atmospheric concentration (C) and the time of exposure (T) or $CT = k$, where k can be death, pulmonary edema, or other biological effects of phosgene exposure (US EPA, 1986). Haber's law has subsequently been shown by other investigators to be valid for both nonlethal and lethal effects within certain limits. There appears to be little species variability with regard to lethality between rats, mice, and guinea pigs, and the $CT = k$ relationship appears to be generally valid (although at very high or very low concentrations or exposure times so short that the animal can hold its breath, the $CT = k$ relationship may not hold).

III. TOXICOKINETICS

Following inhalation exposure, a small portion of phosgene hydrolyzes to hydrochloric acid (HCl) and carbon dioxide (CO_2) in the mucous coating of the upper respiratory tract (Diller, 1985), but in the moist atmosphere of the terminal spaces of the lungs more extensive hydrolysis is thought to occur (Beard, 1982). Although phosgene is only slightly soluble in water, once in solution it rapidly hydrolyzes to HCl and CO_2. However, phosgene reacts even faster with other functional groups such as amino, hydroxyl, and sulfhydryl groups (Jaskot *et al.*, 1991; Diller, 1985). Because of the affinity for lung tissue and the hydrolysis and acylation

TABLE 23.1. Physical and chemical data

Parameter	Data	Reference
Synonyms	Carbonyl chloride, carbon oxychloride, carbonic dichloride, chloroformyl chloride	Lipsett *et al.* (1994); US EPA (1986)
Chemical formula	$COCL_2$	Lipsett *et al.* (1994)
Molecular weight	98.92	Lipsett *et al.* (1994)
CAS registry no.	75-44-5	Lipsett *et al.* (1994)
Physical state	Colorless gas at room temperature	Budavari *et al.* (1989)
Odor threshold	0.5–1.5 ppm	NIOSH (1976)
Odor description	Pleasant, like newly mown hay	Dunlap (2001)
Vapor pressure	1215 mm Hg at 20°C	Budavari *et al.* (1989)
Vapor density	3.5 (air = 1)	NIOSH (2005)
Specific gravity	1.92 (water = 1)	*CRC Handbook* (1988)
Melting/boiling/flash point	−118°C/8.2°C/nonflammable	NIOSH (2005)
Water solubility	Slightly soluble in water, decomposes rapidly ($t_{1/2}$ = 0.26 s)	NIOSH (2005); US EPA (2003)
Reactivity	Reacts with alcohols, alkalis, ammonia, and copper	NIOSH (2005)
Conversion factors in air	1 ppm = 4.1 mg/m^3 1 mg/m^3 = 0.25 ppm	NIOSH (2005)

that take place in the pulmonary system, very little, if any, phosgene is dispersed to other locations in the body.

IV. MECHANISM OF ACTION

The toxicity of phosgene is due to both hydrolysis and acylation, with the latter being most important.

Diller (1985) accumulated data and described the clinical signs associated with phosgene exposure. Phosgene inhaled at concentrations of >1 ppm triggers a transient vagal reflex and causes shallow, rapid respiration with a decrease in respiratory volume and capacity, a decrease in arterial oxygen partial pressure, and bradycardia. The intensity varies greatly between individuals. Phosgene at >3 ppm becomes moderately irritating to the eyes and upper airways (cough) as some of the phosgene undergoes hydrolysis producing HCl.

The acylation reaction of phosgene with nucleophiles, such as amino, hydroxyl, and sulfhydryl groups, also occurs rapidly causing lipid and protein denaturation, irreversible membrane changes, and disruption of enzymatic function. These acylation effects observed at exposures >30 ppm·min produce pulmonary edema as the blood–air barrier becomes more permeable to blood plasma after a clinical latent period (1–24 h). This is called the clinical edema phase which is characterized by increasing inefficiency of gas exchange as more defects in the blood–air barrier occur allowing more accumulation of a protein-rich fluid. Clinical signs in this phase are labored breathing and a frothy expectorant. Progression usually results in death from paralysis of the respiratory center due to anoxemia with secondary cessation of heart function. If anoxemia is controlled, circulatory shock may still occur. At very high concentrations (>200 ppm), phosgene may cause death within a few minutes from ''acute cor pulmonale'' (acute overdistention of the right heart) often before pulmonary edema can develop.

Phosgene depletes lung glutathione, while glutathione reductase and superoxide dismutase increase as a result of the lung's response to injury. Upon exposure to phosgene, cellular glycolysis, oxygen uptake, intracellular ATP, and cyclic AMP are decreased and associated with increased permeability of pulmonary vessels leading to pulmonary edema. Phosgene exposure also causes increased lipid peroxidation and leukotriene synthesis, with no change in cyclooxygenase metabolism (TEMIS, 1997).

V. TOXICITY

A. Human

1. Noncancer

Reports of human phosgene poisonings present a relatively consistent set of clinical effects and sequelae (Stavarakis, 1971; Bradley and Unger, 1982; Misra *et al.*, 1985; Hegler, 1928; Delephine, 1922; Everett and Overholt, 1968; Regan, 1985; Galdston *et al.*, 1947a, b; Diller *et al.*, 1979; Herzog and Pletscher, 1955; Henschler, 1971; Kaerkes, 1992; Wells, 1985; Cordasco *et al.*, 1986). After acute phosgene exposure, brief (20 min) ocular and throat irritation, cough, nausea and vomiting, and dizziness are experienced, followed by a period (24 h) of apparent well-being. After this clinical latent phase, cough accompanied by expectoration,

a sensation of pain or tightness of the chest, shortness of breath, and a choking sensation are experienced. Clinical findings may include hemoconcentration, leukocytosis, rales, and pulmonary edema. After recovery, rapid, shallow breathing, shortness of breath on exertion, and a sense of decreased physical fitness may persist for months. Pulmonary emphysema may occur with repeated exposure to phosgene.

Diller and Zante (1982) identified ocular irritation, throat irritation, and cough as acute irritative effects of phosgene. In a follow-up analysis, Borak and Diller (2001) also performed an extensive literature review concerning human phosgene exposure, and concluded the following: the smell has no warning property; immediate irritation is not prognostic; pulmonary edema can appear several hours after exposure and the length of clinical latency can be used as a prognostic indicator (i.e. the shorter the time to effects, the worse the prognosis). The data also show that while concentration is the primary driver for the onset and severity of symptoms, duration of exposure also plays a role. Information synthesized from these reviews is presented in Table 23.2.

2. Cancer

Epidemiology studies have shown no increase in cancer in workers exposed to phosgene compared to controls. Polednak (1980) and Polednak and Hollis (1985) examined a cohort of chemical workers exposed to chronic low levels of phosgene as well as daily exposures above 1 ppm. Approximately 35 years after exposure to phosgene, no increase in overall mortality or mortality from cancer or respiratory disease was noted.

B. Animal

1. Noncancer

Animal studies with phosgene show a steep concentration–response curve for pulmonary edema and mortality. Acute animal studies also indicate little species variability as rats, mice, sheep, pigs, and dogs exposed developed similar clinical signs (dyspnea, labored breathing, pulmonary edema after a latent period) and histopathological lesions in the lungs. While there are no chronic animal data, subchronic studies indicate little accumulation of phosgene or increased severity of lesions with continuous exposure.

Bronchoalveolar lavage (BAL) fluid analysis may be used to assess pulmonary edema/lung injury following acute inhalation exposure to phosgene (Pauluhn *et al.*, 2007). Maximum protein concentrations in BAL fluid typically occur within one day post-exposure, followed by a latency period up to 15 h. For acute exposures, the *CT* relationship is constant over a wide range of concentrations. However, following noncontinuous, repeated exposure, increased tolerance to subsequent exposures is observed. Although limited, the longer-term data indicate that effects do not increase in severity over time, but do with increased concentration; suggesting that chronic toxicity is dependent on an acute pulmonary threshold dose. A comparison of BAL fluid constituents from acute inhalation studies in rats and dogs suggests that dogs are three to four times less sensitive to phosgene than rats (Pauluhn *et al.*, 2007).

A summary of selected lethal and nonlethal animal toxicity studies is presented in Tables 23.3 and 23.4, respectively.

2. Animal Cancer

A study by Selgrade *et al.* (1989) showed that exposure to very low levels of phosgene enhances the susceptibility of mice to lung tumor formation. Female C57BL/6 mice were exposed for 4 h to 0.01 ($N = 13$), 0.025 ($N = 28$), or 0.05 ppm phosgene ($N = 35$) and injected intravenously with syngeneic B16 melanoma cells on the following day. Controls were injected with tumor cells and exposed to air. The lungs were removed 2–3 weeks after tumor cell injection and the tumors were counted. Compared with controls, there was

TABLE 23.2. Summary of major signs and symptoms of phosgene inhalation exposure in humans (Diller and Zante, 1982; Borak and Diller, 2001)

Exposure					
Concentration	**Duration**	***C* × *T* product (ppm·min)**	**Acute effect**	**Time to onset of pulmonary edema**	**Time to death**
3 ppm	''Acute''	–	Throat irritation	–	–
4 ppm	''Acute''	–	Ocular irritation	–	–
4.8 ppm	''Acute''	–	Cough	–	–
1 ppm	20 min	20	–	–	–
5 ppm	5 min	25	–	–	–
1 ppm	150 min	150	–	10 h	–
50 ppm	5 min	250	–	5 h	–
100 ppm	5 min	500	–	3 h	24 h
1.3 ppm	400 min	520	–	7 h	30 h
300 ppm	2 min	600	–	–	Minutes

TABLE 23.3. Summary of lethal animal inhalation studies with phosgene

Concentration (ppm)	Time	Species	Effect	Reference
Various	30 min	Dog	LC_{50} = 66 ppm	Boyland *et al.* (1946)
Various	30 min	Dog	LC_{50} = 61–70 ppm	Underhill (1920)
Various	60 min	Dog	LC_{50} = 42 ppm	Boyland *et al.* (1946)
Various	8 min	Rat	LC_{50} = 92 ppm	Boyland *et al.* (1946)
12, 37, 75, 80, 88, 93, or 106	10 min	Rat	LC_{50} = 82 ppm	Zwart *et al.* (1990)
41, 44, 52, or 61	10 min	Rat	LC_{50} = 62 ppm	Pauluhn (2006a)
12, 15, 16, 17, or 25	30 min	Rat	LC_{50} = 21 ppm	Zwart *et al.* (1990)
12, 13, 17, or 22	30 min	Rat	LC_{50} = 13.5 ppm	Pauluhn (2006a)
Various	32 min	Rat	LC_{50} = 17 ppm	Boyland *et al.* (1946)
6.4, 8.8, 9.0, or 12	60 min	Rat	LC_{50} = 12 ppm	Zwart *et al.* (1990)
7.3, 9.6, or 12	60 min	Rat	LC_{50} = 7.7 ppm	Pauluhn (2006a)
Various	64 min	Rat	LC_{50} = 11 ppm	Boyland *et al.* (1946)
2.2 or 2.7	240 min	Rat	LC_{50} = 2.1 ppm	Pauluhn (2006a)
10, 15, 25, 35, 50, 70, or 90	5 min	Mouse	LC_{50} = 33 ppm	Kawai (1973)
Various	8 min	Mouse	LC_{50} = 77 ppm	Boyland *et al.* (1946)
12, 37, 75, 80, 88, 93, or 106	10 min	Mouse	LC_{50} = 79 (m) and 60 (f) ppm	Zwart *et al.* (1990)
12, 15, 16, 17, or 25	30 min	Mouse	LC_{50} = 19 (m) and 11.5 (f) ppm	Zwart *et al.* (1990)
1.0, 2.0, 3.0, 6.0, 9.0, or 13.5	30 min	Mouse	LC_{50} = 5.1 ppm	Kawai (1973)
Various	32 min	Mouse	LC_{50} = 15 ppm	Boyland *et al.* (1946)
6.4, 8.8, 9.0, or 12	60 min	Mouse	LC_{50} = 9.5 (m) and 5.0 (f) ppm	Zwart *et al.* (1990)
Various	64 min	Mouse	LC_{50} = 7 ppm	Boyland *et al.* (1946)
Various	8 min	Guinea pig	LC_{50} = 43 ppm	Boyland *et al.* (1946)
Various	32 min	Guinea pig	LC_{50} = 13 ppm	Boyland *et al.* (1946)
Various	64 min	Guinea pig	LC_{50} = 11 ppm	Boyland *et al.* (1946)

a statistically significant ($p < 0.05$) increase in the number of B16 melanoma tumors in the lungs of mice treated with 0.025 or 0.05 ppm phosgene. Exposure to 0.025 ppm was considered the lowest-observed-effect level. Extending the exposure time from 4 to 8 h did not alter the susceptibility to B16 tumors at 0.01 ppm.

VI. RISK ASSESSMENT

Many inhalation regulatory and guideline levels have been derived for phosgene. These values are summarized in Table 23.5, and the definitions and basis for the values are described in the footnotes to Table 23.5.

VII. TREATMENT

Treatments that have been proposed to prevent pulmonary edema in exposed-asymptomatic persons include steroids, ibuprofen, *N*-acetyl cysteine, and positive pressure airway ventilation. However, there is no known antidote for phosgene poisoning, and although animal studies suggest that these treatments may be effective, no clinical data are available to verify efficacy in humans (Borak and Diller, 2001). Asymptomatic individuals exposed to phosgene should be observed and evaluated to determine if symptoms develop. Vital signs and lung auscultation should be evaluated every 30 min and serial chest x-rays should be performed starting 2 h post-exposure. If no clinical signs occur and no signs of pulmonary abnormalities are detected on the x-ray after 8 h, patients may be discharged. If no x-ray is available, patients should be observed for 24 h post-exposure. If signs develop, patients should be treated. Therapy for the cardiogenic pulmonary edema may include positive airway pressure and monitoring (endotracheal intubation and mechanical ventilation with high oxygen concentrations), steroids, theophylline, diuretics, and antibiotics (in patients who develop bacterial pulmonary infections) (Borak and Diller, 2001).

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

Phosgene is a colorless gas at ambient temperature and pressure, and inhalation is the most important route of exposure for phosgene. Phosgene's odor has been described as similar to newly mown hay. Because of its mild upper

TABLE 23.4. Summary of nonlethal animal inhalation studies with phosgene

Concentration (ppm)	Time	Species	Effect	Reference
0 or 60	10 min	Pig	LOAEL $\geq$ 60 ppm, based on ↑ lung wet weight, mortality	Brown *et al.* (2002)
137, 244, 435, or 773	10 min	Sheep	LOAEL $\geq$ 137 ppm based on pulmonary edema, shallow breathing	Keeler *et al.* (1990a)
0 or 490 to 611	10 min	Sheep	LOAEL $\geq$ 490 ppm based on lung edema	Keeler *et al.* (1990b)
0 or 8	20 min	Mouse	LOAEL $\geq$ 8 ppm, based on acidosis, clinical signs and ↓ body wt, ↑ lung ww/dw	Sciuto *et al.* (2001)
0 or 22	20 min	Mouse, rat and guinea pig	LOAEL $\geq$ 22 ppm, based on ↑ LFP	Sciuto (1998)
0, 2.1, 4.3, or 8.8	30 min	Dog	LOAEL = 4.3 ppm, based on ↑ PMNs in BAL fluid NOAEL = 2.1 ppm	Pauluhn (2006c)
.2, .5, 1.0, 2.0, or 4.0	30 min	Rat	LOAEL = 2.0 ppm, based on clinical signs and ↓ body wt, ↑ LFP NOAEL = 1. 0 ppm	Pauluhn (2006b)
0, 0.05, 0.1, 0.2, 0.4, or 1.0	240 min	Rat	LOAEL = 0.2 ppm, based on based on clinical signs and ↓ body wt, ↑ LFP NOAEL = 0.1 ppm	Pauluhn (2006b)
0, 0.1, 0.5, or 1.0	240 min	Rat	LOAEL = 0.5 ppm based on ↓ in NK cell activity NOAEL = 0.1 ppm	Burleson and Keyes (1989)
0 or 1.0	240 min	Rat	LOAEL $\geq$ 1.0 ppm based on ↓ body wt, ↑ lung wts	Ehrlich *et al.* (1989)
0 or 0.5	240 min	Rat	LOAEL $\geq$ 0.5 ppm based on ↑ LFP and lung wts	Jaskot *et al.* (1989)
0, 0.25, or 0.5	240 min	Guinea pig	LOAEL $\geq$ 0.25 ppm based on ↑ LFP	Slade *et al.* (1989)
0.1 to 0.5	240 min	Mouse	LOAEL = 0.15 ppm based on ↑ phenobarbital-induced sleeping times NOAEL = 0.10 ppm	Illing *et al.* (1988)

(*continued*)

TABLE 23.4. (*continued*)

Concentration (ppm)	Time	Species	Effect	Reference
0, 0.125, 0.25, 0.5, or 1.0	240 min	Rat	LOAEL = 0.25 ppm based on ↑ PMNs in lavage fluid NOAEL = 0.125 ppm	Currie *et al.* (1987a)
0, 0.05, 0.125, 0.25, 0.5, or 1.0	240 min	Rat	LOAEL ≥ 0.05 ppm based on ↓ ATP in lungs	Currie *et al.* (1987b)
0, 0.1, 0.2, 0.5, or 1.0	240 min	Rat, mouse and hamster	LOAEL = 0.2 ppm based on ↑ LFP NOAEL = 0.1 ppm	Hatch *et al.* (1986)
0, 0.1, 0.2, 0.5, or 1.0	240 min	Rabbit and guinea pig	LOAEL = 0.5 ppm based on ↑ LFP NOAEL = 0.2 ppm	Hatch *et al.* (1986)
0 or 1.0	240 min	Rat	LOAEL ≥ 1.0 ppm based on ↓ body wt, ↑ lung wts	Franch and Hatch (1986)
0 or 1.0	240 min	Rat	LOAEL ≥ 1.0 ppm based on ↑ pulmonary edema	Frosolono and Currie (1985)
0 or 1.0	420 min	Rat	LOAEL ≥ 1.0 ppm based on ↑ lung wts	Franch and Hatch (1986)
0.125 or 0.25	4 h/day, 5 days/week for 17 days	Rat	LOAEL = 0.25 ppm based on ↑ lung wts and ↑ NPSH and G6PD activity NOAEL = 0.125 ppm	Franch and Hatch (1986)
0, 0.1, 0.2, 0.5, or 1.0	0.1 ppm for 5 days/week; 0.2 ppm for 5 days/week; 0.5 ppm for 2 days/week; 1.0 ppm for 1 day/week All exposed 6 h/day for up to 12 weeks	Rat	LOAEL = 0.1 ppm based on reversible lung histopathology; ↑ lung displacement volume NOAEL = none	Kodavanti *et al.* (1997)
0, 0.1, 0.2, or 0.5	0.1 and 0.2 ppm for 5 days/week; 0.5 ppm for 2 days/week All exposed 6 h/day for up to 12 weeks	Rat	LOAEL = 0.1 ppm based on ↓ bacterial clearance after infection with *S. zooepidemicus* NOAEL = none	Selgrade *et al.* (1995)

LOAEL – lowest observed adverse effect level; LFP – lavage fluid protein; BAL – bronchoalveolar lavage; NOAEL – no observed adverse effect level

TABLE 23.5. Standards and guidelines for phosgene

	Exposure duration				
Guideline	**10 min**	**30 min**	**1 h**	**4 h**	**8 h**
AEGL-1[a]	NA	NA	NA	NA	NA
AEGL-2[a]	0.60 ppm	0.60 ppm	0.30 ppm	0.08 ppm	0.04 ppm
AEGL-3[a]	3.6 ppm	1.5 ppm	0.75 ppm	0.20 ppm	0.09 ppm
ERPG-1[b]	NA				
ERPG-2[b]	0.2 ppm				
ERPG-3[b]	1.0 ppm				
EEGL (NRC)[c]			0.2 ppm		0.02 ppm (24 h)
NIOSH IDLH[d]	2 ppm				
NIOSH STEL[e]	0.2 ppm (15 min ceiling)				
NIOSH REL[e]					0.1 ppm (10 h)
OSHA PEL-TWA[f]					0.1 ppm
ACGIH TLV[g]					0.1 ppm
MAK (Germany)[h]					0.02 ppm
MAC (Netherlands)[i]					0.02 ppm
RfC[j]					7.33×10^{-5} ppm

[a]AEGL (Acute Exposure Guideline Levels) (NRC, 2002) represent threshold exposure limits for the general public and are applicable to emergency exposure periods ranging from 10 min to 8 h. Three levels – AEGL-1, AEGL-2 and AEGL-3 – are developed for each of five exposure periods (10 and 30 min, 1 h, 4 h, and 8 h) and are distinguished by varying degrees of severity of toxic effects. The three AEGLs tiers are defined as follows.

AEGL-1 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience notable discomfort, irritation, or certain asymptomatic, nonsensory effects. However, the effects are not disabling and are transient and reversible upon cessation of exposure. The AEGL-1 is not recommended for phosgene because the odor threshold is at the concentration approaching AEGL-2 values, and odor cannot be used as a warning.

AEGL-2 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience irreversible or other serious, long-lasting adverse health effects or an impaired ability to escape. The AEGL-2 for phosgene is based on chemical pneumonia in rats (Gross *et al.*, 1965).

AEGL-3 is the airborne concentration of a substance above which it is predicted that the general population, including susceptible individuals, could experience life-threatening health effects or death. The AEGL-3 is based on concentrations causing no death in rats or mice (Zwart *et al.*, 1990).

[b]ERPG (Emergency Response Planning Guidelines, American Industrial Hygiene Association) (AIHA, 2006).

The ERPG-1 is the maximum airborne concentration below which it is believed nearly all individuals could be exposed for up to 1 h without experiencing other than mild, transient adverse health effects or without perceiving a clearly defined objectionable odor. The ERPG-1 for phosgene is not derived.

The ERPG-2 is the maximum airborne concentration below which it is believed nearly all individuals could be exposed for up to 1 h without experiencing or developing irreversible or other serious health effects or symptoms that could impair an individual's ability to take protective action. The ERPG-2 for phosgene is based on pulmonary pathology and function studies suggestion that concentrations exceeding 0.2 ppm may produce serious pulmonary effects in some individuals.

The ERPG-3 is the maximum airborne concentration below which it is believed nearly all individuals could be exposed for up to 1 h without experiencing or developing life-threatening health effects. The ERPG-3 for phosgene is based on acute animal inhalation data indicating that concentrations exceeding 1 ppm for 1 h may by expected to produce pulmonary edema and possible mortality in a heterogeneous human population.

[c]EEGL (Emergency Exposure Guidance Levels, National Research Council) (NRC, 1985). The EEGL is the concentration of contaminants that can cause discomfort or other evidence of irritation or intoxication in or around the workplace, but avoids death, other severe acute effects, and long-term or chronic injury. The EEGL for phosgene is based on the ''most relevant animal exposure studies'' (Gross *et al.*, 1965; Rinehart and Hatch, 1964) and studies suggesting that animals do not tolerate phosgene at 0.2 ppm administered 5 h/day for 5 days (Cameron and Foss, 1941; Cameron *et al.*, 1942).

[d]IDLH (Immediately Dangerous to Life and Health, National Institute of Occupational Safety and Health) (NIOSH, 2005) represents the maximum concentration from which one could escape within 30 min without any escape-impairing symptoms, or any irreversible health effects. The IDLH for phosgene is based on acute inhalation toxicity data in humans (Diller, 1978).

[e]NIOSH REL-STEL (Recommended Exposure Limits – Short Term Exposure Limit) (NIOSH, 2005) is defined analogous to the ACGIH TLV-TWA.

[f]OSHA PEL-TWA (Occupational Health and Safety Administration, Permissible Exposure Limits – Time Weighted Average) (NIOSH, 2005) is defined analogous to the ACGIH-TLV-TWA, but is for exposures of no more than 10 h/day, 40 h/week.

[g]ACGIH TLV-TWA (American Conference of Governmental Industrial Hygienists, Threshold Limit Value – Time Weighted Average) (ACGIH, 2006) is the time-weighted average concentration for a normal 8-h workday and a 40-h workweek, to which nearly all workers may be repeatedly exposed, day after day, without adverse effect. The ACGIH TLV-TWA was derived from the marked potential for pulmonary irritation after exposure to phosgene at concentrations greater than 0.1 ppm; this conclusion was based on two studies. Gross *et al.* (1965) found that phosgene at 0.5 ppm for 2 h caused some pathologic changes in the lungs of rats and Cameron *et al.* (1942) found that 0.2 ppm, 5 days/week for 5 consecutive days caused pulmonary edema in 41% of animals exposed (goats, cats, rabbits, guinea pigs, rats, and mice).

[h]MAK [Maximale Argeitsplatzkonzentration (Maximum Workplace Concentration)] Deutsche Forschungsgemeinschaft (German Research Association) 2000 is defined analogous to the ACGIH-TLV-TWA.

[i]MAC [Maximaal Aanvaaarde Concentratie (Maximal Accepted Concentration)]. SDU Uitgevers (under the auspices of the Ministry of Social Affairs and Employment), The Hague, The Netherlands 2000 is defined analogous to the ACGIH-TLV-TWA.

[j]US EPA (US Environmental Protection Agency Reference Concentration) (US EPA, 2005). The RfC is an estimate of a continuous inhalation exposure concentration to people (including sensitive subgroups) that is likely to be without risk of deleterious effects during a lifetime. The RfC was developed by using incidence of the lung histopathological findings from Kodavanti *et al.* (1997) in a benchmark dose analysis.

respiratory, eye, and skin irritancy, and a mildly pleasant odor, an exposed victim may not actively seek an avenue of escape before lower respiratory damage has occurred (Currie *et al.*, 1987a; Lipsett *et al.*, 1994). Pulmonary edema is the cause of death after a clinical latent period of 24 h (Franch and Hatch, 1986). Phosgene exhibits a steep concentration–response curve, and little species variability with regard to lethality. Recent data (Pauluhn, 2006a, b, c; Pauluhn *et al.*, 2007) suggest that with regard to physiology of the respiratory tract and acinar structure of the lung, dogs are more similar to humans than rodents. Thus, it may be most appropriate to base future phosgene risk assessments on data extrapolation from dogs to humans when dog data are available.

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CHAPTER 24

Other Toxic Chemicals as Potential Chemical Warfare Agents

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I. INTRODUCTION

For the development of new chemical weapons (CWs), a number of criteria are necessary: a research base including scientists and equipment, access to information, chemical and arms industries, and of course financial support. It is noteworthy that the development of CWs is possible not only for states but also for terrorists. It is necessary to stress that the intention of this chapter is not to describe new CWs or chemical warfare agents (CWAs) but to comment on a number of trends in toxicology with the aim that these chemicals may be proposed for inclusion in the Chemical Weapons Convention (CWC) verification mechanisms. However, the text of the CWC is comprehensive and covers practically all chemicals that may be misused as CWs.

The objective of this chapter is to briefly describe a number of chemicals that could be used as toxic compounds or CWAs against humans.

II. GENERAL

A. Chemical Weapons Convention: Article II, Definitions and Criteria

1. ''Chemical weapons'' means the following, together or separately:
   (a) Toxic chemicals and their precursors, except where intended for purposes not prohibited under this Convention, as long as the types and quantities are consistent with such purposes;
   (b) Munitions and devices, specifically designed to cause death or other harm through the toxic properties of those toxic chemicals specified in subparagraph (a), which would be released as a result of the employment of such munitions and devices;
   (c) Any equipment specifically designed for use directly in connection with the employment of munitions and devices specified in subparagraph (b).

2. ''Toxic chemical'' means:

Any chemical which through its chemical action on life processes can cause death, temporary incapacitation or permanent harm to humans or animals. This includes all such chemicals, regardless of their origin or their method of production, and regardless of whether they are produced in facilities, in munitions or elsewhere.

Therefore, CWAs can be characterized as toxic chemicals. Initially, it is difficult to differentiate between the research aimed at protection against (defensive) and development of new CWAs (offensive). Both actions deal with the synthesis of chemicals, based on either information or ideas, or incidentally synthesized toxic compounds. A typical example is the synthesis of organophosphates (OPs) by G. Schrader, originally dedicated to the development of new pesticides. Another example of the development of a new CW would be the synthesis of OP compounds of the V series. After synthesis, the compound in question will be characterized chemically and biologically, and sometimes modified to increase its military properties (toxicity, physicochemical properties, e.g. stability, volatility, etc.). Then the compound can be studied in detail for its pharmacological and toxicological characteristics by using more convenient species and routes of administration, etc. At this stage, it is practically impossible to decide if the research is offensive or defensive though some indications would lead to the opinion that the direction is offensive, e.g. when attention is given to its efficacy following percutaneous or inhalation administration. Studied methods of dispersion under field conditions are an indication of an offensive approach; testing for protective qualities under real conditions can be regarded as a defensive approach. However, further steps like production of large quantities and weaponization are clearly offensive. It is useful to compare the time from synthesis to production or use of certain CWs. After the synthesis of phosgene and diphosgene (1812 and 1887), their use in 1916 was observed; a similar situation was observed for mustard (1866–1917); for CS, this period was shortened (synthesis in 1928 and use in 1950); VX was synthesized in the early 1960s and weaponized in 1968. The big question is, what is meant by

large quantities? This can be solved using the approach contained in CWC. Quantities would be also compared with the contamination density prescribed for different CWAs – for yperite it is 19 tons/km^2 for percutaneous administration and 4 tons/km^2; for VX (percutaneous) it is 2 tons/km^2; for sarin and BZ (by inhalation) this value is about 0.5–0.6 tons/km^2 (Robinson, 1985). Of course, it does not apply to the synthesis or production for terroristic purposes.

Apart from this ''classic'' approach, it would be possible to ''improve'' the properties of known CWs, e.g. microencapsulation so that less stable or highly volatile substances can be used. Nanotechnology offers new possibilities, as described recently by Price and Peterson (2008). The other option is to improve penetration using known enhancers like dimethyl sulfoxide (DMSO). While the percutaneous toxicity (expressed as LD_{50} in rats) of one of the toxic organophosphates – *O*-isopropyl *S*-2-diisopropylaminoethyl methyl phosphnothiolate – is 59.1 μg/kg, in mixture with DMSO this value is decreased to 10.1 μg/kg (Bajgar, 1989).

Binary technologies are also acknowledged; however, the development of other methods of synthesis is not excluded (the more steps there are involved in synthesis the more difficult it is to control the process). An alternative is to search for compounds either used or synthesized already. From the groups of highly toxic chemicals these could be fluorophosphorylcholines (unstable) or toxic silatrans. In the group of medicaments, there are also highly toxic chemicals like cardiac glycosides (digoxin), sympathomimetics (noradrenalin), and myorelaxans (succinylcholine, curare derivatives). The other compounds to be included are insulin, cantharidin, aconitin, galantamine, pancuronium, pipecuronium, some derivatives of vitamin D (cholecalcipherol), some antibiotics, cytostatics drugs, etc. It is necessary to point out that their use is limited, e.g. parenteral administration of insulin or delayed acute effect of cytostatics. A possible candidate would be centrally acting alpha 2-adrenergics having antihypertensive and sedative properties. All bioregulators are of great interest especially in connection with the increased possibility of obtaining significantly sufficient quantities for military and terroristic purposes. All these examples are more or less hypothetical and require further testing. There exist some groups of compounds whose misuse is more probable and some of these chemicals are under suspicion (not proved) of being introduced into military arsenals. An illustrative but not exhaustive list of warfare agents is given below.

III. SPECIFIC AGENTS

A. Carbamates

Compounds in this group have a broad spectrum of toxicities – from relatively slightly toxic (carbaryl) to highly toxic compounds comparable with nerve agents (T-1123)

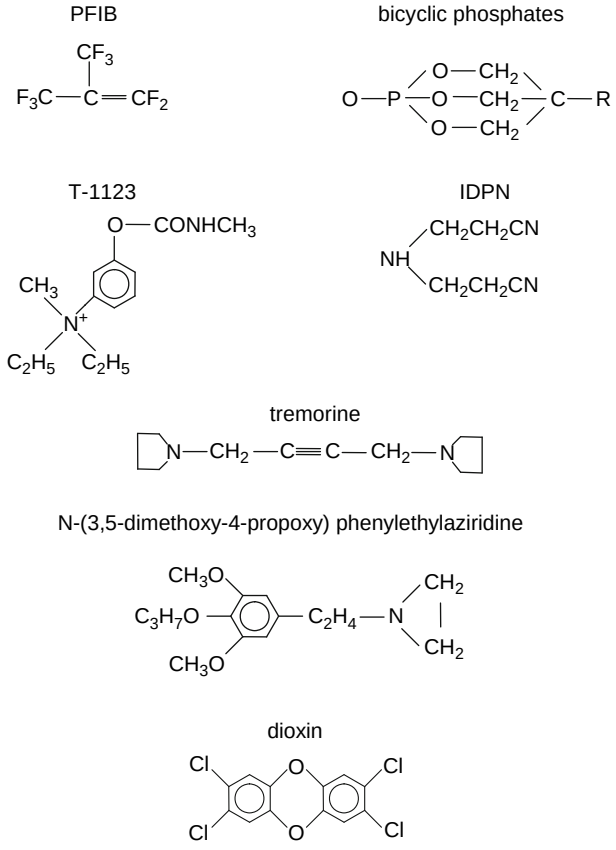


FIGURE 24.1. Chemical formulae of some toxic chemicals.

(Figure 24.1). As described by Robinson (1971), carbamates including T-1123 had been studied by British and Canadians as CW agents since 1940. Other carbamates were described in detail by Badawi and Hassan (1995). They are well absorbed by the lungs, gastrointestinal tract, and the skin. The clinical representation of poisoning is similar to that for nerve agents; perhaps with more expressed peripheral signs because of quaternary nitrogen in the molecule (T-1123) and therefore penetration through blood–brain barrier is difficult (Bajgar and Patocka, 1976; Fusek *et al.*, 1996a). The basic mechanism of action is reversible inhibition of cholinesterases. However, inhibition in case of carbamates is based on carbamylation of the active center of the acetylcholinesterase (AChE). Spontaneous decarbamylation is a relatively quick process (ca 24 h) and carbamylated cholinesterases are resistant to the effect of reactivators. Therefore, the treatment is symptomatic, preferably using only atropine. These difficulties would be reasons for military use (Patocka, 1990).

B. Dioxin

Dioxin is one of the most toxic low molecular weight compounds (Figure 24.1). Its oral LD_{50} for guinea pigs is 2–20 µg/kg, for monkeys 2 µg/kg, for rats 18–60 µg/kg and the dose assessed for humans (subcutaneous administration) is about 107 µg/kg (Bajgar, 2006; Patocka, 2004). Dioxin interferes with the metabolism of porphyrins (and the main symptom of poisoning is derived from an increase of porphyrins in the organism – porphyria cutanea tarda) by induction of delta aminolevulate synthetase. Dioxin also has carcinogenic, hepatotoxic, nephrotoxic, terratogenic, and embryotoxic effects and causes dermal changes (except porphyria cutanea tarda), including chloracne, followed by development of cachexia. There is no specific antidote, making treatment very difficult and symptomatic. Effects of dioxin following acute administration are relatively delayed. This is a limiting factor for its use as a CW. It should be mentioned that dioxin is one of the polychlorinated biphenyls and dibezofuranes that appears to be problematic for the environment (Bajgar, 2006; Sofronov *et al.*, 2001). Dioxin was used to poison Ukraine's President Viktor A. Yushchenko.

C. Bicyclic Phosphates

These compounds were used as flame retardants, antioxidants, stabilizers, or for spectroscopic studies. At present, they are being replaced by other compounds that are not so highly toxic. In a chemical structure (Figure 24.1), when R is substituted by isopropyl, toxicity is very close to sarin ($LD_{50} = 0.18$ mg/kg, i.m. in rat). Bicyclic phosphates act rapidly, in minutes following parenteral administration. Clinical symptoms include behavioral perturbation, muscle weakness, hyperactivity, muscle tremor, and later convulsions passing to paralysis. Intoxication is slightly similar to poisoning with nerve agents but with a different mechanism of action, i.e. probably connected with GABA receptors. Specific antidotal therapy does not exist but a relatively good effect was observed following administration of benzodiazepines (Bajgar, 2006; Patocka, 2004).

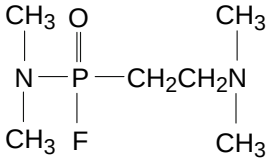
D. PFIB

PFIB is a chemical 2 of the CWC and therefore contained in Schedule 2A. Its chemical structure is shown in Figure 24.1. PFIB is produced by thermal decomposition of Teflon, and has high inhalation toxicity characterized by pulmonary edema. PFIB has been characterized in more detail in an *ASA Newsletter* (Patocka and Bajgar, 1998). Therapy is symptomatic.

E. Organophosphates

There are other known OPs having relatively high toxicity like amiton (Tetram®), Armin®, dimefox (Hanane, Terrasytam®), paraoxon (E 600®), TEPP (Tetron®), etc. These compounds could be used for military and terrorist purposes; however, for the military to replace these substances would be uneconomic. However, a new group of OP compounds has been described and characterized. This class of OPs can be described in general as 2-dialkylaminoalkyl-(dialkylamido)-fluorophosphates. In their chemical formulae, structural similarities with the group of so-called G-compounds (i.e. sarin, soman, tabun) and V-compounds (i.e. VX and others) are found. These chemicals were designated as GP or GV compounds (Bajgar, 1992; Bajgar *et al.*, 1992; Fusek *et al.*, 1996b; Halámek *et al.*, 1995). The toxicities of the most toxic derivatives are shown in Table 24.1. Intoxication with this compound has practically the same syndromes as observed with nerve agents. Treatment with atropine and reactivators is difficult because of the absence of the ability to reactivate

TABLE 24.1. LD_{50} values of GV in mice and rats following various routes of administration

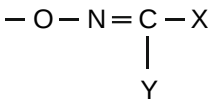


LD_{50} (µg/kg) with their 95% confidence limits

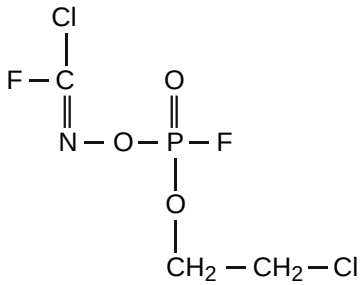
Route of administration	Mice	Rats
i.v.	27.6 (25.6–29.4)	11 (8.5–17.6)
i.m.	30.5 (28–55)	17 (15.5–23.6)
s.c.	32 (29–53)	21 (18–26)
p.o.	222 (194–255)	190 (881–272)
p.c.	not tested	1366 (881–3,138)

inhibited cholinesterases by common oximes (Fusek *et al.*, 1996b; Kassa, 1995; Kassa *et al.*, 2006; Kuca *et al.*, 2006). The lack of reactivation is different from that observed for soman (aging, dealkylation) and it is probably caused by steric hindrance in the cavity of cholinesterase. The volatility of GV compounds is between VX and sarin and therefore these agents are effective when penetrating through uniforms. This is an example of an intermediate volatility agent.

There are sources of information that suggest a new nerve agent known as Novichok or Novichok 5. In 1982, the Soviets began a secret CW development program codenamed Foliant. The program had the apparent goal of developing new binary nerve agent weapons. Novichok has been described as a new toxic agent and it is very difficult to treat the poisoning (practically impossible; the toxicity was about ten times greater than VX agent). Its exact chemical structure is unknown. The Novichok class of chemicals almost certainly belongs to the organophosphorus compounds containing the dihaloformamide group (Bajgar, 2006):



where X and Y are Cl, F, Br or even a stable pseudohalogen like –CN. An example of Novichok could be as follows:



F. Toxins

Toxins are prohibited by the Biological Weapons Convention [Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction, signed in London, Moscow, and Washington on April 10, 1972]. Their isolation from natural sources is sometimes difficult but some toxins are possible to synthesize using biotechnology. Their toxicity is very high, e.g. inhalation LD_{50} of botulinum toxin and tetanotoxin lies in tens of $mg/kg/m^3$. Some other known toxins are saxitoxin, tetrodotoxin, batrachotoxin, etc. They are highly effective by other routes of administration as well. A quick effect is observed during 10–20 min, beginning with muscle weakness, insensitivity of tongue, fingers and mouth, followed by muscle paralysis, including respiratory muscles. Specific therapy is unknown, so it is necessary to save basic life functions (using artificial ventilation, etc.). When death is not observed within the first 24 h after exposure, the prognosis is relatively good.

Other types of toxins are mycotoxins – mostly trichothecenes. Their production is straightforward by fermentation, they are highly stable and therefore may be stored for a long time. They are effective by all routes of administration, including inhalation and percutaneous absorption. Their toxicity is not very high and the effect is prolonged. Symptoms of intoxication are vary greatly, including fever, hemorrhagic eruption, bleeding, necrotic angina, decrease of leukocytes, sepsis, etc. Some toxins are carcinogenic, teratogenic, mutagenic, and hepatotoxic, while some of them also have a neurotoxic effect. There is no specific therapy and treatment is symptomatic only (Patocka, 2004). They caused an epidemic (alimentary toxic aleukia) in the former USSR and were the subject of discussion for possible use in Asia (yellow rain).

In August 1981, based on limited physical evidence, the USA announced that trichothecene mycotoxins had been used – but the findings were less than convincing to the scientific community and the issue became extremely contentious. This controversy was never totally resolved and no definitive evidence was found (Bajgar, 2006).

The question of neurotoxins and neurotoxicity mechanisms was extensively described by Segura-Aguilar and Kostrzewa (2006). Some of them are described below.

1. Aziridines

These are 2-(trisubstituted phenyl) ethyl aziridines that induce changes in behavior and motoric influencing neurotransmission. Their toxicity is not very high and they play a role in modeling a number of diseases. The effect is long lasting, and mostly irreversible without specific antidotal treatment. Some aziridines, e.g. *N*-(3,5-dimethoxy-4-propoxy) phenylethylaziridinium (Figure 24.1), have convulsive properties. Convulsions are treatable with benzodiazepines (Herink, 1977, 1995).

2. Tremorine

This relatively simple compound (Figure 24.1) is known to induce symptoms similar to Parkinson's disease in mice and monkeys. The onset of symptoms, such as salivation, miosis, lacrimation, muscle weakness, bradycardia, was evident within 15–30 min after tremorine administration. Typical symptoms are muscle twitch or fine tremor of the head and extremities, decrease in body temperature, and analgesia. This stage usually lasts for a few hours. Therapy is symptomatic only and not very effective (Bajgar, 2006; Patocka, 2004).

3. IDPN

This is one of the compounds (Figure 24.1) isolated from *Lathyrus sativus*, also called lathyrogenic substances. The toxicity of IDPN (and also of aziridines and tremorine)

expressed as LD_{50} is not very high, as it lies in the range of tens of mg/kg. Following administration of lower doses of IDPN, a condition called ''waltzing syndrome'' is characterized by a circling movement in both directions, and sometimes movement of the head similar to chorea (also hyperkinetic syndrome). High doses of IDPN are known to produce conjunctivitis and edema of the eyelids. In severe cases, it causes hemorrhages in the retina with the possibility of blindness. Hyperkinetic syndrome is irreversible and does not react to therapy (Bajgar, 2006).

G. Bioregulators

A variety of agents have the potential to be used as weapons of bioterrorism. These weapons have been used in wars from the start of recorded history (Metcalfe, 2002). The development of technologies on a modern militarily significant scale was initiated in several countries during the period between the two world wars (Roffey *et al.*, 2002). However, as a result of modern technology, the risks are greater now and the outcomes are more serious (Henderson, 1999). Today, agents include not only different toxins but also a new group of compounds and bioregulators. Bioregulators are naturally occurring organic compounds that regulate diverse cellular processes in all organisms. There are substances normally found in the body that regulate normal biological processes, such as blood pressure, heart rate, breathing, muscle contraction, temperature, mood control, consciousness, sleep, emotions, immune responses, and other critical functions. Their characteristics include activity in extremely low doses and they frequently have rapid effects. Unlike traditional disease-causing biowarfare agents that take hours and days to act, bioregulators can act within minutes after administration. There is comprehensive knowledge available on these compounds, because all these compounds work as regulators and modulators of all vital biochemical pathways, linked with physiological functions of living organisms. If bioregulators were exploited for the purpose of terrorism, they could potentially cause profound pathophysiological effects. The main group of bioregulators under discussion includes different biochemicals such as neurotransmitters, hormones, proteolytic enzymes, and others. The common property of all bioregulators is their ability to induce biological effects and a consequential rapid fall in their concentrations in tissues. The problem is their route of administration (Patocka and Merka, 2004).

A brief description of some bioregulators from a military viewpoint is given below. These peptides have been chosen based on the criteria of bioregulators intended for terrorism and warfare agents (Bokan *et al.*, 2002).

1. Angiotensins

Angiotensins regulate blood pressure and participate in sustaining hypertension (Mazzolai *et al.*, 1998). The principal effect of angiotensin is to stimulate the synthesis of aldosterone and elevate blood pressure via vasoconstriction of smooth muscles in arterioles.

2. Bombesin

Bombesin is a tetradecapeptide isolated from the skin of the amphibian frog *Bombina bombina* (Anastasi *et al.*, 1971). It has been proposed that bombesin-related peptides may be released from the gastrointestinal tract in response to ingested food, and that they bridge the gut and brain via neurocrine means to inhibit further food intake (Merali *et al.*, 1999).

3. Bradykinin

Bradykinin is a vasoactive nonapeptide which is the most important mediator generated by the kinin system and it is involved in inflammation processes (Calixto *et al.*, 2000). Kinins so far identified include bradykinin and kallidin. They cause local increases in the permeability of small blood vessels. Bradykinin is a potent stimulator of pain receptors in the skin and has a powerful influence on stimulating smooth muscle contraction, inducing hypotension, and increasing blood flow and permeability of capillaries (Cyr *et al.*, 2001).

4. Endorphins

Endorphins are peptides which bind to the neuroreceptors in the brain to give relief from pain (Terenius, 1992). Beta-endorphin is the most active, and it is about 20 times more potent than morphine.

5. Endothelins

Endothelins constitute a family of peptides (Hart and Hart, 1992). They are very potent endogenous vasoconstrictors and vasopressors and are secreted by various cells and tissues in the human body. Of the three isoforms, endothelin-1 (ET-1) is one of the most potent contractors of vascular smooth muscles (Miller *et al.*, 1993). Endothelins have very similar structures and biological properties to sarafotoxins (Kloog and Sokolovsky, 1989), and the toxic peptides are obtained from the venom of mole vipers (Atractaspidae).

6. Enkephalins

Enkephalins are endogenous pentapeptides. Two enkephalins have been identified: Met-enkephalin and Leu-enkephalin. Both enkephalins are relatively weak analgesics, which activate all opioid receptors, but appear to have the highest affinity for the delta-receptors. In the CNS, enkephalins have been found in many areas but predominantly those associated with nociception (Przewlocki and Przewlocka, 2001).

7. Histamine Releasing Factor (HRF)

HRF is one of the many immune system protein molecules called cytokines that trigger allergic reactions. Unlike other cytokines, HRF stimulates basophils to release histamine (MacDonald, 1996).

8. Neuropeptide Y (NPY)

NPY is the most abundant neuropeptide in the brain. Its concentration is many times higher than the other neuropeptides. It is a member of a family of proteins that include pancreatic polypeptide, peptide YY, and seminalplasmin. In addition to its function of stimulating feeding behavior, several other physiologic roles have been assigned to NPY, including involvement in circadian rhythms, sexual function, anxiety responses, and vascular resistance (DiBona, 2002; Halford and Blundell, 2000).

9. Neurotensin

Neurotensin is an endogenous peptide neurotransmitter inducing a variety of effects, including analgesia, hypothermia, and increased locomotor activity. It is also involved in regulation of dopamine pathways. Neurotensin is found in endocrine cells of the small intestine, where it leads to secretion and smooth muscle contraction (Moore and Black, 1991).

10. Oxytocin

Oxytocin in a nine amino acid peptide that is synthesized in hypothalamic neurons and transported down through axons of the posterior pituitary for secretion into blood. Oxytocin has three major physiological effects: stimulation of milk ejection, stimulation of uterine smooth muscle contraction at birth, and establishment of maternal behavior.

11. Somatostatin

Somatostatin is a cyclic tetradecapeptide hormone, characterized as the major physiological inhibitor of growth hormone released from the pituitary, but inhibits the release of many other physiologically important compounds, including insulin, glucagon, gastrin, and secretin (Wolkowitz, 1994).

12. Substance P

Substance P is an 11 amino acid polypeptide, and physiologically significant member of a family of three related peptides known as neurokinins. The best known of these is substance P. The specific receptor subtypes corresponding to these three neurokinins are known (Sandberg and Iversen, 1982). These neurotransmitters appear to play a key role in the regulation of emotions, and antagonists of their receptors may be novel psychotropic drugs of the future. Koch *et al.* (1999) demonstrated that substance P, in combination with thiorphan, administered as an aerosol, is highly potent and extremely toxic. Exposure to the substance at extremely low air concentrations may result in incapacitation of humans.

H. Vasopressin

Vasopressin, also called antidiuretic hormone (ADH), is a cyclic nonapeptide hormone, which is released from the posterior pituitary. Its primary function in the body is to regulate extracellular fluid volume by affecting renal handling of water. Specific actions include inhibition of diuresis, contraction of smooth muscles, stimulation of liver glycogenesis, and modulation of ACTH release from pituitary gland. ADH belongs to the family of vasoactive peptides involved in normal and pathological cell growth and differentiation.

I. Thyrotropin, Thyroliberin, or Thyroid-Stimulating Hormone (TSH)

Thyrotropin, thyroliberin, or thyroid-stimulating hormone (TSH) is a peptide released by the anterior pituitary gland that stimulates the thyroid gland to release thyroxine (Ladram *et al.*, 1994). The release of TSH is triggered by the action of thyrotropin-releasing factor (TRF), a peptidic substance found in the hypothalamus of the brain and influencing the secretion of glandula thyroidea.

Not long ago, most bioregulators had been unavailable in the amount needed for terroristic attacks or military operations. However, by the end of the 20th and beginning of the 21st century there had been intensive developments in biomedical sciences, biotechnology, and chemical engineering in the pharmaceutical industry, and because of the revolution in the science and technology of drug discovery, the control of bioregulators will be significantly complicated.

In the near future, genomic and proteomic methods will stimulate increasing use of computer modeling techniques to identify new biologically active compounds and then determine their mode of action. Currently, new compounds are being generated in large numbers by combinatorial methods and assayed for potential activity, and it seems likely that genomic and proteomic methods will make these compounds accessible in amounts necessary for terroristic use. This is a very disagreeable situation and quite a new problem for the control of chemical and biological weapons.

IV. NONLETHAL WEAPONS

Nonlethal weapons can be of a chemical, physical, or pharmacological character (Hess *et al.*, 2005). Nonlethal weapons are designed and primarily employed so as to incapacitate personnel or material while minimizing fatalities, permanent injury to personnel, and undesired damage to property and the environment (Pearson, 2006). Weapons used for physically immobilizing personnel include lasers, microwave impulses, ultrasound, electric current, nets, solidifying foams, and sliding gels. For chemical weapons, immobilizing gases and irritants and smelly bombs can be considered. For pharmacological immobilization, ketamine, benzodiazepines, and onset accelerators are the compounds of real interest. Basic requirements for the use of immobilizing drugs are necessary, such as minimal cardiovascular and respiratory side effects; easy administration, primarily by inhalation; rapid onset; high biological accessibility and well-controlled effects; and specific antagonists which can

be used within a large therapeutic range. These drugs are used for animal immobilization, using narcotizing blowpipes, darts, or guns. This group is also called calmatives. The compounds considered include ketamine and phencyclidine, alpha-2-agonist, opioids – etorphine, fentanyl, carfentanyl – and muscle relaxants (Hess *et al.*, 2005; Patocka and Cabal, 2001; Patocka and Fusek, 2004).

A. Genetic and Ethnic Weapons

Another possible threat is modification of the effects of commonly used chemicals or biological agents. The first administration produces no toxic effect but the second administration of the same or another compound causes damage.

The present genetic material of a human population could be misused. Recent advances in biological research could eventually lead to the creation of new types of chemical weapon targeting a specific group of human beings with common genetic characteristics, as is perhaps the case with certain ethnic groups. Biologically, there are more similarities than differences between human beings. However, differences do exist and if the data on ethnic differences are known, the selective effect of different chemicals to the groups cannot be excluded. Some examples are cited here to support this contention. Glucoso-6-phosphate dehydrogenase, the enzyme that catalyzes dehydrogenation of glucoso-6-phosphate to 6-phosphogluconate, has been genetically determined and found to be low or nonexistent in some groups (more frequently black people or Scandinavians). It is connected with the male chromosome and in these men, hyperbilirubinemia is observed. Following administration of some normal medicaments, like acetylsalicylic acid, sulfadimidine, chinine, chloramphenicol, etc., a hemolytic syndrome is induced. Individuals with other pathologic states, e.g. chronic methemoglobinemia, will be more sensitive to drugs that are able to increase the level of methemoglobin. These drugs include analgesics, antipyretics, nitrates, etc. Deficiency of alpha-1-antitrypsine can cause increased sensitivity to asphyxiation agents. Plasma cholinesterase activity is also genetically determined and individuals with decreased cholinesterase activity are more sensitive to myorelaxants. It is very probable that these people will be more sensitive to nerve agents (Bajgar, 2006).

V. CONCLUDING REMARKS AND FUTURE DIRECTION

This chapter describes that misuse of knowledge of pharmacology and toxicology is possible and can be applied to all activities of humankind. The task is to be well informed in cases where it is important to know the best protection and therapeutic means, and to control all activities connected with the synthesis and possible availability of chemical weapons/chemical warfare agents to nonqualified as well as qualified persons.

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CHAPTER 25

Ricin and Abrin

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I. INTRODUCTION

Human toxicity of castor beans (ricin) and jequirity seeds (abrin) has been acknowledged throughout medical history, worldwide. Ricin and abrin are natural protein toxins isolated from plant seeds *Ricinus communis* and *Abrus precatorius*, respectively (Olsnes *et al.*, 1974). Ricin and abrin share extensive identity and structural similarities of two polypeptide chains: an A-chain subunit and a larger B-chain subunit linked by a disulfide bond (Rutenber and Robertus, 1991; Rutenber *et al.*, 1991; Weston *et al.*, 1994). The mechanisms of toxic action of abrin and ricin are also similar in which the B-chain achieves cellular recognition and binding function to facilitate toxin transport across the cell membrane whereas the A-chain, once internalized by the cell, blocks protein synthesis by catalytically modifying the ribosomes. Both toxins ultimately kill target cells in animal or cell culture models by both necrosis and apoptosis (Rutenber and Robertus, 1991; Rutenber *et al.*, 1991; Weston *et al.*, 1994; Tahirov *et al.*, 1995). The toxicity of ricin and abrin causes severe irritation to the eyes and adnex causing congestion and conjunctivitis after administration of very dilute solutions in microgram quantities of toxins (Audi *et al.*, 2005). Ricin and abrin are toxic to mice by injection in the range 1–20 μg/kg body weight (Stirpe and Battelli, 1990). Along with their toxic potential, the relative ease of ricin and abrin production makes them potential chemical warfare agents or terrorists' weapons.

II. BACKGROUND

A. Botanical Description

Ricin was found by Stillmark in 1889 as the first plant lectin derived from the seeds of the castor plant, *Ricinus communis* L., a member of the Euphorbiaceae or spurge family. Other members of this family include the popular houseplants poinsettia (*E. pulcherrima*), and the croton species. The term ''castor bean'' is used commonly to refer to both the plant and seed of *R. communis*. *Ricinus communis* commonly grows along streams and riverbeds in addition to subtropical locations high in nutrients. *Ricinus communis* is a coarse perennial, 10–13 m tall in the tropics, with a stem 7.5–15 cm in diameter, but usually found as an annual in the temperate regions at 1–3 m tall. Castor beans are ovoid, glossy, 0.5–1.5 cm long, carunculate, and vari-color with base colors white, gray, brownish-yellow, brown, red, or black, with the outer pattern gray or brown to black. The pattern varies from fine to coarse, veined or finely dotted to large splotches (Reed, 1976). Castor beans are glossy, oval in shape, and speckled with black or brown and white spots, and yield an average 50% oil content (Reed, 1976). Castor beans are processed throughout the world to make castor oil. India currently produces the largest amount of castor oil, followed by China and Brazil (Oil World, 2006). Castor oil is yellowish in color and is used for either medicinal applications or industrial processing of paints, varnishes, and sebacic acid (Kim *et al.*, 1999; Jackwerth and Bruening, 2007; Twilley *et al.*, 1967).

Ricin can be made from the waste material left over from processing castor oil. It can be made in either powder, mist, or pellet form, or it can be dissolved in water or weak acid. Ricin is a stable substance under normal conditions, but can be inactivated by heat above 80°C.

Abrin is derived from the plant *Abrus precatorius* L., a member of the Fabaceae (Leguminosae) or pea family. Common names include jequirity seed, rosary pea, crab's eye, precatory pea, and licorice vine. Other names include Gunga or Goonteh or Indian licorice. Originating in India and other parts of tropical Asia, *A. precatorius* is a high-climbing, twining, or trailing woody vine with slender herbaceous branches. The seeds have a remarkably uniform weight of 1/10th of a gram, and were used ancient times for the purpose of weighing gold.

Abrin can be made in the form of a powder, a mist, a pellet, or it can be dissolved in water. Powdered abrin is a stable substance yellowish-white in color.

B. Historical Uses of Ricin and Abrin

Since ancient times, *R. communis* and *A. precatorius* have been used to treat numerous aliments worldwide. Castor beans have been found in Egyptian tombs dating from 4,000 BC and Egyptian temple gardens dating from 1,500 BC (Jacob and Jacob, 1993). In the pharmacopeia of ancient Egypt, castor oil, bean, and root were also mentioned in

medical papyri. The products of castor plant were used in medicinal recipes to treat headaches, stomach ache, tooth ache, skin disease, and constipation. Purified castor oil has also been ingested as a human nutritional supplement, emetic, or purgative (Scarpa and Guerci, 1982; Olsnes, 2004).

In folk medicine, the products of *A. precatorius* have been used orally as a herbal remedy to quicken labor, induce abortion, as an oral contraceptive, and as treatment for diabetes, eye inflammation, kidney inflammation, and pain relief. Jequirity seeds are also used in the manufacture of rosaries, necklaces, and other ornamental articles. However, alongside their beneficial applications for human health, the plants that harbor ricin or abrin are also closely associated with death. Ricin and abrin seeds have been used in Asia for centuries to kill animals, chiefly cattle and other livestock, and have a notorious history as an agent in criminal poisoning (Olsnes, 2004). Over 750 cases of human poisoning with castor beans or ricin, including homicide and suicide attempts, have been reported during the 20th century (Balint, 1974; Rauber and Heard, 1985). One of the most famous cases of ricin poisoning was in 1978 when Georgi Markov, a Bulgarian dissident, was poisoned by an injection of ricin pellet with an improvised umbrella (Balint, 1974; Rauber and Heard, 1985). Most documented case reports involving abrin poisoning involve accidental or intentional ingestion of jequirity seeds.

C. Ricin Use in Warfare

Several aspects of ricin, such as its potent toxicity, lack of specific antidote, and wide availability in large quantities from castor bean meal, have contributed to the international regulation of the toxin as a potential ''weapon of mass destruction''. If made into a partially purified material or refined into a terrorist or warfare agent, ricin could be used to expose people through air, food, or water.

During World War I, the US War Department investigated ricin for chemical warfare use (Audi *et al.*, 2005). At that time it was being considered for use either as a toxic dust or as a coating for bullets and shrapnel. However, the toxic dust concept could not be adequately developed, and the coated bullet/shrapnel concept would violate the Hague Convention of 1899. The war ended before it was weaponized.

During World War II, the USA and Canada resumed the study of ricin, now given the military symbol Whiskey (W), for use in cluster bombs (Audi *et al.*, 2005). Although different bomb concepts were produced, the end conclusion was that it was no more economical than using phosgene, a highly toxic colorless gas used to manufacture pesticides.

Despite ricin's extreme toxicity and utility as an agent of chemical/biological warfare, it is extremely difficult to limit the production of the toxin. Therefore, ricin is currently monitored as a Schedule 1 toxic chemical under the Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on Their Destruction (CWC), and the international use of ricin or abrin as weapons is prohibited under the 1972 Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction (BTWC) Procedural Report and Rolling Text: Ad Hoc Group 23rd session (April 23–May 11, 2001).

In the USA, the Centers for Disease Control and Prevention (CDC) lists ricin on its second highest priority list as an agent for terrorism. Therefore, possession or transfer of ricin, abrin, or genes encoding functional forms of these toxins is regulated by the CDC Select Agents and Toxins Program, US Department of Health and Human Services. Ricin is also categorized as a biological agent under the federal Biological Weapons Anti-Terrorism Act of 1989, which can impose lifetime prison sentences and unspecified fines for its production, acquisition, or possession (CDC, 2003).

III. TOXICOKINETICS

As relatively large proteins, ricin and abrin are not likely to be extensively absorbed from the gastrointestinal tract. In animal studies, ingested ricin was absorbed within 2 h and accumulated mainly in the liver and spleen (Ishiguro *et al.*, 1983, 1992). In mice, intravenously injected ricin was distributed mainly to the spleen, kidneys, heart, and liver, while intramuscularly injected ricin was found to localize in draining lymph nodes (Fodstad *et al.*, 1976; Griffiths *et al.*, 1986). When injected post-intravenously in rodents, 70% of ricin was excreted and less than 2% was recovered in feces or urine over the first 24 h (Blakey *et al.*, 1988; Ramsden *et al.*, 1989). When ricin is post-ingested, approximately 20 to 45% is excreted unchanged in the feces over the first 72 h (Ishiguro *et al.*, 1983, 1992). In rodent studies, ricin was eliminated as degraded proteins, with majority excretion in the urine (Blakey *et al.*, 1988; Ramsden *et al.*, 1989).

Similar to ricin, abrin is poorly absorbed from the intestine, due to its high molecular weight (approximately 65 kDa). Some studies have suggested that abrin is slowly absorbed in which the majority of the toxin was digested within 3 h (Gunsolus, 1955; Lin *et al.*, 1970). However, even a small amount of undigested toxin is still sufficient to cause severe complications and death. After parenteral injection, abrin has been shown to be considerably more toxic than oral administration (Gunsolus, 1955). When injected post-intravenously in mice, abrin is widely distributed in tissues with the greatest percentage found in the liver, followed by the blood, lungs, spleen, kidneys, and heart (Fodstad *et al.*, 1976). When injected into rats via intraperitoneal route, the highest percentage was found predominantly in the liver, followed by the kidneys and blood (Lin *et al.*, 1970). In rodent studies, abrin elimination is almost exclusively renal

as low molecular weight degradation products (Fodstad *et al.*, 1976).

IV. MECHANISM OF ACTION

A. Structural Description

Ricin and abrin share extensive structural similarities. They are composed of two polypeptide chains: an *N*-glycosidase A-chain subunit or effectomere and a larger lectin B-chain subunit or haptomere, linked by a disulfide bond. The A-chains of abrin and ricin have a 102 conserved amino acid homology (Rutenber and Robertus 1991; Rutenber *et al.*, 1991; Weston *et al.*, 1994). Ricin and abrin belong to a group of type 2 RIPs (ribosome-inactivating protein) toxins which inhibit protein synthesis by specifically inactivating eukaryotic 28s ribosomes (Frigerio and Roberts, 1998; Ishiguro *et al.*, 1964a, b; Nicolson and Blaustein, 1972). Other type 2 RIPs toxins include bacterial and plant toxins such as viscumin, pulchellin, modeccin, and volkensin. Abrin-c shares an 86% identical primary amino acid sequence of the A-chains with pulchellin, a highly potent toxin produced in the seeds of *Abrus pulchellus* (Silva *et al.*, 2005; Olsnes, 2004).

Ricin, also known as ricin-D or *R. communis* agglutinin, is a 60–65 kDa globular protein that makes up 1–5% by weight of the castor bean (Ishiguro *et al.*, 1964a, b; Funatsu and Funatsu, 1970; Stirpe and Battelli, 2006). The ricin B-chain is composed of 262 amino acid residues (approximately 34 kDa) containing two lectins capable of binding to galactose-containing glycoproteins and glycolipids expressed on the cellular surface (Lord *et al.*, 1994, 2003). The ricin A-chain is an *N*-glycosidase composed of 267 amino acid residues (approximately 32 kDa) (Lord *et al.*, 1994, 2003). Both chains are glycoproteins containing mannose carbohydrate groups. Ricin A-chain and B-chain are held together by a single interchain disulfide bond, which, upon reduction, remains a relatively weak, noncovalent association (estimated dissociation constant of 10^{-6} M) (Lewis and Youle, 1986).

B. Mechanism of Toxic Action

The mechanisms of toxic action of abrin and ricin are similar. The B-chain attains cell recognition and binding function to facilitate toxin transport across the cell membrane, whereas the A-chain, once internalized by the cell, blocks protein synthesis by catalytically modifying the ribosomes. Both toxins ultimately kill target cells in animal or cell culture models by both necrosis and apoptosis.

Ricin facilitates entry into the cytosol by binding glycoproteins to the cell surface using the galactose binding property of its ricin B-chain (Olsnes, 2004; Olsnes *et al.*, 1974a,b; Sandvig and Olsnes, 1982). The ricin A-chain inhibits protein synthesis by irreversibly inactivating eukaryotic ribosomes through removal of a single adenine residue from the 28S ribosomal RNA loop contained within the 60S subunit. This process prevents chain elongation of polypeptides in protein synthesis and leads to cell death (Olsnes, 2004; Olsnes *et al.*, 1974a,b, 1975; Sandvig and Olsnes, 1982). Ricin toxicity results from the inhibition of protein synthesis, but other mechanisms, including apoptosis pathways, direct cell membrane damage, alteration of membrane structure and function, and release of cytokine inflammatory mediators are also noted (see Figure 25.1) (Day *et al.*, 2002; Hughes *et al.*, 1996).

Abrin exerts its toxic action in the same way as ricin. The abrin B-chain avidly binds to a variety of cell types, in particular reticuloendothelial cells which bear the appropriate mannose receptors. The abrin B-chain also binds to the galactosyl-terminated receptors on the cell membrane to allow the entry of the abrin A-chain. Once internalized, the abrin A-chain is transported from the cell membrane to the ribosomes, where it catalytically inactivates the 60S ribosomal subunit by removing adenine from positions 4 and 324 of 28S rRNA, thereby inhibiting protein synthesis and causing cell death (Stripe and Barbieri, 1986).

Ricin and abrin poisoning can also be explained by hemagglutination. *Ricinus communis* and *A. precatorius* contain the glycoprotein lectins known as ricin communis agglutinin (RCA) and abrus agglutinin, respectively. Their toxic effect is due to its affinity for the red blood cell and other parenchyma cells, such as liver and kidney cells, leading to agglutination and subsequent hemolysis (Lin *et al.*, 1981; Hart, 1963; Lord *et al.*, 1994; Barbieri *et al.*, 1993). Compared to ricin, RCA is a relatively weak cytotoxin due to its poor gastrointestinal absorption (Olsnes *et al.*, 1974a,b); however, it is a powerful hemagglutinin shown to cause clinically significant hemolysis only after intravenous administration (Balint 1974; Olsnes *et al.*, 1975). Another route is endothelial cell damage or vascular leak syndrome, which causes an increase in capillary permeability causing fluid and protein leakage and tissue edema (Baluna *et al.*, 1999). The A-chain causes organ and tissue lesions that might be the result of vascular disturbances induced by the toxin rather than a direct effect of the toxin itself. Regardless of the route of administration, clinical trials have shown that administration of ricin or abrin A-chain immunotoxin caused vascular syndrome characterized by hypoalbuminemia and edema (Baluna *et al.*, 1999).

V. TOXICITY

Numerous laboratory studies in mammals have demonstrated that ricin and abrin are highly toxic and potentially fatal to animals and humans. Major symptoms of both ricin and abrin poisoning are dependent on the route of exposure, the dose (or number of beans) received, or the content of toxin in the seed (or age of seed). Upon ingestion, toxicity is dependent on the degree of mastication: if the bean was

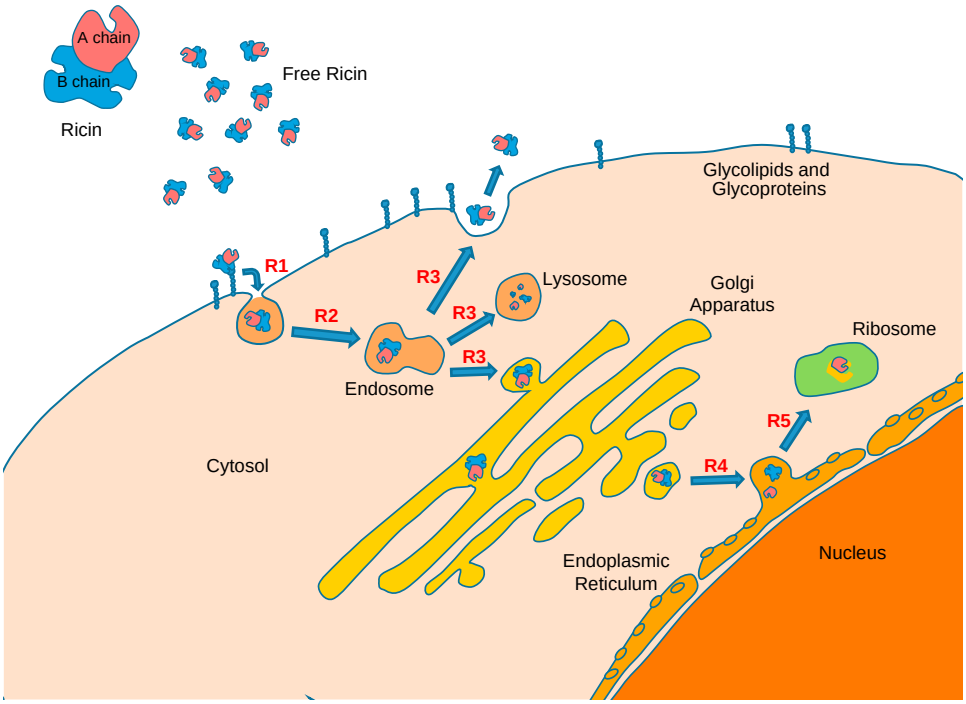

FIGURE 25.1. Mechanism of toxic action of ricin. Ricin B-chain binds to a range of cell surface glycolipids or glycoproteins possessing specific galactose residues and enters the cell by endocytosis (R1); ricin is transported into early endosomes (R2); ricin acquires one of three routes (R3): separation from the endosomal membrane to undergo exocytosis, dissociation from the endosomal binding sites for degradation in the lysosome, or transport to the Golgi apparatus to undergo retrograde transport; from the Golgi apparatus the ricin is transported to the endoplasmic reticulum. The disulfide bond is reduced and the ricin A-chain is separated from the ricin B-chain. It is still unclear as to whether reduction occurs in the endoplasmic reticulum or in the reducing environment of the cytosol (R4) (Lord *et al.*, 2003); ricin A-chain is translocated into the cytosol where it interacts with the ribosome to catalyze the depurination reaction with the rRNA by removing an adenine from position 4324 of the 28S rRNA in the 60S ribosomal subunit. The rRNA is incapable of binding protein elongation factors and thus protein synthesis is disabled (R5).

damaged before consumption or swallowed whole. Intact jequirity seeds are likely to pass through the gastrointestinal tract without harm, due to its impervious shell-like coating, while if the seed was chewed, damaged or even immature, the toxin may be readily absorbed (Davis, 1978; Hart, 1963; Gunsolus, 1955).

A. Experimental/Animal Data

1. Oral Administration

In oral administration of ricin in mice, the approximate LD_{50} dose is 20 mg/kg with time to death up to 85 h (Fodstad *et al.*, 1979). In a rat study, ricin was absorbed in the lymphatic blood vessels and largely distributed in the spleen and liver within 2 h of ingestion (Ishiguro *et al.*, 1983). The study also demonstrated that up to 72 h after ingestion, approximately 20–45% of ricin is excreted in the feces (Ishiguro *et al.*, 1992).

Few animal studies exist on the toxicity of orally administered abrin. The majority of reports indicate jequirity seed poisoning in humans, which is discussed later in the chapter.

2. Parenteral Administration

Ricin and abrin are toxic to mice by parenteral routes in the range 1–20 µg/kg body weight (Stirpe and Battelli, 1990). Injected ricin has been shown to kill laboratory animals in a concentration and time-dependent manner with steep lethality curves. In laboratory mice, the approximate LD_{50} dose and time to death are, respectively, 5 µg/kg and 90 h by intravenous injection, 22 µg/kg and 100 h by intraperitoneal injection, and 24 µg/kg and 100 h by subcutaneous injection (Godal and Fodstad, 1984; Fodstad and Olsnes, 1976). In rats administered 5 µg ricin intramuscularly, LD_{50} dose and time to death are 33–50 µg/kg body weight and a maximum of 35 h, respectively (Leek *et al.*, 1989). The minimum lethal doses via intravenous injection range from 0.7 to 2 µg/kg in mice and 1 to 1.75 µg/kg in dogs (Fodstad *et al.*, 1979). When the dosage of ricin injected intravenously (120 µg/kg body weight) to mice is exceeded, results show hemolytic uremic syndrome, such as thrombotic microangiopathy, hemolytic anemia, and acute renal failure (Fodstad *et al.*, 1979).

With the exception of mice, abrin exposure via intravenous routes demonstrates a significant interspecies variation

in susceptibility: rats 0.35–0.5 μg/kg; guinea pigs 0.4–0.5 μg/kg; rabbits 0.03–0.06 μg/kg; and dogs 1.2–1.35 μg/kg with a dose-dependent survival time of 10 h maximum (Fodstad *et al.*, 1979). The minimum lethal dose in mice was 0.7 μg/kg (Gill, 1982). Intravenous administration of abrin to mice and dogs caused weakness, shivering, anorexia, weight loss, edema, ascites, and rectal bleeding (Fodstad *et al.*, 1979). Intraperitoneal injection of abrin LD_{50} was found to be 0.02 mg/kg body weight in mice (Budavari, 1989). Subcutaneous injection of abrin into mice, guinea pigs and rats caused increases in the AST, ALT, and isocitric dehydrogenase activities are indicative of liver damage (Niyogi, 1977).

3. Inhalation Exposure

Lung deposition and lethality after ricin or abrin inhalation are significantly influenced by particle size, in which low micron-sized particles can deposit deeper into the respiratory tract resulting in higher mortality (Griffiths *et al.*, 1995a, b). Mice exposed to ricin particle sizes less than 5 μm have an LD_{50} about 3–5 μg/kg, while the LD_{50} of rats is 3.7 μg/kg. In laboratory mice, the time to death was 60 h by inhalation (Fodstad *et al.*, 1979). Toxicity results from direct injury to the epithelial membrane with primary toxicity occurring to the type I and II pneumocytes (Griffiths *et al.*, 1995a, b; Brown and White, 1997). In mice, rats, and primates, high doses of inhaled ricin cause death due to hypoxemia resulting from massive pulmonary edema, acute destructive alveolitis as well as necrosis/apoptosis of the lower respiratory tract epithelium (Wilhelmsen and Pitt, 1996; Roy *et al.*, 2003; Gareth *et al.*, 1995). Primates exposed to inhaled ricin developed progressive dyspnea 20–24 h after 21–42 μg/kg dosing of 1–2 μm size particles with mortality occurring within 48 h (Wilhelmsen and Pitt, 1996). Postmortem findings included diffuse pulmonary edema with multifocal areas of necrosis and inflammation (Griffiths *et al.*, 1995a, b; Brown and White, 1997). All injury was significantly worse in the distal airways and alveoli.

The LD_{50} in rats for abrin inhalation was 3.3 μg/kg (Griffiths *et al.*, 1995a, b). Rats exposed to varying concentrations of abrin by inhalation remained clinically well for 18–24 h, then developed general malaise, as well as lethargy, anorexia, piloerectation, and respiratory difficulties. In severely poisoned animals, blood-stained fluid exuded from the nostrils. Examination showed that inhaled abrin caused pulmonary edema, alveolitis, diffuse airway inflammation, necrosis, and diffuse alveolar flooding. Interestingly, ricin caused abundant apoptotic figures in the lung, which were absent following the inhalation of abrin (Griffiths *et al.*, 1995a, b).

4. Dermal and Ocular Exposure

In animal studies, low ricin concentrations caused irritation and the development of conjunctivitis. In ocular exposure, pseudomembranous conjunctivitis occurred following application of ricin solutions in concentrations of 1:1,000–1:10,000 (Audi *et al.*, 2005). In rabbits administered 0.5 μg of ricin, conjunctivitis occurred lasting one week after introduction, while after 1.5 μg of ricin was administrated, corneal lesions with keratitis occurred lasting 11 days after exposure (OSRD, 1946). In dermal exposure of ricin to mice, toxicity was observed at the 50 μg spot. Few animal studies exist on the toxicity of abrin via dermal or ocular exposure. However, clinical reports show abrin exposure to the eye causes severe inflammation of the conjunctiva, along with localized necrosis.

B. Human Data/Clinical Observations

Data about the effects of ricin and abrin poisoning on humans are limited. Due to initial symptoms resembling gastroenteritis or respiratory illness, it is often difficult to discern from other illnesses. Therefore, it is important to observe for epidemiological signs indicative of chemical release.

1. Oral Administration

Most literature reports refer to castor bean or jequirity seed ingestion, with very few reports on ingestion of pure ricin or abrin. As mentioned earlier, toxicity via ingestion of castor bean or jequirity seed is dependent on the degree of mastication. Intact jequirity seeds are likely to pass through the gastrointestinal tract without harm, while if the seed was chewed or damaged the toxin may be readily absorbed (Davis, 1978; Hart, 1963). In human ingestion of castor beans, the lethal dose (LD_{50}) is estimated to be 1–20 mg ricin/kg of body weight, or approximately eight beans for adults (Challoner and McCarron, 1990). In children, one to three seeds can be fatal (Wedin *et al.*, 1986). However, other clinical reports have shown symptomatic poisoning in patients ingesting 15 to 30 beans, and death associated with only two beans ingested (Challoner and McCarron, 1990).

In clinical observations, symptoms may occur approximately within 4–6 h and as late as up to 10 h (Challoner and McCarron, 1990; Klaim and Jaeger, 1990; Rauber and Heard, 1985; Kopferschmitt *et al.*, 1983; Bispham, 1903). Initial symptoms include vomiting, bloody diarrhea, heartburn, abdominal pain, and oropharyngeal irritation. Other less common symptoms include melena and hematemesis. Subsequent features suggest fluid and electrolyte loss, with dehydration, sweating, hypotension, and circulatory collapse. Upon lab testing, abnormalities may include leukocytosis, elevated transaminases and creatinine kinase, hyperbilirubinemia, renal insufficiency, and anemia. In fatal cases, death occurs on the third day or later. Postmortem findings revealed that ingestion of castor beans mainly caused diffuse intestinal hemorrhagic lesions as well as histology consistent with the appearance of apoptotic cell death seen in both humans and animals (Balint, 1974; Challoner and McCarron, 1990; Klaim and Jaeger, 1990;

Rauber and Heard, 1985; Kopferschmitt *et al.*, 1983; Bispham, 1903).

In ingestion of jequirity seeds, the human fatal dose is estimated to be 0.1–1.0 μg/kg of body weight, or approximately one fully masticated jequirity seed may be fatal in either adult or child (Houston, 1982). Children attracted to the brightly-colored seeds are usually exposed to the abrin poison and may chew, suck, or swallow them. However, other reports have shown symptomatic poisoning in patients ingesting 10 to 20 beans, and death associated with only two beans ingested (Swanson-Biearman *et al.*, 1992). In clinical observations, initial symptoms may occur in less than 6 h but usually are delayed for 1–3 days. Early features commonly include nausea, severe vomiting, bloody diarrhea, abdominal pain, and colic. Gastrointestinal bleeding and/or hematemesis may also follow. Patients may exhibit tachycardia, headaches, dilated pupils, irrationality, hallucinations, drowsiness, and weakness. Tetany, tremors, seizures, fever, and dysarrhythmias have also been noted (Davis, 1978; Fernando, 2001). In fatal cases, death has occurred up to 4 days after the onset of symptoms. Postmortem findings revealed that ingestion of jequirity seeds mainly affected the gastrointestinal tract, with hemorrhage, erythema, and edema (Davis, 1978; Fernando, 2001).

2. Parenteral Exposure

Parenteral exposure is one of the most lethal routes of ricin and abrin exposure. In humans, the estimated lethal dose of ricin is 1–10 μg/kg body weight following inhalation or injection (Smallshaw *et al.*, 2002). In clinical observations, onset of symptoms can be delayed for as much as 10–12 h with weakness developing as early as within 5 h of injection. Symptoms can include dizziness, nausea, anorexia, fever, headache, hypotension, and abdominal pain (Fodstad *et al.*, 1976, 1979), and may persist for 8–10 days (CDC, 2006; Fine *et al.*, 1992). Intramuscular injection causes severe local necrosis of muscle and regional lymph nodes with moderate visceral organ involvement. In fatal cases, death has occurred within 3 days. In laboratory testing, results may include increased levels of liver transaminases, amylase, creatinine kinase, hyperbilirubinemia, myoglobinuria, and renal insufficiency (Fine *et al.*, 1992; Targosz *et al.*, 2002). Postmortem findings, in both animal studies and clinical reports, include focal hemorrhage in the intestines, brain, myocardium, and pleura (Godal *et al.*, 1984; Klaim and Jaeger, 1990; Fodstad *et al.*, 1976). Necrosis, hemorrhaging, and edema of the lymph nodes, kidneys, and intestines were also found.

Several cases of ricin exposure via parenteral routes were documented. A 20-year-old man was admitted to the hospital 36 h after subcutaneously injecting castor bean extract. Symptoms were characteristic of nausea, weakness, dizziness, and myalgias. He developed anuria and hypotension, followed by hepatorenal and cardiorespiratory failure, and died 18 h following admission. In another report, a 36-year-old chemist extracted ricin from a castor bean and self-administered intramuscular injections for the purpose of scientific curiosity. He developed fever, nausea, anorexia, mild elevation of liver function tests, and tissue damage at the site of injection. Symptoms persisted for approximately 8–10 days and then improved, at which point he was discharged from the hospital. Finally, the most famous report of ricin poisoning was the ‘‘umbrella murder’’ in which Georgi Markov, a Bulgarian dissident, was thought to have been assassinated with an extremely small amount of ricin. His symptoms included immediate pain at the injection site, weakness within 5 h, and fever and vomiting within 24 h. His clinical course worsened to include shock, multiorgan failure, and death over the next 3 days (Romano *et al.*, 2007).

Human parenteral toxicity for abrin is approximately 0.1–1 μg/kg (Romano *et al.*, 2007). However, based on clinical trials on abrin-immunotoxin use for cancer treatment, the human minimum lethal dose by intravenous injection was estimated to be >0.3 μg/kg without occurrence of serious adverse effects (Gill, 1982).

3. Inhalation Exposure

In humans, inhalation is another one of the most lethal forms of ricin and abrin exposure. In clinical reports, inhalation of ricin typically leads to cough and respiratory distress followed by pulmonary edema, respiratory failure, and multisystem organ dysfunction. Weakness and influenza-like symptoms of fever, myalgia, and arthralgia were also reported (Ellenhorn and Barceloux, 1997; Kortepeter and Parker, 1999; Eitzen *et al.*, 2001). Initial symptoms may occur as early as 4–6 h after exposure, but serious symptoms could also occur as late as 24 h after exposure. The initial symptoms include difficulty breathing, shortness of breath, chest tightness, and cough. The symptoms of ricin poisoning are likely to progress rapidly (generally over 12–24 h) and include problems such as worsening respiratory symptoms and pulmonary edema, and eventually respiratory failure. This rapid progression of symptoms and illness is noticeably different than what typically occurs with most common colds and cough-type illnesses (Ellenhorn and Barceloux, 1997; Kortepeter and Parker, 1999; Eitzen *et al.*, 2001).

A case reported in the 1940s accounted for the unintentional aerosol exposure of ricin in humans. The symptoms were characterized by the onset of the following within 4–8 h: fever, chest tightness, cough, difficulty breathing, nausea and arthralgias, followed by diaphoresis. In another report, workers exposed to castor bean dust from castor oil processing plants developed an allergic set of symptoms which was characterized by nasal and throat congestion, eye irritation, hives and skin irritation, chest tightness, and, in some severe cases, wheezing. Another recent incident occurred in February 2008, in which inhaled ricin resulted in a 57-year-old man becoming comatose for one month. In Las Vegas, Nevada, a large quantity of ricin contained in vials was found in the victim’s hotel room, along with castor beans,

''anarchists' cookbooks'' marked at sections detailing how to make ricin, and firearms. The victim summoned an ambulance, complaining of respiratory distress, where he later developed kidney failure and slipped into a coma for a month. The ricin-exposed man's cousin was later indicted alleging he tried to conceal the production and possession of ricin when attempting to collect the evidence during the investigation (Ellenhorn and Barceloux, 1997; Kortepeter and Parker, 1999; Eitzen *et al.*, 2001; CNN, 2008).

Symptoms of abrin inhalation are similar to ricin exposure. Initial symptoms of abrin poisoning by inhalation may occur within 8 h of exposure. Symptoms include respiratory distress, fever, cough, nausea, and tightness in the chest. Heavy sweating may follow as well as pulmonary edema. Finally, low blood pressure and respiratory failure may occur, leading to death (Ellenhorn and Barceloux, 1997; Kortepeter and Parker, 1999; Eitzen *et al.*, 2001).

4. Dermal and Ocular Exposure

Allergic reaction may occur after handling an intact castor bean plant or exposure to the castor bean dust (Metz *et al.*, 2001; Thorpe *et al.*, 1988). There are no reports of human toxicity by skin contact with abrin. Abrin in powder or mist form can cause redness and pain of the skin and the eyes.

C. Oxidative Stress and DNA Damage by Ricin and Abrin

Preliminary studies indicate that ricin induces an oxidative stress in the livers of mice (Muldoon and Stohs, 1991). Few studies have examined other biochemical alterations that may be associated with ricin toxicity. Oxidative stress, induced by free radicals and reactive oxygen species, has been widely implicated as a mechanism in the toxicity of ricin (Muldoon and Stohs, 1991; Omar *et al.*, 1990).

It is well known that lipid peroxidation, DNA single-strand breaks, and other forms of DNA damage occur in response to oxidative stress (Ames *et al.*, 1982). Depletion of reduced glutathione also commonly precedes or accompanies lipid peroxidation and oxidative stress (Muldoon and Stohs, 1991; Omar *et al.*, 1990). In an *in vivo* study, the effects of ricin administered orally on hepatic lipid peroxidation, nonprotein sulfhydryl content, and DNA single-strand breaks were assessed in mice (Muldoon *et al.*, 1992). The incidence of hepatic DNA damage increased 2.9-, 2.8-, and 2.4-fold relative to control values at 24, 36, and 48 h post-treatment with ricin, respectively. Hepatic nonprotein sulfhydryl concentration decreased significantly from 51 to 65% to control values at 24, 36, and 48 h post-treatment (Figures 25.2 and 25.3).

Earlier studies have also shown that ricin induces oxidative stress in mice, resulting in increased urinary excretion of MDA and formaldehyde (FA) (Muldoon *et al.*, 1994). Other toxicants have been shown to induce oxidative stress by macrophage activation with subsequent release of reactive oxygen species and tumor necrosis factor alpha (TNF-α).

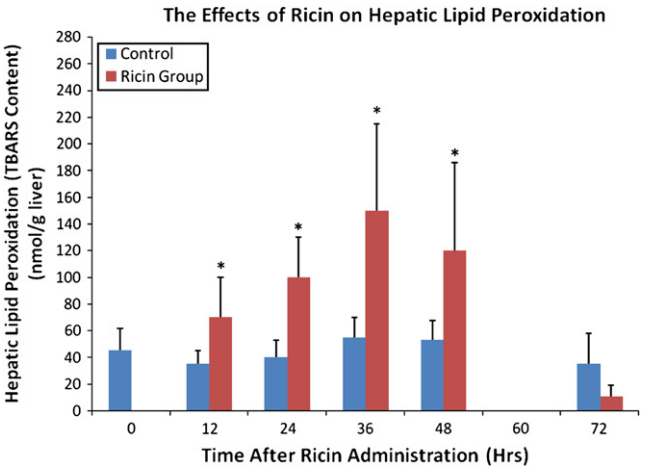

FIGURE 25.2. The effects of ricin on hepatic lipid peroxidation. Concentration of thiobarituric acid reactive substances (TBARS) in the liver was compared between control (untreated) mice and mice treated with 25 μg ricin/kg. Values are the averages of 4–10 animals ± SD. *$p < 0.05$ with respect to corresponding control group.

Therefore, the ability of TNF-α antibody to modulate ricin-induced urinary carbonyl excretion as well as hepatic lipid peroxidation, glutathione depletion, and DNA single-strand breaks was assessed (Muldoon *et al.*, 1994). Ricin-induced urinary MDA and FA were reduced significantly in mice receiving antibody (15,000 U/kg) 2 h before treatment with ricin (5 μg/kg). At 48 h following ricin treatment, MDA and FA concentrations in the urine of TNF antibody-treated

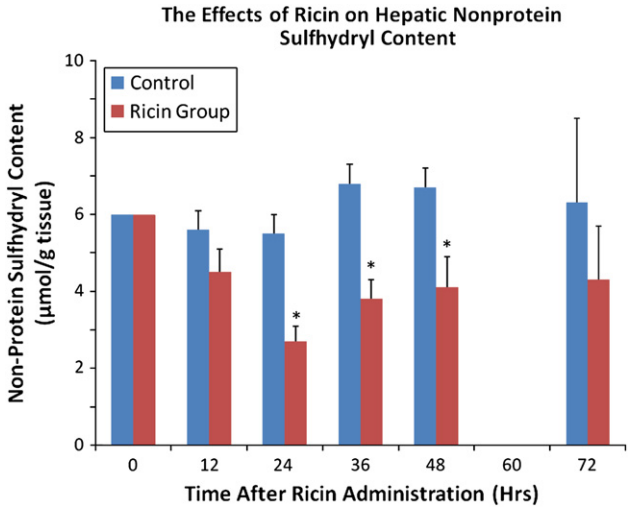

FIGURE 25.3. The effects of ricin on hepatic nonsulfhydryl content. Concentration of nonsulfhydryl content in the liver was compared between control (untreated) mice and mice treated with 25 μg ricin/kg. Protein was removed by precipitation with 2% TCA final concentration. Values are the averages of 3–7 animals ± SD. *$p < 0.05$ with respect to corresponding control group.

mice decreased 25.7 and 53.2%, respectively, relative to ricin-treated mice receiving no antibody (Figures 25.4 and 25.5). In addition, anti-TNF-α (1,500 U/kg) significantly decreased hepatic lipid peroxidation and DNA single-strand breaks, induced by 5 μg ricin/kg, by 49.3 and 44.2%, respectively (Table 25.1). The results suggest that macrophage activation and subsequent release of TNF-α are involved in ricin toxicity.

D. *In Vitro* Studies and Chemoprotectants

The abilities of potential chemoprotectants to inhibit cytotoxicity of ricin have been determined *in vitro*, using the macrophage cell line J744A.1. Six compounds were tested: α- and β-galactopyranosylamine; *N*-bromoacetyl-α-D-galactopyranosylamine; *N*-bromoacetyl-β-D-galactopyranosylamine; *N*-bromoacetylglucopyranosylamine; and *N*-bromoacetylmannopyranosylamine (Figure 25.6) (Hassoun *et al.*, 1992, 1996). Of the six compounds tested, only *N*-bromoacetyl-α-D-galactopyranosylamine and *N*-bromoacetyl-β-D-galactopyranosylamine exhibited significant activity against ricin toxicity, as indicated by the release of lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) (Figures 25.7a and b and 25.8a and b). Enzyme assays were performed within 1 week of media collection. Assays for determining activities of LDH and AST were performed according to the method of Moss *et al.* (1986). The α-isomer provided greater protection against ricin toxicity and also exhibited less inherent cytotoxicity in the absence of ricin, as compared to the β-isomer. Neither the α- and β-galactopyranosylamines nor the glucose and mannose analogs were promising as potential chemoprotectants (Hassoun *et al.*, 1992, 1996).

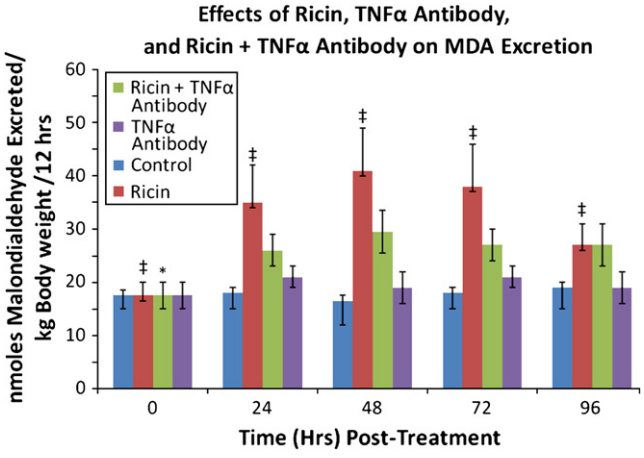

FIGURE 25.4. The effects of ricin (5 μg/kg), TNF-α antibody (15,000 U/kg), and ricin plus TNF-α antibody on malondialdehyde (MDA) excretion. Animals treated with TNF-α antibody plus ricin received the antibody 2 h before ricin. Appropriate vehicle controls were substituted in each of the other regimens. Each value is the mean ± SD of four mice in each group. Values with nonidentical superscripts at each time point are significantly different ($p < 0.05$).

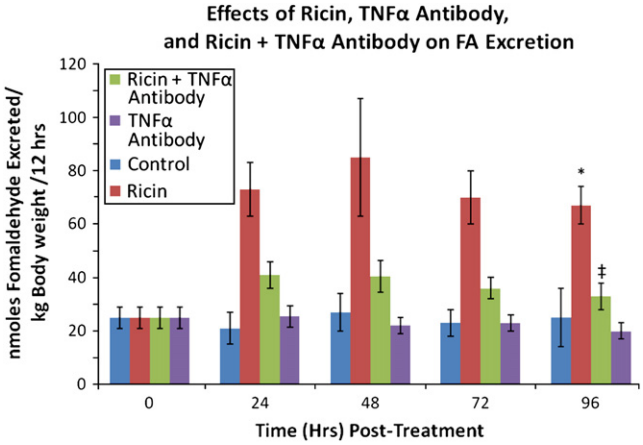

FIGURE 25.5. The effects of ricin (5 μg/kg), TNF-α antibody (15,000 U/kg), and ricin plus TNF-α antibody on formaldehyde (FA) excretion. Animals treated with TNF-α antibody plus ricin received the antibody 2 h before ricin. Appropriate vehicle controls were substituted in each of the other regimens. Each value is the mean ± SD of four mice in each group. Values with nonidentical superscripts at each time point are significantly different ($p < 0.05$).

Previous investigations have suggested that deoxynucleoside analogs may be effective in decreasing the toxicity of ricin (Hassoun *et al.*, 1996). The effects of 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxycytidine (DDC) in decreasing the cytotoxicity of ricin in J774A.1 macrophage and Chinese hamster ovary (CHO) cells in culture by assessing the release of lactate dehydrogenase (LDH) and asparate aminotransferase (AST) from the cells, as well as decreased cell viability, were examined. Results demonstrated that DDC provided partial protection against ricin toxicity in both cell culture systems based on the leakage of LDH and AST. Concentration-dependent effects between 10^{-10} and 10^{-4} g/ml were produced. Under similar conditions, AZT had no effect on the toxicity of ricin in these two cell culture systems. When assessing cell viability, DDC almost doubled the viability of both CHO and J774A.1 cells at a concentration of 10^{-4} g/ml in the presence of ricin. These results demonstrated that DDC but not AZT exhibits a chemoprotective effect against ricin toxicity in the two cell culture systems that were tested (Hassoun *et al.*, 1992, 1996).

VI. RISK ASSESSMENT

Upon exposure to ricin or abrin, the clinical signs, symptoms, and pathological manifestations in intoxicated victims depend on the route of exposure. When inhaled as a small particle aerosol, ricin produces symptoms within 8 h. Respiratory distress, fever, cough, dyspnea, nausea, and chest tightness are followed by profuse sweating, the development of pulmonary edema, cyanosis, hypotension, and finally respiratory failure and circulatory collapse. Ingestion of ricin causes gastrointestinal signs and intestinal

TABLE 25.1. Effects of TNF-α antibody (TNF-α Ab) on ricin-induced lipid peroxidation, NPSH content, and DNA-SSB

Treatment	Lipid peroxidation (nmol MDA/g liver)	DNA-SSB [elution constants ($\times 10^{-3}$)]
Control	64.3 ± 11.9^a	6.0 ± 0.8^a
Ricin (5 μg/kg)	115.9 ± 22.2^b	15.0 ± 2.6^b
Ricin (25 μg/kg)	162.0 ± 4.9^c	19.7 ± 0.9^c
TNF-α Ab (1,500 U/kg)	102.3 ± 41.1^b	6.3 ± 1.2^a
TNF-α Ab (15,000 U/kg)	69.5 ± 8.8^a	5.8 ± 0.9^a
Ricin (5 μg/kg) + TNF-α Ab (1500 U/kg)	60.2 ± 12.8^a	7.8 ± 0.9^c

Each value is the mean ± SD of at least four animals 24 h after treatment. Values with nonidentical superscripts are significantly different from one another ($p < 0.05$)

hemorrhage with necrosis of liver, spleen, and kidneys. Intramuscular intoxication causes severe localized pain, muscle and regional lymph node necrosis, and moderate involvement of visceral organs. Transient leukocytosis appears to be a constant feature in humans, whether ricin intoxication is via injection or oral ingestion. Children are more sensitive than adults to fluid loss, due to vomiting and diarrhea, and can quickly become severely dehydrated and die. If death has not occurred in 3–5 days, the victim usually recovers (CDC, 2008).

The major symptoms of abrin poisoning also depend on the route of exposure and the dose received, though many organs may be affected in severe cases. Initial symptoms of abrin poisoning by inhalation may occur within 8 h of exposure. Following ingestion of abrin, initial symptoms may occur in less than 6 h but usually are delayed for 1–3 days. Within a few h of inhaling significant amounts of abrin, symptoms would be respiratory distress, fever, cough, nausea, and tightness in the chest. Heavy sweating may follow as well as pulmonary edema. Excess fluid in the

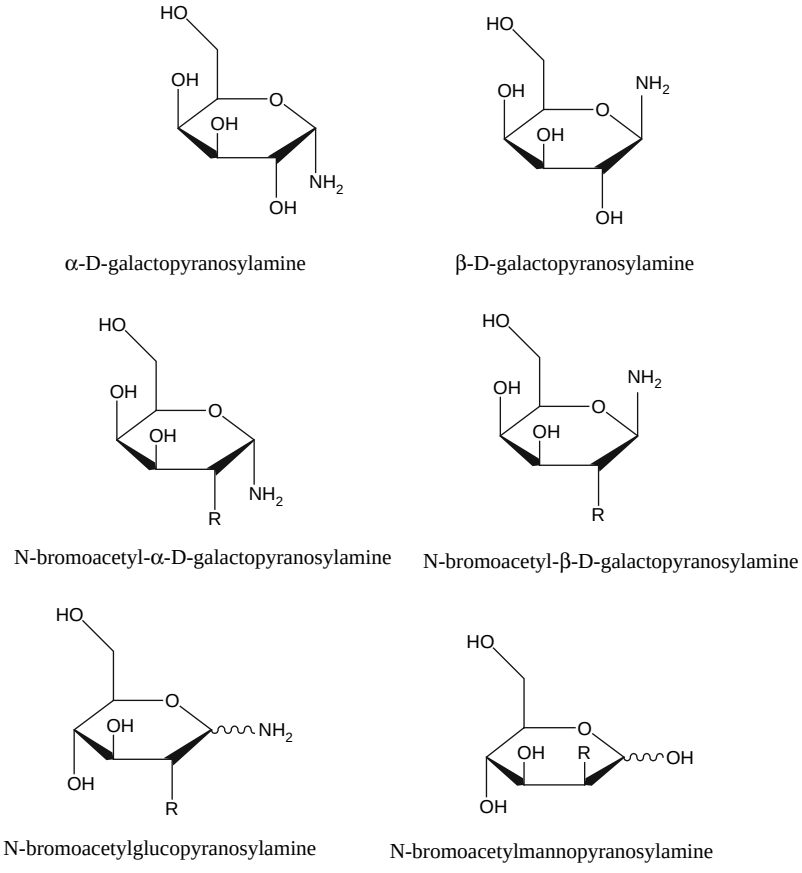

FIGURE 25.6. Chemoprotectants for ricin.

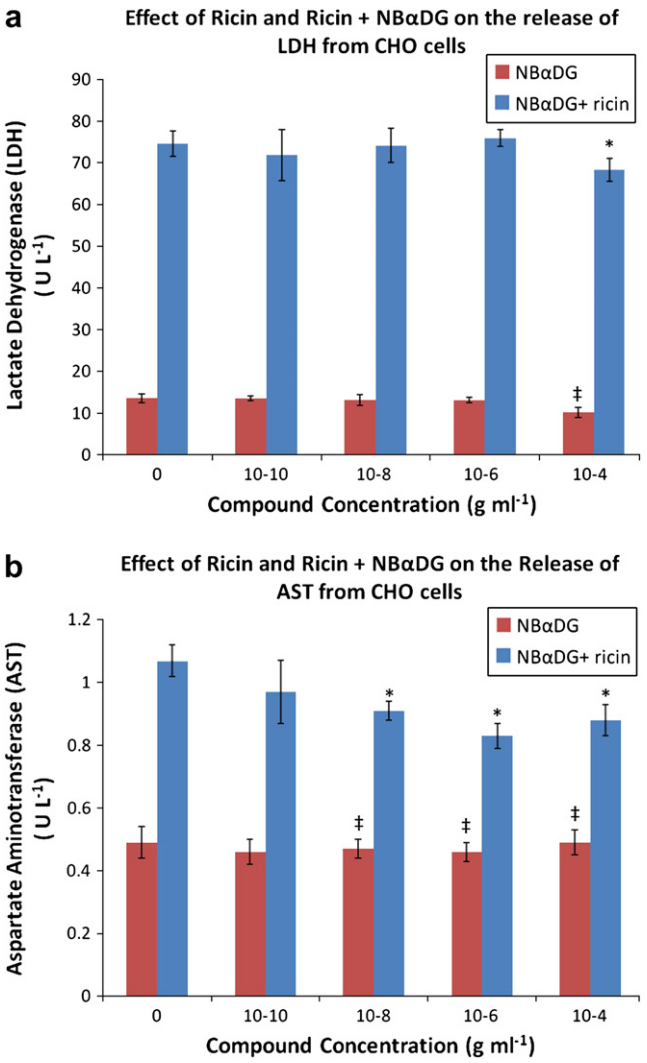

FIGURE 25.7. (a) Ricin (10^{-7} g/ml); various concentrations of the potential chemoprotectant *N*-bromoacetyl-α-D-galactopyranosylamine (NBαDG) and ricin added simultaneously with NBαDG were added to different cultures. The LDH activities were determined in culture media after a 48 h incubation period. Values are the mean ± SD from at least four cultures. Values with nonidentical superscripts are significantly different ($p > 0.05$). (b) Ricin (10^{-7} g/ml); various concentrations of the potential chemoprotectant *N*-bromoacetyl-α-D-galactopyranosylamine (NBαDG) and ricin added simultaneously with NBαDG were added to different cultures. The AST activities were determined in culture media after a 48 h incubation period. Values are the mean ± SD from at least four cultures. Values with nonidentical superscripts are significantly different ($p > 0.05$).

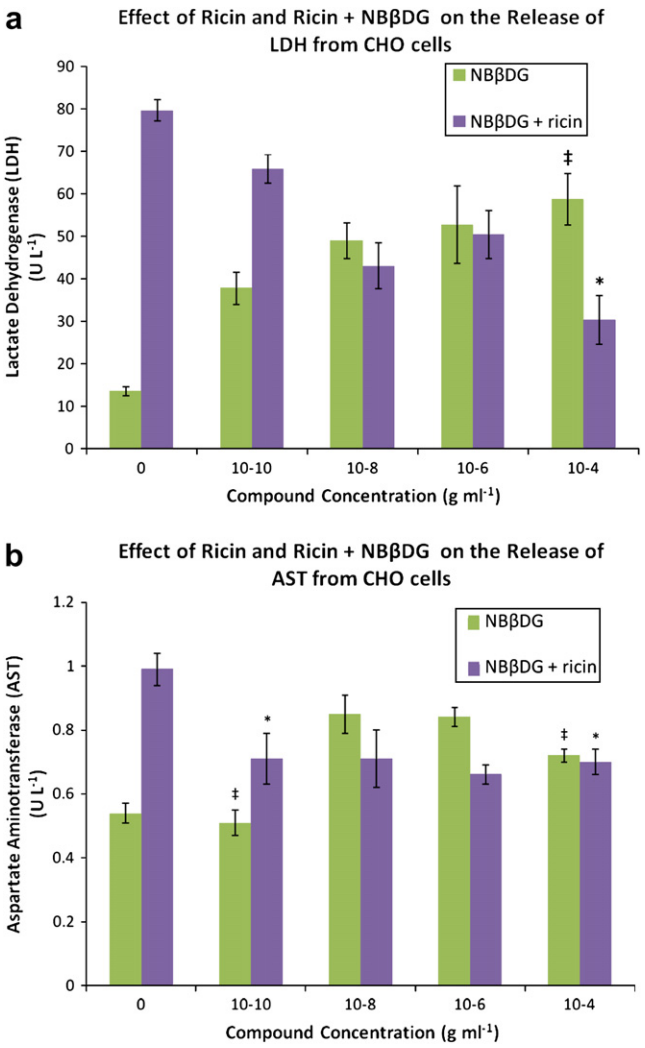

FIGURE 25.8. (a) Ricin (10^{-7} g/ml); various concentrations of the potential chemoprotectant *N*-bromoacetyl-β-D-galactopyranosylamine (NBβDG) and ricin added simultaneously with NBβDG were added to different cultures. The LDH activities were determined in culture media after a 48 h incubation period. Values are the mean ± SD from at least four cultures. Values with nonidentical superscripts are significantly different ($p > 0.05$). (b) Ricin (10^{-7} g/ml), various concentrations of the potential chemoprotectant *N*-bromoacetyl-β-D-galactopyranosylamine (NBβDG) and ricin added simultaneously with NBβDG were added to different cultures. The AST activities were determined in culture media after a 48 h incubation period. Values are the mean ± SD from at least four cultures. Values with nonidentical superscripts are significantly different ($p > 0.05$).

lungs would be diagnosed by x-ray or by listening to the chest with a stethoscope (CDC, 2008). Symptoms of abrin ingestion include bloody vomiting and diarrhea. Severe dehydration may also result, followed by low blood pressure. Other signs or symptoms may include hallucinations, seizures, and blood in the urine. Within several days, impairment of the liver, spleen, and kidneys may occur resulting in death. Symptoms of skin and eye exposure to abrin include redness and pain (CDC, 2008). Symptoms of exposure to inhaled abrin include suddenly developed fever, cough, and excess fluid in the lungs followed by severe breathing problems.

Differential diagnosis of ricin exposure shows early inhalational ricin poisoning and will have primarily respiratory signs and symptoms, whereas ingested ricin will probably present early with gastrointestinal symptoms.

TABLE 25.2. Epidemiological clues for determining the release of concealed ricin, abrin, or any chemical agent or a biological toxin

- an unusual increase in the number of patients seeking a toxin-related illness
- unexplained illness or death among relatively healthy or young people
- detection of unexplained odors on presenting patients
- similar symptoms in groups of people who have common characteristics (i.e. common workplace, common source of drinking water)
- rapid onset of symptoms
- unexplained death of plants, fish or animals
- presence of a particular syndrome known to be associated with a chemical agent or biological toxin

Referenced from: US Public Health Service, Health Studies Branch, National Center for Environmental Health Centers for Disease Control and Prevention. January 27, 2004

Differential diagnoses of inhaled ricin poisoning show staphylococcal enterotoxin B, exposure to pyrolysis byproducts of organofluorines such as Teflon or Kevlar, nitrogen oxides, phosgene, influenza, anthrax, Q-fever, and pneumonic plague (Schier, 2004). Differential diagnosis of ricin poisoning by ingestion shows enteric pathogens such as salmonella or shigella, mushrooms, caustics, iron or other metals, arsenic, and colchicines (Schier, 2004).

Several methods of detection of exposure to ricin include use of enzyme-linked immunosorbent assay (ELISA) testing of blood or other body fluids or immunohistochemical techniques may be useful in confirming ricin intoxication (Poli *et al.*, 1994). No widely available, reliable test exists to detect and confirm exposure to abrin.

In determining the concealed release of ricin, abrin, or any chemical agent or biological toxin, certain epidemiologic clues should be evident (Schier, 2004) (Table 25.2). If clinical findings of ricin- or abrin-associated poisoning are evident, the proper public health authorities including the regional poison control center and local and state health departments should be contacted immediately. The Centers for Disease Control and Prevention and public health laboratories in the Laboratory Response Network are able to detect ricin in environmental samples. Environmental testing may document the potential or affirm the nature of the exposure (Schier, 2004). Unfortunately, effective countermeasures and real-time detection of ricin and abrin are not yet viable options.

VII. TREATMENT

There is no specific treatment for ricin or abrin. Therefore the only recommendation is to rid the body of the toxins as quickly as possible. Ricin and abrin poisoning is treated by giving victims the appropriate supportive medical care to minimize the effects of the poisoning. Cases of gastrointestinal exposure are best managed by vigorous gastric decontamination with activated charcoal, followed by use of cathartics such as magnesium citrate (CDC, 2008). Replacement of gastrointestinal fluid losses is crucial. In cases of aerosol or inhalant exposure, patients are managed for intensive respiratory therapy, fluid and electrolyte replacement, anti-inflammatory agents, and analgesics (CDC, 2008). Other types of supportive medical care would include providing medications to treat conditions such as seizure and low blood pressure, flushing the stomach with activated charcoal, or washing eyes and skin if exposed ocularly or dermally (CDC, 2008).

Gross decontamination of the patient should also follow in the event of a recognized release or exposure. This would include removal of all suspected contaminated clothing, including jewelry and watches, and washing off any obvious contamination with soap and copious amounts of water. Contaminated clothing removed from the victim should be double bagged and labeled as contaminated and then secured in a safe location until it can be safely disposed of. Environmental surfaces or medical equipment can be cleaned with soap and water or a 0.1% sodium hypochlorite solution (CDC, 2008).

Candidate vaccines under development are immunogenic and confer protection against lethal aerosol exposures. Recent animal studies have shown that either active immunization or passive prophylaxis may be effective against intravenous or intraperitoneal intoxication with ricin (Poli *et al.*, 1994). In the case of inhalational exposure, active immunization or prophylactic administration of aerosolized specific anti-ricin antibody may also be effective (Poli *et al.*, 1994). Unfortunately, these applications may not be clinically available since they are still under investigation.

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

Ricin and abrin have played important roles in the history of clinical medicine and biomedical research. Ricin is commonly used as a part of immunotoxins for clinical tumor research and application, although the detailed mechanism of its function is still under investigation (Stirpe and Battelli, 2006; Rao *et al.*, 2005). At present, a major effort in biomedical research is under way to target the toxins and pursue malignant cells. Novel *in vitro* approaches of using ricin toxins in cancer therapy have proven successful; however, further *in vitro* and animal studies are needed to confirm its potential (Wang *et al.*, 2007).

Interestingly, the hope for a vaccination in the treatment against abrin toxicity may be not that far away. In a recent *in vitro* and *in vivo* study, antibodies specific to the recombinant abrin A-chain were shown to rescue cells from toxicity. Importantly, the antibody also protected mice from lethal doses of the toxin. The neutralizing effect of the antibody

was shown to be due to interference with abrin attachment to the cell surface (Surendranath and Karande, 2008). This study along with our understanding of ricin and abrin may bring us closer to the development of a long awaited protective vaccine.

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CHAPTER 26

Trichothecene Mycotoxins

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I. INTRODUCTION

The ideal biologic warfare agent is lethal, easy, and inexpensive to produce in large quantities, stable as an aerosol for dispersion over wide areas, without effective treatment or vaccination, and communicable between people. The trichothecene mycotoxins possess all these properties with the exception of the last. The potent acute toxicity and chemical stability of T-2 toxin, diacetoxyscirpenol (DAS) and, possibly, DON make them candidates for bioterrorism (Stark, 2005). Mycotoxins may already have been used for weapons based on toxicity syndromes, typical for exposure to trichothecene mycotoxins, associated with ''yellow rain'' incidents in southeast Asia and Afghanistan (Heyndrickx *et al.*, 1984; Mirocha *et al.*, 1983; Spyker and Spyker, 1983; Tucker, 2001; Watson *et al.*, 1984). The Soviet Union was alleged to have provided mycotoxins to the armies of Vietnam and Laos for use against resistance forces in Laos and Cambodia and to have used mycotoxins in combat operations in Afghanistan (Haig, 1982; Tucker, 2001). Confirmation of this use of mycotoxins has been difficult and controversial. T-2 toxin, nivalenol, and DON were identified in vegetation at affected sites and both T-2 toxin and HT-2 toxin, a metabolite of T-2 toxin, were found in urine and blood samples from victims of alleged attacks (Mirocha, 1983; Heyndrickx *et al.*, 1984). T-2 toxin was also reportedly found in high concentrations in spots found on rocks and gas masks. While mycotoxins are considered impractical as tactical weapons, crude preparations could be readily produced and used by terrorist organizations to contaminate food or water supplies, or be released as an aerosol in small crowded areas (Stark, 2005).

The government of the USA has created several overlapping lists of potential bioterrorism agents and diseases that are considered a possible threat to the health and safety of the public, animals, and plants. These agents are federally restricted through regulation of their possession, use, and transfer. T-2 toxin is on the current (2007 revision) Overlapping (Human and Livestock) Select Agents and Toxins list for the Center for Disease Control, Department of Human Health Services (CDC, DHHS) and the Animal and Plant Health Inspection Service, United States Department of Agriculture (APHIS, USDA). T-2 toxin is toxic on ingestion with enhanced toxicity by the inhalation route; it is toxic to the skin and can be absorbed across intact skin. Diacetoxyscirpenol (DAS), which is similar to T-2 toxin in mechanism of action and potency, is only on the HHS Select Agents and Toxins list; less information is available about this toxin. DON is on neither list but has been mentioned as a possible bioweapon because its acute toxicity is similar to T-2 toxin. However, it is much less toxic than T-2 toxin (mouse LD_{50} for T-2 toxin is 0.25 mg/kg as compared to 78 mg/kg for DON; Stark, 2005). This chapter will review information on these three trichothecenes with most attention given to T-2 toxin. The review will focus on swine research, since the pig is an excellent model for man, and other information pertinent to human exposure.

II. BACKGROUND

Trichothecene mycotoxins are a family of tetracyclic sesquiterpenoid substances (12,13 epoxytrichothecenes) comprising over 200 compounds of widely varying toxicity. The epoxy group at C-12 and C-13 is considered essential for toxicity. Trichothecenes are broadly divided into two groups, the macrocyclic and nonmacrocyclic, based on the presence or absence of a macrocyclic ring linking C-4 and C-15 with diesters (roridin series) and triesters (verrucarin series). The nonmacrocyclic trichothecenes, such as T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS, anguidine), deoxynivalenol (DON, vomitoxin), and nivalenol, are produced primarily by field fungi of the genus *Fusarium*. The macrocyclic trichothecenes, such as roridins, verrucarins, and satratoxins, are produced primarily by *Stachybotrys* and *Myrothecium* spp. Other genera that have been reported to produce trichothecenes include *Trichoderma*, *Trichothecium*, *Cephalosporium*, *Cylindrocarpon, Verticimonosporium*, and *Phomopsis* (Scott, 1989).

Trichothecenes occur worldwide in grains and other commodities grown in cooler climates. Colonization and toxin production by *Fusarium* spp. occur in the field; however, some toxin production can also occur in storage. Mild temperatures tend to encourage fungal growth and cool temperatures increase toxin production (0–15°C). Trichothecenes tend to be produced in toxic concentrations in years of wet weather when harvests are delayed and prolonged.

The primary fungal species producing the trichothecene mycotoxins are listed in Table 26.1. T-2 toxin is produced primarily by *Fusarium sporotrichioides*, with highest concentrations generally occurring in small grains such as barley and wheat. T-2 toxin may occasionally occur in corn, and forages are sometimes contaminated. Diacetoxyscirpenol is produced by *Fusarium roseum* (*Gibberella zeae*, *F. graminearum*) and several other species of *Fusarium*. Deoxynivalenol (DON, vomitoxin) is also produced primarily by *F. roseum*, with corn, oats, barley, and wheat, as well as other small grains being important sources of exposure to livestock. Macrocyclic trichothecene mycotoxins can be produced by fungi such as *Stachybotrys chartarum* (previously *S. atra*), a black fungus that grows in wet forages and/or straw as well as in water-damaged building materials and wet wood in air ducts.

Trichothecenes are potent inhibitors of protein synthesis due to binding to the 60S ribosomal unit and this is believed to be the main mechanism of toxicity. However, recent experimental data suggest that activation of the mitogen-activated protein kinases (MAPKs) through the ribosomal stress response could be another mechanism that operates via apoptotic and proinflammatory processes.

Trichothecenes can cause problems in livestock and humans ingesting contaminated grains or hay. In the USA and Canada, deoxynivalenol appears to be the most significant member of the group despite its comparatively low toxicity. This is based on agricultural losses in exports and swine production. However, both T-2 toxin and DAS occasionally cause outbreaks of toxicoses in animals in North America as well as Japan. T-2 toxin has been implicated in widespread epidemics of alimentary toxic aleukia (ATA) in humans in the Soviet Union that occurred in the 1930s and 1940s.

Trichothecenes cause apoptosis and/or necrosis in the lymphoid, hematopoietic, and gastrointestinal systems resulting in leukopenia, vomiting, and diarrhea that can be lethal. In addition, trichothecenes are toxic to the skin and testes. Immune suppression and increased susceptibility to infection may occur, especially in the late phase of the disease. The toxic effects from trichothecenes largely resemble those following radiation exposure (radiomimetic) due to effects on rapidly dividing cells in the intestine, bone marrow, and testis.

Lethality due to ingestion of food contaminated by trichothecenes has been reported in horses (Rodricks and Eppley, 1974), cattle (Hsu *et al.*, 1972), and humans (Joffe, 1974). General clinical signs include emesis, food refusal and weight loss, dermal effects, and immune suppression with secondary infection. Clinical signs are dependent on the specific trichothecene involved, the dose, species, route of exposure, as well as the nature of the exposure. Spontaneous and experimental exposures may give somewhat different results, as can exposure to field contaminated materials, when compared to purified toxin. In the case of field contamination or experimental use of crude extracts, multiple mycotoxins, both known and unknown, may be present at the same time.

TABLE 26.1. Partial listing of trichothecene toxins and their comparative toxicity

			Acute LD_{50} values (mg/kg)[a]				Lethal dose (mg/kg)
Group	**Main producer**	**Trichothecene**	**Mouse i.v. or i.p.**	**Mouse oral/p.o.**	**Mouse inhalation[b]**	**Pig i.v.**	**Pig inhalation[c]**
A	*Fusarium* spp.	T-2 toxin	3.0–5.3	3.8–10.5	0.16	1.21	1.5–3.0
		HT-2 toxin	6.5–9.0				
		Diacetoxyscirpenol (DAS)	9.6–23.0	15.5–46.0		0.37	
		Monoacetoxyscirpenol					
B	*Fusarium* spp.	Deoxynivalenol (DON, vomitoxin)	70.0–76.7	46.0			
		Nivalenol	4.0–6.3				
		Fusarenon X	3.4	4.5			
C	*Cephalosporium* spp.	Crotocin	700–810	1,000			
D	*Myrothecium* spp.	Verrucarins					
		Verrucarin A and B	0.5 (A)–7.0 (B)				
		Roridins					
		Roridin A	1.0 (A)				
	Stachybotrys spp.	Satratoxins					

[a]Information largely from Trenholm *et al.* (1989), Ueno (1985)
[b]24 h LD_{50}, in saline solution, Cresia and Lambert (1989)
[c]Estimated retained dose (20–30%) using dry aerosol of T-2 toxin, all animals died within 18 h, Cresia and Lambert (1989)

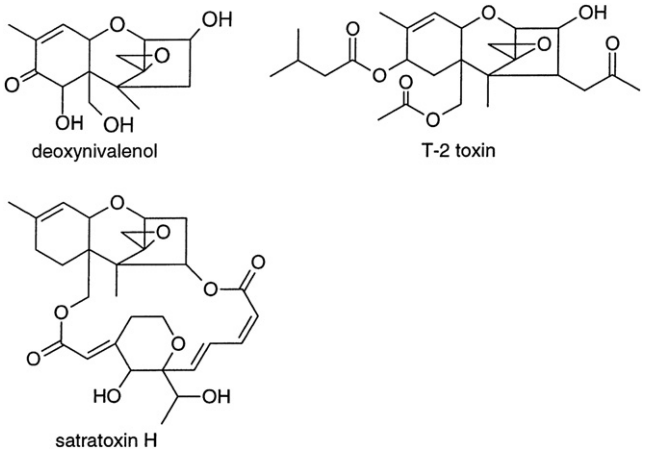

FIGURE 26.1. Chemical structure of trichothecene mycotoxins from group A, T-2 toxin; group B, deoxynivalenol (DON); and group D (satratoxin H). From Haschek *et al.* (2002).

All trichothecene mycotoxins have a basic tetracyclic sesquiterpene structure with a six membered oxygen-containing ring, an epoxide group in the 12,13 position, and an olefinic bond in the 9,10 position. Trichothecenes have been classified into four groups based on substitutions at five positions of the trichothecene skeleton (Figure 26.1). Group A trichothecenes possess hydroxyl or esterified hydroxyls at the C-3, 4, 7, 8, or 15 position and include T-2 toxin, HT-2 toxin, DAS, and monoacetoxyscirpenol. *Fusarium sporotrichoides* and *F. poae* cultures are used most commonly to produce these toxins. Group B trichothecenes contain a keto group at C-8 and a hydroxyl group at C-7, in addition to the other functional groups in group A, and include DON and nivalenol. These trichothecenes are less toxic than those classes without the C-8 keto substitution. Group C trichothecenes typically have a second epoxide ring at C-7, 8, are not produced by *Fusarium* spp., and include crotocin. Group D trichothecenes have a macrocyclic ring linking C-4 and C-15 and include macrocyclic trichothecenes, such as verrucarins and satratoxins, that are produced by *Myrothecium* and *Strachybotrys* spp., respectively. While fungi frequently produce several toxins, most species tend to produce group A, B, or D toxins. However, several different types of toxins may be produced by a single fungal species. For example, *F. roseum*, a primary producer of zearalenone, can synthesize both group A and B toxins.

III. TOXICOKINETICS

Trichothecenes can be absorbed through the gastrointestinal tract, the lungs, and the skin (Madsen, 2001). Most of the information on toxicokinetics is based on i.v. administration because of the powerful emetic effects of these toxins. Limited information is available on oral and inhalation routes of exposure. Inhalation would be the most likely route of exposure for weaponized mycotoxins since this approach would result in many people being affected in a short time. For example, in mice, lethality was present within 5 h when T-toxin was given by inhalation for 10 min (Cresia *et al.*, 1987). Oral or parenteral administration of trichothecenes does not result in their accumulation in the body to any great extent and any residues are rapidly excreted over several days of exposure. In contrast, topical application of T-2 toxin results in delayed absorption and sustained metabolism and excretion due to the skin and subcutaneous fat acting as a reservoir in swine (Pang *et al.*, 1987d).

The trichothecenes undergo all four basic reactions in xenobiotic metabolism, i.e. hydrolysis, oxidation, and reduction in phase I metabolism, and glucuronide conjugation in phase II metabolism. These reactions occur in tissues except for reduction of the 12,13-epoxide which occurs through microbial action in the gastrointestinal tract (Swanson *et al.*, 1988; Swanson and Corley, 1989). Reduced toxicity generally follows T-2 metabolism with deacetylation of T-2 toxin to HT-2 (at C-4), to 4-deacetylneosolaniol, and to T-2 tetraol. T-2 toxin is considered 1.5 to 1.7 times as toxic as HT-2, which is 4.8 times as toxic as T-2 tetraol (Ueno *et al.*, 1973). Both human and bovine liver homogenates can deacetylate T-2 toxin *in vitro* to HT-2 toxin (Ellison and Kotsonis, 1974). Rapid excretion occurs via the biliary system (feces) and urine without significant accumulation of orally administered trichothecenes in the body. Enterohepatic recirculation may occur. Reviews of trichothecene toxicokinetics are provided by Swanson and Corley (1989), and Mostrom and Raisbeck (2007) for agricultural species.

In swine, following intra-aortic administration, the disappearance of parent T-2 toxin followed a two compartment open model with mean elimination phase half-life of 13.8 min and mean apparent specific volume of distribution of 0.366 l/kg (Beasley *et al.*, 1986). Parent T-2 toxin was not detected in plasma, urine, or liver but was transiently present in lymphoid tissues. T-2 toxin and metabolites were eliminated as glucuronide conjugates into bile, undergoing deconjugation in the intestinal tract by microbial action and then underwent enterohepatic recirculation (Corley *et al.*, 1985).

Diacetoxyscirpenol (DAS) is rapidly and extensively metabolized in rats, pigs, and cattle. Pharmacokinetic studies of DAS given i.v. to swine 48 h after anesthesia, indicated a large volume of distribution of 1.58 ml/kg, high total body clearance of 119.4 ml/min/kg, and elimination phase half-life of 10.2 min (Coppock *et al.*, 1987). Less than 1% of parent compound was detected in urine. The major metabolites were monoacetoxyscirpenol and scirpenetriol.

Because deoxynivalenol (DON) commonly contaminates grains and swine are the most susceptible species, toxicokinetics for DON have been extensively studied in this species (Coppock *et al.*, 1985b; Prelusky *et al.*, 1988, 1990; Prelusky and Trenholm, 1991; Goyarts and Danicke, 2006). Intravenously administered DON (1 mg/kg body weight) distributed rapidly to all tissues and body fluids, and declined to negligible levels within 24 h except in urine and bile

(Prelusky and Trenholm, 1991). The half-life of DON in swine was between 2.08 and 3.65 h after i.v. injection of 0.5 mg DON/kg body weight. This suggested that 97% of the DON would be eliminated in 10.1 to 18.3 h (Coppock *et al.*, 1985b). DON concentrations decreased biphasically with terminal elimination half-lives ($t_{1/2}$ β) of between 4.2 and 33.6 h (Goyarts and Danicke, 2006). Orally administered DON is rapidly and nearly completely absorbed from the stomach and proximal small intestine (Danicke *et al.*, 2004). Pigs given DON in one oral dose or chronically dosed (via a diet containing DON at 5.7 mg/kg diet for 4 weeks) rapidly absorbed >50% of administered DON. The toxin rapidly left the blood (apparent volume of distribution exceeded the total body water), with serum elimination half-lives of 5.3 and 6.3 h, respectively, and total elimination time (97%, five elimination half-lives) of 26.5 and 31.5 h, respectively. The majority of the DON was eliminated via the urine and feces with urine excretion of unmetabolized DON accounting for most of the compound.

IV. MECHANISM OF ACTION

Trichothecenes inhibit synthesis of protein, RNA and DNA as well as mitochondrial and electron transport chain function; stimulate lipid peroxidation; alter cell membrane function; induce apoptosis; modulate immune responses; activate mitogen-activated protein kinases (MAPKs) and induce gene expression of numerous chemokines and cytokines; and alter neurotransmitter levels.

Although trichothecenes vary to some extent in their chemical structure and the biological effects that they produce, all trichothecenes target the 60S ribosomal subunit suggesting that the major mechanism of toxicity is translational inhibition. Inhibition of protein synthesis occurs through interference with peptidyl transferase activity, with an intact C-9,10 double bond and the C-12,13 epoxide required for this inhibition (McLaughlin *et al.*, 1977). Inhibition takes place in the translational stage that occurs in the polysomes of the endoplasmic reticulum. All three translational processes, initiation, elongation, and termination, can be affected by the trichothecenes. However, the inhibitory potency and major site of action during translation vary among trichothecenes. Prevention of polypeptide chain initiation or elongation has been demonstrated in cell-free models, and partial translational inhibition was found in cell cultures and in laboratory animals (Azcona-Olivera *et al.*, 1995; Bamburg, 1983; Carter and Cannon, 1977; Thompson and Wannemacher, 1986; Ueno, 1984).

Trichothecenes can be divided into two groups based on their site of action on protein synthesis. Trichothecenes with hydroxyl and acetyl substitutions at both C-3 and C-4, such as T-2 toxin, DAS, verrucarin A, preferentially inhibit initiation while DON, trichodermin, crotocin, and verucarol inhibit elongation and/or termination (McLaughlin *et al.*, 1977). Trichothecenes inhibit both DNA and RNA synthesis in *in vitro* systems, presumably secondary effects of protein synthesis inhibition (Thompson and Wannemacher, 1986). Mitochondrial protein synthesis and electron transport was inhibited both *in vitro* and *in vivo* by high doses of T-2 toxin (Pace, 1983; Pace *et al.*, 1988).

Trichothecenes and other translational inhibitors which bind to ribosomes can also rapidly activate MAPKs and induce apoptosis in a process known as the ''ribotoxic stress response''; this could be another pathway for trichothecene-induced apoptosis (Laskin *et al.*, 2002; Pestka, 2007). MAPKs modulate processes such as cell growth, differentiation, and apoptosis, and are critical for signal transduction in the immune response (Dong *et al.*, 2002). Trichothecenes activate p38, Jun N-terminal kinase (JNK) and extracellular signal regulated kinase (ERK) MAPKs *in vitro* and *in vivo* (Chung *et al.*, 2003; Moon and Pestka, 2003; Moon *et al.*, 2003; Yang *et al.*, 2000; Zhou *et al.*, 2003a, b, 2005a, b). Both proinflammatory cytokine up-regulation as well as apoptosis are mediated by trichothecene-induced MAPK activation (Pestka *et al.*, 2004; Zhou *et al.*, 2003b, 2005b). MAPK activation was correlated with and preceded apoptosis induced by trichothecenes in an *in vitro* system, with cytotoxic potency being satratoxin G, roridin A, verrucarin A >T-2 toxin, satratoxin F, H >nivalenol and DON (Yang *et al.*, 2000).

Apoptosis is now recognized as a major mechanism for trichothecene-induced toxicity. It should be recognized that the majority of studies examining trichothecene toxicity were carried out prior to the recognition of apoptosis as a toxic response. In retrospect, the early morphologic changes described as necrosis in the lymphoid, hematopoietic, and intestinal tract are consistent with apoptosis. Later studies of trichothecene toxicity *in vivo* have verified this for T-2 toxin-induced bone marrow and thymic changes using DNA fragmentation in addition to morphology (Islam *et al.*, 1998, Shinozuka *et al.*, 1998). More recently, T-2 toxin-induced apoptosis of neuroepithelial cells in fetal rats was shown to be preceded by expression of oxidative stress-related genes (e.g. heat shock protein 70) and subsequent activation of MAPKs and caspase-2, important factors in cell signaling pathways for apoptosis (Sehata *et al.*, 2004). Similarly, satratoxin G-induced apoptosis of olfactory sensory neurons was associated with elevated expressions of the proapoptotic genes Fas, FasL, p75NGFR, p53, Bax, caspase-3, and CAD in the olfactory epithelial lined ethmoid turbinate (Islam *et al.*, 2006). Based on these findings, trichothecenes may be able to drive both extrinsic (death receptor-mediated) and intrinsic (mitochondrial-mediated) apoptotic pathways.

Some biological effects may be mediated by reaction of the epoxy groups of trichothecenes with sulfhydryl groups on enzymes and binding of certain trichothecenes to membrane components. T-2 toxin rapidly affected glucose, nucleotide and amino acid transporters as well as calcium/potassium channel activities *in vitro* indicating alteration of cell membrane functions independent of protein synthesis inhibition (Brunner and Morris, 1988). In pigs, DAS was

found to be cytotoxic to cells with specialized ion pumps, namely renal tubular cells, gastric parietal cells, and salivary ducts (Coppock *et al.*, 1985a). Parietal cell toxicity was also identified in rats treated with T-2 toxin (Bratich *et al.*, 1990). Other effects of trichothecenes may be mediated via lipid peroxidation. For example, following oral T-2 toxin administration, rats on antioxidant-deficient diets had increased thiobarbituric acid reactive substances as well as decreased glutathione and superoxide dismutase activity in liver, while mice had increased plasma and organ malondialdehyde (MDA) content and decreased plasma vitamin E levels (Rizzo *et al.*, 1994; Vila *et al.*, 2002).

Recent studies indicate that some trichothecenes can bind covalently. Satratoxin G, a macrocyclic trichothecene, forms covalent adducts with proteins and potentially other cellular macromolecules (Gregory *et al.*, 2004; Yike *et al.*, 2006). The ability of satratoxin G to bind to albumin provides a potential biomarker of exposure to the toxin as well as to *Stachybotrys chartarum*.

Trichothecenes can be immunostimulatory or immunosuppressive depending on dose, exposure frequency, and timing relative to sampling for immune assays (Holt *et al.*, 1988; Pestka *et al.*, 2004; Taylor *et al.*, 1989). Low level exposure promotes, in hormetic fashion, the expression of numerous cytokines and chemokines (Holt *et al.*, 1988; Warner *et al.*, 1994). Both transcriptional and post-transcriptional mechanisms are involved in trichothecene-induced cytokine mRNA expression (Pestka *et al.*, 2004, 2008). One example of cytokine up-regulation is DON-induced IgA dysregulation in mice which results in glomerulonephritis that closely resembles human IgA nephropathy (Pestka *et al.*, 1989). At higher doses, trichothecenes given orally, intravenously, by inhalation, and topically are well known to be cytotoxic to lymphocytes in lymphoid tissues, including the thymus and Peyer's patches, as well as in the leukocytes of bone marrow, collectively resulting in immune suppression (Connor *et al.*, 1986; Coppock *et al.*, 1985a; Islam *et al.*, 1998; Pang *et al.*, 1987a, b, c, 1988; Pestka *et al.*, 2004; Shinozuka *et al.*, 1998). Similar effects have been observed with macrophages, where low doses of trichothecenes up-regulate expression of inflammation related genes including COX-2, proinflammatory cytokines and numerous chemokines, while high doses induce apoptosis thereby suppressing innate immune function (Pang *et al.*, 1987b; Pestka *et al.*, 2004; Yang *et al.*, 2000; Zhou *et al.*, 2005a).

Some trichothecene-induced effects may be due to neurotransmitter alteration in the central nervous system. Emesis or vomiting, which occurs with many of the trichothecenes when given at high doses, has been attributed to the stimulation of the chemoreceptor trigger zone in the area postrema of the medulla oblongata. However, studies with T-2 toxin in cats indicated that other mechanisms, such as the neural afferent pathways from the abdomen, implicated in radiation-induced emesis, may also be involved (Borison and Goodheart, 1989). DON alters serotonin activity in the central nervous system of swine which is important in regulation of food intake (Prelusky *et al.*, 1992). This may be the mechanism for reduction of food consumption found at low levels of DON exposure. In addition, alterations in norepinephrine and dopamine were identified in specific areas of the brain (Prelusky *et al.*, 1992).

V. TOXICITY

LD_{50} values, which may differ based on route of exposure and species exposed, can be used to compare the toxicity of trichothecenes (Table 26.1). For example, the LD_{50} values for i.p. exposure in the mouse are 5.2 mg/kg for T-2 toxin and 23 mg/kg for DAS, while for i.v. exposure in the pig they are 1.21 mg/kg for T-2 toxin and 0.37 mg/kg for DAS (Ueno, 1985). For T-2 toxin, oral LD_{50} values are not markedly different across species (Ueno, 1985); however, T-2 toxin was 10- to 50-fold more toxic when inhaled than when administered orally (Marrs *et al.*, 1986). For DON, pigs are the most sensitive, followed by mice then rats. For DON in pigs, the main clinical effect at high levels is vomiting and at low levels reduced food intake or food refusal. Such responses apparently limit exposure and thus toxic manifestations.

Trichothecene toxicosis is manifested by a broad spectrum of clinical disorders, which vary according to the specific causative toxin or mixture of toxins. Species differences in response are generally related to severity of the response, and young animals are more susceptible than adults. Toxicosis can be acute or chronic, with clinical signs remaining fairly similar. A comprehensive review of the pathophysiology of spontaneous and experimentally induced trichothecene mycotoxicosis is available (Beasley, 1989).

Vomiting (emesis), reduced feed intake, and feed refusal are associated with most trichothecenes, with swine being the most sensitive species (reviewed by Schiefer and Beasley, 1989). Vomiting is an acute reaction that may occur rapidly after either parenteral or oral administration, depending on the dose. Vomiting is believed to be a result of central nervous stimulation, although gastric irritation cannot be ruled out. Reduced food intake and food refusal lead to decreased weight gain. T-2 toxin and DAS cause cytotoxicity in many cell types such as skin or oropharyngeal epithelia that are directly exposed, as well as hematotoxicity and immune suppression due to effects on rapidly dividing cells and, in some cases, neurotoxicity (Beasley, 1989). Abortion and retarded growth rate of offspring have been reported (Francis, 1989).

The ability of the trichothecenes to produce dermal toxicity is rather unusual for toxins. While only the more potent trichothecene mycotoxins have this effect, the cytotoxicity produced on contact with feed may result in lesions on the snout, muzzle, lips, and tongue of animals. Similar dermal effects have been reported in humans and in swine following topical application of T-2 toxin. T-2 toxin, DAS,

and other trichothecenes, especially the macrocyclic toxins produced by *Stachybotrys chartarum* which have been recognized primarily in Europe, have dermal effects although systemic effects are more important. Deoxynivalenol does not produce dermal effects.

Insufficient data are available to draw a firm conclusion regarding the mutagenicity or carcinogenicity of the trichothecenes, including T-2 toxin (reviewed by Haschek, 1989). Trichothecenes are not mutagenic in bacterial assays, although chromatid breaks have been induced by DON in Chinese hamster cells (Hsia *et al.*, 1988). Carcinogenesis bioassays have been largely negative while data from weakly positive studies could be explained by secondary effects of trichothecenes such as altered immune function (Haschek, 1989). Evaluation by IARC (1993, updated online 1997) of available data has concluded that the trichothecenes are not classifiable as to their carcinogenicity to humans (Group 3). Trichothecenes have not been shown to be teratogenic in mammals except at maternally toxic doses. At these doses, embryolethality and anomalies in the nervous and skeletal systems were observed (Francis, 1989).

A. T-2 Toxin and Diacetoxyscirpenol (DAS)

The toxic effects of T-2 toxin and DAS are similar irrespective of route of exposure and species, with ingestion, parenteral administration, and inhalation resulting in lymphoid necrosis, hematotoxicity, and gastrointestinal toxicity (Table 26.2, Figures 26.2 and 26.3). Shock secondary to cardiovascular collapse occurs at high doses. T-2 toxin and DAS also cause dermal and mucous membrane injury on contact.

Ingestion of feed naturally contaminated with T-2 toxin and DAS has caused serious toxicoses mainly in cattle, swine, and poultry, but also occasionally in horses, dogs, cats, and humans. Signs most often include increased incidence of infection, reduced food intake or food refusal, vomiting and diarrhea, and necrosis of skin and oral mucosa. With more severe toxicosis, lesions include hemorrhage and necrosis of the gastrointestinal mucosa (high doses), destruction of hemopoietic tissue and lymphoid necrosis (high doses), and meningeal hemorrhage (massive doses). Shock and death can follow (massive doses). Clotting disorders and reproductive problems also have been reported with T-2 toxin, but the former have not been readily reproduced experimentally. At doses toxic to the dam, embryotoxicity and fetotoxicity, and possibly abortion may occur. In males, testicular damage may occur at high doses.

1. Swine

In early studies, the LD_{50} values for DAS and T-2 toxin administered i.v. in swine were determined to be 0.37 and 1.3 mg/kg, respectively (Weaver *et al.*, 1978a, b). Acute effects included vomiting, lethargy, frequent defecation, and posterior paralysis. Following the ''yellow rain'' incidents in Southeast Asia, extensive studies funded by the Department of Defense were conducted at the University of Illinois in the 1980s using the pig as a model for humans. Exposures were by intravenous, inhalation, and dermal routes. In previous studies of T-toxin and DAS, exposure was mainly by ingestion due to the major concern being natural contamination of food. Using an i.v. LD_{50} dose of 1.2 mg T-2 toxin/kg or above, pigs developed a shock syndrome which was followed by death within 12 to 16 h (Lorenzana *et al.*, 1985a, b). Clinical signs included vomiting, ataxia, collapse, and diarrhea. The same syndrome was induced with DAS at a similar i.v. dose and following inhalation exposure to T-2 toxin. Following i.v. exposure to T-2 toxin, shock was characterized by reduction in cardiac output and aortic blood pressure and increased plasma concentrations of epinephrine, norepinephrine, thromboxane B_2, 6-keto-$PGF_{1\alpha}$, and lactate. Pulmonary vascular resistance and heart rate increased. An initial leukocytosis was followed by leukopenia with both neutrophils and lymphocytes affected. Coagulation assays were normal.

Extensive pathology evaluation was conducted as part of these studies (Pang *et al.*, 1986, 1987c). Lesions included hemorrhages of the lymphoid tissues, gastrointestinal tract, heart, pancreas, adrenal gland, and meninges. Degeneration and necrosis of the lymphoid tissues, necrosis of the surface and crypt epithelium of the gastrointestinal tract (Figures 26.2 and 26.3), and necrosis of the bone marrow confirmed previous findings in pigs and other species. Subsequent reevaluation of lesions supports the findings of apoptotic cell death as well as oncotic necrosis. In addition, scattered necrosis of the pancreatic acinar cells, heart, adrenal cortex, and tubular epithelial cells of the renal medulla were found, as well as a mild interstitial pneumonia. Ultrastructural examination indicated that the endoplasmic reticulum of the pancreatic acinar cells was affected. These changes may have caused release of myocardial depressant factor which would have augmented T-2 toxin-induced cardiac dysfunction. In the heart, myofibrillar lysis and contraction band formation were present (Figure 26.4, Pang *et al.*, 1986). These lesions are consistent with catecholamine-induced changes.

Inhalation of T-2 toxin with endotracheal exposure to a nebulized dose of 9 mg/kg (20–30% retention, 1.8 to 2.7 mg/kg) resulted in clinical signs, and hematologic and morphologic changes similar to those occurring following i.v. exposure but with more severe interstitial pneumonia (Pang *et al.*, 1987b). Two of 17 pigs died or were euthanized (when moribund) within 8 to 10 h of dosing, the remainder were euthanized over a 7 day period. There was a transient reduction in alveolar macrophage viability and phagocytic ability as well as a transient reduction in mitogen-induced blastogenic response by pulmonary, but not peripheral blood, lymphocytes. When pigs were exposed to T-2 toxin at 8 mg/kg and evaluated over 28 days, T-2 toxin did not cause death but caused transient decreases in mitogen-induced (Con A, PHA, and PWM) blastogenic responses of

TABLE 26.2. Target organ toxicity of T-2 toxin and DAS

Tissue	Exposure route	Organ	Clinical signs, hematology	Pathology
Lymphoid	Parenteral, inhalation, ingestion, dermal	Thymus	Immune suppression, lymphopenia	Apoptosis (T-cell), lymphoid depletion
	Parenteral, inhalation, ingestion, dermal	Spleen, lymph nodes	Immune suppression, lymphopenia	Apoptosis, lymphoid depletion, B-cell primarily
	Parenteral, inhalation, dermal	Mucosa associated lymphoid tissue (MALT)	Immune suppression, lymphopenia	Apoptosis, lymphoid depletion, B-cell primarily
Bone marrow	Parenteral, inhalation, ingestion		Leukopenia, anemia, thrombocytopenia	Apoptosis, necrosis – all cell types
Gastrointestinal	Parenteral, inhalation, ingestion	Stomach	Vomiting, dehydration	Ulceration, parietal cell injury
	Parenteral, inhalation, ingestion	Small intestines	Diarrhea, dehydration	Apoptosis, epithelial cell, both surface and crypt
Gall bladder	Parenteral, inhalation, ingestion	Gall bladder		Edema, hemorrhage
Skin	On contact, dermal		Irritation, vesicles, desquamation	Necrotizing dermatitis
Mucous membrane	On contact, oral	Oral and buccal mucosa	Oral/buccal vesicles, ulcers	Stomatitis
Pancreas (pig only)	Intravenous, inhalation, dermal	Exocrine pancreas Endocrine	 Hyper- or hypo-glycemia	Acinar cell necrosis Islet cell necrosis (DAS, i.v.)
Cardiovascular system	Parenteral, inhalation		Decreased cardiac output and aortic blood pressure (T-2 toxin, pig, rat) Hypotension (DAS, human)	Myocardial hemorrhage and necrosis (T-2 toxin, pig i.v., inhalation, and rat i.p.)
Central nervous system	Parenteral, inhalation		?Vomiting	None
	Parenteral, inhalation		Somnolence, confusion, ataxia (DAS, humans), dizziness, vertigo (‘‘yellow rain’’, humans)	Not examined
	Parenteral, dermal		Ataxia, posterior paresis (pig)	Capillary endothelial cell necrosis and hemorrhage (DAS, i.v.)
Reproductive system	Parenteral	Testis	Infertility	Necrosis of germinal epithelium

peripheral blood mononuclear cells and hemagglutination titers to sheep red blood cells (Pang *et al.*, 1988). Therefore, both cell-mediated and humoral immunity were affected as shown in other species.

Trichothecene mycotoxins are highly irritating to the skin and mucous membranes in all species. Skin irritation was experienced by laboratory workers accidentally exposed to trichothecene producing fungal cultures or crude extracts of T-2 toxin. Skin irritation has also been used as a basis for a semiquantitative bioassay to detect these toxins (Bamburg *et al.*, 1968; Ueno *et al.*, 1970). T-2 toxin was applied topically to pigs to characterize its effects by the dermal route and to evaluate therapeutic intervention (Pang *et al.*, 1987a, d). Topical treatment of pigs with T-2 toxin up to 50 mg/kg did not result in any deaths. Clinical signs in pigs followed for 14 days following topical application of T-2 toxin at 15 mg/kg included lethargy, anorexia, posterior weakness or paresis, persistent fever, and pruritis. Severe erythema was present within 24 h and necrotizing dermatitis developed within 3 days (Figure 26.5). By day 14 there was sloughing of

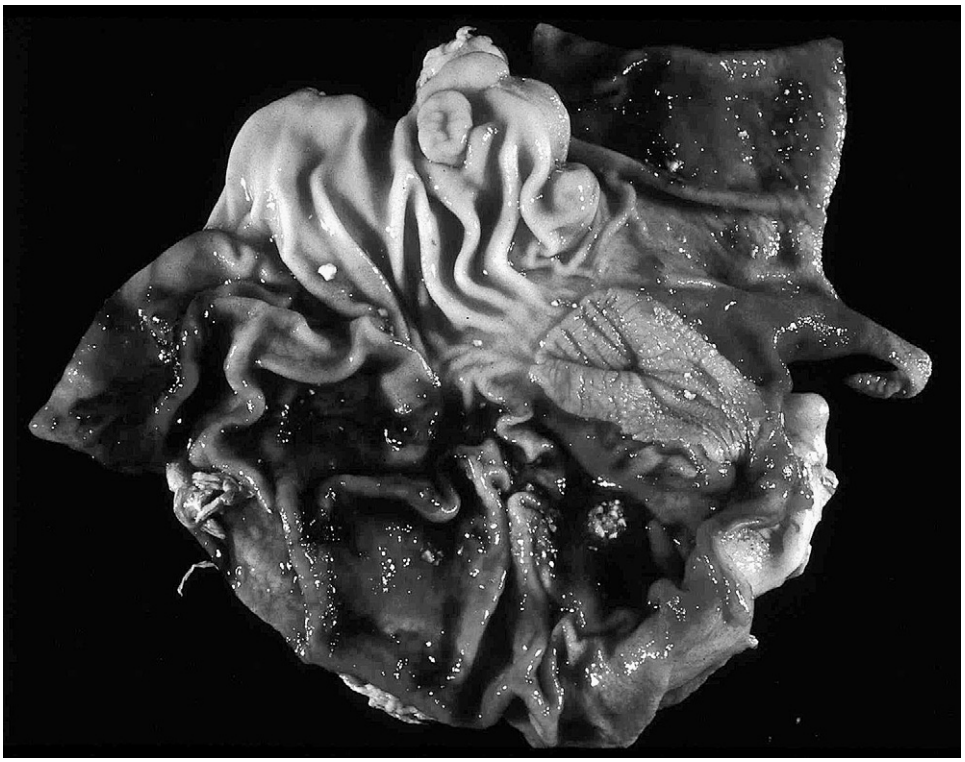

FIGURE 26.2. Stomach from a pig given T-2 toxin at approximately 2 mg/kg as a single inhalation exposure 8 h previously. There is severe necrohemorrhagic gastritis especially in the fundic portion. From Haschek *et al.* (2002).

the necrotic dermis with hyperplasia of the subjacent regenerating epidermis. In addition, there was mild apoptosis of the lymphoid tissues and pancreatic acinar cell necrosis. The skin and subcutaneous tissues appeared to serve as a depot and site of metabolism for T-2 toxin as significant amounts of the toxin and its metabolites were present at the dose site up to 7 days but were not detected systemically (Pang *et al.*, 1987a). In a second study conducted over a 35 day period, the skin lesions had healed by day 21. Prominent leukocytosis, due to neutrophilia, with transient lymphopenia, and increased serum globulin were observed. These leukocyte changes were consistent with a corticosteroid-altered leukogram, potentially related to stress. Decreases in mitogen-induced (Con A, PHA and PWM) blastogenic responses of peripheral blood mononuclear cells were present but not in hemagglutination titers to sheep red blood cells (Pang *et al.*, 1987d). Therefore, cell-mediated immunity was affected by topical application of T-2 toxin.

2. Humans

In Japan, red mold disease or ''Akakabi-byo'' of wheat due to *F. graminearum* infection was reported to cause gastroenteritis in humans (Saito and Ohtsubo, 1974). In China, 60% of people who ate rice contaminated with T-2 toxin were reported to have nausea, dizziness, vomiting, chills, abdominal distension, abdominal pain, thoracic stuffiness, and diarrhea with a latent period of 10 to 30 min (Wang *et al.*, 1993).

Severe disease known as alimentary toxic aleukia (ATA) occurred in humans who had eaten overwintered grains in the former USSR during the 1930s and 1940s, and also earlier (Joffe, 1974, 1983). The primary toxin thought to be responsible for ATA is T-2 toxin although this cannot be confirmed. Four stages have been described with ATA. Stage 1 lasted from 3 to 9 days post-ingestion. It was characterized by a burning sensation of the mouth and irritation of the gastrointestinal tract resulting in hypersalivation, vomiting, diarrhea, dizziness, and tachycardia. This stage correlates with gastrointestinal lesions especially necrosis of crypt epithelial cells. Stage 2 lasted from 2 weeks to 2 months with eventual recovery if exposure stopped. It was characterized by progressive leukopenia with granulocytopenia and a relative lymphocytosis. Anemia, icterus, and impaired resistance to infections were observed. This stage correlates with acute damage to the mitotic cells in the hematopoietic system with clinical signs delayed until the lifespan of the mature cells is exceeded. Abnormalitites in the central and autonomic nervous system were also described. Stage 3 was characterized by more severe leukopenia and anemia, as well as hemorrhages due to thrombocytopenia and fibrinogenemia. Necrotic lesions were observed in the oral cavity with secondary bacterial infections and some individuals died with glottal stenosis, indicating penetrating injury or interference with normal epithelial repair processes. Stage 4 lasted 2 or more months as the hematopoietic system recovered. During this time, individuals were very susceptible to various infections. A similar syndrome was reproduced in cats exposed to repeated doses of T-2 toxin (Lutsky *et al.*, 1978).

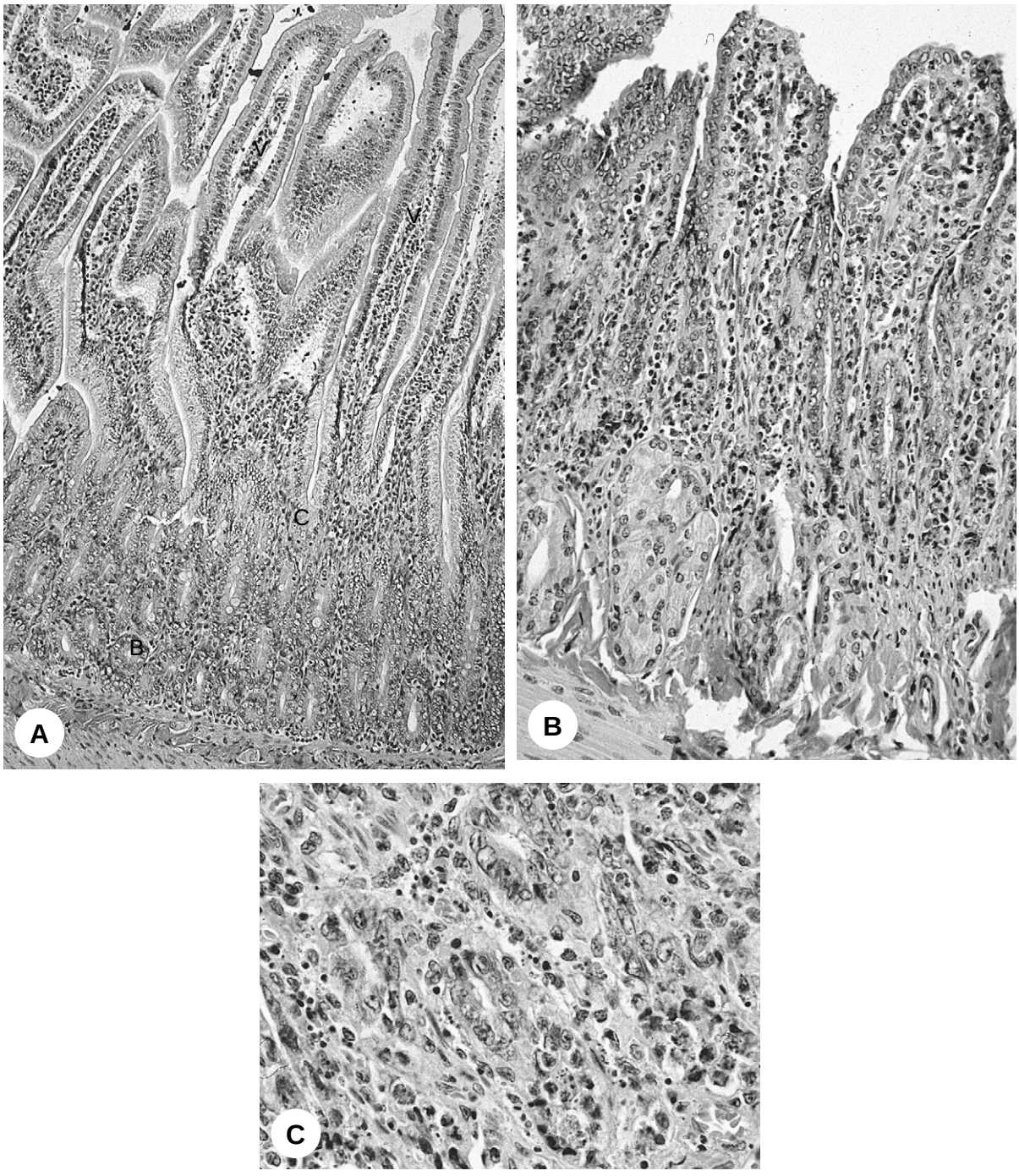

FIGURE 26.3. Intestine from Sprague–Dawley rats given T-2 toxin at 25 mg/kg as a single oral dose 12 h previously (H&E). From Haschek *et al.* (2002). A. Duodenum from a control rat. The mucosa consists of normal elongated villi (V), crypts (C), and Brunner's glands (B) located at the base of the mucosa (×100). B. Duodenum from a treated rat. The intestinal villi are short and blunted. The villous epithelial lining is segmentally ulcerated. Bruner's glands and crypts are largely destroyed (×100). C. Higher magnification of B. Extensive apoptotic necrosis of crypt cells is present. Cellular debris and mononuclear inflammatory cells are present in the lamina propria (×400).

Diacetoxyscirpenol (DAS), also called anguidine, was evaluated as a potential chemotherapeutic agent for cancer treatment (reviewed by Jarvis and Acierto, 1989). While the therapeutic efficacy was insufficient to develop DAS as an anticancer drug, valuable information was obtained in the preclinical toxicology studies, conducted using beagle dogs and rhesus monkeys, and in the phase I and II clinical studies in humans. In the preclinical studies, lethality of DAS was similar in dogs and monkeys based on mg/m^2 but on an mg/kg basis, monkeys tolerated about eight times

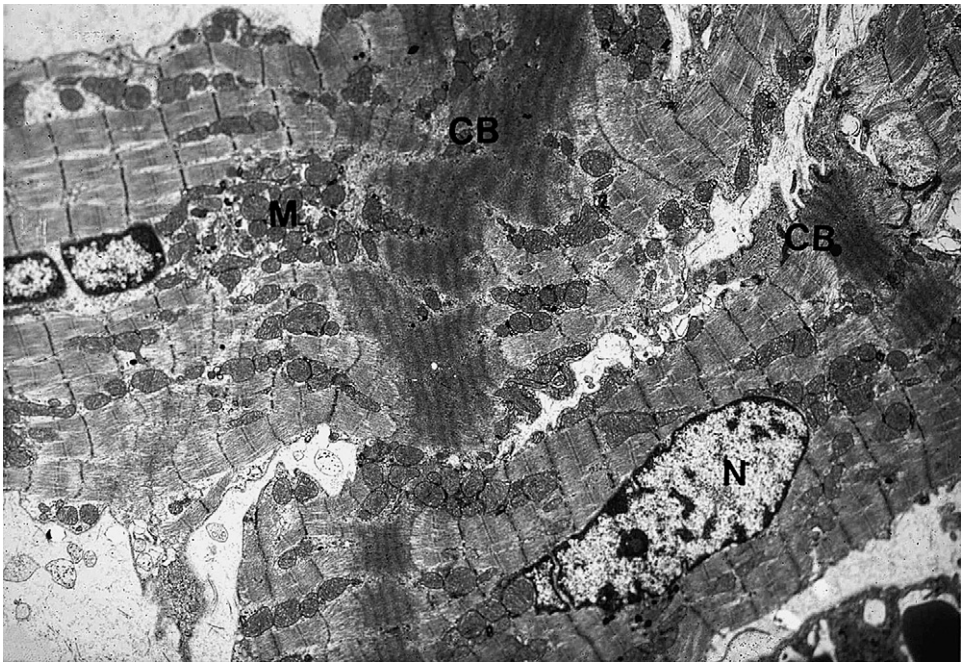

FIGURE 26.4. Heart from a pig given T-2 toxin i.v. at 2.4 mg/kg 4 h previously. Prominent contraction bands (CB) of electron-dense contractile material with adjacent mitochondrial accumulation (M) are present. Edema is present between myocytes (×6,700). From Haschek *et al.* (2002).

more DAS than dogs before lethality occurred. The highest nontoxic dose (HNTD, dose at which no hematologic, chemical, clinical, or morphologic drug-induced alterations occur) in monkeys was 0.125 and for dogs 0.016 mg DAS/kg/injection (Q1DX5, dosed daily for 5 days). Toxic effects were similar to those reported in other species for type A trichothecenes, including vomiting and diarrhea that led to dehydration; and hematopoetic effects that included leukopenia, anemia, and mild thrombocytopenia. All effects were reversible and occurred more consistently in dogs than monkeys.

Cancer patients received DAS i.v. up to 10 mg/m^2 in phase I and II clinical studies (Goodwin *et al.*, 1978; Yap *et al.*, 1979). Deaths were reported but antineoplastic activity was not observed. In phase I clinical trials (Goodwin *et al.*, 1978), toxic effects following i.v. administration of 5 μg/kg to 6 mg/kg body weight included nausea, vomiting, myelosuppression, hypotension, diarrhea, central nervous system dysfunction (including somnolence, confusion, and ataxia), fever, chills, stomatitis, erythema, and thrombocytopenia. CNS toxicity and hypotension were dose limiting. Dosage recommendations for phase II clinical trials were 3.0 to 5.0 mg/m^2 i.v. daily for a period of 5 days. In phase II clinical trials (Yap *et al.*, 1979), hematologic toxicity consisting of mild myelosuppression was deemed substantial but not prohibitive. One patient died of sepsis. Clinical signs were the same as in phase I studies with hypotension being the most significant side effect.

B. Deoxynivalenol (DON, Vomitoxin)

DON causes much less severe toxicity than T-2 toxin. Among domesticated animals, swine appear to be most susceptible, but cattle, horses, dogs, and poultry are all reported to be susceptible (reviewed by Mostrom and Raisbeck, 2007; Pestka, 2007). Though not among the more acutely toxic trichothecenes, deoxynivalenol has caused great economic loss to the livestock industry. The predominant sign associated with contaminated feed is reduced feed intake (or occasionally feed refusal) and decreased rate of gain. In swine, DON in feed at 4 ppm caused a 2% reduction in feed intake while at 40 ppm a 90% reduction was observed. In the field, however, concentrations of DON associated with feed refusal may be as low as 1 ppm. Other signs may include soft stools, diarrhea, failure to thrive, and a predisposition to other disease entities and poor nutrition. Vomiting, which occurs at higher levels of exposure, is infrequently seen. With repeated exposure, pigs may develop a resistance to DON and make compensatory gains. Specific lesions are not observed under field conditions. However, a mild thickening of the squamous mucosa of the stomach has been noted experimentally in swine (Schiefer and Beasley, 1989).

C. Macrocyclic Trichothecenes (Stachybotryotoxicosis)

Stachybotrys chartarum (previously also called *S. atra*), the fungal cause of stachybotryotoxicosis and sick building syndrome, is a black mold. There are two toxic ‘‘chemotypes’’ of *S. chartarum*, one elaborating highly toxic macrocyclic trichothecenes and the other less toxic atranones and simple, but not macrocyclic, trichothecenes (Andersen *et al.*, 2002). Exposure may be by ingestion, e.g. exposure to contaminated straw, or inhalation as when mold grows in water-damaged homes or air ducts.

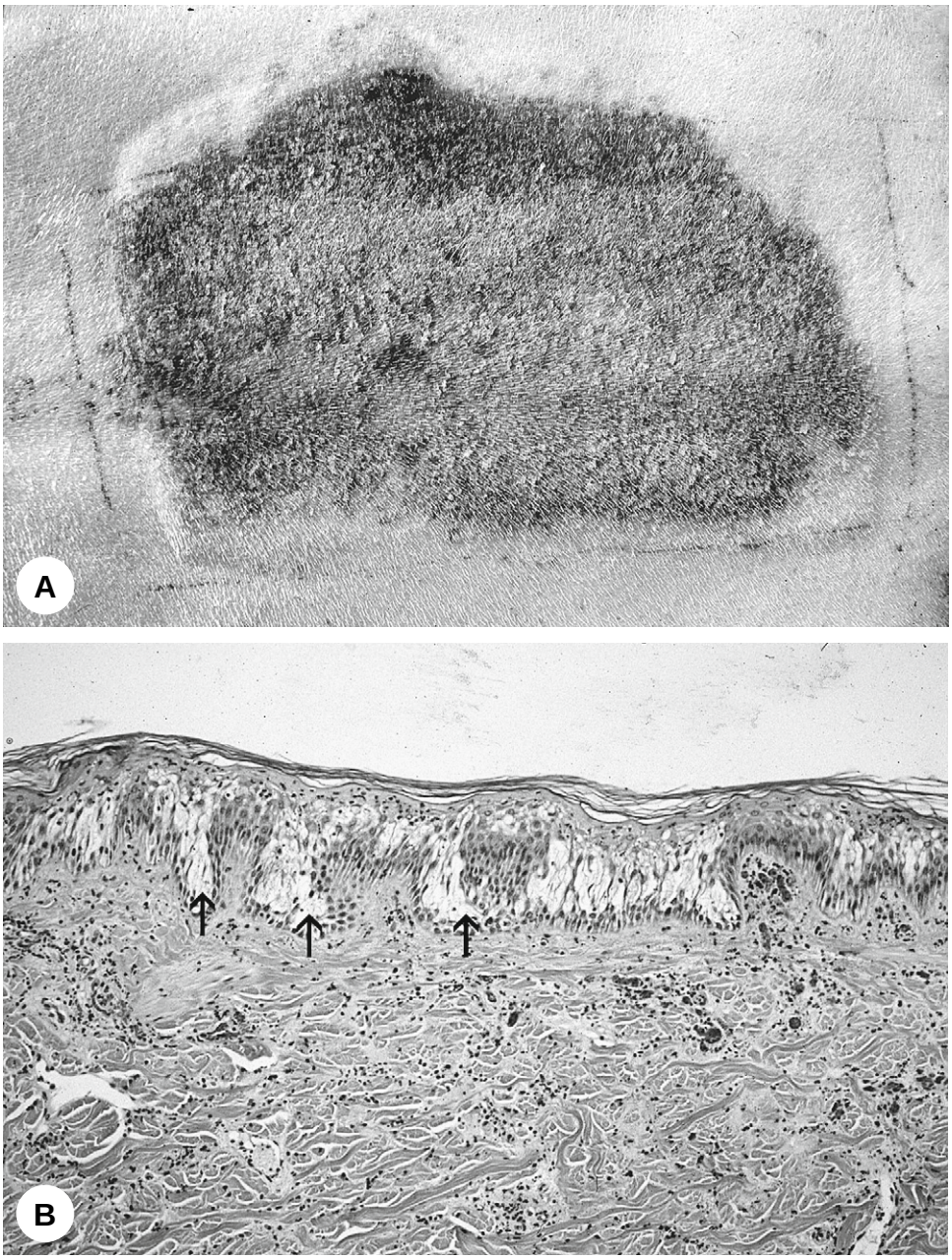

FIGURE 26.5. Skin from a pig given T-2 toxin at 15 mg/kg as a single dermal application. From Haschek *et al.* (2002). A. At 3 days after application, the area was markedly reddened and edematous. B. At 1 day after application, there is multifocal ballooning degeneration of the epidermis, as well as edema and mild cellular infiltration around congested vessels in the dermis (H&E ×10).

Consumption of straw or hay contaminated with the highly toxic trichothecenes has resulted in toxicoses, termed stachybotryotoxicosis, in horses and farm workers (reviewed by Beasley, 1992; Mostrom and Raisbeck, 2007). It has been reported in the USSR and adjacent countries, as well as Finland and South Africa, in humans, horses, ruminants, swine, and poultry. The fungi causing the disease are *Stachybotrys chartarum* and possibly *Myrothecium* and *Dendrodochium* spp., which produce macrocyclic trichothecenes including satratoxins, roridin, and verrucarin on straw or hay. These toxins are directly toxic to mucosal membranes causing necrosis and edema of the lips, tongue, and buccal membranes, and later diarrhea due to gastrointestinal toxicity. Hematopoietic toxicity follows and is characterized by leukopenia, thrombocytopenia, and coagulopathy, which can result in systemic hemorrhage, septicemia, and death. Consumption of large amounts of contaminated feed, at least in horses, results in nervous signs, circulatory collapse, and death (Beasley, 1992). In some affected horses, a ''shocking'' form of stachybotryotoxicosis has been reported. Dermal toxicity due to contact with infected material can occur and has been reported in humans. In addition, respiratory distress, epistaxis (nose bleeds), and eye irritation have been reported in humans, possibly from inhalation exposure.

When mold grows in water-damaged homes or air ducts, exposed humans may show acute or chronic health effects (Peraica *et al.*, 1999; Smoragniewicz *et al.*, 1993).

Stachybotrys chartarum is one of the most commonly noted agents associated with so-called ''sick building'' or ''damp building-related'' syndrome and ''damp building-related illnesses'' (DBRI). While upper and some lower respiratory tract symptoms have been accepted as causally linked to human exposure to moldy damp indoor environments, other reported effects, including airflow obstruction, chronic obstructive pulmonary disease, pulmonary hemorrhage, neurologic effects and cancer, have not (Institute of Medicine, 2004). An excellent recent review of *S. chartarum*, associated trichothecene mycotoxins, and DBRI is available (Pestka *et al.*, 2008).

Experimentally, the macrocyclic trichothecenes satratoxin G, isosatratoxin F, and roridin A have been shown to cause nasal and pulmonary toxicity when administered intranasally or intratracheally to mice. Intranasal exposure of satratoxin G and roridin A induced apoptosis of olfactory sensory neurons resulting in atrophy of the olfactory epithelium and olfactory nerve layer of the olfactory bulb in the frontal brain (Islam *et al.*, 2006, 2007). Alveolar-type II cells and alveolar macrophages were injured following intratracheal instillation of isosatratoxin F or *Stachybotrys* spores with marked changes in surfactant synthesis and secretion (Rand *et al.*, 2002).

VI. RISK ASSESSMENT

The toxic effects of mycotoxins in humans have been reviewed by Peraica (Peraica *et al.*, 1999). Toxins produced by *F. sporotrichioides,* with T-2 toxin believed to be the principal toxin, were implicated with the human disease alimentary toxic aleukia (ATA). Alimentary toxic aleukia was recognized prior to 1900 in the USSR, with thousands of people developing ATA and many dying after consuming cereals that overwintered in fields during and after World War II.

Human exposure to trichothecene mycotoxins was also alleged to occur in Southeast Asia from exposure to a chemical warfare agent named ''yellow rain''. T-2 toxin, DON, nivalenol, and DAS were implicated as components of yellow rain and were detected in low concentrations in blood, urine, and tissue samples of alleged victims (Mirocha *et al.*, 1983; Watson *et al.*, 1984). Clinical signs included vomiting, diarrhea, headache, fatigue, dermatitis with focal alopecia, and generalized malaise. These toxins were also implicated in several outbreaks of gastrointestinal disease in China and India that followed a single ingestion of bread containing contaminated flour or rice (Bhat *et al.*, 1989; Peraica *et al.*, 1999; Wang *et al.*, 1993).

In the 1970s, DAS (anguidine) underwent phase I and II clinical trials in humans for treatment of cancer, but minimal antitumor activity was reported (Goodwin *et al.*, 1978; Yap *et al.*, 1979). This allowed documentation of clinical signs and hematopoetic effects. Clinical signs included nausea, vomiting, diarrhea, hypotension, and CNS disturbances. Myelosuppression was observed but was not the limiting factor in dose setting.

A more recent concern with trichothecenes for humans is stachybotryotoxicosis from macrocyclic toxins produced by *S. atra* and other non-*Fusarium* spp. These fungi grow on organic matter rich in cellulose, such as straw or hay, and in water-damaged homes or airducts (''sick building syndrome''). Sick building syndrome consists of clinical signs including vomiting, diarrhea, headache, fatigue, dermatitis with focal alopecia, and generalized malaise (Peraica *et al.*, 1999; Smoragiewicz *et al.*, 1993). One study from Montreal, Canada, identified T-2 toxin, diacetoxyscirpenol, roridine A, and T-2 tetraol (Smoragiewicz *et al.*, 1993). Farm workers in contact with infected litter or feed developed skin rashes, respiratory distress, nose bleeds, and eye irritation.

Risk assessment for humans is difficult due to limited toxicokinetic and toxicodynamic data available and the paucity of cases of human illness reported from consumption of food derived from heavily contaminated grains. Risk assessment from nondietary routes of exposure in humans is also complicated by lack of epidemiological data.

VII. TREATMENT

Respiratory, skin, and eye protection is required for personnel working with trichothecenes. There are no specific therapies for trichothecene toxicoses. Neither vaccines nor specific antidotes are readily available. Treatment in people and animals is symptomatic and supportive, and the only known prophylactic measure is avoidance of exposure (Fricke and Poppenga, 1989; National Academy of Science, 1983). T-2 toxin is stable in the environment, and resistant to heat and ultraviolet light.

The first step in suspected toxicosis is to stop exposure. If ingestion is the source of contamination, the food source needs to be changed. If exposure is environmental, exposed individuals should have their outer clothing removed and exposed skin decontaminated by washing thoroughly with soap and water. Prompt soap and water wash within 5 to 60 min of exposure significantly reduces the development of dermal effects. Exposed eyes should be treated with copious saline or water irrigation. If exposure has been through ingestion, superactivated charcoal may be used to bind trichothecenes and prevent further absorption.

Various potential treatments have been examined experimentally (reviewed by Fricke and Poppenga, 1989). The only treatments that have shown some success are orally administered activated charcoal (an adsorbent), steroidal anti-inflammatory agents (supportive treatment), and monoclonal antibodies. Activated charcoal has been successful in multiple species, including rats and pigs, when administered prior to or shortly after experimental acute exposure to T-2 toxin (Bratich *et al.*, 1990; Fricke and Poppenga, 1989; Poppenga *et al.*, 1987b). Steroidal

anti-inflammatory agents such as methylprednisolone and dexamethasone have significantly prolonged survival time (Fricke and Poppenga, 1989; Poppenga *et al.*, 1987a). Large doses of monoclonal antibodies directed against T-2 toxin and its metabolites have shown prophylactic and therapeutic efficacy in the rat (Feuerstein *et al.*, 1985). Ascorbic acid was effective in mice but not in rats (Fricke and Poppenga, 1989). For topical T-2 toxin exposure, soap and water applied within 60 min significantly reduced dermal toxicity in swine (Biehl *et al.*, 1989).

Appropriate samples, such as serum, nasopharyngeal swabs and urine, should be collected from exposed individuals and sent for toxin identification or confirmation. Currently, there are no commercially available rapid field diagnostic tests for identification of trichothecene exposure. Confirmation of exposure requires testing of blood, tissue, and environmental samples using GC-MS techniques.

Decontamination of clothing, equipment, and the environment can be performed since T-2 toxin is sensitive to standard household bleach (soaking for 30 min) especially when the solution is alkalinized (Castegnaro *et al.*, 1991; Madsen, 2001). For environmental decontamination, the use of a chlorine bleach solution under alkaline conditions such as 1% sodium hypochlorite (1 part bleach and 4 parts water) and 0.1 M sodium hydroxide solution with a 1 h contact time is recommended. Other trichothecenes should also be sensitive to this decontamination procedure.

In domestic animals, when a history includes food refusal and failure to thrive, mycotoxins should be included in the differential diagnosis, and food should be submitted for a mycotoxin screen. Specific quantitative assays are available for a limited number of trichothecenes, e.g. the Neogen Corporation assay for T-2 toxin has a range of 25 to 250 ppb. Trichothecenes are not found at high concentrations in tissues, although stomach contents may contain detectable levels if the animals were eating contaminated feed prior to death. As for humans, there are no specific antidotes for trichothecene toxicoses in domestic animals. A change in diet and supportive and symptomatic treatment are indicated. For low level chronic exposure, cessation of exposure by removal of contaminated food generally results in recovery. Prevention of absorption of trichothecenes from contaminated feed by use of binders, such as clay and zeolitic products, has not been shown to be effective.

Detection methods for T-2 toxin and other *Fusarium* toxins have been recently reviewed (Krska *et al.*, 2007; Ler *et al.*, 2006). Trichothecene analysis can be done by screening methods such as thin layer chromatography (TLC) and ELISA or analytical methods such as gas chromatography (GC) and high performance liquid chromatography (HPLC). GC instrumentation has been the most frequently used method for experimental work with trichothecenes. Newer methodologies, such as GC-MS and LC-MS, have an excellent lowest level of detection (LOD) of 5 ng/g for T-2 toxin in cereals and food, and wheat flour respectively (Ler *et al.*, 2006). Improved sensitivity for detection of T-2 and HT-2 toxins in cereal grains by HPLC using different fluorescent labeling reagents with similar LOD has also been recently described (Lippolis *et al.*, 2008). For future work LC-MS and LC-MS/MS (liquid chromatography with tandem mass spectrometry) will be the methods of choice given their lack of derivitization requirement, high sensitivity, high selectivity, high specificity, and capability to identify and quantify unknown agents simultaneously in a short time frame. However, the sophisticated instrumentation and high cost are major drawbacks to use in many laboratories. Emerging methods include fluorescence polarization immunoassays and dipsticks, and even newer methods such as biosensors and noninvasive methods based on infrared and acoustic techniques are being developed (Krska *et al.*, 2007).

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

Our understanding of the mode of action and effects of trichothecene mycotoxins has been greatly expanded over the last few years. This has been primarily due to the importance of these compounds in agriculture, e.g. DON, and in human health, e.g. toxins produced by *Stachybotrys* spp. However, the major expansion of knowledge of the trichothecene toxins, especially T-2 toxin, was in the 1980s because of their potential use in chemical warfare. Major gaps still remain in our knowledge base and this has been emphasized by the concern of their potential use for bioterrorism. The primary trichothecene toxins of concern are T-2 toxin and DAS which are on the select agents list.

Recent trichothecene research in the USA has focused on DON since it is the most commonly encountered and most economically important agricultural mycotoxin (Pestka, 2007). The elegant studies by Pestka and colleagues have made important strides in unraveling the mechanism of toxicity for this toxin. It is presumed that similar mechanisms operate for other trichothecenes such as T-2 toxin and DAS; however, additional work needs to be done to evaluate the relevancy of these mechanisms. T-2 toxin is of greater concern agriculturally in Europe than in the USA since increased levels of T-2 toxin have been recently found in grain. This is attributed to the increased use of no till farming and to climate change. Because of the increasing human health concerns related to wet and moldy buildings, e.g. following hurricane Katrina in Louisiana, emphasis has also been placed on toxins produced by *Stachybotrys* spp. Recent advances in the understanding of the mechanisms of toxicity caused by *Stachybotrys* toxins have been made by Pestka and Harkema, but much remains to be done in this area (Pestka *et al.*, 2008). Appropriate therapeutic regimens can only be developed once mechanisms of toxicity are understood. This would also allow development of methods for the early diagnosis of the health effects of mycotoxins,

particularly the detection of early changes that occur before the development of irreversible effects.

General research needs lie in the areas of detection, decontamination, and treatment. Better and cheaper methods need to be developed for the rapid detection and measurement of mycotoxin levels be it in food or tissues. In regard to bioterrorism, better methods need to be developed for the identification and measurement of mycotoxins in human and animal tissues, body fluids, and feces. Availability of mycotoxin reference samples to provide comparability of analytical results obtained between laboratories within a country as well as in different parts of the world would be helpful.

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CHAPTER 27

Toxins of Cyanobacteria

J.F. HUMBERT

I. INTRODUCTION

Cyanobacteria (or blue-green algae) are photosynthetic prokaryotes, which like the higher plants contain pigments that allow them to perform oxygenic photosynthesis. These microorganisms are distributed worldwide, and they colonize terrestrial, marine, and freshwater (rivers, lakes, ponds, and estuaries) ecosystems (Mur *et al.*, 1999; Whitton and Potts, 2000). Many cyanobacterial species are able to synthesize a wide range of toxins. These toxins have been involved in cases of fatal human poisoning, for example in a recent case affecting a hemodialysis center in Brazil (Azevedo *et al.*, 2002; Carmichael *et al.*, 2001; Jochimsen *et al.*, 1998). Moreover, many cases of poisoning events leading to the death of nonhuman mammals have also been reported in the past few years (see reviews by Briand *et al.*, 2003; Puschner and Humbert, 2007).

In aquatic ecosystems, cyanobacteria abundance can reach very high levels when environmental conditions are appropriate for their growth. Cyanobacterial blooms usually occur in eutrophic environments, which are characterized by high concentrations of mineral nutrients, and more particularly of phosphorus (Humbert *et al.*, 2001). When toxic cyanobacterial species proliferate they can synthesize large quantities of cyanotoxins, in particular of microcystins, during blooms, and these could be potentially extracted. It is also easy to cultivate some cyanobacterial species intensively, and this has already been done, for example, for nontoxic *Spirulina* strains, which are used to produce a human food supplement. However, some toxic strains belonging to the *Anabaena* and *Aphanizomenon* genera could be also cultivated under the same conditions or in smaller bioreactors, in order to obtain large amounts of cyanotoxins (Miyamoto *et al.*, 1988; Moreno *et al.*, 2003). Large biomasses of cyanobacteria can also be harvested from natural ecosystems, as described by Carmichael *et al.* (2000) for *Aphanizomenon flos-aquae* from Lake Klamath.

Saxitoxin is the only cyanobacterial toxin that is included in Schedule 1 of the Chemical Weapons Convention. However, other cyanotoxins would in fact be easier to obtain, due to the fact that they are produced by a wider range of species, and occur in many aquatic ecosystems. For this reason, in this chapter we will consider all the hepatoxins and neurotoxins synthesized by cyanobacteria.

II. BACKGROUND

The morphology of cyanobacteria varies widely, and they include spherical, ovoid, and cylindrical unicellular species, as well as multicellular colonial and filamentous forms (e.g. Couté and Bernard, 2001). Some species differentiate to form various specialized cells, such as heterocysts, which are able to fix nitrogen in water under N-limited conditions, and akinetes, which allow them to survive when environmental conditions are not favorable for growth. This high phenotypic plasticity makes it rather difficult to identify cyanobacteria to the species level.

In aquatic ecosystems, cyanobacteria can grow in the water column (pelagic species) or live attached to rocks, sand, or plants (benthic species). The parameters that can limit their growth are light (due to their photosynthetic metabolism), nutrients (particularly phosphorus), water temperature, and water column stability. When environmental conditions are favorable for their growth, blooms can produce very large biomasses in freshwater ecosystems within a few weeks. During these blooms, the vertical and horizontal distribution of cyanobacteria is often very heterogeneous, which makes it difficult to survey blooms and estimate biomasses.

It is also impossible to predict the potential toxicity of a cyanobacterial proliferation since even within a given species known to be potentially microcystin producing, for example, genotypes known to be toxin-producing and nontoxin-producing can occur in proportions that vary considerably not only from one ecosystem to another, but also over the course of a proliferation (Briand *et al.*, 2008). In addition, the quantity of microcystins produced by the toxin-producing cells can vary considerably, depending on the rate of cell growth (Sivonen, 1990; Briand *et al.*, 2005). Little information is available about other cyanotoxins, but it also looks as though here too the toxin-producing capacity can vary considerably from one strain to another in the species known to produce toxins.

Cyanobacteria can be fairly cultured under laboratory conditions, ranging from small batch cultures to high-volume bioreactors (Marxen *et al.*, 2005; Richmond *et al.*, 1993), but they can also be grown outdoors, in tubular bioreactors or large ponds. For example, the nontoxic species *Arthrospira platensis* (also known as Spirulina) has been cultured in

450 m^2 ponds in the south of Spain (Jimenez *et al.*, 2003), demonstrating that the outdoor industrial production of this species is possible in the Mediterranean climate, and several papers have reported different systems for outdoor mass cultures (see the review of Chaumont, 1993). Toxic genera, such as *Microcystis* or *Planktothrix* are more difficult to produce in large quantities, but high biomasses of these genera very frequently occur in aquatic ecosystems, which could be used to provide large quantities of toxins.

III. STRUCTURE, MECHANISM OF ACTION, AND TOXICITY OF CYANOTOXINS

The two main families of cyanotoxins that could potentially be used for chemical warfare are the hepatotoxins and the neurotoxins. Chemical structures of some of these toxins are shown in Figure 27.1.

A. Hepatotoxins

Three families of toxins mainly target the liver: the microcystins, which include more than 80 variants, the nodularins, and the cylindrospermopsins.

1. Microcystins and Nodularins

Microcystins (MC) are widely distributed cyanotoxins, and have often been implicated in accidental human and animal poisonings. They are produced by several genera, including the planktonic *Microcystis*, *Planktothrix*, *Anabaena* species, and the benthic *Oscillatoria*. Nodularins are only produced by the species *Nodularia spumigena,* which occurs in brackish waters, essentially in the Baltic Sea, Australia, and New Zealand.

These two families of toxins are both cyclic peptides (Figure 27.1A and B), with the same basic cyclic structure involving an amino acid known as ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), and four (nodularins) or six (microcystins) other amino acids (Chorus and Bartram, 1999). Only six nodularins have so far been identified. In contrast, microcystins have two variable amino acids (X and Z), two groups (R1, R2) and two demethylated positions (3 and 7), and as a result there are more than 80 known microcystins, ranging in molecular weight from 800 to 1,100 daltons.

These hepatotoxins range from extremely toxic compounds, such as microcystin-LR ($LD_{50} = 50$ µg/kg body weight, i.p. in mice) (Chorus and Bartram, 1999; Wolf and Frank, 2002) and nodularin ($LD_{50} = 30$ µg/kg body weight, i.p. in mice), to nontoxic microcystins (Rinehart *et al.*, 1994). The oral toxicity in mice has been reported to be 30- to 100-fold lower than the toxicity after i.p. injection, depending on the microcystin(s) present (Falconer *et al.*, 1994).

Clinical signs of microcystin or nodularin intoxication in mammals are diarrhea, vomiting, piloerection, weakness, and pallor (Elleman *et al.*, 1978; Falconer *et al.*, 1981). Acute episodes of gastroenteritis due to oral contamination by microcystins have been observed in Australia and Brazil (Rao *et al.*, 2002). Moreover, in one case, 76 human deaths were recorded in Brazil after dialysis patients had been exposed to water contaminated by microcystins and cylindrospermopsin (Azevedo *et al.*, 2002; Carmichael *et al.*, 2001; Jochimsen *et al.*, 1998). We are not aware of any case of human poisoning attributable to nodularins.

Microcystins probably enter the boodstream from the ileum via the bile acid transporter system (Eriksson *et al.*, 1990; Runnegar *et al.*, 1991, 1995). Microcystins and nodularins have been shown to be potent inhibitors of serine/threonine protein phosphatases 1 and 2A (Honkanan *et al.*, 1994; MacKintosh *et al.*, 1990), and this leads to hyperphosphorylation of the proteins associated with the cytoskeleton in hepatocytes (Toivola *et al.*, 1997). The rapid loss of the sinusoidal architecture and of cell attachment is followed by intrahepatic hemorrhage, and progressive liver necrosis leading to the death if the dose is sufficient. Lower doses of microcystins cause progressive changes in liver tissue, including chronic inflammation, focal degeneration of hepatocytes, and the accumulation of metabolites such as bilirubin in the blood (Elleman *et al.*, 1978; Hermansky *et al.*, 1990a).

The liver appears to be the main target organ for microcystin accumulation, but microcystins may also be found in the intestine and kidneys after i.p. and i.v. administration of microcystin-LR. On the other hand, following oral administration of microcystins in mice, less than 1% of microcystins are found in the liver, where they are detoxified in three metabolic products (Chorus and Bartram, 1999).

Microcystins do not seem to be genotoxic, but they do have carcinogenic activity as demonstrated in various rat and mouse studies. For example, Ito *et al.* (1997) demonstrated that neoplastic nodules were induced in mouse liver by repeated intraperitoneal injections of MC-LR, and Humpage *et al.* (2000) detected an increase in the area of aberrant colon crypt foci in the mouse after chronic oral ingestion of MC for several months. In the same way, nodularin may have hepatic tumorigenicity, as detected in rats by Ohta *et al.* (1994).

2. Cylindrospermopsins

To date, cylindrospermopsins are only known to be produced by *Cylindrospermopsis raciborskii* (Hawkins *et al.*, 1985), *Aphanizomenon ovalisporum* (Banker *et al.*, 2000; Shaw *et al.*, 1999), *Umezakia natans* (Harada *et al.*, 1994), and *Raphidiopsis curvata* (Li *et al.*, 2001), mainly in tropical areas. Cylindrospermopsin (CYN) has been identified in New Zealand field extracts, but the toxin-producing cyanobacterium was not identified (Stirling and Quilliam, 2001).

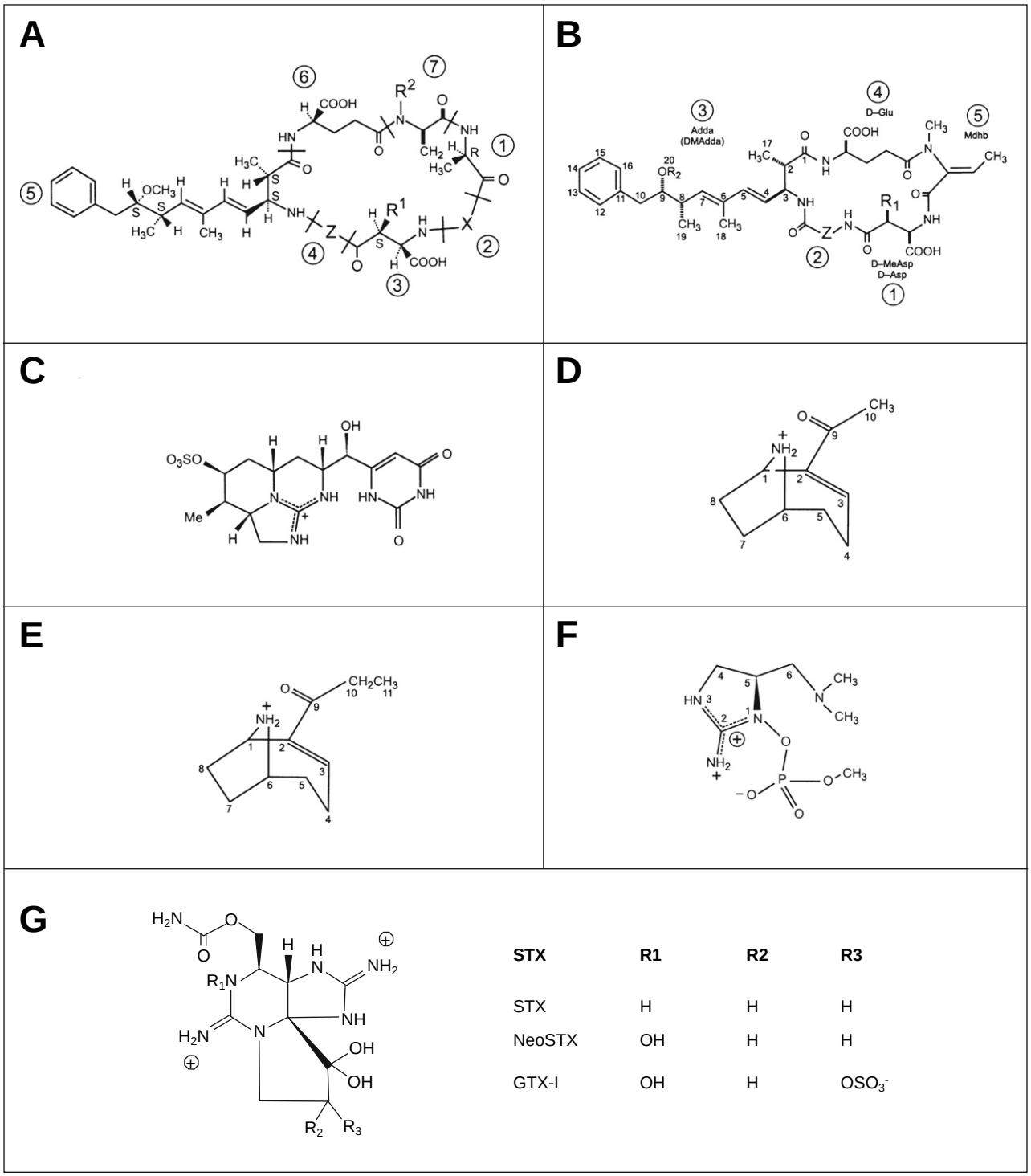

FIGURE 27.1. Chemical structures of A: microcystins, B: nodularins (X and Z are variable amino acids, R = H or CH_3), C: cylindrospermopsin, D: anatoxin-a, E: homoanatoxin-a, F: anatoxin-a(s), G: saxitoxin (STX), neosaxitoxin (NeoSTX); and gonyautoxin-I (GTX-I).

Cylindrospermopsin (CYN) is an alkaloid containing a tricyclic guanidine combined with hydroxymethyl uracyl (Figure 27.1C) with a molecular weight of 415 daltons. Intraperitoneal injection of cells containing CYN in mice is followed by diarrhea, anorexia, irregular respiration, and death if the dose is sufficient (Hawkins *et al.*, 1985). This molecule acts mainly by inhibiting protein synthesis, but other actions have also been described (Falconer, 1998; Terao *et al.*, 1994). The LD_{50} of CYN is 2,100 µg/kg body weight in mice at 24 h, but only 200 µg/kg at 5–6 days by

i.p. injection (Ohtani *et al.*, 1992). However, in the present context it should be noted that a number of papers have reported that crude extracts of *Cylindrospermopsis raciborskii* can have greater 24-h toxicities than would be expected from the known CYN content (Falconer *et al.*, 1999; Hawkins *et al.*, 1997). The oral toxicity is about 30-fold less than the i.p. toxicity (Seawright *et al.*, 1999). CYN has two known derivatives, one of which is toxic, 7-epicylindrospermopsin (Banker *et al.*, 1997), and the other virtually nontoxic, deoxy-cylindrospermosin (Norris *et al.*, 1999).

The main target of this toxin is the liver, but unlike the microcystins, CYN can also affect other organs such as the kidneys (tubular necrosis), thymus (atrophy), or heart (subepicardial and myocardial hemorrhage). Gastroenteritis and hepatitis can be generated by oral exposure (Duy *et al.*, 2000), and acute poisoning induces death probably due to heart failure, as suggested by Seawright *et al.* (1999). At toxic concentrations, the development of lesions is relatively minor compared to the rate of clinical progress. Recently, CYN has been found to display genotoxic activity (Humpage *et al.*, 2000; Shaw *et al.*, 2000). From the experimental data available, CYN appears to be slower acting and less toxic than microcystins.

B. Neurotoxins

The neurotoxins known to be produced by freshwater cyanobacteria include anatoxin-a and homoanatoxin-a, anatoxin-a(s), and saxitoxins. Their target is the neuromuscular system, and they can paralyze peripheral, skeletal muscle, including respiratory muscles. Death ensues as a result of respiratory arrest within a few minutes to a few hours (see reviews by Duy *et al.*, 2000; Kuiper-Goodman *et al.*, 1999).

1. Anatoxins

Anatoxins are produced mainly by *Anabaena* species, but also by *Aphanizomenon*, *Planktothrix*, *Cylindrospermum*, *Microcystis*, and the benthic *Oscillatoria* and *Phormidium* (Sivonen and Jones, 1999).

Anatoxin-a is a bicyclic secondary amine (Figure 27.1D) with a molecular weight of 165 daltons. It is a cholinergic agonist that binds to nicotinic acetylcholine receptors (nAChRs) in nerves and at neuromuscular junctions (NMJs). Subsequent depolarization that opens voltage-sensitive Ca^{2+} and Na^{+} channels can lead to muscle paralysis and death by asphyxiation (Falconer, 1998). The LD_{50} is 200 µg/kg body weight in mice by i.p. injection. The oral toxicity of a sonicated suspension of anatoxin-a-containing Anabaena cells is about 100 to 1,000 times higher (Carmichael, 1992), and the LD_{50} values in several species range from 1 to 10 mg/kg. Homoanatoxin-a is an anatoxin-a homolog (Figure 27.1E), with a molecular weight of 179 daltons, and lower potency ($LD_{50} = 250$ µg/kg body weight in mice by i.p. injection).

No documented case of human anatoxin-a poisoning has been found in the literature, whereas numerous cases of animal poisoning have been reported. In France, for example, the death of several dogs has been attributed to the consumption of benthic anatoxin-a-producing cyanobacteria (Gugger *et al.*, 2005).

The chemical structure of anatoxin-a(s) is not related to that of anatoxin-a because it is a unique *N*-hydroxyguanidine methyl phosphate ester (Figure 27.1F) with a molecular weight of 252 daltons. Anatoxin-a(s) is an acethylcholinesterase (AChE) inhibitor with a mechanism similar to that of the organophosphorus (OP) insecticides. There is no oral study of this metabolite, but it appears to be considerably more toxic than anatoxin-a, with an LD_{50} in mice of only 20 µg/kg body weight by i.p. injection (Falconer, 1998). The neurological effects in mice given acute doses are muscle weakness, respiratory distress (dyspnea), and convulsions (effect on seizure threshold), culminating in death. In pigs and mice, anatoxin-a(s) can cause viscous mucoid hypersalivation. Death often occurs as a result of respiratory arrest (Matsunaga *et al.*, 1989). The ''s'' in the name of the toxin stands for ''salivation'', because of the additional hypersalivation observed in mice.

2. Saxitoxins

Saxitoxins are well-known marine toxins produced by dinoflagellates, especially *Alexandrium* spp. and *Gymnodinium* spp. (Van Dolah, 2000; Wright and Cembella, 1998), and by some heterotrophic bacteria (Gallacher *et al.*, 1997). PSPs (paralytic shellfish poisons) have recently been identified in five freshwater cyanobacterial species: *Aphanizomenon flos-aquae* (Ferreira *et al.*, 2001; Ikawa *et al.*, 1982; Mahmood and Carmichael, 1986; Pereira *et al.*, 2000), *Anabaena circinalis* (Humpage *et al.*, 1994; Negri *et al.*, 1995), *Lyngbya wollei* (Carmichael *et al.*, 1997; Onodera *et al.*, 1997), *Cylindrospermopsis raciborskii* (Lagos *et al.*, 1999), and *Planktothrix* sp. (Pomati *et al.*, 2000). Saxitoxins (STXs) have also been identified in Danish freshwater field extracts, but without unambiguous identification of toxin-producing species (however, *Anabaena lemmermannii* is very common in these lakes) (Kaas and Henriksen, 2000).

The saxitoxins or paralytic shellfish poisons (PSPs) consist of a family of carbamate alkaloids (Figure 27.1G) containing more than 20 molecules with a tetrahydropurine structure (molecular weights from ranging from 241 to 491 daltons). They can be divided into four groups, depending on the substitutions in the five variable positions R1 to R5: saxitoxins (STX, dcSTX, neoSTX) (Humpage *et al.*, 1994; Ikawa *et al.*, 1982; Kaas and Henriksen, 2000; Lagos *et al.*, 1999; Mahmood and Carmichael, 1986; Negri *et al.*, 1995; Pereira *et al.*, 2000; Pomati *et al.*, 2000), gonyautoxins (GTX 1 to 6) (Humpage *et al.*, 1994; Kaas and Henriksen, 2000; Lagos *et al.*, 1999; Negri *et al.*, 1995; Pereira *et al.*, 2000), and dcGTX 2 and 3 (Humpage *et al.*, 1994; Negri *et al.*, 1995; Onodera *et al.*, 1997), C-toxins (C1 and 2) (Ferreira *et al.*, 2001; Humpage *et al.*, 1994; Negri and

Jones, 1995), and variants identified in American strains of *Lyngbya wollei* (LWTX 1 to 6) (Onodera *et al.*, 1997). Depending on the variants, the toxicity in the mice can differ considerably. Saxitoxin is the most potent PSP ($LD_{50} = 10$ µg/kg mouse, i.p.), and LWTX 1, 4, and 6 can be more than 165 times less toxic (Oshima, 1995).

All these toxins act in the same way: nervous transmission is blocked when the PSP binds to site 1 of the sodium channels (Catterall, 1980), and this induces muscle paralysis. In animals, typical neurological effects induced by this toxin include nervousness, jumping, jerking, ataxia, convulsions, and paralysis. The paralysis of respiratory muscles is fatal within a few minutes (Runnegar *et al.*, 1988). Saxitoxins are toxic both by ingestion and by inhalation, and they could be dispersed as aerosols and inhaled, and so lead to rapid respiratory collapse and death.

Saxitoxins have been involved in numerous cases of human poisoning as a result of consuming marine shellfish, but no documented cases have been recorded in freshwater environments.

IV. RISK ASSESSMENT

A. What are the Potential Routes of Contamination?

The two routes by which human beings could be deliberately contaminated by cyanotoxins involve either the ingestion of cells containing these toxins, or the direct ingestion of the free toxins in water. This means that for the purpose of deliberate poisoning with these toxins, the potential routes of contamination consist essentially of water or other dietary constituents to which cyanotoxins have been added, or the ingestion of cells in the form of a powder or capsules, for instance, after replacing a nontoxic strain with a toxin-producing strain.

In the case of the water-borne route, it would not be possible to envisage manipulating the cyanobacterial populations in natural aquatic ecosystems so as to replace nontoxic populations of cyanobacteria by toxic populations. Similarly, attempting to add toxins to these ecosystems would require far too much toxin to produce a sufficient level of risk; even without taking into account the fact that a fair proportion would be destroyed either naturally or during water treatment processes. The greatest threat would therefore seem to be likely to come either from the contamination of potable water supplies intended for human populations after they have been treated, or contaminating water intended for dialysis of hospital patients, for instance. The contamination of liquids intended for human consumption is another possible threat. The stability of these toxins is variable, depending on the compound concerned. In the natural setting, the microcystins seem to be fairly stable, and they are not very vulnerable to photodegradation, whereas anatoxin-a is much more sensitive to light, and this makes it much less stable. In a natural environment, microcystins have been detected in an ecosystem 21 days after they had been released into the water (Van Apeldoorn *et al.*, 2007).

With regard to dietary products, it would be possible to culture toxin-producing strains of cyanobacteria in bioreactors, or to harvest them from ecosystems undergoing natural cyanobacterial blooms, and then to process the biomasses obtained so that they can be supplied as dietary complements for use in slimming diets or high-protein diets, in the same way as spiruline is already administered. Furthermore, marketing these products online, and the lack of toxicity tests could make it even easier to use toxic strains of cyanobacteria to cause deliberate harm. Finally, the threat is increased by the fact that intracellular toxins are very stable once the cells have been freeze-dried, which means that they can pose a toxic risk extending over a very long period of time.

B. How can Cyanotoxins be Detected?

The methods required to detect and identify cyanotoxins can be time consuming, complicated, and expensive. In the case of the microcystins, for a long time, the method usually used involved first extracting the toxins in 100% methanol, and then using high-performance liquid chromatography (HPLC) coupled to a photo diode array detector (or possibly a mass spectrometer) to carry out specific identifications of the different variants (Nicholson and Burch, 2001). The main problem encountered in using HPLC was linked to the fact that only a fairly limited range of microcystin standards was available on the market, despite the large number of known variants. Subsequently, ELISA tests were used to carry out immunodiagnosis of these substances, and an indirect test was used to measure the toxicity by estimating the inhibition of the protein phosphatase PP2A. Very recently, a quick microcystin immunodetection kit has come onto the market, which takes less than 30 min to test a liquid sample and find out whether the concentrations of microcystins are above a threshold value. The detection of nodularins, which have a molecular structure similar to that of the microcystins, is based on the same type of approach.

The methods for detecting anatoxin-a and homoanatoxin-a are based either on bioassays, either a rather nonspecific mouse test or a slightly more specific neuroblastoma tests, or on chromatography after extracting these substances. There are several different methods of extracting these substances, as well as several different chromatographic methods for assaying them, based either on the use of HPLC, coupled with a UV or fluorescence (FL) detector, or with a mass spectometer, or on gas chromatography coupled with a mass spectrometer. There is an excellent review of all these methodologies in the paper of Osswald *et al.* (2007).

Finally, in the case of the saxitoxins (STXs), their extraction is based on the use of acetic acid, and preliminary concentration can be carried out on graphitized carbon black

cartridges (Nicholson and Burch, 2001). The identification and quantification are based on the use of HPLC-FL using either precolumn or postcolumn oxidation, and derivatization of the STXs. Hydrophilic interaction LC (HILIC)-MS and LC-EISI-MS-based methods are increasingly being used to assay these substances in water or in cells (Dell' Aversano *et al.*, 2004).

V. TREATMENT

Very few data are available about possible treatment protocols for use following cyanotoxin poisoning. Thus, in the case of the microcystins, Rifampin seems to be an effective chemoprotectant and antidote against MCLR toxicity, as shown by Hermansky *et al.* (1990b). Other drugs have also demonstrated protective effects, such as cyclosporin-A and silyramin (Rao *et al.*, 2004). However, in a recent article, Weng *et al.* (2007) point out that there is no effective treatment available for the liver damage caused by microcystins. Physostigmine and high concentrations of 2-PAM appear to be the only effective antagonists against a lethal dose of anatoxin-a(s) (Hyde and Carmichael, 1991). Finally, there is no specific treatment or antidote for saxitoxins, although Benton *et al.* (1998) have shown that saxitoxin-induced lethality can be reversed by 4-aminopyridine administered during the early stages of respiratory depression.

VI. CONCLUDING REMARKS AND FUTURE DIRECTION

No terrorist attack involving cyanotoxins has been reported to date. However, cyanobacteria and their toxins cannot be discounted as a potential threat, because large quantities of toxins sometimes exist under natural conditions in aquatic ecosystems, and in most cases, it would be quite easy to extract them. However, it is not always easy to culture cyanobacteria on a large scale, and this could considerably restrict their large-scale use. Furthermore, the lower toxicity observed after oral ingestion of several cyanotoxins, compared to their toxicity by the i.p. route, means that large quantities of toxins would potentially be required to contaminate large drinking water reservoirs, and this constitutes a second restriction on their use by terrorists. The main threat from cyanotoxins would therefore seem to be their use in relatively small-scale terrorist attacks, for example involving the contamination of food supplies or of small reservoirs of drinking water.

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CHAPTER 28

Radiation and Health Effects

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I. INTRODUCTION

Radiation cannot be seen; however, its interaction with matter is partially understood at a theoretical and an experimental level. Its effects in humans have been extensively explored in large studies (Harley, 2001, 2008). These studies were those in which large numbers of people had been exposed; the doses and responses were as well known as in any branch of toxicology or chemical safety (Harley, 2008). These studies included the radium dial painters, survivors of the atomic bomb, patients with ankylosing spondylitis, children treated for ringworm (tinea captis), the workers and residents around Chernobyl and uranium miners exposed to radon soil gas, and to the short-lived radon daughter isotopes. The major health effect so far at the lowest doses has been cancer. Only at higher doses [>0.5 seivert (Sv); 500 milliSv (mSv); 50 roentgen equivalent in man (rem)] have some heart and digestive diseases been noted in atom bomb survivors (Harley, 2001, 2008).

Models of radiation damage have been explored and are well developed. For many years, accidental releases of ionizing radiation have been universally feared. Alternatively, radiation has been used in controlled, defined amounts as a therapy for certain tumors in both companion animals and humans. Taken together, all studies provide a cohesive and comprehensive picture of radiation toxicity. There are sufficient details of radiation effects to make credible estimates of risk resulting from radiation exposure (Harley 2001, 2008).

Since acute radiation toxicity responses become apparent shortly after exposure, history is an important criterion in determining whether the radiation is related to the cause of a particular complication or adverse effect. As with any attempt to specify a dose–response relationship, the dose is an important component. In contrast, late radiation toxicity in organs such as the kidneys, liver, or central nervous system (CNS) will not be seen until months or perhaps even years after radiation exposure (Center for Drug Evaluation, 2005). The integrated response is often to the radiation response and attempts to heal any radiation damage that has been caused.

Natural background radiation is substantial. Attempts to determine if there were subtle effects from this background irradiation have only begun to yield information about these worldwide effects; techniques for such estimation have increased in sensitivity and reliability.

II. BASIC RADIATION CONCEPTS

The four main types of irradiation are x-rays, gamma rays, electrons (negatively charged beta particles or positively charged positrons), and alpha particles. An atom can decay by the loss of mass (helium – loss of two protons and neutrons), or the loss of positively or negatively charged electrons (beta particles or positrons). Gamma irradiation occurs when excess energy is released from the nucleus, usually after an alpha, beta, or positron transition (Harley, 2001, 2008).

A. Radiation Energy

$$\text{Energy} = \left\{\frac{1}{2}\right\}(\text{Particle mass})(\text{Particle velocity})^2$$

Both high amounts of mass and high velocity can be used experimentally (artificially) to contrive releases of large amounts of energy such as neutron activation by bombarding an atom.

As particles approach the speed of light estimations of their energy must be corrected for relativity using

$$\text{E} = 0.511(1 - \text{Particle velocity}^2/\text{Light velocity}^2) + 0.511$$

Alpha particles have sufficiently low velocity relative to the speed of light that no correction is required for relativity (the velocities ratio is very small and approaches 0).

Gamma rays are pure electromagnetic radiation with energy = (6.626×10^{-34} joules/second – Planck's constant) (frequency of the radiation). Conventional units of radiation to be considered are one electron volt (eV) = 1.6×10^{-19} joules and one million electron volts (MeV) (Harley, 2001, 2008).

B. Alpha Particles

Alpha particles are a helium nucleus (two protons and two neutrons) ejected from the nucleus of a radioactive element

$$^{226}\text{Radium}_{86} \text{ decomposes to } ^{222}\text{Radon}_{84}$$
$$\text{and an alpha particle } (^{2}\text{He}_{2}^{++}) + \text{Energy (5.2 MeV)}$$

Most alpha particles are in the range 4–8 MeV. There are several, discrete monoenergetic alphas emitted from most alpha emitters, not just a continuous spectrum of emissions (Harley, 2001, 2008).

C. Beta Particles, Positron and Electron Capture

Beta particles are emitted when a neutron is converted to a proton plus an electron and the electron is lost. Unlike the discrete energy emissions from the decay of alpha particles, beta particles are emitted along a spectrum of energies, because energies are shared between positive and negative electrons. Positrons are emitted when a proton becomes a neutron and decays by beta emission or an electron is captured. These are competing processes, and both occur with about the same frequency (Harley, 2001, 2008).

D. Gamma Ray (Photon) Emission

Gamma ray emission is mostly secondary to an alpha, beta, or positron emission or electron capture. When all of the energy is not used by the emission, the nucleus is in an excited state with energy available for emission. This excitation energy is emitted as a photon when the particle is emitted. In many cases the photon will not be emitted, but bind to an electron which is ejected as a mono-energetic particle equal to the photon energy minus the binding energy. This latter process is called internal conversion (Harley, 2001, 2008).

III. INTERACTION OF RADIATION WITH MATTER

Ionizing radiation loses energy by producing ion pairs (an electron and a positively charged atom). About 33.85 eV is needed to produce an ion pair. This is twice the energy needed for ionization, which is lost in the ion pair formation. Alpha and beta particles and gamma rays lose energy in somewhat different ways (Harley 2001, Harley 2008).

A. Alpha Particles

An alpha particle is heavily charged with a mass equal to 7,300 times that of an electron. Yet energy can be transferred to particles going only twice as fast as the alpha particle. Since it requires 33.85 eV to produce an electron pair, a 5 MeV can produce ~7,400 pairs within 1 micron (micrometer) of the decay. One micron (1/1,000,000 meters; 1/1,000 millimeters; 15% of the diameter of a human red blood cell) is much thinner than a sheet of paper. This linear energy transfer (LET) is said to be much greater than is needed to have DNA strand breakage. Thus, it is said to have a high LET.

B. Beta Particles

Energy loss in matter cannot be simplified as it can in alpha particles because:

1. Even low energy beta emitters are traveling near the speed of light and must be corrected for mass increase by the term

$$0.511(1 - \text{Particle velocity}^2/\text{Light velocity}^2) + 0.511$$

2. Electrons are interacting with particles of the same mass; large energy losses are possible
3. Radiative (Bremstrahlung) energy losses from particles slowing down are appreciable (Harley, 2001, 2008), while with alpha particles they are not.

C. Gamma Rays

$$\text{E in MeV } \text{m}^2/\text{kg} = \text{Mass energy absorption coefficients } (\text{m}^2\ \text{g}^{-2})(\text{initial photon energy})$$

Mass energy absorption coefficients in air and in muscle are somewhat similar between 0.01 to 3.0 MeV. Energy loss/unit length is obtained by multiplying absorption coefficients by density (kg/m^3). This LET particle can penetrate much further into tissue. Thus, radiation dose is distributed over much greater areas, tending toward diffuse as opposed to focal distribution (Harley, 2001, 2008).

If lead shielding were used, density would be much greater, and penetration would be much less beyond the lead shield. If energy emitted is sufficiently low penetration can be taken to be negligible. A lead shield is often used when obtaining diagnostic x-rays as a safety feature which greatly reduces the exposure to gamma irradiation. Some fraction of energetic gamma emitters (e.g. ^{60}cobalt) irradiation can penetrate even lead and added earth shielding. This radiation leakage is usually measured to determine external radiation from a source and shielding. It may be as high as 1–10% of the source strength depending on the amount of shielding used if therapeutic gamma instead of x-irradiation is used.

IV. ABSORBED DOSE

A. Total Dose and Dose Rate

Total dose is the total energy absorbed per unit of mass; this measurement is independent of the time taken to deliver it (the dose rate) (Harley, 2001, 2008). If the dose is delivered quickly in a single exposure at a high dose rate, there is almost no time to repair radiation damage and damage/unit of dose absorbed is high (Harley, 2008). Alternatively, if the total dose is delivered quite slowly at a lower dose rate, or in divided dose fractions, say over the lifespan of the animal,

there is more time to repair radiation damage and damage/unit of dose is much lower.

Very little time is required to initiate repair. For example, if multiple fractionated doses are delivered per hour, the damage/unit of total dose decreases significantly. In this instance, the radiation therapist can deliver higher doses to the area, but less total rem/h from the source or x-radiation machine. Because charges are frequently per rad, the use of fractionated radiotherapy is small, but does occur. Fractionated radiotherapy leads to the emergence of a more homogeneous population of live cells from cell repopulation and by the high degree of radiosensitivity of the G2 cells that is a decrease in the percentage of G2 cells.

Variations in the chromatin pattern of the cells may be explained by DNA repair processes (El-Khattabi *et al.*, 1997; Murata *et al.*, 1998; Harley, 2001). While these data may be relevant to higher radiation doses required for therapy where present tolerable doses are merely palliative, protraction does lengthen the time and therefore the cost required to deliver that dose.

Absorbed dose is a function of the mass and density of the media and is equal to the mean energy deposited in mass divided by the mass of the target organ. Sometimes absorbed dose is called kerma (kinetic energy released in matter). Dose is delivered to target organs. Because exposure and dose are often used interchangeably, dose is often confused with exposure level. For comparison, exposure level is radiation in air = total radiation ions (one sign) per unit mass of air (Harley, 2001, 2008).

B. Equivalent Dose and Cancer Risk

Alpha particles with large mass produce intense ionization tracks per unit distance relative to beta particles. Beta particles produce more intense ionization than do gamma rays. Linear energy transfer (LET) measures this transfer of energy per unit distance traveled by the emission from the radiation particle. In situations where dose is considered to be proportional to response, that dose must be normalized for LET. For a particular endpoint (e.g. cell death in mouse fibroblasts), frequently one calculates relative biological effectiveness (RBE). RBE = dose of radiation under study relative to the dose of gamma rays to achieve the same effect. Radiation with high LET will be expected to have an RBE of greater than 1.

We calculate equivalent dose as:

$$\text{Equivalent dose} = (\text{Dose})(\text{Weighting factor or relative biological effect (RBE)})$$

Weighting factors recommended are approximately 1 for x-rays, gamma rays, beta particles, and electrons and 20 for conventional neutrons (>>>0.1 to 2 MeV), protons, alpha particles and charged particles of unknown energy.

For example, the weighting factor and RBE of plutonium-238 (^{238}Pu) is about 10–30. Thus, the roentgen equivalent in man (rem) is 10–30 roentgen dose in air (rads). This radiation dose pattern following alpha emission is confined to an immensely small area because alpha irradiation travels only a short distance in tissue, but has a high LET. Because the dose is in a small area it is said to be focal as opposed to being diffuse if lower energy transfer were operating and penetration was greater. It is not uncommon to have focal dose correspond to focal response in alpha particle exposure (Harley, 2001, 2008). Thus, one might see millions of ''burned out zones'' or indicators of cell death following alpha emission if microscopic resolution were sufficiently good.

The term effective dose allows comparison of cancer and genetic risks from different partial body and whole body doses to be integrated for the purposes of estimating a whole body response. Some tissues (gonads weighting factor = 0.2) are more sensitive than others (red bone marrow, colon, stomach, and lung weighting factors = 0.12; bladder, breast, liver, esophagus, and thyroid weighting factors = 0.05). Skin and bone surface are the least sensitive (weighting factors = 0.01). Other tissues not specified are taken to have a weighting factor of 0.05 as a conservative estimate (Harley, 2001, 2008).

It is useful to calculate doses using the tissue weighting factor in protracted exposures (exposures over significant periods of time). Most nonexperimental companion or domestic animals do not receive protracted exposures. A major exception to this is the animal receiving repeated fractionated doses as radiation therapy, or the relatively rare animals in which radiation has been implanted so that it acts as an internal emitter of radioactivity (Harley, 2001).

C. Committed Equivalent Dose

Once radioactivity is placed internally it becomes an internal emitter. Internal emitters are used infrequently either therapeutically (actually implanted in a tumor) or with accidental exposure via ingestion or inhalation or penetration through skin; internal emitters may be removed only with varying degrees of difficulty (Harley, 2001). Usually, they cannot be removed simply or easily and an irreversible committed equivalent dose is said to exist.

For radionuclides with half-lives zero to 3 months the equivalent dose is equal to the annual dose of the year of intake (Harley, 2001). It is often convenient to estimate the fraction of the committed equivalent dose which remains. For a radionuclide with a half-life of 3 months, after 1 year of exposure, it had undergone the passage of four half-lives so that fraction of dose delivered was $(1 - ½^4) \sim 94\%$ of the total dose for all time. Thus, 94% of the possible dose had been delivered and only 6% remains to be delivered. Those with shorter half-lives would approach 100% more closely (half-life of 1 month $(1 - ½) \sim 99.98\%$ of the total dose for all time). In both cases, any effort to remove the particle will reduce the internal dose only minimally.

Alternatively, if the half-life was long, say 1, 10, or even 5,000 years, considerably less radioactive decay would have occurred. Thus, considerably more of the total dose would remain to be delivered $(1 - ½) \sim 50\%$, $(1 - ½^{1/10}) > 0\%$, and $(1 - ½^{1/5,000}) >>> 50\%$ and nearly 100%, respectively at the end of 1 year, making removal of the radioactive particulates of greater advantage at that time to the reduction of risk. It is a good idea to perform such analyses with each radionuclide to see how much of the dose remains to be delivered; thus, one can assess the relative advantage of going to considerable effort to remove the remaining dose.

D. Negligible Individual Risk Level (Negligible Dose)

Radiation is feared because its delivery cannot be seen, and is often not measured, because it is only in retrospect that one realizes that radiation exposure has occurred. We accept the concept of linear, nonthreshold cancer induction from ionizing radiation (Harley, 2001, 2008); this allows calculation of cancer risk regardless of how small (or large) the dose may be. Calculating very low risks leads to calculating a risk that one would consider acceptably low, that is a risk below the ability to detect a response (Harley, 2001).

The National Council for Radiation Protection (NCRP) has identified a negligible individual risk level (NIRL) as a level of annual excess risk of health effects attributable to irradiation below which further effort to reduce radiation to the individual is unwarranted. The NCRP emphasized that this level should not be confused with an acceptable risk level, a level of significance or a standard. The NCRP recommended a level around half the natural background radiation level; the final recommended NIRL level is 1 mrem; this level is now called negligible individual dose (NID) level (Harley, 2001, 2008).

Effective dose was a parameter used to assess biological risk related to radiation exposure, from dual-energy X-ray absorptiometry (DXA). Children are a worst case estimate because they absorb higher doses than adults. With the exception of the hip scans in 1- and 5-year-old children, the effective doses were below the negligible individual dose limit of 1 mrem per year (Thomas *et al.*, 2005).

The NCRP recommends an annual effective dose for continuous members of the public in some circumstances of 1 mSV (100 mrem). This value is in addition to natural background level of irradiation approximately twice that (2 mSv; 200 mrem). In this context, the NIRL was taken to be 1/100 of this level, or 0.01 mSv/year (1 mrem/year). This level of exposure was low enough to have a risk of <1 cancer/1,000,000 and the risk for lung cancer $<1/10,000,000$. The notation is negligible individual dose (NID) (Harley 2001, 2008).

E. Radiation Safety for Diagnostic Imaging

Diagnostic imaging with radiation must be performed with proper protection (Kahn and Line, 2005). Although exposure factors are substantially lower than with past diagnostic imaging, a considerable radiation dose may be delivered over time if proper protection is not worn. Lead gloves will lower external radiation dose $\sim$1,000-fold from scatter radiation, not in the direct radiation beam, but only 10-fold from radiation when directly in the beam of irradiation. Thyroid shields and eye shields are recommended. For example, upper limb and skull studies are especially likely to result in substantial exposure to anyone holding the film or restraining the animal (Kahn and Line, 2005; Pickrell, 2007).

V. MECHANISMS OF DNA DAMAGE AND MUTAGENESIS

A. Energy Deposition in the Cell Nucleus

Radiation is released and slows down by forming ion pairs (Harley, 2001, 2008). Different ion densities result in the formation of alpha particles, beta particles, or gamma rays. Tract structure is roughly characterized into sparsely populated (low LET) and densely populated (high LET) tracts. Each tract from the x-irradiation of gamma rays results in 70 events across the width of the nucleus for $\sim$0.5 rads [5 mGrey (mGy); 500 mrads]. Alternatively, a 400 MeV alpha track may produce 30,000 events across the same nucleus for $\sim$300 rads (3 Gy). Within the nucleus even low LET gamma radiation may produce some microregions of relatively dense ionizations in the DNA region. The threshold is very low for some single strand breaks (Panajotovic *et al.*, 2006, cited in Harley, 2008).

The ability to capture environmental CO_2 to form backbones of macromolecules such as DNA or proteins needed to be preserved robustly to sustain both memory (faithful reproduction) and contingency (variation), if both were to be permitted and preserved (Smith, 2008). A significant percentage, but far from all of the volume of the nucleus is expressed as fibers (flexible cylinders; worms) of DNA, indicating a higher level of organization (Jones, 2004). Both genetic and epigenetic processes occur in the nucleus.

Within a cell indirect effects occur at sites within nanometers of the direct effects. Bigger tracts are caused by radiation that is a high LET. Low doses of high LET result in a few cells being hit by a single track. Low LET radiation tends to be spread out over the cells more evenly. One gray of radiation will produce 1,000 single strand breaks and fewer double strand breaks ($\sim$40); intermediate levels of damage were seen in base damage (500 breaks) and deoxyribonucleic acid (DNA) protein crosslinks (150 breaks). About 30% of the double strand breaks are complex because of additional breaks (Harley, 2001, 2008).

In the past 15 years, research on epigenetic (nontargeted) effects has shown that the nucleus need not be irradiated for health effects to occur (Little, 2006, cited in Harley, 2008). Epigenetic effects include: genomic instability – increased genomic alterations; bystander effects – effects in cells not traversed by radiation, but very close by; cytoplasmic/membrane effects – bystander effects following cytoplasmic irradiation; clastogenic effects – detrimental effects from irradiated plasma; abscopal effects – whole organ damage resulting from partial volume irradiation; and trans-generational effects – instability passed through the germ line. Risk calculations are based on observed exposure response in human and animal populations. Thus, while contributing greatly to mechanistic and biological understanding, this new epigenetic information should not affect the risk calculations (Little, 2006, cited in Harley, 2008).

B. *In Vitro* Irradiation Studies

Small lymphocytes from horses were transformed into larger radioresistant lymphoblast-like cells following stimulation by phytohemagglutinin *in vitro*. One hundred rads x-irradiation killed all the small lymphocytes, but only around one-third of the lymphoblast-like cells (Dewey and Brannon, 1976). Horse lymphocytes were more radiosensitive than human lymphocytes. The ratios between doses inducing the same effect are 1.3, 1.7, and 9.4 for the number of binucleated cells with micronuclei, micronucleus frequency in binucleated cells, and DNA synthesis inhibition, respectively (Catena *et al.*, 1997).

In cattle, at any LET value, repair is much slower after heavy ion exposure than after x-irradiation. For ions with an LET of less than 10,000 keV/μm more than 90% of the strand breaks induced are repaired within 24 h. At the highest LET value (16,300 keV/μm) no significant repair is observed (Baumstark-Khan *et al.*, 2003). Thus, a steep dose–response effect is seen at higher doses.

The baseline frequency of SCEs was similar in the three species and no significant variation in human, cattle, or muntjac (deer) cells was seen even after administration of 400 rad of x-rays (Das and Sharma, 1983). Preirradiation of artificial vessel walls substantially decreased the wall's capabilities to resist fibrosarcoma-induced lysis and abilities of blood vessels to limit extravasation and perhaps invasion (Heisel *et al.*, 1984).

X-rays induce DNA damage including strand breaks that lead to the formation of micronuclei and chromosomal aberrations; x-irradiation was associated with an increased number of apoptotic cells (Konopacka and Rzeszowska-Wolny, 2006). This phenomenon was termed ''bystander effect''. A number of studies suggest that bystander effect appears to be associated with up-regulation of oxidative metabolism. The factors causing micronucleation by x-irradiation, oxidative DNA damage, and incomplete repair may be regulated by apoptosis-independent pathways (Konopacka and Rzeszowska-Wolny, 2006).

Immature B lymphocytes in the chicken bursa of Fabricius have previously been reported to undergo apoptosis by low doses of ionizing radiation. Increase in the number of pyknotic cells was observed at a dose of as little as 1 Gy. The chicken bursal cells are hypersensitive to x-irradiation with regard to induction of apoptosis, and the apoptotic bursal cells exhibit most of the ultrastructural features known to be typical of apoptosis (Arai *et al.*, 1996).

VI. ANIMAL EXPOSURES AND RADIATION TOXICITY

Animals may show acute irradiation-induced changes from high single doses of radiation from either external or internal emitters. Alternatively, irradiation regimens may be devised which have high radiation dose rates. Cells likely to be most sensitive to high doses of irradiation may be used to model the changes expected to be seen from these radiation doses. High external doses are most commonly accumulated from either an x-irradiation source or a ^{60}cobalt gamma ray source. Usually diagnostic x-irradiation will not be sufficiently high to produce acute symptoms. However, if therapeutic irradiation is given as a single dose high doses of external irradiation may be sustained. Most often very high doses of external irradiation given for therapeutic purposes are fractionated over several to many doses, allowing a more total radiation dose to be sustained without causing clinical signs of radiation toxicity to appear, because of the lower radiation dose rate. Mammary tumors are sometimes treated with external irradiation. The radiation field commonly includes the areas occupied by the intestines and lungs. Less commonly, fugitive releases (reactor accidents) may release radioactive iodine which localizes to irradiate the thyroid glands.

The highest levels of this type of radiation produce CNS changes. It is unlikely that anything but a gross miscalculation of radiation dose rate would lead to this high level of radiation. At ultra-high external irradiation doses, humans and animals die quickly (<1 week) with associated CNS nervous signs. Disruption of the blood–brain barrier (BBB) occurs after radiation injury; it is detectable by magnetic resonance imaging or horseradish peroxidase studies. For example, rats, irradiated at 60 Gy, that were serially sacrificed at 2 to 24 weeks showed a detectable disruption of the BBB at 2 weeks post-irradiation; disruption of BBB preceded white matter necrosis (Rubin *et al.*, 1994). In humans, one pilot study administered 55–60 Gy which as a single exposure would have been fatal; however, this dose was hyperfractionated in 110 cGy fractions. The hyperfractionation allowed completion of the course of radiotherapy. Tumor regression was noted, with minimal recurrence of tumors and no overt radiation toxicity (Fontanesi *et al.*, 1995). Thus, hyperfractionation allows delivery of high doses that were therapeutic to tumors.

At high levels of this type of irradiation, but below the levels capable of inducing CNS signs, the short-lived intestinal epithelial cells die in massive amounts and gastrointestinal (GI) mucosal changes are said to occur. Death usually occurs in 1–2 weeks. Again, only the worst radiation accident or miscalculation of dose would lead to exposures of this level. For example, Chernobyl had fatalities in 30 of ~3,000 staff members, but no record of GI changes being the predominant cause of death (Harley, 2001).

At lower levels there can still be appreciable clinical contributions from gastrointestinal mucosal changes; great effort is made to minimize the importance of these to people or animal survival. One such way of improving chances of survival from radiation therapy is directing the radiation toward the field of the tumor; we call this involved field radiation therapy (IFRT) (Albuquerque *et al.*, 2005). This is relatively simple to effect in large animals such as humans; it is more difficult to focus the beam sufficiently for the same advantages in smaller dogs. Twenty ovarian cancer patients treated with 50.4 Gy of directed radiation were 66% 5-year recurrence free and 33% disease free. Thus, directed beam radiation therapy that would have been fatal if not directed minimized the consequences of human ovarian cancer (Albuquerque *et al.*, 2005). After radiation treatment for gynecological cancer, patients gained more body fat than expected, e.g. Chilean women around menopause; the loss of fat-free mass observed during radiation treatment was probably associated with infrequent physical activity (Pia de la Maza *et al.*, 2004). The extent to which this effect occurs in older dogs has not been determined.

At still lower doses failure to renew blood cells from bone marrow aplasia or hypoplasia may occur. These occur at low enough doses that radiotherapy may induce them clinically. Hyperfractionation of radiation dose helps minimize the consequences of these changes by lowering the overall dose rate. When human external beam irradiation 50–70 Gy was combined with cisplatin chemotherapy to treat head and neck cancer 6% of the patients had groin hematomas requiring no treatment. In addition, 6% of the patients had mucosal events; hematologic changes were less frequent at 2% (Gemmette, 2003). Intensive induction chemotherapy combined with external beam irradiation therapy of children with brain stem gliomas produced objective improvements in 50% of cases; this treatment course also produced severe but manageable hematologic toxicities. Data suggested that future radiation therapy regimens may require hematologic toxicities to be managed (Benesch *et al.*, 2001). IL-17A is a T cell-derived proinflammatory cytokine required for microbial host defense (Tan *et al.*, 2006). *In vivo* expression profoundly stimulates granulopoiesis. Knockout mice establish IL-17A as an inducible mechanism that is required for recovery of granulopoiesis after radiation injury (Tan *et al.*, 2006).

Below 10 mSv/day (1,000 mrem; 1 rem per day) organisms show some indicators of response (Fliedner and Graessle, 2008). Between 10 and 100 mSv/day animals are capable of dealing with excessive cell loss. Above 100 mSv/day dogs have progressive shortening of their lifespan. The mechanisms are compatible with an excess cell loss (relative to steady state) that is a function of the daily dose rate. Once the stem cell pool is approaching exhaustion level, a ''turbulence region'' is reached and takes only a small amount of stress for the system to fail. It is important to understand the pathophysiology of the cell renewal systems in order to allow one to predict the development of radiation-induced lesions (Fliedner and Graessle, 2008).

Depending on dose, external irradiation may lead to different types of reproductive toxicity in the male companion animal (Scialli *et al.*, 1995, cited in Ellington and Wilker, 2006; De Celis *et al.*, 1996, cited in Ellington and Wilker, 2006). At very high doses, it may lead to permanent aspermia. At intermediate doses it may lead to reduction in sperm numbers. Finally, at lower doses external irradiation may lead to DNA alterations in sperm cells (Scialli *et al.*, 1995, cited in Ellington and Wilker, 2006; De Celis *et al.*, 1996, cited in Ellington and Wilker, 2006).

Finally, at lower levels, but with continuing irradiation, degenerative changes, for example cancer, are of the greatest concern. Usually, these changes are caused by fugitive releases of radiation (reactor accidents). The Chernobyl reactor accident released massive quantities of ^{131}iodine causing increased thyroid cancer in Belarus (>100 thyroid cancers/year). Less than one-third of this increase was seen in Ukraine and the Russian Federation (Harley, 2001). No such tumors are reported for companion dogs, but they were similarly exposed and some tumors may have occurred. Widespread radioactivity was released across much of Europe and has been recorded >10,000 miles around the world at Fiji atoll. Increased levels of ^{90}strontium and ^{131}iodine were in European cows' milk near the time of Chernobyl.

Thirteen dogs with invasive thyroid carcinoma were treated by four once-weekly fractions of 9 Gy of 4 MeV x-rays of external beam irradiation (Brearley *et al.*, 1999). Four of the dogs died from primary thyroid carcinoma and four from metastatic spread. Of the remaining five dogs, three died of unrelated problems, and two were still alive at the time of the census. Median survival time from first dose to death from either primary or metastatic disease was 96 weeks, with a range of 6 to 247 weeks. Radiation therapy should be considered an important modality for the control of invasive canine thyroid carcinoma (Brearley *et al.*, 1999).

Animals receiving fractionated external radiation therapy for mammary tumors may develop chronic pulmonary degenerative disease because the thorax is irradiated. To achieve the desired therapeutic effect, the dose may be too high to heal by primary intent; thus, they will heal by secondary intent – fibrosis. Type II cell proliferation is a common early event in radiation toxicity to the lungs.

Following 10–15 Gy of x-irradiation, the first wave of type II cell proliferation correlated with an increase in surfactant in alveolar fluids (Coggle, 1987). The second wave follows an additional delay allowing the alveolar epithelial continuity to be sufficiently compromised by the low rates of type I alveolar pneumocyte loss; type I cell loss triggered a compensatory wave of type II cell divisions that contrasted with the dramatic and immediate hyperplastic responses which many toxic irritants produce in type II epithelial cells (Coggle, 1987). At later times, fractionated external thoracic irradiation 1–2 times weekly to achieve 4,000 to 6,000 rads modestly increased total lung collagen (Pickrell *et al.*, 1975, 1983). Collagen production was initially increased, leading to an increase in total lung collagen; collagen metabolism returned to a more normal level at later times (Pickrell *et al.*, 1975, 1983).

Dogs exposed to ^{226}radium to establish coefficients for ^{239}plutonium produced data that showed the best way to translate data to humans was to use a two-mutation model to calculate radiation risks to supplement published risk estimates (Bijwaard, 2006; Bijwaard and Dekkers, 2007). The total exposure resulting from a 5 mCi administration of 18F fluoroethyl cyanophenoxy methyl piperidine 18F SFE as a tracer show it to be safe in human positron emission tomography (PET) imaging studies (Waterhouse *et al.*, 2006).

The relation of static compliance of excised lungs to collagen accumulation and histologic fibrosis was examined in Syrian hamsters inhaling sufficiently high LET $^{238}PuO_2$ particles to achieve initial lung burdens of 50 or 100 nCi (Pickrell *et al.*, 1983). Hamsters exposed to 50 nCi $^{238}PuO_2$ showed normal collagen content and static lung compliance with minimal histologic fibrosis 288 days after exposure. In contrast, hamsters exposed to 100 nCi had significant pulmonary fibrosis at that time and the highest incidence of dense scars at any time period. Such findings are consistent with a stiffening of lung parenchyma. Hamsters had increased total lung collagen, reduced lung compliance, and histologic evidence of diffuse interstitial fibrosis. The diffuse interstitial fibrosis developed by this injury resolves spontaneously as indicated by total lung collagen, compliance, and histology; dense fibrous scars, however, do not resolve (Pickrell *et al.*, 1983; Pickrell and Abdel-Mageed, 1995).

Total body irradiation-induced changes in expression of CD25 and CD71 activation markers on the surface of lymphocytes suggest that radiation may alter tumor surveillance. Taken together, the relative percentages and activation status of immune cell compartments support the conclusion that these total body irradiation-induced changes function to slow tumor progression.

VII. HUMAN EXPOSURES AND RADIATION TOXICITY

The major health effects at the lowest doses have been the production of cancer – such production has often been influenced by exposure to tobacco smoke. Only at higher doses [>0.5 seivert (Sv); 500 milliSv (mSv); 50 roentgen equivalent in man (rem)] have some heart and digestive diseases been noted in atom bomb survivors (Harley, 2001, 2008).

Six major studies in which dose and response were well characterized have been reviewed by Harley (2001, 2008). The worker and environmental populations have been studied to make certain that there is consistency in radiation risk data extrapolated from higher exposures.

A. Radium Exposures

The highest effects were seen in the radium dial painters who tipped their paint brushes during painting. The only late effect was osteogenic sarcoma; no leukemia was noted. Radium was retained in the body >1% after ~1,000 days (>2.5 years); loss of radium from the body matched the simple power function in several studies

$$R = 0.54t^{-0.52}$$

where t is the total time in days. The fit was good except at long times after exposure (Norris cited in Harley, 2008). Radium once taken up is somewhat similar to calcium. It is incorporated on the surface of the bone into the bone matrix. Raabe *et al.* (1980; cited in Harley, 2008) modeled dose and risk and found a practical threshold (a level sufficiently low that bone cancer will not appear) from these radium exposures in the normal human lifespan to be 0.04 Gy (4 rads/day) or a total dose of 0.8 Gy (80 rads) to the skeleton.

European children and adults were treated with ^{224}radium for tuberculosis or ankylosing spondylitis. Data analyzed together showed that they had an overall risk of 0.01 per Gy (100 rads) at a total dose of 1 Gy, which nearly doubled by a total dose of 10 Gy to 0.02 per Gy; no excess leukemias were noted (Harley, 2008).

B. Atom Bomb Survivors

The survivors from atomic bombs dropped in August 1945 were analyzed. Within 1 km of the epicenter 64,000 people were killed by the blast. From 1–2 km of the epicenter, people received doses as high as several Sv. At more than 2.5 km, irradiation was not significantly above background. Risk estimates are important because they are used for occupational exposure guidelines. The latest updates report that 5% of the solid cancer deaths and 0.8% of the noncancer deaths were estimated to be due to radiation exposure (>9,000 cancer deaths; >31,000 noncancer deaths; 47 years of follow-up).

Projected cancer incidences (>9,000 cancer deaths) for 100 mSv (0.1 Sv) were 0.8 and 1.3% (1.2 and 1.8% for a DS86 subcohort) for men and women, respectively. Looking at specific organs in this subcohort, incidence was as high as 0.52% for breast cancer in females and 0.23% for both liver and lung in males. Examination of smoking

interaction with lung cancer showed no interaction, that is smoking and atomic bomb radiation acted independently and not multiplicatively in lung cancer induction.

More than 31,000 excessive noncancer deaths were seen at doses higher than 0.5 Sv (500 mSv) as significant excesses of heart disease, stroke, digestive diseases, and respiratory diseases.

C. Children Irradiated with X-Irradiation for Ringworm (Tinea Captis)

More than 2,000 children who were irradiated for ringworm at New York University Medical Center over 20 years (age range 1–15 years) were compared to 1,380 control children. An additional 11,000 children (mean ages 7 to 8 years) irradiated for ringworm in Israel were also compared to control children. Significant increases in skin cancer, cancer of the scalp, and thyroid malignancies were noted. Risk of basal cell carcinoma of the skin was highest at 0.32 per Gy (100 rads). Total incidence was 0.01 per Gy for males and 0.04 per Gy for females. Total mortality from all sites was ~10% of the incidence (0.001 for males; 0.004 for females).

D. Chernobyl and Thyroid Cancers

The Chernobyl incident (April 1986) was the largest radiation exposure of recent times. Chernobyl resulted in a rapid increase in reactor power, vaporization of water in the reactor that blew the reactor core apart and destroyed most of the building. During that year, 220,000 people from areas near or surrounding the reactor were evacuated. Approximately 250,000 people were relocated from Belarus, the Russian Federation, and Ukraine.

About 280,000 persons were initially given status of liquidators; these were workers who took part in reactor mitigation or clean-up. This number would later increase to ~600,000.

Within the following few weeks 28 of the 32 acute deaths of exposed employees were judged due to radiation exposure. Thyroid cancer in children under 18 rose from an incidence of 0.5 of 100,000 (1986–1988; baseline) to more than ten times that level (5–8 per 100,000) in Belarus (1993–2002). Increases were just as consistent, but of less magnitude for Ukraine, going from 0.2 per 100,000 from 1986 to 1988 (baseline) to 5–10 times that level (1 to 2.2 per 100,000) from 1993 to 2002. There is little doubt that Chernobyl radiation caused thyroid cancer. In 2,000 there were 4,000 cases of thyroid cancer in children under 18 drinking milk contaminated with ^{131}I; in 2002 there were 12 deaths related to these exposures.

Many countries had large supplies of potassium iodide (KI) to block the uptake of ^{131}I. Had KI been available, and use of the contaminated milk been discontinued in favor of other sources, it is doubtful that large numbers of thyroid tumors would have occurred.

E. Patients Irradiated with X-Rays for Ankylosing Spondylitis

About 14,000 persons, mostly men, were exposed to x-rays to relieve and/or cure ankylosing spondylitis. Organs were partially and variably shielded; cancers occurred 6–20 years after exposure. Leukemia and cancer to the esophagus were significantly elevated relative to the control population but had relatively low risks of cancer [0.0011 and 0.007 per Gy (100 rads)]. Risk of lung cancer was about 2–3 times as high as that for esophagus or leukemia at 0.0022 per Gy. Noncancer deaths were about 30% higher than a carefully matched control population related to this cohort (Harley, 2008). Control populations were closely matched to those exposed, so that radiation effects might be investigated.

F. Underground Miners Exposed to Radon

Most cancers from radon were produced by radon daughter decay products (polonium – 3 isotopes; bismuth – 1 isotope; and lead – 3 isotopes). ERR (excessive relative risks) of cancers varied, ranging from 0.002 to 0.08 per working level months (WLM) of 170 h of exposure. One WLM is about 200 pCi per liter in a home and 300 pCi per liter in an underground mine. Relative risk increases from 1 to 10 at 2,500 WLM and 16 at twice that exposure level (5,000 WLM) in the Colorado cohort (Harley, 2008).

Aerosol size has an effect on the lung dose. Fine particles breathed through the mouth or the nose from 100 nm to 1,000 nm median size had 5–20 nGy per becquerel. Below 100 nm, this number more than doubled at 25–65 nGy per becquerel at 20 nm median size. Median sizes had an approximate geometric standard deviation of 2, so that the 95% confidence interval of particle sizes ranged from 0.25 to 4 times the stated median size. Very small particles deposited more efficiently in the airways. Lung cancer was related to radiation dose. These dose estimates are important determinants of lung cancer.

The greatest risk was at 5–15 years after initial exposure; later risk fell to 0.5 of that level and in those aged 55–64 to 0.4 of that level. Cancer fatalities were thought to be 0.0003 per working level month. Smoking may have had a modest effect. For example, there are 15,000 lung cancer cases in people exposed to radon and radon daughters, 10,000 in those who have smoked and 5,000 in those who have never smoked (Harley, 2008).

G. Natural Radioactivity and Background Radiation

The occupational, accidental, and wartime experiences have provided the basis for the estimation of risk to humans following radiation exposure. However, cosmic, cosmogenic, inhaled, and in-body radiation deliver total body effective doses of 3 becquerel per year total effective dose. The average radon concentration indoors is 40 becquerel

per m^3, resulting in an inhalation of 2 of the 2.1 (lung) and 3 (total tissue) mSv per year total effective dose (Harley, 2008). Thus, indoor air pollution is responsible for most of our total effective radon and total effective radiation dose from natural radioactivity and background radiation. Since radon is different for each indoor dwelling, this figure may vary considerably. This variation has led to the measuring of radon concentration in houses. Usually, natural radiation is not sufficiently high to produce clinical signs.

VIII. RADIATION HORMESIS

Hormesis is defined, originally in the field of toxicology, as a phenomenon in which a harmful substance gives stimulating (beneficial) effects to living organisms when the quantity is small (Sakai, 2006). Although radiation was thought to be harmful no matter how low the dose, recent investigations have revealed that radio resistance is induced by low dose irradiation given in advance. The stimulation of bioprotective functions includes antioxidant capacity, DNA repair functions, apoptosis, and immune functions as adaptive processes. The adaptive response is effective for chromosomal induction, acute death, and tumorigenesis induced by high doses of radiation. Radiation hormesis and adaptive responses provide a new scope in the risk assessment and medical application of ionizing radiation (Sakai, 2006).

The effect of low-dose irradiation of the immune system was investigated in mice (Ren *et al.*, 2006). When a 0.2 Gy (20 rad) dose of x-irradiation was administered every other day for a total of four times, the number of lymphocytes yielded by the liver, spleen, and thymus decreased by 10 days, but increased above the level of the control mice by day 28. The population of NK cells dramatically expanded, especially in the liver where primordial lymphocytes were present. Functional and phenotypic activation of these cells occurred at the recovery stage, raising the possibility that an initial activation of macrophages by low-dose irradiation mediated the innate immune system (Ren *et al.*, 2006).

Induction of hormesis and adaptive responses by low dose radiation have been extensively studied (Wang *et al.*, 2008). Pre-exposure of diabetic prone mice to low dose radiation (LDR) has reduced the incidence of alloxan diabetes and delayed the onset of hyperglycemia, although the mechanisms are unclear (Wang *et al.*, 2008). Low intensity laser (LIL) has stimulated healing in diabetes (Wang *et al.*, 2008).

Pre-exposure B6C3F1 hybrid male mice with low dose $^{12}C^{6+}$ ion beam or ^{60}Co gray (0.05 Gy; 5 rad) significantly alleviated the harmful effects of subsequent high dose irradiation (2 Gy; 200 rad) – reduction in serum follicle stimulating hormone, luteinizing hormone, testosterone, testis weight, sperm count, and sperm morphology (Zhang *et al.*, 2006). The effects were greater and the reversal nearly the same if $^{12}C^{6+}$ were used rather than ^{60}Co for irradiation. These data suggested that the low dose radiation pre-exposure could induce adaptive response(s) (Zhang *et al.*, 2006).

Thus, it would appear that low dose irradiation is fully capable of inducing adaptive responses so that the overall response is below that when the adaptive response was not induced. Advocates of hormesis suggest that we may be ignoring the benefits of radiation hormesis (the low level that produces adaptation necessary to combat higher levels of radiation) if we set our allowable radiation limits too high. The next study addresses this concept.

The hormesis theory proposes the low-dose beneficial and high-dose detrimental pattern existing for radiation has not been borne out at the level of our present radiation protection criteria (Zapponi and Marcello, 2006). A study of 400,000 power plant workers has shown there is a small risk at the limits of radiation protection and possibly below those limits. Thus, the hormesis theory-based criticism of current radiation protection, assumed to be excessively conservative, is not justified. Also not justified is the criticism that by dismissing hormesis, regulatory agencies such as the US EPA deny the public the opportunity for optimal health and avoidance of diseases. Analogical considerations are not necessarily logical ones and the single result should be considered in the whole context of radiation hormesis (Zapponi and Marcello, 2006).

Zapponi and Marcello (2006) report an interesting study, which says that if our low level adaptive response were to occur at just the level of the allowable radiation exposure limit, then the authors calculate that there is still some risk at or possibly below that level. To be a true adaptive response one would want to go below the level where there is significant risk. As these data point out, this level may be difficult to calculate. Moreover, it may be easy to interpret data in a variety of different ways.

Incidentally, the author is hardly unbiased with respect to hormesis and was far from convinced about its benefits (Pickrell and Oehme, 2002). After being asked to provide a critique he became a reluctant convert as he learned more about it (Pickrell and Oehme, 2002, 2005, 2006).

This leaves us with two possibilities; the first is that hormetic protection and adaptation do not exist. Three papers argue against this position (Zhang *et al.*, 2006; Wang *et al.*, 2008; Ren *et al.*, 2006). An alternative position might be that to achieve radiation hormesis, one might want to go below the allowable radiation protection limit. In three specific instances, the low level protective dose can produce beneficial responses (Zhang *et al.*, 2006; Wang *et al.*, 2008; Ren *et al.*, 2006). In the case of Zhang *et al.* (2006) the beneficial responses were phenotypical, and the measurements seemed relevant to the arguments of Zapponi and Marcello (2006).

Zapponi and Mastrello argue that at low level the allowable exposure limit would not provide the protection described by hormesis. I tend to agree. They go on to say that the critique which says that US EPA, by setting these limitations, is denying us the protection of radiation

hormesis and the opportunity for optimal health is not correct. Reluctantly, I must agree with them again, it is a matter of wording, and they are correct. However, their data do not say that there is not such a level, in this case below the allowable radiation exposure limit that would initiate an adaptive response and provide the protection of radiation hormesis and facilitate optimal health. In the interest of fairness, their data do not say that such a level exists either. However, the other three studies (Zhang *et al.*, 2006; Wang *et al.*, 2008; Ren *et al.*, 2006) I cited, especially that of Zhang *et al.* (2006), argue for this point and provide examples, of such a level, with measurements relevant to radiation risk assessments. Although future research will be needed to completely assess these arguments, we should keep our minds open to assessing the additional data.

IX. CONSEQUENCES OF RADIATION THERAPY

When x-ray therapy (XRT) has been given, radiation injury is often limited to organs within the radiation beams. The risk of radiation injury to an organ is determined by the organ's radiosensitivity and by the concentration time–activity curve of the agents in that organ or at a specific anatomical target. For example, late radiation effects can occur if the kidneys receive a significant radiation absorbed dose. The kidneys are known to have a relatively low radiation tolerance dose (23 Gy for conventionally fractionated XRT); therefore, late radiation nephritis may be a dose-limiting toxicity. Radiation fibrosis is a common sequela to XRT for lung cancer (Pickrell *et al.*, 1975, 1978; Pickrell and Mageed, 1995; Center for Drug Evaluation, 2005).

It is possible to predict the effect of treatment in cancer in a noninvasive manner by apoptosis imaging *in vivo* after radiotherapy by using ^{125}I-Annexin V. Brain tumors were implanted in susceptible (nude) mice. By 6 h after receiving levels of x-irradiation as low as 2 Gy both autoradiography and immunohistochemical staining showed more apoptosis in the tumors of irradiated groups than in the control group (Watanabe *et al.*, 2006).

Therapeutic brain irradiation can cause progressive decline in cognitive function, particularly in children, but the reason for this effect is unclear. Neocortical neurons of very young mice are more susceptible to radiation-induced apoptosis than are older mice. However, this sensitivity decreases rapidly after birth. By 14 days after parturition, acute cell loss due to radiation occurs primarily in non-neuronal populations (Nakaya *et al.*, 2005).

Poly (ADP-ribose) polymerase-1 (PARP-1) facilitates the repair of DNA strand breaks (Calabrese *et al.*, 2004). Inhibiting PARP-1 increases the cytotoxicity of DNA-damaging chemotherapy and radiation therapy *in vitro*. But classical PARP-1 inhibitors have limited clinical utility. AG14361 is, to our knowledge, the first high-potency PARP-1 inhibitor with the specificity and *in vivo* activity to enhance chemotherapy and radiation therapy of human cancer (Calabrese *et al.*, 2004). GR205171 has the most potent anti-emetic activity of any tachykinin NK1 receptor antagonist described to date. The compound is orally active in the ferret and dog, long-lasting, and warrants further investigation as a potential broad-spectrum anti-emetic agent (Gardner *et al.*, 1996).

Three-dimensional dose–rate/time/response surfaces for chronic exposure to carcinogens and ionizing radiation clarify the interactive roles of competing risks (Raabe, 1987). The three dimensions are average dose rate, exposure time, and risk. The improved conceptualization afforded by them contributes to the planning and evaluation of epidemiological analyses and experimental studies involving chronic exposure to radiation toxicants (Raabe, 1987).

X. CONCLUDING REMARKS AND FUTURE DIRECTION

Radiation is of concern because it cannot be seen, exposure is frequently not painful, but it can cause significant biological effects. Alpha and neutron particles are the largest radiation particles, they penetrate the least and deliver the highest radiation energy per unit distance. Their dose patterns resemble multiple tiny foci of radiation damage. When irradiation interacts with matter, alpha and neutron particles have 20 times the injurious cancer causing potential than beta irradiation and gamma rays have.

Cancer causing or apoptotic potentials are related to the interaction of ionizing radiation (neutron, alpha, beta, or gamma) with the critical target cell nucleus. In veterinary medicine, the greatest potential for acute radiation damage lies in accidents causing release of the contents of nuclear reactors such as Chernobyl in Belarus, radiation cancer therapy, most commonly for dog mammary tumors, or a gross miscalculation of irradiation dose needed for diagnostic imaging. Radiation fatalities at the highest dose can affect the CNS, perhaps by damaging the blood–brain barrier. At a lower dose they can damage intestinal epithelium with rapid turnover, causing the gastrointestinal syndrome. At a still lower level than can damage blood cells causing the hematologic syndrome.

Fractionating the radiation dose dramatically reduces the probability of an acute reaction from radiation therapy. If the dose is protracted over long time periods (months instead of hours to minutes), there is danger of chronic degenerative disease. For example, children who receive relatively low radiation doses appear to lose measurable cognitive function. The degree to which this is a lower dose manifestation of the CNS syndrome is not understood. When radiation is in the lung area, pulmonary fibrosis may develop; post-radiation nephritis is not uncommon. Dr Raabe's 1987 model for three-dimensional dose–rate/time/response surfaces after chronic exposure to ionizing

radiation by dose fractionation has helped us to understand the interactive roles of competing risks from therapeutic irradiation.

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CHAPTER 29

Depleted Uranium

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I. INTRODUCTION

Uranium (U) is a naturally occurring heavy metal that is both radioactive and ubiquitous (ATSDR, 1999; Harley *et al.*, 1999; Jiang and Aschner, 2006). Depleted uranium (DU) is the by-product of the enrichment process of uranium for its more radioactive isotopes. Enriched uranium (EU) is used for nuclear energy, whereas DU is used in other applications which require a heavy metal, such as aviation counterweights and naval ballasts. Because of its pyrophoric and dense metallic properties, DU is used primarily by the military in armor and ammunitions. DU retains 60% of its natural radioactivity and as such there are health hazards associated with exposure to the retained inherent radioactivity, but the radiological risks are less than those of natural uranium. However, the chemical toxicity of DU cannot be overlooked and the possibility that it exerts specific chemical toxicological effects separate from its radiological effects has been a subject of intense investigation.

There has been significant public concern regarding the use of DU by the military, and it has been hypothesized that DU may be a cause of Gulf War Syndrome. The public concern also stems from the lack of awareness regarding the specific physical chemistry and hazards of DU, and the belief that DU is still a form of uranium and therefore radiologically hazardous. These concerns have given rise to the belief that DU may be used as a weapon of mass destruction in the form of a dirty bomb, or as an agent of bioterrorism.

II. BACKGROUND

Small amounts of uranium are found in rock, soil, air, water, and food, and it is estimated that total annual intake of uranium by human adults approximates 460 μg by ingestion of food and water, and 0.6 μg by inhalation (Fisenne *et al.*, 1987; Pietrzak-Flis *et al.*, 2001; UNSCEAR, 2000a, b). Natural uranium is composed of three isotopes, ^{234}U, ^{235}U, and ^{238}U, in the proportions shown in Table 29.1.

DU is formed as a by-product of the enrichment of naturally occurring uranium for its most radioactive isotope ^{235}U. As such, DU contains significantly less ^{235}U and more ^{238}U. Enriched uranium (EU) contains much greater levels of ^{235}U, anywhere ranging from 3% to greater than 90%, while its ^{238}U content is decreased from anywhere less than 10% to 97%. For every one kilogram of uranium enriched to 3% ^{235}U during the enrichment process about 5 kg of DU (as a fluoride, UF_6) is produced (Bem and Bou-Rabee, 2004; Jiang and Aschner, 2006). It is estimated that 700,000 tons of UF_6 are stored in the USA, and that each year the mass of accumulated DU increases by 30,000 tons (Bem and Bou-Rabee, 2004; Hartmann *et al.*, 2000; Jiang and Aschner, 2006).

DU has 40% less radioactivity than natural uranium, but may contain trace levels of plutonium, neptunium, americium, technetium, and ^{236}U, which increase the radioactivity by 1% but are insignificant with respect to chemical and radiological toxicity (Force Health Protection & Readiness Policy & Programs, 2008; Sztajnkrycer and Otten, 2004; WHO, 2001). Because of the decreased radioactivity of DU, it is believed that DU is a safer form than natural uranium, while maintaining the same chemical properties. As the heaviest occurring element, uranium is extremely dense and both uranium and DU are often used in applications which require such dense metals.

This chapter describes depleted uranium and its applications in weapons of mass destruction. The DU exposure pathways, pharmacokinetics, health effects, toxicity, and available treatments are also reported.

A. Civilian Uses of DU

DU in civilian applications is marginal compared to its use in military applications (Bem and Bou-Rabee, 2004; Jiang and Aschner, 2006). As a heavy metal, DU is used as ballasts in yachts, counterbalances in commercial jets (Mould, 2001; Sztajnkrycer and Otten, 2004), shielding in radiation therapy, and as containers for transportation of radioactive materials (Jiang and Aschner, 2006). Considering ingestion, inhalation, and dermal exposure as the typical routes of exposure, typical civilian exposure to uranium from food, water, and air is considered minimal under normal circumstances (Fisenne *et al.*, 1988). These exposures fall well below the daily tolerable intake levels for soluble uranium (0.5 μg/kg body weight), insoluble uranium (5 μg/kg body weight), and inhaled uranium

TABLE 29.1. Forms of uranium and their respective isotope percentages

	Form of uranium		
Isotope	**Natural**	**Depleted**	**Enriched**
^{238}U	99.275%	99.8%	<10% to 97%
^{235}U	0.720%	0.2%	3% to >90%
^{234}U	0.0055%	0.001%	0.03%

(1 μg/m^3 in the respirable fraction), as set forth by both the WHO (2001) and ATSDR (1999). Because uranium is present in greater levels in war-ravaged regions, it is believed that uranium may pose a potentially greater exposure danger to civilians in these regions (Bem and Bou-Rabee, 2004).

B. Military Uses of DU

The major use of DU is by the military as an alloy in armor and ammunition. These applications take advantage of the unique metallic properties of DU, specifically the density and pyrophoric properties. Uranium is the heaviest naturally occurring element and is extremely dense. Uranium has a density 1.7 times the density of lead, and rods made of uranium are resistant to deformation (Bem and Bou-Rabee, 2004). Uranium shielding is therefore used in the armor of military armored vehicles, allowing the deflection of enemy projectiles. Furthermore, alloys of uranium that contain 2% molybdenum or 0.75% tungsten have a unique attribute such that they will sharpen themselves on impact with a hard target, which allows for greater penetration of uranium-based projectiles compared to traditional tungsten-based alloys (Jiang and Aschner, 2006). DU is also pyrophoric, such that particles will ignite at relatively low temperatures. Fine particles will burn rapidly at relatively low temperatures (150–175°C), while particles will spontaneously burn violently with air above 600°C, releasing heat and uranium oxide aerosols (Bem and Bou-Rabee, 2004; Harley *et al.*, 1999). These unique properties of uranium make it an excellent material for military applications.

During the 1991 Gulf War (Operation Desert Storm), it is estimated that 300 tons of DU were used in the aircraft rounds and tank-fired shells in Kuwait and southern Iraq over an area of 20,000 km^3 (Bem and Bou-Rabee, 2004). Studies have shown that DU penetrators hitting armored targets convert 17–28% of a projectile's mass into DU aerosols (Bem and Bou-Rabee, 2004; Force Health Protection & Readiness Policy & Programs, 2008; Harley *et al.*, 1999; Parkhurst, 2003). Of these aerosols, 83% are S-type (S for slow dissolution) oxides, while 17% are M-type (M for medium dissolution) oxides, and the respirable fraction (diameter <10 μm) may be 50% of the total mass of the aerosol (Harley *et al.*, 1999; Jiang and Aschner, 2006). This would mean that of 50 tons of tank-fired and 25 tons of aircraft-fired DU munitions, 10 tons of uranium oxides would have been released into the air in the form of respirable uranium (Jiang and Aschner, 2006).

The US Department of Defense (DOD) has conducted studies to evaluate distribution of aerosolized DU after destruction of an armored tank to assist in determing DU health risk (Force Health Protection & Readiness Policy & Programs, 2008; Harley *et al.*, 1999). The concentration of uranium inside a tank can reach hundreds or thousands mg/m^3 just after an explosion caused by a DU penetrator (Force Health Protection & Readiness Policy & Programs, 2008; Harley *et al.*, 1999). As uranium has such a high density, the aerosolized DU particles fall within 10 m of a burning tank. The US DOD estimates that the DU intake of a person in the vicinity of a tank hit by a single 120 mm DU-containing projectile is 0.1 mg uranium, and the maximum intake in traversing a cloud of smoke plume that is 200 m in length is 0.8 μg uranium (Bem and Bou-Rabee, 2004; Force Health Protection & Readiness Policy & Programs, 2008; Harley *et al.*, 1999; Parkhurst, 2003). These US DOD studies demonstrate that the risks of uranium inhalation are greatest for crews of damaged tanks and rescue teams (Jiang and Aschner, 2006). However, as the DU particulate matter can be carried by winds and deposited in soil and water, there are concerns that both civilian and military units may be exposed to DU dust, vapors, and aerosols, particularly as military conflicts continue. The concerns regarding the unknown exposure data of these individuals have spurred recent studies on war veterans and civilians living in war-ravaged regions to try to determine if DU has human health and environmental impacts.

III. EXPOSURE PATHWAYS AND BODY RETENTION OF DU

The chemical toxicity of DU is only an issue if the metal is internalized. The three traditional pathways of exposure are inhalation, ingestion, and dermal contact (Figure 29.1). Typically, in nonmilitary situations, the main routes of natural uranium uptake are by inhalation and ingestion. Because of the use of DU in ammunitions and armor by the military, the more important routes of exposure are inhalation and internalization of the DU (Figure 29.1). This internalization of the DU results from embedding of DU projectile fragments (shrapnel) due to explosions of DU-containing armor and ammunitions. Follow-up studies of 1991 Gulf War veterans with embedded DU shrapnel have attempted to determine if there are adverse health effects associated with internalized DU (Hodge *et al.*, 2001; McDiarmid *et al.*, 2000, 2001a, b, 2002, 2004a, 2006, 2007; Squibb and McDiarmid, 2006).

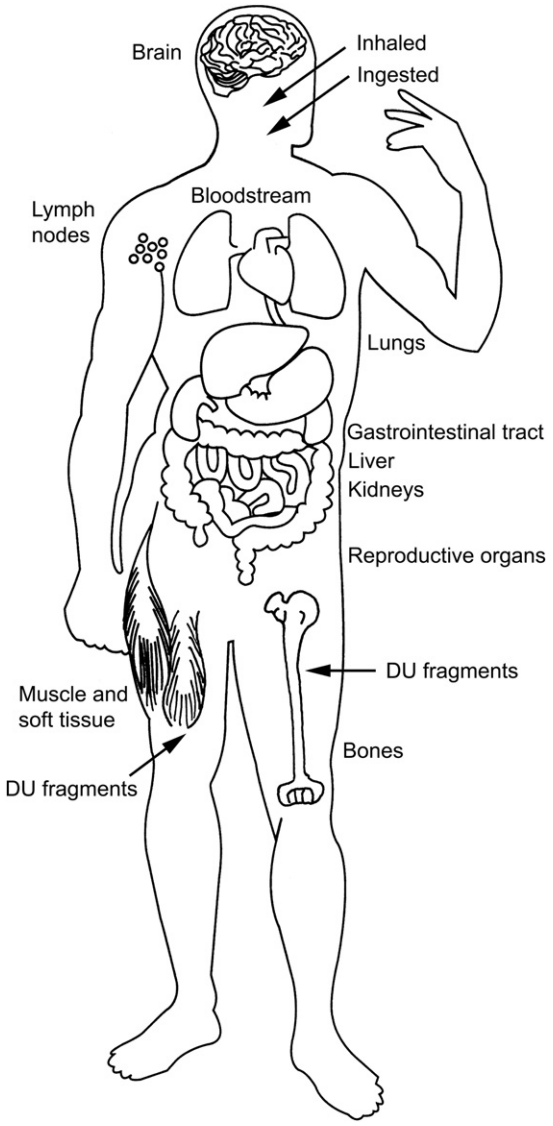

FIGURE 29.1. Routes of depleted uranium entry into the body and the corresponding target organs where DU has been shown to accumulate.

A. Inhalation

Inhalation is a likely route of intake of DU primarily due to its use in DU munitions, whereas naturally occurring uranium dust particles are only inhaled in very small quantities (ATSDR, 1999; Jiang and Aschner, 2006). Studies on people in New York City show that about 1 µg of uranium is inhaled each year by each person (ATSDR, 1999; Fisenne *et al.*, 1987; Harley *et al.*, 1999). Inhalation risk from DU is due to the aerosolization following the impact of ammunitions, forming DU oxides and particles. These particles become suspended in the air by the wind, or settle into the environment for later resuspension. For example, it was determined that the estimated mean annual concentration of suspended matter in ambient air in Kuwait following the 1991 Gulf War was 200 µg/dm^3, one of the highest concentrations in the world (Bem and Bou-Rabee, 2004).

The particle size of the aerosolized DU, and its correlated physical and chemical properties (e.g. solubility), have a significant impact on how far into the lungs the particles will penetrate, and if they will result in deposition in the respiratory tract (ATSDR, 1999; Jiang and Aschner, 2006). Only small particles less than 10 µm in diameter will reach and accumulate in the bronchioles and alveoli, while larger particles are effectively cleared by mucociliary clearance (Gwiazda *et al.*, 2004; Harley *et al.*, 1999; WHO, 2001). These respirable particles present a potential health hazard from uranium inhalation, but studies have shown than less than 1% of inhaled uranium actually reaches the kidneys (Gwiazda *et al.*, 2004; Harley *et al.*, 1999; Spencer *et al.*, 1990; WHO, 2001). These studies have demonstrated that of the uranium that is inhaled, 75% is exhaled and 25% is retained in the respiratory tract and lungs. Of the retained fraction of uranium in the lungs, 80% is removed by bronchial clearance, 15% is deposited in lymph nodes, and 5% actually enters the blood (Harley *et al.*, 1999; Spencer *et al.*, 1990).

B. Ingestion

Soil ingestion by children is an important exposure pathway, but typically for humans ingestion is only an important route of entry if food and drinking water are contaminated by DU (Jiang and Aschner, 2006). The average daily intake of uranium from food and water is estimated to be between 1 and 5 µg/day from uncontaminated areas (ATSDR, 1999; Harley *et al.*, 1999). In uranium mining areas, ingestion has been found to range between 13 and 18 µg/day (ATSDR, 1999; Harley *et al.*, 1999). Obviously, these concerns of adverse health effects from DU-contaminated food and drinking water are amplified in battlefield arenas, such as Iraq, Kuwait, the Balkans, and Afghanistan. A number of studies have demonstrated increased uranium levels in the soil in these sites several years after the conflict (Bem and Bou-Rabee, 2004; Danesi *et al.*, 2003; Di Lella *et al.*, 2004; Durante and Pugliese, 2003; Mitchel and Sunder, 2004; Oliver *et al.*, 2007; Sansone *et al.*, 2001; Spencer *et al.*, 1990). To date, there have been a number of studies that have attempted to link higher malignancy rates and genotoxic effects in these regions with varying degrees of correlation (Milacic *et al.*, 2004; Obralic *et al.*, 2004). Furthermore, bone ash data indicate significant baseline uranium differences across countries (Fisenne et al., 1980, 1983, 1987; Fisenne and Welford, 1986), and there is no clear indication as yet that these different levels of uranium have led to any adverse health consequences (ATSDR, 1999; Harley *et al.*, 1999).

The solubility of the uranium compound is an important consideration in determining adsorption and distribution as toxicity is related to uptake efficiency of the gastrointestinal (GI) tract. Generally, absorption is increased with the

increased solubility of the compound. Uranium oxides (U_3O_8 and UO_2) are relatively insoluble (Types M and S; M for medium and S for slow dissolution) while uranium trioxide (UO_3) is more soluble (between Type M and F; F for fast dissolution). These more insoluble forms are less likely to enter the bloodstream and cause toxicity. On the other hand, uranyl nitrate hexahydrate, uranium hexafluoride, or uranyl fluoride are all relatively soluble forms and tend to be absorbed through the GI epithelium. However, unlike other heavy metals, uranium is not efficiently absorbed through the intestinal lumen (ATSDR, 1999; Harley *et al.*, 1999; Spencer *et al.*, 1990). Studies in humans ingesting uranium nitrate in spiked grapefruit juice demonstrate only 0.5–5% absorption of the original dose (Karpas *et al.*, 1998) (IARC publication). Only 0.2–2.0% of uranium in food and water is absorbed by the GI tract, and of the amount that is absorbed, 67% will be filtered out by the kidneys and excreted in the urine within the first 24 h (ATSDR, 1999; Harley *et al.*, 1999; WHO, 2001).

C. Dermal Contact and Embedded Fragments

Dermal absorption has not been characterized in human studies, and there have not been any dermal effects reported from uranium miners, millers, or processors (ATSDR, 1999). There have also been no human deaths related to dermal exposure of uranium. Dermal contact is not an important route of exposure since DU does not pass through the skin into the blood unless there are open wounds or embedded fragments (Harley *et al.*, 1999; WHO, 2001).

Embedded DU-containing shrapnel, if not removed, is a permanent exposure source within the body, and will allow the DU to enter the systemic circulation. The DU Follow-Up Program at the Baltimore Veterans Administration Medical Center has been surveying a small population of approximately 230 soldiers since the 1991 Gulf War and reports from this program have shown that the uranium leaches into the circulation resulting in elevated urinary uranium levels compared to control patients (Ejnik *et al.*, 2000, 2005; Gwiazda *et al.*, 2004; Hodge *et al.*, 2001; McDiarmid, 2001; McDiarmid *et al.*, 2000, 2001a, b, 2002, 2004a, b, 2006, 2007; Squibb *et al.*, 2005; Squibb and McDiarmid, 2006).

IV. PHARMACOKINETICS

A. Adsorption

1. Ingestion

Uranium ingested from food and water ingestion ranges from 1–5 μg uranium in uncontaminated regions to 13–18 μg uranium in uranium mining areas (ATSDR, 1999). The absorption of uranium across the gastrointestinal tract is related to the solubility of the compound and generally increases with increased solubility. Only a small fraction is absorbed of even the relatively soluble uranium compounds, such as uranyl nitrate hexahydrate, uranium hexafluoride, or uranyl fluoride. For example, studies have shown that humans ingesting uranium nitrate hexahydrate or uranyl nitrate absorb only 0.5–5% of the original dose (Hursh *et al.*, 1969; Karpas *et al.*, 1998). In animal studies with uranyl nitrate and uranium dioxide directly intubated into the GI tract, only 0.77% and 0.11% of the total doses, respectively, were absorbed (Harrison and Stather, 1981). Studies in rats with an intragastrically applied relatively insoluble form of uranium, UO_2 (Type S dissolution), showed that uranium could not be detected in liver, kidney, muscle, bone, brain, blood, and urine, and that uranium was not absorbed or retained significantly in the epithelial cells of the intestinal wall (Lang and Raunemaa, 1991).

2. Inhalation

Typically, uranium is present in limited concentrations in the air, and uranium particle inhalation is minimal (ATSDR, 1999; Harley *et al.*, 1999). Uranium particle deposition in the respiratory tract is governed by the physical forces that effect particle behavior in the air, as well as the anatomy of the respiratory tract (ATSDR, 1999; Bleise *et al.*, 2003; Phalen and Oldham, 2006). The anatomy of the lungs is important as this affects the clearance mechanisms available to deal with deposited particles, and the degree of actual uranium absorption that will occur. In addition to the aerodynamic diameter (AD) of the particle, the solubility of the inhaled uranium is an important determinant as to how much uranium will be absorbed (Eidson, 1994; Lang *et al.*, 1994).

Most inhaled uranium particles have an AD that does not permit them to be carried to the deep lungs (ATSDR, 1999; Igarashi *et al.*, 1987). Studies have shown that only small uranium particles less than 10 μm in diameter will reach and accumulate in the bronchioles and alveoli, while larger particles are effectively cleared by mucociliary clearance (Gwiazda *et al.*, 2004; Harley *et al.*, 1999; WHO, 2001). These inhaled respirable particles do present a potential health hazard from uranium inhalation, but studies have shown than less than 1% of inhaled uranium actually reaches the kidneys (Gwiazda *et al.*, 2004; Harley *et al.*, 1999; Lang *et al.*, 1994; Morris *et al.*, 1990; Spencer *et al.*, 1990; WHO, 2001). Studies have demonstrated that of the uranium that is inhaled, 75% is exhaled and 25% is retained in the respiratory tract and lungs. Of the retained fraction of uranium in the lungs, 80% is removed by bronchial clearance, 15% is deposited in lymph nodes, and 5% actually enters the blood (Harley *et al.*, 1999; Spencer *et al.*, 1990).

3. Dermal

In animal studies, soluble uranium compounds have been shown to penetrate the skin of rats within 15 min of application (de Rey *et al.*, 1983). The penetration was as high as 7 g/kg body weight for ammonium uranyl tricarbonate while uranyl nitrate hexahydrate ranged from 0.5 to 7 g/kg body

weight. No penetration through the skin was observed when uranium dioxide, a more insoluble form, was applied (de Rey *et al.*, 1983). Two days after exposure, demally absorbed uranium was no longer localized to the epithelium, and rats either had significant weight loss or had died. Other studies have demonstrated that other uranium compounds (uranium tetrafluoride, uranium tetrachloride, and uranium trioxide) are absorbed through the skin of mice, rats, and guinea pigs at a rate of 0.1% of determally applied uranium, a relatively low absorption rate (Orcutt, 1949). These studies show that soluble uranium compounds can be absorbed through the skin. However, the concentrations of uranium applied to the skin were extremely high, and it is unlikely that humans would typically experience such exposures. Dermal absorption has not been characterized in humans (ATSDR, 1999).

B. Distribution

Once absorbed into the system circulation, uranium undergoes chemical transformations to complex with the blood. Uranium in the trivalent form will oxidize to the hexavalent species to form uranyl ions, which form soluble complexes with bicarbonate, citrate, or proteins in the plasma (Chevari and Likhner, 1968; Cooper *et al.*, 1982; Stevens *et al.*, 1980). The distribution of uranium in the blood is approximately 47% complexed with bicarbonate in plasma, 32% bound to plasma proteins, and 20% bound to erythrocytes (Chevari and Likhner, 1968, 1969).

Uranium is then distributed by the systemic circulation primarily to the bones and kidneys. Of the total absorbed uranium, 85% is associated with bone, where uranium replaces calcium in the hydroxyapatite complex (Donoghue *et al.*, 1972). Of the remaining 15% uranium, over 90% is associated with the kidneys, accumulating primarily in the proximal tubule (ATSDR, 1999). Uranium also distributes in detectable amounds to the liver, brain, testes, and spleen (Fitsanakis *et al.*, 2006; Leggett and Pellmar, 2003; Pellmar *et al.*, 1999a). The embedded uranium fragments continuously release uranium into the circulation, and the fragment size diminished with time.

Less soluble uranium compounds (Type M and Type S dissolution), which are not effectively absorbed, are distributed in bronchial lymph nodes as well as the lung tissue itself (Leach *et al.*, 1970).

C. Metabolism and Excretion

Once uranium is in the systemic circulation, it is transported as various complexes in the blood. The predominant form of uranium is as a bicarbonate complex, the stability of which is highly dependent on the pH of the solution (ATSDR, 1999; Chevari and Likhner, 1968). In the kidneys, the bicarbonate complex is filtered at the renal glomerulus and excreted in the urine (Adams and Spoor, 1974; ATSDR, 1999; Blantz, 1975; Bowman and Foulkes, 1970; Brady *et al.*, 1989; Foulkes, 1971). Protein-bound uranium will remain in the blood since little protein passes through the glomerulus.

Studies in humans have shown that approximately 66% of an intravenous injection of uranium is eliminated from the plasma within 6 min, while 99% of the uranium is eliminated from the plasma 20 hours after injection (ATSDR, 1999; Harley *et al.*, 1999; Luessenhop *et al.*, 1958). Another study has shown that the kidneys excrete over 90% of intravenously injected soluble hexavalent uranium salt, with less than 1% excreted in the feces; approximately 70% of the dose is excreted within the first 24 h (Bassett *et al.*, 1948).

1. Oral Exposure

The average gastrointestinal uptake of uranium is limited, and ranges from 1 to 5% in adult humans (ATSDR, 1999; Leggett and Harrison, 1995). Absorption generally increases with increasing solubility of the compound. It is highest for the soluble uranium compounds; nevertheless, only a small fraction of uranium is absorbed across the gastrointestinal epithelium. Uranium absorption takes place predominantly in the small intestine, with no absorption from the buccal cavity, stomach, or large intestine (Dublineau *et al.*, 2005). Measured with *ex vivo* techniques, the apparent uranium permeability was shown to be similar in various parts of the small intestine (Dublineau *et al.*, 2005). Uranium transport across the gastrointestinal epithelium likely occurs via a transcellular pathway. Approximately 90% of the ingested uranium in humans is excreted in the feces without being absorbed while the remainder is excreted in the urine (Oeh *et al.*, 2007a, b; Spencer *et al.*, 1990; Wrenn *et al.*, 1985). Studies in rats show similar excretion patterns to humans, of which the majority of ingested uranium (99%) is eliminated in the feces, and 95% of the absorbed uranium is excreted in urine within a 1 week of exposure, with a half-life of 2–6 days (Dublineau *et al.*, 2005; La Touche *et al.*, 1987; Wrenn *et al.*, 1985).

2. Inhalation Exposure

The rate of deposition and clearance of uranium-containing particles from the lung depends upon its chemical form and particle size. As previously discussed in the adsorption section, most of the larger uranium particles are transported out of the respiratory system by mucocilliary action, or swallowed and eliminated in the feces. Smaller particles with higher solubilities are more rapidly absorbed into the systemic circulation but can then be excreted in the urine.

The aerosol by-products of exploded DU munitions are primarily the uranium oxides with varying dissolution rates. Uranium trioxide (UO_3) is soluble like uranyl salts, and systemic absorption accounts for more than 20% of the exposure burden, with 20% of the excreted uranium being in the urine (Morrow *et al.*, 1964, 1972, 1982). UO_3, being soluble, has a fast dissolution rate (Type F), and is rapidly removed from the lung (half-life of 4.7 days). Uranium

dioxide (UO_2) and triuranium octaoxide (U_3O_8) are relatively insoluble and have slow dissolution rates (Type S) resulting in pulmonary clearance rates dominated by particle size and mucocilliary transport out of the lungs. In humans, studies of these insoluble uranium compounds suggest a two-phase clearance process, consisting of a short phase with a biological half-time of between 11 and 100 days and a slow phase of clearance with a biological half-time between 120 and 1,500 days (Eidson, 1994; Hodge, 1973; Taylor and Taylor, 1997).

3. Embedded Fragments Exposure

DU fragments from embedded shrapnel act as reservoirs to continuously release uranium into the circulation (Fitsanakis *et al.*, 2006; Jiang and Aschner, 2006; Leggett and Pellmar, 2003; Pellmar *et al.*, 1999a). Significant percutaneous diffusion of soluble uranium through intact skin has been described (de Rey *et al.*, 1983; Lopez *et al.*, 2000; Petitot *et al.*, 2007). In rats, the absorption of uranium via deep wounds was shown to depend on the chemical form of uranium. Upon intramuscular injection of uranium nitrate, approximately 98% of the uranium was in the blood (Houpert *et al.*, 1999). In contrast, after UO_4, 70 and 85% of instilled uranium was found in the blood after 1 and 3 days, respectively (Houpert *et al.*, 1999).

Surgical removal of fragments attempts to remove as many fragments as possible, but often there will be residual fragments that cannot be removed due to location and/or size. Over time the fragment sizes will decrease as the uranium leaches from these fragments; the body has no other way to effectively remove such embedded fragments.

V. MECHANISM OF ACTION

There have been many *in vitro* studies evaluating the toxic effects of uranium in different cell types, many of which demonstrate cytotoxicity. There is no specific mechanism that emerges to explain all the results, but the weight of evidence suggests that DU-induced cytotoxicity may result from oxidative stress and eventual cell death. Uranyl compounds have high affinity for phosphate, carboxyl, and hydroxyl groups, and easily combine with proteins and nucleotides to form stable complexes (Weir, 2004). DU can cause oxidative DNA damage by catalyzing hydrogen peroxide and ascorbate reactions, resulting in single strand breaks in plasmid DNA *in vitro* (Miller *et al.*, 2002a; Yazzie *et al.*, 2003). Evidence for induction of oxidative stress and reactive oxygen species (ROS) by DU has been shown by increases in NO (Abou-Donia *et al.*, 2002), lipid oxidation (Briner and Murray, 2005; Ghosh *et al.*, 2007; Jiang *et al.*, 2007), transcriptomic, and proteomic changes (Malard *et al.*, 2005; Prat *et al.*, 2005). Moreover, some studies indicate that heat shock proteins may be involved in the cellular response to DU exposure and acquired resistance to uranium rechallenge (Furuya *et al.*, 1997; Mizuno *et al.*, 1997; Tolson *et al.*, 2005). These data strongly suggest the possibility that uranium may result in the formation of reactive oxygen species, leading to cell death. An apoptotic mechanism has even been suggested following a study where significant apoptotic events were seen in mouse macrophage cells treated with 100 μM DU (Kalinich *et al.*, 2002).

VI. TOXICITY OF DEPLETED URANIUM EXPOSURE

Scientists in the mid-19th century first believed that natural uranium had homeopathic properties, and could be an effective treatment for diabetes and albuminuria after promising results in animals and humans dosed with uranium (Hodge, 1973; Jiang and Aschner, 2006). However, animal studies in the early 20th century showed that uranium was not therapeutic, and the misuse of uranium in humans was halted (Hodge, 1973). There is extensive literature on the toxicology of uranium, which now includes studies specifically on DU. The following section highlights a number of key *in vitro*, animal, and human studies of DU, as they relate to health effects from uranium exposure. Chemical toxicity of uranium only poses a threat if the metal is internalized.

A. Nephrotoxicity

The kidney is the major target organ for uranium toxicity (ATSDR, 1999; Harley *et al.*, 1999; Jiang and Aschner, 2006). Renal toxicity associated with uranium exposure results as the kidneys work to eliminate internalized uranium and this has been known for two centuries (Goodman, 1995). In the kidneys, the site of action is the proximal tubule where proton secretion degrades the bicarbonate complex of the uranyl ion. Uranium can then react with apical cell membranes of the tubule epithelium (Goodman, 1995; Harley *et al.*, 1999). Nephrotoxicity is clearly associated with uranium exposure and has been documented in animal studies at high exposure levels (Harley *et al.*, 1999; Leggett, 1989; Sztajnkrycer and Otten, 2004). Recent *in vitro* studies on renal cells demonstrated a concentration-dependent uranium toxicity (Carriere *et al.*, 2004; L'Azou *et al.*, 2002; Thiebault *et al.*, 2007). Any observed uranium nephrotoxicity results from acute exposure. Currently, there is no evidence that DU has any long-term effects on renal function, or that long-term renal impairments will develop if no acute effects are seen (ATSDR, 1999; Harley *et al.*, 1999; Jiang and Aschner, 2006). There is even evidence in animals and humans that there may be repair of damaged tubular epithelial tissue (Diamond *et al.*, 1989; Goodman, 1995; Harley *et al.*, 1999).

B. Carcinogenicity

1. Bones

Bones are the secondary target organ of uranium toxicity (ATSDR, 1999). The majority of absorbed uranium is distributed to the bones and not surprisingly the carcinogenic potential of uranium in bones has been explored in uranium workers and personnel living in or having served in the war-ravaged regions potentially exposed to DU (Abu-Qare and Abou-Donia, 2002; Boice *et al.*, 2003, 2007; Harley *et al.*, 1999; Obralic *et al.*, 2004; Storm *et al.*, 2006).

Animal studies to mimic Gulf War veterans' injuries and assess the chronic effects of internalized DU have utilized rats implanted with DU and control tantalum pellets (Arfsten *et al.*, 2007; de Rey *et al.*, 1984; Fitsanakis *et al.*, 2006; Hahn *et al.*, 2002; Leggett and Pellmar, 2003; Pellmar *et al.*, 1999a). Carcinogenicity of these surgically implanted DU fragments has been evaluated, and is related to the size of the fragments – DU fragments of sufficient size can cause localized proliferative reactions and soft tissue sarcomas (Hahn *et al.*, 2002). Urine and serum mutagenicity studies with rats implanted with DU demonstrated enhanced mutagenic activity in *Salmonella* TA98 strain and Ames II mixed strain (TA7001-7006) in a dose-dependent manner with excreted urinary concentration (Miller *et al.*, 1998b). There are numerous other studies which demonstrate the deleterious effects of DU on viability, micronuclei, chromosomal instability, and sister chromatid exchanges by human bronchial, bone marrow, and Chinese hamster ovary cells (Kadhim *et al.*, 1992, 1994; Kennedy and Saluga, 1970; Lin *et al.*, 1993; Miller *et al.*, 1998a, 2003; Nagasawa and Little, 1992; Schroder *et al.*, 2003). However, it is still unclear if the mutagenic and carcinogenic effects are a result of the chemical or radiological effects of DU, but it is believed that these effects are primarily due to the latter. Osteosarcoma risk has clearly been demonstrated to be related to the amount of radiation exposure and there is a wealth of *in vitro* studies on bone cells and in animal models that illustrate this (Ibrulj *et al.*, 2004, 2007; Miller et al., 2002b, 2003, 2005). However, many scientists believe that the amount of radiation emitted by DU is insufficient to raise a significant risk in humans, but may be more of a concern for children whose bones are growing rapidly (Brugge *et al.*, 2005; Jiang and Aschner, 2006).

2. Lungs

It is often difficult to pinpoint specific cancerous agents using epidemiological studies (Priest, 2001), but as the lungs are the primary portal of inhaled uranium numerous studies have examined the health effects of inhaled uranium. It has been known since the 1940s that the most soluble uranium compounds are the most toxic (Voegtlin and Hodge, 1949a, b). Indeed, early animal studies did not reveal significant animal mortality from inhalation unless very soluble forms of uranium were used. However, most of the effects of uranium on the lungs occur due to the insoluble forms of uranium. Researchers have found that the lungs and tracheobronchial lymph nodes are the major sites of uranium accumulation for large particles and relatively insoluble forms of uranium, and fibrotic changes in the lung tissue have been observed suggestive of radiation injury (Leach *et al.*, 1970, 1973). These insoluble particles deposited in the lungs have a long residual time and may result in increased risk for cancer (Hartmann *et al.*, 2000; Houpert *et al.*, 1999). Indeed, typical health effects seen in uranium inhalation studies are the development of pneumonia and chemically irritated passages, which are considered as early signs of lung cancer (Voegtlin and Hodge, 1949a, b, 1953a, b).

The mechanisms associated with lung injury in high-level uranium exposure are not well understood. Treatment of rat lung epithelial cells with uranyl (VI) acetate was shown to result in increased oxidative stress and decreased cell proliferation, which was attributed to loss of cellular redox (Periyakaruppan *et al.*, 2007). Effects of uranium on cytokine secretion and on the proteasome–ubiquitin system have also been advanced (Gazin *et al.*, 2004; Malard *et al.*, 2005). Several studies have also established the deleterious effects of DU on viability, micronuclei, chromosomal instability, and sister chromatid exchanges in cells (Lin *et al.*, 1993; McDiarmid *et al.*, 2001b; Miller *et al.*, 1998a, 2002c; Prabhavathi *et al.*, 1995; Schroder *et al.*, 2003; Wolf *et al.*, 2004). These effects are believed to be due more to the radiological properties of uranium than to the chemical effects (Bolton and Foster, 2002; Mould, 2001).

C. Reproductive/Developmental Toxicity

The reproductive effects of DU have recently been studied in various animal models (Arfsten *et al.*, 2001, 2005, 2006; Domingo, 2001; Linares *et al.*, 2005; Llobet *et al.*, 1991; Paternain *et al.*, 1989). Uranium has been shown to be a developmental toxicant when given orally or subcutaneously, resulting in decreased fertility, embryo/fetal toxicity including teratogenicity, and reduced growth of offspring, following uranium exposure at different gestation periods (Arfsten *et al.*, 2001, 2005; Bosque *et al.*, 1993; Domingo, 2001; Domingo *et al.*, 1989a, b; Ortega *et al.*, 1989b). It is still unclear what the exact mechanism of action is, although *in vitro* studies in Chinese hamster ovary cells showed DU-induced genotoxicity and cytotoxicity (Lin *et al.*, 1993). In humans, studies have evaluated the reproductive effects in male miners, uranium processors, and Gulf War veterans and show that these individuals have uranium in their semen, but do not otherwise show any detrimental reproductive effects (Abu-Qare and Abou-Donia, 2002; Arfsten *et al.*, 2006; Domingo, 2001; Harley *et al.*, 1999; Houpert *et al.*, 2007; Linares *et al.*, 2005; McDiarmid *et al.*, 2000; Priest, 2001; Squibb and McDiarmid, 2006; Voegtlin and Hodge, 1953a, b).

D. Neurotoxicity

As concerns mounted with regards to the cause of Gulf War Syndrome, a growing body of evidence suggested that DU may indeed be affecting the central nervous system (CNS). Animal studies demonstrated that uranium crosses the blood–brain barrier (BBB) and readily accumulates in the brain (Fitsanakis *et al.*, 2006; Gilman *et al.*, 1998a, b, c; Houpert *et al.*, 2007; Leggett and Pellmar, 2003; Lemercier *et al.*, 2003; Monleau *et al.*, 2005; Pellmar *et al.*, 1999a, b). Furthermore, studies in rats, and some follow-up studies in Gulf War veterans, suggested that DU may cause subtle changes in CNS function without any corresponding nephrotoxicity (McDiarmid, 2001; McDiarmid *et al.*, 2000, 2001b, 2002, 2004a, 2007; Pellmar *et al.*, 1999a, b). The public concern regarding the potential neurotoxic effects of DU has spurred recent novel scientific research to extensively evaluate if there are reasons to be concerned about DU exposure and neurotoxicity.

One of the earliest studies evaluating the specific effects of DU on the CNS demonstrated that uranyl nitrate facilitated the release of acetylcholine from the nerve terminals to potentiate muscle contraction in phrenic nerve preparations from mice (Lin *et al.*, 1988). It was then shown that uranium could cross the blood– brain barrier (BBB) *in situ* using rat brain perfusions (Lemercier *et al.*, 2003). Significant amounts of uranium accumulated in the brain after only a 2 min perfusion. Studies in rats embedded with DU and/or control tantalum pellets for 1 day, 6, 12, and 18 months confirmed previous biodistribution data that demonstrated kidneys and bone as the primary target organs, but also identified other sites in the lymphatic, respiratory, reproductive, and central nervous systems (Pellmar *et al.*, 1999a). High-dose (20 pellets) DU implantation resulted in significantly increased uranium levels in the skull (12.5 ng U/g skull tissue) after only 1 day compared to controls (1.41 ng U/g skull tissue) (Pellmar *et al.*, 1999a). By 6 months, there was a significant difference in the amount of uranium in brain samples from high-dose rats (approximately 5,000 ng U/g tissue) compared to tantalum control samples (approximately 50 ng U/g tissue) (Pellmar *et al.*, 1999a). Significant differences in brain region distribution of uranium were also found. Differences were also seen in electrophysiological studies in hippocampal slices of rats implanted with DU, the hippocampus being important in learning, memory consolidation, and spatial orientation functions. The 6-month and 12-month high-dose groups exhibited decreased neuronal excitability compared to controls, whereas rats in the 18-month DU-treatment groups did not show significant changes in neuronal excitability perhaps obscured by the aged rats (Pellmar *et al.*, 1999b). No significant differences in hippocampal weights of DU treated animals compared to controls were seen.

Generation of nitric oxide (NO) and evaluation of the central cholinergic system of male Sprague-Dawley rats following uranium exposure have also been studied (Abou-Donia *et al.*, 2002). Intramuscular injection of 0.1 and 1.0 mg/kg for 7 days, followed by a 30-day observational period, resulted in sensorimotor deficits in rat behavior, differential levels of NO, and increased acetylcholinesterase activity in the cortex of animals dosed with 1 mg/kg, suggesting multiple exposures to low doses of uranyl acetate caused prolonged neurobehavioral deficits in rats after the initial exposure has ceased, similar to the exposures of Gulf War veterans (Abou-Donia *et al.*, 2002).

Short-term and long-term differences in brain lipid oxidation and open-field behavioral differences in rats exposed to DU have been shown (Briner and Murray, 2005). After 2 weeks of DU exposure, brain lipid oxidation was increased and correlated with increases in line crossing and rearing behavior. While open-field behavior differences remained after 6 months of DU exposure, brain lipid oxidation could no longer be clearly correlated with the behavioral changes (Briner and Murray, 2005). Male rats were also more sensitive to the behavioral effects of DU compared to female rats (Briner and Murray, 2005). The gender differences may warrant further study to allay the fears of the public as the US armed forces deployment to Kosovo, Bosnia, and the Persian Gulf in 2000 were approximately 91% males (US Department of Defense, 2002). The results of both of these studies support previous studies which indicate that DU is a toxicant capable of crossing the BBB and producing prolonged behavioral/neurological changes.

Studies from our laboratory have recently addressed the cytotoxicity of uranyl acetate in primary rat cortical neuron cultures. We found no evidence that uranyl acetate at concentrations below 100 μM caused cytotoxicity (Jiang *et al.*, 2007). Furthermore, we failed to document a significant change in the levels of F_2-isoprostanes, biomarkers of oxidative stress, as well as thiol metabolite levels upon treatment with uranium. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assays and lactate dehydrogenase (LDH) activity were also unchanged in neurons treated with uranyl acetate. In complementary studies in the nematode *C. elegans*, we found no evidence for neuronal degeneration using green fluorescent protein (GFP)-reporter strains corroborating the neuron culture results (Jiang *et al.*, 2007).

With respect to human subjects, Gulf War veterans subjected to friendly fire and embedded with shrapnel from DU projectiles have been followed (Hooper *et al.*, 1999; McDiarmid *et al.*, 2000, 2001a, b, 2002, 2004a, b, 2006, 2007). The fragments are a permanent exposure source inside the body and these veterans have demonstrated consistently higher urine levels of uranium, compared to controls without DU shrapnel, even after 10 years of exposure (Hooper *et al.*, 1999; McDiarmid *et al.*, 2000, 2001a, b, 2002, 2004a, b, 2006, 2007). These Gulf War veterans do not show evidence of kidney damage or dysfunction, but there was an indication that increased uranium exposure may be marginally correlated with decreased neurocognitive performance, as

measured by paper and pencil, and automated tests. In more recent follow-up studies, the initially identified neurological disabilities can no longer be detected. Many groups have called for further studies to examine the potential relationship between DU exposure and cognition (Bem and Bou-Rabee, 2004; Durakovic, 2001, 2003; Harley *et al.*, 1999; Priest, 2001; Sztajnkrycer and Otten, 2004; The Royal Society, 2001, 2002; US Department of Defense, 2000; WHO, 2001).

VII. TREATMENT

Currently, the treatments for uranium exposure are limited. Chelation therapy is used to prevent acute toxicity of high doses of uranium in the systemic circulation, typically resulting from some sort of ingestion. Chelating agents are used to competitively compete for the uranyl ion. Numerous studies have tested the efficacy of different chelating agents, such as gallic acid, 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron), diethylenetriaminepentaacetic acid (DTPA), 5-aminosalicylic acid (5-AS), catechol-3,6-bis(methyleneiminodiacetic acid) (CBMIDA), and ethane-1-hydroxy-1,1-bisphoshonate (EHBP) (Durbin *et al.*, 2000, 1997; Fukuda, 2005; Fukuda *et al.*, 2005; Ortega *et al.*, 1989a). These chelating agents can be effective in removing uranium and providing protective effects from actute uranium toxicity with differing efficacy. However, the length of time before initiating chelation therapy for acute uranium intoxication greatly influences the effectiveness of this therapy (Domingo *et al.*, 1990).

For embedded DU fragments surgical removal is the best option to get rid of as much of the shrapnel as possible. Because shrapnel can be of different sizes and dispersed in multiple locations, it is often difficult to remove every piece. Large fragments and fragments that are easily accessible, such as in soft tissues, are removed where possible to minimize the amount of uranium that will remain in fragments in the body.

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

Depleted uranium is an excellent metallic substrate for radiation shielding and for armor and ammunition by the military due to its density and pyrophoric properties. Furthermore, the unique ability of uranium-based ammunitions to sharpen themselves upon impact, allowing for deeper penetration of the ammunitions, also makes DU a better substrate for weapons of mass destruction. As such, it is not suprising that the use of DU in military applications is expected to grow. This increased use will no doubt be bolstered by recent scientific studies showing that DU exposure has relatively low adverse health effects, contrary to the public backlash and belief that DU is a harmful chemical and may be the cause of Gulf War Syndrome.

There has also been speculation that there is a potential black market use of DU as a weapon for bioterrorism, or as part of a dirty bomb. However, a thorough evaluation of the toxicology of uranium indicates that DU is relatively inert, compared to other potential materials and organisms that could be used to make an effective bioterrorism weapon or dirty bomb. Indeed, the reason why DU may still be considered to be an excellent bioterrorism agent is the public perception that DU is a harmful chemical, even though the toxicology of the chemical and radiological effects are relatively mild.

Recent studies have provided a great deal of new information about DU but there are still gaps in our understanding. For example, although we now know that uranium will cross the blood–brain barrier and accumulate in the brain, the functional changes associated with brain uranium accumulation remain to be seen. As such, although the evidence suggests that DU is relatively inert, the controversy still remains as to whether DU may be an agent that causes Gulf War Syndrome. Additionally, although there is not a great demand for pharmacological interventions, potential development of better chelating agents would be beneficial to assist in the treatment of uranium exposures, both acute and chronic.

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CHAPTER 30

Botulinum Toxin

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Opinions, interpretations, conclusions, and recommendations are those of the author(s) and are not necessarily endorsed by the US Army.

I. INTRODUCTION

Botulism is a disease caused by anaerobic, spore-forming bacteria found in soil. Disease results from the actions of chemical toxins produced by these bacteria. The most common forms of human botulism include foodborne, infant, and wound. The main etiology of botulism in humans is foodborne; this form is caused by eating foods contaminated with botulinum spores, which germinate and multiply into bacteria to produce neurotoxin in the food. Commonly contaminated foods include improperly preserved home-processed foods such as honey, corn, green beans, and beets. Less likely sources are fish products and other commercially processed foods. Infant botulism is often associated with eating honey contaminated with spores, but new evidence suggests that soil and dust brought into the house from the outside may be a significant source of botulinum spores. Wound botulism occurs when spores contaminate a wound, germinate, and produce toxins absorbed into the bloodstream. Regardless of the form of botulism, disease results from the intoxicating effects of potent neurotoxins.

Botulinum neurotoxins (BoNTs) comprise a family of seven distinct neurotoxic proteins produced by immunologically discrete strains of the anaerobic bacteria. These spore forming, Gram-positive bacteria secrete deadly toxins with an estimated human LD_{50} of 1–3 ng/kg (Simpson, 2004; Sobel *et al.*, 2004). In fact, BoNTs are the most potent substances known to humankind. Due to their extremely high potency, ease of production and previous history of weaponization, the BoNTs have been designated as category A threat agents by the US Centers for Disease Control and Prevention (CDC). Category A agents are defined by the CDC as those that ''… result in high mortality rates and have the potential for major public health impact; might cause public panic and social disruption and require special action for public health preparedness''.

While there are currently seven known antigenic serotypes of BoNT, only serotypes A, B, and E are predominantly associated with human intoxication. Intoxication by BoNTs leads to bilateral flaccid paralysis, involving skeletal muscle and structures innervated by autonomic fibers (Habermann and Dreyer, 1986; Simpson, 1986; Shapiro *et al.*, 1998). Death is inevitable if left untreated. The toxicity of BoNTs leading to flaccid paralysis of skeletal muscle is due to their ability to block acetylcholine (ACh) release from peripheral cholinergic nerve endings. Paralysis could persist for weeks to months depending on the serotype, and the available treatment consists of supportive care including fluids, total parenteral nutrition (TPN), and mechanical ventilation. Death occurs when the diaphragm and intercostal muscles become sufficiently compromised to impair ventilation or when patients succumb to secondary infections following long periods of intensive care (Hatheway *et al.*, 1984; Shapiro *et al.*, 1998; Robinson and Nahata, 2003).

BoNTs pose a serious concern to our national security. The toxins are highly lethal, easy to isolate, and easy to deliver by terrorists. Activities of hostile nations, international terrorists, and antigovernment groups make the threat of BoNTs a serious problem for both our military and civilian populations. Development of BoNTs as weapons of mass destruction began over 50 years ago. Nonstate-sponsored terrorists have an interest in BoNTs; members from the Aum Shinrikyo cult attempted to disperse BoNT in Tokyo and at US military installations throughout Japan on at least three occasions in the 1990s (Brackett, 1996). Furthermore, in the years following Operation Desert Storm (1990–1991), it was discovered that Iraq produced thousands of liters of concentrated BoNT in their weapons program. Moreover, approximately half of that volume was already loaded into military weapons. The BoNT generated by Iraq has yet to be fully accounted for. An act by terrorists to release BoNT into a civilian population through contamination of products of consumption or inhalation would pose a serious threat to national security and public safety. Wein and Liu (2005) modeled the threat of deliberate BoNT release into a milk-processing facility. The actions by such a terrorist group could lead to massive casualties. Another study estimated that dispersal of BoNT via inhalation could kill 10% of an exposed population within 0.5 km downwind of the incident.

II. BACKGROUND

A. Toxin Structure and Molecular Function

1. Background

The highly potent neurotoxins synthesized by the *Clostridium botulinum* microorganism and several related clostridial species (*C. baratii*, *C. butyricum*, and *C. argentinense*) are the causative agents of botulism, a potentially lethal disease historically associated with the ingestion of contaminated food products. Seven different BoNTs, designated A through G, are currently known to be produced by various strains of clostridial bacteria; these neurotoxins are antigenically distinct but comparable in basic structure. The BoNTs are members of a superfamily of homologous proteins that also include tetanus neurotoxin. BoNTs are generated as single-chain polypeptides which are then post-translationally modified (proteolytically nicked) to yield a disulfide bond-linked dichain structure composed of a heavy chain (H-chain or HC) and a light chain (L-chain or LC). Enzymes synthesized by these microorganisms themselves often mediate this cleavage although the gastrointestinal enzymes of the host can also generate the dichain structure from the ingested toxin. The three-dimensional dichain protein structure of the purified toxin is provided (see Figure 30.1).

2. Function of Heavy and Light Chains

The HC and LC of BoNTs each play critical roles in toxicity. HC is thought to mediate binding and internalization of the toxin at peripheral nerve synapses. LC, the toxic moiety, inhibits neurotransmitter exocytosis through its zinc-dependent endoproteolytic activity. The LCs of the various BoNT serotypes differ in their distinct molecular targets within the peripheral cholinergic nerve terminals (Schiavo *et al.*, 1992, 1993a, b, 1994; Blasi *et al.*, 1993; Yamasaki *et al.*, 1994). The endoproteolytic activities of the different toxin LCs produce similar flaccid paralytic effects, despite their distinct targets.

BoNT serotype A is the most well characterized of the different serotypes in terms of both structure and function.

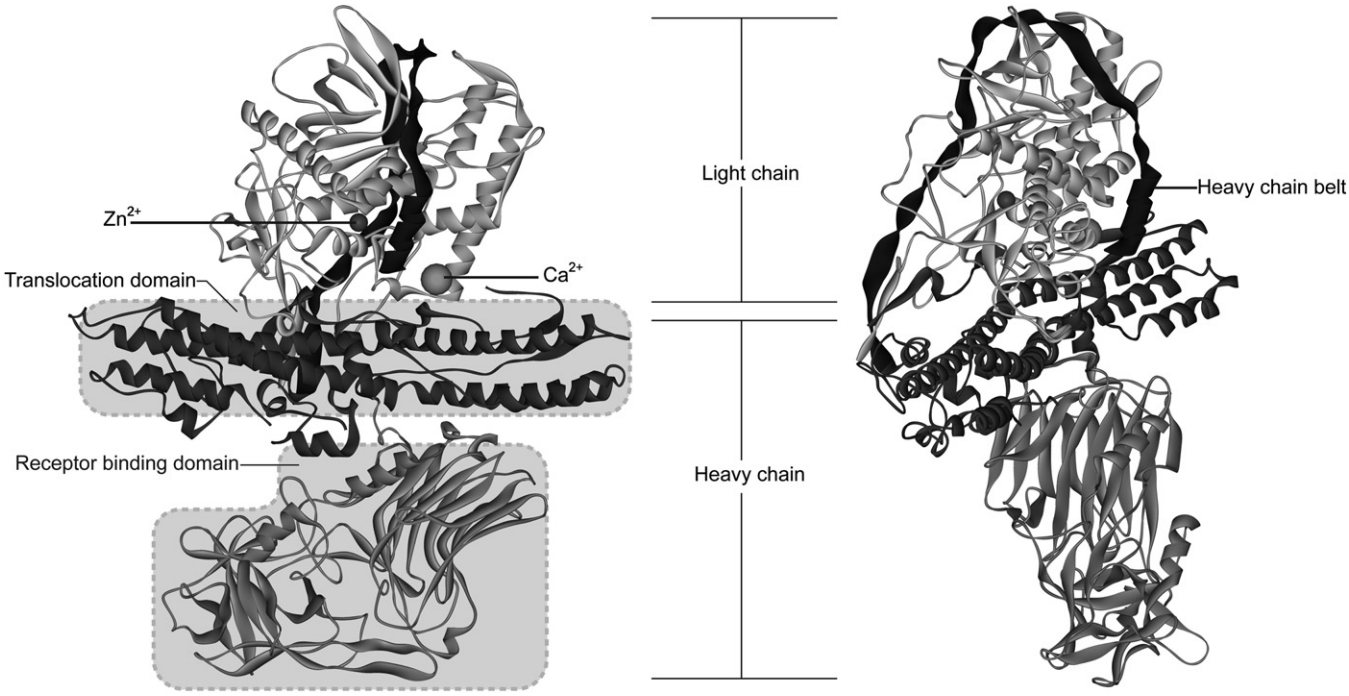

FIGURE 30.1. Three-dimensional structure of botulinum toxin serotype A (BoNT/A). BoNT/A (1,296 amino acids), rendered as a ribbon structure, is depicted in two views. BoNTs are synthesized as a single polypeptide and nicked by bacterial proteases to form a dichain molecule. The 50 kDa light chain (LC), 448 residues, and the 100 kDa heavy chain (HC), 848 residues, are linked by a dilsulfide bond. All BoNTs comprise three major domains: a receptor binding domain (C-terminal end of HC), a translocation domain (N-terminal end of HC), and a zinc-binding metalloprotease domain on LC. All seven BoNTs exhibit conserved sequence, but are also antigenically distinct at the same time. The LC seems to be held in place by the translocation belt of HC (Brunger *et al.*, 2007). The belt spans residues 492–545 in BoNT/A and 481–532 for BoNT/B and wraps around the catalytic domain of LC. Brunger *et al.* (2007) suggest that the belt acts as a surrogate pseudosubstrate inhibitor of the LC protease and acts as a chaperone during translocation across the endosome membrane into the cytosol. The belt occludes access to the active site of LC, thereby holding the unreduced holotoxin in its catalytically inactive state. The sphere represents the bound Zn^{2+} at the LC active site. The structure of BoNT/A holotoxin was provided free of copyright restrictions from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (Berman *et al.*, 2000; PDB ID: 2nz9; Garcia-Rodriguez *et al.*, 2007) and rendered using Accelrys DS Visualizer 2.0 software.

Early biochemical efforts led to its crystallization, and this crystalline form was used in numerous animal studies on the pathogenesis of botulism. Crystalline type A toxin has a sedimentation constant of 19S (around 900 kDa), which is far larger than the combined size of the HC and LC components (Simpson, 1981). This large ''progenitor toxin'' form was shown to dissociate under moderately alkaline conditions, releasing the ''derivative'' (7S) or neurotoxin component (Heckly *et al.*, 1960; Sugii *et al.*, 1977a, b; Chen *et al.*, 1998). The derivative neurotoxin has a molecular weight of 150 to 160 kDa, representing the combined size of the heavy and light chains. Additional work led to the finding that the dichain molecules comprising the various neurotoxins are often released as higher-order polypeptide complexes. The crystalline type A progenitor toxin consists of the 7S neurotoxin and one or more noncovalently linked accessory proteins. These nontoxic components of the progenitor toxin complex were later identified as three different hemagglutinins (HAs) and a nontoxic nonhemagglutinin (NTNH) protein.

3. Accessory Proteins of the Progenitor Toxin Complex

The accessory proteins of the progenitor toxin serve to enhance the stability of the toxin to ensure uptake from the gut. The NTNH component of the multimeric type A toxin complex is encoded by a single gene upstream of the neurotoxin locus while three different HA proteins have been characterized in association with BoNTs. All *C. botulinum* serotypes have been shown to produce neurotoxin complexes with the NTNH and HA proteins.

B. Overview of BoNT Action

After gaining entry to the lymphatics and circulation via the gastrointestinal tract, BoNTs function as potent neuromuscular blockers. The cellular and molecular mechanisms involved in toxin absorption, transit to specific target tissues, escape from the vasculature, and uptake within peripheral cholinergic nerve terminals have yet to be fully characterized. However, each of the neurotoxins has been shown to block vesicular neurotransmitter release at peripheral cholinergic synapse through the endoproteolytic cleaveage of proteins associated with the exocytosis machinery (Schiavo *et al.*, 1995, 1993a, b; Blasi *et al.*, 1993). At peripheral cholinergic nerve endings, BoNT binding to high-affinity receptor(s) leads to acceptor-mediated endocytosis and low pH-induced translocation across the endosomal membrane into the cytosol. The carboxy-terminal region of the toxin heavy chain appears to mediate binding at the nerve synapse while the amino-terminal domain controls translocation. The LC is held in close association with the HC by an amino acid belt. The LC of each toxin functions as a zinc-dependent endoprotease, cleaving at least one of three soluble *N*-ethylmaleimide-sensitive fusion (NSF) protein attachment receptor (SNARE) proteins involved in neurotransmitter release.

Stimulus-evoked, calcium-dependent release of acetylcholine (ACh) from the cholinergic synapse normally occurs through the formation of a fusion complex between ACh-containing vesicles and the intracellular leaflet of the nerve terminal membrane (Arnon *et al.*, 2001). This synaptic vesicle fusion complex consists of several proteins of the SNARE family, including a 25 kDa synaptosomal associated protein (SNAP-25), vesicle-associated membrane protein (VAMP, or synaptobrevin), and the synaptic membrane protein syntaxin. Other SNARE proteins have been identified as components of membrane transport systems in yeast and mammals but have not been implicated as targets for BoNTs. Meanwhile, type A and E neurotoxins cleave SNAP-25 while types B, D, F, and G act on VAMP and type C1 toxin cleaves both syntaxin and SNAP-25. Neurotoxin-mediated cleavage of any of these substrates disrupts the processes involved in the exocytotic release of ACh and leads to flaccid paralysis of the affected skeletal muscles.

C. Clinical Forms of Botulism in Humans and Animals

Exposure to BoNTs can produce lethal disease in humans and other animal species. Six different clinical forms of human botulism have been described in the literature (see Table 30.1). These include: (1) foodborne botulism, (2) infant botulism, (3) wound botulism, (4) an adult form of infant botulism, (5) inadvertent systemic botulism, and (6) inhalation botulism (reviewed by Cherington, 1998; Middlebrook and Franz, 2000; Arnon *et al.*, 2001). Botulism is the result of either an infectious process, involving elaboration of toxin from the colonizing clostridial organism, or a noninfectious process. Infant and wound botulism are the most prevalent infectious forms of botulism. Although rare, an adult form of infant botulism has been documented; gastrointestinal colonization in adults may be enabled by alterations in normal gastrointestinal flora resulting from antibiotic treatment (Cherington, 1998). Foodborne and inhalational have noninfectious etiologies and are the result of ingesting or inhaling preformed toxin. Although only one inhalational botulism incident has been reported in humans, this incident demonstrates that humans are susceptible to respiratory intoxication similar to that which has been experimentally produced in many laboratory species (Holzer, 1962; reviewed by Middlebrook and Franz, 2000). Finally, the emergence of a multitude of therapeutic applications for BoNTs has led to infrequent cases of inadvertent systemic botulism resulting from local toxin injection (reviewed by Arnon *et al.*, 2001).

D. Infectious Forms of Botulism

1. Infant Botulism

Infectious botulism is a consequence of ingesting or inhaling clostridial spores which colonize the large intestines,

TABLE 30.1. Clinical forms of botulism

	Infectious			Noninfectious		
	Infant	**Wound**	**Adult colonization**	**Foodborne**	**Inhalational**	**Inadvertent systemic**
Cause	Colonization of immature intestinal tract	Wound colonization resulting from contact with contaminated material	Intestinal colonization secondary to disruption of normal intestinal flora	Ingestion of preformed toxin in contaminated food products	Respiratory exposure to toxin aerosols or droplets	Systemic toxin uptake after therapeutic toxin administration
Susceptibility	Young infants (2 to 4 months of age) prior to establishment of normal intestinal flora	Self-administering users of intravenous drugs (often black tar heroin)	Antibiotic-treated patients	All exposed individuals	All exposed individuals	Patients treated with local toxin injections

germinate, and elaborate toxin into the bloodstream. Infant and wound botulism are the most prevalent infectious forms of botulism. Infant botulism comprises the majority (72%) of reported human botulism cases in the USA, while most of the remaining cases involve foodborne and wound botulism (Mackle *et al.*, 2001). Young infants are especially susceptible to infant botulism. Infants were found to be uniquely susceptible to gastrointestinal colonization due to a lack of well-established competing gut flora (Arnon, 1995). While infant botulism can be acquired by inhalation of spores, this differs markedly from inhalational botulism, which results from inhaling preformed aerosolized toxin and not spores. Clostridial spores do not pose a threat in older infants or most adults (Arnon, 1995, 1998; Cox and Hinkle, 2002).

2. Wound Botulism

Wound botulism involves growth of *C. botulinum* spores in a contaminated wound with *in vivo* toxin production (Weber *et al.*, 1993). It accounts for less than 25% of all botulism cases (Sandrock and Murin, 2001). The majority of wound botulism cases are caused by serotype A and the remainder by serotype B (Shapiro *et al.*, 1998). The neurological symptoms of wound botulism differ little from those of foodborne botulism except for the general absence of gastrointestinal symptoms. From its discovery in 1943 until 1996, only 111 incidents of wound botulism were documented (CDC, 1998; Merson and Dowell, 1973; Shapiro *et al.*, 1998); among the 100 laboratory confirmed cases, 83 cases were type A, 16 cases type B, and one a mixture of type A- and B-producing organisms (Hatheway, 1988; CDC, 1998). Risk factors for wound botulism include deep wounds, avascular areas, compound fractures, and crush injuries of the hand. Although a rare form of naturally occurring BoNT intoxication, it most recently occurred in Maryland as a result of a construction worker receiving a contaminated, compound fracture of the femur after falling into an excavated pit (Hilmas, personal observation). Wound botulism also occurs in intravenous drug users as a result of bacterial colonization at needle puncture sites or nasal/sinus lesions secondary to cocaine snorting (MacDonald *et al.*, 1985). From 1986 through 1996, 78 cases of wound botulism were reported, and the majority of cases were linked to black tar heroin, introduced intravenously.

3. Child or Adult Botulism from Intestinal Colonization

Gastrointestinal colonization in adults or children by clostridial bacteria does not typically take place except under circumstances where the normal flora has been altered by antibiotic treatment (Cherington, 1998). Botulism results from *in vivo* production of toxin, analogous to the pathogenesis of infant botulism (McCroskey and Hatheway, 1988; Chia *et al.*, 1986). Support for this form of botulism is provided by demonstration of prolonged excretion of toxin and *C. botulinum* in stool and/or by the demonstration of *C. botulinum* spores but not preformed toxin in suspected foods.

E. Noninfectious Forms of Botulism

1. Foodborne Botulism

Worldwide, BoNT intoxication is most commonly associated with food poisoning. In the early 19th century, the effects of botulism were observed to be associated with the consumption and handling of meat products. Thus German physician Justinus Kerner described what he termed ''sausage poisoning'' (Erbguth, 2004). It was later in the 19th century that the term ''botulism'' was used, from the Latin *botulus* for ''sausage''. Foodborne botulism results from ingesting preformed toxin in food contaminated with toxin spores. Inadvertent and inhalational botulism, two other noninfectious forms of botulism, also involve

exposure to preformed toxin. Outbreaks of foodborne botulism in the USA result from eating improperly preserved home-canned foods (CDC, 1995). The majority of cases of foodborne botulism are due to serotypes A, B, and E. From 1990 through 1996, type A accounted for 44.6% of foodborne outbreaks in the USA, followed by type E (35.7%) and type B (12.5%) (CDC, 1998). The prompt recognition of such outbreaks in the USA and early treatment with serotype-specific botulinum antitoxin has limited the number of casualties, severity of the disease, and the case-to-fatality ratio. Mortality from foodborne botulism has declined from 60% (CDC, 1998) in 1950 to less than 10% of clinical cases (Shapiro *et al.*, 1998).

2. Inhalational

Because humans are relatively resistant to gut colonization by the *C. botulinum* microorganism, oral and inhalational exposures to preformed neurotoxin are likely to present the greatest threats with respect to intentional dissemination. The ability for inhaled botulinum neurotoxins to produce illness has been documented in humans and in several experimental species. Only one incident involving inhalational intoxication in humans has been reported. Three laboratory workers presented with physical and neurological symptoms after accidental respiratory exposure to aerosolized type A toxin (Holzer, 1962; Middlebrook and Franz, 2000; Arnon *et al.*, 2001). These patients were all successfully treated with antiserum, gradually recovering from their weakness and visual disturbances over the next several days. After inhalational exposure, the neurotoxins are absorbed from the respiratory tract into the lymphatics and circulation for transport to peripheral cholinergic synapses (reviewed by Simpson, 2004). The pathogenesis following neurotoxin absorption is thought to be similar for both the respiratory and gastrointestinal exposure routes. Thus, the primary neurophysiological signs and symptoms associated with respiratory exposure parallel those observed in cases of foodborne botulism.

3. Inadvertent Systemic Botulism

The therapeutic indications for BoNTs are numerous. They are used in the treatment of ophthalmological disorders (strabismus, Duane's syndrome, esotropia/exotropia), movement disorders (focal dystonias, blepharospasm), spasticity, neuromuscular disorders, pain (headache, myofacial pain), disorders of the pelvic floor (anal fissures), ear/nose/throat disorders, cosmetic applications (wrinkles), and hyperhidrosis. The recent explosion in new indications for BoNTs in the treatment of a wide range of medical conditions also brings the possibility for medical errors in BoNT dosing. Systemic botulism may result from injection of excessive doses of the potent neurotoxin. The most infamous case of systemic botulism involved the paralysis of four Florida patients, including the doctor, treated with BoNTs for wrinkles. The physician used non-FDA approved formulations of type A from Toxin Research International, Inc. The research grade type A neurotoxin was apparently sold to the doctor and reconstituted to be thousands of times more potent than the typical dose used in BOTOX® for paralyzing facial muscles. Later testing estimated that the raw bulk toxin used contained between 20,000 and 10 million units of botulinum toxin. In comparison, a typical vial of BOTOX from Allergan, Inc. contains only 100 units (CIDRAP, 2004). All three patients and the physician were injected with the toxin preparation; they developed severe systemic botulism requiring mechanical ventilation. While all four survived the super-lethal dose of type A toxin, several of the patients have experienced a syndrome involving chronic gastrointestinal symptoms and discomfort months after exposure.

F. Human Intoxication

The basic syndrome of BoNT intoxication is similar for all naturally occurring forms, as well as for inhalation exposure and does not vary appreciably among serotypes (Simpson, 1986; Habermann and Dreyer, 1986; Hatheway *et al.*, 1984; Jankovic and Brin, 1997). Based upon documented laboratory evidence, human BoNT intoxication is caused by exposure primarily to serotypes A, B, E, and to a much lesser extent to serotype F; disease manifests mostly as a result of foodborne, infant, and wound botulism (Habermann and Dreyer, 1986; Simpson, 1986). BoNTs are also lethal from inhalation of aerosolized toxin, although this form is not generally observed in nature.

The various toxin serotypes are usually associated with analogous clinical presentations. Paralysis proceeds in a descending fashion after an initial bulbar involvement. The earliest symptoms of botulism typically include visual disturbances, followed by dysphagia, dysphonia, and dysarthria, reflecting an especially high susceptibility of cranial efferent terminals to BoNT action (Habermann and Dreyer, 1986; Jankovic and Brin, 1997). A descending generalized skeletal muscle weakness may then develop, progressing from the upper to lower extremities. Involvement of the diaphragm and intercostal muscles can lead to ventilatory failure and death unless appropriate supportive care is provided (Shapiro *et al.*, 1998; Robinson and Nahata, 2003).

III. EPIDEMIOLOGY

A. Foodborne Botulism

Human foodborne botulism outbreaks have typically been linked to the consumption of toxin-contaminated home-prepared or home-preserved foods (Maselli, 1998). The vast majority of foodborne botulism cases are attributed to toxin types A, B, or E. Maselli (1998) reports that type B is the most prevalent (52%) in the USA, followed by type A (34%) and type E (12%), while the CDC (1998) suggests 37.6% of all foodborne botulism outbreaks since 1950 were caused by

type A, 13.7% by type B, 15.1% by type E, 0.7% by type F, and 32.9% were unidentified with respect to toxin type. Outbreaks of type F and G botulism are rare (Sonnabend *et al.*, 1981; Maselli, 1998; Richardson *et al.*, 2004), and only anecdotal reports of isolated type C1 and D botulism cases can be found in the published literature (Lamanna, 1959).

The natural epidemiology of foodborne botulism provides additional insight into the similarities and discrepancies between the human disease and that represented in various animal models. In the USA, around 25% of reported human botulism cases are classified as foodborne and 72% are infant (Mackle *et al.*, 2001). Human type A and B foodborne botulism cases occur worldwide and constitute the vast majority of reported human intoxications (Maselli, 1998). The majority of other botulism cases are attributed to serotype E and are typically associated with the consumption of contaminated seafood. Generalizations have been made regarding the geographic distribution of the most common *C. botulinum* strains within the USA. Most human foodborne botulism outbreaks occurring west of the Mississippi are due to type A toxin; type B strains are more prevalent east of the Mississippi while type E strains are typically isolated to Alaska and the Pacific Northwest (Arnon *et al.*, 2001; Richardson *et al.*, 2004).

Several clinical and epidemiological reports have evaluated the worldwide geographic distributions of human foodborne botulism cases. A review of 13 outbreaks between 1970 and 1984 identified geographic differences in the toxin serotypes associated with human foodborne botulism cases. Type B botulism was predominant in Portugal, Spain, France, and several other European countries (Lecour *et al.*, 1988). Interestingly, the low mortality rate associated with human type B foodborne botulism (8.8% versus 24.3% for type A and 30.8% for type E in the USA from 1950 to 1979) did not correlate with the high oral toxicity for type B toxin in mice (Ohishi *et al.*, 1977; Sugii *et al.*, 1977a, b, c; Ohishi, 1984). Serotype E was linked to botulism outbreaks in select regions such as the Baltic countries (Lecour *et al.*, 1988) and typically resulted from the consumption of contaminated fish (Maselli, 1998).

Type F toxin was only associated with two reported outbreaks of human foodborne botulism prior to 1998 (Maselli, 1998). The first of these outbreaks occurred in Denmark (on the Island of Langeland) and was attributed to a contaminated liver paste product (Muller and Scheibel, 1960; Richardson *et al.*, 2004). The second outbreak, in 1966, affected three individuals in California and was associated with home-made venison jerky (Midura *et al.*, 1972; Richardson *et al.*, 2004). While a few other type F botulism cases have been reported, they are generally thought to have resulted from intestinal colonization and type F toxin production by another related species, *C. baratii* (Hall *et al.*, 1985; Richardson *et al.*, 2004). A recent report of a type F botulism case in California provided some additional insight into this uncommon toxin serotype and the associated clinical disease (Richardson *et al.*, 2004). The patient described in this report presented with typical signs and symptoms including ptosis, dysphagia, and weak extremities. Although the source of the ingested toxin was not conclusively determined, the exposure was tentatively linked to shellfish consumption, and type F toxin was subsequently detected in the patient's stool (Richardson *et al.*, 2004). Human type F botulism cases may have been underreported in the past since some laboratories did not test culture isolates for the presence of *C. baratii*, which also produces type F toxin.

Type G toxin-producing clostridial organisms (*C. argentinense*) have been detected in several soil samples from a South American cornfield (Gimenez and Cicarelli, 1970; Maselli, 1998), but only one reported outbreak of type G botulism (in Switzerland) has been identified in the published literature (Sonnabend *et al.*, 1981). Certain aspects of this outbreak draw questions as to whether it was truly associated with type G intoxication. Type G organisms were isolated from all four affected adults and an 18-week-old infant, suggesting that the intoxications were due to ingestion and subsequent colonization by type G spores (Sonnabend *et al.*, 1981). Type G toxin was detected at low levels of 2–7 mouse intraperitoneal lethal dose 50 ($MIPLD_{50}$)/ml in the serum of three out of the four lethally intoxicated adults, all of whom died suddenly sometime after the presumed foodborne intoxication. Type A toxin was also detected in two of these individuals, suggesting that the intoxications may have involved colonization either by a mixed set of clostridia or by a unique strain producing multiple toxins (Sonnabend *et al.*, 1981). Alternatively, detection of dual serotypes could have been an artifact of the culture or testing methods. Soil samples taken from the area indicated the presence of only type A clostridial organisms (Sonnabend *et al.*, 1981). Regardless, the occurrence of human type G botulism is rare, and the relative susceptibility of humans to colonization and intoxication from this serotype is not clear.

Species-specific patterns of susceptibility to different toxin types are common in both naturally occurring and experimental foodborne botulism. These differences do not necessarily facilitate identification of the most appropriate animal models from the human condition, but they may help to eliminate highly variant species. For example, mink were reported to be relatively resistant to toxin types A, B, and E (Yndestad and Loftsgard, 1970), which are responsible for the vast majority of human botulism outbreaks (Maselli, 1998; Arnon *et al.*, 2001). Weanling pigs, on the other hand, were shown to be moderately resistant to types A, C1, E, and F and highly resistant to type D toxin (Smith *et al.*, 1971). Experimental and epidemiological studies have identified one persistent difference in the epidemiology of botulism in humans compared to many other animal species. Few reports citing human outbreaks of type C and D are available. One of these reports mentions two human type C outbreaks and one type D outbreak but provides no

reference for these cases (Lamanna, 1959). More recent reports of human type C or D botulism have not been found in the literature, and it is widely assumed that human foodborne intoxications are rarely, if ever, associated with these toxin types. In contrast, naturally occurring botulism of both types is quite common among domestic and wild animal species, and several studies have established the susceptibility of various laboratory species to experimental type C and D botulism.

The authors of this chapter have studied the effect of C and D toxin serotypes, as well as A, B, and E, on human intercostal muscle (Hilmas, unpublished data). All serotypes showed a similar ability to produce complete muscular paralysis in *ex vivo* human intercostal muscle. Intercostal muscle was excised from patients receiving a thoracotomy and intercostal muscle flap procedure. The muscle was removed tendon to tendon by surgical excision without electrocautery and dissected into multiple bundles with their associated intercostal nerves. The nerve–muscle units were placed in a vertical twitch bath and stimulated at 0.03 Hz (0.2 ms pulses of supramaximal strength) using a novel nerve clamp electrode to illicit an indirect muscle twitch. Potent toxins (1 nM) from various serotypes were added to the bath after confirming the stability of control muscle responses. In each case, twitch tensions declined to negligible amplitudes by 1 h after direct toxin application to the tissue bath.

Several nonhuman primate species are known to be susceptible to type C1 and D toxins both in nature and as experimental models. A large natural outbreak of type C botulism was reported in a troop of captive *hamadryas* baboons in 1989 (Lewis *et al.*, 1990). The outbreak resulted in the deaths of 36 animals, including 3 adult males, 6 sub-adult males, 17 adult females, and 10 sub-adult females. Additional animals displayed mild to moderate symptoms that resolved over a period of several days (Lewis *et al.*, 1990). As with human foodborne botulism, various age groups and both sexes were affected, and no macroscopic lesions were apparent. Serum samples and gastric contents taken from ill animals contained type C1 toxin, although the source of the toxin was not identified (Lewis *et al.*, 1990). The authors speculated that man is probably also susceptible to type C1 toxin. The reason for the relative lack of human type C botulism cases remains unknown. It has been suggested that serotype C is often associated with carrion, providing a possible explanation for the absence of reported human cases. At least two other outbreaks of naturally occurring type C botulism in nonhuman primates were previously reported, one in squirrel monkeys (*Saimiri sciureus*) and capuchin monkeys (*Cebus capucinus* and *Cebus olivaceus*) (Smart *et al.*, 1980) and the other in gibbons (*Hylobates lar*) (Smith *et al.*, 1985).

In addition to nonhuman primates, most other animal species that show some sensitivity to botulinum intoxication are in fact susceptible to toxin serotypes C1 and D. Several rodent species are susceptible to oral intoxication with most botulinum toxins, including types C1 and D (Matveev, 1959; Jemski, 1961a, b; Cardella *et al.*, 1963; Sergeyeva, 1966; Sugiyama *et al.*, 1974; Smith, 1986; Fujinaga *et al.*, 1997; Gelzleichter *et al.* 1999; Middlebrook and Franz, 2000). The majority of botulism outbreaks in cattle have also been attributed to toxins C1 and D (Schocken-Iturrino *et al.*, 1990). Cattle intoxication is typically associated with the ingestion of contaminated bones and other carcass remains; their apparent susceptibility to type C and D botulism might simply be due to frequent ingestion of decaying material that is primarily contaminated with these toxin types. A recent study indicated that cows are also uniquely sensitive to intravenous injection of type C1 toxin (Moeller *et al.*, 2003). The high susceptibility of cattle to type C botulism is not dependent on exposure route although the specific factors contributing to their sensitivity are not known.

A recent outbreak of type C botulism among farmed mink and foxes in Finland underscores the need to consider not only the quantitative susceptibility of various species to the toxins but also the potential epidemiological significance of interspecies differences in dietary patterns. Lindstrom *et al.* (2004) reviewed the Finland incident, which was the largest documented type C botulism outbreak in fur production animals. Over 52,000 animals developed illness after consumption of feed product that was contaminated with over 600 MLD of type C1 toxin per gram. This feed consisted of acidified slaughter by-products from poultry, beef, and fish (Lindstrom *et al.*, 2004). According to national regulations, these by-products were acidified with an organic acid to yield a final pH of 4.0 or lower. Such processing would inhibit the growth of many microorganisms but would not necessarily result in significant toxin inactivation. Over 44,000 of the 52,000 affected animals died, and the death rate among all potentially exposed animals was almost 22% (Lindstrom *et al.*, 2004).

The large number of animals affected and the high lethality associated with the outbreak could be considered indicative of the high susceptibility of the affected species to foodborne type C botulism. This high susceptibility might appear to be in stark contrast to that of man due to the scarcity of human type C cases. However, the Finland outbreak provides a clear indication that dietary differences between species may play a significant role in these epidemiological patterns. Humans would be far less likely to consume slaughter by-products (including intestinal tissues) as opposed to the higher quality beef, poultry, and fish products. Moreover, preparation of such products for human consumption would generally involve cooking rather than acidification. Thus, the influence of dietary habits must be taken into consideration when evaluating interspecies differences in epidemiological patterns for the various toxin serotypes.

It remains possible that humans generally do not consume the types of foods that are typically subject to contamination with type C1 and D toxins. Some researchers continue to speculate that humans are likely to be susceptible to both serotypes because they lead to botulism in monkeys both in nature and after experimental oral

exposure. Alternatively, humans might display a unique pattern of physiological susceptibility to the different toxin types. Lack of human susceptibility to type C1 and D intoxication could be attributed either to poor absorption of these specific toxins from the human gastrointestinal tract or to resistance of human cholinergic nerve terminals to the activity of these toxins. One cell culture study provided some support for the latter explanation. Type C1 neurotoxin was shown to bind with high efficiency to mouse neuroblastoma cells and to hybridomas of mouse neuroblastomas and rat gliomas, but not to human neuroblastoma cell lines (Yokosawa *et al.*, 1989). Yokosawa *et al.* (1989) suggested that reduced binding of type C1 toxin to human versus mouse neuroblastoma cells could provide one explanation for the lack of human type C botulism cases.

Another potential explanation for the unique epidemiology of human botulism was provided in a study of botulinum toxin binding and transcytosis across polarized monolayers of two human colon carcinoma cell lines (T-84 and Caco-2). Substantial binding of iodinated BoNT/A and BoNT/B to human colon carcinoma cells was observed while minimal binding of type C1 neurotoxin was detected (Maksymowych and Simpson, 1998). Both type A and B neurotoxins were also efficiently taken up, transcytosed, and released, by the polarized human carcinoma cells, whereas minimal transcytosis of type C1 neurotoxin was observed. The patterns of neurotoxin transcytosis (A and B but not C1) observed in these human gut epithelial cell lines correlate with human susceptibility to foodborne botulism (Maksymowych and Simpson, 1998). The authors speculated that since human tissues are fully sensitive to the neuromuscular blocking properties of C1 neurotoxin (Coffield *et al.*, 1997; Eleopra *et al.*, 2004), the relative absence of human foodborne type C botulism could be due to the inability of this toxin to penetrate from the gut to the general circulation. Human susceptibility to type C1 and D neurotoxins remains unclear; however, clarification of this issue will be important in interpreting data derived both from *in vitro* studies on toxin transcytosis and from animal models for oral intoxication.

IV. PATHOGENESIS

A. Overview of Pathogenesis

BoNTs are a group of immunologically distinct but closely related bacterial proteins that act as potent inhibitors of synaptic transmission in skeletal muscle. Inhibition of ACh release from the presynaptic terminal of the neuromuscular junction (NMJ) is thought to be the sole mechanism involved in the toxins' lethal action (Sellin, 1985; Simpson, 1986) and therefore the cause of botulism. The pathogenesis of intoxication is not completely understood but is generally thought to involve a multistep process to interrupt normal vesicular release of ACh from the presynaptic motor nerve terminal. In a process of transcytosis, ingested or inhaled BoNT must first cross a barrier (either intestinal or pulmonary epithelial cells) to gain access to the circulation (see intestinal absorption of BoNT, Figure 30.2). Once in the circulation, BoNT travels to its major target, located on presynaptic membranes of alpha motor neurons at NMJs and neuroeffector junctions. Toxin binding through its heavy chain to a cell surface receptor on the presynaptic motor nerve ending is followed by internalization via an endocytotic vesicle, acidification of the endosome, conformational change allowing cleavage of the enzymatically active light chain (LC) from bound heavy chain (HC), and release of light chain toxin into the cytoplasm. Here the light chain cleaves one of the integral members of the SNARE complex (SNAP-25, VAMP, or syntaxin), proteins involved in exocytosis of ACh (Simpson, 1986, 2004; Black and Dolly, 1986; Blasi *et al.*, 1993; Montecucco *et al.*, 1994; Schiavo *et al.*, 1995). BoNT thereby prevents docking of synaptic vesicles with presynaptic plasma membrane by selective proteolysis of synaptic proteins. Each stage in BoNT action provides a potential point for pharmacological intervention.

B. Toxin Stability

1. Biological Stability of the Toxins in the Gastrointestinal Tract

A major factor to consider in botulism is the stability of both the organism and the toxin. A variety of factors can affect the stability of ingested BoNTs within the gastrointestinal tract. The oral potency of the toxins is closely related to their ability to withstand the conditions found in these biological compartments prior to absorption into the lymphatics and general circulation. The stability of BoNT preparations has therefore been examined in the gastrointestinal compartments of intoxicated animals, as well as under different enzymatic and acidic conditions *in vitro*. Some of the earliest work on BoNT intoxication indicated that the stability and resulting potency of type A toxin vary both qualitatively and quantitatively in different rodent species (Minervin and Morgunov, 1941).

Several groups have evaluated the influence of ingested foods on the gastrointestinal stability and potency of BoNTs. Lamanna and Meyers (1959) reported that the ingestion of protein- or fat-containing foods prior to oral type A exposure in mice resulted in moderate increase in toxicity. The mechanisms by which food intake enhanced toxin potency were not clarified; however, the relatively small observed increases (two-fold) could have been to normal experimental variation in determining oral toxicity values. The same study demonstrated that fluorescein-labeled type A toxin was quickly destroyed in the stomach of mice. Crystalline and purified toxins form stable complexes with albumin and other proteins found in food and serum (Lamanna and Meyers, 1959). Albumin was later shown to prevent loss of potency when type A toxin was

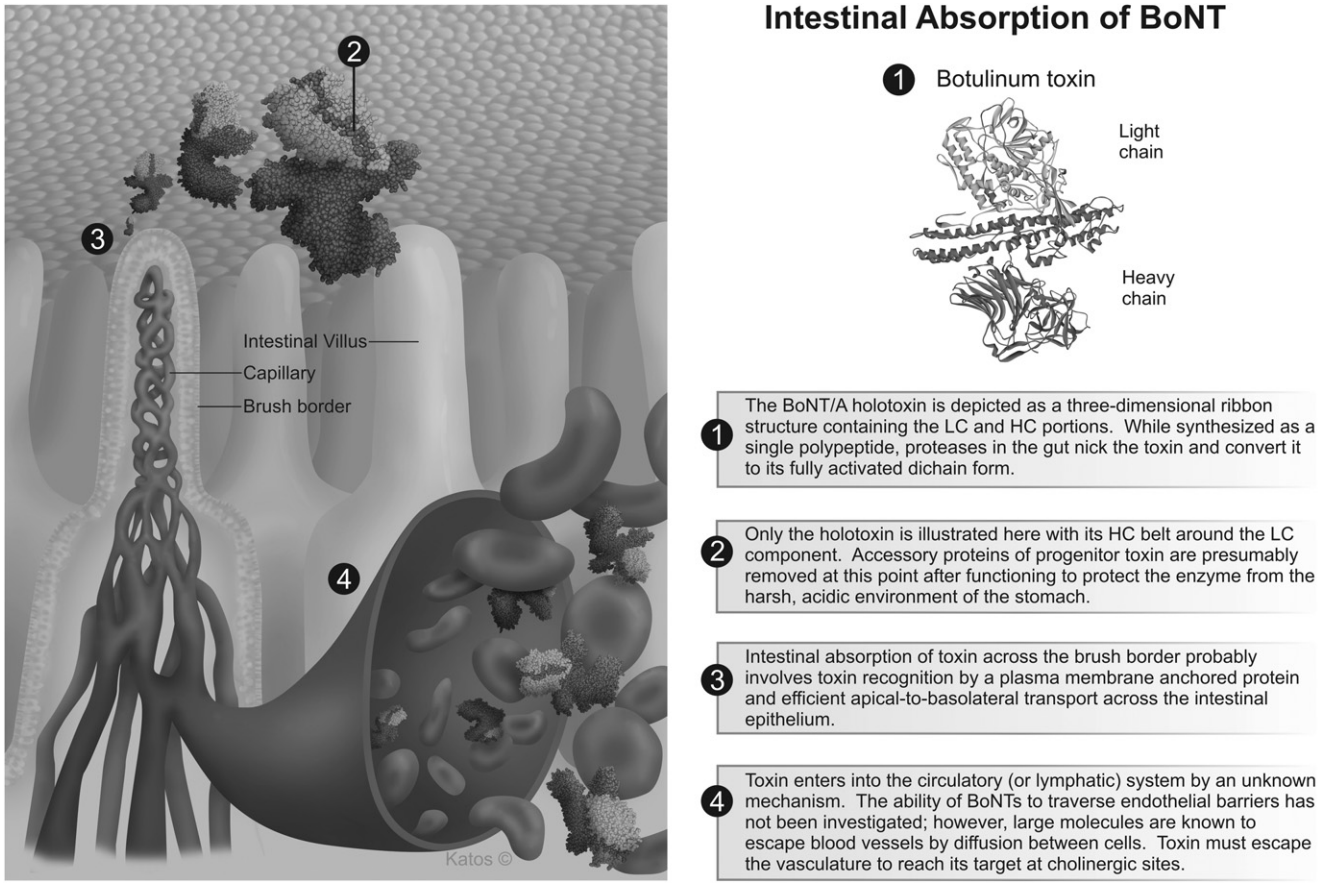

FIGURE 30.2. Intestinal absorption of botulinum neurotoxin. Neurotoxin present in the gut lumen as a result of foodborne (ingested toxin) or infant botulism (toxin synthesized by clostridial spores) must cross the epithelial membrane to reach the circulatory and lymphatic systems. The toxin presumably binds to an as yet unidentified receptor on the intestinal villus epithethium that is linked to an efficient transport process. Progenitor toxin contains nontoxic HA and NTNH accessory proteins which are probably shed from the protein prior to entry across the intestine brush border. Progenitor toxin is thought to be too large for any significant rate of paracellular diffusion (Simpson, 2004). The ability of botulinum holotoxins without accessory proteins to traverse endothelial barriers has not been investigated; however, large molecules are known to escape blood vessels by diffusion between cells. Toxin escapes the circulatory system and reaches peripheral (and possibly central) cholinergic sites. These include neuromuscular junctions, ganglia of the sympathetic and parasympathetic nervous system, postganglionic parasympathetic sites, and postganglionic sympathetic sites that release ACh. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

exposed to a wide range of pH values (Zacks and Scheff, 1967). This observation was consistent with the more recent finding that the enzymatic activity of BoNT A was enhanced in the presence of albumin (Schmidt and Bostian, 1997).

Subsequent studies expanded upon this early work by investigating the stability of other toxin types and preparations in solutions having different pH values and other conditions similar to those encountered in the gastrointestinal tract. Type C1 toxin was stable in most acidic and basic environments, as significant inactivation (as indicated by loss of toxicity in a mouse lethality assay) was only observed following exposure to extreme pH values (pH 1.8 and pH 12) (Halouzka and Hubalek, 1992). Progenitor type E toxin was more stable than its derivative (purified) form, which was subject to rapid inactivation when exposed to pH values less than 4.0 (Sakaguchi and Sakaguchi, 1974). This study demonstrated that type E progenitor toxin dissociated either during or after gastrointestinal absorption in mice, as only the derivative component could be detected in the blood and lymph following oral administration of the progenitor form.

Similar findings on the relatively high stability of progenitor versus derivative forms of the other toxin serotypes have been reported in other studies. Types A, B, and F progenitor toxins were more stable under conditions of low pH, as well as more resistant to digestion by pepsin and papain, than their corresponding derivative toxins (Sugii *et al.*, 1977a, b).

The derivative forms of toxins A and B were almost completely inactivated after 10 min of peptic digestion at pH 2.0, while the progenitor forms retained over 60% toxicity after an 80 min treatment (Sugii *et al.*, 1977a). Crystalline type A toxin was shown to be partially resistant to proteolysis by trypsin, retaining 25% of the potency of

control-treated toxin even after a 72 h trypsin digestion at 37°C (Coleman, 1954). The crystalline toxin was more readily inactivated by digestion with pepsin at pH 1.4 and chymotrypsin at pH 6.5. Interestingly, another group reported that the potency of type A toxin was weakened by 80% after a 5 h incubation in phosphate buffer (pH 7.5), while toxins C1 and D maintained 100% toxicity under the same conditions (Miyazaki and Sakaguchi, 1978). These findings demonstrate both serotype- and enzyme-dependent effects on the *in vitro* stability of the BoNTs that are also likely to impact their persistence in the gastrointestinal tract. A similar pattern of stability among the various BoNT forms in gastrointestinal juices isolated from different animal species has been demonstrated (Sugii *et al.*, 1977a). The progenitor forms for all toxin serotypes retained significant toxicity in comparison to their derived holotoxin counterparts.

Epitope mapping experiments suggested that the nontoxic component of the intact progenitor toxin complex covers a large portion of the binding domain of the neurotoxin (Chen *et al.*, 1998). Toxin interaction studies also revealed that the purified neurotoxin adheres to lipid monolayers while the progenitor complex is not subject to significant adsorption to the same monolayers. This observation led to speculation that the protective nontoxic components (HA and NTNH) may also facilitate progenitor toxin transit through the gastrointestinal tract by minimizing neurotoxin adherence to lipid membranes (Chen *et al.*, 1998). On the other hand, toxicity studies suggest that adherence to lipid membranes is not critical for neurotoxin absorption since the intact progenitor complex is generally much more potent by the oral route than the purified neurotoxin. Moreover, the neurotoxin has been shown to protect the agglutination capacity of the associated nontoxic HA components within the progenitor toxin complex (Chen *et al.*, 1998).

Importantly, the nontoxic HA components of the progenitor complex appear to protect the neurotoxin from proteolysis and degradation under pH extremes while the agglutinating activity of the nontoxic component is maintained by the presence of the neurotoxin. The type A progenitor toxin complex contains several HA components that might contribute to protecting the neurotoxin from degradation. One of these HA components, referred to here as HA-33 (or HA1 in some studies), was shown to interact directly with type A neurotoxin and to significantly increase its resistance to enzymatic proteolysis *in vitro* (Sharma and Singh, 2004). The authors of this work hypothesized that HA-33 provides protection against enzymatic degradation either by blocking the accessibility of protease-sensitive sites on BoNTs or by inducing structural changes within the neurotoxin itself.

Collectively, these studies offer important insight into the relative stability of the BoNTs within the gastrointestinal tract based upon their resistance to inactivation under various enzymatic conditions. In general, toxin stability directly correlates with the presence of the accessory HA and NTNH components of the multimeric progenitor toxin complex. These proteins appear to function in protecting the neurotoxin from degradation or inactivation. The various toxin serotypes also display unique resistances to enzymatic digestion although the basis for these differences is not known.

C. Oral Intoxication: Toxin Absorption from the Gastrointestinal Tract

1. Role of Progenitor Toxin Accessory Proteins

The role of the nontoxic accessory proteins within the progenitor toxin complexes is not fully understood. They appear to function in protecting the ingested toxins from degradation and in facilitating absorption from the gastrointestinal tract. Functional characterization of the HA and NTNH proteins has been advanced by biochemical techniques for generating toxin preparations containing only select components of the progenitor complex. These 7S toxins are relatively sensitive to proteolytic degradation and denaturation in the stomach (Schiavo *et al.*, 1992). The auxiliary HA and NTNH proteins within the multimeric complex have been shown to dramatically increase the stability of the associated neurotoxin during transit through the gastrointestinal tract (Ohishi *et al.*, 1977; Fujinaga *et al.*, 1997; Sugii *et al.*, 1977a, b, c). The multimeric complex is then thought to readily dissociate either in the intestine or after absorption into the circulation. Most studies suggest that the accessory proteins do not appear to have any involvement in the activity of the toxins at peripheral nerve terminals. Thus, the HA and NTNH components are likely to be dispensable in disease pathogenesis after parenteral or respiratory exposure, where the toxins bypass the harsh conditions of the gastrointestinal tract.

2. Role of Enterocytes

Both absorptive enterocytes and Peyer's patch-associated M cells have been implicated in toxin transcytosis from the gastrointestinal tract after oral exposure. Peyer's patches are collections of lymphoid tissue that are part of the gut-associated lymphoid tissue. M cells are found not only in the intestinal tract, but also in the respiratory epithelium overlying bronchus-associated lymphoid tissue. Unpublished work by Park and Simpson (2003) indicates that knockout mice deficient in Peyer's patch M cell complexes are still susceptible to both oral and respiratory botulinum intoxication and the development of HC-specific antibody responses. Based upon these results, the authors suggest that M cells are not likely to be involved in toxin uptake and processing from the respiratory tract. In addition, both cell types have comparable transcytosis rates (M cells are five times as efficient in transcytosis than intestinal enterocytes), but enterocytes greatly outnumber M cells; therefore, gastrointestinal enterocytes are the predominant cell types involved in toxin uptake and processing from the gastrointestinal tract.

Maksymowych and Simpson (1998) used transwell culture systems with various transformed epithelial cell lines to evaluate the fate of the HA components of type A progenitor toxin complex. Radiolabeled preparations of both BoNT/A and HA were taken up by cultured T-84 human colon carcinoma cells by bulk endocytosis. However, efficient delivery across the T-84 cells was only observed for the neurotoxin.

D. Respiratory Intoxication

The potential threat posed by aerosolized botulinum toxins is emphasized by their ease of production, their extremely high potency relative to other biological toxins, and their use in various weaponization programs over the past several decades (Arnon *et al.*, 2001). This threat, along with the relative lack of information on respiratory toxicity and pathogenesis in humans, has fueled research on inhalational botulism in several animal models including mice, rabbits, guinea pigs, mongrel dogs, and rhesus monkeys.

1. Toxin Absorption from Respiratory Tract

The relative persistence and absorption of the toxins following experimental respiratory exposure have been investigated in a few animal species. An early literature review suggests that type A toxin is more potent in mice by the respiratory route than by subcutaneous (SC) administration but less potent by the intraperitoneal (IP) route (Morton, 1961).

Guinea pigs were shown to be highly sensitive to inhaled botulinum toxins when compared to other rodent species. Respiratory penetration and retention of inhaled toxin are higher in guinea pigs than mice (Lamanna, 1961). Toxin could be detected in the lungs of guinea pigs after intranasal (IN) administration of only 2 mouse lethal doses of type E toxin although detection in the blood or liver required higher doses (Sergeyeva, 1962, 1966). Guinea pigs were also reportedly more susceptible to type A toxin by inhalation than mice because shorter incubation periods were observed in guinea pigs prior to the onset of acute disease (Iakovlev, 1958).

Although inhalational botulinum intoxication was investigated in other animal species, these studies have not provided specific data on toxin absorption. The behavior of BoNTs in the respiratory tract was only recently investigated. Park and Simpson (2003) studied the properties of pure BoNT/A neurotoxin both *in vivo* and *in vitro* using mice and pulmonary cell culture models, respectively. Mean survival times were compared in mice receiving various doses of pure BoNT/A either IN or IP. Pure BoNT/A was found to be a potent intranasal poison, although the toxicity (as determined by mean survival time) associated with IP administration was somewhat higher. Mean survival times in mice were less than 100 (IP) or 600 min (IN) after administration of 0.1 μg pure toxin; 75 (IP) or 400 min (IN) for 1 μg toxin; and 120 min (IN) for 10 μg toxin (Park and Simpson, 2003). As seen with oral and parenteral routes, a linear relationship existed between the log of the intranasal dose administered and the geometric mean survival time. The HA and NTNH component of the progenitor toxin did not enhance toxicity, establishing different requirements for the stability and absorption of inhaled versus ingested toxin (Park and Simpson, 2003).

Transwell experiments were also performed to investigate BoNT/A transcytosis across a human pulmonary adenocarcinoma cell line (Calu-3), the MDCK cell line, and a primary rat alveolar epithelial cell line (Park and Simpson, 2003). Efficient BoNT/A transcytosis in both directions across polarized Calu-3 monolayers was observed, while toxin transcytosis occurred at a much lower rate across MDCK cells. These findings were in agreement with previous work demonstrating that the efficiency of BoNT/A transcytosis across MDCK monolayers was much lower than that observed across gut epithelial cells (Maksymowych and Simpson, 1998). BoNT/A transcytosis was also observed across primary rat alveolar cells, although at a slightly slower rate than that seen for the human adenocarcinoma cells (Park and Simpson, 2003). While the light chain of BoNT/A was not essential for transcytosis, heavy chain apical–basolateral (A-B) and basolateral–apical (B-A) transcytosis rates were somewhat lower than those of intact BoNT/A for both Calu-3 cells (HC 53% lower than BoNT/A for A→B; 45% lower for B→A) and rat alveolar cells (HC 62% lower for A→B; 17% lower for B→A). The transcytosis process was shown to involve an active-energy-dependent mechanism and was significantly inhibited by toxin preincubation with immune serum (Park and Simpson, 2003).

An important caveat to consider when evaluating the relevance of these *in vitro* studies is the use of pulmonary adenocarcinoma and alveolar epithelial cell lines in modeling respiratory absorption. It is generally believed that systemic absorption of inhaled particles is more likely to occur within the distal regions of the respiratory tract; therefore, particles must pass through thinner membranes in the deep lung and are less susceptible to nonabsorptive particle clearance (Palm *et al.*, 1956; Lamanna, 1961; Schlesinger, 1989). Some potential also exists for significant particle absorption from the nasopharyngeal and tracheobronchial regions of the respiratory tract; the cell lines utilized in these *in vitro* studies clearly do not account for this absorption potential. Importantly, investigators in the field have recently sought to characterize the specific cell types involved in toxin absorption from the respiratory tract.

M cells are found not only in the intestinal tract, but also in the respiratory epithelium overlying bronchus-associated lymphoid tissue. The studies of Park and Simpson (2003) indicate that M cells are not the major players in transepithelial transport of toxin across the respiratory epithelium. Additional studies directly investigating the absorption of inhaled BoNTs do not exist.

E. Toxin Binding and Uptake into Target Tissues

The remaining steps of BoNT pathogenesis following neurotoxin absorption are thought to be similar for both the respiratory and gastrointestinal exposure routes. After oral or inhalational exposure, the neurotoxins are absorbed from the gut or respiratory tract, respectively, into the lymphatics and circulation for transport to peripheral cholinergic synapses (Simpson, 2004). Figure 30.3 illustrates the neuromuscular junction, a major target for the actions of BoNTs. BoNTs are taken up presynaptically at the endplate region of neuromuscular junctions (Verderio *et al.*, 1999) and at other cholinergic synapses. Toxin binding involves high-affinity presynaptic receptors. These receptors have recently been identified as a combination of poly-sialogangliosides, synaptic vesicle (SV) protein 2 (SV2), and synaptotagmin (Verderio *et al.*, 2006). Each serotype displays an affinity for a unique combination of receptors. For example, BoNT/B recognizes synaptotagmin II (and I) and ganglioside lipids (Jin *et al.*, 2006; Nishiki *et al.*, 1996; Dong *et al.*, 2003) (see Figure 30.3). BoNT/A involves recognition of SV2C, SV2A, and SV2B (Dong et al., 2003); binding to SV2C also involves a lipid. After toxin binding,

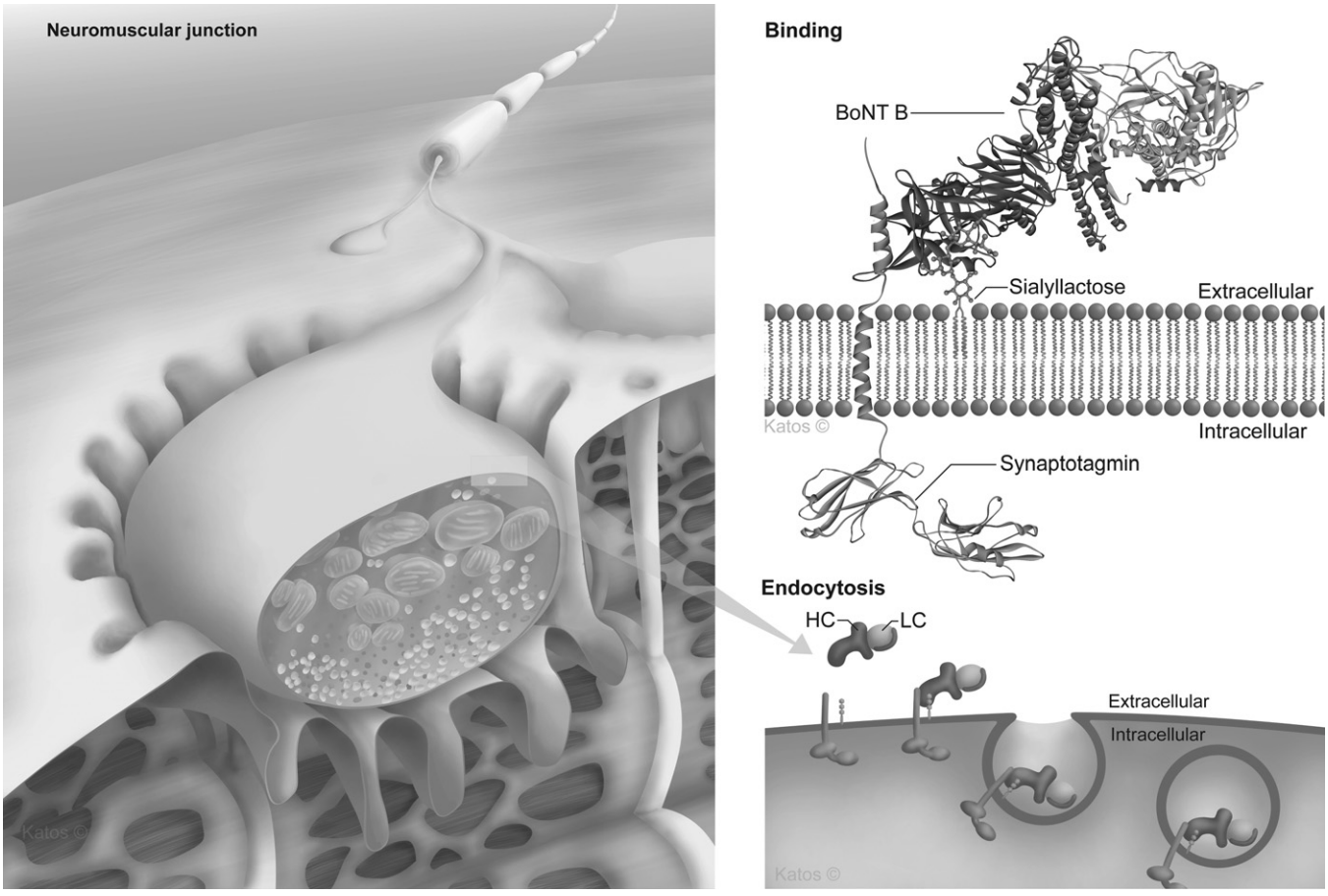

FIGURE 30.3. Toxin binding and internalization at the neuromuscular junction. Left panel: A mammalian neuromuscular junction is illustrated with the alpha motor neuron innervating skeletal muscle at specialized junctional folds in the membrane (Couteaux, 1973). Invaginations of the T system are also illustrated at the level of transition between the A and I bands. The axon loses its myelin sheath and dilates to establish an irregular contact with the muscle fiber. Muscle contraction begins with the release of ACh from synaptic vesicles (tiny spheres) at the motor endplate region. ACh binds to post-synaptic muscle-type nicotinic ACh receptors and causes an increase in the permeability of the sarcolemma. This process is propagated to the rest of the sarcomlemma and ultimately to the sarcoplasmic reticulum (SR) by the T system. An increase in SR permeability liberates calcium ion (Ca^{2+}) stores, resulting in sliding of illustrated muscle filaments and muscle contraction. BoNTs bind and internalize at the presynaptic side of the neuromuscular junction (Verderio *et al.*, 1999). Release of BoNTs into the cytosol results in inhibition of ACh release and flaccid paralysis of innervated muscle. Top right panel (Toxin binding): A three-dimensional ribbon structure of BoNT/B is illustrated. The receptor binding domain of BoNT/B HC binds to synaptotagmin (Syt-II) and ganglioside (sialyllactose) receptors of the presynaptic motor endplate. Each BoNT serotype binds to a different set of receptors in the membrane (Verderio *et al.*, 2006). Bottom right panel (endocytosis): Receptor-mediated endocytosis of BoNT/B holotoxin is illustrated in this panel. The remaining steps in BoNT toxicity involve acidification of the endosome, pH-induced conformational change in the toxin, translocation of BoNT LC across the endosomal membrane with the aid of BoNT HC, and proteolytic degradation of target SNARE proteins by LC. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

the complex is internalized by what is believed to be a clathrin-mediated endocytotic process.

V. TOXICOKINETICS

The onset of symptoms in botulism depends upon the amount of toxin ingested or inhaled and the related kinetics of absorption. Time to onset can range from as early as 2 h to as long as 8 days, although symptoms typically appear between 12 and 72 h after consumption of toxin-contaminated food (Lecour *et al.*, 1988; Arnon *et al.*, 2001). In a review of 13 foodborne botulism outbreaks involving 50 patients from 1970 to 1984, the incubation period ranged from 10 h to 6 days (Lecour *et al.*, 1988).

A. Foodborne Toxicity

1. Toxin Persistence in Circulation and Transit to Target Tissues

Case reports of human foodborne botulism incidents offer some information on toxin persistence and transit after oral exposure in humans. Koenig *et al.* (1964) reported that circulating toxin was detected in five out of six patients suffering from type E botulism after consuming contaminated fish by the mouse lethality assay on serum samples collected from the patients from 1 to 10 days after foodborne exposure. Serum from one of the patients who rapidly succumbed to disease contained approximately 8 MIPLD$_{50}$/ml; extrapolation of this value yields an estimate that 20,000 to 24,000 human LD$_{50}$s were in this individual's circulation (Koenig *et al.*, 1964). The toxin isolated from the serum of these clinically ill patients was not further activated *in vitro* by trypsin treatment. This observation was in agreement with other studies demonstrating cleavage of the single-chain prototoxin to the active dichain form within the gastrointestinal tract. Importantly, circulating toxin was not detected in a patient with minimal disease 11 days after ingestion of contaminated food (Koenig *et al.*, 1964). Six out of seven individuals who had consumed the contaminated fish but did not develop clinical illness also lacked circulating toxin. Circulating toxin was therefore detected much more consistently in symptomatic patients associated with this outbreak than in subjects who were unaffected after toxin ingestion (Koenig *et al.*, 1964).

Koenig *et al.* (1964) also reviewed previously published literature on the detection of circulating toxin in botulism patients. Circulating toxin (primarily serotype B) had been detected in select patients from 2 to 25 days after consumption of contaminated food, and was rarely detected in type A botulism patients (Koenig *et al.*, 1964). The authors suggested that serotype-specific differences in the persistence of circulating toxin might be attributed to their unique avidities to target tissues. Circulating toxin is generally detected only at very low levels at or immediately prior to death in lethally intoxicated patients (Ono *et al.*, 1970).

Efforts have been made to determine the kinetics of the accessory components of the progenitor toxin complexes after systemic absorption of BoNTs. Iida *et al.* (1970) found that circulating type E toxin was shown to exist in the 7S form after oral administration of the progenitor toxin to rabbits, suggesting that the larger toxin complex dissociated at some point during or after absorption from the gastrointestinal tract. Similar findings were reported on the absorption and persistence of progenitor type A toxin in the rat; the mean sedimentation value of toxin in the lymph after intraduodenal instillation was 7.9S, significantly lower than that of the crystalline toxin (Heckly *et al.*, 1960).

B. Inhalation Toxicity

1. Toxin Persistence in Circulation and Transit to Target Tissues

Very limited data are available on the persistence of BoNTs in circulation following inhalation exposure in any animal species. These data indicate that circulating toxin can be detected soon after exposure but is subsequently cleared rapidly from the circulation. Park and Simpson (2003) reported on the time course for appearance (and amount) of either purified BoNT/A or type A HC in the circulation of mice after intranasal exposure. Maximum serum levels were observed at 2 h post-exposure for both proteins although the peak values were higher for BoNT/A than for HC. Rapid clearance was observed over the next few hours.

An earlier study showed that type A toxin could be detected primarily in the lungs and liver rather than the serum of guinea pigs after intranasal exposure (Sergeyeva, 1962). The same group reported on the correlation between administered toxin dose and detection of toxin in the blood, lungs, and liver of guinea pigs intoxicated via the IN route (Sergeyeva, 1966). Type E toxin was detected in the lungs of guinea pigs after IN administration of two lethal doses, while toxin only appeared in the blood or liver following IN administration of at least five lethal doses. The organ distribution patterns were similar in guinea pigs after inhalation exposure to types A, B, or C toxins (Sergeyeva, 1966). These studies did not address the potential for persistent toxin detection in the lymph after inhalational intoxication, despite the fact that other routes of exposure result in significant absorption into the lymphatics.

While scant literature is available on persistence and distribution after inhalation exposure, several studies have evaluated the systemic behavior of parenterally administered toxins. One group investigated toxin persistence in serum and tissue distribution in white mice following intravenous (IV) administration of 1,000 lethal doses of ^{35}S-labeled type B toxin (Pak and Bulatova, 1962). Mice were sacrificed at 20, 60, and 150 min after toxin administration, and blood and tissues were harvested for toxin distribution analysis. These mice showed symptoms of severe intoxication, including atypical breathing patterns and paralysis, at 150 min post-exposure. Toxin levels (as determined by

radioactivity) were highest in the lung 20 min after toxin injection, followed by the liver, heart, kidneys, intestines, and brain (Pak and Bulatova, 1962). Radioactivity levels in the blood, as well as the liver, heart, intestines, and brain, were further reduced after 60 min post-toxin injection. Serum toxin concentrations were lower than those detected in any other tissue at all time points (Pak and Bulatova, 1962). The authors concluded that the toxin rapidly escaped from blood to various other tissues, suggesting the capacity for unimpeded passage of the toxin through the vasculature and cellular membranes.

Somewhat slower kinetics for toxin clearance from the circulation were observed in dogs following parenteral [IV, IP, or intramuscular (IM)] exposure to type A toxin (House *et al.*, 1964). Serum toxin persistence was evaluated in mongrel dogs receiving 8,000 to 10,000 mouse units/kg of type A toxin. Peak serum toxin levels were detected 5 h after IP administration (13% of injected dose), 12 h after IM administration (9% of injected dose), and within only 3 min after IV administration (79% of injected dose) (House *et al.*, 1964). The relative clearance kinetics were slower after IM and IP exposure than for IV administration, as serum toxin levels were identical 22 h after injection via all three rounds (approximately 6% of injected dose). Some serum toxin activity could be detected by the mouse lethality assay for 2 to 4 days after parenteral administration. Serum toxin patterns were also evaluated in rhesus monkeys following IV administration of type A toxin (Stookey *et al.*, 1965). Serum toxin levels dropped by about 50% of maximum within 16 to 24 h after IV injection, and previous exposure did not affect toxin clearance rates after the administration of subsequent doses.

Another study investigated circulating toxin levels in weanling pigs (5 to 12 weeks old) following parenteral administration of toxin types A, B, C1, and D (Smith *et al.*, 1971). Toxin was cleared from circulation less than 24 h after IV injection of type B (560 $MIPLD_{50}$/kg), type C1 (5,000 $MIPLD_{50}$/kg), or type D (60,000 $MIPLD_{50}$/kg) toxin. In contrast, toxin could consistently be detected in the serum over the entire 4 day period prior to death in pigs injected with serotype A (21,400 $MIPLD_{50}$/kg) (Smith *et al.*, 1971). Serum toxin levels were 100 MIP_{50}/ml at 24 and 48 h after injection of type A toxin, 30 $MIPLD_{50}$/ml after 3 days, and 10 $MIPLD_{50}$/ml after 4 days. These findings indicated serotype-specific differences in the persistence of circulating BoNTs, at least in systemically intoxicated pigs.

Although these studies provide some insight into the persistence of circulating toxin after parenteral administration, they do not necessarily reflect the behavior of absorbed toxin after respiratory exposure. The route of administration may not have a significant impact on the behavior of toxin once absorbed into the serum and lymph, but the patterns and kinetics of absorption into the circulation might be quite different after inhalational versus parenteral exposure. Respiratory exposure could lead to a different proportion of toxin taken up into the circulation and/or lymphatics over a given time period than that seen after systemic injection. Such discrepancies might impact both the quantitative persistence of circulating toxin and its transit to peripheral target tissues. At this point, information is not yet available on the homing and distribution of toxins to target nerve tissues. The available literature also provides no insight on the mechanisms by which toxin is removed from the circulation, either through extravasation and uptake in target tissues or by metabolic processes. In the future, such data will be important in characterizing the pathogenesis of botulism after respiratory intoxication and other routes of exposure.

VI. MECHANISM OF ACTION

ACh release from presynaptic vesicles is dependent upon a propagated action potential, localized depolarization at the presynaptic motor endplate, proper SNARE complex formation (i.e. SNAP-25, syntaxin, and synaptobrevin), and synaptic vesicle docking with the presynaptic membrane (see neuromuscular transmission in absence of BoNT; Figure 30.4). Regardless of the exposure route, BoNTs lead to inhibition in the release of ACh from peripheral cholinergic nerve terminals resulting in flaccid paralysis (Habermann and Dreyer, 1986; Simpson, 1986; see Figure 30.5). The specific target for BoNT/A and/E is the 25 kDa vesicle docking protein SNAP-25; BoNT/A cleaves the last nine residues, whereas BoNT/E cleaves a larger 26 residue fragment from the C-terminus of this protein (Blasi *et al.*, 1993; Montecucco *et al.*, 1994). The target of BoNT/B is the small transmembrane protein synaptobrevin/VAMP located on the surface of small synaptic vesicles (Schiavo *et al.*, 1995). The enzymatically active portion of the 150 kDa BoNT is the 50 kDa light chain; the role of the 100 kDa heavy chain involves binding to cholinergic nerve endings and intracellular penetration via receptor mediated endocytosis (Simpson, 1986, 2004; Monteccuco *et al.*, 1994).

A. Heavy Chain (HC)

The heavy chain of the botulinum neurotoxins has been shown to mediate toxin binding and internalization at cholinergic nerve terminals (Daniels-Holgate and Dolly, 1996; Lalli *et al.*, 1999; Maruta *et al.*, 2004; Simpson, 2004). The mostly β-strand-containing carboxy-terminus of the heavy chain (Hc) appears to be directly involved in toxin binding while the mostly α-helical amino-terminal region (Hn) mediates translocation across the endosomal membrane (Lalli *et al.*, 1999; Simpson, 2004). Through mechanisms that have yet to be fully characterized, the toxins gain entry into the nerve terminal through receptor-mediated endocytosis followed by pH-induced translocation from the endosome to the cytosol. The ability of the Hn region to form transmembrane ion channels raises the possibility that they are intimately involved in translocating the toxic moiety into the cytoplasm (Koriazova and Montal,

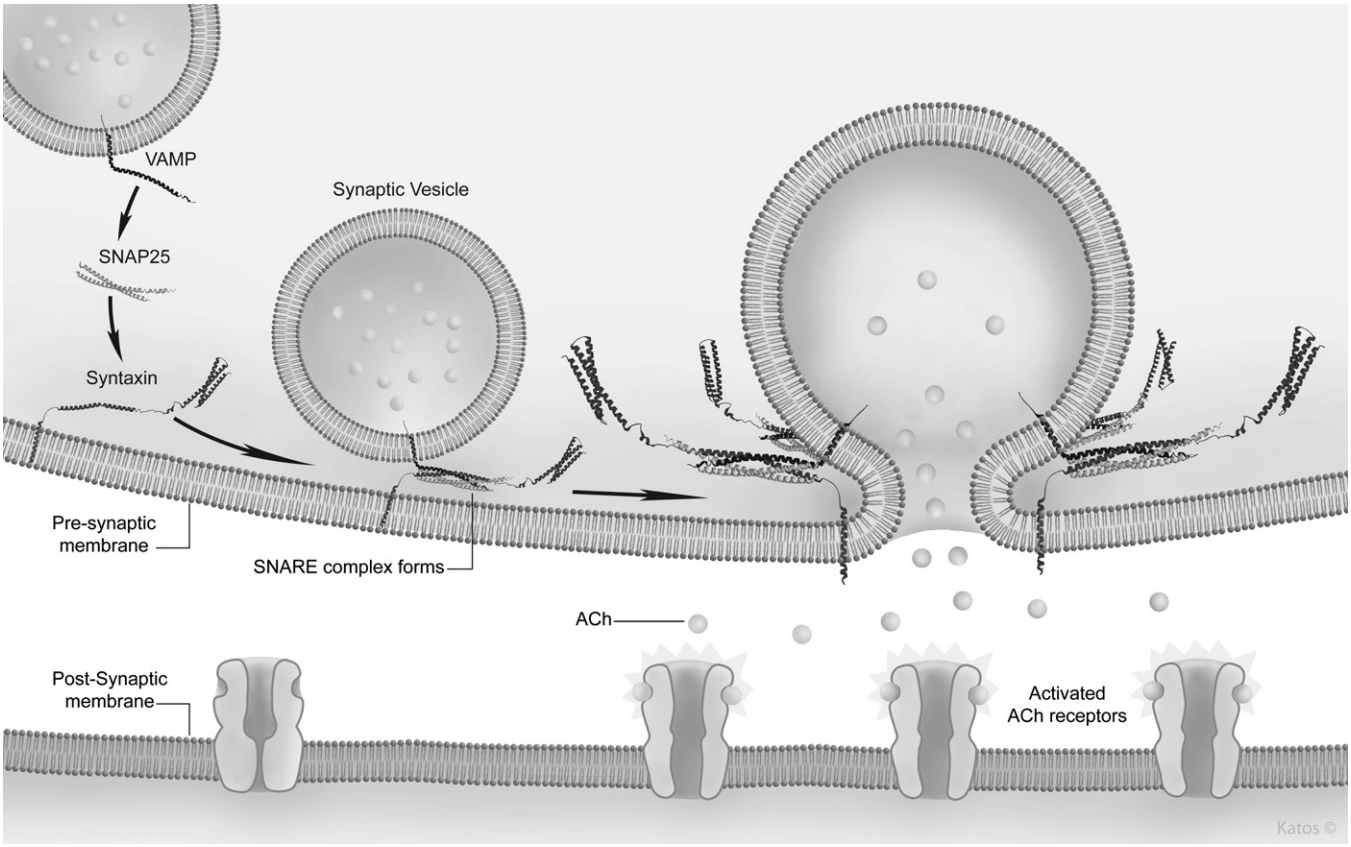

FIGURE 30.4. Neuromuscular transmission in the absence of BoNT. A nerve impulse is transmitted to the effector (muscle) cell by neurotransmitter liberated at the synapse. When the action potential arrives at the axonal terminus to depolarize the presynaptic membrane, Ca^{2+} ions enter through voltage-dependent Ca^{2+} channels. Ca^{2+} ions facilitate the fusion of synaptic vesicles, containing the neurotransmitter ACh, with the presynaptic membrane. Three SNARE proteins (syntaxin, synaptobrevin, and SNAP-25) are critical for synaptic vesicle fusion. As long as the SNARE complex is intact, ACh releases by exocytosis, diffuses across the synaptic cleft, and binds to postsynaptic muscle-type nicotinic ACh receptors. Binding of ACh makes the sarcolemma of the muscle cell more permeable to sodium, which results in membrane depolarization. Excess ACh is hydrolyzed by the enzyme cholinesterase bound to the synaptic cleft basal lamina. ACh breakdown is necessary to avoid prolonged activation of ACh receptors. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

2003). Once translocated into the cytosol, the toxic fragments exert their paralytic effects by inhibiting ACh release from neuromuscular junctions as well as other peripheral cholinergic sites, including sympathetic and parasympathetic ganglia and post-ganglionic parasympathetic synapses (Lamanna, 1959; Vincenzi, 1967; Simpson, 2004).

B. Light Chain (LC)

These paralytic effects have been attributed to the proteolytic activity of BoNT light chain (LC) on protein substrates required for vesicular exocytosis. BoNT LC inhibits neurotransmitter exocytosis through its zinc-dependent endoproteolytic activity. The LCs of the various neurotoxin serotypes possess distinct molecular targets within the peripheral cholinergic nerve termainals (Schiavo *et al.*, 1992, 1993a, b, 1994, 1995; Yamasaki *et al.*, 1994). The endoproteolytic activities of the different toxin LCs produce similar flaccid paralytic effects.

The intracellular proteins SNAP-25, syntaxin, and synaptobrevin (or vesicle-associated membrane protein, VAMP) normally interact with each other in mediating neurotransmitter release from cholinergic and other nerve terminals (see Figure 30.4). Toxin types B, D, F, and G cleave the VAMP proteins while types A, C1, and E act on SNAP-25; type C1 toxin also cleaves syntaxin (Dong *et al.*, 2003). The functions of the various neurotoxins are even more specialized in that one toxin type can cleave its substrate at a different site than that targeted by other toxin serotypes. For example, BoNT/A cleaves SNAP-25 between residues 197 and 198 (resulting in the loss of nine amino acids), while BoNT/E cleaves the same protein between residues 180 and 181 (thereby removing 26 amino acids) (Schiavo *et al.*, 1993a).

Although the LCs of both BoNT/A and/E target SNAP-25, these two serotypes exert significantly different potencies and paralytic profiles in cultured neurons and *in vivo*. A potential molecular basis for this discrepancy was

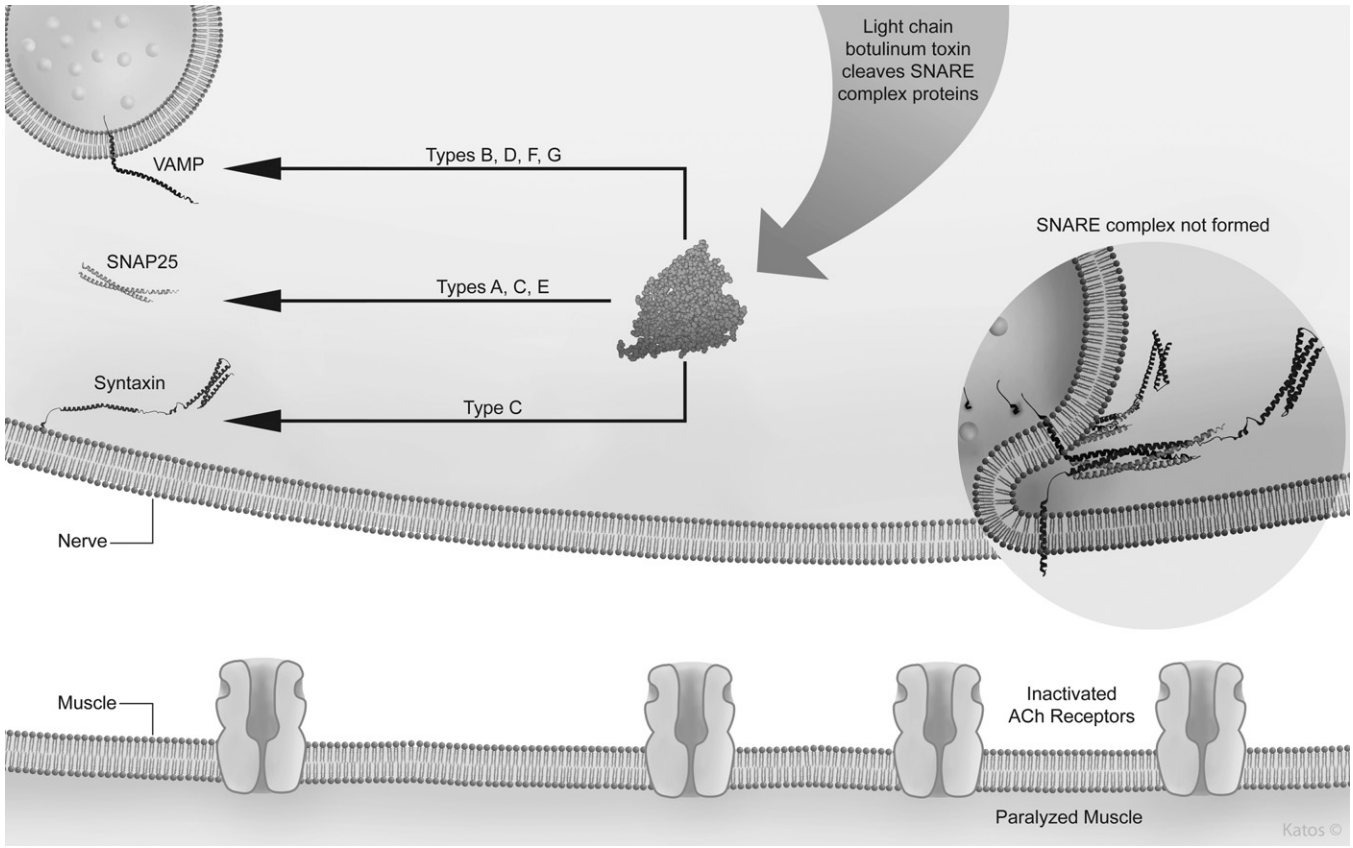

FIGURE 30.5. Neuromuscular transmission in the presence of BoNT. The catalytic LCs of the various serotypes cleave specific SNARE proteins. The SNARE complex does not form if any of the proteins are cleaved. In the presence of BoNT LC inside the axonal terminus, synaptic vesicles will not fuse with the presynaptic membrane, ACh will not be released, and the muscle will not contract, resulting in paralysis at the neuromuscular junction. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

established by the finding that these neurotoxins target different cleavage sites within the SNAP-25 protein. BoNT/A cleavage generates a 197-residue truncated protein (P197) by cleaving the last nine amino acids from the C-terminus of SNAP-25 while BoNT/E cleavage produces a 180-residue species (P180) by removing the final 26 residues (Schiavo *et al.*, 1993a). A series of studies by Keller *et al.* (1999) and Keller and Neale (2001) provided additional insight into the molecular mechanisms associated with the potent and persistent action of type A neurotoxin relative to type E.

Serotype-specific cleavage events provide insights into the differential activities of the toxins at nerve terminals. In some cases, substrate cleavage studies also revealed important information regarding interspecies differences in the activity of certain toxins. BoNT/B was shown to block neuromuscular transmission by cleaving VAMP proteins between residues Q76 and F77 (in humans and mice) (Bakry *et al.*, 1997). However, the rat VAMP1 (synaptobrevin) protein sequence differs at this critical cleavage site in that the glutamine at position 76 is replaced by a valine, rendering the region more resistant to proteolysis by BoNT/B (Bakry *et al.*, 1997; Verderio *et al.*, 2006; Callaway, 2004). On the other hand, rats and mice were shown to have similar susceptibilities (body weight adjusted) to IM injection of type A toxin; rats have also been shown to be much more resistant than mice to type F toxin (Kauffman *et al.*, 1985).

The specific paralytic profiles associated with each of the BoNTs are typically attributed to their unique proteolytic activities within the nerve terminal. These activities are known to be mediated by the LC components of the various neurotoxins. The various nontoxic components within the multimeric progenitor toxin complexes have traditionally been considered accessory proteins that primarily function to increase neurotoxin stability and, in some cases, to facilitate absorption. Yet studies over the past few years have suggested a potential role for the HA constituents in enhancing the endopeptidase activity of the LC (Cai *et al.*, 1999; Sharma and Singh, 2004). It is widely believed that pure BoNT/A requires either proteolytic ‘‘nicking’’ or chemical reduction for significant SNAP-25 cleavage activity. However, new evidence suggests that the type A progenitor toxin complex is apparently highly active even in nonreduced form (Cai *et al.*, 1999). Further research is needed to substantiate this preliminary work and to establish a more detailed understanding of the prerequisites for LC proteolytic activity.

A recent study by Sharma and Singh (2004) provided additional support for the expanded roles of at least one

neurotoxin-associated protein within the type A progenitor complex. The HA-33 component, representing 25% of the accessory protein content of progenitor neurotoxin, significantly increases the proteolytic activity of both BoNT/A and/E *in vitro* and in rat synaptosome preparations. The addition of HA-33 to nonreduced BoNT/A leads to a 21-fold increase in GST-SNAP25 fusion protein cleavage activity *in vitro* and a 13-fold enhancement of endopeptidase activity in rat synaptosomes (Sharma and Singh, 2004). Similar enhancement of proteolytic activity was seen when HA-33 was added to BoNT/E both *in vitro* and in rat brain synaptosomes.

The enhancement of SNAP-25 cleavage activity by HA-33 in rat brain synaptosomes was taken as evidence that the neurotoxin and the accessory protein both gain entry to the nerve terminal (Sharma and Singh, 2004). The possibility that an accessory component of the progenitor toxin complex could exert direct effects on LC endopeptidase activity within the nerve terminal could have important implications with respect to neurotoxin function *in vivo*.

Two recent reports revealed additional layers of complexity regarding the mechanisms involved in the distinct durations of action associated with the different toxin serotypes. Fernández-Salas *et al.* (2004) investigated the subcellular localization of BoNT/A, /B, and /E LC-GFP fusion proteins following overexpression in several different mammalian cell lines. The LC/A fusion protein was shown to localize within discrete plasma membrane compartments in both neuronal (PC12) and nonneuronal (HEK293, HeLa, HIT-T15) cell lines, while LC/B was detected throughout the cell and LC/E was primarily found within the cytosol.

VII. TOXICITY

A. Lethality

BoNTs are the most potent substances known to man. A comparison of the lethal nature of BoNTs in relation to other toxic chemicals and substances discussed throughout this textbook is provided in Table 30.2. The toxicity associated with oral exposure of a given species to BoNTs is significantly lower than that resulting from parenteral administration (see Table 30.3). The susceptibility of various animal species to parenteral intoxication does not provide adequate indication of their sensitivity to gastrointestinal exposure (Lamanna, 1961). The estimated human LD_{50} of approximately 1 ng/kg for parenteral botulinum intoxication is similar to that reported for most laboratory animals (Arnon, 1995; Middlebrook and Franz, 2000). In contrast, the relative susceptibilities of humans and other animal species to oral intoxication vary significantly (Morton, 1961). A recent clinical review of human botulism reported that the ingestion of as little as 0.05 to 0.1 μg of BoNT A may be sufficient to cause death in humans (Cherington, 1998). Human lethal

TABLE 30.2. Comparison of toxic chemicals and substances

Chemical or toxin	Mouse IV LD_{50}* (mg/kg)	Chemical or toxin	Mouse IV LD_{50}* (mg/kg)
Botulinum toxin	0.00001	Strychnine	0.41
Batrachotoxin	0.002	Potassium cyanide	2.60
Anthrax lethal toxin	0.003–0.005	Mustard[b]	3.30
Ricin	0.005	Aflatoxin	9.5
Tetrodotoxin	0.01	Heroin	21.8
Saxitoxin	0.01	CR[c]	37
VX	0.012	Marijuana[d]	42
Abrin	0.02	BZ[e]	46
GD	0.066	CS[f]	48
GB	0.10	Caffeine[g]	62
GA	0.15	CN	81
TCDD[a]	0.182	Thujone[h] (absinthe)	134.2
Capsaicin	0.40	PAVA[i]	224

*Intravenous dose that is lethal to 50% of mice
[a]2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin (TCDD), a contaminant of the defoliant and herbicide Agent Orange
[b]Mustard gas, 1,1′-thiobis[2-chloroethane]
[c]Riot control agent [dibenz-(b,f)-1,4-oxazepine]
[d]Delta-3,4-trans-tetrahydrocannabinol
[e]Incapacitating agent, 3-quinuclidinyl benzilate
[f]Riot control agent (*o*-chlorobenzylidene malononitrile)
[g]Riot control agent (chloroacetophenone)
[h]Constituent of wormwood absinthe, a popular emerald liquor, 4-methyl-1-(1 methylethyl) bicycle [3.1.0] hexan-3-one
[i]Riot control agent (pelargonic acid vanillylamide)

TABLE 30.3. Comparison of the lethality of serotypes A–G by various routes of administration in the guinea pig[†]

Route of intoxication	Botulinum toxin serotype						
	A	**B**	**C**	**D**	**E**	**F**	**G**
Oral	717	306	177	436	–	–	–
IP[‡]	3.1[a]–5.2[b]	4.2[b]–6.5[a]	1.6[b]–3.2[a]	3.0[c]–6.4[a]	34.3[b]–78[c]	–	40–100[d]
IM[††]	4.3[a]	6.9[a]	3.1[a]	8.7[a]	102[a]	–	–
SC[‡‡]	6[e]–30[f]	–	–	3[g]	100[h]	30–30[g]	–
Aerosol	141	350	87	186	778	–	–

[†]The doses are normalized to mouse IP LD_{50} units
[‡]Intraperitoneal administration
[††]Intramuscular administration
[‡‡]Subcutaneous administration
[a]Gelzleichter *et al.* (1998a)
[b]Cardella *et al.* (1963)
[c]Lamanna (1961)
[d]Cicarelli *et al.* (1977)
[e]Morton (1961)
[f]Sergeyeva (1962)
[g]Dolman and Murakami (1961)
[h]Sergeyeva (1966)

doses have also been extrapolated from primate studies, yielding an oral lethal dose of approximately 70 µg for crystalline type A toxin for a 70 kg human (Arnon *et al.*, 2001). The lethal human respiratory dose is estimated to be 0.7–0.9 µg and the intravenous or intramuscular dose is 0.05–0.15 µg (Middlebrook and Franz, 2000; Arnon *et al.*, 2001).

B. Oral Toxicity

An earlier report suggested that humans are more susceptible than monkeys to type A toxin by the oral route based on toxin dose estimates in foodborne botulism case studies (Morton, 1961). Morton (1961) also summarized the susceptibility of numerous other animal species to oral botulinum intoxication. Mice, monkeys, and guinea pigs were considered highly susceptible while chickens, rabbits, horses, dogs, rats, and cattle were classified as more resistant, and ferrets, minks, and hogs were deemed resistant. The oral lethal doses of type A toxin in guinea pigs (1,000–3,000 MIPMLD) and monkeys (2,000 MIPMLD) (Morton, 1961) are similar to the estimated oral lethal dose for humans (7,000 MIPMLD).

Morton (1961) provided evidence for an estimated human oral lethal dose of much less than 3,500 MIPMLD for type B toxin. This estimate was based upon an earlier report describing a fatal type B human botulism case resulting from the ingestion of 3,500 MIPMLD in toxin-contaminated cheese (Meyer and Eddie, 1951). A man weighing 104 kg consumed approximately 70 g of contaminated cheese; repeated tests of the cheese indicated that it contained only 50 MLD/g of type B toxin. The patient first developed somewhat atypical disease symptoms of nausea, vomiting, diplopia, dysphagia, phagodynia, and instability within 7 h of exposure (Meyer and Eddie, 1951). The man was later hospitalized and developed symptoms more characteristic of foodborne botulism within 18 to 20 h. He died 57 h after toxin ingestion, despite receiving 35,000 units each of both type A and B antitoxins (Meyer and Eddie, 1951). Therefore, it was determined that 3,500 MIPMLD is in great excess of one minimum lethal dose of type B toxin for humans due to the rapid onset of illness and severity of disease (Morton, 1961). The same study reported a woman who died from botulism 42 h after consuming a small piece of toxin-contaminated pear.

C. Inhalation Toxicity

Naturally occurring botulism cases in humans and other animal species are almost exclusively associated with the ingestion of toxin- or spore-contaminated foods. The level of knowledge in the published literature on toxin absorption following inhalational exposure is therefore much more limited than that associated with gastrointestinal intoxication. The potencies of inhaled BoNTs have been investigated in several experimental animal species. In an early literature review, Morton (1961) reported comparatively similar ratios (5.9:1) of oral to respiratory toxicity (the comparative lethal doses for toxin administered via the oral versus the respiratory route) for type A toxin in guinea pigs and mice. Iakovlev (1958) concluded that guinea pigs were more susceptible than mice to type A toxin by inhalation because they succumbed to intoxication after a shorter incubation period (1–2 days versus 3–4 days for mice). Several technical reports established more specific guinea pig inhalation toxicity data for serotypes A through E (Jemski, 1960, 1961b).

D. Clinical Toxicity

The natural occurrence of human foodborne and infant botulism translates into a wealth of information on the clinical signs and symptoms of disease. This information can be compared to the array of physiological and pathological findings in various species of experimental animals after oral administration of BoNTs. The ability for inhaled BoNTs to produce illness has also been documented in

humans and in several experimental species. The primary neurophysiological signs and symptoms associated with respiratory exposure parallel those observed in cases of foodborne botulism; however, infants display a unique clinical picture of botulism. In addition, the various toxin serotypes are usually associated with analogous clinical presentations, with the most severe cases of foodborne botulism being caused by the ingestion of type A toxin.

Exposure to BoNT via oral or inhalational routes results in symptoms indicative of an inactive peripheral cholinergic system due to inhibition of ACh release from the nerve terminal. The time to onset of disease is dependent on the amount of toxin ingested and ranges from several hours to a few days after oral exposure (Lecour *et al.*, 1988; Arnon *et al.*, 2001). Prominent signs and symptoms of intoxication common to all serotypes and various routes of exposure include, in order of descending frequency: dysphagia, xerostomia, diplopia, dysarthria, fatigue, ptosis of the eyelids, constipation, arm weakness, leg weakness, gaze paralysis, blurred vision, diminished gag reflex, nausea, facial palsy, dyspnea, emesis, tongue weakness, sore throat, dizziness, dilated or fixed pupils, abdominal cramps, reduced or failed reflexes, nystagmus or involuntary rapid eye movement, diarrhea, ataxia, and paresthesia (reviewed by Arnon *et al.*, 2001).

1. Foodborne Botulism

Human foodborne botulism presents as an acute, symmetric, flaccid paralysis that generally involves multiple cranial nerve (CN) palsies initially, termed bulbar involvement. Early symptoms involve paralysis of the motor components of the CNs. The motor components are derived from cell bodies located in the brain with axons that exit the cranium to control muscles, glandular tissue, or specialized muscle in the heart and gastrointestinal tract. Paralysis by BoNTs lead to ptosis and dilated pupils (CN III), disconjugate gaze and blurred vision (CN III, IV, VI), facial droop or palsy (CN VII), dysphagia, dysarthria, and absence of gag reflex (CN IX, X), tongue weakness (CN XII), and weakness of neck strap muscles (CN XI). Botulism patients typically develop difficulty in seeing, speaking, or swallowing in the early phases of intoxication. As paralysis extends caudally, toxic signs and symptoms include loss of head control, hypotonia, generalized weakness, and flaccid paralysis or floppy appearance (infants and children). In infants and young children exposed to BoNT, floppy appearance and constipation may be the only presenting signs to warrant a diagnosis of BoNT exposure, since obtaining a reliable history may not be possible in this population.

Loss of the gag reflex and dysphagia may require intubation and mechanical ventilation. Deep tendon reflexes are often lost during later stages of intoxication, and death in untreated patients results from airway obstruction or inadequate tidal volume (Arnon *et al.*, 2001). Respiratory failure is the most serious clinical manifestation of botulism, and the decline in mortality associated with foodborne botulism is primarily due to improvements in ventilatory support (Lecour *et al.*, 1988). Around 60% of botulism patients in the USA require mechanical ventilation at some point during their hospitalization and treatment (Varma *et al.*, 2004). In severe botulism cases, as in the Florida physician case involving research grade type A toxin instead of BOTOX® for facial muscle paralysis, respiratory support may be required for prolonged periods of time and autonomic dysfunction may persist for months to years (Mackle *et al.*, 2001).

Other clinical forms of the disease share many of these signs and symptoms. The presentation and duration of disease are coupled to the relative persistence of the toxin in blocking the release of ACh at peripheral nerve synapses. Although untreated botulism is potentially deadly, the availability of antiserum has dramatically reduced the mortality rates for the common clinical manifestations of the disease. Severe cases of foodborne botulism may still require ventilatory support for over a month, and neurological symptoms can sometimes persist for more than a year (Mackle *et al.*, 2001).

2. Infant Botulism

The characteristic symptoms of infant botulism are poor sucking, constipation, generalized weakness, floppy appearance, and respiratory insufficiency (Cox and Hinkle, 2002). Infant botulism may quickly progress to respiratory failure if not treated. The development of the intestinal flora has been demonstrated to suppress germination and growth of *Clostridium botulinum* spores in mice (Sugiyama and Mills, 1978). Ingestion of honey by infants is the classic scenario cited in infant botulism; honey is therefore not recommended in this susceptible population (Spika *et al.*, 1989; Arnon, 1998).

VIII. RISK ASSESSMENT

BoNTs present a very real threat to the public health and are the most toxic substances known to humankind. In a military or bioterrorist incident, intoxication by BoNT is likely to occur by inhalation of aerosolized toxin or by ingestion of contaminated food or beverages (Franz, 1997; Sobel *et al.*, 2004). Although the municipal water systems are considered to be safe from BoNT attacks, due to chlorination and dilution, it is not known whether current water treatments adequately decontaminate the toxin. Furthermore, bottled mineral water and milk (Sobel *et al.*, 2004; Kalb *et al.*, 2005) are obvious targets for terrorist groups. The vulnerability of the nation's milk supply was highlighted in a recent modeling study, where its complex distribution system would magnify the consequences of poisoning by BoNT (Wein and Liu, 2005; Kalb *et al.*, 2005). BoNTs are a serious threat to our national security due to their potency, remarkable stability, and persistence in the body.

Wein and Liu (2005) modeled a bioterror attack using BoNTs on the nation's milk supply. Modeling of toxin for dispersal in a liquid medium has been previously computed in a terrorist scenario (Dembek, 2005) involving a water fountain and contamination at a recreational center (CDC, 1999). Wein and Liu's assessment estimates the amount of toxin required, critically evaluates entry points into the milk supply industry, and details deficiencies in our current detection capabilities required to thwart such an attack (Wein and Liu, 2005).

The most prevalent BoNTs isolated in human botulism are serotypes A, B, and E. The ability of serotypes C and D, in addition to F, to paralyze human skeletal muscle should also be noted (Hilmas, unpublished). Complicating matters is the fact that all BoNTs remain stable in common beverages and retain significant potency for prolonged periods of time (>90 days) at room temperature and in biological fluids (human whole blood and serum) at physiological temperatures (Hilmas *et al.*, 2006b; Williams *et al.*, 2007). In addition, BoNTs possess a remarkable ability to remain within the nerve terminal for extended periods. Keller *et al.* (1999) showed BoNT protein detectable by western blot for 90 days in rat spinal cord cultures.

Stability of the BoNT protein should be considered in an assessment of the threat posed by intentional release of the toxins. In addition to the remarkable persistence of the toxin in biological fluids and beverages described above, BoNT remains a potent environmental threat. BoNT/A was subjected to desiccation to simulate the residue of an intentional release. Following 28 days of drying, the toxin still possessed remarkable paralytic properties (Williams *et al.*, 2007).

The duration of muscle paralysis following intoxication by BoNT/A exceeds that resulting from exposure to other BoNT serotypes (Keller *et al.*, 1999; Robinson and Nahata, 2003; Fernández-Salas *et al.*, 2004). The remarkable persistence of BoNT/A action has led to its widespread use in the treatment of disorders of muscle tone and movement (Jankovic and Brin, 1997). Although a long duration is desirable in clinical use, the prolonged action of BoNT/A would also make intoxication by this serotype difficult to treat, particularly if used as a bioweapon (Franz, 1997). The duration of intoxication by BoNT/E is relatively brief (several weeks), whereas BoNT/B is of intermediate duration (Keller *et al.*, 1999; Blanes-Mira *et al.*, 2004). The basis for the differences in serotype persistence is currently unknown. In any case, a bioterrorist attack, involving the most lethal substance known to humankind, would overwhelm the limited resources (i.e. mechanical ventilators) available to treat botulism patients.

IX. TREATMENT

There are currently seven known antigenic serotypes of botulinum toxin, designated with the letters A through G, whereby antitoxin to one type does not cross-neutralize any of the others. Only early administration of antitoxin antibody in cases of suspected botulism will minimize the neurologic damage but will not reverse any existing paralysis. Paralysis could persist for weeks to months, and the available treatment consists of supportive care including fluids, total parenteral nutrition (TPN), and mechanical ventilation.

A. Antitoxin

The administration of heterologous antitoxin was one of the first therapeutic approaches developed for botulism patients and remains the most effective when initiated in the early stages of intoxication. The primary limitation of antitoxin treatment was established in some of the earliest published reports on experimental botulism. One of these reports evaluated the pathogenesis of oral intoxication and the efficacy of antitoxin therapy in monkeys (Dack and Wood, 1928). Antitoxin treatment was not effective when administered after symptoms of botulism were already apparent, despite the fact that circulating toxin could still be detected in many of the animals.

Oberst *et al.* (1967) investigated the effectiveness of antitoxin therapy, artificial respiration, and supportive treatment in rhesus monkeys after IV type A toxin injection. These treatments were administered to the animals either alone or in combination after signs of intoxication were observed. Only one in six monkeys survived after receiving antitoxin injections alone as treatment for overt intoxication with 2.5 LD_{50} of type A toxin (Oberst *et al.*, 1967). A combination of antitoxin therapy and supportive treatment initiated soon after the development of toxic signs protected eight of ten animals from death after IV injection of 4 to 5 LD_{50}. Artificial respiration prolonged survival in monkeys with respiratory paralysis but was not effective as a primary treatment after lethal intoxication; no animals receiving only artificial respiration survived intoxication with 5 to 24 LD_{50} (Oberst *et al.*, 1967). Untreated animals developed overt signs of intoxication within 20 to 38 h and died 32 to 135 h after toxin injection.

While antitoxin treatment was generally ineffective in experimental animals displaying significant clinical signs, several case studies of foodborne botulism indicated that antitoxin therapy remained potentially beneficial in humans even after the onset of illness. Iida (1963) reviewed the high efficacy of antitoxin therapy in type E botulism outbreaks associated with contaminated fish consumption in Japan. A mortality rate of only 3.5% was observed among 85 antitoxin-treated patients in nine recent foodborne botulism outbreaks, while a rate of 28.9% was reported among 135 untreated patients in 19 previous outbreaks. Iida noted that all moderately and seriously ill patients in a 1962 foodborne type E botulism outbreak survived after antitoxin treatment. Hatheway *et al.* (1984) reported on the effectiveness of trivalent (ABE) antitoxin therapy during a 1978 outbreak of type A botulism. Four of seven patients with confirmed

disease from type A toxin ingestion were treated with two to four vials of trivalent antitoxin (Hatheway *et al.*, 1984). All four treated patients survived, although one of these individuals continued to suffer from severe paralysis and required ventilatory assistance for several months.

The current Centers for Disease Control and Prevention (CDC) therapy for the public is an FDA-approved, bivalent, botulinum equine antitoxin against serotypes A and B. The trivalent antitoxin against types A, B, and E is no longer available. In cases of exposure to any of the other botulinum toxin serotypes, the US Army can provide an investigational heptavalent (ABCDEFG) equine antitoxin, but the time required for typing a toxin subtype would limit its effectiveness in such cases as an outbreak. A parenteral vaccine against the toxin is currently available, but the need exists for newer nonparenteral vaccines that could be administered orally or via inhalation.

B. Treatment for Infant Botulism

Administration of equine antitoxin is not recommended for preexposure prophylaxis. The heterologous serum of antitoxin therapy can lead to a high frequency of adverse reactions. The equine antitoxin available for use in humans has been reported to cause adverse reactions such as anaphylaxis in over 20% of treated patients (Lewis and Metzger, 1980). This problem has been circumvented in the development of a safer approach to the treatment of infant botulism using plasma isolated from human subjects repeatedly immunized with pentavalent toxoid. Equine antitoxin is not used as a treatment for infant botulism due to the high risk of serious adverse reactions and the possibility of long-term sensitization to horse serum-based therapeutics (Arnon, 1998). An antiserum product termed BabyBIG (botulism immune globulin), derived from human volunteers immunized with pentavalent toxoid, is available for infant botulism patients. Intravenous BabyBIG therapy has proven extremely effective in counteracting the toxic effects of *C. botulinum* colonization in infants and in avoiding the risk of adverse reactions to equine antitoxin. It is also most effective when administered within 24 h of a high-dose aerosol exposure to the toxin (Gelzleichter *et al.*, 1998a, b, 1999).

C. Vaccines

There are as yet no FDA-approved vaccines to prevent botulism. An investigational pentavalent botulinum toxoid (PBT) product, developed at Fort Detrick, is available for persons at risk for botulism (i.e. laboratory workers, warfighters). While determined to be safe and immunogenic, PBT is not useful or recommended for post-exposure prophylaxis. Antitoxin titers do not develop until a month after the third dose in the vaccine schedule. PBT is reserved for employees at high risk for BoNT exposure but not the general population. Several factors limit the usefulness of PBT as a vaccine for inoculating the general population. These include a declining potency and immunogenicity in recent years, the need to take multiple doses to maintain titers, and the limited supply of the vaccine.

X. CONCLUDING REMARKS AND FUTURE DIRECTION

The toxicity of botulinum toxins leading to paralysis is due to their ability to block ACh release from peripheral cholinergic nerve endings (Simpson, 2004). Once ingested or inhaled, the toxin binds to epithelial cells, transports to target tissues via the circulatory system, targets the NMJ, and penetrates cellular and intracellular membranes. BoNTs bind to the lipid bilayer of the neuronal cell surface, gain access by receptor-mediated endocytosis, and cleave polypeptides involved in exocytosis of ACh. As a result, botulism leads to a descending flaccid paralysis, starting usually in the bulbar musculature to involve deficits in sight, speech, and swallowing. Paralysis eventually progresses beyond cranial nerve (CN) palsies to include generalized muscle weakness and loss of critical accessory muscles of respiration. If untreated, death is inevitable from airway obstruction secondary to paralysis of pharyngeal, diaphragm, and accessory respiratory muscles, as well as loss of the protective gag reflex.

The CDC recommended therapy for the public is a trivalent equine antitoxin against types A, B, and E. In cases of exposure to other BoNT serotypes, the US Army can provide an investigational heptavalent (ABCDEFG) antitoxin. However, the antitoxins are in limited supply and would need to be retrieved from stockpiles. Therefore, the development of safe and effective post-exposure therapeutic compounds for BoNT intoxication is of paramount importance to serve the requirements of the military and civilian populations. In conjunction with drug discovery efforts, there is a parallel exigency to develop appropriate animal models to test the usefulness of various strategies for protection against BoNT intoxication.

A. Development of Animal Model Test Systems

1. Inadequacies of Current Animal Model Test Systems

Currently, a large number of animal models (mice, rats, guinea pigs, rabbits, and nonhuman primates) have been used for BoNT research, and it is not clear which species is the most appropriate. This is especially problematic since there are marked species differences in the relative potencies of the different serotypes and in their latency of action (effect of BoNT/B in mice, rabbits, guinea pigs vs rats; Erdal *et al.*, 1995; McLellan *et al.*, 1996; Hilmas *et al.*, 2006a). Mice, in particular, are desirable in BoNT research because they offer the most favorable balance between the scientific needs of the experiment and consistency with the

existing literature. A variety of mouse strains and sexes have been used for other BoNT studies. The mouse LD_{50} is still used to quantitate the purity of BoNT batches and is the basis of the international standard used in serum neutralization assays of BoNT antitoxin. The mouse phrenic nerve–hemidiaphragm assay has been used to measure the effect of BoNTs on skeletal muscle contraction and the doses necessary for inhibition are well characterized. The mouse has further advantages over other rodent species like rats.

Rats are not a valuable test system for BoNTs as they are widely recognized as being insensitive to serotype B (Verderio *et al.*, 2006). On the other hand, skeletal muscles of CD-1 mice, Hartley guinea pigs, and New Zealand white rabbits have similarities to humans in that their muscles are sensitive to serotypes A, B, C, D, and E (Hilmas *et al.*, 2006b). *In vivo* and *in vitro* physiological assessments of BoNT action in rat have also proved to show inconsistent and erroneous results. *In vivo* experiments using the rat extensor digitorum longus (EDL) muscle assay showed sensitivities of rat muscle to the B serotype at low doses (10 MU, corresponding to approximately 1–10 pM) (Adler *et al.*, 1996), despite the wide body of literature on the rat to the contrary. In addition, *ex vivo* rat phrenic nerve–hemidiaphragm preparations are insensitive to BoNT/B at even very high concentrations in the nanomolar range (Williams *et al.*, 2007).

Another physiological model to evaluate therapeutic candidates against BoNT intoxication is the rat toe spread assay. The rat toe spread assay is problematic as a model test system. First, it will not allow for the evaluation of therapeutic candidates against the B serotype since rats are insensitve to BoNT/B. Second, the rat toe spread assay does not involve focal application of BoNT; neighboring muscles are paralyzed due to local diffusion of toxin from the site of intramuscular injection. Toe spread in the rat is mediated predominantly by digiti minimi abductor muscles and to a lesser extent by the EDL, the actual muscle injected in the assay. Intramuscular injection of rat EDLs with BoNT will primarily paralyze the EDL and to a lesser extent the digiti mini muscles, the true abductors of toe spread, by local diffusion. Therefore, EDL muscles injected with BoNTs would tend to show an erroneously early recovery of toe spread as the primary effectors of toe spread recover sooner compared to injected EDL muscles. To date, there is no acceptable *in vivo* model to test the efficacy of inhibitory compounds.

2. Advantages of the Mouse Hemidiaphragm Assay

Current approaches to the inhibition of BoNT activity involve a number of strategies, each with potential advantages and disadvantages. Ultimately, model test systems that can incorporate each of these potential approaches are needed to evaluate the relative merit of potential therapeutic compounds. Since the presynaptic terminal is the primary target for BoNTs, a test system based on toxin action at presynaptic terminals is indicated. Such systems should permit testing of all relevant aspects of toxin (internalization, activity, overcoming inhibition of transmitter release), should be simple and reliable, and should permit rapid evaluation of novel therapeutics or their precursor compounds.

Due to the high sensitivity of mammalian synapses to the actions of BoNTs, due in part to the presence of high-affinity binding sites for toxin on the cell surface and to the intracellular presence of the appropriate enzymatic substrates, the test model systems should be of mammalian origin. Muscle is the ideal test system for BoNT since it is the most sensitive *in vivo* target for neurotoxin action. In addition, inhibition of the diaphragm muscle is the proximal cause of death in botulism (Habermann and Dreyer, 1986; Simpson, 1986). Furthermore, a positive result with BoNT on muscle implies that the toxin is correctly folded and the binding, catalytic, and translocation domains are all intact. Enzyme linked immunosorbent assays (ELISAs), on the other hand, detect only components of the toxin and may provide positive results when the toxin has in fact lost its ability to intoxicate (Kalb *et al.*, 2005). The mouse phrenic nerve–hemidiaphragm assay is a favorable model test system to evaluate therapeutics against BoNT-induced paralysis.

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CHAPTER 31

Anthrax

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Opinions, interpretations, conclusions, and recommendations are those of the author(s) and are not necessarily endorsed by the US Army.

I. INTRODUCTION

Anthrax is a virulent, contagious, and potentially fatal disease. The first accounts of anthrax infection were written by the Roman poet Vergil in early antiquity. While its lethal effects were ascribed to the actions of an exotoxin over a half century ago, the pathogenesis and mechanism of anthrax toxicity continue to be refined. Depending on the route of exposure, anthrax can cause a different disease, including inhalational, cutaneous, and oral/ingestional forms. Anthrax infection involves a complex set of steps in its pathogenesis from spore uptake by immune cells, germination, transport to local lymph nodes, production of deadly toxins, systemic spread, and ultimately death of the host. The details of each step are continually being debated. Even today debate ensues regarding the mechanisms of macrophage killing by the toxins and the importance of other cell types involved in toxin-induced fatality.

While anthrax infection causes high numbers of bacilli and overt septicemia, it is the exotoxins which are responsible for the intoxicating symptoms and death. Birth of the molecular age in modern research led to the identification and enzymatic characterization of three proteins that constitute two anthrax exotoxins. These are the protective antigen (PA), edema factor (EF), and lethal factor (LF). A better understanding of the precise mechanisms of toxicity initiated by anthrax toxins will uncover many of the unsolved mysteries surrounding *Bacillus anthracis* infection. Moreover, nature's clever engineering will be evident from the sometimes contradictory actions of the spore form, vegetative bacilli, and bacterial toxins.

II. HISTORY

Anthrax has plagued man and beast since early recorded history. Scholars have attributed several plagues in antiquity to anthrax. The Plague of Athens (430–427 BC) and two of the plagues of Egypt (the fifth – death of livestock – and sixth plagues – boils), during the time of the Israelites' captivity have both been ascribed to outbreaks of anthrax (McSherry and Kilpatrick, 1992). Publius Vergilius Maro or Vergil (70–19 BC) provided the earliest, definitive, and detailed descriptions of an anthrax epidemic in his four-volume *Georgics*, a narrative on agriculture and animal husbandry (Sternbach, 2003). Vergil described the same disease ravaging sheep, horses, cattle, dogs, and various other animals. In addition to signs of toxicity, he provided insight into knowledge of how the disease was transmitted, namely wool. He even noted the virulent and contagious nature of anthrax, as well as its ability to spread to humans (Dirckx, 1981).

> The pelts of diseased animals were useless, and neither water nor fire could cleanse the taint from their flesh. The sheepmen could not shear the fleece, which was riddled with disease and corruption, nor did they dare even to touch the rotting strands. If anyone wore garments made from tained wool, his limbs were soon attacked by inflamed papules and foul exudates. (Dirckx, 1981)

During the course of the next 1,500 years, Europe witnessed sporadic outbreaks of anthrax as they occurred in 14th century Germany and 17th century central Europe and Russia. The disease was classified as anthrax or charbon malin (Morens, 2003) in 1769 by the French physician Nicholas Fournier (Fournier, 1769; Morens, 2003). The name is derived from the black eschar lesions, the hallmark of cutaneous infection. Fournier also noted a link between those who worked with raw animal hair or wool and an increased susceptibility to disease. In the 18th century, an epidemic destroyed half of the sheep in Europe, possible evidence that anthrax was a major problem. Inhalation anthrax became known in the Victorian era as woolsorters' disease; however, infection was more often the result of contact with goat hair or alpaca than wool.

In 1850, Pierre-Francoise Olive Rayer (Rayer, 1850) and Casimir-Joseph Davaine (Davaine, 1863) reported the presence of ''small filiform bodies'' in the blood of anthrax-infected sheep (Carter, 1988). By 1855, Franz Aloys Antoine Pollender confirmed this discovery and implicated their role in producing anthrax disease (Pollender, 1855). In 1858, Freidrich August Brauell noted the ''bodies'' to be absent from healthy animals or animals infected with diseases other than anthrax. Brauell also noted their inability to be transmitted from pregnant sheep to fetus (Brauell, 1857).

In the 1870s, Robert Koch, a Prussian physician, isolated the anthrax bacillus and traced the complete life cycle using suspended-drop culture methods. Koch determined the bacillus could form spores which remained viable, even in hostile environments (Koch, 1876). Louis Pasteur provided demonstration of infectious disease transmission. He inoculated one cohort of cattle with live attenuated vaccine and a control cohort without vaccine. When all animals were injected with virulent anthrax, only the control cattle died.

A. Modern History: Weaponizing Anthrax and Terrorism

Research into the utilization of anthrax spores as a biological weapon began in the early 20th century. During World War I, German development of *B. anthracis* and other disease-causing organisms gave rise to covert programs intended to infect livestock and animal feed to be exported to the Allies. These plans included contaminating feed for horses and cattle to be exported from the USA to England, infecting sheep from Romania to be exported to Russia, and exporting contaminated livestock from Argentina to various Allied nations (Hugh-Jones, 1992; Merck, 1946).

During World War II, the pace of anthrax and biological weapons research in general accelerated. Imperial Japan had a large, active bioweapons program that included a substantial anthrax component. The central Japanese research facility was located in Manchuria, known as Unit 731. It is believed that in excess of 10,000 prisoners of war died either by direct experimental exposure to *B. anthracis*, among other pathogens, or by execution following exposure (Harris, 1992, 1994). Much of the Japanese bioweapons research transitioned to the battlefield. Operations such as Nomonhan in 1939, where Japanese troops entered the Soviet Union to infect Russian herds, met with only partial success. As it turned out, Japanese troops were unprepared to operate in a biological weapons environment. Their actions resulted in many inadvertent friendly casualties (Harris, 1992).

The Allies were pursuing biological weapons programs at the same time as the Axis powers. In 1942, the United Kingdom conducted anthrax experiments off the coast of Scotland at Gruinard Island. British scientists working at the biological weapons laboratory at Porton Down had demonstrated the lethality and military utility of the bacillus. Spores persisted and remained theoretically capable of infection for decades afterwards. A subsequent decontamination effort took nearly 10 years to clean up the island (Carter, 1992). The USA began developing anthrax as a biological weapon in 1943. A civilian agency, the War Reserve Service, constructed a research facility at Camp Detrick (later Fort Detrick in 1956), and conducted research into a number of pathogens, including *B. anthracis*.

In April and May of 1979 an anthrax epidemic occurred in Sverdlovsk, a city of then 1.2 million people, 1400 km east of Moscow. The Soviet medical community reported an outbreak in livestock south of the city, and human exposures by ingestion of infected meat and contact with diseased animals led to cases of gastrointestinal and cutaneous anthrax. According to Soviet medical reports, 96 cases of human anthrax were identified, of these 79 were said to be gastrointestional and 17 cutaneous. These cases resulted in 64 deaths, all reported to be gastrointestional exposures (Meselson *et al.*, 1994). In 1986, the Soviet Union invited a group of American scientists to visit Russia and investigate the outbreak. In collaboration with the Russian clinicians who treated the victims, the panel concluded that the outbreak was the result of inhalation exposure due to the accidental release of an estimated 10 kg of military-grade anthrax from the Soviet military microbiological facility in Sverdlovsk. This event remains the largest documented outbreak of inhalation anthrax (Sternbach, 2003).

In 2001, the first case of intentional anthrax release in the USA occurred. In October and November of that year, 11 confirmed cases of inhalation anthrax and 11 confirmed or suspected cases of cutaneous anthrax were reported in postal workers and others who handled mail that had been deliberately contaminated with anthrax spores (Abalakin *et al.*, 1990). These contaminated letters were mailed anonymously to several news media and Federal government offices. The letters contained handwritten threats as well as cryptic references to the terrorist attacks on September 11 of that year. The anthrax spores were analyzed and determined to be of the ''Ames'' variety, the strain which originated in the USA and had been acquired by Army research institutes for vaccine development.

Anthrax remains both a serious public health hazard and a very real biological weapon threat. A deliberate release of an anthrax weapon in a populated area could have catastrophic implications. An economic model developed by the Centers for Disease Control and Prevention (CDC) suggested a cost of $26.2 billion to treat 100,000 people exposed to anthrax (Kaufmann *et al.*, 1997). A risk assessment, provided at the end of this chapter, will serve to highlight the dangers of a realistic scenario involving anthrax spores.

III. EPIDEMIOLOGY

A. Persistence

Bacillus anthracis can remain for extended periods of time in soil. The mechanism responsible for its persistence is unclear. Therefore, persistence may involve multiplication cycles and sporulation. It may involve multiplication of the organism in the soil or bacterial amplification in infected animals prior to soil contamination by the carcass. Spores may germinate simply upon application of water to soil (Hanna and Ireland, 1999; Oncul *et al.*, 2002).

B. Infection

Infection typically results from herbivores grazing on soil or feed contaminated with spores. Oral consumption may

produce oropharyngeal or gastrointestinal infection, an invariably fatal condition. Terminally ill herbivores generally bleed from the nose, mouth, and bowel, resulting in further contamination of the soil or drinking source (Shafazand *et al.*, 1999). While the actual number of cells or spores shed by an infected animal is unknown, studies have shown that counts of 10^4 to 10^6 spores/g of soil can be found near infected carcasses (Turnbull *et al.*, 1998).

C. Dissemination

Dissemination of anthrax spores may result from biting flies or vultures (de Vos, 1990; Hugh-Jones and De Vos, 2002; Davies, 1983; Turell and Knudson, 1987). Flies and mosquitoes contaminated with the vegetative cells, as a result of feeding on blood, can remain infectious for hours. The infectious material is deposited onto leaves through defecation, leading to contamination of herbivore species such as cattle, sheep, horses, and goats. Vultures often feed on contaminated carcasses and disseminate the organism to other birds or common drinking sources shared by various animals.

D. Forms of Anthrax Disease

Anthrax in humans is associated with agricultural, horticultural, or industrial exposure to infected animals or animal products. Three forms of anthrax can be diagnosed in humans: cutaneous, gastrointestinal, and inhalational. Cutaneous anthrax infection can occur through handling of contaminated hides, wool, bones, and carcasses. Cutaneous anthrax is the most common form of natural human infection, consisting of >95% of anthrax cases, and is treatable if recognized early. Infection occurs as a result of direct contact with infected animals or animal products. The skin is typically damaged or abraded prior to establishment of successful skin lesions by the bacteria. The persons most at risk of a natural cutaneous exposure are industrial or agricultural workers, such as herders, butchers, slaughterhouse workers, or processing mill workers (Pile *et al.*, 1998). Infection can also occur through an insect bite (Spencer, 2003). While cutaneous anthrax is rarely fatal (mortality <1% in treated cases; Anon, 2000), it can progress to a systemic infection with a mortality rate of 5–20% in untreated cases (Pile *et al.*, 1998). In general, cutaneous anthrax is not as life threatening as the inhalational form, which results in much higher mortality. However, it is still important to study dermal pathogenesis models, particularly because cutaneous anthrax cases may result from an aerosol release (Inglesby *et al.*, 1999), a method most likely to be used by bioterrorists.

Inhalational anthrax may occur after inhaling aerosols of spores, formed from processing contaminated animal products (wool) or as the result of direct bioweaponization. Inhalational anthrax contributes to only 5% of all reported cases but is by far the most lethal form. The estimated mortality is approximately 90% in untreated patients (Atlas, 2002; Bales *et al.*, 2002; Dixon *et al.*, 1999; Friedlander, 1999, 2000; Meselson *et al.*, 1994; Oncu *et al.*, 2003). Inhalational anthrax is typically reported in industrial settings where animal products are handled in enclosed spaces, allowing for exposure to aerosolized spores. Individuals passing by these industrial facilities have been stricken with inhalational anthrax. In one study of 27 anthrax cases involving textile mills, 21 cases were cutaneous and six were inhalational. All but one of the inhalational cases was fatal. One case of inhalational anthrax occurred in a secretary at a goat hair-processing facility.

Gastrointestinal anthrax is far less common than inhalational anthrax, but the mortality rate is extremely high, from 50 to 75%, even with early treatment (Mansour-Ghanaei *et al.*, 2002). Oropharyngeal or gastrointestinal forms of anthrax can result from ingestion of contaminated meat (Atlas, 2002; Bales *et al.*, 2002; Dixon *et al.*, 1999; Friedlander, 1999). Gastrointestinal anthrax has never been confirmed in the USA, but this may be the result of cases being unreported in rural communities, where physicians may not be aware of this form (Atlas, 2002; Bales *et al.*, 2002; Dixon *et al.*, 1999; Friedlander, 1999; Oncu *et al.*, 2003; Pile *et al.*, 1998; Shafazand *et al.*, 1999). Two out of 53 persons in a 1998 Kazakhstan outbreak developed the gastrointestinal form, resulting from the consumption of contaminated raw meat. In Minnesota, several family members consumed steer meat and fell ill with gastrointestinal symptoms (Bales *et al.*, 2002). The meat was later confirmed to contain *B. anthracis*, but the bacteria could not be cultured to confirm the presence of the gastrointestinal pathogen. According to one case of a 15-year-old male who was infected after ingesting half-cooked sheep meat, the incubation period for gastrointestinal anthrax varies from 2 to 5 days (Mansour-Ghanaei *et al.*, 2002).

Worldwide, the annual incidence of human anthrax infection is estimated between 20,000 and 100,000 (Oncu *et al.*, 2003; Pile *et al.*, 1998). The vast majority are cutaneous anthrax. In the USA, less than one case is diagnosed per year, as compared to 127 cases/year diagnosed in the early 20th century (Oncu *et al.*, 2003; Pile *et al.*, 1998; Shafazand *et al.*, 1999). Occasionally, outbreaks of anthrax will occur as a result of breakdown in public health standards and practices or lack of public health services.

IV. PATHOGENESIS

A. Overview

Themes common among all anthrax infections are the following: (1) uptake by macrophages and other immune cells, (2) germination to the vegetative form at or near the site of inoculation prior to transit to target tissues, (3) time course of transport to target organs, (4) organs targeted for toxicity, (5) overwhelming septicemia, and (6) release of

soluble factors responsible for death. Two types of *B. anthracis* have been identified: a spore form and a vegetative form. Unless stated otherwise, the vegetative bacillus will be referred to as *B. anthracis* because this is the form that produces the deadly toxins; however, both forms will be discussed at length. The spore form is essential for uptake by host cells.

B. Uptake of Spores

Anthrax infection typically results from entry of *B. anthracis* spores into the host through a minor abrasion, insect bite, ingestion of contaminated meat, or inhalation of airborne spores. These routes of exposure lead to cutaneous, intestinal/oropharyngeal, and inhalational anthrax diseases (Figure 31.1). While *B. anthracis* is not categorized as an intracellular pathogen, it uses tissue macrophages as a sanctuary where the engulfed spores can germinate during the early phase of infection (Guidi-Rontani and Mock, 2002). Successful infection and eventual disease requires uptake of spores. While spores are resistant to phagosomal superoxide (Baillie *et al.*, 2005), they have evolved to recognize receptors contained on host phagocytic cells through their pathogen-associated molecular patterns (PAMPs). While most studies have shown uptake of the bacterium by macrophages, one study showed that human dendritic cells can be triggered to internalize *B. anthracis* spores (Brittingham *et al.*, 2005). This led to the hypothesis that these cells may take part in bacterial transport to the lymph nodes, similar to macrophages. Spore PAMPs are antigenic determinants containing highly conserved molecules which interact with cell-surface Toll-like receptors (TLRs) on tissue macrophages, dendritic cells, polymorphonuclear leukocytes (PMNs), and other cell types (Janeway and Medzhitov, 2002). PAMPs typically include lipopolysaccaride (LPS) from Gram-negative bacteria and lipoteichoic acid and peptidoglycan from the cell wall of Gram-positive bacteria such as *B. anthracis*. Anthrax spores can trigger a strong inflammatory response by activating TLR4 on antigen presenting cells of the immune system at the entry site into the host (Hsu *et al.*, 2004). In addition, anthrolysin O, a protein secreted by *B. anthracis*, is a potent agonist for TLR4 (Park *et al.*, 2004).

C. Uptake Via Lungs

Anthrax spores are approximately 1 to 2 μm in diameter, optimal for inhalation and deposition in the alveolar spaces (Brachman *et al.*, 1966; Penn and Klotz, 1997; Brachman, 1970, 1980). In the case of inhalational anthrax, inhaled spores reach the respiratory bronchioles and alveoli (Figure 31.2). While most spores are internalized rapidly into phagolysosomes by resident macrophages in the alveolar space (Guidi-Rontani *et al.*, 1999b; Ross, 1957), the exosporium layer of anthrax, discovered by Flügge (1886) prevents its degradation. It should be noted that the exosporium is not present on vegetative forms of anthrax bacteria and therefore only spores contain the antigenic

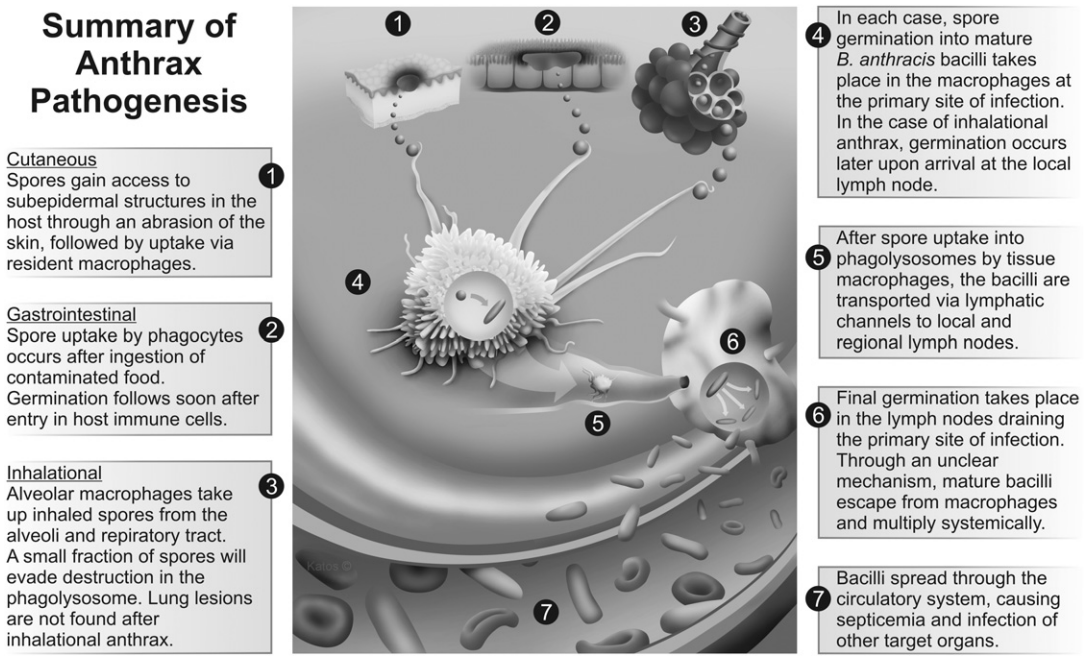


FIGURE 31.1. Overview of anthrax disease and pathogenesis. The steps of anthrax intoxication including spore uptake by macrophages, germination to the vegetative form, migration to lymph nodes, bacillus multiplication, release into the circulatory system, and septicemia are illustrated. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

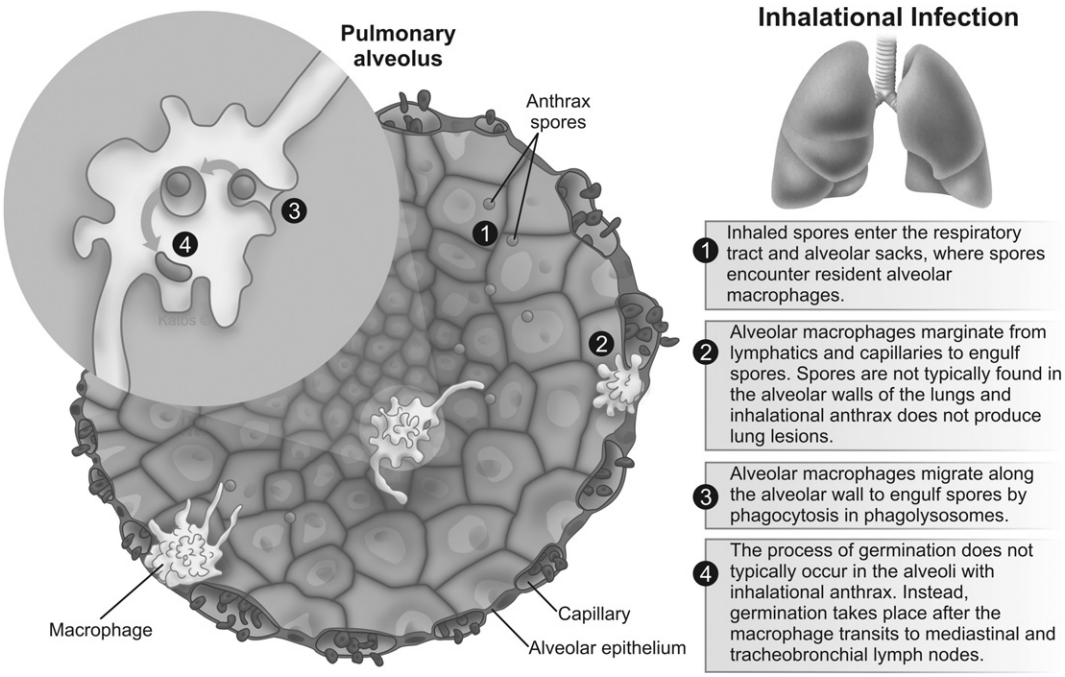


FIGURE 31.2. Spore uptake in inhalational anthrax disease. The steps involving spore deposition into a pulmonary alveolus, margination of alveolar macrophages out of capillaries into the alveolar space, spore uptake, and entry into lymphatic channels are illustrated. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

determinants recognized by TLR4 receptors of immune cells. In any case, spores will escape destruction due to their resistance to superoxide and enzymes of the phagolysosome. Spore-bearing alveolar macrophages migrate along lymphatic channels to mediastinal, peribronchial, and tracheobronchial lymph nodes (Ross, 1957; Lincoln *et al.*, 1965), while germinating en route.

D. Uptake Via Skin

The cutaneous infection process initiates as a result of anthrax spores colonizing an abrasion in the skin (Figure 31.3). A small eruption or lesion develops into a painless, black eschar. Eschars usually develop within 2–5 days following exposure. During this stage of infection, low-level spore germination can occur at the primary site of infection, leading to localized edema and necrosis. While this infection often remains localized, some patients experience systemic symptoms.

Systemic disease, a rare secondary occurrence of cutaneous anthrax, is likely due to phagocytosis by macrophages in the dermis that marginate out of lymphatic channels and blood capillaries (Figure 31.3). In cutaneous anthrax, germination typically occurs immediately inside the host macrophage. Macrophages carrying *B. anthracis* cells migrate back into lymphatic ducts *en route* to regional lymph nodes draining the primary site of infection (Dixon *et al.*, 1999). The anthrax bacilli spread through the blood and lymph and proliferate to high concentrations, creating acute septicemia.

E. Uptake Via Gastrointestinal Route

The infection process of gastrointestinal anthrax starts with ingestion of spore-contaminated food/drink or ingestion of inhaled spores. In general, gastrointestinal anthrax is similar to cutaneous anthrax, but gastrointestinal anthrax occurs in the intestinal mucosa. As in cutaneous anthrax, the organisms probably invade the mucosa through a preexisting wound or lesion or possibly through interaction with a cell-surface receptor on epithelial cells. Presumably, the mucosal lining is the entry point for the endospores (Figure 31.4), but the exact germination location is yet unknown in cases of gastrointestinal anthrax infection. The bacteria may spread from the mucosal lesion to the lymphatic system by way of macrophages migrating to the primary site of infection. Germination can occur inside host macrophages after uptake prior to macrophage migration back to lymphatic channels draining to lymph nodes (Figure 31.4). Ulcer formation is a typical symptom during gastrointestinal anthrax, and ulcers may be present at various locations along the gastrointestinal tract from the oral cavity to the cecum. However, it is not known whether ulceration occurs only at sites of bacterial infection, or if it is caused by the anthrax toxins and therefore has a less restricted occurrence (Dixon *et al.*, 1999).

F. Spore Function

Several studies have examined the molecular mechanisms by which *B. anthracis* spores undergo phagocytosis,

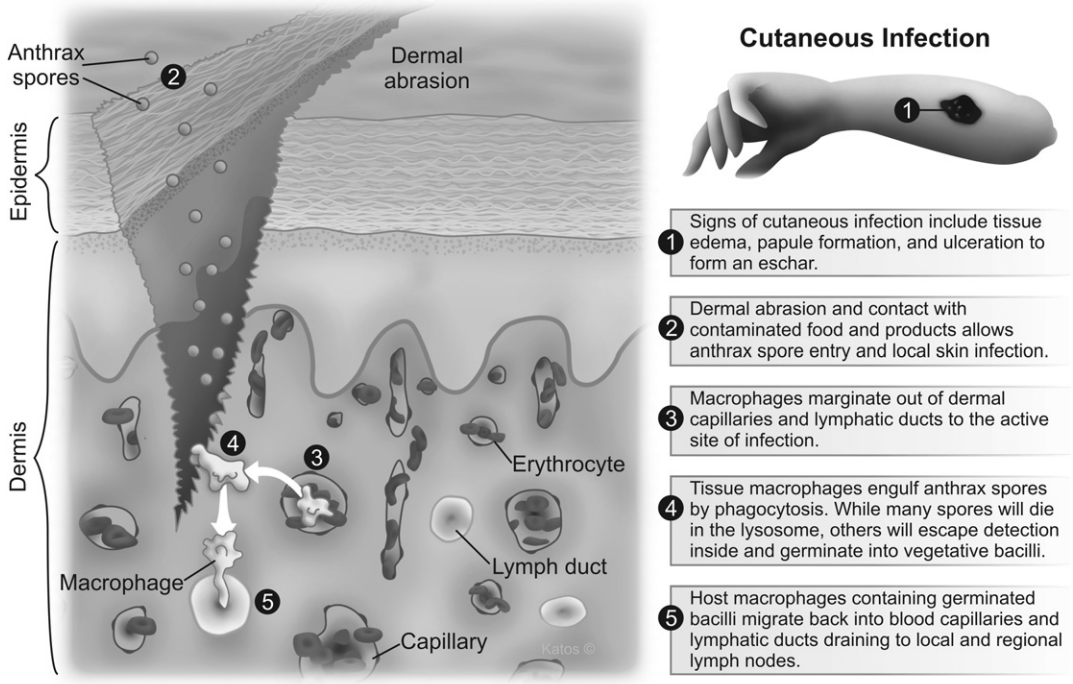


FIGURE 31.3. Spore uptake in cutaneous anthrax disease. The steps involving spore uptake at the primary site of local infection on the skin, germination inside the macrophage, and migration back into lymphatic ducts are illustrated. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

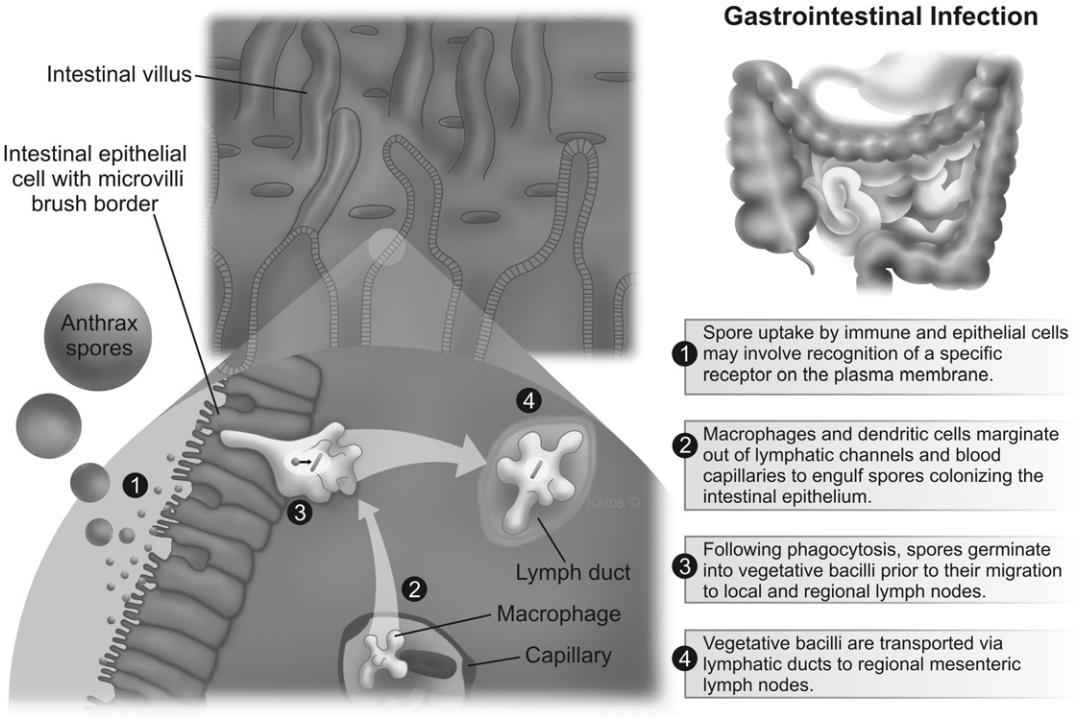


FIGURE 31.4. Spore uptake in gastrointestinal anthrax disease. The steps involving spore colonization of the gastrointestinal mucosa, uptake by phagocytes, germination, and lymphatic spread are illustrated. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

germination, and subsequent escape to mediate systemic infection. Time-lapse microscopy suggested that the number of spores per macrophage can affect whether any spores are able to outlast the macrophage's inhibitory action of the bacteria. Macrophages that have engulfed a larger number of spores are more likely to have a few that survive to germinate and become vegetative bacilli (Ruthel *et al.*, 2004). Inside the macrophage, the spores must first avoid cellular nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activation. This enzyme, which is present in the phagosomal membrane, initiates the reduction of oxygen to superoxide anion through NADPH oxidation. The superoxide anion is further converted to other reactive oxygen species (ROS), including hydrogen peroxide. To date, no *B. anthracis* enzymes have been discovered that are involved in the removal of ROS (Guidi-Rontani and Mock, 2002). After surviving the initial membrane oxidative burst, the anthrax spores must contend with the acid environment of the phagolysosome to further germinate and multiply. Several pathogens have shown an ability to evade phagolysosomal activity; however, the mechanism by which anthrax spores avoid hydrolysis in the phagolysosome is unclear (Guidi-Rontani and Mock, 2002).

Anthrax spores promote expression of interleukins (ILs) and other pro-inflammatory cytokines in macrophages and dendritic cells (Pickering and Merkel, 2004; Pickering *et al.*, 2004). Macrophages are important mediators of the inflammatory response, and produce tumor necrosis factor alpha (TNFα), interleukin-1β, and IL-6 in response to infection. While some spores will be killed by immune cells, many will evade the host cell detection only to initiate germination. The purpose of promoting inflammation here by anthrax spores is to increase their chance for internalization by tissue macrophages and dendritic cells, in order to germinate into the vegetative form of the bacteria (Welkos *et al.*, 1989).

An intact mitogen-activated protein kinase (MAPK) cascade and pro-inflammatory response by macrophages is also necessary for host cell migration to regional lymph nodes and recruitment of additional macrophages to the primary site of infection. Without functional immune cells, the toxin would not be able to enter lymphatics or the general circulation. In fact, dendritic cells which engulf *B. anthracis* spores change their pattern of chemokine-receptor expression. Specifically, they lose tissue-retaining receptors (CCR2 and CCR5) and up-regulate lymph node homing receptors (CCR7 and CD11c) (Brittingham *et al.*, 2005).

G. Time Course of Spore Germination

In cutaneous and gastrointestinal anthrax, spore germination takes place at or near the inoculation site following uptake by the macrophage or dendritic cell. It is the vegetative form, not the spore, which produces the deadly factors. After transformation to the vegetative bacilli, these bacteria are free to mediate toxicity at the inoculation site in skin and gastrointestinal tract (Beatty *et al.*, 2003). In inhalational anthrax, germination does not occur until the spores have been transported to the local lymphatics and mediastinal lymph nodes (Riedel, 2005). In the case of inhalational anthrax, spores are not believed to germinate at the site of infection in the alveoli or bronchioles. Instead, they germinate en route or after migrating to local nodes of the lymphatic system.

H. Spore Germination

While the anthrax endospore has no measurable metabolism, spore germination is a predictor of productive anthrax infection. Similar to endospores from other species, anthrax spores seem to represent a biologically inert organism with the ability to transform into one of the most lethal organisms on earth. Germination inside the host immune cell is the key step toward this transformation from benign bystander to active infection. The spores have little or no water, no ATP production, no macromolecular synthesis, and no active enzymes. Thus, endospores can remain stable in the environment under adverse conditions for decades. Once inside a host cell, spores start to germinate and initiate early synchronous *de novo* expression of genes vital for infection, as well as expression of genes necessary for vegetative growth (Hanna and Ireland, 1999; Oncul *et al.*, 2002).

Several putative germination proteins have been identified (Guidi-Rontani *et al.*, 1999a, b; Huang *et al.*, 2004), but their roles in spore germination have yet to be clarified. L-alanine appears to be essential in initiating germination (Foster and Johnstone, 1990), but the mechanism that leads to spore germination is unclear. A germinant receptor locus (*ger S*) essential to *B. anthracis* germination has been identified; without it, the organism could not germinate in macrophages (Ireland and Hanna, 2002). Once the spore has germinated, the vegetative bacilli are free to synthesize deadly bacterial toxins; however, the precise manner in which vegetative bacilli or spores break free from immune cells is still poorly understood. A recently proposed model suggests that an interaction between anthrax toxin from newly germinated spores inside the phagolysosome of macrophages and anthrax toxin receptor 2 promotes escape of the bacilli from the cells (Banks *et al.*, 2005).

I. Vegetative Anthrax and its Capsule

The role of the vegetative bacillus is in stark contrast to that of the spore form (Table 31.1). The vegetative form functions mainly to produce the deadly toxins lethal toxin (LT) and edema toxin (ET). Germination of *B. anthracis* within macrophages is closely followed by expression of the toxin genes (Guidi-Rontani *et al.*, 1999a). *Bacillus anthracis* contains no capsule *in vitro*, but capsule synthesis begins upon host infection (Preisz, 1909). Host signals, including carbon dioxide concentrations above 5%, are thought to induce transcription of capsule and toxin genes (Uchida *et al.*, 1997; Koehler *et al.*, 1994; Sirard *et al.*, 1994).

TABLE 31.1. Differences between spore and vegetative forms of *Bacillus anthracis*

Spore form	Vegetative form
• Contains an exosporium which promotes immune host detection and uptake	• Contains poly-D-glutamic acid capsule, essential for virulence and avoidance of immune detection
• Exosporium promotes internalization by macrophages	• Capsule avoids phagocytosis by macrophages
• Requires phagocytosis, internalization by macrophages, and germination to become pathogenic	• Survives as extracellular pathogen within the body of the host
• Does not produce anthrax toxins	• Produces lethal and edema toxins to kill the target host cell
• Requires target host cells to remain intact so they can circulate to local lymph nodes	• Kills the host cell
• Promotes TNF-α and cytokine production	• Inhibits TNF-α and cytokines
• Requires target host cells to remain intact so they can circulate to local lymph nodes	• Kills the host cell
• Enhances host inflammatory response	• Suppresses the immune system
• Can remain dormant for months	• Unable to survive outside the host for any appreciable length of time

In vitro studies by Ezzell and Abshire (1995) suggest that encapsulated vegetative cells appear within 30 min after germination *in vitro*. The capsule enables vegetative forms to survive as extracellular pathogens and avoid phagocytosis by macrophages (Guidi-Rontani and Mock, 2002). Indeed, *B. anthracis* isolates lacking capsules are significantly less virulent. Vegetative cells move through the bloodstream and lymphatics to cause systemic infection.

J. Systemic Infection and Septicemia

Significant numbers of bacilli in the blood are characteristic of the late stages of infection (Atlas, 2002; Shafazand *et al.*, 1999; Oncu *et al.*, 2003; Riedel, 2005). Without an activated immune response due to the release of soluble anthrax toxins which suppress immune function, *B. anthracis* is free to multiply to high concentrations. It is currently unclear whether *B. anthracis* multiplies within the macrophage as some studies suggest (Shafa *et al.*, 1966) or the bloodstream. Cultured blood from infected rabbits and guinea pigs suggest a continuous rise in bacterial counts until the final hours of life; numbers increase sharply to 10^7 and 10^9 organisms per milliliter of blood for rabbits (Bloom *et al.*, 1947) and guinea pigs (Smith and Keppie, 1954; Keppie *et al.*, 1955), respectively. Death was shown to be dependent on bacteremia, but death was not shown to be a consequence of mechanical obstruction by large bacterial counts. Regardless of the route of infection, systemic spread results in similar colonization. The lungs and gastrointestinal tract are particular targets of system infection by mature bacilli, with the spleen, brain, liver, and almost any other organ being colonized within hours or days (Riedel, 2005). *Bacillus anthracis* has been isolated from stool specimens of both animals and humans, reflecting its presence in the bowel (Beatty *et al.*, 2003). While septicemia is important, death is the direct result of soluble factors (discussed below) secreted by the vegetative bacilli which weaken the immune response and initiate cell death.

K. Anthrax Infection Cycle

An infection cycle for *B. anthracis* has been proposed by Hanna and colleagues (Hanna and Ireland, 1999). Phagocytosis of spores by macrophages, germination, vegetative cell proliferation, and toxin release occur within hours after exposure to *B. anthracis* in a productive infection. During the middle stages of infection, the bacilli grow extracellularly and express toxins and other virulence factors. During the final stages of infection, the bacilli, having depleted nutrients from the host, form endospores, which return to the environment for the next cycle of infection (Hanna and Ireland, 1999; Oncul *et al.*, 2002). The precise sequence of events leading to successful establishment of infection is not completely understood. After germination, anthrax bacilli multiply in the lymph nodes, causing an immediate lymphadenitis, hemorrhagic mediastinitis, and spread throughout the body via the circulatory system (Dutz and Kohout, 1971; Albrink, 1961).

L. Release of Soluble Factors

Bacillus anthracis possesses four known virulence factors, including an antiphagocytic capsule of the vegetative form, lethal factor (LF, 90 kDa), edema factor (EF, 89 kDa), and a protective antigen (PA, 83 kDa). These virulence factors acting together play a key role in pathogenesis but are not toxic when present individually. In combination, PA, LF, and EF induce a dangerous cascade of events upon cell entry. As early as 1953, Smith and colleagues implicated toxic factors as the cause of death from anthrax infection (Smith *et al.*, 1953, 1955; Keppie *et al.*, 1953, 1955). It has since been shown that these three factors combine to form the two protein exotoxins of anthrax LT and ET.

PA binds to an as yet ill-defined cell receptor/lipid raft, mediating the entry of the other two components inside the cytoplasm via the endosomal pathway. Edema factor is a Ca^{2+}/calmodulin-dependent adenylate cyclase involved in producing a generalized accumulation of fluid into the interstitium, characteristic of anthrax. Lethal factor is a proteolytic enzyme involved in the inactivation of MAPK kinases (MAPKK), key players in secondary messenger signal transduction cascades. Both toxins are translocated into the cytosol of target cells by way of endosomes through a pore formed from a heptamer of PA molecules. They interfere with vital cellular responses to bacterial infection, disabling host immunity and promoting bacterial dissemination. As the disease progresses, the toxins accumulate to higher levels, causing respiratory distress, shock, widespread hemorrhage, and death.

V. TOXICOKINETICS

A. Inhalational Anthrax

To date, mice, rats, guinea pigs, rabbits, nonhuman primates, dogs, swine, and sheep have been used in inhalational animal model studies for *B. anthracis*. Routes of administration have included aerosol, intranasal, and intratracheal methods. In addition, various animal species differ in their natural resistance to infection (Welkos and Friedlander, 1988). Some of the most common findings in human inhalational anthrax concern the respiratory tract. A majority of patients experience mediastinal widening (70%), pulmonary infiltrates (70%), and pleural effusion (80%). These signs demarcate inhalational anthrax cases from influenza-like illnesses (Oncu *et al.*, 2003). Pathological findings from inhalational anthrax patients in a bioterrorism-related outbreak cited hemorrhage and necrosis in mediastinal lymph nodes, hemorrhage or inflammation of the pleurae and interhilar septae, and prominent intra-alveolar macrophages or inflammation in the lung parenchyma. There were no skin lesions. Bacilli were located in the lung, mediastinal tissues, and thoracic tissues, as identified by staining (Guarner *et al.*, 2003).

Early studies with mice were unable to identify the vegetative form of *B. anthracis* in the alveolar walls of the lungs. Aerosol exposure was achieved by exposing mice (mouse strain unreported) to clouds of spores (Albrink, 1961). Other aerosol studies involving mouse models indicated evidence of the spore form in lung sections but not the vegetative form (Young *et al.*, 1946; Barnes, 1947). The Barnes study highlighted the fact that not all of an aerosolized dose ends up in the lungs; spores can be swallowed to pass through or infect the alimentary tract.

Intratracheal administration of anthrax has demonstrated a respiratory lesion in mice (Lyons *et al.*, 2004). Inoculation of the Ames strain (5,000 vs 10,000 vs 50,000 spores) in BALB/c mice does not produce signs of early pulmonary lesions. Significant numbers of anthrax colony forming units were found in lung-associated lymph nodes harvested 5 h post-inoculation in a dose-dependent manner (Lyons *et al*, 2004). Airway damage did occur after 24 h post-inoculation with all anthrax doses; signs of toxicity include widespread edema vacuole formation, degeneration, airway epithelial cell sloughing, and necrosis. After 48 h, bacterial rods (vegetative form) were located within alveolar capillaries, suggesting active bacterial uptake from the respiratory tract.

Drysdale *et al.* (2005) investigated the role of *B. anthracis* capsule in uptake from the respiratory tract. These investigators deleted the capsule operon *capBCAD* in mutant strains of *B. anthracis* and inoculated female BALB/c mice intratracheally with ~4×10^4 mutant spores. All capsule-deficient strains germinated in the lungs (Drysdale *et al.*, 2005), indicating no role for the capsule at this particular stage in pathogenesis. This contradicts the accepted hypothesis that germination takes place in the lymph nodes for inhalational anthrax exposure.

In the only intranasal mouse model, Guidi-Rontani *et al.* (1999b) demonstrated uptake of *B. anthracis* by alveolar macrophages. A dose of 2.5×10^7 Sterne strain spores was given to Balb/c mice. Bronchial alveolar lavage (BAL) fluids were subsequently collected at 1, 3, and 24 h post-inoculation. Germination of spores in the fluid was assessed by exposing the material to heat (65°C) for 30 min. Ungerminated spores are resistant to this treatment. Germination was significantly evident in the alveolar macrophage fraction of BAL fluid by 24 h post-inoculation. Therefore, the uptake of bacteria by alveolar macrophages occurs rapidly in this system (Guidi-Rontani *et al.*, 1999b).

To date, evidence of respiratory lesions in humans does not exist. Spore cloud exposure studies by Ross (1957) determined the pathogenicity of anthrax in guinea pigs. Guinea pigs were exposed to high numbers of M.36 strain spores for 20 min and sacrificed at various time points to assess spore location. After 1 h, spores were indeed found in alveolar macrophages. Free spores were not, however, isolated from surrounding lymph nodes at this early time point. However, after 18–24 h, bacilli were observed throughout the lymphatic system. The results of these studies led to the idea that spore germination takes place in regional lymphatic nodes (i.e. tracheobronchial nodes) before spreading to the rest of the body via the blood circulatory system (Ross, 1957).

In a guinea pig/aerosol model, 6×10^5 spores of the vaccine anthrax strain STI were detected in the lungs 1 h after infection from a dose of 2.43×10^6 spores. *Bacillus anthracis* was not detectable in the tracheobronchial lymph nodes until 2 days post-infection. On day 36 post-infection, lung levels were down to 10^3 spores and tracheobronchial lymph nodes were again negative.

B. Cutaneous Anthrax

Dermal *B. anthracis* pathogenesis has been studied in mice, rats, hamsters, rabbits, guinea pigs, nonhuman primates, and

dogs. Several methods of inoculation have been tested, including subcutaneous, intradermal, epicutaneous, footpad, and scarification. Data on uptake/absorption from the skin, persistence in the circulation, transit to target tissues, and lethality will be discussed. The only animal models that have been used in bacterial uptake studies following dermal inoculation to date are the mouse and rabbit.

An extensive study on the interaction of *B. anthracis* with mouse skin following epicutaneous inoculation was conducted by Hahn *et al.* (2005). C57BL/6 and DBA/2 mice were inoculated with the Sterne strain. Epicutaneous inoculations were performed by applying an inoculum of 10^7 spores onto the shaved, tape-stripped, or abraded area of the mouse. A difference in foci development was observed, according to skin treatment prior to inoculation. Shaved-only inoculation sites did not have foci of vegetative bacilli. In contrast, abraded inoculation sites had readily apparent foci. Germination and proliferation occurred at the skin surface and in the epidermis and hair follicles. Hair follicles had deeper foci of infection, $>200\,\mu m$ below the skin surface. In animals inoculated with 2×10^8 spores onto unshaved skin, foci appeared only in the hair follicles and not in the epidermis or dermis (Hahn *et al.*, 2005). According to reviews of human infection, germination and proliferation also occur, to some degree, at the site of inoculation (Cranmer and Martinez, 2001). Proliferation also occurs in the draining lymph nodes near the site of inoculation (Anon, 2000). Zaucha *et al.* (1998) subcutaneously inoculated New Zealand white rabbits with 43 to 1.56×10^5 CFU of the Ames strain of *B. anthracis*. At the inoculation site, the main sign of bacterial uptake involved dermal and subcutaneous edema, signs observed in human cutaneous anthrax.

C. Gastrointestinal Anthrax

Despite the rarity in documented human cases of gastrointestinal anthrax (Beatty *et al.*, 2003), this form is common in underdeveloped areas of the world where infected carcasses are consumed (Sirisanthana and Brown, 2002). Gastrointestinal anthrax infection carries a 25–60% mortality rate (Mansour-Ghansei *et al.*, 2002). Gastrointestinal anthrax can be divided into intestinal and oropharyngeal forms. In the intestinal form, following an incubation period of 1–7 days, there is severe abdominal pain, hematemesis, melena and/or hematochezia, ascites and watery diarrhea. Intestinal anthrax carries a greater risk of mortality. In contrast, the milder, oropharyngeal form can be contracted following consumption of infected cattle and water buffalo. In this form, there is marked neck edema and ulcerative lesions in the oropharynx. Clinical signs include nausea, loss of appetite, emesis, and fever. In one natural outbreak of oropharyngeal anthrax, only three out of 24 patients died (Anon, 2000; Pile *et al.*, 1998).

Limited data regarding the pathogenesis of gastrointestinal *B. antracis* infection in any animal model system are available. Barnes (1947) observed that 1 h following inhalational exposure to anthrax spores, the majority of spores were found in the stomach. Therefore, regardless of the route of exposure, there is a high risk of spores transiting to the gastrointestinal tract. Unfortunately, further pathophysiology was absent from the study.

The guinea pig is the only animal model to date for which there are data following oral exposure. There is a need for more oral animal models of *B. anthracis* infection. Gastrointestinal anthrax is perhaps far underdiagnosed in humans; however, it remains an important disease due to the significant risk of ingesting spores after exposure to inhalation anthrax. Since there are limited data regarding pathogenesis of gastrointestinal anthrax in humans, evaluation of future animal models will be difficult. Stability of the organism as a function of pH in the various compartments of the gastrointestinal tract, method of inoculation, presence of stabilizers, and gastrointestinal physiology will become important factors to analyze for oral anthrax.

Aloni-Grinstein *et al.* (2005) assessed the stability of spores and vegetative cells in the gastric fluid and gastrointestinal tract. Female Hartley guinea pigs were given 5×10^9 spores or 5×10^8 vegetative cells of the anthrax MASC-13 variety *per os*. This vaccine strain is nontoxigenic, devoid of capsule, and lacking a nonfunctional form of PA. Bacteria in feces and gastric fluid were charted according to days post-ingestion. Gastric fluid incubations were conducted at 37°C with fluid taken from the guinea pig stomach. Anthrax spores exhibited much greater stability to the harsh environment created by the gastric mucosa than their alternate form. Vegetative cells were barely detectable on day 1 post-ingestion in feces and gastric fluid. There are no animal models or human studies that have examined bacterial persistence in circulation and transit to target tissues following oral exposure to *B. anthracis*. Similarly, there are no animal models that examine oral lethality, clinical signs, or epidemiology following oral exposure.

VI. MECHANISM OF TOXICITY

The significance of the capsule in virulence was demonstrated early last century when anthrax strains lacking a capsule were shown to be avirulent (Bail and Weil, 1911; Bail, cited by Sterne, 1959). The genes encoding synthesis of the capsule were found to be encoded on a 110-kilobase (kb) plasmid. Anthrax strains lacking the plasmid no longer produced the capsule and were attenuated (Ivins *et al.*, 1986), confirming the role of the capsule in pathogenesis.

The capsule contains a polymer of poly-D-glutamic acid, conferring resistance to phagocytosis by macrophages (Keppie *et al.*, 1963). This linear polymer of the capsule is weakly immunogenic (Goodman and Nitecki, 1967). The negatively charged capsule enables the bacterium to inhibit phagocytosis of bacilli by macrophages (Tomcsik and Szongott, 1933; Sterne, 1937; Keppie *et al.*, 1963; Ezzell

and Welkos, 1999). In conjunction with LF and EF, whose target cells include macrophages, dendritic cells, and other immune cells, the capsule allows *B. anthracis* to grow virtually unimpeded in the infected host. The capsule functions as a ''one-way'' filter, allowing bacilli to diffuse the other three virulence factors through the capsule, resulting in host cell intoxication without causing self-harm.

A. Protective Antigen

Bacillus anthracis secretes three plasmid-encoded soluble toxin proteins collectively referred to as anthrax toxin; these are PA, LF, and EF. LF and EF function individually and in combination as catalytic enzymes in susceptible host cells. In an unusual twist of nature, they both have evolved to share PA as a common receptor binding moiety for translocation into the cytosol of the host. PA is a dominant component of the three-part protein toxin secreted by *B. anthracis* (Liddington *et al.*, 1999; Petosa *et al.*, 1997). The mature form of PA, a secreted 735 amino acid protein, has a molecular weight of 83 kDa. The ribbon structure is illustrated in Figure 31.5 along with a detailed description of its functional domains. PA forms a membrane-insertion heptamer that translocates other toxic enzymes (EF and LF) into the cytosol of host cells.

The mechanisms by which *B. anthracis* toxins work at the cellular level are illustrated in Figure 31.6 and are described elsewhere in great detail (Mock and Fouet, 2001; Duesbery and Vande Woude, 1999). In this model, PA released from the vegetative form of the bacilli binds to cell surface receptors, namely tumor endothelium marker (TEM) 8 and capillary morphogenesis protein (CMG) 2, which are expressed as different isoforms by many cell types, including immune cells (Collier and Young, 2003; Bradley *et al.*, 2001; Liu and Leppla, 2003; Scobie *et al.*, 2003; Baldari *et al.*, 2006). Upon binding to the receptor, PA is cleaved into a 20 kDa C-terminal domain (PA_{20}) and a 63 kDa fragment (PA_{63}) by furin or similar host cell surface-associated protease (Klimpel *et al.*, 1992; Molloy *et al.*, 1992). The PA_{20} fragment is released, resulting in spontaneous oligomerization of truncated PA (PA_{63}) into heptamers that bind to EF and LF. It was once thought that a PA heptamer complex may bind up to seven molecules of LF and/or EF (Beauregard *et al.*, 2000; Duesbery *et al.*,

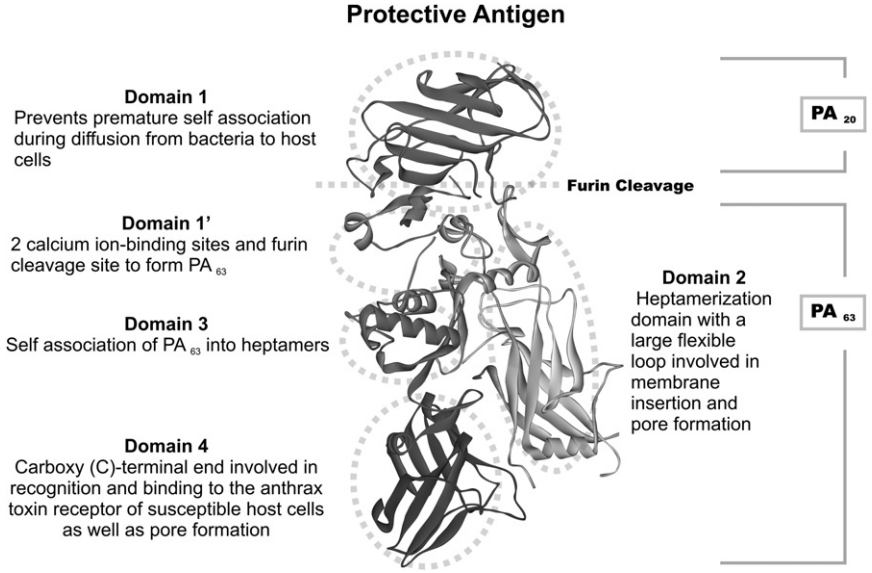


FIGURE 31.5. Three-dimensional ribbon structure of protective antigen (PA). PA, an 83 kDa protein with 735 residues, is organized into antiparallel β-sheets comprising four major domains (Petosa *et al.*, 1997). Domain 1 contains the first 258 residues and the furin enzyme cleavage site between residues 164 and 167. Prior to translocation of soluble factors into the host cell, PA is cleaved by a cell-surface protease (furin enzyme) into PA20 (20 kDa portion containing residues 1–167) and PA63. PA20 is responsible for maintaining PA as a soluble monomer and preventing premature self-association (Collier and Young, 2003). Domain 1′ (residues 168–258) designates the N-terminal end of PA63. This domain contains two charged calcium (Ca^{2+}) atoms which function to maintain PA63 in a conformation capable of self-association into heptamers and binding to the host ligand anthrax toxin receptor (Gao-Sheridan *et al.*, 2003, Petosa *et al.*, 1997; Collier and Young, 2003. Domain 2, comprising residues 259–487, is illustrated with a β-barrel core structure and large flexible loops. This structure enables membrane insertion and pore formation (Petosa *et al.*, 1997; Benson *et al.*, 1998). Domain 3, comprising residues 488–595, is involved in self-association of PA63 into heptamers. Domain 4 contains the remaining C-terminal end of PA63 (residues 596–735) and binds to the anthrax toxin receptor on the host cell membrane (Singh *et al.*, 1991; Collier and Young, 2003). The various domains of PA63 enable the protein anchor to the plasma membrane of the host, form heptamers, bind EF or LF, and translocate these soluble factors into the cytosol through a pore. The structure of PA was provided free of copyright restrictions from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (Berman *et al.*, 2000; PDB ID: 1acc; Petosa *et al.*, 1997) and rendered using Accelrys DS Visualizer 2.0 software.

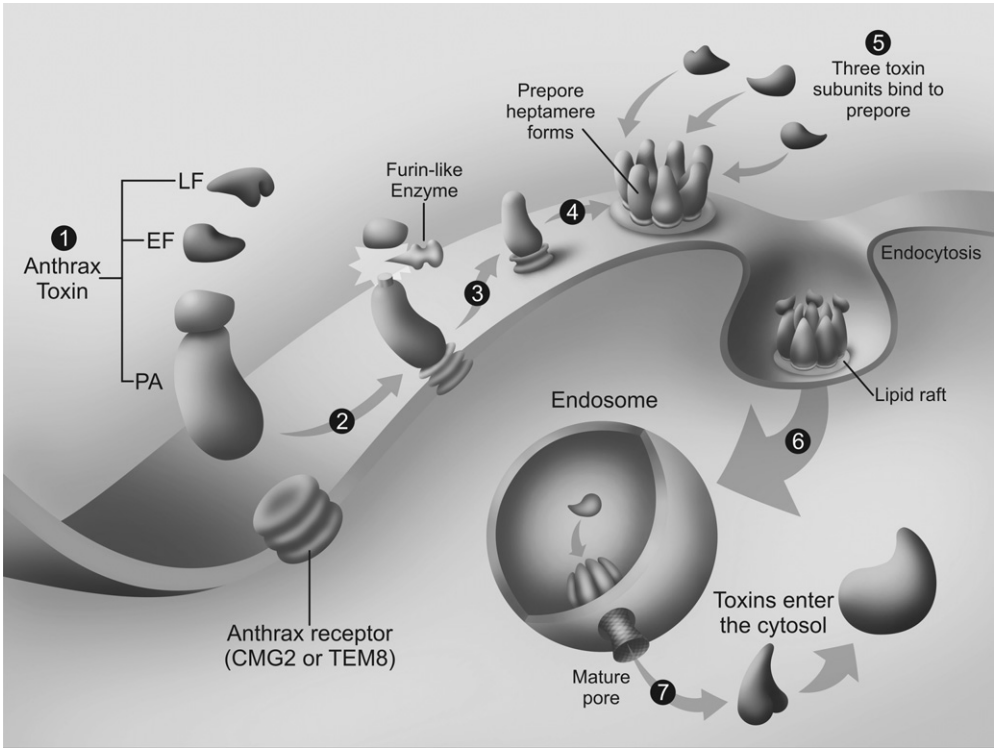


FIGURE 31.6. Anthrax toxin entry. Cellular entry of LF and EF into susceptible host cells involves an unusual mechanism for sharing PA. Binding of PA to the host cell, cleavage to form PA63, heptamerization of PA in the plasma membrane, organization into lipid rafts, binding of EF and LF to PA, internalization, PA pore formation, and translocation of anthrax soluble factors across the vesicle membrane are illustrated. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

1998; Mock and Fuet, 2001); however, evidence suggests that LF or EF binding sites on the PA_{63} heptamer span the interface between adjacent PA_{67} subunits. Therefore, it is believed that a single heptamer can interact with a maximum of three EF or LF molecules (Baldari *et al.*, 2006). The heptamer PA-LF and PA-EF complexes enter lipid rafts, illustrated as a shaded disc in Figure 31.6, in the membrane. Binding and oligomerization trigger a receptor-mediated endocytotic event followed by internalization of the hetero-oligomeric toxin complex in a membrane-bound vesicle (Beauregard *et al.*, 2000) via clathrin-dependent, receptor-mediated endocytosis. The internalized vesicle becomes an acidic endosome. The acidic pH of the endosome triggers a conformational change in the complex, leading to insertion of a flexible loop of each PA molecule into the lipid bilayer to form a pore. Formation of the pore allows for translocation of LF and EF out of the late endosome and into the cytoplasm of the host cell. Once inside the cytoplasm, LF and EF reach their respective targets.

B. Structure and Activity: Edema Factor

EF (89 kDa) is a calmodulin (CaM)-dependent adenylate cyclase, increasing intracellular cyclic adenosine monophosphate (cAMP) levels in the infected host cell cytosol. The first 261 N-terminal residues of EF are responsible for CaM binding (Duesbery and Vande Woude, 1999; Labruyere, 1990). The catalytic domain resides in amino acids 265–570 of the EF peptide sequence (Betsou *et al.*, 1995; Escuyer *et al.*, 1988). The ribbon structure of EF is illustrated in Figure 31.7. The N-terminal sequence of EF is highly conserved to a similar region in LF; this domain is essential for binding to PA. Fusion of this highly conserved N-terminal sequence to other toxins, such as Shiga and diphtheria, can cause toxic effects in mammalian cells (Arora and Leppla, 1994). Edema toxin (ET), the combination of PA and EF, causes edema when injected into the skin of experimental animals (Stanley and Smith, 1961; Beall *et al.*, 1962). Injection of EF alone, in contrast, has no toxic activity.

C. Structure and Activity: Lethal Factor

LF has a molecular weight of 90 kDA and is one of the key agents of anthrax disease. The structure of LF is illustrated in Figure 31.8. LF consists of several domains, including a PA-binding domain, a Zn^{2+}-binding domain, an imperfect repeat region, and a catalytic domain. LF functions as a highly specific Zn^{2+}-binding metalloprotease that can cleave MAPKKs near their amino (N)-termini. This cleavage reaction can potentially inactivate one or more host cellular signaling pathways (Pannifer *et al.*, 2001). Similar to EF, the LF N-terminal residues (1–254) serve as the binding domain for PA (Arora and Leppla, 1994). Adjacent to the PA-binding domain is a region of imperfect repeats containing 19 amino acids each. This region was demonstrated to be essential for toxic activity (Arora and Leppla, 1993). Deletion of the first of the four imperfect repeats of residues 308–383 eliminated LF toxicity. The LF catalytic domain resides in the C-terminus where a zinc-metalloprotease consensus sequence has been identified

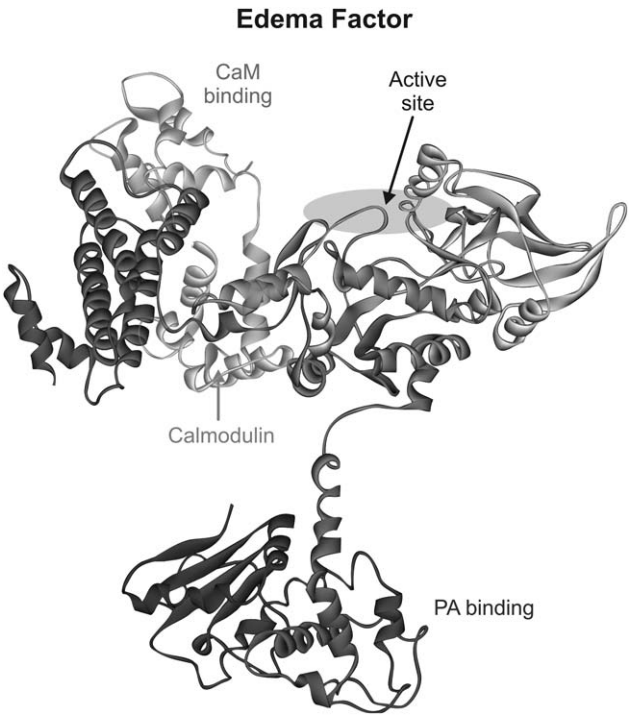


FIGURE 31.7. Three-dimensional ribbon structure of edema factor (EF). The purified and crystallized structure of EF has been previously reported (Shen *et al.*, 2005). EF is a calmodulin (CaM)-activated adenylyl cyclase and is another key factor in anthrax pathogenesis. EF contains a finger-like projection comprising a CaM-binding region, an active site to bind Mg^{2+} and 3′ deoxy-ATP, and a separate PA63 binding region. The structure of EF with bound CaM is illustrated. CaM binding causes a conformational change and activation of EF. The PA63 binding region of EF is separate from the remainder of the protein. It is shown in the same approximate position as the homologous PA-binding domain of LF in Figure 31.8 for comparison. The structure of EF was provided free of copyright restrictions from the RCSB PDB (Berman *et al.*, 2000; PDB ID: 1xfv; Shen *et al.*, 2005) and rendered using Accelrys DS Visualizer 2.0 software.

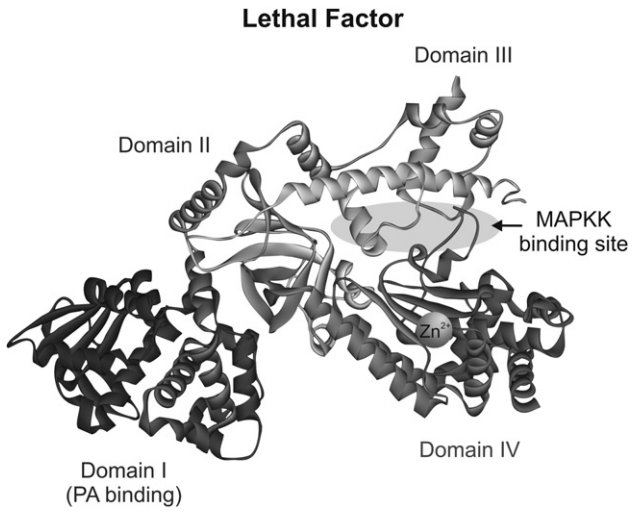


FIGURE 31.8. Three-dimensional ribbon structure of lethal factor (LF). The purified and crystallized LF has been reported (Bernardi *et al.*, 2000; Pannifer *et al.*, 2001). LF contains four recognized regions, termed domains I–IV. Residues 28–263 comprising domain I are illustrated as being entirely separate from the rest of the protein. Domain I binds the membrane-translocation component of PA63 (Lacy *et al.*, 2002). It is homologous to the same PA binding domain of edema factor (EF). Domains II–IV function together to create a long, deep pocket that holds the 16-residue N-terminal tail of MAPKK before the cleavage reaction takes place. Members of the MAPKK proteins are the only known cellular substrates of LF. The cleavage reaction removes the docking sequence for the downstream mitogen-activated protein kinase (MAPK) and therefore blocks cellular signaling via the MAPK pathway. Domain II has an ADP-ribosyltransferase active site. Domain III contains an α-helical bundle, and domain IV contains both a Zn^{2+}-binding motif and a catalytic center of the protease. The structure of LF was provided free of copyright restrictions from the RCSB PDB (Berman *et al.*, 2000; PDB ID: 1jky; Pannifer *et al.*, 2001) and rendered using Accelrys DS Visualizer 2.0 software.

within residues 686–692 (Klimpel *et al.*, 1994). The zinc metalloprotease activity is responsible for the cytotoxicity of LF. LF binds Zn^{2+} (Klimpel *et al.*, 1994; Kochi *et al.*, 1994), and mutations in LF which decrease zinc binding are poorly cytotoxic to cultured cells (Brossier *et al.*, 2000; Klimpel *et al.*, 1994).

LF interferes in the MAPK pathway which relays environmental signals to the machinery required for transcription in the nucleus and therefore modulates gene expression and protein synthesis. Specifically, LF inhibits MAPKKs; the identified substrates for LF enzymatic activity are MAPKK1, MAPKK2, MAPKK3 (Duesbery *et al.*, 1998; Pellizzari *et al.*, 1999; Vitale *et al.*, 1998), MAPKK4, MAPKK6, and MAPKK7 (Vitale *et al.*, 2000); MAPKK5 has never been demonstrated to be cleaved by LF. MAPKK cleavage occurs within the N-terminal proline-rich region preceding the kinase domain, subsequently inhibiting protein–protein interactions essential for assembly of host cell signaling complexes (Hammond and Hanna, 1998).

D. Mechanism of Toxicity: Edema Toxin

Edema toxin does not produce major tissue damage. In fact, its major role is to impair phagocyte function (Leppla, 2000). This is consistent with other toxins which function to elevate cAMP concentrations. Edema toxin inhibits phagocytosis of spores by human PMNs (O'Brien *et al.*, 1985) similar to LF; this is in contrast to spores which promote immune cell uptake (see Table 31.1). Increased intracellular cAMP induced by EF inhibits neutrophil chemotaxis, phagocytosis, superoxide production, and microbicidal activity (Crawford *et al.*, 2006; Turk, 2007; O'Brien *et al.*, 1985; Friedman *et al.*, 1987; O'Dowd *et al.*, 2004; Ahmed *et al.*, 1995). EF has been shown to inhibit TNFα and increase IL-6 production (Hoover *et al.*, 1994). Increased cAMP levels also block LPS-induced activation

of extracellular signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) MAPK pathways in monocytes through protein kinase A (PKA) activation. This might explain suppression of cytokine production by EF (Willis and Nisen, 1996; Dziarski *et al.*, 1996; Delgado and Ganea, 2000). EF also activates guanine nucleotide-exchange proteins directly activated by cAMP 1, known as Epac1, through elevated cAMP levels. Epac1 inhibits phagocytosis by both alveolar macrophages and monocyte-derived macrophages (Aronoff *et al.*, 2005; Bryn *et al.*, 2006). EF also inhibits T-cell proliferation and subsequent cytokine production through inhibition of ERK and JNK MAPK pathways (Comer *et al.*, 2005). Therefore, EF impairs the adaptive immune response as well. EF affects other cell types; it causes endothelial cell barrier dysregulation, coagulopathy, and RBC death (Banks *et al.*, 2006).

E. Mechanism of Toxicity: Lethal Toxin

The mechanism of death accredited to lethal toxin is unclear. A summary of the cellular targets and effects of lethal and edema toxins is provided in Table 31.2. LF has similar actions on phagocytes; it disables phagocytosis by macrophages and dendritic cells. Once internalized, LF cleaves the N-termini of MAPKKs, kinases for MAPKs and ERKs, and MEKs (MAPKs and ERKs) (Turk, 2007; Collier and Young, 2003). As a result, LF blocks three critical cell signaling pathways downstream of MAPKKs and MEKs. These include the ERK1/2, JNK, and p38 MAPK pathways. In addition, LF has additional targets within host cells.

There is evidence that LT suppresses pro-inflammatory cytokine production in macrophages (Erwin *et al.*, 2001) and decreases TNFα (Pellizzari *et al.*, 1999). These reports, which suggest impairment of innate immunity in the host cell, are in contrast to those which suggest LT functions to increase cytokines by macrophages (Hanna *et al.*, 1993). LT has also been shown to cause an increase in ion permeability and rapid depletion of ATP in J774 macrophage-like cells, leading to cell lysis by osmotic mechanisms (Hanna *et al.*, 1992). Inhibition of MAPKKs may block induction of NF-κB target genes, causing apoptosis of activated macrophages (Park *et al.*, 2002).

LF effects on monocytes and macrophages are multiple. LF seems to decrease innate immune responses by blocking maturation of monocytes, which differentiate into both macrophages and dendritic cells, and promote death of activated macrophages (Banks *et al.*, 2006). LF causes reductions in pro-inflammatory cytokines and inhibits the ability of dendritic cells to activate T cells *in vivo* (Agrawal *et al.*, 2003). This negates an important mechanism of inducing adaptive immunity. Alileche *et al.* (2005) demonstrated that LF can be cytotoxic to human and murine dendritic cells. Cytotoxicity was demonstrated to be through either a caspase-dependent apoptotic mechanism in the case of human dendritic cells or a necrotic pathway. LF alters PMNs, important mediators of the adaptive and innate immune response. LT also slows neutrophil mobility (During *et al.*, 2005).

LF promotes lysis of red blood cells (RBCs) when PMNs are present (Banks *et al.*, 2006). It is believed that LT induces these immune cells to release toxic factors that are hemolytic, causing RBC lysis (Wu *et al.*, 2003). Vascular damage is pathognomonic of anthrax disease. Signs of endothelial damage include hemorrhages and leaky blood vessels, leading to vascular collapse, shock, and death. LF appears to induce a caspase-dependent apoptotic pathway in endothelial cells derived from large vessels or so-called human umbilical vein endothelial cells; LF has been shown to cause similar apoptosis in small vessels, namely neonatal dermal vascular endothelial cells (Kirby, 2004).

F. Interactions Between Lethal and Edema Toxins

Since their expression is coordinately regulated, both anthrax toxins circulate together during infection (Turk, 2007). These toxins have distinct mechanisms of toxicity, yet they target similar cell types. Both LF and EF cause endothelial cell barrier dysregulation, coagulopathy, RBC death, inhibition of neutrophil mobility and phagocytosis, and alterations in cytokine modulation. Acting together, EF and LF inhibit superoxide production by neutrophils and cytokine production by dendritic cells (Crawford *et al.*, 2006; Tournier *et al.*, 2005). EF up-regulates PA receptors, TEM 8 and CMG 2, in macrophages and increases their sensitivity to LF cytotoxicity (Comer *et al.*, 2005; Maldonado-Arocho *et al.*, 2006). Finally, the appearance of black pigmentation characteristic of anthrax disease might be explained by the combined effects of LF and EF on melanocytes. LF has been shown to produce melanin in melanoma cell lines, an effect enhanced by the addition of EF (Koo *et al.*, 2002).

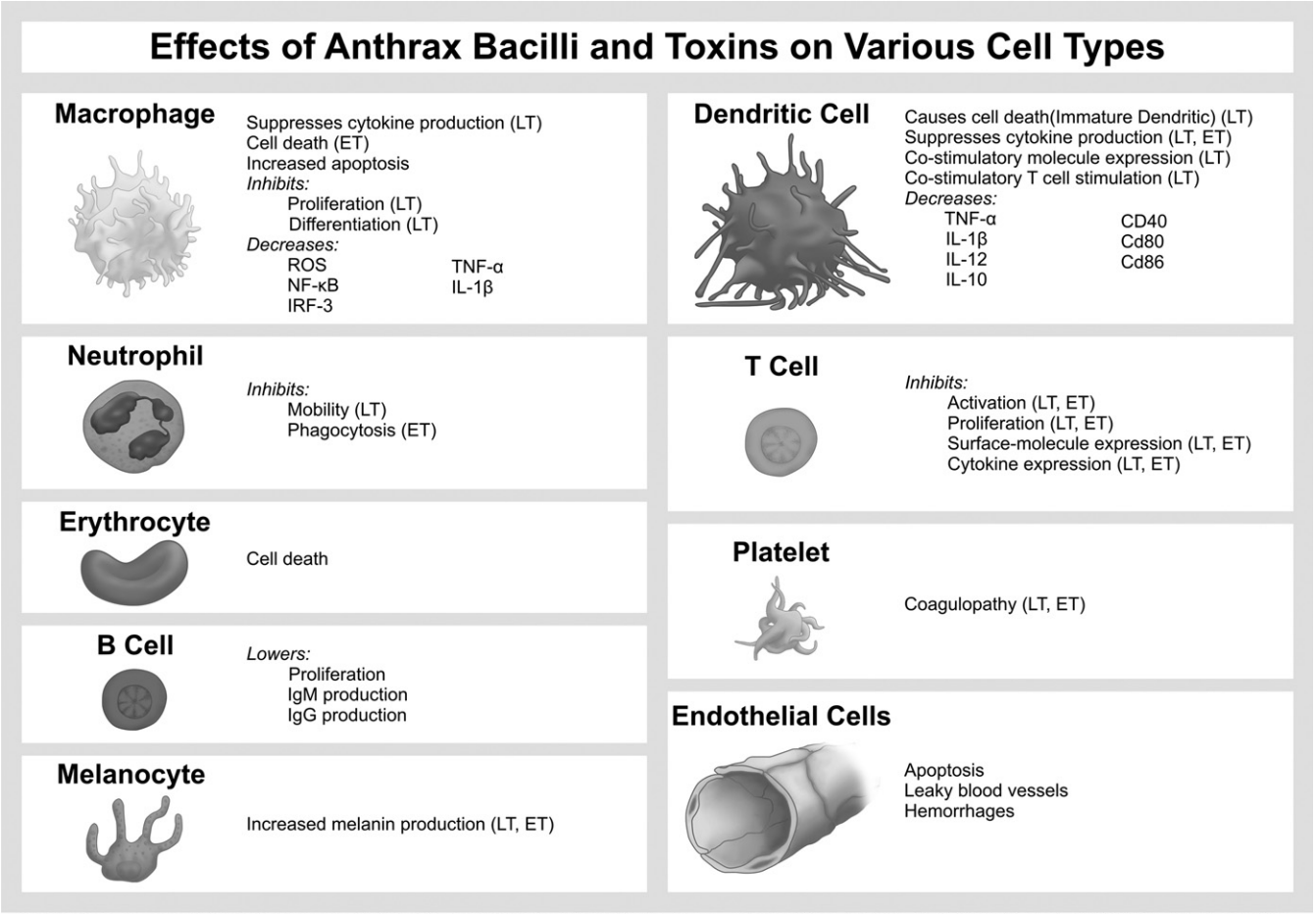
VII. TOXICITY

A. Cutaneous Anthrax

Cutaneous anthrax is the most common form of anthrax encountered worldwide, consisting of >95% of anthrax cases. The most obvious sign of bacterial uptake/absorption from the skin in human anthrax cases is the developing papule. This papule progresses to become vesicular in nature and then ulcerative. Finally, a black eschar forms, which is the hallmark of human cutaneous anthrax infection (Bell *et al.*, 2002). A more detailed look at the lesion discloses subepidermal edema, vessel thrombosis, tissue destruction, and hemorrhagic interstitium (Oncu *et al.*, 2003).

Symptoms of cutaneous anthrax infection begin with a painless papule at the site of infection 3–5 days after exposure. After 24–36 h, the papule progresses to a vesicle 1–2 cm in diameter. Once the lesion ruptures, it slowly

TABLE 31.2. Effects of anthrax bacilli and toxins on various cell types



Illustrations are copyright protected and printed with permission by Alexandre M. Katos. Adapted from (Baldari *et al.* 2006; Turk *et al.* 2007; Banks *et al.* 2006; Koo *et al.* 2002).

erodes, leaving a necrotic ulcer with a black central scab, termed an eschar. Eschar lesions typically form at exposed areas of the body (arms, hands, neck, and face). The formation of edema around the eschar lesion is also characteristic of cutaneous anthrax. Two to three weeks later, the eschar sloughs off the skin. Other signs and symptoms of cutaneous disease in humans include fever, headache, malaise, toxemia, and regional lymphadenopathy. Painful swelling of regional adrenal glands can occur.

B. Inhalational Anthrax

Signs and symptoms of inhalational anthrax follow a biphasic course. The initial phase is characterized by fever, malaise, and unproductive cough characteristic of an upper respiratory infection. Following this flu-like phase, the patient will typically recover after 2–4 days. The second phase proceeds rapidly with the following constellation of signs and symptoms: acute dyspnea, pleural effusion, fever, progressively worsening respiratory failure, cyanosis, circulatory collapse, shock, and death, if left untreated. Death occurs 24 h after the onset of this second phase secondary to toxemia and suffocation (Atlas, 2002; Bales, 2002; Dixon *et al.*, 1999; Friedlander, 1999). Although the lung is the primary site of infection here, inhalational anthrax is not considered a true pneumonia. In most, but not all cases, there is no infection in the lungs (Abramova *et al.*, 1993; Albrink, 1961). An infectious dose is estimated at 8,000 to 50,000 spores via aerosol (Franz *et al.*, 1997).

C. Gastrointestinal and Oropharyngeal Anthrax

Although extremely rare, gastrointestinal anthrax has a high estimated mortality rate (25–60%). Symptoms occur 1–7 days post-ingestion, presenting as either oropharyngeal or intestinal anthrax. Oropharyngeal anthrax presents as tongue lesions, lymphadenopathy, fever, and dysphagia. Intestinal anthrax presents 2–5 days after infection with abdominal pain, fever, nausea, emesis, and diarrhea. Abdominal pain and diarrhea are a direct result of ulceration, edema, and hemorrhaging of the gastrointestinal tract

due to edema and lethal factors (Friedlander, 1997). Therefore, diarrhea may be bloody with extensive fluid loss and circulatory collapse. If treatment is not started early, toxemia and shock may develop, resulting in death. Oropharyngeal anthrax produces a milder infection than the intestinal form and leads to a better outcome. Treatment for either form reduces symptoms and typically leads to full recovery 10–14 days after infection.

D. Meningitis

Meningitis is a complication of all anthrax infections and is associated with high mortality. Anthrax meningitis occurs through either a hematogenous or lymphatic spread from the infection site to the central nervous system (CNS) (Sejvar, 2005). High bacterial counts from anthrax bacteremia allow for *B. anthracis* to cross the blood–brain barrier into the CNS to infect the meninges and cerebrospinal fluid. Anthrax meningitis is most common with inhalational anthrax and seen in ~50% of those cases. Development of anthrax-associated meningitis is a very grave sign and invariably fatal. Even with antibiotic treatment, death occurs approximately 1–6 days after signs and symptoms of meningitis appear (Dixon *et al.*, 1999), and the mortality rate is estimated to be 94% (Sejvar, 2005).

Symptoms at the onset of anthrax meningitis include fever, headaches, nausea, vomiting, chills, malaise, agitation, and nuchal rigidity (Sejvar, 2005). Delirium, coma, refractory seizures, and neurological degeneration occur within 2–4 days (Kim *et al.*, 2001; Sejvar, 2005). Neurological degeneration can be seen, with signs that include cranial nerve palsies, myoclonus, fasciculations, decerebrate posturing, and papilloedema (Sejvar, 2005). A notable feature of anthrax meningitis infection includes subarachnoid and intraparenchymal hemorrhages. Hemorrhage can be observed in the cerebrospinal fluid, as well as polymorphonuclear pleocytosis, an increase in protein concentration, and a decrease in glucose concentration. *Bacillus anthracis* can be observed in the cerebrospinal fluid (CSF), meninges, subarachnoid space, and brain parenchyma.

VIII. DETECTION AND DIAGNOSIS

A. Detection

Detection and diagnosis of anthrax infection can be difficult. Diagnosis is made predominantly on a positive history of exposure to contaminated animal products and a physical exam of the presenting signs and symptoms. Tests to detect *B. anthracis* are typically never ordered unless there is evidence of exposure. The disease typically progresses to an advanced stage prior to initiating appropriate treatment. The formation of a black eschar with hyperemic and edematous borders is a hallmark of cutaneous anthrax infection. Unfortunately, the black eschar occurs in the later stages of infection. The appearance of painless, pruritic papules with edematous borders is suggestive of possible cutaneous anthrax infection. Further tests to identify *B. anthracis* as the infectious agent should be attempted but should not delay initiation of antibiotic treatment. Evidence of pulmonary involvement on chest x-ray can provide suspicion of inhalational anthrax. The common triad of findings is mediastinal widening, pulmonary infiltrates, and/or pleural effusions (Friedlander, 1997; Dixon *et al.*, 1999). Without a proper diagnosis, progression of some forms (i.e. inhalational anthrax) to the second stage of infection is nearly 100% fatal.

Depending on the form of disease suspected, certain specimens should be collected. If cutaneous anthrax is suspected, swabs of the lesion are warranted. A sterile, dry swab should be used to collect vesicular fluid if the lesion is in the vesicular stage. If the lesion is in the eschar stage, the edge of the eschar should be lifted and the swab should rotate underneath for 2 to 3 seconds. However, if the lesion is not in either the vesicular or eschar stage, the base of the ulcer can be swabbed with a sterile swab moistened with saline. Blood cultures should be collected regardless of the route of exposure or signs of disease (Beatty *et al.*, 2003). If gastrointestinal anthrax is suspected, ascitic fluid should be taken, and if oropharyngeal anthrax is suspected, swabs from oropharyngeal lesions should be taken (Beatty *et al.*, 2003). If pleural effusions are seen, pleural fluid should be collected, and if meningeal signs/symptoms are present, CSF should be collected.

B. Diagnostics

Laboratory tests for anthrax can include Gram staining, differential plating, γ-bacteriophage plaque assay, blood cultures, motility tests, enzyme-linked immunosorbent assays (ELISA), and fluorescent covalent microsphere immunoassay (FCMIA). Although microbial tests take 24 h to perform, often delaying the diagnosis, these tests may be necessary to confirm a diagnosis of anthrax. If there is a high index of suspicion for anthrax, initiation of therapy should not be delayed for results of these confirmatory tests.

1. Microbiological Tests

Simple tests to rule out anthrax infection are Gram staining of cultured bacteria, differential plating, and a γ-bacteriophage plaque assay. Gram staining is nearly always used in the identification of bacteria (CDC, ASM, APHL, 2002). *Bacillus anthracis* will appear as large, Gram-positive rods in short chains. The size of the bacterium is approximately 1–1.5 by 3–5 μm. *Bacillus anthracis* capsules will not take up India ink stain and will cause the bacilli to appear as clear zones on a black, stained background. *Bacillus anthracis* can be distinguished from other *Bacillus* species by culturing the organism on blood agar plates. It is the only *Bacillus* species that does not cause hemolysis. In addition,

B. anthracis cells are lysed by the bacterial virus γ-bacteriophage to form small plaques on nutrient agar plates.

Blood cultures should follow normal laboratory protocol. Specimens collected for cutaneous anthrax can be plated on 5% sheep blood agar (SBA), MacConkey agar (MAC), or any media normally used for surface wounds (CDC, ASM, APHL, 2002). Stool specimens from gastrointestinal anthrax cases should be plated on phenylethyl alcohol agar (PEA), MAC, and SBA. Sputum specimens from inhalational anthrax patients should be plated on chocolate agar, SBA, and MAC. Cultures will show isolated *B. anthracis* colonies 2–5 mm in diameter, flat or slightly curved, with a wavy border after 15–24 h. *Bacillus anthracis* shows growth on SBA and not on MAC. Colonies of *B. anthracis* plated on SBA will appear as a ''beaten egg white'' when teased with a loop (basic diagnostic testing). These blood cultures are useful, especially in the differential diagnosis of anthrax, and should be completed before antibiotic treatment is given.

A motility test is useful because *B. anthracis* is a nonmotile bacterium. Two motility tests available are the wet mount and motility medium variety. In a wet-mount preparation, organisms with Brownian movement or no movement will support the presence of *B. anthracis*. The presence of *B. anthracis* in a motility medium preparation would be a single line of growth along the original inoculum stab (CDC, ASM, APHL, 2002).

2. Molecular Tests

Acute and convalescent serum samples for serological ELISA testing should be collected for diagnosis. The CDC developed an ELISA for the detection of *B. anthracis* protective antigen (PA) using immunoglobulin G (IgG) antibodies in response to the bioterrorist anthrax plot in 2001. ELISA proved extremely useful in the detection of cutaneous and inhalational anthrax (Quinn *et al.*, 2002). The diagnostic sensitivity and diagnostic specificity for this test are 97.6% and 94.2%, respectively.

A more recent study reports another tool that detects anthrax better than ELISA detection of PA (Biagini *et al.*, 2004). The FCMIA can be multiplexed, meaning that numerous analytes can be measured simultaneously. In anthrax cases, anti-PA and anti-LF can be examined in serum samples at the same time. FCMIA is more sensitive, rapid, and reliable than ELISA.

3. Histopathology

Punch biopsies can confirm cutaneous anthrax if Gram stain and culture results are negative, due to antibiotic treatment (Godyn *et al.*, 2005), and a suspicion of cutaneous anthrax remains (Celia, 2002). The center of the eschar, the erythematous region, and the skin margin should all be included in the biopsy when an eschar is present (Godyn *et al.*, 2005).

4. Radiology

Radiological tests are available to narrow the differential diagnosis for anthrax. A chest radiograph will almost always exhibit a widened mediastinal contour that includes unilateral or bilateral hilar enlargement, as well as prominent periobronchovascular markings and pleural effusions (Frazier *et al.*, 2006). The size of the widened mediastinum contours and pleural effusion can increase rapidly over hours/days. However, findings from the radiograph might be subtle and even appear normal in the early stages of infection (Frazier *et al.*, 2006). Noncontrast CT will exhibit high-density mediastinal and hilar lymphadenopathy, which can rapidly increase in size over days (Friedlander, 1997; Frazier *et al.*, 2006). This indicates both hemorrhage and edema in the mediastinal lymph nodes. In addition, contrast-enhanced CT on mediastinal nodes can display rim enhancement and central hypodensity (Frazier *et al.*, 2006). The role of radiological tests has not been identified in the diagnosis of gastrointestinal/oropharyngeal anthrax (Beatty *et al.*, 2003). Radiographs of patients with gastrointestinal anthrax displayed findings indicative of obstruction; however, further studies need to be completed to determine if radiology can be used to help diagnose gastrointestinal anthrax (Beatty *et al.*, 2003). Patients with anthrax disease can also acquire complications of anthrax meningitis. CT or magnetic resonance imaging (MRI) shows hemorrhages in the deep gray matter, subarachnoid space, and ventricles (Sejvar *et al.*, 2005).

IX. RISK ASSESSMENT

The risks posed by an intentional outbreak of anthrax cannot be minimized, as the potential effects go beyond merely the medical. The primary costs of a biological terrorism-related anthrax event in terms of lives lost, medical resources required to treat affected individuals, and economic burden to decontaminate spores are significant. Certainly, the subsequent psychological and societal costs could be as high or higher, based on the US Federal government experience with the 2001 anthrax letters. ''On a collective level, a major epidemic of anthrax can destroy the social order'' (Guillemin, 1999).

The distribution of *B. anthracis* endospores in mailings through the US Postal Service in the fall of 2001 served to ignite public awareness concerning anthrax as a weapon of mass destruction. Deliberate contamination of the mail resulted in 22 cases of anthrax (11 inhalational and 11 cutaneous). These mailings led to five deaths among the inhalational anthrax cases and an enormous economic burden associated with decontamination. The media coverage and public fallout unveiled the deficiencies in our current risk assessment of anthrax.

The effects on public order, as well as economic and social effects, should be considered in an assessment of weaponized anthrax risk. Decontamination of the Hart

Senate Office Building in Washington, DC required 7 months at a cost of $23 million. Decontaminaton of the postal facilities that processed the ''anthrax letters'' in Brentwood, DC and Hamilton Township, NJ required more than a year at a cost in excess of $100 million (Fernandez, 2002). The psychological effects of an anthrax attack could exceed the medical or economic effects, especially in the short term. Panic caused by seemingly random outbreaks of symptoms in apparently unrelated persons or events ''could degenerate into panic, flight, communications breakdown, general societal dysfunction'' (Wein *et al.*, 2003).

A comprehensive risk assessment is dependent in part on determining the health risk posed to the individual by this biological agent. Animal models play a critical role by providing key sources of information for predicting consequences of human exposure since comparable naturally occurring human indices are rare. These animal models have been reviewed in previous sections throughout this chapter. Another method to assess risk is to use an anthrax stimulant. The Defense Research Establishment Suffield (DRES) in Canada undertook a series of experiments to assess the risk of envelopes filled with anthrax spores (Kournikakis *et al.*, 2001). In their experiments, envelopes containing spores of the nonpathogenic *Bacillus globigii* were opened in a mock mail room inside an aerosol test chamber to estimate aersol release from the envelopes. Investigators showed that dispersal by passive letter opening was far more effective than initially thought. A lethal dose could be inhaled within seconds of opening the content (Kournikakis *et al.*, 2001). Not only would the mail handler opening the envelope receive a lethal dose (LD) of between 500 and 3,000 LD_{50}s, but other workers in the room would inhale lethal numbers of spores.

In silico models of anthrax release can be used to bridge the gap to help predict consequences of human exposure in the event of an outbreak or biological attack scenario. Several modeling studies have been published (Wein *et al.*, 2003) in attempts to assess anthrax risk. The model of Wein *et al.* (2003) assumes a point-release of 1 kg of spores at a height of 100 m over a city of 10 million inhabitants. The effects on mortality of different antibiotic treatment strategies in an urbanized anthrax release are examined. This *in silico* biological weapon attack results in >100,000 deaths, given both symptomatic and asymptomatic persons receive antibiotics and the model incorporates availability and distribution parameters. In the base case, an approximation of current treatment and distribution strategies, deaths are estimated at >1% (Wein *et al.*, 2003). The model predicts significantly higher death rates (≥7 times) in cases where less aggressive distribution or administration is modeled (Webb, 2003).

One of the greatest fears of a scenario involving anthrax is the application of genetic engineering to enhance its pathogenicity. Several studies have demonstrated the ability to generate antibiotic-resistant anthrax (Pomerantsev *et al.*, 1992, 1993). In addition to developing multiple antibiotic-resistant strains of *B. anthracis*, biotechnology offers a highly likely scenario that the very nature of *B. anthracis* could be altered. It is possible that *B. anthracis* can be engineered to produce novel toxin activity (Mesnage *et al.*, 1999; Sirard *et al.*, 1997a, b). Other approaches may focus on the modification of PA such that it remains functional but immunologically distinct from the PA used in current vaccines.

X. TREATMENT

A. Overview

Treatment of any form of anthrax infection is generally the same: aggressive antibiotics and supportive care. Rapid definitive diagnosis of anthrax is critical for effective treatment, though in cases where anthrax is suspected prior to confirmation antibiotic therapy should not be withheld pending test results. Post-exposure treatment should be given in cases of putative exposure to anthrax spores to prevent systemic disease. Antibiotics and supportive management are the mainstays of treatment to prevent septic shock, fluid and electrolyte imbalance, and dyspnea associated with systemic anthrax disease. Initial administration of ciprofloxacin or doxycyclin is recommended (USFDA, 2001; CDC, 2001a, b, c). A duration of 60 days has been recommended for prophylaxis, though the most efficacious duration has not been determined (Brook, 2002).

Intravenous ciprofloxacin or doxycycline are recommended for treatment of anthrax, usually as part of a cocktail of antibiotics (CDC, 2001a, b). Multiple antibiotics are usually indicated in anthrax cases with signs of septicemia, extensive edema, or for cases with cutaneous lesions in the head and neck (Brook, 2002). Penicillin may be included in the antibiotic cocktail, but is not recommended as a standalone therapy due to *B. anthracis* β-lactamase production. *Bacillus anthracis* can express β-lactamase variants, penicillinases and cephalosporinases, which would undermine a lone-penicillin therapy (Lightfoot *et al.*, 1990). *Bacillus anthracis* has shown *in vitro* resistance to cephaloporins and trimethoprim-sulfamethoxazole (Inglesby *et al.*, 2002). Corticosteroid therapy may help treat edema from head and neck lesions or prevent airway obstruction. Table 31.3 contains therapeutic guidelines, based on CDC recommendations (CDC, 2001a, b, c), for pharmacologic management of *B. anthracis* infection.

B. Inhalational, Oral, and Gastrointestinal Anthrax

At the time of writing, the recommended initial therapy for inhalational, oral, and gastrointestinal anthrax in adults is 400 mg of ciprofloxacin every 12 h or 100 mg of doxycycline every 12 h, administered intravenously (Inglesby *et al.*, 2002). In addition to these treatments, one or two

TABLE 31.3. Anthrax therapeutic guidelines[a]

Therapy		Adults		Children		Pregnant women	
		Initial therapy[e] (intravenous dosing)	**Oral dosing**	**Initial therapy (intravenous)**	**Oral dosing**	**Initial therapy**	**Duration**
Recommended first line treatment – inhalational, GI, and oropharyngeal anthrax[b]	Ciprofloxacin (use in combination with one or two additional antimicrobials listed below)	IV treatment initially (400 mg q 12 h) in combination with one or two additional antimicrobials[f]	Switch to oral dosing when appropriate (500 mg PO BID)	IV treatment initially (10–15 mg/kg q 12 h)[h] in combination with one or two additional antimicrobials	Switch to oral when appropriate (500 mg PO BID)	Same for adults	
		Duration: Continue for 60 days total (IV and PO combined)[i]					
	Doxycycline[d] (use in combination with one or two additional antimicrobials listed below)	IV treatment initially (100 mg q 12 h) in combination with one or two additional antimicrobials[f]	Switch to oral when appropriate (100 mg PO BID)	IV treatment initially >8 yrs & >45 kg (100 mg q 12 h) >8 yrs & <45 kg (2.2 mg/kg q 12 h) <8 yrs (2.2 mg/kg q 12 h)	Switch to oral therapy when appropriate >8 yrs & >45 kg (100 mg PO BID) >8 yrs & <45 kg (2.2 mg/kg PO BID) <8 yrs (2.2 mg/kg PO BID)		
		Duration: Continue for 60 days total (IV and PO combined)[i]					

Therapy		Adults		Children		Pregnant women	
		Initial therapy (oral)[g]	**Duration**	**Initial therapy (oral)**	**Duration**	**Initial therapy**	**Duration**
Recommended first line treatment – cutaneous anthrax[b]	Ciprofloxacin	500 mg BID	60 days[i]	10–15 mg/kg q 12 h (not to exceed 1 g/day)	60 days[i]	500 mg BID	60 days[i]
	Doxycycline [d]	100 mg BID		>8 yrs & >45 kg (100 mg q 12 h) >8 yrs & <45 kg (2.2 mg/kg q 12 h) <8 yrs (2.2 mg/kg q 12 h)		100 mg BID	
Alternative option in cutaneous anthrax[c]	Amoxicillin		500 mg PO TID		80 mg/kg/day divided every 8 h		

[a]Therapeutic treatment protocol for immunocompromised persons is the same for immunocompromised adults and children
[b]Ciprofloxacin or doxycycline are first line therapeutics for all forms of anthrax
[c]Amoxicillin is an alternative option in adults and children for completion of therapy only after clinical improvement
[d]If meningitis is suspected, doxycycline may be less optimal due to poor central nervous system penetration
[e]Steroids may be considered in as adjunct therapy for patients with severe edema and for meningitis. [Dexamethosone: Adults (0.75–0.90 mg/kg/day orally, i.v., or i.m. in divided doses every 6 h); Children (0.25–0.50 mg/kg every 6 h)] [Prednisone: Adults (1–2 mg/kg or 5–60 mg orally/day); Children (0.5–2 mg/kg/day)]
[f]Other agents with *in vitro* activity include rifampin, vamcomycin, penicillin, ampicillin, chloramphenicol, imipenem, clindamycin, and clarithromycin. Because of concerns for beta-lactamases in *Bacillus anthracis*, penicillin and ampicillin should not be used alone. Consultation with an infectious disease specialist is advised
[g]Cutaneous anthrax with signs of systemic involvement, extensive edema, or lesions of the head or neck require intravenous therapy, and a multidrug therapeutic approach
[h]In children, ciprofloxacin dosage should not exceed 1 g/day
[i]Due to the potential for spores to persist after an aerosol exposure, antimicrobial therapy should be continued for 60 days

additional antimicrobials should be administered (list mentioned above) (Inglesby *et al.*, 2002). These guidelines should be followed until the condition of the patient improves. The treatment should then be switched to either 500 mg of ciprofloxacin twice a day or 100 mg of doxycycline twice a day (Inglesby *et al.*, 2002). This stage of treatment should begin intravenously and switch to oral dosing when appropriate (Inglesby *et al.*, 2002). Treatment should continue for 60 days (Inglesby *et al.*, 2002). Oral amoxicillin can be used as an alternative in adults and children for completion of therapy only after clinical improvement (Inglesby *et al.*, 2002; CDC, 2001a, b). Pediatric guidelines for medical management of anthrax are provided in Table 31.3, in addition to recommendations for pregnant women and immunocompromised patients.

C. Cutaneous Anthrax

The recommended initial therapy for adults with cutaneous anthrax is either 500 mg of ciprofloxacin orally administered twice a day or 100 mg of doxycycline orally administrated twice a day (Inglesby *et al.*, 2002). The duration of therapy is suggested to be 60 days (CDC, 2001a, b), although previous guidelines recommend 7 to 10 days (Inglesby *et al.*, 2002). Intravenous therapy and a multidrug regimen approach are recommended for patients with signs of extensive edema, or head/neck lesions (CDC, 2001c). The guidelines for pharmacologic management of cutaneous anthrax in pediatric patients and pregnant women are also provided (Table 31.3). With early treatment, systemic disease does not occur, and laboratory cultures will yield negative results for *B. anthracis*; however, treatment will not curtail formation of the black eschar and progression of the edematous skin ulcer (CDC, 2001c).

D. Bacteremia

Bacteremia often occurs with anthrax infection, especially with gastrointestinal/oropharyngeal and inhalational anthrax. A multidrug approach consisting of ciprofloxacin or doxycycline, along with one or two other antimicrobials, is suggested when bacteremia is suspected (CDC, 2001c). Other drugs that are recommended for use with ciprofloxacin or doxycycline include rifampin, vancomycin, imipenem, chloramphenicol, penicillin/ampicillin, clindamycin, and clarithromycin (CDC, 2001c).

E. Anthrax Meningitis

There is limited clinical experience for treating patients with anthrax meningitis (Sejvar *et al.*, 2005). However, the recommended therapy is also a multidrug treatment, including the use of a fluoroquinolone and two additional drugs with excellent CNS penetration. The fluoroquinolone that is recommended for use is ciprofloxacin. The ciprofloxacin level in CSF is 26–50% of serum levels. Other fluoroquinolones include levofloxacin, gatifloxacin, moxfloxacin, and ofloxacin. Doxycycline is not recommended as a first-line agent for anthrax meningitis. Although doxycycline has a low MIC (0.03 mg/l), it has low CNS penetration and a lower percentage in the CSF of serum levels than ciprofloxacin (10–26%). These drugs have been shown to work against *B. anthracis*; however, these drugs have not been observed in humans and have not been tested in animal models. Drugs with significant CNS penetration include ampicillin, meropenem, rifampicin, or vancomycin. Although penicillin is a beta-lactam, like ampicilin and meropenem, it is not suggested for use against anthrax meningitis for reasons stated above. Similar to the treatment suggestions above, the recommended duration of therapy is 60 days. Even with the effectiveness of antibiotic treatment, there is no guarantee that anthrax will be diagnosed in time to initiate therapy. Therefore, anthrax represents a significant risk to the public because it is lethal, potent, and induces public anxiety.

F. Vaccines

While vaccines are promising, further research needs to be conducted with the goal of updating vaccine technology. Conclusive demonstration of seroconversion against an anthrax infection was demonstrated in animals previously immunized with an active anti-PA vaccine and later given post-exposure antibiotic therapy (Friedlander *et al.*, 1993). Therefore, other therapies, such as injection of anthrax-specific monoclonal antibodies (Maynard *et al.*, 2002), may lead to a promising treatment following exposure to *B. anthracis*. Both of the current anthrax vaccines, *anthrax vaccine adsorbed* (AVA) and a newer one based on recombinant PA (rPA), offer long-lasting protection against an aerosol challenge of *B. anthracis* in rhesus macaques. Efficacy of both vaccines is due primarily to the PA. The AVA vaccine offers similar protection from challenge in rabbits; however, the duration of immunity is unknown (Phipps *et al.*, 2004). Various negative side effects have been reported after administration of the current anthrax vaccine to humans, and it would be beneficial to develop a vaccine that has fewer side effects before instituting a massive vaccination plan. Recent reports suggest that the addition of CpG oligonucleotides may offer an improved response. These CpG motifs interact with various receptors on B cells and dendritic cells, which improve antigen presentation and up-regulate pro-inflammatory cytokine expression. After co-administration of CpG oligonucleotides with the AVA vaccine, this combination triggered a fast and higher-titer immune response in comparison with the vaccine alone (Klinman *et al.*, 2004). These findings suggest a potential for using oligonucleotides as an adjuvant in a post-exposure vaccine.

Other studies have used an adenoviral delivery system to invoke a protective immune response in mice (Tan *et al.*,

2003). Immunization with this form of vaccine demonstrated a rapid anti-PA antibody response at a higher level than that of the current vaccine. Further, this method of delivery offered a longer protection time in comparison to that of the rPA vaccine. However, current public fears of using a virus as an immunization vector coupled with the lack of similar findings with other model systems make it imperative to further research this potential area of vaccine technology.

XI. CONCLUDING REMARKS AND FUTURE DIRECTION

The fear of *B. anthracis* has only been heightened in recent years as a result of the terrorist events in 2001 and the increase in acts of terrorism worldwide. There are few infectious agents that are more feared or notorious than *B. anthracis*, the causative agent of the zoonotic disease anthrax. *Bacillus anthracis* would be an ideal weapon for a terrorist attack not only because of the disease pathology but also because this organism can sustain desiccation and survive long term outside of its host as a dormant spore. Moreover, infection of humans by inhalation of spores has been reported. Thus, *B. anthracis* disseminated in an aerosol could be a feasible means by which to target and infect a large population.

The processes of *B. anthracis* infection and pathogenesis have been studied in various animal models. During the initial phases of infection, regardless of the route of infection (i.e. inhalational, dermal, or oral), the host animal may display general symptoms that are characteristic of numerous infections or disease states, making a positive diagnosis difficult. Pathogenesis involves initial uptake of dormant spores by phagocytes. Germination takes place soon afterwards either at the site of inoculation or later after transport to the lymph nodes. It is the vegetative cell that moves through the circulatory system and lymphatics to infect other target organs. Lungs and gastrointestinal tract are major targets for vegetative bacilli. Later the spleen, brain, liver, and almost every other organ in the body can be colonized. In systemic anthrax, there is overwhelming septicemia. While septicemia is important, lethality from *B. anthracis* is believed to be mediated by the actions of anthrax toxins (LT and ET). The toxins are thought to be primarily responsible for causing immune system suppression, necrosis of critical cells, vascular leakage, hemorrhage, shock, and death.

There are numerous gaps in our understanding of anthrax. While the general pathogenesis of *B. anthracis* has been studied in a variety of animals, a number of important questions still remain unanswered. Although spore uptake has been demonstrated in macrophages and dendritic cells, the molecular interactions involving uptake, spore germination, and escape require additional research. Further *in vitro* and *in vivo* studies will help to elucidate these mechanisms. Understanding of the molecular mechanisms of TLR signaling and its relationship to LT-mediated apoptosis may lead to new therapies. The inhibition of TLR-mediated signaling of apoptosis may inhibit macrophage cell death and assist in controlling the pathogen.

Several studies have shown bacilli in many organs of the body following various routes of exposure. However, the time course of transit to target organs and the targeting order of these organs may be helpful in further defining therapeutic windows of opportunity. Lastly, the molecular mechanisms of toxicity and death caused by LT and ET remain unclear. There is no direct evidence that proves the importance of macrophage apoptosis in the course of disease progression. Recent reports suggest that LT may induce apoptosis of endothelial cells, which suggest that macrophages are not the sole cell type affected by LT (Kirby, 2004). In addition, reports which demonstrate LT reduces cytokine expression (Erwin *et al.*, 2001; Moayeri and Leppla, 2004; Pellizzari *et al.*, 1999) suggest that death may not be due to an inflammatory reaction.

Several animal model studies have focused on the immune response to infection and also to vaccination. Most studies identify antibody titer, but some also show cytokine production or possible mechanisms of bacterial evasion. A focus on immune response studies will help to further define *B. anthracis* pathogenesis and provide insight into the design of future vaccines and therapeutics. Most countermeasure studies have been conducted on vaccines, as antibiotic studies have typically been performed *in vitro*. Primary concerns are the length of required treatment, efficient prophylaxis methods, and less complicated dosing regimens for vaccines. In a theoretical bioterrorist attack scenario, it will be essential to distribute effective treatment and prophylaxis to infected or potentially exposed persons in an expedient manner.

The choice of an appropriate animal model system that most closely mimics the human response to *B. anthracis* infection is crucial to the development of improved treatments and more effective vaccines. Such development relies on testing in appropriate animal models in which disease, infection, and progression mimic that seen in humans. The importance of selecting an appropriate animal model for human anthrax is magnified by the potential of a genetically engineered *B. anthracis* bacterium made more virulent than its predecessor. The addition of antibiotic resistance genes would render prophylactic treatment against infection by modified organisms difficult, if not futile. Further, the inclusion of genes that encode toxins not naturally found in *B. anthracis* may assist the bacterium in escaping host immunosurveillance. Therefore, defining the pathogenesis of *B. anthracis* and designing appropriate countermeasures is a critical necessity for treatment of those exposed in occupational settings, national security, and the global community. Whether medical systems would be able to provide treatment early enough to prevent widespread disease in the event of a bioterrorist attack involving anthrax remains questionable.

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CHAPTER 32

The Nervous System as a Target for Chemical Warfare Agents

LINNZI WRIGHT, CAREY POPE, AND JING LIU

I. INTRODUCTION

Chemical warfare agents (CWAs) comprise a widely diverse group of chemicals. As outlined in this and the following chapters, multiple organ systems can be targeted by CWAs, and some types of CWAs can affect one or more of the major organ systems. One of the earliest forms of CWA was possibly natural toxins placed on the tips of arrows. Indeed, the importance of arrow poisons (the Greek word *toxikos* roughly means ''pertaining to the bow'') had a prominent influence on the early development of the field of toxicology. Evidence suggests that some types of CWAs have been used for centuries. Their use in ''modern'' warfare, however, dates back only to World War I (WWI, 1914–1919). The prominent CWAs used during WWI (i.e. chlorine gas, mustard gas, and phosgene) were primarily dermal and/or pulmonary toxicants. Mustard gas was again used during WWII. During the 1930s and 1940s, a number of organophosphorus (OP) chemicals, which primarily act on the nervous system, were synthesized as potential CWAs. There remains widespread public recognition of the neurotoxic potential of the OP ''nerve agents'', reinforced by their military use in the Iran–Iraq War of 1980–1988 and their use by terrorists in Japan in 1994 and 1995. Other CWAs also have prominent effects on nervous system function. This chapter will provide a brief overview of the nervous system, highlighting some of the factors contributing to its often unique sensitivity to CWAs. This sensitivity can be due to both direct interactions with elements of the nervous system and indirect actions on other organ systems. Specific information on the effects of selected CWAs on the nervous system is provided for illustration.

II. OVERVIEW OF THE NERVOUS SYSTEM

The nervous system can be divided into two major structural divisions: the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS consists of the brain and spinal cord, whereas the PNS consists of the autonomic and somatic systems. Communication within and among these various systems is absolutely essential for physiological coordination.

There are two basic types of cells in the nervous system: neurons and glia (Vander *et al.*, 2001). Neurons are highly specialized cells designed for the conduction of electrical impulses to other nerve cells and to innervated tissues such as glands and muscles. There are several types of neurons in the nervous system, but in all types there exists a cell body (soma), smaller branching structures (dendrites), and an electricity-conducting fiber (axon), which gives rise to specialized areas of intracellular communication (synapses). Afferent neurons conduct electrical impulses from innervated tissues into the CNS; efferent neurons conduct electrical impulses from the CNS to innervated tissues while interneurons transmit information between neurons. Despite outnumbering neurons in the nervous system (Jehee and Murre, 2008), the primary functions of glia (i.e. astrocytes, microglia, and oligodendrocytes) have historically been considered to be the metabolic and physical support of neurons. Astrocytes are thought to aid in the maintenance of homeostatic conditions in the extracellular fluid surrounding neurons by removing excess potassium ions (K^+) from the synapse (Leis *et al.*, 2005). Microglia participate in the phagocytosis of foreign materials following neuronal damage/degeneration (Jordan and Thomas, 1988). Oligodendrocytes (CNS) and Schwann cells (PNS) sheath axons with a lipid-rich material (myelin) (Bunge *et al.*, 1978), which increases the speed of electrical signals down the axon and impairs the spread of electrical impulses between juxtaposed axons. While the covering of neurons with myelin increases conduction velocity, the rate of conduction also depends on the diameter of the axon, i.e. large axons conduct impulses faster than smaller axons (Govind and Lang, 1976).

Neurons (as all cells) maintain a resting potential across their plasma membranes, typically −60 to −75 mV relative to the extracellular fluid. This resting potential is generated by the active and passive diffusion of ions across the plasma membrane. In the resting state, the sodium ion (Na^+) concentration is higher in the extracellular fluid than in the intracellular fluid. In contrast, the K^+ concentration is higher

on the inside of the cell. If the membrane potential of a neuron exceeds a certain voltage (the threshold potential), a series of events are triggered that result in the transmission of an impulse (the action potential) along the length of an axon. Each action potential begins with a rapid reversal of voltage from the negative resting potential to a positive potential (depolarization phase) and then ends with an almost equally rapid change to a negative potential (repolarization phase). The depolarization phase of an action potential is due to the opening of voltage-gated Na^+ channels, which dramatically increases the membrane permeability to Na^+. Na^+ then passively moves into the neuron down the steep concentration gradient. The repolarization phase of an action potential is due to the inactivation (closure) of voltage-gated Na^+ channels and the subsequent opening of voltage-gated K^+ channels. K^+ moves out of the neuron, also down the concentration gradient, and contributes to the restoration of resting voltage conditions.

Conduction is the movement of an impulse down an axon within a single neuron. Once initiated, the action potential is self-propagated to the end of the neuron in an ''all-or-none'' fashion, i.e. either there is an action potential generated or there is not, and one action potential has the same characteristic as another action potential within that particular neuron. When an action potential reaches the distal terminal region of the axon, voltage-gated Na^+ channels become less involved while voltage-gated calcium ion (Ca^{2+}) channels assume prominent importance. Impulses are conducted from one neuron (presynaptic neuron) to another (postsynaptic neuron) by the release of a neurotransmitter into the synaptic cleft. When an action potential in the presynaptic neuron reaches the synapse, Ca^{2+} channels open. Due to the extensive concentration difference within and outside the terminal, Ca^{2+} then moves into the neuron and triggers a cascade of biochemical reactions (exocytosis), in which vesicles containing neurotransmitter molecules fuse with the plasma membrane and release their contents into the synaptic cleft. The neurotransmitter molecules diffuse within the synaptic cleft and interact with specific membrane receptors on the postsynaptic neuron. This neurotransmitter–receptor interaction typically either alters ion conductance in the plasma membrane influencing the postsynaptic cell's membrane polarity (either depolarizing or hyperpolarizing) or affects the synthesis of intracellular signaling molecules (second messengers), which can alter a host of intracellular signaling pathways and functions.

III. SPECIAL FEATURES OF THE NERVOUS SYSTEM

Table 32.1 lists special features of the nervous system that may contribute to its sensitivity to CWAs. For example, the nervous system is comprised of different cell types interconnected by a complex communication mechanism dependent upon chemical transmission. Each of these special features often carries with it metabolic requirements and unique vulnerabilities to toxic agents. Given that the nervous system has only a modest capacity for repair/regeneration (Freed *et al.*, 1985), subtle damage may have long-lasting effects.

A. Architecture

The cell body (soma) of neurons is responsible for supplying the metabolic needs of the elongated processes (axons and dendrites). Two interrelated demands placed on the neuron are the maintenance of a much larger cellular volume than most cells and the transport of intracellular materials over great distances (Cavanagh, 1984). Nissl substance, found exclusively in cell body of neurons (Liu and Bahu, 1975), is formed by clusters of ribosomal complexes and is involved in the synthesis of large amounts of protein necessary to maintain the neuron. The process of transporting materials from the soma to the distal aspect of the axon (anterograde axonal transport) can be an important site of toxic action.

B. Blood–Brain Barrier

The mature brain is functionally separated from the circulation by a continuous lining of endothelial cells whose apposed surfaces contain specialized tight junctions (Reese and Karnovsky, 1967) that, along with associated glial cells (Tao-Cheng and Brightman, 1988), constitute a barrier to

TABLE 32.1. Special features of the nervous system that contribute to sensitivity to CWAs

Feature	Description
Architecture	The soma of a neuron is responsible for supplying the metabolic needs of axons and dendrites, which can extend great distances
Blood–brain barrier	The brain is separated from the circulation by a lining of endothelial cells whose apposed surfaces contain specialized tight junctions
Electrochemical communication	The arrival of an action potential at the synapse activates a cascade of events that culminates in the release of neurotransmitters into the synaptic cleft
Energy requirement	Neurons require the continuous supply of glucose and oxygen in order to generate the large amount of ATP needed to support various cellular processes

the free passage of molecules. In humans, this blood–brain barrier is incompletely developed at birth (Saunders *et al.*, 1999) and even less so in premature infants (Graziani *et al.*, 1992). It also does not cover the choroid plexus and certain regions of the brain that typically have neurosecretory functions, i.e. the area postrema, hypophysis, hypothalamic regions, pineal body, and the supraoptic crest (Duvernoy and Risold, 2007). Because of the presence of these endothelial cell tight junctions, the entry of most exogenous molecules into the brain is largely limited by their lipid solubility and thus their ability to diffuse through the plasma membrane (Oldendorf, 1974). Therefore, the blood–brain barrier plays a major role in maintaining the microenvironment of the brain both by excluding hydrophilic molecules (e.g. dopamine) and by participating in the specific transport of required molecules (e.g. glucose). Certain abnormal states, whether induced (e.g. anesthesia), metabolic (e.g. adrenocorticoid hypertension), pathological (e.g. meningitis) or stress related, lead to loss of blood–brain barrier integrity and can thereby promote the leakage of molecules into the brain (Pollay and Roberts, 1980).

C. Electrochemical Communication

As noted above, the arrival of an impulse at the synapse activates a cascade of events that culminates in the release of neurotransmitters into the synaptic cleft, the diffusion of neurotransmitters to membrane-bound receptors on a neighboring neuron or cell, and the removal of neurotransmitter molecules from the synaptic cleft. These events are readily disrupted by a number of toxicants and can lead to both subtle and severe functional deficits.

D. Energy Requirements

Neurons have an absolute requirement for the continuous supply of glucose and oxygen in order to generate aerobically the large amount of ATP needed to support ionic membrane pumps, intracellular transport mechanisms, and energy-requiring signaling processes (Magistretti and Pellerin, 1999). The high oxygen requirement of the human brain takes about 20% of the total cardiac output (Hoyer, 1982). Toxic agents that interrupt the supply of oxygen [e.g. carbon monoxide (Penney, 1990)] or the utilization of oxygen by neurons [e.g. cyanide (Brierley *et al.*, 1976)] can produce catastrophic cellular damage in susceptible brain regions. Neuronal damage under these conditions can thus be a combination of direct effects on the nervous tissue and secondary damage from hypoxia/ischemia (Kristian, 2004).

IV. TYPES OF NEUROTOXICANTS

Neurotoxicants are any chemical agents that disrupt the normal function of the nervous system, in the presence or absence of visible structural damage. Hundreds of agents are recognized as having neurotoxic potential including animal venoms [e.g. green mamba snake venom (Dajas *et al.*, 1987)], drugs of abuse [e.g. cocaine (Rowbotham and Lowenstein, 1990)], industrial solvents [e.g. toluene (Filley *et al.*, 2004)], gases [e.g. carbon monoxide (Penney, 1990)], metals [e.g. arsenic (Vahidnia *et al.*, 2007)], nerve agents [e.g. soman, sarin, tabun, VX (Fernando *et al.*, 1985; Gupta *et al.*, 1987, 1991)], pesticides [e.g. parathion (Liu *et al.*, 2005)], and pharmaceutical drugs [e.g. doxorubicin (Lopes *et al.*, 2008)]. Some of these agents act directly on the nervous system, whereas others interfere with metabolic processes on which the nervous system is dependent. Table 32.2 lists four cellular mechanisms of neurotoxicity for direct acting toxicants, i.e. chemicals that cause neuronopathies, axonopathies, or myelinopathies, or that affect synaptic neurotransmission (Anthony *et al.*, 2001).

The first category of neurotoxicants [e.g. doxorubicin (England *et al.*, 1988), MPTP (Langston and Irwin, 1986), and trimethyltin (Bouldin *et al.*, 1981)] results in a neuronopathy or death of a neuron, with subsequent degeneration of its associated axon, dendrites, and myelin sheath when present.

TABLE 32.2. Cellular mechanisms of neurotoxicity

Mechanism	Description	Example
Neuronopathy	Loss of neurons with subsequent degeneration of axons, dendrites, and myelin sheath	Doxorubicin MPTP Trimethyltin
Axonopathy	Loss of axons with subsequent degeneration of myelin sheath	Acrylamide Some organophosphates Pyridinethione
Myelinopathy	Loss of myelin sheath or separation of myelin lamellae	Hexachlorophene Lead Tellurium
Disruption of neurotransmission	Alteration of synthesis, transport, storage, release, metabolism, reuptake, signaling of neurotransmitters	Cocaine Nicotine Cholinesterase inhibitors

Such neuronal degeneration is irreversible and can result in an encephalopathy with global dysfunction or, if only certain neuronal populations are affected, the interruption of particular functionalities. The second category of neurotoxicants [e.g. acrylamide (LoPachin *et al.*, 2003), organophosphorus esters (Lotti and Moretto, 2005) and pyridinethione (Ross and Lawhorn, 1990)] results in the loss of axons that later can include the degeneration of all associated myelin sheaths. Cell bodies, however, remain intact. Such axonal degeneration is reversible in the PNS and results in a clinical condition known as peripheral neuropathy in which sensations and motor strength are first impaired in the hands and feet, later progressing to other areas of the body. However, axonal degeneration is irreversible in the CNS (Richardson *et al.*, 1980). The third category of neurotoxicants [e.g. hexachlorophene (Powell *et al.*, 1973), lead (Dyck *et al.*, 1977), and tellurium (Said and Duckett, 1981)] results in the loss of myelin (i.e. demyelination) or the separation of the myelin lamellae (i.e. intramyelinic edema). While neurons are structurally unaffected by such myelin degeneration, their functions may be altered. Remyelination of axons occurs in the PNS, but only to a limited extent in the CNS (Ludwin, 1988). The fourth category of neurotoxicants [e.g. cocaine (Rowbotham and Lowenstein, 1990) and nicotine (Benowitz, 1986)] interferes with neurotransmission. These neurotoxicants can inhibit or increase the release of neurotransmitters, alter the removal of neurotransmitters from the synaptic cleft, or act as agonists or antagonists at specific receptors. Their effects are usually reversible, but nevertheless of toxicological relevance as they may result in severe acute toxicity or even death.

V. CELLULAR MAINTENANCE

All cells must assemble macromolecular complexes, maintain the intracellular environment, produce energy, and synthesize endogenous molecules in order to survive. Xenobiotics with neurotoxic potential can interfere with key aspects of cellular maintenance including adenosine triphosphate (ATP) synthesis and Ca^{2+} homeostasis.

A. ATP Synthesis

ATP plays a central role in cellular maintenance both as a chemical for biosynthesis of macromolecules and as the major source of energy for all cellular metabolism. ATP is utilized in numerous biochemical reactions including the citric acid cycle, fatty acid oxidation, gluconeogenesis, glycolysis, and pyruvate dehydrogenase. ATP also drives ion transporters such as Ca^{2+}-ATPase in the endoplasmic reticulum and plasma membranes, H^{+}-ATPase in the lysosomal membrane, and Na^{+}/K^{+}-ATPase in the plasma membrane. Chemical energy (30.5 kJ/mol) is released by the hydrolysis of ATP to adenosine diphosphate (ADP).

Oxidative phosphorylation is the process by which ATP is formed as electrons are transferred from the reduced forms of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) to molecular oxygen (O_2) by a series of electron transporters (i.e. the electron transport chain). ATP is required for cell division, fueling cellular motility, the maintenance of cell morphology, muscle contraction, the polymerization of the cytoskeleton and vesicular transport, and other vital functions. Impairment of oxidative phosphorylation results in the depletion of ATP and compromises these cellular processes. ATP depletion decreases neuronal membrane potential and thereby enhances the activation of glutamate receptors (Wang *et al.*, 1994). Oxidative phosphorylation can be affected by various neurotoxic agents, which are divided into four classifications (Gregus and Klaassen, 2001). Class I agents [e.g. ethanol (Li *et al.*, 1995) and fluoroacetate (Saito, 1990)] interfere with the delivery of hydrogen to the electron transport chain. Class II agents [e.g. cyanide (Piantadosi *et al.*, 1983)] inhibit the transfer of electrons along the electron transport chain to O_2. Class III agents [e.g. carbon monoxide (Piantadosi *et al.*, 1988) and cocaine (Yuan and Acosta, 2000)] interfere with the delivery of O_2 to the electron transport chain. Class IV agents [e.g. chlordecone (Jinna *et al.*, 1989)] inhibit ADP phosphorylation. These agents have a propensity to induce striatal damage, which results in large part from the supraphysiological excitation of neurons equipped with glutamate receptors (i.e. excitotoxicity) and the failure of energy-dependent homeostatic mechanisms (Del Rio *et al.*, 2007).

B. Calcium Homeostasis

Intracellular Ca^{2+} levels are highly regulated by the impermeability of the plasma membrane to Ca^{2+} and by efflux pumps that transport Ca^{2+} from the cytoplasm. Ca^{2+} is actively pumped from the cytosol both to the extracellular fluid and into storage vesicles in the endoplasmic reticulum and mitochondria. Ca^{2+} homeostasis can be affected by various neurotoxic agents, which are divided into four classifications (Gregus and Klaassen, 2001). Class I agents [e.g. glutamate (Choi, 1987)] induce Ca^{2+} influx into the cytoplasm. Class II agents [e.g. chloroform (Levitt, 1975)] inhibit Ca^{2+} export from the cytoplasm. Class III agents [e.g. carbon monoxide (Piantadosi *et al.*, 1988) and cyanide (Piantadosi *et al.*, 1983)] impair ATP synthesis. Class IV agents [e.g. MPTP (Singer *et al.*, 1988)] induce the hydrolysis of $NADP^{+}$. Sustained elevations in intracellular Ca^{2+} levels trigger a series of potentially neurotoxic events including mitochondrial dysfunction, oxidative stress and mitogen-activated protein kinase (MAPK) pathway activation.

There are three types of MAPKs: extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. Sustained elevations in intracellular Ca^{2+} levels stimulate p38 activation, which inactivates B-cell leukemia 2 (BCL-2) and decreases its antiapoptotic potential

(De Chiara *et al.*, 2006). The inactivation of BCL-2 triggers cytochrome c release and activation of the caspase cascade. Caspase-3 also cleaves transcription factors associated with myocyte enhancer factor-2 (MEF2) contributing to neuronal injury (Okamoto *et al.*, 2002).

Sustained elevations in intracellular Ca^{2+} levels promote mitochondrial Ca^{2+} uptake, which decreases the mitochondrial membrane potential and blocks the electron transport chain leading to ATP depletion. Large increases in mitochondrial Ca^{2+} uptake also increase mitochondrial membrane permeability (Dubinsky and Levi, 1998) resulting in the release of proapoptotic factors such as cytochrome c and apoptosis inducing factor (AIF) (Luetjens *et al.*, 2000). Cytochrome c binds to apoptotic protease-activation factor 1 (APAF1) and procaspase-9, forming a multiprotein complex known as the apoptosome that activates the caspase cascade. The apoptosome activates caspase-9, which in turn activates caspase-3. Caspase-3 activates caspase-activated DNase (CAD), resulting in DNA fragmentation, characteristic of apoptosis.

Prolonged elevation of intracellular Ca^{2+} levels also interrupts the electron transport chain, leading to accumulation of hydrogen peroxide and superoxide, which further react to form hydroxyl radicals. Superoxide also reacts with nitric oxide to form peroxynitrite, contributing to excitotoxicity associated with ischemia (Dawson and Dawson, 1998) and leading to lipid peroxidation and protein nitration.

VI. VOLTAGE-GATED ION CHANNELS

Voltage-gated ion channels are formed by integral membrane proteins that belong to a superfamily of proteins necessary for the formation of channels that are ''gated'' (i.e. opened or closed) by changes in membrane potential (Conley and Brammar, 1999). Many xenobiotics with neurotoxic potential interfere with ion channel functions, and they tend to have rapid and sometimes dramatic effects on sensory and neuromuscular functions.

A. Calcium Ion (Ca^{2+}) Channels

Ca^{2+} channels play a critical role in the release of neurotransmitters from the presynaptic neuron into the synaptic cleft. Four types of Ca^{2+} channels (L, N, P, and T) have been identified based on biophysical and pharmacological criteria. L-type Ca^{2+} channels function in the excitation–secretion coupling of endocrine cells and some neurons. N-type Ca^{2+} channels are restricted to neurons where they function in neurotransmitter release. P-type Ca^{2+} channels are restricted to Purkinje cells where they mediate depolarization-induced repetitive spikes. T-type Ca^{2+} channels, which deactivate more slowly than any other Ca^{2+} channel, mediate depolarization-induced repetitive spikes in endocrine cells and neurons. L-type Ca^{2+} channels are pharmacologically blocked by diltiazem, nifedipine, and verapamil (Triggle, 2006), N-type Ca^{2+} channels are blocked by ω-conotoxin (Nielsen *et al.*, 2000), and P-type Ca^{2+} channels are blocked by ω-agatoxin (Adams *et al.*, 1993).

B. Chloride Ion (Cl^-) Channels

Cl^- channels underlie a wide range of physiological processes including cell volume regulation, intravesicular acidification and the maintenance of electrical excitability in muscle and nerve membranes. Nine types of Cl^- channels have been identified, and common pharmacological blockers include 4,4′-diisothiocyano-2,2′-stilbenedisulfonate (DIDS), 9-anthracene-carboxylic acid (9-AC) and p-chlorophenoxy-propionic acid (CPP) (Pusch *et al.*, 2006).

C. Potassium Ion (K^+) Channels

K^+ channels are responsible for maintaining the resting potential of a neuron as well as its repolarization following an action potential. Six types of K^+ channels have been identified including delayed rectifier (K_V) channels, which are activated with some delay following membrane depolarization; rapid-delayed rectifier (K_{VR}) channels, which are activated rapidly following membrane depolarization; slow-delayed rectifier (K_{VS}) channels, which are activated after a long delay following membrane depolarization; A (K_A) channels, which are activated by membrane depolarization after a period of hyperpolarization; inward rectifier (K_{IR}) channels, which are activated at resting potential; and sarcoplasmic reticulum (K_{SR}) channels, which are located in the sarcoplasmic reticulum and have a low selectivity for K^+. Dendrotoxin, a toxin found in the venom of green mamba snakes, blocks K^+ channels (Harvey and Anderson, 1985). Other classical K^+ channel blockers include the bee toxin apamin and 4-aminopyridine.

D. Sodium Ion (Na^+) Channels

Na^+ channels are responsible for the generation of the rising phase of an action potential, which is the result of a rapid diffusion of Na^+ across the axonal membrane. Na^+ channels also influence the threshold for action potential generation and the frequency of neuronal firing. Two types of Na^+ channels have been identified based on their sensitivity to tetrodotoxin (TTX). TTX-resistant Na^+ channels are predominantly expressed in the neonatal brain, whereas TTX-sensitive Na^+ channels are predominantly expressed in the adult brain (Novakovic *et al.*, 2001). Tetrodotoxin is the classical sodium channel blocker, while saxitoxin, a cyanobacterial toxin that contaminates shellfish, also blocks Na^+ channels (Llewellyn, 2006).

VII. NEUROTRANSMITTER SYSTEMS

Many xenobiotics with neurotoxic potential target macromolecules/processes involved in the metabolism, release, reuptake, signaling, storage, synthesis, and transport of neurotransmitters (Cooper *et al.*, 2003). The major neurotransmitter systems are briefly described below.

A. Acetylcholine (ACh)

ACh is synthesized in cholinergic nerve terminals from acetyl CoA and choline in a reaction catalyzed by choline acetyltransferase (ChAT). Acetyl CoA is synthesized from acetate, citrate, or glucose in the mitochondria of the presynaptic cell. The transport of choline from the extracellular fluid into the presynaptic neuron is the rate-limiting step in the synthesis of ACh. Once synthesized, ACh molecules are packaged in synaptic vesicles by the vesicular ACh transporter along with ATP and proteoglycan. This transport of ACh into the synaptic vesicles can be pharmacologically blocked by vesamicol (Prior *et al.*, 1992). Upon terminal depolarization, vesicles fuse with the plasma membrane of the presynaptic cell and release their contents into the synaptic cleft. Black widow spider venom (Hurlbut and Ceccarelli, 1979) and β-bungarotoxin (Spokes and Dolly, 1980) promote the release of ACh molecules from vesicles, whereas botulinum toxin inhibits the release of ACh molecules from vesicles (Sellin and Thesleff, 1981). In the synapse, ACh molecules interact specifically with postsynaptic cholinergic (muscarinic and nicotinic) receptors on the postsynaptic cell membrane to modulate the function of that cell. Muscarinic receptors are G protein-coupled receptors that affect the formation of second messengers [e.g. cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), diacylglycerol (DAG), and inositol triphosphate (IP3)]. To date, five muscarinic receptors (M1–M5) have been cloned. M1, M3, and M5 receptors are coupled to phospholipase C (PLC) via G_q and increase the formation of DAG and IP3, whereas M2 and M4 receptors are coupled to adenylyl cyclase via G_i and decrease the formation of cAMP. Nicotinic receptors are ligand-gated ion channels that increase Na^+ influx. ACh molecules can also interact with presynaptic receptors that modulate the function of the presynaptic cell (i.e. release of ACh molecules) in a ''feedback'' manner. AChE is located within the synaptic cleft where it hydrolyzes ACh to choline and acetic acid. Nerve agents and other OP esters inhibit AChE to elicit cholinergic toxicity (Taylor, 2001). Because AChE is one of the most efficient enzymes in the body [each molecule of enzyme can hydrolyze approximately 5,000 molecules of ACh per second (Cooper, 1994)], ACh molecules have a very short half-life in the synaptic cleft and only a transient ability to activate cholinergic receptors. Choline is transported back into the presynaptic cell by a high-affinity choline uptake process, which can be pharmacologically blocked by hemicholinium-3 (Freeman *et al.*, 1979).

B. Dopamine (DA)

DA is synthesized in dopaminergic nerve terminals from tyrosine in a series of reactions catalyzed by tyrosine hydroxylase (TH) and L-aromatic acid decarboxylase, respectively. The rate-limiting step in the synthesis of DA is the conversion of tyrosine to 3,4-dihydroxy-L-phenylalanine (L-DOPA) by TH. L-DOPA is subsequently converted to DA by L-aromatic acid decarboxylase. DA molecules are packaged along with ATP into synaptic vesicles by the vesicular monoamine transporter. The transport of DA molecules into synaptic vesicles is pharmacologically blocked by reserpine (Henry *et al.*, 1998). Upon terminal depolarization, vesicles fuse with the plasma membrane of the presynaptic cell and release their contents into the synaptic cleft. γ-Hydroxybutyrate (GHB) inhibits the release of DA molecules from the vesicles (Feigenbaum and Howard, 1996). In the synapse, DA molecules interact with postsynaptic receptors to modulate the function of the postsynaptic cell. Dopamine D1 receptors are coupled to adenylyl cyclase via Gs and increase the formation of cAMP. Dopamine D2 receptors are coupled to adenylyl cyclase via G_i/G_o and decrease the formation of cAMP, decrease Ca^{2+} influx, and increase K^+ efflux. DA molecules can also interact with presynaptic receptors to modulate the function of the presynaptic cell. Dopamine D2 receptors on the postsynaptic cell have a micromolar affinity for DA, whereas dopamine D2 receptors on the presynaptic cell have a nanomolar affinity for DA molecules (Creese *et al.*, 1984). Catechol-O-methyl transferase (COMT) is located within the synaptic cleft where it converts DA to 3-methoxytryptamine, which is then converted to homovanillic acid (HVA) by monoamine oxidase (MAO). Tropolone is an inhibitor of COMT (Broch, 1972), whereas pargyline is an inhibitor of MAO (Finberg and Youdim, 1983). DA molecules may also be taken back into the presynaptic cell by a high-affinity DA transporter. Amphetamine blocks the reuptake of DA into the presynaptic cell (Heikkila *et al.*, 1975). In the presynaptic cell, MAO converts DA to dihydroxyphenyl acetaldehyde, which is subsequently converted to dihydroxyphenyl acetic acid (DOPAC) by aldehyde dehydrogenase. DOPAC is converted to HVA by COMT. DOPAC is the major metabolite in rats (Wilk *et al.*, 1975), whereas HVA is the major metabolite in humans (Wilk and Stanley, 1978).

C. γ-Aminobutyric Acid (GABA)

GABA is synthesized in GABAergic nerve terminals from glutamic acid in an irreversible reaction catalyzed by glutamic acid decarboxylase (GAD) using pyridoxine as a cofactor. GABA is also synthesized from α-ketoglutarate in a reversible reaction catalyzed by GABA transaminase (GABA-T). GABA molecules are packaged into synaptic

vesicles by a vesicular transporter. Upon terminal depolarization, vesicles fuse with the plasma membrane of the presynaptic cell and release their contents into the synaptic cleft. In the synapse, GABA molecules interact with postsynaptic receptors ($GABA_A$ or $GABA_B$ receptors) in order to modulate the function of the postsynaptic cell. $GABA_A$ receptors are ligand-gated ion channels that increase Cl^- influx. $GABA_B$ receptors, which can be distinguished pharmacologically from $GABA_A$ receptors by their selective affinity for baclofen, are coupled to adenylyl cyclase via G_i/G_o and decrease the formation of cAMP, decrease Ca^{2+} influx and increase K^+ efflux. GABA molecules can also interact with presynaptic receptors to modulate the function of the presynaptic cell. GABA-T and succinic semialdehyde dehydrogenase (SSADH) are located within the synaptic cleft. GABA-T converts GABA to succinic semialdehyde, which is then converted to succinic acid by SSADH. Aminooxyacetic acid is an inhibitor of GABA-T (Stoof and Mulder, 1977). GABA molecules may also be taken back into the presynaptic cell by a high-affinity GABA transporter.

D. Glutamate (Glu)

Glu is synthesized in glutamatergic nerve terminals from α-ketoglutarate in a reaction catalyzed by GABA-T and from glutamine that is synthesized in glial cells, transported into nerve terminals and locally converted by glutaminase. Glu molecules are packaged into synaptic vesicles by a vesicular transporter. Upon terminal depolarization, vesicles fuse with the plasma membrane of the presynaptic cell and release their contents into the synaptic cleft. In the synaptic cleft, Glu molecules interact with postsynaptic receptors [α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainic acid (KA) and *N*-methyl-D-aspartate (NMDA) receptors] to modulate the function of the postsynaptic cell. Both AMPA and KA receptors are ligand-gated ion channels that increase Na^+ influx to mediate fast excitatory neurotransmission. Metabotropic glutamate (mGlu) receptors are coupled to PLC via G_q and increase the formation of DAG and IP3. NMDA receptors are ligand-gated ion channels that increase Ca^{2+} influx in order to mediate slow excitatory neurotransmission. As with other transmitters, Glu can also interact with presynaptic receptors to modulate the function of the presynaptic cell. Glu molecules are taken back into either glial or presynaptic cells by a number of high-affinity Glu transporters (EAAT1–EAAT5). Glu molecules that are transported into glial cells are converted by glutamine synthase into glutamine, which can then be transported into neighboring nerve terminals to act as a precursor for the synthesis of Glu.

E. Glycine (Gly)

Gly is synthesized in glycinergic nerve terminals from serine in a reaction catalyzed by serine hydroxymethyl transferase. Gly molecules are packaged into synaptic vesicles by a vesicular transporter. Upon terminal depolarization, vesicles fuse with the plasma membrane of the presynaptic cell and release their contents into the synaptic cleft. In the synapse, Gly molecules interact with postsynaptic receptors to modulate the function of the postsynaptic cell. Gly receptors are ligand-gated ion channels that increase Cl^- influx. Gly molecules may be taken back into the presynaptic cell by two high-affinity Gly transporters (GLYT-1 and GLYT-2). GLYT-1 is found primarily in glial cells, whereas GLTY-2 is found primarily in neuronal cells (Zafra *et al.*, 1995).

F. Norepinephrine (NE)

NE is synthesized in adrenergic nerve terminals from tyrosine in a series of reactions catalyzed by tyrosine hydroxylase (TH), L-aromatic acid decarboxylase, and dopamine-β-hydroxylase, respectively. As with DA, the rate-limiting step in the synthesis of NE is the conversion of tyrosine to 3,4-dihydroxy-L-phenylalanine (L-DOPA) by TH. L-DOPA is subsequently converted to DA by L-aromatic acid decarboxylase. DA molecules are packaged into synaptic vesicles by a vesicular monoamine transporter and rapidly converted into NE by dopamine-β-hydroxylase with ascorbic acid and molecular oxygen as cofactors. Upon terminal depolarization, vesicles fuse with the plasma membrane of the presynaptic cell and release their contents into the synaptic cleft. In the synapse, NE molecules interact with postsynaptic receptors (various subtypes of α- and β-adrenergic receptors) to modulate the function of the postsynaptic cell. α_1-Adrenergic receptors are coupled to PLC via G_q and increase the formation of DAG and IP3, whereas α_2-adrenergic receptors are coupled to adenylyl cyclase via G_i and decrease the formation of cAMP. Both β_1- and β_2-adrenergic receptors are coupled to adenylyl cyclase via G_s and increase the formation of cAMP. Other subtypes exist. NE molecules can also interact with presynaptic receptors to modulate the function of the presynaptic cell. COMT is located within the synaptic cleft, and converts NE to normetanephrine, which is then converted to 3-methoxy-4-dehydroxyphenylglycolaldehyde by MAO. 3-Methoxy-4-dehydroxyphenylglycoaldehyde is subsequently converted to 3-methoxy-4-hydroxymandelic acid (VMA) by aldehyde dehydrogenase. NE molecules may also be taken back into the presynaptic cell by a high-affinity NE transporter. In the presynaptic cell, MAO converts NE to 3,4-dihydroxyphenylglyceraldehyde, which is then converted to 3,4-dihydroxymandelic acid (DOMA) by aldehyde dehydrogenase. DOMA is subsequently converted to VMA by COMT.

G. Serotonin (5-HT)

5-HT is synthesized in serotonergic nerve terminals from tryptophan in a series of reactions catalyzed by tryptophan hydroxylase and L-aromatic acid decarboxylase,

respectively. Tryptophan, which is the rate-limiting factor in the synthesis of 5-HT, is transported from the extracellular compartment into the presynaptic cell by a neutral amino acid transporter and converted into 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase. 5-HTP is subsequently converted to 5-HT by L-aromatic acid decarboxylase. 5-HT molecules are packaged into synaptic vesicles by a vesicular monoamine transporter. Upon terminal depolarization, vesicles fuse with the plasma membrane of the presynaptic cell and release their contents into the synaptic cleft. In the synapse, 5-HT molecules interact with postsynaptic receptors (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7 receptors) to modulate the function of the postsynaptic cell. 5-HT_1 receptors are coupled to adenylyl cyclase via G_i/G_o and decrease the formation of cAMP, open K^+ channels, and close Ca^{2+} channels. 5-HT_2 receptors are coupled to PLC via G_q and increase the formation of DAG and IP3. 5-HT_3 receptors are ligand-gated ion channels that increase Na^+ influx. 5-HT_4, 5-HT_6, and 5-HT_7 receptors are coupled to adenylyl cyclase via G_s and increase the formation of cAMP. 5-HT_5 receptors are G protein-coupled receptors with an unknown second messenger system. 5-HT molecules may be taken back into the presynaptic cell by a 5-HT membrane transporter, which is inhibited by fluoxetine and sertraline (Rickels and Schweizer, 1990). In the presynaptic cell, MAO converts 5-HT to 5-hydroxyindole acetaldehyde, which is subsequently converted to 5-hydroxyindole acetic acid (5-HIAA) by aldehyde dehydrogenase.

VIII. SELECTED CWAs THAT AFFECT NERVOUS SYSTEM FUNCTION

As briefly described above, the nervous system has some unique biochemical and physical characteristics that can contribute to its relative sensitivity to xenobiotics including CWAs. Many CWAs adversely affect the nervous system, and some of these have been covered in detail in subsequent chapters. This chapter provides a discussion to illustrate how some CWAs may directly or indirectly target nervous system functions.

A. Cyanides

Cyanogen chloride (CK) and hydrogen cyanide (AC) are the only cyanide-containing compounds designated as CWAs. Due to their high volatility, these two compounds rarely achieve lethal atmospheric concentrations except in enclosed spaces (Lee, 1997). When the Nazis used hydrogen cyanide in concentration camps during WWII, it was used in enclosed chambers and not on the battlefield. Although they are not CWAs, potassium and sodium cyanide salts have been used for equally malicious purposes. In the fall of 1982, seven people in the Chicago area of the USA died after ingesting Extra Strength Tylenol capsules that had been adulterated with potassium cyanide (Dunea, 1983).

Signs of cyanide exposure include agitation, dizziness, headache, and mental confusion followed by cardiac disturbances, loss of consciousness, respiratory distress and seizures. While cyanide exposure is often fatal, there are reports in the literature on its long-term effects. Finelli (1981) described a 30-year-old male who attempted suicide with cyanide. Over a year after intoxication, the patient developed choreiform movements of his extremities and an impairment in the movement of his left hand. Similarly, Carella *et al.* (1988) described a 46-year-old woman who drank a beverage poisoned with cyanide and later developed a dystonic posture of the mouth and tongue, which was deviated to the right and twisted. Computed tomography scans showed that both individuals had lesions in the basal ganglia.

Although there are a number of reports in the literature on the neuropathological consequences of cyanide exposure including necrotic lesions in the cerebellar gray matter of dogs (Haymaker *et al.*, 1952) and demyelinative lesions in the corpus callosum and optic nerves of rats (Lessell, 1971), its neurobehavioral consequences have received little attention. D'Mello (1986) reported that a single exposure to sodium cyanide (4 mg/kg, s.c.) impaired swimming ability in guinea pigs. Mathangi and Namasivayam (2000) reported that repeated exposure to sodium cyanide (2 mg/kg, i.p.) impaired the memory aspects of T-maze performance in rats.

The main mechanism of action underlying cyanide-induced toxicity is inhibition of cytochrome c oxidase, the terminal enzyme of the electron transport chain, resulting in a cytotoxic hypoxia with a shift from aerobic to anaerobic metabolism, a decrease in ATP production and an increase in lactic acid production (Way, 1984). The CNS is particularly sensitive to the toxic effects of cyanide due to its limited anaerobic metabolic capacity and high energy dependence. Ikegaya *et al.* (2001) showed that the inhibition of cytochrome c oxidase activity following the oral administration of potassium cyanide (10 mg/kg) was highest in rat brain compared to other organs. Thus, not only is the CNS particularly sensitive to the action of cyanide, but the target enzyme itself may be more sensitive to inhibition compared to other tissues.

Oxidative stress may also play a critical role in cyanide-induced toxicity. Potassium cyanide (7 mg/kg, s.c.) decreased the activities of catalase, glutathione peroxidase, and superoxide dismutase in mouse brain (Ardelt *et al.*, 1989). Potassium cyanide also stimulated the formation of reactive oxygen species (ROS) and increased levels of malondialdehyde in a number of neuronal cell lines including cerebellar granule cells (Gunasekar *et al.*, 1996), primary cortical cells (Li *et al.*, 2002), and rat pheochromocytoma (PC-12) cells (Kanthasamy *et al.*, 1997). Johnson and colleagues (1986) proposed that a rise in intracellular Ca^{2+} levels following potassium cyanide exposure is

responsible for the formation of ROS. In support of this proposal, Gunasekar and colleagues (1996) showed that the removal of Ca^{2+} from the culture medium blocked the formation of ROS in cerebellar granule cells exposed to potassium cyanide (100 μM). Antioxidants (Muller and Krieglstein, 1995), Ca^{2+} channel blockers (Johnson *et al.*, 1987), cyclooxygenase-2 (COX-2) inhibitors (Li *et al.*, 2002), NMDA receptor antagonists (Gunasekar *et al.*, 1996), and phospholipase A2 inhibitors (Kanthasamy *et al.*, 1997) all blocked the formation of ROS in neuronal cell lines exposed to potassium cyanide.

Cyanide-induced cell death involves the selective activation of apoptosis or necrosis in different neuronal populations. Mills *et al.* (1999) reported that cyanide-induced cell death occurred via apoptosis in the cortical region of a mouse brain and via necrosis in the substantia nigra. Similarly, Prabhakaran *et al.* (2002) reported that cyanide-induced cell death occurred via apoptosis in primary cortical cells and via necrosis in primary mesencephalic cells. While exposure to potassium cyanide (400 μM) increased the formation of ROS in both cell types, the rates of formation and the nature of ROS varied. For example, catalase and superoxide dismutase decreased the formation of ROS in cortical cells but not in mesencephalic cells. These findings suggest that the selective vulnerability of these neuronal populations to cyanide may be related to differences in their susceptibility to oxidative stress.

Dopaminergic neurons have an enhanced sensitivity to oxidative stress possibly due to the autoxidation of DA to quinones and ROS (Basma *et al.*, 1995; Ben-Shachar *et al.*, 1995). Kanthasamy *et al.* (1994) reported that mice repeatedly exposed to potassium cyanide (6 mg/kg, s.c.) had a reduced number of TH-positive cells indicating a loss of dopaminergic neurons in the substantia nigra. Approximately 30% of the cyanide-treated mice exhibited decreased locomotor activity and akinesia, which were suppressed by the administration of L-DOPA (100 mg/kg, i.p.). Cassel and Persson (1992) reported that striatal DA and HVA levels were rapidly decreased in rats exposed to sodium cyanide (20 mg/kg, i.p.). However, the *in vivo* synthesis of DA, measured as the rate of L-DOPA accumulation after neuronal decarboxylase inhibition, was increased in these rats suggesting an increase in striatal DA release. In support of this hypothesis, Kiuchi *et al.* (1992) reported that perfusion of sodium cyanide (2 mM) into rat striatum produced a transient but marked increase in DA release. In addition, Cassel *et al.* (1993) reported that the administration of sodium cyanide (2 mg/kg, i.p.) to rats decreased dopamine D1 and D2 receptor binding in the striatum. Given that Parkinson's disease is a condition characterized by the selective loss of dopaminergic neurons in the substantia nigra and the depletion of striatal DA levels (German *et al.*, 1989), these findings suggest that decreased dopaminergic activity may play an important role in the development of Parkinsonian-like signs following cyanide exposure.

Glutamatergic neurons also have an enhanced susceptibility to excitotoxicity and oxidative stress (Coyle and Puttfarcken, 1993). Excitotoxicity is the pathological process by which high concentrations of Glu overexcite and damage neurons through excessive activation of ionotropic Glu receptors (Olney, 1969). At NMDA receptors, high concentrations of Glu cause excitotoxicity by allowing an excessive amount of Ca^{2+} to enter into the neuron (Choi, 1987). Patel *et al.* (1992) demonstrated that NMDA receptor activation was responsible for the rise in intracellular Ca^{2+} levels contributing to cyanide-induced toxicity in cultured hippocampal neurons. Yamamoto and Tang (1998) showed that the morphological changes observed in cerebrocortical neurons exposed to potassium cyanide (1 mM) were blocked by 2-amino-7-phosphonoheptanoic acid (AP7; 1 mM), a selective NMDA receptor antagonist. Given that there is a strong correlation between whole-brain Ca^{2+} levels and cyanide-induced seizures in mice (Johnson *et al.*, 1986), increased glutamatergic activity may contribute to the development of seizures following cyanide exposure. Furthermore, Yamamoto and Tang (1996) showed that cyanide-induced seizures in mice were blocked by MK-801 (2 mg/kg, s.c.), a selective NMDA receptor antagonist.

Decreased GABAergic activity may also contribute to the development of seizures following cyanide exposure. Persson *et al.* (1985) reported that striatal GABA, Glu, and glutamine levels were decreased in rats exposed to sodium cyanide (20 mg/kg, i.p.), and Cassel *et al.* (1991) showed that these decrements were associated with an increased susceptibility to seizures. Similarly, Yamamoto (1990) reported that whole-brain GABA levels were markedly decreased in cyanide-treated mice exhibiting seizures. The decrease in GABA and the associated seizures were blocked by α-ketoglutarate (500 mg/kg, i.p.).

B. Sulfur Mustard

Sulfur mustard (2,2′-dichlorethyl sulfide; HD) was first used as a CWA in WWI during a German attack on British troops at Ypres, Belgium, in 1915. Subsequently, it has been used in a number of military conflicts including the Iran–Iraq War of 1980–1988 (Marshall, 1984). During this conflict, Iraqi troops employed sulfur mustard against both military personnel and civilians. Approximately 40,000 victims of sulfur mustard have been documented among Iranian and Kurdish populations (Hay, 2000; Khateri *et al.*, 2003), and a majority of those victims sent to European hospitals for medical treatment had mild CNS complaints including anxiety, confusion, headache, and lethargy (Balali-Mood and Hefazi, 2006).

Sulfur mustard exerts a local action on the eyes, respiratory tract, and skin followed by a systemic action on the central nervous, gastrointestinal and hematopoietic systems (Dacre and Goldman, 1996). A moderate exposure to sulfur mustard can cause blisters, conjunctivitis, erythema, lacrimation, nausea, and respiratory inflammation; whereas

a more severe exposure can cause blindness, bronchitis, bronchopneumonia, corneal damage, leucopenia, seizures, and vesication. The most accepted theory as to the basis of these clinical signs of exposure is alkylation of DNA, which activates chromosomal poly(ADP-ribose) polymerase (PARP), reducing the intracellular supply of NAD^+ and thus inhibiting glycolysis and causing cell death (Papirmeister *et al.*, 1985). However, more recent hypotheses suggest that oxidative stress, via the formation of ROS, plays a critical role in sulfur mustard-induced toxicity (Naghii, 2002). In support of this proposal, Jafari (2007) demonstrated that high doses of sulfur mustard (>10 mg/kg, i.p.) decreased the activities of catalase, glutathione peroxidase, glutathione *S*-transferase, and superoxide dismutase in rat brain leading to impairment of the antioxidant defense system.

While the literature on the effects of sulfur mustard on the central nervous system is sparse, Lynch *et al.* (1918) reported that lethal doses of sulfur mustard (>10 mg/kg, s.c.) caused hyperexcitability followed by unsteadiness of gait, muscular weakness and seizures in dogs. Philips and Thiersch (1950) reported that lethal doses of 2,4,6-tris (ethylenimino)-*S*-triazine (>125 mg/kg, i.p.), which has an ethylenimine moiety analogous to sulfur mustard, caused similar effects in mice. Although the mechanism responsible for these effects is entirely unknown, it is interesting to note that sulfur mustard (3.15 μM) induced acetylcholinesterase activity in neuroblastoma cell cultures suggesting that cholinergic activity may play a role in the central nervous system effects of sulfur mustard (Lanks *et al.*, 1975). Thus, while sulfur mustard is typically thought of as a dermal toxicant, clinical and experimental data suggest it has the potential to cause neurotoxicity possibly mediated by direct action in the nervous system elicited by oxidative stress.

C. Botulinum Toxins

The bacterial group *Clostridium botulinum* produces seven immunologically distinct toxins (types A–G) that are the most potent neurotoxins known to humankind (Gill, 1982). These toxins inhibit spontaneous and impulse-dependent ACh release (Kao *et al.*, 1976) resulting in a potentially fatal neuroparalytic illness (i.e. botulism). The typical manifestation of botulism is a flaccid paralysis involving skeletal muscle and structures innervated by autonomic ganglia (Habermann and Dreyer, 1986; Merz *et al.*, 2003). Other clinical features include a decrease in intestinal motility and tone, the inhibition of bronchial and salivary secretions and sweating, mydriasis, and respiratory collapse. Human intoxication is caused by botulinum toxin types A, B, and E and is generally manifested as foodborne, intestinal (infant), and wound botulism (Simpson, 1981).

Botulinum toxins are synthesized as 150 kDa single-chain, inactive progenitors that undergo proteolytic cleavage to form two chains coupled by a disulfide bond (de Paiva *et al.*, 1993). The heavy chain (100 kDa) is responsible for the binding and translocation of the light chain (50 kDa) into the neuronal cell; whereas the light chain is responsible for the inhibition of one of three proteins in the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex that are required for neurotransmitter exocytosis (Rizo and Sudhof, 1998; Simpson *et al.*, 2004). Botulinum toxin types B, D, F, and G cleave synaptobrevin (Sollner *et al.*, 1993), botulinum toxin types A and E cleave synaptosomal-associated proteins of 25 kDa (SNAP-25) (Otto *et al.*, 1995), and botulinum toxin type C cleaves SNAP-25 and syntaxin (Schiavo *et al.*, 1995). Botulinum toxin thus acts directly and specifically in the nervous system, disrupting the fusion of synaptic vesicles and blocking cholinergic neurotransmission.

D. Organophosphorus Anticholinesterases

As noted above, the organophosphorus (OP) nerve agents are widely recognized as CWAs. Cyclosarin (*o*-cyclohexylmethyl phosphonofluoridate; GF), sarin (isopropyl methyl phosphonofluoridate; GB), soman (pinacolyl methyl phosphonofluoridate; GD), tabun (ethyl *N,N*-dimethylphosphoramidocyanidate; GA), and VX (*o*-ethyl *S*-2-diisopropylaminoethyl methylphosphonothioate), commonly referred to as nerve agents, were originally synthesized between 1936 and 1958 in various attempts to create more effective pesticides. Nerve agents are among the most lethal CWAs ever developed, and tons of these agents were previously stockpiled for military use by the USA and the Soviet Union during the Cold War. However, the first documented use of nerve agents was not until the Iran–Iraq War of 1980–1988 (Newmark, 2004). During this conflict, Iraqi troops employed sarin against both military personnel and civilians, confirmed by detection of trace amounts of a breakdown product, isopropyl methylphosphonic acid, in soil samples taken from a Kurdish village in northern Iraq (Macilwain, 1993).

More than 100,000 US military personnel participating in the Gulf War were potentially exposed to low levels of cyclosarin and sarin following the destruction of a munitions storage facility at Khamisiyah, Iraq, in March 1991 (Smith *et al.*, 2003). Reviews of medical records for these veterans and of published field accounts revealed no clinical indications of cyclosarin or sarin acute intoxication at this time (Riddle *et al.*, 2003). However, deficits in motor dexterity and visuospatial abilities (Proctor *et al.*, 2006) and a two-fold increase in the incidence of brain cancer deaths (Bullman *et al.*, 2005) were reported in these veterans 4 years after possible exposure. In addition, Heaton *et al.* (2007) reported a reduction in the overall volume of white matter and an enlargement of the lateral ventricles in Gulf War veterans. A number of persistent neurological symptoms are still expressed in many Gulf War veterans, and

substantial evidence suggests exposure to AChE-inhibiting neurotoxicants may have played a role (Binns *et al.*, 2004).

A Japanese cult named Aum Shinrikyo (Divine Truth) employed sarin in two separate terrorist attacks in the mid-1990s. The first attack, which occurred on June 27, 1994 in a residential neighborhood in Matsumoto, Japan, resulted in 56 hospitalizations and seven deaths, whereas the second attack, which occurred on March 20, 1995 in several subway cars in Tokyo, Japan, resulted in 796 hospitalizations and 12 deaths (Yanagisawa *et al.*, 2006). Although few abnormalities were observed in survivors 3 months after the attacks (Okumura *et al.*, 1996), changes in psychomotor functioning (Yokoyama *et al.*, 1998b), impairments in postural sway of low frequency (Yokoyama *et al.*, 1998a), and neurophysiological alterations as measured by visual evoked potentials (Murata *et al.*, 1997) were observed in survivors 6–8 months after the attacks. Yamasue *et al.* (2007) observed smaller regional white matter volumes and diffuse bilateral disruption of white matter integrity in survivors 5–6 years after the attacks, and Miyaki *et al.* (2005) observed deficits in attention and gross motor speed in survivors 7 years after the attacks.

Nerve agents exert their toxicological effects by inhibiting AChE in the central and peripheral nervous systems. Changes in other cholinergic neurochemical signaling components may be directly or indirectly elicited. Fosbraey *et al.* (1990) reported that AChE levels were maximally inhibited by 90% in guinea pigs treated with soman (31.2 μg/kg, s.c.), and Whalley and Shih (1989) reported a 30–40% decrease in high-affinity choline uptake in the cortex and hippocampus of rats treated with sarin (135 μg/kg, s.c.) or soman (120 μg/kg, s.c.). The accumulation of ACh within the synaptic cleft following AChE inhibition causes the prolonged activation of muscarinic and nicotinic receptors on postsynaptic cells. Shih (1982) reported a 320% increase in ACh levels in the cortex of rats treated with soman (120 μg/kg, s.c.), and Khan and colleagues (2000) reported a 10% increase in muscarinic receptor binding and a 132% increase in nicotinic receptor binding in the cortex of rats treated with sarin (100 μg/kg, i.m.). In the PNS, the prolonged activation of cholinergic receptors results in hypersecretions (e.g. defecation, lacrimation, rhinorrhea, salivation, and urination), miosis, muscle fasciculations, and tremors (Ecobichon, 2001). In the CNS, the prolonged activation of cholinergic receptors can lead to loss of consciousness, respiratory distress, and seizures/convulsions.

Respiratory distress was observed in approximately 63% of the casualties from the terrorist attack in Tokyo, Japan (Okumura *et al.*, 1996). Rickett *et al.* (1986) demonstrated that one of the first signs of nerve agent-induced respiratory distress in cats was disruption of the normal firing of respiratory-related neurons in the pons medulla followed by changes in airflow, diaphragm contraction, diaphragm electromyogram (EMG), and phrenic nerve activity. Spectral analysis of diaphragm EMG activities showed that the functional integrity of the diaphragm in guinea pigs following soman exposure (15 μg/kg, s.c.) was not sufficiently compromised to produce respiratory distress despite signs of fatigue (Chang *et al.*, 1990). These studies suggest that respiratory depression following nerve agent exposure is affected through disruption of central cholinergic signaling. Bajgar *et al.* (2007) showed that the pons medulla was particularly sensitive to AChE inhibition by nerve agents.

Seizures were observed in approximately 3% of the casualties from the terrorist attack in Tokyo, Japan (Okumura *et al.*, 1996). Shih and McDonough (1999) demonstrated that all five of the classic nerve agents (i.e. cyclosarin, sarin, soman, tabun, and VX) were capable of inducing seizures at lethal doses ($2.0 \times LD_{50}$ in guinea pigs and $1.6 \times LD_{50}$ in rats). In the case of VX exposure, however, the latency of seizure development was 3–5 times longer than with the other nerve agents. Tonduli *et al.* (2001) reported that soman-induced seizures occurred in rats only when the cortical AChE inhibition was over 65%, and Churchill *et al.* (1985) showed that early body weight loss was a nonlethal indicator of the severity of soman-induced seizures in rats. Both anticholinergics (atropine sulfate, biperiden HCl, and trihexyphenidyl HCl) and benzodiazepines (diazepam and midazolam) were able to terminate nerve agent-induced seizures in guinea pigs when administered within 5 min of seizure onset, but a higher dose of each was typically required to terminate seizures induced by soman than by other nerve agents (Shih *et al.*, 2003).

Prolonged seizure activity (i.e. *status epilepticus*), following nerve agent exposure, has been shown in several animal models to cause neuropathological lesions that are associated with long-term deficits in cognitive function and other behaviors (Geller *et al.*, 1985; Raffaele *et al.*, 1987; Rylands, 1982). Shih *et al.* (2003) demonstrated that all five of the classic nerve agents were capable of inducing neuropathology when they were administered to guinea pigs at doses that produced seizures. Moderate to severe neuropathological lesions were reported in 70% of sarin- (Kadar *et al.*, 1995) and 98% of soman-treated (McDonough *et al.*, 1995) rats exhibiting seizures for 20 min or longer. Lesions were primarily noted in the hippocampus, piriform cortex, and thalamus, but pathology later progressed to other brain regions. Filliat *et al.* (1999) reported that spatial memory performance in the Morris water maze was directly related to the severity of hippocampal lesions in soman-treated rats. Interestingly, no memory impairment was observed below a threshold of 15% neuronal loss. Soman-induced deficits were also reported in active (Romano *et al.*, 1985) and passive (Buccafusco *et al.*, 1990) avoidance, brightness discrimination (Myhrer *et al.*, 2005), differential reinforcement of low rate (DRL) acquisition (McDonough *et al.*, 1986), match-to-sample discrimination (Gause *et al.*, 1985), maze learning (Raffaele *et al.*, 1987), and various schedule-controlled behaviors (Brezenoff *et al.*, 1985; Hymowitz *et al.*, 1985).

Seizures followed by the cessation of respiration are important components of severe nerve agent intoxication. Neuropathological lesions are proposed to occur via ''recruitment'' of a glutamate-mediated excitotoxic mechanism; nerve cells targeted by glutamatergic neurons experience an unrelenting stimulation (excitotoxicity) that results in a failure of their homeostatic mechanisms, cytoplasmic changes and cell death (Solberg and Belkin, 1997). Lallement *et al.* (1991) observed a 78% increase in extracellular Glu levels in the CA3 region of the hippocampus within 30 min of seizure onset in soman-treated rats. In the hippocampal CA1 region, they observed a 180% increase in extracellular Glu levels within 50 min of seizure onset. Sparenborg *et al.* (1992) found that post-treatment with MK-801 (30, 100, or 300 μg/kg, i.m.) following soman administration arrested seizure activity in a dose-dependent manner suggesting that NMDA receptor activation may play a critical role in the spread and maintenance of nerve agent-induced seizures. Given that excitotoxicity is also induced by decreasing the amount of oxygen-derived energy available to sustain cellular maintenance, it seems likely that the glutamatergic effects of nerve agents are exacerbated by their indirect actions on the respiratory control center and on peripheral neuromuscular sites involved in ventilation. Oxidative phosphorylation would also be interrupted by anoxia and hypoxia associated with nerve agent-induced seizures, and excessive bronchial secretions may interrupt airflow and contribute to cyanosis.

Thus, the OP nerve agents illustrate an example of neurotoxicity in which a specific (cholinergic) neurotransmitter system is initially affected to disrupt neuronal function. As opposed to botulinum toxins that block cholinergic neurotransmission, the OP nerve agents enhance cholinergic signaling by disrupting ACh degradation. These actions can be life-threatening and can involve disruption in the cholinergic regulation of higher functions (e.g. cognition), ventilation, cardiac function, and somatic motor and autonomic functions. Persistent neurological sequelae may result from acute intoxications. While generally thought to be at least initially selective for cholinergic neurotransmission, noncholinesterase actions and other neurotransmitter systems may be involved, and oxidative stress may play a role in both acute and persistent toxic consequences.

E. 3-Quinuclidinyl Benzilate (QNB)

QNB (1-azabicyclo[2.2.2]oct-3-yl hydroxy(diphenyl)acetate; BZ) is a potent, atropine-like glycolic acid ester that blocks muscarinic receptors in the CNS and PNS (Spencer, 2000). During the 1950s QNB was explored by both the USA and the Soviet Union as a potential antidote for OP anti-ChEs. However, the marked hallucinogenic actions of QNB led to its potential use as a CWA (Marshall, 1979). In July 1995 the Yugoslav People's Army allegedly used an incapacitating agent that caused hallucinations and irrational behavior against Bosnian refugees fleeing from the town of Srebrenica (Hay, 1998). While many believe that this incapacitating agent was QNB, there is no hard evidence to support this allegation.

QNB acts as a potent, competitive inhibitor of ACh at muscarinic receptor sites located in brain, autonomic ganglia, exocrine glands, and smooth muscles, resulting in a confusional state with delusions, a decrease in intestinal motility and tone, inhibition of bronchial and salivary secretions and sweating, erratic behavior, hallucinations, mental slowing, mydriasis, and tachycardia (Ketchum, 1963). Early studies demonstrated that QNB was a potent and selective ligand for muscarinic receptors. Yamamura and Snyder (1974) showed that the inhibition of [^{3}H]-QNB binding to homogenates of rat brain by muscarinic drugs correlated with their pharmacological potencies, whereas nicotinic and noncholinergic drugs had negligible affinity. Kuhar and Yamamura (1976) reported that the injection of [^{3}H-]QNB into rat brain led to its localization to muscarinic receptors in the cerebral cortex, hippocampus, nucleus accumbens, and striatum. In addition, Jovic and Zupanc (1973) reported that the subcutaneous administration of QNB (5–20 mg/kg) to rats decreased oxygen consumption in the cerebral cortex and medulla oblongata. Interestingly, while QNB as a radioligand appears to bind to all known subtypes of muscarinic receptors, some evidence suggests that it has selectivity for the M2 subtype of muscarinic receptors when administered *in vivo* (McRee *et al.*, 1995). QNB thus can produce a variety of neurological effects presumably through the antagonism of cholinergic muscarinic receptor signaling in the CNS and PNS.

IX. CONCLUDING REMARKS AND FUTURE DIRECTION

The nervous system has a number of biochemical and structural characteristics that can make it particularly sensitive to CWAs. Indeed, many of the different types of CWAs can elicit neurotoxic responses. In some cases, these neurotoxic effects are elicited by direct interaction with specific target molecules within the nervous system, while in other cases the target is less specific but the tissue itself is more sensitive, and in yet other cases actions on other organ systems can lead to indirect impairment of neurologic function. The global importance of the continuous neuronal regulation of vital processes throughout the body makes the nervous system an effective target for disruption by CWAs. Thus, knowledge of mechanisms of neurotoxicity for CWAs can aid in understanding how to counteract such adverse neurological effects.

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CHAPTER 33

Behavioral Toxicity of Nerve Agents

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I. INTRODUCTION

Behavioral changes in humans exposed to highly toxic organophosphorus compounds, called nerve agents, have been discussed in numerous reports. The incidence of behavioral effects is higher in individuals who have been severely exposed to nerve agents, but they may occur in individuals who have received a small exposure and have no or minimal physical signs and symptoms. The behavioral effects usually start within a few hours and last from several days to several weeks or months. The most frequent symptoms include feelings of uneasiness, tenseness, and fatigue. Exposed individuals may be forgetful and generally display impaired memory and learning, poor comprehension, decreased ability to communicate, or occasional mild confusion.

There are a few reports describing behavioral changes in subjects accidentally exposed to nerve agents. They reported sleep disturbance, mood changes, fatigue, jitteriness or tenseness, an inability to read with comprehension, difficulties with thinking and expression, forgetfulness, a feeling of being mentally slowed, depression, irritability, giddiness, poor performance in arithmetic tests, minor difficulties in orientation, and frightening dreams. It was observed that the complex of central nervous system (CNS) symptoms may not fully develop until 24 h after exposure. In addition, no correlations between the presence or severity of symptoms and the degree of acetylcholinesterase inhibition were seen. Most of the effects of exposure disappear within 3 days. It was concluded that not only severe but also mild intoxication of nerve agents may cause behavioral and psychological disturbances. In general, the behavioral effects have not been permanent but have lasted from weeks to several months, or possibly several years. Long-term behavioral effects after poisoning with nerve agents or organophosphorus insecticides have been reported (Karczmar, 1984; Levin and Rodnitzki, 1976; Sidell, 1974). These reports are based on clinical observations, which are occasionally supported by psychological studies.

II. THE METHODS USED TO EVALUATE BEHAVIORAL EFFECTS OF NERVE AGENTS

A. Functional Observatory Battery

The functional observatory battery (FOB) is a noninvasive and relatively sensitive type of neurobehavioral examination of 40 sensory, motor, and automatic nervous functions. Some of them are scored (Table 33.1), and the others are measured in absolute units (Frantik and Hornychova, 1995; Slechta, 1989). The first evaluation is made when nerve agent-exposed or control rats are in the home cage. The observer evaluates each animal's posture, palpebral closure and gait, and the presence or absence of convulsions is noted. Each rat is then removed from the home cage and briefly held in the hand. The presence or absence of spontaneous vocalization, piloerection, and other fur and skin abnormalities as well as irritability is noted. Lacrimation and salivation are also observed. Other signs such as exophthalmus, crustiness around the eyes, or emaciation are recorded too. The rats are then placed on a flat surface which serves as an open field. A timer is started for 3 min during which the frequency of rearing responses is recorded. At the same time, gait characteristics are noted and ranked, and activity, tremor, convulsions, and abnormal posture are evaluated. At the end of the third minute, the number of fecal boluses and urine pools on the absorbent pad is registered. Then, a reflex test that consists of recording each rat's response to the frontal approach of the blunt end of a pen, a touch of the pen to the posterior flank, and an auditory click stimulus is used. The responsiveness to a pinch on the tail and the ability of pupils to constrict in response to light are then assessed. These measurements are followed by a test for the aerial righting reflex, then by the measurements of forelimb and hindlimb grip strength, body weight, rectal temperature, and finally hindlimb landing foot splay. The whole battery of tests requires approximately 6–8 min per rat. Motor activity data are collected using an apparatus for testing

TABLE 33.1. Functional observational battery (FOB)

	Scored values only									
Marker	**−2**	**−1**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Posture				Sitting or standing	*Rearing*	*Asleep*	Flattened	Lying on side	Crouched over	Head bobbing
Catch difficulty				Passive	*Normal*	Defense	Flight	Escape	Aggression	
Ease of handling				Very easy	Easy	Moderately difficult	Difficult			
Muscular tonus	Atonia	Hypotonia	*Normal*	Hypertonia	Rigidity	Fasciculations				
Lacrimation			*None*	Slight	Severe	Crusta	Colored crusta			
Palpebral closure				*Open*	Slightly	Half-way	Completely shut	Ptosis		
Endoexophthalmus		Endo	*Normal*	Exo						
Piloerection			*No*	Yes						
Skin abnormalities			*Normal*	Pale	Erythema	Cyanosis	Pigmented	Cold	Injury	
Salivation			*None*	Slight	Severe					
Nose secretion			*None*	Slight	Severe	Colored				
Clonic movements			*Normal*	Repetitive	Nonrhythmic	Mild tremors	Severe tremors	Myoclonic	Clonic	
Tonic movements			*Normal*	Contraction of extensors	Opisthotonus	Emprosthotonus	Explosive jumps	Tonic convulsions		
Gait			*Normal*	Ataxia	Overcompensation of hindlimb movement	Feet point outwards from body	Forelimbs are extended	Walks on tiptoes	Hunched body	Body is flattened against surface

Gait score			*Normal*	Slightly impaired	Somewhat impaired	Totally impaired		
Mobility score			*Normal*	Slightly impaired	Somewhat impaired	Totally impaired		
Arousal (level of unprovoked activity)			Very low	Sporadic	Reduced	*Normal*	Enhanced	Permanent
Tension		*None*	Partial (ears)	Stupor				
Stereotypy		*None*	Head weaving	Body weaving	Grooming	Circling	Others	
Bizarre behavior		*None*	Head	Body	Self-mutilation	Abnormal movements	Others	
Approach response			No reaction	*Normal*	Slow reaction	Energetic reaction	Exaggerated reaction	
Touch response			No reaction	*Normal*	Slow reaction	Energetic reaction	Exaggerated reaction	
Click response			No reaction	*Normal*	Slow reaction	Energetic reaction	Exaggerated reaction	
Tail-pinch response			No reaction	*Normal*	Slow reaction	Energetic reaction	Exaggerated reaction	
Pupil size	Miosis	*Normal*	Mydriasis					
Pupil response		No reaction	*Normal reaction*					
Righting reflex			*Normal*	Slightly uncoordinated	Lands on side	Lands on back		

a spontaneous motor activity of laboratory animals. The animals are placed for a short period (10 min) in the measuring cage and their movement (total horizontal activity, stereotypical activity, rearing, jumping, scratching, and total vertical activity) is recorded.

B. Performance on the RAM Task

RAM sessions are conducted using an eight-arm commercially available radial maze measuring 137.2 cm in diameter. The center of the maze is a plastic octagon hub measuring 26.67 cm across, with a Plexiglass lid and wire grid floor. A Plexiglass arm with a wire mesh floor is attached to each of the eight sides of the hub. The entrance to each arm contains a motorized guillotine door allowing access to and from the hub. Each arm's runway contains two floor-mounted switches, which are depressed by the weight of the rat when present in the proximal and distal portion of the runway, respectively. The terminal portion of each arm contains a food dispenser, for delivering food pellets, connected to a trough that is outfitted with the photoemitter/detector unit that can detect access by the rats. Experiments are controlled and monitored using a commercial hardware interface and a microcomputer using the L2T2S software control system (Coulbourn Instruments). For the RAM task, four of eight arms are ''baited''. That is, a single food pellet is available upon a nose-poke into the food trough at the terminal portion of four arms. Each rat is randomly assigned a maze configuration of four baited arms from 37 possible configurations which excludes more than two consecutive baited arms. Thus, the same configuration of baited arms is used for a particular rat for each of the sessions, but different configurations can be used for different rats. Sessions begin with the rat placed in the center hub compartment and the doors to the eight arms are raised. The rat is then free to explore the maze to obtain the food rewards available from the four baited arms. The session is terminated when a rat obtains all four food rewards or 15 min have elapsed. If a rat does not complete the maze within 15 min, a completion time of 15 min is assigned and errors are analyzed. Failure to complete the maze, however, is infrequent and only occurs during the initial few sessions on the maze. No familiarity training with the maze is conducted prior to the first session. The major dependent variables characterizing performance on the RAM task are the time to complete the maze and the number of errors made. Errors are designated as occurring when a rat chooses an unbaited arm (reference memory error) or when a rat returns to a baited arm after obtaining the food reward (working memory error) (Genovese *et al.*, 2006).

C. Acoustic Startle Response and Pre-pulse Inhibition

The animals are tested for acoustic startle response (ASR) and pre-pulse inhibition (PPI) in the SM100 Startle Monitor system. The system is usually programmed for six types of white-noise burst stimulus trials: no stimulus (background, 60 dB), pre-pulse (70 dB), pulse (100 dB and 120 dB), pre-pulse plus pulse (70 dB + 100 dB and 70 dB + 120 dB). Each trial type is presented ten times in ten blocks. Stimuli are presented in random order to avoid order effects and habituation. The inter-trial interval can vary from 9 to 16 s. All animals are regularly handled before individual tests in order to minimize handling-related stress. Animals are pair matched according to baseline values into the experimental groups using the average of the response to 100 dB and 120 dB. The tested animals are restrained loosely in holders that are placed on a sensing plate transforming movements of the body (jerks) into an analog signal through an interface. Finally, the percentage pre-pulse inhibition measures are calculated as the difference between the pulse alone and multiplied by 100. Percentage scores are typically used to minimize the effect of individual variation of startle amplitude on pre-pulse inhibition (Mach *et al.*, 2008).

D. Performance on Y-Maze

Cognitive functioning can be tested using a Y-maze with averse motivation by a strong electric footshock, evaluating learning and spatial memory (Koupilova *et al.*, 1995). The Y-maze is a fully automated apparatus used for the study of behavior of laboratory rats. It is a plastic box consisting of a square start area (285 × 480 mm) separated by a Plexiglass sliding door from two trapezoid, black and white arms – choice area (140 × 324 mm). The grid-floor in the start and choice area is electrifiable. The animal (usually rat) is placed on the start area and after 48 s electric footshocks (60 V, 50 Hz, duration 0.5 s) are applied at 5 s intervals. The rats try to avoid the shock by escaping to one of two arms. In the case of a rat moving to the wrong (dark) arm, the rat fails to avoid further footshock. The animals are taught spatial discrimination with the preference of the black or white arm in the Y-maze. The latency to enter the correct arm is measured and the number of wrong entries is counted. Before exposure to nerve agent, the rats are trained to avoid footshock by moving to the correct (white) arm in the Y-maze. It usually takes 4 weeks of training to reach the criterion which was 80% or more correct averse behavior (moving to the correct arm) within less than 1.5 s. During the training, ten sessions (two trials/session) per week lasting 4 min are realized. The exposure starts the day after the animals reached this criterion. The latency time to enter the correct arm by nerve agent-exposed rats and the number of entry errors are compared to the values obtained from the control rats exposed to the pure air instead of nerve agent.

E. Performance on T-Maze

Cognitive functioning can be also tested using a T-maze, consisting of five segments, a starting and a goal compartment

to evaluate learning, spatial memory and spatial orientation (Koupilova and Herink, 1995). The rats are trained, with the food reward, to run through the maze in less than 10 s without entering the side arm. The time necessary to reach the goal box is recorded. Before exposure to nerve agent, the rats are trained to reach the goal box as soon as possible by moving to the correct segment in the T-maze. It usually takes 4–6 weeks of training to reach the criterion which was 80% or more correct behavior. The exposure starts the day after the animals reached this criterion. The time of reaching the goal box by nerve agent-exposed rats is compared to the values obtained from the same rats immediately before nerve agent exposure and from control rats exposed to pure air instead of nerve agent.

F. Performance on Morris Water Maze

The water maze is often used for the evaluation of effects of various compounds on memory functions, i.e. memory formation, consolidation and retrieval effects due to its advantages and broad utilization. The Morris water maze (WM) is a widely used measurement of visuospatial learning that has been demonstrated to have high validity in identifying cognitive effects of various brain lesions and the effects of drugs used to treat cognitive deficits (Morris, 1984; Myhrer, 2003). Special motivation such as food and water deprivation is not required for the WM performance. The effect of odor cue is eliminated in the WM. In addition, rats are forced to swim in the WM. They cannot choose whether or not to move, so failure to respond is not a confound (Shukitt-Hale *et al.*, 2004). The place learning version with submerged platform can be used for working memory tests (Myhrer, 2003). The WM can be used to measure spatial learning and memory in the case of the evaluation of cognitive impairment in rats because of mentioned advantages.

The rats perform cognitive tasks that require spatial learning and memory – the ability to acquire a cognitive representation of location in space and the ability to effectively navigate the environment in the WM (Shukitt-Hale *et al.*, 2004). Memory alterations appear to occur mostly in secondary memory systems and are reflected in the storage of newly acquired information (Bartus *et al.*, 1989; Joseph, 1992). It is thought that hippocampus mediates allocentric spatial navigation (i.e. place learning) and prefrontal cortex is critical to acquiring the rules that govern performance in particular tasks (i.e. procedural knowledge), while the dorsomedial striatum mediates egocentric spatial orientation (i.e. response and cue learning) (McDonald and White, 1994; Oliviera *et al.*, 1997).

The water maze consists of a black circular pool (180 cm diameter × 80 cm high) filled to a depth of 25 cm with water of room temperature (Raveh *et al.*, 2002). The pool is imaginarily divided into four same compartments numbered 1–4 clockwise. The black antireflective circular escape platform (15 cm diameter) is placed into compartment no. 1 or 4, 20 cm off the pool wall. The platform is sunk 2 cm below water surface, so it is not visible from the rats' view owing to the water mirror effect. The yellow rectangle (30 cm × 40 cm) is fixed on the pool wall, immediately close to the platform, as the spatial conditional cue (Robinson *et al.*, 2004). Its place is variable according to the platform. Another dark rectangle is randomly fixed on the pool wall in different compartments (without platform) as the negative conditional cue. Round the pool, there are several stable extramaze cues in the room that the rat could use to navigate the maze (Morris, 1984). However, the impact of extramaze cues is not significant due to high maze walls.

III. LONG-TERM BEHAVIORAL EFFECTS OF ACUTE HIGH-LEVEL EXPOSURE TO NERVE AGENTS

Much of the data regarding long-term neurological sequelae to exposures to cholinesterase inhibitors in humans have been gathered following accidental exposures to organophosphorus (OP) compounds (pesticides as well as nerve agents). Nevertheless, the extrapolation from these exposures to prediction of effects from nerve agent is difficult because:

- the cholinergic crisis caused by acute, severe intoxication with the OP pesticides is generally much longer than that caused by OP nerve agents
- OP pesticides-induced delayed peripheral neuropathy can be caused by nerve agents only at doses many times greater than the LD_{50} (Davis *et al.*, 1960)
- a delayed manifestation of OP poisoning has not been described after administration of nerve agents to animals or in the instances of nerve agent poisoning in humans (Sidell, 1997).

There have been descriptions of the acute effects in humans that follow high-dose exposure ($\geq LD_{50}$) to nerve agents soman, sarin, and VX (Inoue, 1995; Nakajima *et al.*, 1997; Nozaki *et al.*, 1995; Sidell, 1974). The similar cluster of behavioral symptoms (anxiety, psychomotor depression, intellectual impairment, and sleep disturbance) was observed in the immediate period following resolution of the acute signs of intoxication and then slowly faded with time, sometimes taking months to be fully resolved. The CNS symptoms noted following short-term exposure of humans to diisopropyl fluorophosphate (DFP) were excessive dreaming, insomnia, jitteriness and restlessness, increased tension, emotional lability, subjective tremulousness, nightmares, giddiness, drowsiness, and mental confusion. CNS symptoms were correlated with the depression of red blood cell acetylcholinesterase (AChE; EC 3.1.1.7) to 70 and 60% of original activity and they disappeared within 1 to 4 days (Grob *et al.*, 1947). It was also noted that more severely exposed individuals and those with multiple exposures tended to display persistent symptoms that included forgetfulness, irritability, and confused

thinking, although the duration of these persistent symptoms was never clearly defined (Holmes and Gaon, 1956). These CNS symptoms are virtually identical to those that have been reported to occur following high-level exposure to nerve agents. It was shown in the study of human sarin poisoning that sarin-induced behavioral effects were virtually identical to those reported for DFP. These effects coincided with the depression of plasma ChE and red blood cell AChE activity to approximately 60 and 50% of original activity (Grob and Harvey, 1958). The behavioral symptoms such as anxiety, psychomotor depression, a general intellectual impairment consisting of difficulties in concentration and retention, and sleep impairment generally involving insomnia due to excessive dreaming were also described during human poisoning with nerve agent VX (Bowers *et al.*, 1976).

The exposure to high doses of OP compounds including nerve agents has been demonstrated to result in severe brain neuropathology that involves not only neuronal degeneration and necrosis of various brain regions (Lemercier *et al.*, 1983; McLeod *et al.*, 1982; Petras, 1981) but also persistent severe alteration in behavior and cognitive incapacitation especially impairments of learning and memory (Bushnell *et al.*, 1991; McDonald *et al.*, 1988). The most significant injury caused by OP poisoning is neuronal degeneration of the hippocampus that is associated with spatial learning and memory. Therefore, impairment of cognitive functions, especially incapacitation of learning and memory, belongs to the most frequent central signs of acute OP poisoning (Marrs, 1993; McDonald *et al.*, 1988). In addition, the adverse effects of OP compounds on cognition functions, such as learning and memory, may persist for quite some time after termination of toxicant exposure. The results from several studies have demonstrated the presence of OP compounds-induced learning impairments several days after the classic signs of OP toxicity have subsided (Buccafusco *et al.*, 1990; Bushnell *et al.*, 1991; McDonald *et al.*, 1988). Behavioral effects are typically evident before the occurrence of physical symptoms. These effects were associated with whole blood ChE inhibitions of >60%.

Several studies of the long-term effects of the sarin exposure victims from Japan have been published. Exposure to nerve agents in humans was found to produce effects that include cognitive deficits and memory loss (Hatta *et al.*, 1996; Hood, 2001; Okudera, 2002). Eighteen victims of the Tokyo subway incident were evaluated at 6 to 8 months after exposure (Yokoyama *et al.*, 1998). Sarin-exposed individuals scored significantly lower than controls on a digit symbol substitution test, and scored significantly higher than controls on a general health questionnaire (GHQ, psychiatric symptoms) and a profile of mood states (POMS, fatigue). The elevated scores on the GHQ and POMS were positively related to the increased PTSD (post-traumatic stress disorder) scores and were considered to be due to PTSD (Yokoyama *et al.*, 1998). There have been two brief reports of severely poisoned nerve agent victims (one sarin and one VX) in Japan who experienced retrograde amnesia, possibly due to prolonged periods of seizures and/or hypoxia (Hatta *et al.*, 1996; Nozaki *et al.*, 1995). Symptoms related to sarin exposure in Japan still exist 1–3 years after the incident and include fatigue, asthenia, shoulder stiffness, and blurred vision (Abu-Qare and Abou-Donia, 2002).

The existence of long-term behavioral effects following acute exposure to high doses of nerve agents was many times verified with the help of laboratory experiments on animals. There are numerous studies in animals showing that survivors of high-level OP exposure can experience subtle but significant long-term neurological and neuropsychological outcomes that are detectable months or even years following the recovery from acute poisoning (Brown and Kelley, 1998). Exposure of animals to nerve agents was shown to produce neurotoxicity in the CNS areas associated with cognition and memory functions (Koplovitz *et al.*, 1992; Petras, 1994). There are a few studies that revealed changes in the brain following sublethal nerve agent exposure that involve not only the cholinergic system but also the glutamatergic system (Lallement *et al.*, 1992; McDonough and Shih, 1997). Excitotoxic injury caused by increased levels of glutamate has repeatedly been shown to cause cognitive dysfunction (O'Dell *et al.*, 2000). Therefore, the disruption of cognitive functions, especially spatial and working memory, seems to be the most frequent and the most observable behavioral effect of nerve agent poisoning. The studies show changes in the brain following sublethal nerve agent exposure that lead to memory and attention deficits that normally involve the hippocampus (Hatta *et al.*, 1996; Miyaki *et al.*, 2005; Nishikawi *et al.*, 2001). The role of hippocampus in complex visuospatial learning and memory has been well established. The high concentration of NMDA and AMPA glutamate receptors, which play a key role in hippocampal-mediated learning and memory, also makes the hippocampus highly vulnerable to glutamate-induced excitotoxic injury from nerve agent poisoning (Filliat *et al.*, 2007; Lallement *et al.*, 1992; Shih *et al.*, 1990).

Following a high-dose exposure (above 0.5 LD_{50}) seizures are a prominent sign of nerve agent intoxication and these prolonged seizures can produce neural lesions (McDonough and Shih, 1997). Thus, neurological and behavioral deficits are predictable long-term effects following exposure to such doses of nerve agents. Animals exposed to high (convulsive) doses of nerve agent can develop spontaneous seizures, and display hyperactive and aggressive behavior and profound deficits in learning and/or performance of a variety of behavioral tasks. Animal studies have demonstrated deficits in acquisition of several types of operant tasks, performance of serial probe recognition task, maze learning, and passive avoidance learning following acute poisoning with nerve agents (McDonough *et al.*, 1986; Modrow and Jaax, 1989; Raffaele *et al.*, 1987).

The inhalation exposure to high-level sarin induced in rats impaired memory processes seen at 1 month post-exposure

with no recovery of cognitive function during the 6 month follow-up period. In the open field, sarin-exposed rats showed a significant increase in overall activity with no habituation over days. In a working memory paradigm in the water maze, the same rats showed impaired working and reference memory processes with no recovery. These data suggest long lasting impairment of brain functions in surviving rats following a single sarin exposure. Animals that seem to fully recover from the exposure, and even animals that initially show no toxicity signs, develop some adverse neurobehavioral changes with time (Grauer *et al.*, 2008). These findings are in accord with reports on long-term behavioral impairment following exposure to OP pesticides used in agriculture (Wesseling *et al.*, 2002). Similarly, long-term follow-up of victims of the sarin attacks in Japan demonstrated neurological as well as emotional and cognitive changes up to 7 years post-exposure (Miyaki *et al.*, 2005; Ohbu *et al.*, 1997; Yokoyama *et al.*, 1998).

Generally, according to high-dose exposure studies, animals exposed to nerve agents that exhibit seizures that are not promptly controlled develop brain damage and subsequent neurobehavioral problems. Animals that do not develop seizures or those that are rapidly and effectively treated with drugs that stop the seizures suffer no brain lesion and display no long-term neurobehavioral deficits.

IV. CHRONIC BEHAVIORAL EFFECTS OF SINGLE OR REPEATED LOW-LEVEL EXPOSURE TO NERVE AGENTS

Anticholinesterase compounds such as nerve agents can alter behavioral functions even after small subtoxic doses. There are very few data on human exposures. Based on the data describing the signs and symptoms in accidentally exposed humans, some long-term health effects, including behavioral effects of repeated subclinical exposures to OP compounds, were observed (Wesseling *et al.*, 2002). When the workers were exposed to small amounts of nerve agents they showed mild toxic signs of exposure including CNS effects such as insomnia, excessive dreaming, restlessness, drowsiness, and weakness (Craig and Freeman, 1953). It was shown that psychological symptoms are probably more common than usually recognized and may persist in more subtle forms for much longer (days, weeks) than physical symptoms (Sidell and Hurst, 1997). Recently, a dose–response association was found between low-dose exposure to sarin and cyclosarin inhalation during the 1991 Gulf War and impaired neurobehavioral functioning as well as subtle CNS pathology as revealed by MRI study (Heaton *et al.*, 2007; Proctor *et al.*, 2006). It is interesting that functional impairments were detected even in people who initially developed only mild or no signs of sarin or cyclosarin toxicity. These data correspond to the published epidemiological studies showing alterations in cognitive functions, impaired memory, and concentrations in humans after chronic low-dosage occupational exposure to OP insecticides (Parrón *et al.*, 1996; Stephens *et al.*, 1995). Increased reported forgetfulness and difficulties in thinking, exposure-related increases in work-related tension, sleep disturbance, restlessness, and nervousness have been documented among sheep farmers exposed to OP pesticides (Beach *et al.*, 1996; Stephens *et al.* 1995).

Based on the experimental animal data, the progression of signs, their neuropharmacological basis, and toxic consequence elicited from acute high-dose exposures have been well characterized (McDonough and Shih, 1993; Shih *et al.*, 2003). However, much less is known about the long-term effects of repeated low-dose nerve agent exposure. Several comprehensive reviews on the long-term health effects of exposure to low-level nerve agent exposure have been published (Moore 1998; Romano *et al.*, 2001).

It is known that a significant, clinically manifested AChE inhibition in the central nervous system leading to the neuronal degeneration of some brain regions including the hippocampus, associated with spatial learning and memory, is not necessary for clinically manifested cognitive impairments. This fact corresponds with earlier published data about neurological and neurophysiological outcomes detectable months or even years following recovery from acute OP poisoning (Savage *et al.*, 1988; Yokoyama *et al.*, 1998). It is very difficult to find the real reason for the memory impairments in the case of low-level nerve agent exposure. Recently, a temporal relationship has been demonstrated between OP-induced impairment in performance of a spatial memory task and the protracted decrease in the expression of cholinergic receptors in specific brain regions (including the hippocampus) following the asymptomatic exposure to OP compounds (Stone *et al.*, 2000). Nerve agent-induced impairment of cognitive functions is probably caused by subsequent desensitization and internalization of cholinergic receptors as a reaction of nerve agent-exposed organisms on hyperstimulation of cholinergic receptors, especially in parts of the brain with a high density of cholinergic synapses such as the hippocampus (McDonald *et al.*, 1988; Stone *et al.*, 2000). This means that a decrease in the number of cholinergic receptors in the hippocampus following low-level exposure to OPs without significant AChE inhibition could cause memory impairments.

In the available literature on repeated low-dose exposure to nerve agents, soman is the nerve agent studied most often. Mice, rats, guinea pigs, and primates were used to investigate repeated low-dose soman exposure. The effects of repeated soman exposures ranged from performance decrements on a well-learned compensatory tracking task (Blick *et al.*, 1994b) to development of attention deficits (Gause *et al.*, 1985) and hyperreactive responses to handling (Shih *et al.*, 1990). Unlike soman, the amount of literature regarding the effects of repeated low-level exposure to sarin is rather sparse and sometimes conflicting. Rhesus monkeys exposed to low levels of intramuscular sarin showed no signs of adverse health or long-term

behavioral effects (Burchfiel *et al.*, 1976). In contrast, it has been observed in rats and mice that intraperitoneal injections of subtoxic doses of sarin or soman decreased locomotor activity, altered behavior on the plus-maze and elevated horizontal bridge tests (Baille *et al.*, 2001; Nieminen *et al.*, 1990; Sirkka *et al.*, 1990). It was also shown that repeated low-level sarin inhalation in rats at clinically asymptomatic doses was disruptive to neurophysiological function and caused long-term memory impairments (Kassa *et al.*, 2001a, b).

The results of the study related to the measurement of sarin-induced alteration of behavioral and neurophysiological functions at 3 months following low-level sarin inhalation exposure of rats showed a significant alteration of mobile activity and gait characterized by ataxia and an increase in stereotypical behavior. These signs were observed in rats repeatedly exposed to sarin at clinically asymptomatic doses or singly exposed to sarin at doses causing mild muscarinic signs of exposure. These animals had awkward hindlimbs and their mobility was markedly diminished (Kassa *et al.*, 2001d). Spatial discrimination in the Y-maze was also altered in rats exposed to low levels of sarin. While spatial orientation of rats singly exposed to clinically asymptomatic doses of sarin was significantly influenced for a short time only (1 or 2 h following exposure), the rats repeatedly exposed to clinically asymptomatic doses of sarin showed a decrease in Y-maze performance for a relatively long time (until the third week following the exposure) (Kassa *et al.*, 2004). The significant impairment of spatial memory of rats exposed to clinically asymptomatic concentrations of sarin was also observed when cognitive functions were evaluated with the help of T-maze performance. Rats exposed to low-level sarin showed a significant decrease in T-maze performance for a short time (until the first day following the exposure). In addition, the effects of low-level sarin inhalation exposure were dose dependent. When the rats were exposed to low-level sarin causing moderate signs of poisoning, their time of passage through the maze was more lengthened at 1 and 2 h following the inhalation exposure compared to the rats exposed to clinically asymptomatic levels of sarin (Kassa *et al.*, 2001c).

A single exposure to another nerve agent, cyclosarin, at concentrations that do not produce convulsions or severe clinical signs of toxicity can also produce performance deficits on learned behavioral tasks. However, with repeated exposure, the deficits are not persistent and recovery is complete. In addition, exposure concentrations not producing any evaluated clinical signs of toxicity, other than temporary miosis (in the case of inhalation exposure), do not produce performance deficits on the behavioral tasks (Genovese *et al.*, 2006).

Reports in the literature of animal studies show that nerve agents can be administered repeatedly with minimal overt neurobehavioral effects if care is taken in choosing the dose and the time between doses (Sterri *et al.*, 1980, 1981). The repeated low-level nerve agent exposure made the cognitive impairments longer and higher compared to the single nerve agent exposure. The repeated exposure to low doses of soman can produce small, transient performance decrements only, probably due to the development of a physiological and behavioral tolerance to low levels of ChE activity (Blick *et al.*, 1994a, b). Nevertheless, a progressive and long-lasting inhibition of ChE in CNS following repeated administration of low doses of nerve agent soman was demonstrated (Hartgraves and Murphy, 1992). This study was corroborated by Olson using nerve agent sarin (Olson *et al.*, 2000). Generally, repeated or long-term exposure to low levels of nerve agents can cause neurophysiological and behavioral alterations (Abu-Quare and Abou-Donia, 2002).

The rats repeatedly exposed to sarin at doses corresponding to $0.5 \times LD_{50}$ (three times per week, s.c.) showed an increase in acoustic startle and a decrease in distance explored in the open field 2 weeks after sarin exposure. On the other hand, no effect of sarin exposure on passive avoidance was noted at the same time after sarin poisoning. Brain regional AChE was not affected at any time after sarin exposure, but muscarinic receptors were down-regulated in the hippocampus, caudate putamen, and mesencephalon in the sarin group at 2 weeks after sarin exposure. Thus, down-regulation of muscarinic receptors in the hippocampus as a reaction to acetylcholine accumulation at muscarinic receptor sites based on AChE inhibition can be considered a cause of behavior performance deficits, especially disruption of cognitive functions (Scremin *et al.*, 2003). In addition, protracted impairment of cognitive functions in rats exposed repeatedly to low-level organophosphorus compounds may be associated with a decreased rate of AChE recovery in the hippocampus (Prendergast *et al.*, 1997).

The results from several studies have demonstrated the presence of OP-induced learning impairments several days after the behavioral signs of OP toxicity have subsided (Bushnell *et al.*, 1991, McDonald *et al.*, 1988). Chronic exposure to OP compounds can also result in specific long-term cognitive deficits even when signs and symptoms of excessive cholinergic activity are not present (Prendergast *et al.*, 1998). Thus, the significant, clinically manifested AChE inhibition in the CNS leading to the neuronal degeneration of some brain regions including the hippocampus is not necessary for the clinically manifested cognitive impairments. This conclusion corresponds with earlier published data about neurological and neurophysiological outcomes detectable months or even years following recovery from acute OP poisoning (Savage *et al.*, 1988; Yokoyama *et al.*, 1998). A current study attempts to show a temporal relationship between OP-induced impairment in performance of a spatial memory task and the protracted decrease in the expression of cholinergic receptors in specific brain regions caused by asymptomatic exposure to an OP compound (Stone *et al.*, 2000). In addition, low-level

OP-induced memory impairment may be associated with a decreased AChE recovery in the hippocampus relative to the cortex. This decreased rate of enzyme recovery may contribute to hippocampal toxicity undelying protracted impairment of working memory and other cognitive functions (Prendergast *et al.*, 1997).

Repeated or chronic low-level nerve agent exposure can cause a prolonged inhibition of extracellular AChE leading to a prolonged increase in extracellular acetylcholine (ACh). The prolonged availability of ACh in the synaptic clefts results in feedback inhibition on muscarinic, presynaptic receptors to decrease further ACh release (Russell *et al.*, 1985). The greater ACh release in the nerve agent-exposed group may be due to the known down-regulation of muscarinic receptors in response to chronic nerve agent exposure (Churchill *et al.*, 1984). Neurochemical analyses showed that the normal brain neurotransmitter and receptor homeostasis is disrupted even at 10–12 days after 2 weeks of chronic nerve agent exposure at least in the striatum but probably throughout the whole cholinergic system in the brain (Shih *et al.*, 2006).

V. CONCLUDING REMARKS AND FUTURE DIRECTION

Exposure to high doses of nerve agents has been demonstrated to result in severe brain neuropathology that involves not only neuronal degeneration and necrosis of various brain regions but also persistent severe alterations in behavior and cognitive functions, especially impairment of learning and memory. The most significant injury caused by nerve agent poisoning is neuronal degeneration of the hippocampus which is associated with spatial learning and memory. Therefore, impairment of cognitive functions, especially incapacitation of learning and memory, belongs to the most frequent central signs of acute nerve agent poisoning. In addition, the adverse effects of nerve agents on cognitive functions, such as learning and memory, may persist for a relatively long time following the termination of nerve agent exposure.

Behavioral alterations and impairments of cognitive functions were found following acute exposure to nerve agents with the absence of any classic signs of cholinergic toxicity. It was shown based on the experimental results that not only convulsive doses but also clinically asymptomatic doses of nerve agents can cause subtle long-term neurophysiological and neurobehavioral dysfunctions. The neurological and neurophysiological outcomes are detectable months or even years following the recovery from acute poisoning. This probably means that systems other than the cholinergic nervous system can be involved in nerve agent-induced long-term signs of alteration of neurological and neurophysiological functions. Thus, it is necessary in the future to find new markers describing noncholinergic outcomes of low-level nerve agent exposure.

The long-term behavioral toxicity of nerve agents, especially the alteration of cognitive functions (T-maze, Y-maze, Morris maze test) due to nerve agent-induced delayed toxicity, seems to be connected with the neuropathological damage observed in the hippocampus. Thus, neuropathology of the hippocampus connected with the alteration of cognitive functions can occur after high-level as well as repeated or long-term low-level nerve agent exposure.

Neurochemical analysis of repeated or low-level nerve agent exposure provokes the suggestion that the prolonged nerve agent-induced alteration in brain chemistry may be a pharmacological basis for neurobehavioral changes. Thus, it is necessary to follow brain homeostasis during acute as well as chronic nerve agent exposure.

Repeated or long-term exposure to low levels of nerve agents can cause neurophysiological and behavioral alterations due to down-regulation of muscarinic receptors in the hippocampus as a reaction to acetylcholine accumulation at muscarinic receptor sites based on AChE inhibition. This phenomenon is considered to be the cause of behavior performance deficits, especially disruption of cognitive functions.

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CHAPTER 34

Cardiovascular System as a Target of Chemical Warfare Agents

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The views and opinions expressed are the author's and do not constitute official views of the US Department of the Army.

I. INTRODUCTION

Upon contact with toxicants, the heart reacts immediately by changes in its electrical homeostasis. Ancillary indicators, i.e. changes in the metabolites in body fluids, are time delayed and initially less useful for diagnosis and for selection of countermeasures. This chapter describes how the heart is affected by and reacts to various chemical warfare agents (Balazs, 1981; Baskin, 1991; Acosta, 2001; Simeonova and Fishbein, 2004). The first section gives an overview of the anatomy and functioning of the heart (Opie, 1998; Murphy and Lloyd, 2007) with emphasis on the constituents most vulnerable to toxic presence. The importance of cardiac neuronal changes as possible indicators of exogenous effects is underscored. The normal electrical state and signature of cardiac toxicity are described. This is followed in detail by the palette of military warfare agents, their modes of action at the cellular level, including description of their expression in the changed morphology of the tissue and modified electrical state (Romano *et al.*, 2008; Ellison, 2008). The effect on the heart of the different poisons is strongly dose related. The references give a good summary of the expected effects of each of the anticipated conditions. Electrocardiograms (ECGs) (Wexler *et al.*, 1947; Holstege *et al.*, 2006; Karki *et al.*, 2004; Delk *et al.*, 2007), illustrating the effect of the toxicants, are given where available. Recommended antidotes for the discussed toxicants are given.

II. BACKGROUND

A. Cardiac Anatomy

The timed delivery of oxygenated blood to all parts of the body is the function of the four-chambered pump, the heart. It is enclosed in a double-layered sac, the pericardium, with the inner layer, the visceral pericardium, anchoring the heart and the outer layer attached to the sternum. A cardiac skeleton anchors the four heart valves and the atria and ventricles. The thick wall separating the two ventricles, the interventricular septum, houses the Purkinje fibers, which play an important part in the electrical activity of the heart (Murphy and Lloyd, 2007).

Deoxygenated blood from the body enters the heart by the vena cavae and empties into the right atrium. Through the tricuspid valve blood then transits into the right ventricle and via the pulmonic valve enters the lungs, where it is oxygenated. It returns to the heart through the pulmonary veins and the atrium to the left ventricle. This muscular pump redistributes the oxygenated blood to all parts of the body.

The heart is mediated by an electrical system that is easily disturbed by toxicants changing the timing, flow, and magnitude of the electrical pulses and thus interfering with the required pumping action. The activity of the heart is myogenic, i.e. the activity is initiated by the heart itself. Various components of the heart, the sinoatrial (SA) node, atrioventricular (AV) node, and the Purkinje fibers are capable of pacemaker activity. Healthy atrial and ventricular tissue does not engage in pacemaker activity. The primary pacemaker is the SA node, but in diseased or affected states the secondary pacemakers take over.

The SA node lies in the right atrium, in an epicardial location and functions as the primary source of the electrical impulse formation. It consists of specialized muscle cells, smaller than ventricular cells containing few contractile elements. Due to its location, the action potential generated traverses the atria first, causing the primary contraction. The action potential is relayed to the AV node, the conduit for the electrification of the ventricles. The signal must pass through the AV node, otherwise the atria and the ventricles are electrically insulated from each other. A time delay in transmission is needed for the atria to be emptied of blood.

The AV node is a subendocardial right atrial construct that is located within a fibrous stroma in the triangle of Koch. It is connected to both sympathetic and parasympathetic nerves. The bundle of His arises from the distal section of the AV node and goes to the summit of the

ventricular septum. It is the only nonpathologic electrical conduction between the ventricles. The bundle of His divides into the left and right bundle branches which then become the Purkinje fibers that interweave the contractile cells of the ventricle and speed the excitation throughout the ventricles.

Action potential of the cardiac myocytes is initiated by inward sodium current in atria and ventricles with inward calcium currents contributing to the upstroke in the SA and AV nodes. Slow inward currents result in lower resting and activation potentials. In the latter, membrane potentials range from −40 to −70 mV and activation threshold lies in the range −30 to −40 mV and shows phase 4 depolarization. These cells are modulated by acetylcholine (ACh) and catecholamine. The duration of the action potential depends on the outgoing potassium current durations with the inward calcium currents of lesser importance. It is longest in the Purkinje fibers. Resting potentials of atrial and ventricular cells range from −80 to −90 mV, with activation thresholds in the −60 to −70 mV range. Impulse conduction ranges up to 300 V/s in atrial and ventricular cells and up to 900 V/s in the Purkinje tissue.

B. Innervation of the Heart

The autonomic nervous system guides the electrical and mechanical functions of the heart. The heart is innervated by both the sympathetic and parasympathetic systems, which have opposite effects and are activated reciprocally. They play important roles in arrhythmia susceptibility. Sympathetic stimulation originates from the intermediolateral column of the thoracic spinal cord. Its neurotransmitter, norepinephrine, is released from neurons of postganglionic fibers of stellate ganglia and epinephrine is released from the adrenal medulla. Both of these act on cardiac β adrenergic receptors. Sympathetic nerves are predominantly on the epicardial surface. Receptors for norepinephrine on cardiac muscle are of the β-adrenergic kind. Postganglionic sympathetic neurons innervate the SA and AV nodes, the conduction system and the myocardial fibers in the ventricles. Epinephrine, an amine, is the primary endogenous catecholamine produced in the adrenal medulla and regulates organic metabolism. It stimulates β_1 receptors, enhances ventricular contractility and enhances SA nodal cell phase 4 depolarization, that is, impulse generation (Lilly, 2003).

The cholinergic, i.e. parasympathetic system, acts through the vagal nerves by release of acetylcholine (ACh) that opposes the sympathetic stimulation. Parasympathetic preganglionic neurons originate in the medulla. Parasympathetic fibers terminate mainly on cells of the atria. Parasympathetic innervation is denser in the SA and AV nodes than the left ventricle. The right vagus nerve innervates the SA node. The neurotransmitter ACh and adenosine promote susceptibility to atrial fibrillation, and shorten atrial refractoriness. Excessive stimulation causes bradyarrhythmia. The left vagus nerve innervates the AV node where excessive stimulation results in AV block. Receptors for ACh are of the M2 muscarinic type and ACh binding to muscarinic receptors inhibits cAMP production. ACh has a negative chronotropic effect (slows heart), slows conduction (negative dromotropic effect), and also has a negative inotropic effect (decreases strength of contraction). The latter is through the activation of the current $I_{K,ACh}$ resulting also in shortening the action potential. ACh has three actions on cardiac muscle: (1) it activates ACh-sensitive K-current, $I_{K,ACh}$, (2) it inhibits the voltage, time-dependent inward calcium current I_{Ca}, and (3) it inhibits the hyperpolarization-activated inward current I_f, important for pacemaking. Ventricular muscle is not affected by vagal stimulation.

There are anatomical physiological differences between the sympathetic and parasympathetic systems. The parasympathetic ganglia lie within or close to the organ that postganglionic nerves innervate, while sympathetic ganglia lie closer to the spinal cord. There is limited parasympathetic innervation of the ventricles compared to the atria. The vagal nerve fibers are mostly intramural. The heightened adrenergic activation in the ventricles is potentially arrhythmogenic.

All catecholamine receptors are metabotropic. They act by initiating metabolic processes affecting cellular functions. β-adrenergic receptors, receptors for epinephrine, and norepinephrine act by stimulatory G proteins to increase cAMP in the post-synaptic cell. cAMP binds to and activates protein-kinase enzyme.

Disturbed balance between the parasympathetic and the sympathetic systems can result in disturbances in cardiac function. As discussed later, chemical warfare agents play an important role in disturbing this balance.

C. Neuropeptides

Vasoactive intestinal peptide (VIP), a neurotransmitter, is found in extrinsic and intrinsic nerves of the heart. VIP is released by the vagal nerve, and its effect is to increase I_f and pacemaker rates (Chang *et al.*, 1994; Accili *et al.*, 1996). VIP release takes place under high-frequency stimulation. As an internal brake, it limits the ability of ACh to excessively suppress the sinus node and other pacemakers. It also has an effect on the calcium-activated potassium channel.

VIP acts as a parasympathetic neurotransmitter in its involvement in the post-synaptic control of the heart. VIP is exactly opposite in its action to that of ACh. Vagal activity, causing release of ACh, slows the heart but co-release of VIP leads to tachycardia due to VIP preferring receptors leading to an increase in adenylate cyclase and accumulation of cAMP.

Neuropeptide Y (NPY) suppresses the pacemaker current I_f. Colocalized with norepinephrine in sympathetic nerve terminals on the heart, it is released with catecholamines during sympathetic neural activation. NPYs on cardiomyocytes have surface membrane binding sites and

suppress contractility in concentrations from 10^{-9} and above. In ventricular myocytes, the L-type Ca^{2+} current, as well as the delayed rectifier potassium currents, are suppressed.

D. Energetics of the Heart

The heart requires a continuous supply of energy to be able to sustain its pumping action. Most of the energy is derived from fatty acids. Under ischemic or anaerobic conditions, glycolysis comes into play consuming large amounts of glucose with the adverse effect of the formation of lactic acid (Jafri *et al.*, 2001).

More than 95% of the metabolic energy is used in the form of adenosine triphosphate (ATP). Its concentration in a myocyte is about 10 mM. ATP is synthesized by oxidative phosphorylation in the mitochondria. There, acetyl CoA is broken down to CO_2 and hydrogen atoms. Electrons are pumped out to form a proton gradient across the mitochondrial membrane. The protons reenter the mitochondria and combine with oxygen, eventually forming water.

The glycolytic pathway under hypoxic conditions produces only a limited amount of ATP. Pyruvate formed in glycolysis is transported into the mitochondria where CO_2 is formed through cellular respiration. From each glucose molecule, 28 ATP molecules are formed in anaerobic glucose metabolism. The heart also has an energy reserve in the form of phosphocreatine (PCr), which is an immediate precursor of ATP. In the reverse Lohman reaction, creatine kinase (CK) favors the maintenance of the ATP concentration at required levels. The energy released during hydrolysis of the phosphoanhydride bond in ATP powers energetically unfavorable processes, such as the transport of molecules against a concentration gradient.

During conditions of hypoxia, ATP is first degraded to adenosine diphosphate (ADP), then to adenosine monophosphate (AMP), and then to adenosine. The latter diffuses into the circulating blood where adenosine concentration rapidly increases under conditions of cellular damage. The action of adenosine is anti-inflammatory as well as inhibitory.

E. Electrophysiology

The sinoatrial node (SA), consisting of spindle-shaped cells, initiates the electrical activity of the heart. From its location in the right atrium in proximity to the superior vena cava, the electrical activity spreads to the atria whose cells are larger than those of the SA. The pulse from the atria spreads to the atrioventricular node (AV), the gateway to the ventricles. The atria and the ventricles are electrically isolated. The AV node also slows down the electrical activity giving the atria time to fill. The bundle of His is the upper end of the electrical path, which through the Purkinje fibers allows the electrical signal to activate the ventricles and thus to pump the blood.

Each cell's activity goes through four phases of the action potential. The morphology of each type of cell is different. Also, the kinds of electrolytes moved, and their quantity and speed are dictated by environmental conditions, detailed in Carmeliet and Vereecke (2002). Xenobiotic interference with the transmission of the ions changes the electrical homeostasis, and commences breakdown in the state of the tissue. The membrane currents, pump, and exchanger of importance in this regard are given in Table 34.1. Anionic chloride currents, especially swelling activated, play an important role in the case of cyanide intoxication (Baumgarten and Clemio, 2003).

III. SIGNATURES OF CARDIAC TOXICITY

A. The Electrocardiogram as Diagnostic Tool for Poisoning

1. Recorded Morphological Changes on the ECG

At present, morphological changes of ECG are insufficient to make a definite diagnosis on the nature of a poisoning. Without access to and comparison with a pre-incident ECG, the deductions may be erroneous. On the other hand, poisons cause definite changes in timing and morphology of the ECG of affected individuals (Delk *et al.*, 2007; Holstege *et al.*, 2006; Gussak *et al.*, 2004; Surawicz, 1995; Surawicz

TABLE 34.1. Membrane currents, pump and exchanger of importance

Current	Function/origin	Effect
I_{Na}	Voltage-gated Na^+ current	Depolarization
I_{K^+}	Voltage-gated K^+ channel	
$I_{Ca^{2+}}$	L-type Ca^{2+} current	
$I_{Cl^-\,swell}$	Activated by cell swelling	Cell swelling
$I_{K,ATP}$	Activated by fall in intracellular ATP	Fall in ATP
I_f	Hyperpolarization-activated current carried by Na^+ and K^+ in sinoatrial and AV node cells and His-Purkinje cells and contributing to phase 4 depolarization	
$I_{K,ACh}$	Parasympathetic control of the heart	ACh
Na^+/K^+-ATPase	Moves Na^+ out, K^+ into cell against concentration gradient using ATP for energy	Stimulated by α, β recap. via PKA, PKC
Na/Ca	Exchanges intracellular Ca^{2+} for extracellular Na^+	Responsible for DAD

and Knilans, 2008; Dalvi *et al.*, 1986; Zoltani and Baskin, 2007).

Disequilibrium in the electrolyte balance can provide diagnostic clues. For example, hyperkalemia causes tall T-waves in leads II, III, V_2 to V_4, when the potassium balance exceeds 5.5 mmol/l. In conjunction, the amplitude of the P wave is reduced and QRS is widened. Hyperkalemia is usually present when the amplitude of the T-wave is higher than that of the R-wave. With increasing potassium concentration, P-waves widen and eventually disappear. Accentuated hyperkalemia results in asystole.

Hypokalemia results in decreased T-wave amplitude and ST-segment depression; however, accurate QT interval measurement is difficult. Malignant ventricular arrhythmias result when potassium concentrations become very low. Hypercalcemia shortens the QT interval while hypocalcemia produces ST-segment prolongation.

Left bundle branch block (LBBB) is characteristic of poisoning and is defined by Zimetbaum *et al.* (2004) as QRS $>$ 0.12 s with delayed intrinsicoid deflection in the V_1, V_5, and V_6 leads greater than 0.05 s. The risk of arrhythmia is greatest when QRS is $\geq$0.11 s. Right bundle branch block (RBBB) greater than 0.12 s is a fairly good predictor of arrhythmic death.

Blockade of potassium, sodium, calcium channels, β-adrenergic receptor sites, and the Na^+/K^+-ATPase pump change the ECG and may give an indication of the type of poisoning present in the tissue (Delk *et al.*, 2007).

Reductions in the outward potassium ion flow prolong QT, a harbinger of Torsade de Pointes (TdP), and ventricular fibrillation (VF). Sodium channel blockers delay the entry of sodium ions, widening the QRS complex. In extreme cases, asystole ensues. A subsidiary event may be ventricular tachycardia degenerating into VF.

For control of tachyarrhythmia, calcium channel blockers are used. Blockade of these channels decreases contractility and SA and AV node depolarization. Blocking β_1 receptor sites within the myocardium reduces the intracellular calcium concentration and the contractility. The potassium concentration is increased, an undesirable effect when the β_2 receptors are blocked.

Inhibition of the Na^+/K^+-ATPase pump results in $[K^+]_o$ and intercellular $[Na^+]_i$ increases in turn increasing the intracellular calcium concentrations.

2. Long QT

An important predictor of arrhythmia is changes in the duration of the QT trace, the time for ventricular repolarization, displacement of the ST-segment, and changes in the pattern of T-waves that may sometimes be seen as a T and U wave (Roden, 2004, 2008). It can be linked to ventricular tachycardia, including TdP. Lengthened QT increases the time available for intracellular calcium accumulation, enabling early after-depolarization (EAD) in the Purkinje fibers, and activates calmodulin (CaM) and calmodulin kinase (CaMK). CaMK is believed to enhance after-depolarization, triggering TdP. CaMK increases L-type calcium channel activity. Anderson (2006) reported that inhibition of CaMKII can prevent cellular arrhythmia where QT-prolongation is present (Zoltani and Baskin, 2007).

During the plateau phase of the action potential, there is a delicate balance between the L-type Ca^{2+} channels and the slowly and rapidly activating cardiac delayed rectifier currents, I_{Ks} and $I_{Kr.}$ Due to the slow onset of I_{Ks} activation during the rectification of $I_{Kr,}$ a limited amount of repolarizing current flows. The L-type Ca^{2+} channels are time-dependently inactivated while an outflowing potassium current takes place. With a net outflow of ions, repolarization takes place. It is critical that the I_{Kr} deactivates slowly. Thus small changes in the inward or outward conductance, i.e. the amount of current leaving or entering, has a deciding influence on the length of the repolarization. The effect of poisons on these processes plays an important role in determining the QT-segment length.

B. Biochemical Markers of Tissue Injury

Chemical markers are released into the bloodstream following injury of the cardiac muscle. Three types of troponin, as well as elevated creatine kinase–MB (CK–MB) levels, are indicators of cardiac tissue damage (Apak *et al.*, 2005; O'Brien, 2008).

Cardiac troponin complex consists of three parts. Troponin T facilitates contraction, troponin I (cTnI) inhibits actin–myosin interactions and troponin C binds to calcium ions. Troponin I and T are specific to the heart. In the course of cell damage, cardiac troponin is released from myocytes, facilitated by increased membrane permeability that allows smaller troponin fragments to traverse the membrane.

In cancer patients undergoing high-dose chemotherapy, elevated troponin I and CK–MB are predictors of ventricular systolic dysfunction, and thus indicators of cardiac damage. Immunological ultraviolet-array is used for the determination of the level of CK–MB, where an elevation greater than twice normal (Hochholzer *et al.*, 2008) is taken as an indicator of myocardial infarction. Cardiac troponin is measured by electrochemiluminescent immunoassay. According to the European Society of Cardiology/American College of Cardiology, troponin values greater than 0.03 μg/l are taken as pathogenic. Cardiac impairment is indicated by the presence of troponin. The release of the enzymes has been linked to a mismatch between oxygen demand and expenditure in the tissue, lessening of the time available for diastolic coronary perfusion, catecholamine release, and intense sympathetic stimulation. Warfare agent associated cardiac toxicity is characterized by these conditions.

Is the increased level of troponin due to damage to the heart muscle? In-depth studies by Jensen *et al.* (2007) and by Blich *et al.* (2008) note that stroke victims without acute myocardial infarction have elevated troponin levels. Jensen and colleagues, in fact, state that there is ''no convincing evidence that the release of troponin is due to neurologically

mediated microvascular change'' in cases where there were no cardiac symptoms before the stroke. Thus, until convincing proof is presented, increase in the presence of marker enzymes can be taken as an indicator of damage due to toxins in the cardiac tissue.

Creatine kinase is a protein necessary for ATP generation. One of its forms, CK–MB, is found mainly in the myocardium and upon tissue damage, such as myocardial infarction (MI), becomes elevated. It takes up to 24 h for the elevated level to reach its peak. A difficulty resides in the fact that unlike troponin, an assay does not allow us to distinguish between cardiac and skeletal muscle damage. Also, in about a third of MI cases while CK–MB levels stay neutral, troponin elevation is noted. The normal level of troponin in the blood is less than 0.3 μg/l while for CK–MB it is less than 3.0 ng/ml. For humans, the CK is in the range of 55–170 IU/l (international units per liter) and is less specific than CK–MB for cardiac tissue damage.

IV. INDICES OF THE TOXICITY OF WARFARE AGENTS

A. Classes of Warfare Agents

Three broad classes of warfare agents are considered: organophosphate nerve agents (Newmark, 2007; Munro *et al.*, 1994), the cyanides (Baskin *et al.*, 2009), and a third general category that is less weaponized but whose effects are important as potential terror agents, the arsenics and ricin.

The classes of nerve agents of primary interest are the ''G'' agents, first synthesized in Germany. Denser than air, these agents hug the ground and represent a vapor hazard due to their volatility. Tabun (GA), sarin (GB), and primarily soman (GD) are the ones most widely used. ''V'' (venomous) agents were developed in the United Kingdom. VX is more toxic than the G agents. Cyanides are less weaponized but also of concern. Arsenic and ricin are more terror threats than battlefield weapons. Novichok, developed in the Soviet Union, is the most potent nerve agent but on which there is little publicly available information.

B. Background

Chemically induced cardiac failure has been the subject of a number of works. Balazs and Ferrans (1978), Baskin (1991), and Acosta (2001) give an overview of the subject. Hypoxia is one of the effects of the decreased availability of ATP which depresses contraction in the muscle. The energy that is supplied by the phosphate bonds is possible only as long as aerobic glycolysis and oxidative phosphorylation are maintained. Under anoxic conditions, this is no longer possible and with the adrenergic stimulus continuing, calcium accumulation in the mitochondria leads to impairment of function, eventually causing dose-related lesions (Suzuki, 1968).

C. Signatures of Toxicity

Warfare agent-supplied xenobiotics disturb cardiac homeostasis. The main changes of concern include the following:

- morphological tissue changes
- enhanced neural stimulation
- release of neuropeptides
- activation of usually dormant cardiac currents
- alteration in the storing and movement of ions
- disturbances in energy use and storage.

Ferrans *et al.* (1969) noted ultrastructural changes in myofibrils, including mitochondrial swelling and disorganization. Catecholamines also produce changes in the activity of oxidative enzymes. The decline in the activity progresses to the point where necrosis is evident. Ancillary effects include loss of myocardial potassium and an increase in interstitial fluid. Intracellular calcium overload also develops as noted by Fleckenstein *et al.* (1974). It has been hypothesized that necrosis due to catecholamine overload may be caused by a defect in energy supply needed for the maintenance of cellular processes.

At low concentrations, the catecholamines, epinephrine, and norepinephrine exert positive inotropic effects on the myocardium. High concentrations, however, can cause cardiac lesions (Balazs and Ferrans, 1978; Inoue *et al.*, 1998). Even physiologic concentrations, when extended over time, lead to cardiac damage as shown by Szakacs and Mellman (1960). The LD_{50} of norepinephrine in rats is 680 mg/kg, but at doses as low as 0.02 mg/kg, focal necrotic lesions are produced.

The oxidation product of catecholamine is adrenochrome whose accumulation has also been linked to myocardial necrosis, and morphological and subcellular alteration (Yates and Dhalla, 1975). Singal *et al.* (1982) showed that administration of adrenochrome to rats also induced heart arrhythmias. Free radicals may also contribute to these processes. It has been suggested that catecholamines may activate β-adrenergic receptors, stimulating adenylate cyclase, and thus elevating cAMP. This in turn activates protein kinase, increasing the phosphorylation of slow calcium channels possibly resulting in overflow of calcium leading to necrosis preceded by swollen sarcoplasmic reticulum (SR), altered enzymatic activities, and lower ATP.

Cyanide primarily blocks oxidative phosphorylation and ATP production. Every heart beat uses up to 2% of the energy available to the cell. Arsenic primarily causes long QT (LQT) interval on the ECG, by blocking the fast potassium current, an action that is a precursor to ventricular fibrillation.

The activation of dormant currents by the presence of xenobiotics, including cell swelling, radically changes the electrical homeostasis of the tissue (Zoltani and Baskin, 2007). Toxicities of major weaponized agents are given in Table 34.2.

D. Nerve Agents

There is a difference in distribution of nerve agents for different organs of the body as well as different locations within the heart. Roth *et al.* (1993) detail effects on the heart. Also, soman is deposited in ''depots'' where time-release effects cause unsuspected difficulties in treatment.

1. Mechanism of Action

Nerve agents are OP compounds, which irreversibly inhibit AChE, leading to ACh accumulation, and cause over-stimulation of muscarinic and nicotinic ACh receptors. The effect at the SA node, the primary heart control site, is inhibitory and bradycardia results. VX primarily affects neurotransmitter receptors, those of norepinephrine, and also affects the central nervous system (CNS) not related to AChE inhibition.

Toxicants change the homeostatic distribution and timing of the presence of electrolytes, enzymes and other constituents of myocytes and their environment. These changes affect and determine the current flow, in turn affecting the electrical state and possibly signaling the onset of electrical instability. Table 34.2 shows toxicities of weaponized nerve agents.

2. Electrocardiographic Signature of OPs

Disturbances in the electrical activity of the heart caused by xenobiotics are readily discernible in a surface electrocardiogram (Yurumez *et al.*, 2008; Dalvi *et al.*, 1986; Chuang *et al.*, 1996; Chhabra *et al.*, 1970).

OPs cause QT prolongation on the ECG that subsequently can degenerate into TdP. In one reported OP case, 79.7% had QT prolongations with ST segment and T wave abnormalities (Karki *et al.*, 2004; Rubinshtein *et al.*, 2002; Saadeh, 2001; Saadeh *et al.*, 1997). Changes are ultimately expressed in arrhythmia, ventricular fibrillation and TdP, and severe disturbance of the energy homeostasis of the heart.

TABLE 34.2. Toxicities of weaponized agents

Agent	LCt_{50} (mg-min/m^3)	ICt_{50} (mg-min/m^3)	LD_{50} (mg/kg)
GA	400	300	14.28
GB	100	75	24.28
GD	70		5.0
VX	50	35	0.1428
Cyanide	2500–5000		1.1

An initial indicator of an impending arrhythmia is prolongation of the QT interval. The lengthening can be caused by reduction in the outward currents or increased inward currents, i.e. imbalance of inward and outward currents during the second and third phases of the cardiac cycle, i.e. the $I_{Kr,}$ $I_{Ks,}$ the $I_{Ca(L)}$, and I_{Na} currents. These currents can generate early after-depolarization (EAD) and trigger activity at the end of repolarization. Primary sites are the Purkinje fibers and the mid-myocardial M cells. In the Purkinje fibers, at higher positive resting potentials than the ventricles, blockade is voltage dependent with increased block in the depolarized tissue. The failure of complete repolarization leads to dispersion of refractoriness and enhanced arrhythmogenicity. Acquired LQT involves pause-dependent or short–long–short RR interval sequences on the ECG, and enhancement of sympathetic nervous system tone. LQT is favored in cases of severe bradycardia, hypokalemia, and conditions that lead to early after-depolarization. LQT may not be a sufficient condition for TdP. Stimulation of adrenergic receptors plays a significant role but it can enhance or inhibit after-depolarization.

The electrical activity of the heart is modulated by hormones and neurotransmitters. Xenobiotics disturb their balance. The parasympathetic system releases ACh and the sympathetic system releases catecholamines (norepinephrine and epinephrine). These bind to α and β types of receptors. α_1-receptors are present on the post-synaptic member of the organ and mediate vasoconstriction and stimulation of Na^+/K^+-ATPase, the Na^+/Ca^{2+} exchanger, and the Na^+/H^+ exchanger. This affects the I_{KATP} and inhibits the I_{Na^+} and I_{to}. The α-receptor stimulation thus effectuates depolarization, and the α_2-receptor inhibits norepinephrine release.

Cardiac β_1-receptors and atrial and ventricular β2-receptors take part in positive inotropic response. Inhibition of catecholamines at β-adrenergic receptor sites interrupts the production of cAMP and inhibits calcium influx producing a negative inotropic effect that yields a reduction in the heart rate.

The main effect of adrenergic stimulation is to enhance the intracellular adenylyl cyclase activity. This in turn increases cyclic adenosine monophosphate levels. Protein kinase A is activated which modulates, i.e. phosphorylates, calcium and potassium channels. Phosphorylation of the calcium channel increases the inward current leading to early after-depolarization.

β_1-Adrenergic stimulation increases the activity of the Na^+/K^+ pump and inhibits early after-depolarization. Hyperpolarization of resting membrane potential counteracts automaticity, leading to a decrease in heart rate. α_1-Adrenergic stimulation hyperpolarizes membranes, and enables early after-depolarization and TdP by blocking potassium channels. Release of norepinephrine enhances α_1-adrenergic stimulation facilitating early after-depolarization (Schomig *et al.*, 1984). In long QT, increased sympathetic discharge may induce both bradycardia and TdP. Increased adenosine

increases ventricular refractoriness and sympathetic tone setting the conditions for re-entrant arrhythmias. Hypokalemia prolongs cardiac action potential and may be the precursor of early after-depolarization. The net outward potassium currents are reduced and the inward calcium current is increased.

3. Toxic Effects of OPs on the Heart

The anticholinesterase effects of OP nerve agents depend on the sites where they act. The muscarinic effects in the heart act through the parasympathetic system, while the nicotinic effects act through the sympathetic system. Agents like tabun, sarin, and soman, analogous to acetylcholine, are capable of changing the receptor sites, which increases the conductance of electrophysiological signals related to the enhancement of neuromuscular function. VX, on the other hand, counteracts the effect of ACh, interrupting neuromuscular function.

ACh is a neurotransmitter that is present in the parasympathetic nervous system and is stored in vesicles. Its function, under normal circumstances, is terminated by AChE that is present in both the pre- and post-synaptic membranes. OPs inhibit AChE by electrophilic (a reactant which accepts an electron pair from a molecule with which it forms a covalent bond) attack of the enzyme. The neurotoxicity of OPs was exploited in the development of nerve gases. The key to the understanding of OP inhibition of AChE is the enzyme's serine hydroxyl group behavior. Normally, it attacks ACh at the carboxyl carbon yielding a covalent bond between the enzyme and the ACh substrate. The transiently acetylated enzyme is next hydrolyzed and the active enzyme site is regenerated. The active serine hydroxyl group is attacked by the electrophilic phosphorus of the OP instead of the ACh. A covalent, nonhydrolyzable bond is formed between the enzyme and the OP, leaving the enzyme in an inactivated form. Release of an alkyl chain further strengthens the phosphorus–enzyme bond. With AChE inactivated, the amount of ACh present increases, resulting in overstimulation of the tissue. The pathophysiological effects that result can be explained by the overabundance of the ACh. Overstimulation of muscarinic receptors in the heart leads to bradycardia.

V. SPECIFIC WARFARE AGENTS OF CONCERN REGARDING THE HEART

A. Nerve Agents

The heart may be affected by both muscarinic and nicotinic effects. In the former, stimulation of the parasympathetic nerve endings, while in the latter, excess ACh on the nicotinic receptors, is of importance. The cardiovascular effects are tachycardia caused by the overstimulation of the sympathetic system, bradyarrhythmias, atrioventricular block, hypotension and QT prolongation, VF, and TdP (Grmec *et al.*, 2004).

OP intoxication manifests itself in three phases. First, a nicotinic phase hypertension and sinus tachycardia occurs. This is followed by sinus bradycardia and parasympathetic overstimulation and ST-segment changes on the ECG and rhythm disturbances. During the last phase TdP and sudden cardiac death occur. According to Ludomirsky *et al.* (1982), a QTc of 580 ms signals high probability of sudden cardiac death. Roth *et al.* (1993) and Kiss and Fazekas (1979) give further insight from actual cases.

A breakdown of actual cardiac symptoms for OP poisoning in hospital admissions is given by Karki *et al.* (2004) and also by Saadeh *et al.* (1997). Sixty-seven percent of the acute OP cases had QT prolongation, 24% experienced ST-segment elevation, and 17% had inverted T-waves. Nine percent had atrial tachycardia, 9% ventricular tachycardia, and 4% had ventricular fibrillation. Sinus tachycardia was observed in 35% of admissions while sinus bradycardia was noted in 28%. Noting that acidosis and electrolyte derangement play a major role in the development of cardiac events, they recommend ''atropine in adequate doses very early in the course of the illness'' as the strategy to be implemented.

OPs are known to induce time-delayed neurotoxicity. This is due to the inhibition of an esterase in nerve tissue, neuropathy target esterase (NTE), that is also found in muscle and blood cells. The NTE level in the blood is an indicator of the inhibition of the enzyme. Inhibition of NTE and aging, the process of following the OP binding to an active esterase site that prevents the reactivation of the site, is important for selection of an antidote against certain OP nerve agents. It is of primary concern for Novichok agent. There is little information available on OP-caused neurotoxicity and the cardiac toxicity.

1. VX

The most toxic nerve agent available in the West is VX. It is an inhibitor of AChE, which acts by increasing the ACh at the nerve synapses. Toxicity sets in when more than 50% of the AChE enzyme is inhibited. The $AChE_{50}$ value for VX is taken as 0.023 mg/kg for an oral dose (Sidell, 1974). Activity of RBC-AChE and plasma butyrylcholinesterase (BChE) are other markers of toxicity of OPs. In tests on human volunteers 1 μg of VX/kg by i.v. infusion showed a depression in AChE activity of more than 50% but for these tests no cardiac symptoms were recorded. VX produces intense stimulation of nicotinic ACh receptor ion channels and muscarinic ACh receptors. Though not recorded in human volunteer tests, cardiac effects are known to take place, based on animal studies. VX exposure produces positive inotropic effects. Arrhythmia was noted in rats and dogs (Robineau, 1987; Robineau and Guittin, 1987). In guinea pigs treated with VX, delayed after-depolarization was found (Corbier and Robineau, 1989). The effect of VX is ascribed to inhibition of the rat cardiac Na^+/K^+-ATPase α_1 isoform. At 1 μM concentration, the inhibition is 35% (Robineau *et al.*, 1991). Physostigmine, hyoscine, and HI-6 are under investigation as antidotes (Wetherell *et al.*, 2006, 2007; Kuca *et al.*, 2007; Munro *et al.*, 1994).

TABLE 34.3. Effect of OP on the electrophysiology of cardiac tissue

	Lesions				
AChE	**Anoxia**	**Acidosis**	**Modulated ion concentration**	**Release of catecholamines**	**Second messenger (VIP, others)**
ACh overload causes bradycardia, slows conduction in AV, prevents hydrolysis $[Ca^{2+}] \uparrow \rightarrow I_{K,ACh} \uparrow$	Lowers ATP, cAMP; ATPase inhibition $I_{K,ATP} \uparrow$ (activated), AP shortened	pH ↓, Na^+/H^+ exchange ↑, [ATP] ↓, $[K^+]_o$ ↑, reduces I_{Kr} by increasing rate of deactivation, shifts voltage dependence of activation to more positive potentials, $g_K \uparrow$	$[K^+]_o \uparrow$ effect on velocity of propagation, inexcitability	Prolongs AP, $[Na^+] \uparrow$, Na^+/K^+-ATPase antagonized DAD enhanced difference for α, β receptors	Adenylate cyclase activation
Antagonizes adenylyl cyclase		Cytoplasmic $[Ca^{2+}] \uparrow$ slows repolarization, reduces max. diastolic potential	$[Na^+] \uparrow Na^+/K^+$ pump inhibition, Na^+/Ca^{2+} exchanger $\rightarrow Ca^{2+}$ influx	α stimulation: reperfusion arrhythmia, calcium overload, gap junction conductance ↓, exchanger stimulation, activitates Na^+/K^+ pump	$I_f \uparrow$, cAMP ↑, → HR ↑OP reduces cAMP $\rightarrow Ca^{2+}$ influx, inhibits adenylate cyclase, stimulates ATP, $I_K \uparrow$, affects $I_{Ca(L)}$, EAD, DAD → arrhythmia
Arrests cAMP synthesis, depresses I_f (pacemaker current)		$I_{Ca(L)} \downarrow$, $I_{Na} \downarrow$ (inactivation of fast Na^+ channel), decreased excitability, CO_2 accumulation	$[Ca^{2+}] \uparrow Na^+/Ca^{2+}$ exchanger ↓reduced SR uptake		
ACh inhibits adenylate cyclase			$[Mg^{2+}] \uparrow$(hydrolysis of ATP), activates enzymes, reduces $I_{Ca(L)}$, I_{K1}, $I_{K,ACh}$, I_{KATP}, I_{Ks}	β stimulation: adenylate cyclase, elevates cAMP, increases calcium influx, I_f activation, triggered activity improves modal conduction	
			Intermediate stage		
			AF, VF		

g – conductivity of the tissue; [..] – concentration; $I_{(.)}$ – ionic current with the subscript denoting the channel type; ↑ – increase; ↓ – decrease; → – yields

2. Tabun (GA)

GA, a unitary chemical munition, inhibits AChE, the enzyme responsible for the breakdown of the neurotransmitter ACh. When inhaled, its toxicity is half that of sarin. It depresses plasma and RBC-AChE activities significantly in the blood. At 20–25% of red blood cell AChE baseline, the effect of the nerve agent becomes noticeable. There is no evidence of systemic toxicity other than the cholinesterase activity (Parker *et al.*, 1990; Munro *et al.*, 1994). GA has not been shown to produce OPIDN except at extremely high doses. The cardiac effect of GA conforms to OP-caused arrhythmias and AV block.

3. Sarin (GB)

Sarin was involved in terrorist attacks in Japan (Okumura *et al.*, 2003; Okudera, 2002). The increase in sympathetic and parasympathetic tone results in tachycardia, ST-segment modulation (Abraham *et al.*, 2001), and arrhythmia. Inhibition of cholinesterase within the neuroeffector junction also affects nerve impulse transmission by direct action. Direct action on muscarinic or nicotinic ACh receptors (Somani *et al.*, 1992) is observed when the blood level of sarin exceeds the micromolar level. Sarin inhibits RBC-AChE 80–100% as well as plasma-BChE between 30 and 50% (Grob and Harvey, 1958). It also binds to aliesterase, an enzyme that contributes to ester-link hydrolysis.

Sarin exhibits OP-delayed cardiotoxicity. Allon *et al.* (2005) suggest epinephrine-induced arrhythmias as a possible cause in cases of sarin intoxication. The hypothesis is supported by Khositseth *et al.* (2005) who showed that epinephrine changes T-waves in the ECG where AT prolongation already exists.

4. Soman (GD)

The most widely used nerve agent, soman (GD), exerts a defining effect on cardiovascular function. Myocardial degeneration and necrosis was noted by Britt *et al.* (2000) in soman-exposed rhesus macaques. Generally, upon contact, bradycardia and modulated cardiac output are followed by hypotension and changes in the ECG. Notably, AV conduction modulation, QT extension, T-wave and ST-segment changes characteristic of myocardial infarction, and ACh-induced coronary vasospasm are noted. For OP's cardiac effects, important references include Sidell (1974), Luzhnikov *et al.* (1975), Kiss and Fazekas (1979, 1983), and Anastassiades and Ionnides (1984). McKenzie *et al.* (1996) also showed that in swine a dose of $2 \times LD_{50}$ soman i.v. increased coronary sinus plasma ACh by 314% and decreased coronary blood flow to 55% of control. The evidence indicates OP-caused deaths are due to ACh-caused coronary vasospasms that culminate in MI. This is seen as VF on the ECGs. Recently, the Food and Drug Administration (FDA) approved pyridostigmine bromide as a pretreatment for soman poisoning (Newmark, 2007).

5. Novichok

In the open literature little is known about these agents developed in the Soviet Union. They are assessed to be five to ten times more toxic than VX (Ellison, 2008; Smithson *et al.*, 1995). The toxicity of these binary agents does not rely primarily on the inhibition of AChE, but it is thought that it causes permanent neuropathy. Consequently, conventional nerve agent antidotes may not work. Reactive oximes such as potassium 2,3-butanedione monoximate may be useful in detoxification. No published information is available on cardiac pathologies caused by Novichok agents.

B. Antidotes for OP Nerve Agents

Enzymatic hydrolysis is a primary route for elimination of nerve agents. Specifically, treatment for OP intoxication includes atropine, a muscarinic receptor antagonist, an anticonvulsant such as diazepam, and a cholinesterase reactivator, an oxime. It has been found that drug-induced inhibition of ACh release and accumulation in the synaptic cleft, such as adenosine receptor antagonist early in the OP intoxication, improves the chances of survival. Some AChE reactivators, such as bispyridinum oximes, HI 6 and HLö 7 with atropine, are quite effective.

C. Cyanide

Classified as a blood agent, cyanide is usually deployed as hydrogen cyanide and cyanogen chloride. Considerable literature exists on the effects of cyanide (Meredith *et al.*, 1993; Suzuki, 1968; Vick and Froehlich, 1985; Baskin *et al.*, 2009). Cyanide binds irreversibly to its target sites. In the human host it preferentially accumulates in the hypothalamus and neural tissue. Its concentration in red blood cells is much greater than in plasma. Lethal dose is of the order of 1 mg/kg or inhalation of 50 ml of hydrogen cyanide gas.

1. Toxicity

Cyanide binds to Fe^{3+} in heme-containing proteins. This inhibits the terminal cytochrome complex IV of the electron transport chain. The blocklock of complex IV by cyanide depletes ATP culminating in cell death. Oxygen is unable to reoxidize the reduced cytochrome a3. Thus cellular respiration is inhibited as well as ATP production, in essence depriving the cells, tissue, and ultimately the whole body of oxygen. Hypoxia evolves into metabolic acidosis and decreased oxygen saturation. The extent of lactic acidosis indicates the severity of the cyanide poisoning (Naughton, 1974). In a collapsed individual, plasma lactate is an indicator of cyanide poisoning (Baud *et al.*, 2002). In severe cyanide poisonings, up to 98% of the cyanide in the bloodstream is tightly bound to red blood cells. The ancillary response is myocardial depression and decline in cardiac output. Bradycardia, hypotension, and cardiac arrhythmia then develops into ventricular fibrillation and cardiovascular collapse.

In severe cyanide poisonings, autonomic shock due to the release of biogenic amines plays a role. The coronary arterial vasoconstriction, resulting in an increase in central venous pressure, leads to the observed shock-like state that is not attributable to inhibition of cytochrome oxidase (Vick and Froehlich, 1985). In the liver CN is metabolized by rhodanese to thiocyanate, which is excreted in the urine.

Hypoxia is one of the signatures of cyanide poisoning. One of its markers is that ATP declines to about 10% of the normal value. Since ATP forms the substrate for adenylate cyclase, the slow channels are affected lowering the slow calcium current and depressing the contraction. In CN poisoning, as in ischemia, oxidative metabolism is blocked and acidosis is enhanced. Acidosis depresses contractility and metabolism while sparing ATP supplies.

A dose of 0.54 mg of hydrogen cyanide per kg of body weight is fatal, with an average of 1.4 mg as shown by Gettler and Baine (1938). Their data indicate that the heart absorbs the second most amount of cyanide per organ weight. On the other hand, in sublethal exposure, cyanide-fed rabbits (Okolie and Osagie, 2000) do not show the hemorrhaging in the cardiac tissue noted by Suzuki (1968). Cyanide also causes decline in $[K^+]_i$, i.e. significant hypokalemia, and an increase in $[Na^+]_i$. These changes were not reflected in the skeletal muscle (Karzel *et al.*, 1974). Cyanide caused decline in ATP, the energy source of the cell, to less than 10% of the normal value, which activates the otherwise dormant potassium channel and the outward current I_{KATP}. These changes result in shortening of the AP and decrease in the contraction.

Cyanide also causes endogenous catecholamine release (Kanthasamy *et al.*, 1991; Kawada *et al.*, 2000; Schomig *et al.*, 1995; Inoue *et al.*, 1998). Inoue *et al.* (1998) also point out that cyanide-produced depolarization increases intracellular calcium due to the suppression of the potassium channels and activation of the voltage-dependent calcium channel. Anoxia induces suppression of the sodium pump and activates cation channels due to the decrease in ATP. A further consequence of the presence of cyanide in the tissue is inhibition of the Na^+/Ca^{2+} exchanger (NCX) (Ju and Allen, 2005). NCX is important for the pacemaker currents. Metabolic inhibition of NCX reduces the firing rate of pacemaker cells.

Cyanide poisoning is marked by metabolic acidosis and a large anion gap. The latter is a consequence of the blocked oxidative phosphorylation and the increased rate of glycolysis. Maduh *et al.* (1990) showed that cyanide also affects H^+ and thus the pH of the tissues. In turn, the Ca^{2+} transport process is disrupted, leading to a rise in cystolic $[Ca^{2+}]$. Acidification depolarizes the cell membrane and changes the potassium conductance.

Cyanide also exerts a strong influence on the vagus nerve and thus on the VIP that under normal conditions exerts a strong inotropic and chronotropic effect. VIP stimulates adenylyl cyclase activity. In ventricular myocytes, VIP potentiates voltage-gated Ca^{2+} channel currents and also acts on pacemaker currents.

As shown by Lederer *et al.* (1989) and later by Goldhaber *et al.* (1991), contractile failure, i.e. twitch shortening, is caused by cyanide. It is said to be due to failure of activation of the Ca^{2+} current. Electrocardiographic manifestations of CN poisoning are shown in Katzman and Penney (1993) and Wexler *et al.* (1947), described in Zoltani *et al.* (2004), and summarized in Table 34.4.

The ECG of an individual executed by inhalation of cyanic acid revealed that initially, between the first and third minutes, a heart rate slowing was discernible with the disappearance of the P-wave. Later the heart rate increased slightly. T-waves showed an increase in amplitude and a marked shortening of the ST-segment. One subject, unlike some others in this cohort, showed normal AV conduction until ventricular tachycardia and ventricular fibrillation developed.

2. Antidotes for CN Poisoning

Bhattacharya *et al.* (1995) and Cummings (2004) described the following approaches:

1. Use of a nitrite (oxidizing agent such as sodium nitrite) to change the ferrous ion of hemoglobin to a ferric ion. The created methemoglobin binds cyanide forming cyanmethemoglobin. One drawback, however, is that impairment of oxygen transport occurs, i.e. the amount of hemoglobin available is reduced. Amyl nitrite to generate methemoglobin is no longer preferred, since it does not bind enough cyanide.
2. Sulfur donors for conversion of cyanide to thiocyanate by rhodanese or other sulfur transferases, that is, a source of sulfur. For moderate poisoning, sodium thiosulfate is the usual choice.
3. Use of cobalt chemistry that chelates the cyanide directly such as hydroxycobalamin.
4. Hydroxocobalamins, precursors to vitamin B_{12}, are preferred in France and elsewhere in Europe. Hydroxocobalamin binds cyanide to form cyanocobalamin. It does not interfere with tissue oxygenation but large doses are required to be effective.
5. In the UK, dicobalt edentate, which chelates the cyanide directly, is preferred, but assurance that cyanide poisoning is present is needed since this antidote contains cobalt, which can be toxic.

VI. OTHER TERROR AGENTS

A. Arsenic

Arsine blood agents were first developed for battlefield use during World War I. Due to difficulties with dispersion, they were never used. Arsenic, however, has potential use as a terror agent (Ellison, 2008; Sidell *et al.*, 1997).

Arsine is the simplest compound of arsenic. It is colorless and 2.5 times denser than air with an odor resembling garlic. Arsine binds to hemoglobin of the red blood cells destroying them. Poisoning kills by allosteric inhibition of metabolic

TABLE 34.4. Effect of CN on the electrophysiology of cardiac tissue

P	QRS	ST	T	Remarks	Reference
Disappearance (auricular arrest)	Change in amplitude, right axis deviation	Shortening, disappear	Incr. amplitude, origin of T-waves on QRS	0.11–0.20 mg/kg NaCN, inhalation, AV dissociation, HR↓, BBB, initial bradycardia, AV block, asystole	Wexler *et al.* (1947) (man)
	Lengthened	Absent or depressed, elevated after 20 s	Increased, decreased over time, T starts on top of R, diphasic, negative	0.4–0.8 mg/kg NaCN HR↓, bradycardia, Wenckebach, heart block, V-flutter, VF	Leimdorfer (1950) (cats)
		Short, shifted toward T-wave	Tall T, surpassing R, sometimes inverted	0.1 mg/kg NaCN, bradycardia, incomplete AV blockade, VF	Paulet (1955) (dog)
	Abnormal	Elevated, shortened	T-wave beginning high on QRS	0.3–0.6 sodium nitrite as antidote with sodium thiosulfate, atrial fibrillation	DeBusk *et al.* (1969) (man)
	Narrow QRS	Nonspecific ST-T changes in anterior chest leads, shortened ST-segment	Fusion of T-wave into QRS, sharp rise of T-wave	Sinus tachycardia, acidosis with high anion gap	Chin *et al.* (2000) (man)
	Q-wave present in lead III, persisted after treatment of acidosis	Deviation of 4 mm in leads II, III, aVF, 2 mm in V_6, ST depression of 2–4 mm in V_1–V_4, I, aVL leads, normalization of ST-segment in precordial leads	T-wave inversion in II, III, aVF, V_5 and V_6 leads	Acidosis, CN level of 3 μg/ml, anion gap present	Sanchez *et al.* (2001) (man)

↑ – increase; ↓ – decrease.

enzymes. Arsenic disrupts ATP production. Arsenic inhibits pyruvate dehydrogenese, uncoupling oxidation phosphorylation. Arsenic poisoning also occurs through arsenic–oxygen compounds, especially arsenic trioxide, As_2O_3 (ATO), which is 500 times more toxic than pure arsenic.

ATO has been effectively used as a remedy for relapsed acute promyeloctic leukemia, but with the side effect that it causes QT interval prolongation, possibly heralding ventricular arrhythmia (Chiang *et al.*, 2002). Abnormalities in $I_{Ca(L)}$ in myocytes were also noted (Sun *et al.*, 2006). ATO's direct effect on cardiac repolarization with its effect on I_{Kr} was also noted by Haverkamp *et al.* (2000). ATO also causes cellular Ca^{2+} overload and augments APD (Yamazaki *et al.*, 2006). Chronic arsenic exposure leads to QT prolongation blockage of I_{Kr}, TdP, and T-U alternans, and changes in the T-wave result (Little *et al.*, 1990; Ficker *et al.*, 2004).

It has been suggested that potassium ion channel alteration induced by arsenic may be related to hERG trafficking defects. ECG changes in arsenic poisonings have been reported by Fennel (1981) and also by Glazener *et al.* (1968). The T-waves are domed. ECG changes, especially where arsenic involvement is not severe, are reversible. Hemodialysis and BAL (dimercaprol) therapy has been found to be effective. Sun *et al.* (2006) in a recent publication suggest that choline can normalize QT interval abnormality by inhibiting $[Ca^{2+}]_i$ and $I_{Ca(L)}$ in ventricular myocytes when ATO is present. Arsenic intoxication results in widened QRS by 0.06 s and prolonged QT (Ahmad *et al.*, 2006). Ventricular tachycardia and VF have been reported by Goldsmith and From (1980) and St Petery *et al.* (1970).

B. Ricin

Ricin, a toxic glycoprotein derived from the castor bean, causes hypotension and myocardial hemorrhage. The Center for Disease Control and Prevention (CDC) lists it as a category B agent due to its easy availability as a terrorist weapon. Only a limited amount of information is available

in the open literature on ricin's effect on the heart. The medical files of Georgi Markov, the Bulgarian journalist, assassinated in London with what was assumed to be ricin, are not publicly available (Crompton, 1980).

Ricin is a glycoprotein made up of two chains linked by a disulfide bond. Its toxicity results from one of the chains inhibiting protein synthesis by irreversibly inactivating eukaryotic ribosomes (Audi *et al.*, 2005). The lethal dose of ricin has been set at 1 to 20 mg/kg of body weight (Bradberry *et al.*, 2003). Christiansen *et al.* (1994) and Ma *et al.* (1996, 1995) performed extensive experiments on rabbits. Their main findings include the following:

1. Ricin caused vasodilatation and increased endothelial-dependent vascular relaxation resulting in hypotension.
2. Ricin disturbed calcium homeostasis leading to cell necrosis.
3. Ricin reduced both systolic and diastolic left ventricular function.
4. Ricin caused myocardial hemorrhage.

Balint (1974) and later Zhang *et al.* (1994) found that at the lethal dose in rabbits, ricin caused hemorrhage and necrosis. Christiansen *et al.* (1994) found that the release of norepinephrine from sympathetic nerves in the vasculature is not impaired by ricin. The CDC, under signs and symptoms of ricin poisoning that may be encountered, cite cardiovascular collapse (hypovolemic shock).

ECG abnormalities in children who ingested castor beans have been noted by Kaszas and Papp (1960). These include QT interval lengthening, repolarization changes, and intraventricular conduction disturbances. Crompton (1980) later reported on experiments on pigs that experienced hemorrhagic lesions and an abnormal ECG due to ricin. At the present time, no antidotes or effective therapy are available to counteract the effects of ricin.

VII. CONCLUDING REMARKS AND FUTURE DIRECTION

Per organ weight, the heart is the second most preferred depository for several of the warfare agents used. Disturbance of the electrical homeostasis, the cellular energy production, and breakdown of the electrical control network within the tissue are mostly affected.

Areas that need further insight include the following:

- time-delayed effect of OPs
- means of reactivation, i.e. overcoming ''aging'' of OP-based agents
- the effect of genetic factors and state of the fitness of the cardiac system in combating the effect of xenobiotics
- mechanism of toxicity involving neuropathy target esterase (NTE).

Human data for various cardiotoxic scenarios are unavailable. These include, at the time of this writing, ricin poisoning. The means of prompt identification of the particular xenobiotic causing poisoning remains an urgent task. Progress in metabolomics, the identification biomarkers that result from metabolic changes caused by the presence of xenobiotics, will enable the development of chip-based rapid-responding assaying devices.

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CHAPTER 35

Skeletal Muscle

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I. INTRODUCTION

Skeletal muscle is a target organ for a variety of chemicals. Adverse or toxic effects on skeletal muscles can range from minor muscle weakness or slight pain to complete paralysis. Next to the brain, skeletal muscles are major targets for the toxicity of organophosphate (OP) nerve agents. Morbidity and mortality associated with OP intoxication is due to the effects of these compounds on skeletal muscles in general and muscles of respiration in particular. Deaths from overdose of OPs are due in part to respiratory paralysis by depolarizing neuromuscular blockade. Understanding the skeletal muscle system in the context of OP poisoning is interesting, yet very complex because muscles containing different fiber types often respond differently even to the same OP compound. The distinct features of slow and fast muscles are the most fascinating aspects of skeletal muscles in this area of research.

Skeletal muscles are enriched with cholinergic as well as noncholinergic elements that are directly or indirectly modulated by OP nerve agents. Motor innervation plays an important role in the regulation of many properties of skeletal muscles, including neuromuscular activity. Changes in the activities of acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) appear to greatly modulate neuromuscular activity and can modifies neuromuscular transmission. At the cholinergic synapse, AChE plays an important role in the removal of acetylcholine (ACh) from the synaptic cleft. Inhibition of this enzyme by compounds such as OP nerve agents profoundly modifies neuromuscular transmission as seen in twitch potentiation, fasciculation, muscular weakness, and muscle cell death by necrosis or apoptosis. Being rich in metabolism, skeletal muscles are very vulnerable to OP-induced oxidative/nitrosative stress due to excess free radical generation. In the past two decades, interest in skeletal muscles has been enormous because of their involvement in intermediate syndrome and tolerance development related to the toxicity of OPs. OP-induced effects on skeletal muscles can occur at one or multiple sites (the nerve fiber, the nerve terminal, the junctional cleft, the motor endplate, or the myofibrils). This chapter describes structural and functional aspects of skeletal muscles in the context of OP nerve agents' toxicity.

II. BEHAVIORAL EFFECTS

In general, exposure to sublethal signs-producing doses of an OP nerve agent exerts prominent motor, behavioral, and autonomic symptoms. The motor symptoms are fasciculations, fibrillations, and body tremors. Fasciculations and fibrillations are due to antidromic neural discharge from excess junctional ACh, while tremors are of a central origin (Gupta *et al.*, 1986; Misulis *et al.*, 1987). A comparative description of behavioral toxicity of OP nerve agents (soman, sarin, tabun, and VX) from experimental studies is provided here in brief. Different nerve agents require different concentrations to produce equitoxic effects, degree of AChE inhibition, and myonecrosis. Acute symptoms of equal severity in male Sprague-Dawley rats by soman, sarin, tabun, and VX can be achieved at a dose of 100 μg/kg, 110 μg/kg, 200 μg/kg, and 12 μg/kg, s.c., respectively (Gupta *et al.*, 1987a, b, 1991). Based on the equitoxic doses, VX is the most toxic and tabun is the least toxic OP nerve agent.

Rats receiving an acute dose of soman (100 μg/kg, s.c.) exhibit onset of toxicity such as salivation, muscle fasciculations, and severe tremors within 5–15 min. Within 20–30 min, signs of toxicity are maximal and can persist for 4–6 h. Thereafter, the intensity is reduced to a mild form but the signs of toxicity can still be observed after 24 h. Rats usually become free of obvert signs after 72 h. With VX, onset of symptoms and appearance of maximal severity are delayed by approximately 20 min compared to other nerve agents (Gupta *et al.*, 1987a, b, 1991). During peak toxicity of soman, signs such as complex posturing movements and tremors are indicative of pronounced CNS effects as compared to the moderate peripheral muscle fasciculations. Muscle fasciculations superimposed upon this activity are less prominent than the signs of gross motor unit activity. This is in contrast to the OP compound diisopropylphosphorofluoridate (DFP), which exerts greater peripheral activity with fasciculations and fewer central toxicity signs (Gupta *et al.*, 1985, 1986, 1987a, b). This suggests that AChE inhibitors differ in their propensity to produce central or peripheral effects and that peripheral effects are required for muscle necrosis (Gupta *et al.*, 1985, 1986, 1987a, b; Misulis *et al.*, 1987).

III. CHOLINERGIC SYSTEM

Key elements of the cholinergic system include a neurotransmitter, ACh, an enzyme, AChE, that hydrolyzes ACh, and an enzyme, ChAT, that synthesizes ACh. All skeletal muscles contain these cholinergic components, but their quantities can significantly vary from muscle to muscle, i.e. fast fiber containing muscle has greater values than the slow fiber or mixed fiber containing muscle.

A. Normal Activity of AChE and its Molecular Forms

Muscle acetylcholinesterase (AChE, E.C. 3.1.1.7) is partly concentrated in the endplate region, i.e. 20–40% of the total amount found in the whole muscle (Hall, 1973; Younkin *et al.*, 1982). Using histochemical technique, Müntener and Zenker (1986) demonstrated the presence of AChE in the sarcoplasm of limited areas in sections of normal rat muscles. Normal activity of AChE varies from muscle to muscle, i.e. higher AChE activity is found in the fast fiber (type II fibers in general, and type IIB fibers in particular) than in the slow fiber (type I fiber) containing muscle. Enzyme activity in mixed fiber muscle is found in between the values of slow and fast muscles (Table 35.1). AChE plays an essential role in the removal of ACh at the neuromuscular junction (NMJ). Inhibition of AChE activity, which results in accumulation of ACh, profoundly modifies neuromuscular transmission by producing fasciculations and twitch potentiation. At high rates of stimulation, the muscle is unable to maintain a normal contraction, and muscle hyperactivity often ensues in muscle fiber necrosis.

It is well established that AChE exists in nerves and muscles in a range of globular and asymmetric molecular forms. A wide variety of sedimentation profiles have been established for AChE molecular forms in different mammalian muscles (Massoulie and Bon, 1982). The variations seen in the ratios of these molecular forms between different muscles are wide and complex. Qualitative and quantitative variations exist among different species, as well as young versus adult (Barnard *et al.*, 1984). In the rat extensor digitorum longus (EDL, a fast twitch muscle), the G_1 (4S), G_4 (10S), and A_{12} (16 S) molecular forms are predominant, while in soleus (SOL, a slow twitch muscle) and diaphragm (a mixed muscle) a fourth major molecular form is also present, the A_8 (12S). In rat SOL, the majority of the total AChE activity is contributed by the 12S and 16S forms; whereas in the diaphragm 4S and 10S and in the EDL 4S (Grosswald and Dettbarn, 1983a, b; Patterson *et al.*, 1987) (Table 35.2).

In the rat, the 16S form is found in high concentration at the endplates and it is thought to be involved in neuromuscular transmission (Hall, 1973). The different molecular forms of AChE in SOL and EDL have apparent K_m values similar to that previously found in the diaphragm muscle (Hall, 1973; Grosswald and Dettbarn, 1983a, b). There appears to be no difference between catalytic sites of the molecular forms of AChE in fast EDL and slow SOL muscles, despite the different molecular form patterns and activity in three muscles.

B. Inhibition of AChE and its Molecular Forms by Nerve Agents

In general, following an acute exposure to OP nerve agents, signs of cholinergic toxicity appear within a few minutes and are caused by irreversible inhibition of AChE activity in neuronal tissues. Inhibition of AChE causes excess accumulation of ACh at central and peripheral synaptic sites leading to failure of neuromuscular, respiratory, and cardiovascular functions. For some OP compounds, a close relationship exists between severity of the toxic signs and the inhibition of AChE during the acute phase of intoxication. The observable toxic effects usually do not persist for more than 4–6 h, while recovery of AChE activity occurs at a much slower rate, such as 7–14 days or even 3–4 weeks depending on the tissue and the inhibitor.

Gupta *et al.* (1987a) demonstrated that selective inhibition of AChE activity in skeletal muscles was apparent within 1 h of soman administration (100 μg/kg, s.c.) in rats when SOL showed the maximum inhibition (87%), whereas EDL showed the least inhibition (47%). AChE activity in skeletal muscles was maximally depressed during the following 3–6 h, the time when animals showed severe signs

TABLE 35.1. Normal values of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity in rat skeletal muscles

Muscle	AChE activity (μmol substrate/g/h)	BuChE activity (μmol substrate/g/h)
EDL (fast fiber muscle)	105.8 ± 1.0	9.7 ± 0.8
Soleus (slow fiber muscle)	60.1± 1.2	10.3 ± 0.3
Diaphragm (mixed fiber muscle)	78.3± 1.6	10.2 ± 0.2

The substrates used were acetylthiocholine iodide and butyrylthiocholine iodide for AChE and BuChE activity, respectively

Each value represents mean ± SEM ($n = 5$–6)

TABLE 35.2. Total AChE activity and percent contribution of molecular forms to total AChE activity in rat skeletal muscles

			Molecular forms			
		Total activity	**A_{12} (16S)**	**A_8 (12S)**	**G_4 (10S)**	**G_1 (4S)**
EDL	w/ PI	93.8 ± 7.5	18.7 ± 1.8	–	26.4 ± 3.5	54.9 ± 4.1
	w/o PI	102.9 ± 4.7	18.7 ± 1.6	–	30.2 ± 2.8	51.1 ± 3.1
Soleus	w/ PI	53.6 ± 4.7	26.8 ± 1.9	33.7 ± 2.3	15.2 ± 1.0	24.3 ± 0.9
	w/o PI	61.4 ± 5.3	25.9 ± 2.2	33.0 ± 2.2	16.3 ± 1.3	24.8 ± 1.4
Diaphragm	w/ PI	81.4 ± 6.1	26.9 ± 1.4	14.2 ± 0.9	28.8 ± 0.3	30.1 ± 1.7
	w/o PI	89.5 ± 4.9	24.6 ± 1.8	14.8 ± 0.9	29.7 ± 1.7	30.9 ± 1.5

Activity values are expressed as μmol ACh hydrolyzed/g/h tissue for total activity and as a percentage of the total distribution for molecular forms. Values are the mean ± SD ($n = 6$ to 8 muscles), w/ PI – protease inhibitors present; w/o PI – protease inhibitors absent. No statistical significance was found comparing the presence vs absence of protease inhibitors for values of the individual molecular forms or total activities

of toxicity. Recovery was clearly apparent during 3–7 days in all three skeletal muscles (SOL, EDL, and diaphragm). Seven days after soman treatment, enzyme recovery was >90% in EDL and diaphragm compared to 75% in SOL (Gupta *et al.*, 1987a). Within 1 h, all molecular forms of AChE were reduced to less than 10% of control in SOL and diaphragm. In EDL the 16S form, mainly localized at the NMJ, was not affected at this time, while the 10S form was completely inhibited and the 4S form was reduced to 50%. Further inhibition was seen after 24 h but even then the 16S form was the least inhibited in EDL. After 3 days of soman treatment, the AChE molecular forms in all three muscles showed signs of recovery. This was particularly evident in the 16S form in EDL and diaphragm where it appeared to be approximately 75% of controls. In the SOL the 16S form had recovered to only about one-third of the control when measured after 3 days. Seven days after soman, the 16S and 4S forms in the EDL and diaphragm had fully recovered. The activities of the 10S EDL form and the 10S and 12S diaphragm forms were still reduced, while in the SOL the activity of all forms remained below the control.

In similar experiments, tabun (200 μg/kg, s.c.) produced differential AChE inhibition in various skeletal muscles (SOL = diaphragm > EDL) which was similar to that seen with soman (Gupta *et al.*, 1987a, b). The varying degrees of AChE inhibition in the skeletal muscles treated with tabun, however, correlate well with the observed difference in the number of myonecrotic lesions, i.e. the greater the AChE inhibition (during the initial 24 h period), the higher the number of lesions found in diaphragm and SOL. EDL, with a low level of AChE inactivation, had the lowest number of lesions. This observation was in agreement with a similar pattern of histochemical observations following soman and sarin (Meshul *et al.*, 1985; Gupta *et al.*, 1987a, 1991).

The reason for this difference in susceptibility to a particular nerve agent may be due to: (1) variations in location of AChE in different muscles (Grosswald and Dettbarn, 1983a), (2) changes in ACh release due to

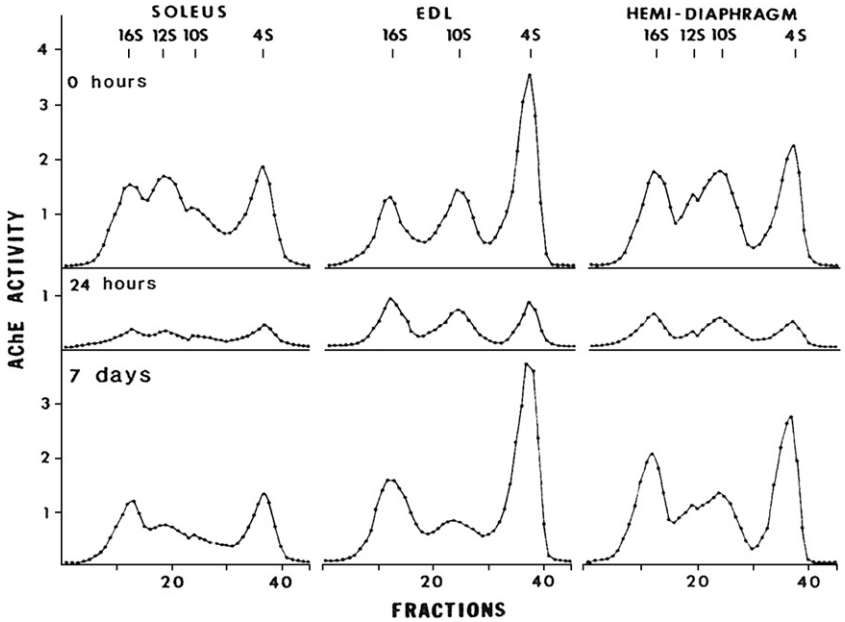

FIGURE 35.1. Representative profiles of the activity of the AChE molecular forms in soleus, EDL, and hemidiaphragm muscles. Profiles at the top of each column are from untreated muscles followed by profiles of activity of AChE molecular forms of muscles 24 h and 7 days, respectively, after receiving an acute dose of soman (100 μg/kg, s.c.). The AChE activity scale is in arbitrary units based on the μmole substrate hydrolyzed/min by the enzyme activity in each fraction. The sedimentation values of the AChE molecular forms are given in the profiles of untreated muscles above the associated peaks. Sedimentation values were determined by the location of the added sedimentation standards, β-galactosidase (16.0 S), catalase (11.1 S), and alkaline phosphatase (6.1 S), following velocity sedimentation of the gradients.

a different firing pattern (Misulis *et al.*, 1987), and (3) pharmacokinetic variables which influence the delivery of a particular OP compound. With tabun toxicity, inhibition and recovery of AChE molecular forms, especially the 16S form associated with the neuromuscular junction (endplate region), corresponded well with the appearance and disappearance of necrotic lesions. The delay in the molecular form's return to normal is due to the fact that the heavier molecular forms (16S, 12S, and 10S) are based on an assembly of the monomeric 4S form. Synthesis of the 4S form is increased when the assembly of the heavier forms lags behind.

Following an acute exposure to sarin (110 µg/kg, s.c.), AChE activity in skeletal muscles was reduced to 23% in SOL and 48% in diaphragm within 1 h, while EDL AChE was significantly unaffected. By 24 h, however, a still greater inhibition was seen for these muscles, and in EDL, AChE activity was reduced to 43%. In an early phase, recovery of AChE was rapid, but still not complete when measured after 7 days of treatment. Activities of the AChE molecular forms, after 1 h of sarin injection, were significantly depressed in SOL and diaphragm, while those in the EDL showed significant inhibition only after 24 h. By day 7 in SOL, activities of the 4S and 10S molecular forms of AChE had recovered to higher than control levels, while in diaphragm a significant shift toward the 4S molecular form had taken place so that the AChE profile resembled that of a control EDL. In EDL, the activity of 4S and 16S molecular forms recovered at a faster rate than the 10S molecular form.

Within 1 h of VX administration (12 µg/kg, s.c.), AChE activity of all three skeletal muscles was reduced to between 8 and 17% of control with incomplete recovery by the end of 7 days. At the same time, VX caused significant inhibition in the activity of all molecular forms in all three muscles. In EDL, the activity of 4S and 10S showed significant recovery 24 h later, while those of SOL and diaphragm remained inhibited. By day 7, the activity of all forms had recovered and an excess activity was seen in the 4S form of EDL and diaphragm, shifting the latter profile toward that of the EDL and contributing a higher protection to the total AChE activity.

C. Butyrylcholinesterase

Compared to AChE activity, butyrylcholinesterase (BuChE, E.C. 3.1.1.8) activity in skeletal muscles is significantly less (Table 35.1). Also, unlike variable AChE activity, all three

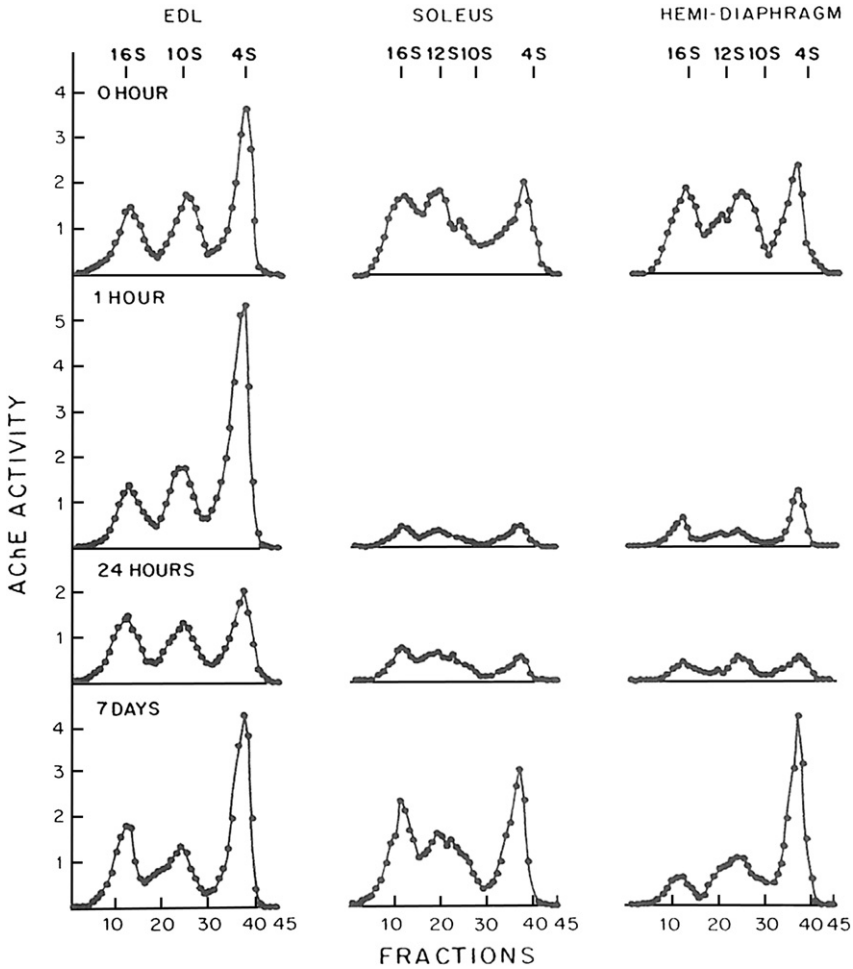

FIGURE 35.2. Representative profiles of the AChE molecular forms in EDL, soleus, and hemidiaphragm muscles from rats following an acute sublethal injection of sarin (110 µg/kg, s.c.). Profiles at the top of each column are from untreated muscles. Subsequent profiles are of the activity of the AChE molecular forms 1 h, 24 h, and 7 days, respectively, after sarin treatment. For further details, see legend to Figure 35.1.

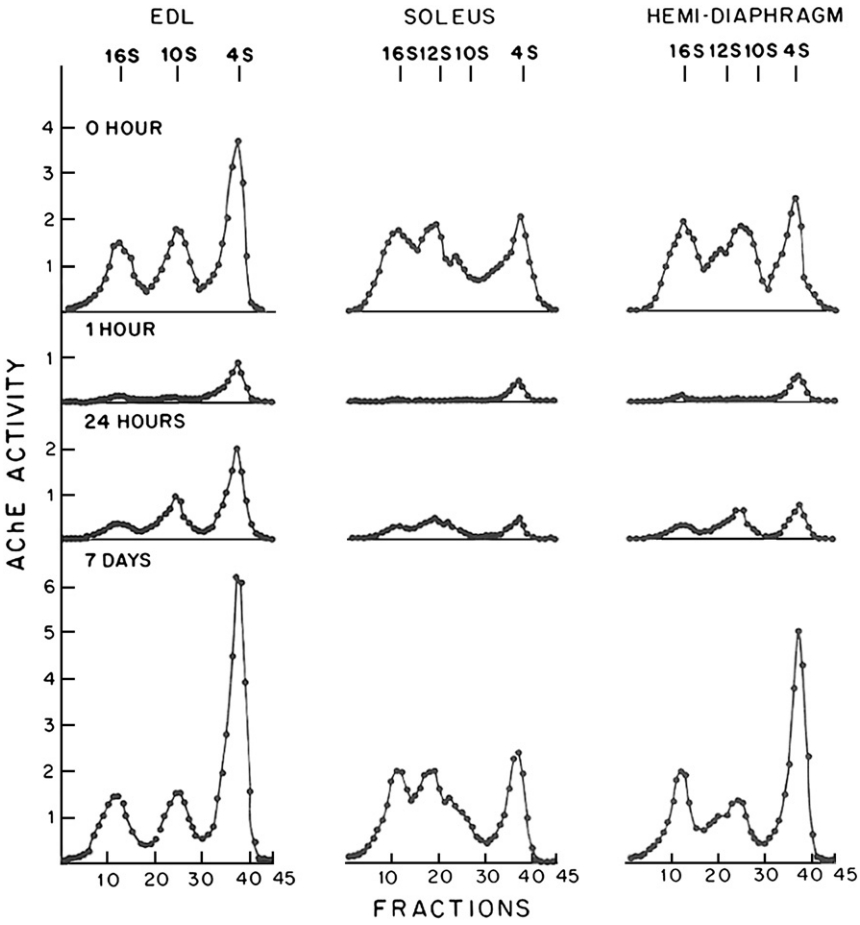

FIGURE 35.3. Representative profiles of the activity of the AChE molecular forms in EDL, soleus, and hemidiaphragm muscles from rats following an acute sublethal injection of VX (12 µg/kg, s.c.). For further details, see legend to Figure 35.1.

muscles (SOL, EDL, and diaphragm) contain equal levels of BuChE activity. Following soman administration (100 µg/kg, s.c.) in rats, BuChE activity in skeletal muscles was maximally inhibited only after 24 h. At this time, SOL was greatly affected (98%), followed by diaphragm (87%) and EDL (60%). A rapid recovery of BuChE was noticed during 48–72 h after soman treatment and the enzyme activity returned to the baseline values when measured after day 7 (Gupta *et al.*, 1987a).

Tabun (200 µg/kg, s.c.) caused significant inhibition of BuChE activity within 1 h in skeletal muscles (SOL, EDL, and diaphragm; 14%, 50%, and 35% remaining activity, respectively), but the maximal inhibition appeared after 3 h in SOL (6% remaining activity), and after 24 h in diaphragm and EDL (7 and 11% remaining activity, respectively) (Gupta *et al.*, 1987b). Unlike AChE, the recovery rate of BuChE appeared to be rapid, as the enzyme activity in diaphragm and EDL recovered to baseline values by day 7. At this time, SOL BuChE activity still remained significantly inhibited. After 1 h of sarin administration (110 µg/kg, s.c.), BuChE activity was significantly inhibited in SOL and EDL (78 and 79% remaining activity, respectively), but not in diaphragm. With sarin, maximal BuChE inhibition was noted after 24 h in SOL, EDL, and diaphragm (39, 71, and 43% remaining activity, respectively). In similar studies, within 1 h VX (12 µg/kg, s.c.) caused significant and maximal inhibition of BuChE in all three muscles (29, 60, and 60% remaining activity, respectively). When measured after 24 h, EDL and diaphragm showed marked recovery, while SOL still had 41% inhibition. All three muscles showed complete recovery of BuChE when measured after 7 days.

D. Choline Acetyltransferase

Existence of choline acetyltransferase (ChAT, acetyl-CoA-choline *O*-acetyltransferase, EC 2.3.1.6) in skeletal muscles is probably of neural origin, and its activity varies among skeletal muscles. ChAT activity can be altered by increased or decreased neuromuscular activity. It appears that neuromuscular activity exerts a regulatory influence on neuronal production of ChAT. Alterations in ChAT activity in response to variations in muscular activity represent changes in enzyme synthesis, although effects on catabolism of the enzyme or on exoplasmic transport of enzyme to the nerve terminal cannot be ruled out. Acute exposure to an OP compound DFP (1.5 mg/kg, s.c.) caused insignificant increase, while repeated administration at low dosage (0.5 mg/kg/day for 5 days) resulted in significant increase in ChAT activity (diaphragm 140%, EDL 150%, and SOL

156%). Significant increase in ChAT activity was noted even after 2 weeks of repeated administration.

E. Acetylcholine Receptors

Acetylcholine receptors (AChRs) are of two types: muscarinic (mAChR) and nicotinic (nAChR) based on the agonist activities of the natural alkaloids, muscarine and nicotine, respectively. These receptors are functionally different. The muscarinic type being G-protein coupled receptors mediate a slow metabolic response via second messenger cascades, while the nicotinic type are ligand-gated ion channels, which mediate a fast synaptic transmission of the neurotransmitter. Skeletal muscles are enriched with nAChRs and are devoid of mAChRs.

The nAChRs, with a molecular mass of 290 kDa, are composed of five receptor subunits (α, β, δ, ϵ, γ) arranged symmetrically around the central pore. Adult muscle is composed of $\alpha 2\beta\alpha\delta\gamma$, whereas fetal muscle has $\alpha 2\beta\delta\epsilon$ (Mishina *et al.*, 1986). Experimental studies of the late 1970s revealed that in relatively immature muscle cells, the AChRs are distributed uniformly over the entire sarcolemma (Bevan and Steinbach, 1977). At about the time of the initial innervation, aggregation/clusters of AChRs appear, in addition to the relatively low-density receptors (Braithwaite and Harris, 1979). During the course of embryonic development, the low-density receptors disappear and almost all AChRs in the adult muscle fibers are localized to the region of the NMJ (Fambrough, 1979). Sohal and Boydston (1982) suggested that neither the total receptor content nor the ability of the receptors to cluster seems an essential element for the formation and maintenance of the morphological aspects of the NMJs or for the growth and maturation of the muscle. It is yet to be established what exactly, if any, role nAChRs have in the formation of the NMJ.

At the mammalian NMJ, where nAChRs are localized, stimulation of the presynaptic nerve causes the release of the neurotransmitter ACh from the nerve terminals. Released ACh diffuses across the gap, separating nerve and muscle cells and interacts with specific receptors associated with the postsynaptic muscle membrane to produce increased membrane permeability to some cations. When two molecules of ACh bind to an nAChR, a conformational change occurs in the receptor, resulting in the formation of an ion pore. The nAChRs are a family of cationic channels whose opening is controlled by ACh and nAChR agonists and they are the key molecules in cholinergic nicotinic transmission at the NMJ. At this site, the opening of a pore produces a rapid increase in the cellular permeability of Na^+ and K^+ ions, resulting in the depolarization and excitation of the muscle cell, thereby producing a muscular contraction. A number of studies have shown that adult vertebrate muscle fibers are highly sensitive to ACh only in the region of the NMJ. Muscle fibers of fetal and neonatal rats are also sensitive to ACh in regions outside the NMJ.

It is established that excessive ACh or long-acting cholinomimetic agents can produce muscle paralysis due to prolonged depolarization of the endplate, a phenomenon referred to as depolarization block. Prolonged transmitter (ACh) receptor (nAChR) interactions, as a result of the high concentrations of accumulated ACh, produce a depolarization block, similar to that seen with agents such as decamethonium. Furthermore, there is a possibility of the same post-junctional area being activated repeatedly, resulting in desensitization of receptors (desensitization block). These forms of neuromuscular block usually produce muscle weakness and paralysis.

The binding site for cholinergic effectors is believed to reside primarily in the α-subunits. Evidence for this fact is based on affinity labeling experiments in which analogs of ACh (bromoacetylcholine and 4-(-*N*-maleimidophenyl) trimethylammonium) have been shown to label only the α-subunits. Receptors also demonstrate high-affinity binding of the polypeptide α-neurotoxins, such as α-bungarotoxin (αBT), which interacts specifically and in an essentially irreversible manner with nAChRs. At the nAChRs, αBT competes for binding with both cholinergic agonists (e.g. carbamylcholine chloride and decamethonium bromide) and antagonists (e.g. curare) (Karlin, 1980). Berg *et al.* (1972) reported binding of αBT to AChRs in rat diaphragm muscle and found that 90% of the binding that occurred was ''endplate-specific''.

Toxicity of OP nerve agents and pesticides is primarily due to inhibition of AChE, but some of the effects are unrelated to this mechanism. The exact mechanism by which OP nerve agents and other anti-AChE compounds affect nAChRs is yet to be elucidated. Some of the biochemical and morphological alterations have been attributed to an excess amount of ACh in the synaptic cleft resulting from AChE inactivation (Roberts and Thesleff, 1969; Chang *et al.*, 1973; Kawabuchi *et al.*, 1976). Many anti-AChE agents have been shown to produce postsynaptic morphological, biochemical, and electrophysiological alterations in adult mammalian skeletal muscle after acute and long- term treatment (Fenichel *et al.*, 1972; Laskowski *et al.*, 1977; Hudson *et al.*, 1978; Kawabuchi *et al.*, 1976). Normally, at the motor endplate, the small electrical nerve impulse releases the chemical transmitter ACh which diffuses across the synaptic membrane and attaches to the postsynaptic receptors on the muscle membrane. But during AChE inhibition by OPs and CMs, unhydrolyzed ACh does not diffuse from the cleft, but repeatedly combines with post-synaptic receptors. The prolonged presence of ACh in the synaptic area appears to cause some of the myopathic changes. This was supported by observations of paraoxon causing antidromic firing and increasing spontaneous miniature endplate potential frequency to 38 times control rates (Laskowski and Dettbarn, 1971).

In an early study, Gupta *et al.* (1986) demonstrated that an acute exposure to DFP (1.5 mg/kg, s.c.) changed neither nAChR density (Bmax) nor the affinity constant (K_D) in the

diaphragm muscle. Subchronic DFP treatment (0.5 mg/kg/day, s.c. for 5 days) caused marked decrease in the B_{max} (56%), without significant change in K_D. Chronic treatment with anti-AChEs reduced the total number of nAChRs in the range 42–45% in the endplate region (Chang *et al.*, 1973; Noble *et al.*, 1979). Later studies revealed that in sublethal doses, OPs induce symptoms which cannot be solely attributed to AChE inhibition, indicating a direct interaction with postsynaptic nAChRs (Menking *et al.*, 1990). Tattersall (1990) investigated the effects of DFP, sarin, soman, VX, and ecothiophate on the nAChR ion channel at the adult mouse muscle endplate by using single channel recording techniques. DFP, sarin, and soman had no effect on open times at concentrations up to 100 µM, but ecothiophate and VX were found to have voltage- and concentration-dependent open-channel-blocking actions at concentrations of 1–50 µM. In similar experiments, Bakry *et al.* (1988) examined direct actions of OP anti-ChEs on nAChRs. These authors found that OP nerve agents and ecothiophate bind to AChRs, inhibit or modulate binding of radioactive ligands to these receptors, and modify events regulated by them. The OP anti-ChEs also bound to allosteric sites on the nAChR (identified by inhibition of ^{3}H-phencyclidine bonding), but some bound also to the receptor's recognition site (identified by inhibition of ^{125}I-αBT binding). Soman and ecothiophate in micromolar concentrations acted as partial agonists of the nAChR and induced receptor desensitization. On the other hand, VX acted as an open channel blocker of the activated receptor and also enhanced receptor desensitization. Membrane fragments from Torpedo electric organs were used to determine these interactions using ^{3}H-phencyclidine as a probe. The results were consistent with the hypothesis that OPs bind to, and irreversibly phosphorylate, an allosteric site on the ion channel associated with the nAChR.

In the context of OP toxicities, it needs to be emphasized that for a normal muscle contraction, the ratio of AChE to nAChRs is crucial in determining the minimum AChR density. Since the AChE recovers at almost the same rate as do the AChRs, a balance of AChE to AChR is maintained over the postsynaptic surface during recovery. A relatively constant ratio of AChE to AChR is very important for maintaining normal neuromuscular function.

IV. NONCHOLINERGIC SYSTEM

A. Muscle Excitotoxicity

Involvement of the cholinergic neurotransmitter ACh in muscle excitotoxicity has been known for a long time. Waerhaug and Ottersen (1993), by using quantitative electron immunocytochemistry, demonstrated the glutamate-like immunoreactivity at rat NMJ, and it was suggested that glutamate and ACh are co-released at the NMJ. Glutamate might be a mediator or modulator of neuromuscular transmission. It has also been established that glutamate receptors present at the NMJ are predominantly an *N*-methyl-D-aspartate (NMDA) subtype. In an early study, Koyuncuoglu *et al.* (1998) demonstrated that the blockade of NMDA receptors, the inhibition of glutamate release, or the suppression of glutamate production, attenuates the contractions of the rat-isolated hemidiaphragms elicited by indirect electrical stimulation. Furthermore, it has also been demonstrated in *in vivo* studies that the noncompetitive NMDA receptor antagonist, memantine, blocks the AChE inhibitors (DFP, soman, sarin, tabun, and VX)-induced muscle fasciculations, suggesting an involvement of NMDA receptors (Gupta and Dettbarn, 1992; McLean *et al.*, 1992). Of course, memantine also exerts several other pharmacological actions, including nicotinic ACh receptor blockage. Unlike the well-understood role of cholinergic excitotoxicity, the role of glutamate excitotoxicity is yet to be established in AChE inhibitor-induced muscle toxicity.

B. Oxidative/Nitrosative Stress

Although the exact mechanism underlying skeletal muscle damage by OP nerve agent-induced hyperactivity still remains unclear, mounting evidence indicates that free radicals play an important role. During normal conditions, free radicals are generated at a low rate and subsequently taken care of by the well-developed scavenger and antioxidant systems. However, during exhaustive hyperactivity of the skeletal muscles caused by anti-ChE, excessive amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (hereafter referred to as ROS) are generated and exceed the capacity of the muscle defense system, thus producing oxidative stress. Excessively generated ROS can cause muscle injury by reacting with cellular components, such as membrane phospholipids, mitochondrial enzymes/proteins and nucleic acids. Consequently, this leads to skeletal muscle cell apoptosis/necrosis, inflammation, and loss of cell viability.

It appears that a causal relationship exists between excitotoxicity, ROS, lipid peroxidation, and muscle cell injury/death. One of the well-recognized targets of ROS-induced injury is peroxidation of lipids and the formation of prostaglandin F_2-like compounds, such as F_2-isoprostanes (F_2-IsoPs). A number of studies have shown that the assay of these compounds provides an accurate measure of lipid peroxidation both *in vitro* and *in vivo* (Morrow *et al.*, 1992; Roberts and Morrow, 2000; Dettbarn *et al.*, 2001). Quantification of F_2-IsoPs and nitric oxide (NO) in skeletal muscles (SOL, EDL, and diaphragm) has provided strong evidence that AChE inhibitor-induced toxicity initiates lipid peroxidation and muscle cell injury (Dettbarn *et al.*, 2001; Gupta *et al.*, 2001a, b; Milatovic *et al.*, 2001). In an earlier study, Yang and Dettbarn (1998) reported a significant increase of F_2-IsoPs in diaphragm muscle (156%) 1 h after DFP injection (1.7 mg/kg, s.c.), at the time when muscle hyperactivity was maximal.

The primary reason for the increased generation of ROS appears to be the decreased rate of ATP synthesis in the mitochondria, which is related to a loss of cytochrome oxidase (COX) activity. COX is the terminal complex in the mitochondrial respiratory chain, which generates ATP by oxidative phosphorylation. During intense muscle hyperactivity, the activity of COX is reduced, leading to an increase in the electron pressure within the electron transport chain and to increased ROS production (Soussi *et al.*, 1989; Gollinick *et al.*, 1990; Yang and Dettbarn, 1998; Zivin *et al.*, 1999). More than 90% of the O_2 consumption in the cells is catalyzed by COX. The chance of intermediary products, such as superoxide anion, hydrogen peroxide, and the hydroxyl radical, escaping is small under conditions where COX remains active. A reduced capacity of this enzyme, however, increases the risk for an incomplete reduction of O_2 and further O_2 radical formation (Sjodin *et al.*, 1990).

Another enzyme contributing to increased ROS generation is xanthine oxidase (XO). During normal conditions, 80–90% of native XO exists as xanthine dehydrogenase (XD), but during metabolic stress and increased Ca^{2+}, XD is converted to a reversible oxidase form. XO uses molecular O_2 instead of NAD^+ as an electron acceptor. Molecular O_2 is thereby reduced and the superoxide radical (O_2^-) is formed. During hyperactivity of the muscle by AChE inhibitors, regeneration of ATP is insufficient, not due to the lack of O_2, but due to greater utilization and impaired synthesis of ATP (Gupta *et al.*, 1994, 2000a, b, 2001a, 2002). Unlike ischemia, O_2 is present during oxidative stress caused by prolonged contractile activity. This suggests that subsequent to the conversion of XD to XO, O_2 is continuously univalently reduced to superoxide anions. This occurs during oxidative stress when ATP utilization exceeds the rate of ATP synthesis during increased muscle activity (Gollinick *et al.*, 1990). Yang and Dettbarn (1998) provided direct evidence for the role of COX and XO in muscle injury by its hyperactivity, showing that during DFP-induced muscle hyperactivity, a decrease in COX activity and an increase in XO activity occurred. Blockage of muscle fasciculations provided prevention of these enzyme changes.

Another ROS contributing to oxidative stress is peroxynitrite ($ONOO^-$), which is formed by the reaction of nitric oxide (NO) with superoxide (O_2^-) (Huie and Padmaja, 1993). $ONOO^-$ has the potential to modify biomolecules through several different mechanisms, and is a good candidate for mediation of the NO-dependent pathophysiological process (Brunelli *et al.*, 1995). Under normal conditions, NO, which is a free radical gas (synthesized in a reaction catalyzed by NO synthase), is widely regarded as a multifunctional messenger/signaling molecule, and is thought to have two physiological functions in skeletal muscle. One, to promote relaxation through the cGMP pathway (Schmidt *et al.*, 1993), and two, to modulate muscle contractility that is dependent on reactive oxygen intermediates (Abramson and Salama, 1989). At the NMJ, NO appears to be a mediator of: (1) early synaptic protein clustering, (2) synaptic receptor activity and transmitter release, and (3) downstream signaling for transcriptional control (Blottner and Luck, 2001). NO has also been demonstrated to modulate excitation–contraction coupling in the diaphragm muscle (Reid *et al.*, 1998). Within skeletal muscle cells, all three known NO synthase (NOS) isoforms (neuronal, nNOS; endothelial, e-NOS; and inducible, iNOS) are present, but nNOS, which is Ca^{2+} dependent, seems to predominate, and is concentrated at the sarcolemma and postsynaptic surface of the NMJ (Silvango *et al.*, 1996; Stamler and Meissner, 2001; Wang *et al.*, 2001). In a recent study, NOS has been demonstrated to play the role of mechanosensor in skeletal muscle fibers (Smith *et al.*, 2002). Neuronal NOS in skeletal muscles is involved in the regulation of metabolism and muscle contractility (Stamler and Meissner, 2001). Kobzik *et al.* (1994) reported for the first time that the NOS activity of individual muscles could be correlated primarily with type II fibers (being highest in the EDL), while Grozdanovic *et al.* (1995) and Frandsen *et al.* (1996) found the distribution of nNOS homogeneous.

Data presented in Table 35.3 show the levels of citrulline (determinant of NO/NOS) in skeletal muscles of control rats and those treated with an acute dose of DFP (1.5 mg/kg, s.c.). Analyses of control muscles revealed markedly higher citrulline levels in the soleus, followed by the EDL and diaphragm. The observed higher level of NO in the soleus could be due to a greater activity of NOS. Within 1 h of DFP exposure, when rats exhibited signs of peak severity and maximal reduction of AChE activity (90–96%), the levels of citrulline were maximally increased (272–288%), and remained significantly elevated in all three muscles (>twofold) when measured after 2 h. The finding of elevated NO by DFP-induced muscle hyperactivity (Gupta *et al.*, 2002) was supported by previous studies that increased muscle contractility generates significantly greater quantities of ROS/RNS (Yang and Dettbarn, 1996; Narayan *et al.*, 1997; Clanton *et al.*, 1999). A significant increase in NO is known to cause inhibition of mitochondrial function and thereby appears to be the cause of the impaired synthesis of ATP.

TABLE 35.3. Citrulline levels (nmol/g) in skeletal muscles of rats after 1 h of DFP (1.5 mg/kg, s.c.) injection

Treatment	Soleus	EDL	Diaphragm
Control	451.2 ± 5.3 (100%)	381.3 ± 8.2 (100%)	331.2 ± 9.9 (100%)
DFP	1227.1 ± 47.2^a (272%)	1061.0 ± 38.9^a (278%)	952.3 ± 49.8^a (288%)

Values are means ± SEM ($n = 4$–5). [a]Significant difference between control and DFP-treated rats ($p < 0.05$)

C. High-Energy Phosphates Depletion and Myonecrosis

In the recent past, interest in the role of energy metabolites (especially ATP) in chemical-induced mitochondrial/cytotoxicity has been reinvigorated, since ATP appears to be a switch to decide whether cells die from apoptosis or necrosis. Muscle necrosis is probably caused by increased contractile activity in individual muscle fibers. In normal cells, the mitochondrial Ca^{2+} content is relatively low compared to that in the cytoplasm. However, abnormally high cytosolic Ca^{2+} levels (due to NMDA receptor activation and impaired Ca^{2+} extrusion which is an energy-dependent process) perturb many cellular processes. The major changes include: (1) reduced cytochrome-c oxidase activity, (2) increased xanthine oxidase activity, (3) mitochondrial damage due to excessive mitochondrial Ca^{2+} accumulation, (4) reduced ATP synthesis, and (5) increased production of O_2^- and NO^-, resulting in increased $OONO^-$ formation. The extreme vesiculation and disruption of the sarcoplasmic reticulum (SR) under the endplate as well as the swollen mitochondria may reflect Ca^{2+} overloading of the muscle Ca^{2+}-binding capacity, which could result in high sarcoplasmic Ca^{2+} levels leading to the necrosis (Salpeter *et al.*, 1979, 1982). The control of intracellular Ca^{2+} concentration is of great importance to muscle fibers and a transient rise leads to contraction. An increased net influence of Ca^{2+} forces the mitochondria and SR to maintain Ca^{2+} homeostasis by sequestering the excessive amounts of this ion. This is an energy consuming process and occurs in preference to ATP formation. The ensuing lack of energy ultimately causes the free sarcoplasmic Ca^{2+} to rise and excessive ROS formation, leading to necrosis. The energy required for muscle contraction is derived from the breakdown of ATP. As soon as ATP is broken down, it is promptly restored in the so-called ''Lohman reaction'' at the expense of phosphocreatine (PCr).

Data presented in Table 35.4 show the levels of high-energy phosphates (ATP and PCr) and their metabolites in skeletal muscles of control rats and those treated with an acute dose of DFP (1.5 mg/kg, s.c.). Analyses of control muscles revealed the levels of ATP and PCr to be higher in the EDL, followed by the diaphragm and the soleus. The values of energy charge potential (ECP = ATP + 0.5ADP/TAN) in soleus, EDL, and diaphragm were $0.86 \pm < 0.01$, $0.91 \pm < 0.01$, and $0.86 \pm < 0.01$, respectively. At the time of maximal severity, i.e. 1 h after DFP exposure, the levels of ATP, total adenine nucleotides (TAN = ATP + ADP + AMP), PCr, and total creatine compounds (TCC = PCr + Cr) were maximally reduced in all three muscles, and remained reduced to the same degree when determined after 2 h. Similar results were found with soman toxicity.

AChE inhibitors cause depletion of energy-rich phosphates (ATP and PCr) due to a combination of impaired synthesis and greater utilization of ATP during muscle hyperactivity. In fact, the time course of necrosis correlates with the reduced levels of PCr, the reduction of which may have been the result of an increased demand for energy and a low rate of ADP phosphorylation, caused by an increased level of sarcoplasmic Ca^{2+}. AChE inhibitor-induced increases in NO (Table 35.3) can exert cellular toxicity primarily by depleting energy stores through multiple mechanisms: (1) by prolonging poly-(ADP-ribose) polymerase activation, (2) by inhibiting mitochondrial enzymes, such as COX, aconitase, creatine kinase, and (3) by

TABLE 35.4. Energy phosphates and their metabolites in muscles of rats intoxicated with DFP (1.5 mg/kg, s.c.)

		μmol/g (means ± SEM; n = 4–5)			
	Treatment	**ATP**	**TAN**	**PCr**	**TCC**
Soleus	Control	3.42 ± 0.05 (100)	4.42 ± 0.04 (100)	6.38 ± 0.19 (100)	20.41 ± 0.37 (100)
	DFP	2.06 ± 0.06[a] (60)	2.78 ± 0.08[a] (63)	3.67 ± 0.19[a] (57)	12.87 ± 1.01[a] (63)
EDL	Control	4.91 ± 0.37[b] (100)	5.80 ± 0.38[b] (100)	9.63 ± 0.59[b] (100)	26.30 ± 1.64[b] (100)
	DFP	3.42 ± 0.02[a] (70)	4.15 ± 0.02[a] (72)	6.82 ± 0.23[a] (71)	21.74 ± 0.33[a] (83)
Diaphragm	Control	4.47 ± 0.23 (100)	5.76 ± 0.26 (100)	6.18 ± 0.03 (100)	23.34 ± 0.17 (100)
	DFP	2.74 ± 0.43[a] (61)	3.66 ± 0.05[a] (64)	3.96 ± 0.12[a] (64)	15.86 ± 0.45[a] (68)

[a]Significant difference between control rats and DFP-treated rats ($p < 0.05$)
[b]Significant difference between soleus and EDL values of control rats ($p < 0.05$)
Numbers in parentheses are percent remaining values compared to controls (100%)

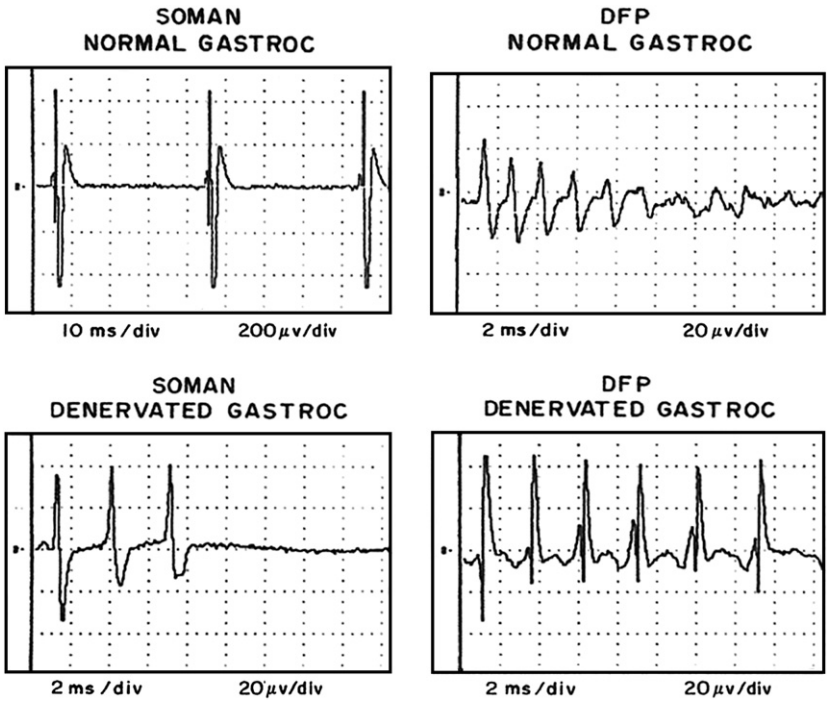

FIGURE 35.4. Electromyographic recordings from normal and acutely denervated lateral gastrocnemius in response to soman (100 μg/kg, s.c.) or DFP (1.5 mg/kg, s.c.). Left, soman; right, DFP; top, nondenervated muscle; bottom, denervated muscle. Note the difference in time base and voltage scale. The differing morphology of the normal DFP waveform is due to electrode orientation and distance from the muscle fiber and does not represent a systematic difference between the effects of individual agents.

inhibiting the glycolytic enzyme phosphofructokinase. NO, at nanomolar concentrations, can directly and specifically inhibit mitochondrial respiration by competing with molecular O_2 for binding to COX, thereby causing inhibition of ATP synthesis (Brown and Cooper, 1994; Giulivi, 1998). Other factors which contribute to the decline of energy metabolites may include damage to mitochondria, a higher rate of ATP utilization needed to generate NAD^+ in the ADP-ribosylation of nuclear proteins, enhanced influx of sarcoplasmic Ca^{2+}, and an increased number of contractile protein cross-bridges, in addition to the release of ATP in concert with ACh from the nerve terminals. The net effect of AChE inhibitor-induced muscle hyperactivity is a reduced cellular energy level.

V. MUSCLE ACTIVITY – EMG

AChE inhibitors (DFP, 1.5 mg/kg, s.c. or soman, 100 μg/kg, s.c.) at a toxic signs-producing sublethal dose elicit prominent motor, behavioral, and autonomic symptoms. As mentioned earlier, the motor symptoms are fasciculations, fibrillations, and body tremors. Fasciculations and fibrillations are due to antidromic neural discharge from excess junctional ACh, while tremors are of central origin. Misulis *et al.* (1987) observed a difference in the pattern of motor symptoms in rats treated with soman or DFP. Soman produces complex posturing movements and tremors affecting virtually the entire body, while DFP produces movements that are similar to fasciculations or myokymia. Muscle fiber necrosis is also more frequent at symptom-producing doses of DFP than equivalent doses of soman. This suggests that the AChE inhibitors differ in their propensity to produce central or peripheral effects and that peripheral effects are required for muscle necrosis.

Electromyographic (EMG) findings indicate that soman and DFP produce different responses (Figure 35.4). The majority of the motor symptoms induced by soman are due to impulses descending from the CNS, and a proportion of these symptoms are epileptiform activity. This activity is not generated at spinal levels. In contrast, DFP produces motor symptoms mainly by peripheral action that is dependent on a functioning nerve terminal (Misulis *et al.*, 1987). Anderson (1987) also demonstrated that DFP and soman have opposite effects on skeletal muscle contracture during tetanic stimulation. DFP significantly decreased the ability of rat skeletal muscle to maintain a contracture. Soman, on the other hand, increased muscle force in a frequency-dependent manner. The opposite actions of DFP and soman on muscle contractility might be the consequence of a difference in effects of these two agents on postsynaptic muscle ACh receptors. The finding of Anderson (1987), i.e. increase in contracture following soman, was somewhat surprising in view of the work of Dettbarn (1984) and Gupta *et al.* (1987a, b), who reported that soman is as effective as other AChE inhibitors in producing muscle necrosis (Table 35.6). However, Meshul *et al.* (1985) reported that soman appears to spare motor endplates from structural damage and thereby may account for maintaining muscle function (Anderson, 1987). In essence, different AChE inhibitors can produce opposite effects on muscle contracture.

VI. MUSCLE FIBER HISTOPATHOLOGY

By using actomyosin ATPase reaction, analysis of untreated rat slow muscle soleus (SOL) revealed predominantly type 1 fibers with a few type 2A and 2B fibers. In contrast, the fast muscle EDL is composed predominantly of type 2 fibers with few type 1 fibers. Total fiber numbers are approximately 1,800 in SOL and 2,500 in EDL (Gupta *et al.*, 1989).

It is well established that OP pesticides and nerve agents and some carbamates in a single sublethal dose are sufficient to cause muscle fasciculations, and induce myopathy (histopathological changes) in the diaphragm, soleus, EDL, sternomastoid, gastrocnemius, triceps, and tibialis muscles of experimental animals. Pathological changes in skeletal muscles by AChE inhibitors are observed in the region adjacent to the motor endplate. Patterson *et al.* (1987) determined the necrotic lesions by assessing endplates in skeletal muscles of rats acutely intoxicated with DFP (1.5 mg/kg, s.c.). In SOL, these are only found in the midsection of the muscle, while in EDL the endplates are found throughout the muscle length. In EDL, the fibers are innervated at different levels throughout the length of the muscle, while in SOL the nerve makes functional contact in the midsection only. No endplates or lesions are found outside this area.

Gupta *et al.* (1985, 1986) found the highest number of lesions in all three muscles (diaphragm, SOL, and EDL) of rats within 24 to 48 h after a single injection of DFP (1.5 mg/kg, s.c.), when the inhibitory effect on AChE activity was also maximal (Table 35.5). The diaphragm muscle had the highest number of lesions, followed by the EDL and SOL.

Data on soman (100 µg/kg), sarin (110 µg/kg), tabun (200 µg/kg), and VX (12 µg/kg) are presented in Table 35.6. Following soman treatment, the numbers of necrotic fibers in the SOL and the diaphragm were found to increase up to 24 h and no new lesions appeared subsequently. By day 7, these two muscles appeared to have fully recovered from the soman effect, since no necrotic fibers were seen. In EDL, no morphological changes were seen at any time. While the acute toxic effects of tabun are more or less similar to soman, there are also some significant differences between these two agents. Unlike soman, tabun caused prolonged AChE inhibition and progressive development of muscle necrosis over a period of 3 days. While the primary toxic effect of tabun is due to inhibition of AChE activity, some of the differences seen in regard to other nerve agents may be due to additional actions of the cyanide group that is released during AChE inhibition. Effects such as prolonged inhibition of AChE and increasing number of lesions seen up to the third day may be due to the leaving acidic group CN. The inhibitory effect of CN on oxidative metabolism could result in reduced *de novo* synthesis, delaying synthesis of AChE and prolonged inhibition of Ca^{2+} sequestration into the sarcoplasmic reticulum of skeletal muscles. Therefore, in the context of OP nerve agent-induced myopathy, soman, sarin, tabun, or VX caused the greatest number of lesions in diaphragm, followed by SOL, and least in the EDL during 24 to 48 h (Gupta *et al.*, 1987a, b, 1991). With all four nerve agents, the greatest number of lesions occurred in animals exhibiting severe muscle fasciculations (Gupta *et al.*, 1986, 1987a, b, 1991; Inns *et al.*, 1990; Bright *et al.*, 1991). These authors also demonstrated that by day 7, muscles recovered from soman-induced myopathy but not from any other nerve agents since the necrotic lesions were still evident.

The earliest lesions are focal areas of abnormality in the subjunctional section of the muscle fiber adjacent to the motor endplate including eosinophilia and sarcoplasmic swelling (Laskowski *et al.*, 1977; Gupta *et al.*, 1985, 1986; Patterson *et al.*, 1987). Mitochondria are disrupted as evidenced by clumping of highly reactive material of the lactate dehydrogenase (LDH) and reduced nicotinamide adenine dinucleotide (NADH) reactions. These focal changes progress to a breakdown of subjunctional fiber architecture, loss of striations, followed by phagocytosis. Longitudinal sections reveal that the necrosis affects only a small segment of fiber lengths. During latter stages, progressively greater lengths of muscle fibers are affected

TABLE 35.5. Number of necrotic fibers following a single sublethal injection of DFP (1.5 mg/kg, s.c.)

Time after DFP administration	Soleus		EDL		Diaphragm	
	AChE	Lesions	AChE	Lesions	AChE	Lesions
Control	100%	0	100%	0	100%	0
1 day	24%	27 ± 6^a	17%	64 ± 11^a	13%	308 ± 58^a
2 day	24%	39 ± 9^a	14%	71 ± 12^a	13%	333 ± 63^a
3 day	39%	5 ± 1^a	31%	49 ± 5^a	25%	174 ± 35^a
7 day	65%	0	53%	0	69%	0

Values of AChE activity are expressed as % remaining activity of control (100%)
Numbers of lesions are presented per cross-section from the mid-belly region of muscle
Data are presented as the means ± SEM ($n = 5$–10)
[a]Significant difference between control and DFP-treated rats ($p < 0.05$)

TABLE 35.6. Necrotic fibers in skeletal muscles of rats following a sublethal dose of soman, sarin, tabun, or VX

Time (post-treatment)	Muscle	Number of lesions/1,000 fibers			
		Soman (100 µg/kg, s.c.)	Sarin (110 µg/kg, s.c.)	Tabun (200 µg/kg, s.c.)	VX (12 µg/kg, s.c.)
1h	DIA	0	9 ± 1	6 ± 2	26 ± 5
	SOL	0	12 ± 4	9 ± 2	16 ± 3
	EDL	0	0	0	0
6h	DIA	3 ± 2	100 ± 30	48 ± 12	233 ± 45
	SOL	13 ± 3	111 ± 38	21 ± 4	97 ± 17
	EDL	0	0	0	23 ± 10
24h	DIA	260 ± 48	435 ± 154	302 ± 32	322 ± 49
	SOL	48 ± 9	101 ± 24	66 ± 14	99 ± 20
	EDL	1 ± 0	103 ± 45	33 ± 5	28 ± 14
3 days	DIA	74 ± 24	490 ± 66	541 ± 31	305 ± 78
	SOL	9 ± 3	135 ± 42	192 ± 29	186 ± 37
	EDL	0	18 ± 17	66 ± 12	79 ± 16
7 days	DIA	0	216 ± 69	40 ± 10	75 ± 12
	SOL	0	237 ± 61	28 ± 4	62 ± 7
	EDL	0	30 ± 11	3 ± 1	27 ± 11

Value are means ± SEM of necrotic lesions (n=5)

(Gupta *et al.*, 1986; Patterson *et al.*, 1987). Serial cross-sections of 10 µm thickness indicate that the number of lesions correlates with the greatest density of endplates (Patterson *et al.*, 1987). The longer the delay between injection and sacrifice, the greater the special extent of the lesions. A significant increase in blood creatine kinase (CK) activity coincides with appearance of myonecrosis, indicating destruction of the muscle membrane (Sket *et al.*, 1989; Gupta *et al.*, 1991, 1994).

Subjunctional changes in the muscle fibers, such as super-contraction of subjunctional sarcomeres, as well as disruption of cytoarchitectural organization, are always present (Gupta *et al.*, 1986, 1987a, b, 1991; Inns *et al.*, 1990; Bright *et al.*, 1991). The initial changes are in the mitochondria which first swell and then show lysis of the central cristae. Myelin figures beneath the endplate are frequently observed while the region more distal to the endplate is less affected. The nucleoli of the muscle cell nuclei are enlarged and move to the periphery of the nucleus. This myopathy can be induced with OP prototype compound DFP (Leonard and Salpeter, 1979; Gupta *et al.*, 1986; Misulis *et al.*, 1987; Patterson *et al.*, 1987, 1988; Sket *et al.*, 1991a, b), or OP nerve agents soman, sarin, tabun, and VX (Gupta *et al.*, 1987a, b, 1991; Inns *et al.*, 1990; Gupta and Dettbarn, 1992).

Laskowski *et al.* (1975) reported the ultrastructural changes in the subsynaptic folds that were quite varied even between muscle fibers from the same diaphragm of rats acutely treated with paraoxon. The fact that some endplates were totally degenerated after 2 days of paraoxon treatment while others appeared almost normal even after 5 days indicates that some endplates are more resistant than others. The most consistent change at the endplates was the presence of vesicular structures in the synaptic clefts. Some regions of the subsynaptic folds contained varying sizes of vacuoles and vesicles. There appears to be a wide variation in the severity of the lesions in the subsynaptic folds even in the same muscle. After 2 days of paraoxon treatment, the muscle surrounding the motor endplate often showed less cytoarchitectural organization than control muscles. Myelin figures beneath the endplate were frequently observed, while the region of muscle distal to the endplate was less severely affected.

Despite the diversity of structures of chemicals described above, the induced myopathic changes are the same, suggesting involvement of a common mechanism. This mechanism is inhibition of AChE. From several studies, it is evident that OP-induced myopathy is dose dependent and depends on both a critical duration and degree of enzyme inhibition. It has been demonstrated that the faster the rate of AChE inactivation, the higher the number of necrotic lesions found indicating a correlation between AChE inhibition and the number of necrotic fibers, thus supporting the role of ACh in the generation of myopathy (Wecker *et al.*, 1978a, b; Gupta *et al.*, 1986, 1987a, b, 1991). Muscle fiber necrosis depends on a rapid, critical reduction and duration of suppressed AChE activity. Regardless of the inhibitor involved, AChE inhibition >80% for about 2 h is necessary to initiate severe muscle fiber necrosis. AChE inhibition resulting in an excess of acetylcholine (ACh) and its prolonged functional

interactions with the ACh receptors is responsible for producing lesions. However, some anti-ChEs have been shown to interact directly with the nicotinic receptors (Tattersall, 1992). The OP compounds acting directly on postsynaptic membranes or on components of the muscle cell were also considered in the etiology of myopathy (Laskowski and Dettbarn, 1971). However, the finding that prior injection of either tubocurarine or α-bungarotoxin (Koelle and Gilman, 1949; Salpeter *et al.*, 1979) prevented the development of the myopathy proved conclusively that neither the OP agent *per se* nor ACh by itself caused the damage, but that the damage was the result of changes that followed transmitter–receptor interaction. Tubocurarine and alpha-bungarotoxin, by occupying the post-junctional receptors, prevent the ACh from interacting with the receptor. Furthermore, studies have shown that increased ACh may be an important trophic neurotransmitter involved in the pathophysiology of myopathy. The primary defect is at both presynaptic and postsynaptic sites. This not only supports the concept of an abnormal neurotrophism as a mechanism of myopathy but also allows the possibility that the primary defect is in the cholinergic system of nerve and muscle.

There is ample evidence that ACh accumulation is involved in causing Ca^{2+} influx into skeletal muscle fibers during anti-ChE poisoning. Evidence for the involvement of locally elevated levels of ACh was further confirmed since denervation prevented myopathy (Ariens *et al.*, 1969; Fenichel *et al.*, 1972; Wecker and Dettbarn, 1976; Dettbarn, 1984) or nicotinic ACh receptor blockade with alpha-bungarotoxin (Leonard and Salpeter, 1979). Wecker and Dettbarn (1976) clearly demonstrated that transection of the phrenic nerve to the rat hemidiaphragm prevented myopathic development, while in the contralateral innervated hemidiaphragm the number of lesions was increased. Thus, the common denominator is muscle hyperactivity, such as fasciculations (Gupta *et al.*, 1986, 1987a, b, 1991; Adler *et al.*, 1992). In similar experiments, Fenichel *et al.* (1972) observed that the number of lesions produced by paraoxon in rat soleus, gastrocnemius, and quadriceps muscles were greatly reduced by prior sciatic nerve section or by prior administration of hemicholinium. In addition to inhibition of AChE activity, paraoxon produced a rapid transient increase in ACh release from motor nerve terminals in the rat diaphragm (Laskowski *et al.*, 1977).

Accumulation of ACh in the nerve terminal stresses the metabolic capacity of the muscle, initially enhancing muscle contraction, but then surpassing the ability of the muscle to maintain functional and structural integrity (Salpeter *et al.*, 1979; Dettbarn, 1984; Gupta *et al.*, 1986; Gupta and Dettbarn, 1987). The longer the muscle hyperactivity lasts, the greater the number of necrotic muscle fibers found (Dettbarn, 1984; Gupta *et al.*, 1985, 1986, 1987a, b).

In the context of OP-induced myopathy, the role of Ca^{2+} as a cell death trigger is very important. *In vitro* experiments with the nonhydrolyzable cholinergic agonist carbamylcholine produce similar alterations to those observed with the AChE inhibitors *in vivo*. In these experiments, excessive Ca^{2+} influx was thought to contribute to the myopathy (Leonard and Salpeter, 1979, 1982). The increased rate of postsynaptic stimulation that accompanies fasciculations and the concomitant influx of Ca^{2+} may initiate the changes in subjunctional mitochondrial morphology. These authors found that both alpha-bungarotoxin and Ca^{2+} depletion prevented DFP-induced myopathy in a nerve–muscle preparation *in vitro.* The swelling of mitochondria with a parallel release of sequestered Ca^{2+} thus evokes contraction of subjunctional sarcomeres and leads to subjunctional fiber damage. *In vivo* experiments in mice with sarin (25–150 μg/kg, s.c.) also revealed a similar role of Ca^{2+} in muscle fiber damage (Inns *et al.*, 1990). Ca^{2+} was found in the diaphragm of those mice to which sarin had been administered at doses of 50 μg/kg or above. Calcium accumulation, which was confined to the region of the motor endplates, occurred earliest and remained the longest in the diaphragm from those animals receiving the highest doses of sarin. Generally, Ca^{2+} accumulation can be detected on day 1 after injection and none after day 7. This observation coincides with the duration of appearance and disappearance of muscle lesions. These authors suggested that Ca^{2+} is among a series of events which ultimately lead to myonecrosis. Mitochondrial damage by excessive Ca^{2+} is expected to cause a partial or complete depletion of ATP synthesis and consequently excessive generation of oxygen- and nitrogen-free radicals and eventually myotoxicity (Gupta and Dettbarn, 1987, 1992; Gupta *et al.*, 2001a, b, 2002). In conclusion, following an acute exposure to an OP compound at a higher dose, a causal relationship exists between a critical level of AChE inhibition, Ca^{2+} accumulation, and appearance of muscle fiber necrosis.

Chronic exposure (30–60 days) of rats to paraoxon, at doses (0.05–0.1 mg/kg, s.c./day) that do not produce parasympathomimetic effects, led to necrosis of muscle fibers in diaphragm muscle which was qualitatively similar to that following the administration of a single high dose of paraoxon (0.23 mg/kg, s.c.). The lesion was characterized by the presence of central nuclei, fiber splitting, and breakdown of fiber architecture followed by phagocytosis and necrosis. With either dose, AChE inhibition occurred in the endplate regions and not in the nonendplate regions (Wecker and Stouse, 1985).

VII. MUSCLE CYTOTOXICITY BIOMARKERS

A. Creatine Kinase (CK) and CK Isoenzymes

CK catalyzes the synthesis of ATP and PCr in a reversible Lohmann reaction. Data presented in Table 35.7 show the normal distribution of CK and its isoenzymes in skeletal muscles of untreated control rats. The findings revealed that the fast muscle EDL had the maximal CK activity, followed

TABLE 35.7. Normal distribution of CK and CK isoenzymes in skeletal muscles and serum of rats

	Total CK	CK isoenzymes		
		CK-BB (CK-1)	CK-MB (CK-2)	CK-MM (CK-3)
Soleus	2,062,800 ± 71,065 (100)	ND	ND	2,062,800 ± 71,065 (100)
EDL	4,659,125 ± 185,583 (100)[a,b]	ND	ND	4,659,125 ± 185,583 (100)[a,b]
Diaphragm	3,018,240 ± 110,777 (100)	ND	ND	3,018,240 ± 110,777 (100)
Serum	3,769 ± 240 (100)	576 ± 45 (15.3)	148 ± 17 (3.9)	3,086 ± 209(80.8)

Values expressed in terms of IU/L are presented as means ± SEM ($n = 4$–6)
Numbers in parentheses are percentages of isoenzymes to total CK activity (100%)
ND – none detected
[a]Significant difference between EDL and soleus ($p < 0.001$)
[b]Significant difference between EDL and diaphragm ($p < 0.001$)

by diaphragm, with the lowest in the soleus. Electrophoretic separation of CK isoenzymes in all three muscles revealed the existence of only CK-MM isoenzyme. Further separation of CK-MM isoenzyme for subforms showed only CK-MM3 in all three muscles. Compared to muscles, serum had very little CK activity; however, the CK consisted of three distinct isoenzymes: CK-BB (15.3%), CK-MB (3.9%), and CK-MM (81.8%). Further electrophoresis of serum CK-MM isoenzyme revealed the presence of three subforms: CK-MM1 (6.3%), CK-MM2 (24%), and CK-MM3 (69.7%) (Gupta *et al.*, 1994). Literature abounds showing that the CK-MM3 subform secretes from muscles into the plasma, where it converts into the MM2 and MM1 subforms by carboxypeptidase-N2.

Within 1 h of exposure to carbofuran (1.5 mg/kg, s.c.), CK activity was significantly reduced in soleus, while increased in diaphragm. At the same time, activities of CK and all three CK isoenzymes were significantly elevated in serum. An important finding was that carbofuran or methyl parathion caused a shift in the serum CK-MM subform, i.e. higher sequential conversions of CK-MM3 subform to CK-MM2 and CK-MM2 to CK-MM1, possibly due to enhanced carboxypeptidase-N2 activity (Gupta *et al.*, 1994).

B. Lactate Dehydrogenase (LDH) and LDH Isoenzymes

LDH catalyzes the synthesis of lactate and pyruvate in a reversible reaction, and is commonly used as a biomarker of cell damage or death. Normal distribution of LDH and its isoenzymes in skeletal muscles of rats revealed that in controls, LDH activity was found to be highest in the EDL, followed by diaphragm, and lowest in the soleus (Table 35.8). Compared to muscles, the enzyme activity was meager in serum. It was interesting to note that all three muscles and serum contained all five electrophoretically distinct LDH isoenzymes, although with varying quantities. Each muscle presented a characteristic LDH isoenzyme pattern. For example, EDL contained a large proportion of LDH-5 (62.6% of total activity) and very little LDH-1 (3.5%). Soleus, on the other hand, had a very small amount of LDH-5 (11.3%) and about 20% each of the other four isoenzymes (LDH-1 through LDH-4). In general, values of LDH isoenzymes in diaphragm were intermediate to the EDL and soleus. Diaphragm had predominantly LDH-5 and LDH-4 (40% and 27.7%, respectively). In control serum, isoenzyme LDH-5 was 87% of the total LDH activity (100%).

Total LDH activity was significantly enhanced in EDL, diaphragm, and serum by carbofuran (1.5 mg/kg, s.c.) or methyl parathion (5 mg/kg, i.p.) within 1 h of injection. Each AChE inhibitor caused marked elevation of all five isoenzymes in serum, with maximum increases in LDH-1 and LDH-4 (three-fold). Unlike serum, muscle LDH isoenzymes depicted variable patterns by carbofuran or methyl parathion intoxication. A significant decline in ATP appears to be the mechanism involved in leakage of cytoplasmic/mitochondrial enzymes into circulation (Gupta *et al.*, 1994).

VIII. SKELETAL MUSCLES AND TOLERANCE DEVELOPMENT

Repeated application for a prolonged period of OP nerve agents or pesticides in concentrations that initially do not produce obvious symptoms of toxicity can lead to a limited degree of adaptation as seen in a reduction of the duration and intensity of muscle fasciculations, behavioral tolerance, and muscle fiber necrosis (Wecker and Dettbarn, 1976; Gupta *et al.*, 1985, 1986). Various cholinergic mechanisms in skeletal muscles underlying this phenomenon have been well documented, including: (1) reduced cholinergic binding sites of muscarinic and nicotinic receptors, (2) reduced uptake of choline, and (3) stimulation of AChE synthesis (Gupta *et al.*, 1985, 1986). In an *in vivo* subchronic study (DFP, 0.5 mg/kg, s.c./day for 14 days) in male rats, Gupta and Dettbarn (1986) demonstrated complete recovery of protein synthesis in skeletal muscles (soleus, EDL, and diaphragm) during the tolerance phase (day 14) from inhibition of protein synthesis observed during the toxicity phase (day 5). Further, during the toxicity phase (day 5),

TABLE 35.8. Normal distribution of LDH and LDH isoenzymes in skeletal muscles and serum of rats

		LDH isoenzymes				
	Total LDH	**LDH-1 HHHH**	**LDH-2 HHHM**	**LDH-3 HHMM**	**LDH-4 HMMM**	**LDH-5 MMMM**
Soleus	72,720 ± 2,484 (100)	14,962 ± 476 (20.7)	19,054 ± 617 (26.3)	16,118 ± 692 (22.2)	14,254 ± 1,332 (19.5)	8,316 ± 890 (11.3)
EDL	207,300 ± 22,945 (100)[a,b]	7,290 ± 1,890 (3.5)[a,b]	12,300 ± 2,033 (5.9)[a,b]	18,105 ± 1,498 (8.7)	40,420 ± 4,100 (19.5)[a,b]	129,870 ± 15,298 (62.6)[a,b]
Diaphragm	129,120 ± 4,828 (100)	17,020 ± 504 (13.2)	17,498 ± 571 (13.5)	16,190 ± 780 (12.6)	26,738 ± 1,080 (27.7)	51,680 ± 3,081 (40.0)
Serum	740 ± 28 (100)	9.8 ± 1.4 (1.3)	17.4 ± 2.0 (2.3)	10.2 ± 1.2 (1.4)	38.0 ± 3.8 (5.2)	643 ± 65 (87.0)

Values expressed in terms of IU/l are presented as means ± SEM ($n = 4$–6)
Numbers in parentheses are percentages of isoenzymes to total LDH activity (100%)
[a]Significant difference between EDL and soleus ($p < 0.01$)
[b]Significant difference between EDL and diaphragm ($p < 0.01$)

inhibition of *in vivo* protein synthesis was comparable to that seen with an acute dose of DFP (1.5 mg/kg, s.c.). Additional mechanisms that may contribute to general tolerance are the availability of other serine active site enzymes such as carboxylesterase (CarbE) and butyrylcholinesterase (BuChE). Although the functional role of these enzymes is unknown, binding to and inhibition of these enzymes reduce the free concentration of inhibitors otherwise available to interact with AChE (Gupta *et al.*, 1985). This is supported by studies showing that the toxicity of OPs can be potentiated by inhibition of CarbE, and tolerance to soman develops when plasma CarbEs recover during chronic exposure (Sterri *et al.*, 1981).

A major mechanism causing adaptation to the necrotic action of DFP at the NMJ is the reduction of nicotinic ACh receptors (B_{max}, 56%) without significant change in affinity (K_D). This loss of receptors can also explain the disappearance of fasciculations that was observed between days 3 and 5 of treatment (Gupta *et al.*, 1986). Whether presynaptic receptors regulating fasciculations are involved in this process remains to be determined. Changes in the postsynaptic receptor density could occur as an adaptation mechanism of the cell to the excessive cholinergic stimulation caused by AChE inhibition. In an earlier report, Chang *et al.* (1973) demonstrated a significant decrease in nicotinic ACh receptor density in rat diaphragm following repeated injections of neostigmine. The loss of ACh receptors may be caused by a reduction of junctional folds (Laskowski *et al.*, 1975) on which most of the nicotinic ACh receptors are located. Changes in the ionic milieu, especially increases in Ca^{2+} caused by the increased neuromuscular activity during the DFP-induced fasciculations, may be involved in the loss of secondary junctional folds. In essence, at the NMJ, the two major mechanisms leading to adaptation are: (1) an increased recovery of AChE activity as a result of *de novo* synthesis (Gupta *et al.*, 1986), and (2) a reduction in nicotinic ACh receptor binding sites (Gupta *et al.*, 1985, 1986, 1987a, b). The other mechanism supporting this tolerance development is an increase in nonspecific binding sites for DFP as seen in the recovery of CarbEs activity during days 5–14 (Gupta *et al.*, 1985, 1986). Lastly, mechanisms such as modification of ACh release from presynaptic sites may be an additional contributing factor (Carlson and Dettbarn, 1983, 1987, 1988).

IX. SKELETAL MUSCLE INVOLVEMENT IN INTERMEDIATE SYNDROME

The organophosphate (OP) insecticide-induced intermediate syndrome (IMS) was reported for the first time in human patients in Sri Lanka in 1987 (Senanayake and Karalliede, 1987; Karalliede and Henry, 1993). Thereafter, this syndrome has been reported in South Africa (1989), Turkey (1990), Belgium (1992), India (2003), and many other countries. IMS is clearly a separate entity from acute

TABLE 35.9. Protective effect of PBN against DFP-induced fasciculations, AChE inhibition, and necrosis in rat EDL

	Control	DFP[a]	PBN + DFP[b]	DFP + PBN[c]
Fasciculations	−	+	−	+
Necrotic fibers/cross-section of muscle	2 ± 2	82.55 ± 2.98*	0**	67.0 ± 4.59
AChE activity	12.17 ± 0.73 (100%)	3.70 ± 0.35* (30%)	10.85 ± 0.59** (89%)	2.97 ± 0.17 (24%)

[a]DFP (1.7 mg/kg, s.c.) was given 1 h before sacrificing the rats
[b]PBN (300 mg/kg, i.p.) was given 30 min before DFP (1.7 mg/kg, s.c.)
[c]PBN (300 mg/kg, i.p.) was given 20 min after DFP (1.7 mg/kg, s.c.) administration
Activity of AChE is expressed in terms of nmol/mg protein/min and number of necrotic fibers/1,000 muscle fibers. Ranking of fasciculations: −, absent; +, high frequency affecting all muscles. Values are means ± SEM of 5 muscles
*Significant difference between control and DFP-treated rats ($p < 0.01$)
**Significant difference between DFP-treated rats and PBN + DFP-treated rats ($p < 0.01$)

cholinergic crisis and delayed neuropathy. IMS is a life-threatening complication of OP poisoning, which most commonly occurs 48–72 h after exposure. In a recent case, Paul and Mannathukkaran (2005) reported IMS with the carbamate insecticide carbofuran. So far, IMS has not been documented with OP nerve agents. Clinically, IMS is characterized by acute respiratory paralysis and weakness in the territories of several cranial motor nerves, neck flexors, facial, extraocular, palatal, nuchal, and proximal limb muscles. Despite severe AChE inhibition, muscle fasciculations and muscarinic receptor-associated hypersecretory activities are absent. Based on EMG findings from OP-poisoned patients and experimental studies on laboratory animals, scientists have found that the defect in IMS is at the neuromuscular endplate and postsynaptic level, but the effects of neural and central components in muscular weakness have not been ruled out. EMG in the early stages reveals marked decrements at low rates of repetitive nerve stimulation and increments at a high rate, suggesting diverse types of impaired neuromuscular transmission. IMS seems to be due to persistent AChE inhibition at the endplate, presumably leading to combined pre- and postsynaptic impairment of neuromuscular transmission. Currently, very little is known about the type of damage at the motor endplate or about risk factors associated with IMS. For details on the involvement of muscles in IMS, readers are referred to a recent publication by De Bleecker (2006).

X. PREVENTION/TREATMENT OF MYOPATHY

Poisoning by anticholinesterase OP nerve agents is often characterized by abnormal muscle activity (such as fasciculations) and myopathy (muscle fiber necrosis). The severity of necrosis appears to be correlated with the frequency and duration of the fasciculations. Gupta *et al.* (1985, 1986, 1987) and Misulis *et al.* (1987) demonstrated that both soman and DFP produced muscle toxicity, although soman had its predominant action through CNS stimulation while DFP had prominent PNS as well as CNS activation. The fact remains that excess activation of muscle fibers due to sustained interaction between ACh and the ACh receptor, regardless of whether it arises indirectly from CNS or directly from PNS stimulation, results in muscle lesions (Gupta *et al.*, 1986, 1987a, b).

Various pharmacologic and therapeutic drugs have been tested to prevent or treat myopathy. Drugs effective in treatment of neuromuscular signs of anti-ChE toxicity include: (1) oximes that reactivate the phosphorylated AChE (Wecker *et al.*, 1978a), (2) subparalyzing as well as paralyzing doses of *d*-tubocurarine (Patterson *et al.*, 1987; Clinton and Dettbarn, 1987), (3) atropine sulfate/atropine methyl nitrate (Patterson *et al.*, 1987; Clinton *et al.*, 1988), (4) diazepam, and (5) creatine phosphate (Clinton *et al.*, 1988). In several studies, reversible AChE inhibiting carbamates, atropine, or anticonvulsants were given in combination with oxime to achieve an optimal effect. Some of the drugs, including AChE reactivators, muscarinic and nicotinic ACh receptor blockers, NMDA receptor antagonists, anticonvulsants, antioxidants, etc., are described here in brief for their prophylactic/therapeutic efficacy. It should be noted that some of these agents, however, produce toxicity when given for an extended period of time, thereby limiting their utility.

A. AChE Reactivators and ACh Receptor Blockers

The standard treatment of nerve agent-induced muscle toxicity calls for: (1) reactivation of the phosphorylated AChE with an oxime, and (2) blockage of the nicotinic ACh receptor sites from the stimulating action of ACh with *d*-tubocurarine. Oximes such as obidoxime, pralidoxime (2-pyridine aldoxime methochloride, 2-PAM) and a few others have been found very effective when given in combination with other drugs such as atropine, *d*-tubocurarine, diazepam, etc. Effectiveness of pretreatment with oximes varies with the chemical structures of the nerve agents and depends on the time after exposure. For example, it has been

established that HI-6 effectively activates AChE inhibited by soman, while TMB-4 is known as one of the most efficient reactivators of tabun-inhibited enzyme. In *in vivo* studies, Jovanovic (1983) evaluated two bis-pyridinium oximes (BDB-27 and HGG-12) on neuromuscular blockade induced by nerve agents (sarin, soman, tabun, and VX) in rats, and effectiveness was compared with the two most potent oximes (HI-6 and TMB-4). It was found that BDB-27 was equal or superior to HI-6 in sarin, soman, and VX and to TMB-4 in tabun-poisoned animals. The potency of HGG-12 was equal to HI-6 only in soman poisoning, but much less pronounced against neuromuscular blockade induced by the other nerve agents. In general, oxime therapy becomes progressively ineffective with time due to changes in the enzyme–inhibitor complex which loses an alkyl radical. This so-called aged phosphorylated enzyme complex is resistant to oxime reactivation (Radic and Taylor, 2006). Thus, prevention of formation of the enzyme–inhibitor complex seems to be one of the preferred strategies in treatment of myopathy.

The best pretreatment should include the agents that prevent access of the nerve agent to AChE without affecting its activity or a combination of the drugs that would decrease the release of ACh from nerve terminals and/or partially block access to the nicotinic as well as the pre- and postsynaptic muscarinic receptors. So far, timely administration of atropine sulfate or atropine methyl nitrate in combination with an oxime, such as 2-PAM or diazepam, is currently the treatment of choice for OP poisoning. Atropine has been shown to modify ACh receptor interaction at the NMJ postsynaptically by shortening the opening time of the ion channel and possibly by lowering Na^+ conductance (Katz and Miledi, 1973). Despite the fact that atropine is up to 2,000 times less potent than *d*-tubocurarine at nicotinic sites (Beraneck and Vyskocil, 1967), atropine sulfate has been observed to reduce motor activity and muscle necrosis caused by AChE inhibitors (Clinton and Dettbarn, 1987). Atropine methyl nitrate is four times more potent than atropine sulfate in its peripheral effects (Malick and Barnett, 1975; Lonnerholm and Widerlov, 1975) and as expected has been confirmed to ameliorate peripheral motor activity caused by AChE inhibitors than atropine sulfate (Clinton *et al.*, 1988). Atropine methyl nitrate (16 mg/kg, s.c.) failed to ameliorate the central effects of both soman and DFP (Clinton *et al.*, 1988) due to its quaternary structure that hinders its passage across the blood–brain barrier (Lonnerholm and Widerlov, 1975).

Earlier studies with *d*-tubocurarine and atropine indicated that nicotinic and muscarinic ACh receptor blockers can modulate the release of ACh induced by an inhibitor of AChE (Carlson and Dettbarn, 1987, 1988). The effects of these drugs, whether inhibiting or stimulating ACh release, are concentration dependent and determined by the frequency of nerve activity (Bowman, 1980; Wessler *et al.*, 1987a, b). Therefore, drugs that reduce axonal hyperexcitability by decreasing amount of ACh released from the nerve terminals without interfering with normal transmission provide another pretreatment possibility. Protection against DFP-induced myopathy was achieved by using small concentrations of atropine sulfate (16 mg/kg, s.c.) or atropine methyl nitrate (16 mg/kg, s.c.) and *d*-tubocurarine (75 µg/kg, s.c.) that prevented fasciculations and muscle necrosis without interfering with the normal neuromuscular function (Patterson *et al.*, 1987). In this study, pretreatment agents acted presynaptically by preventing DFP-induced backfiring and muscle fasciculations possibly by reducing the release of ACh. The protective drugs in the concentrations used had no significant effect on the normal characteristics of conduction and transmission. Carlson and Dettbarn (1987) showed that *d*-tubocurarine or atropine sulfate in subparalytic concentrations prevented the increases in miniature endplate potential (MEPP) frequency when given prior to an AChE inhibitor or attenuated the increased frequency to normal when given after the increase of frequency. At a subparalytic dose *d*-tubocurarine (50 µg/kg, i.v.) is known to suppress fasciculations by preventing repetitive firing of nerve terminals. Furthermore, *d*-tubocurarine and atropine sulfate reduced Ca^{2+} influx and ACh release (through presynaptic receptors), which is associated with repetitive activity as seen during AChE inhibitor-induced antidromic firing.

B. NMDA Receptor Antagonist

In a series of *in vivo* experiments, rats receiving a sublethal dose of soman (100 µg/kg, s.c.), sarin (110 µg/kg, s.c.), tabun (200 µg/kg, s.c.), VX (12 µg/kg, s.c.), or DFP (1.5 mg/kg, s.c.) developed seizures and severe muscle fasciculations within 15–20 min that lasted for about 4–6 h. Marked inhibition of AChE activity and necrotic lesions in skeletal muscles (soleus, EDL, and diaphragm) became evident between 1 and 24 h post-injection and persisted for several days. Pretreatment of rats with NMDA receptor antagonist memantine (18 mg/kg, s.c.) together with atropine sulfate (16 mg/kg, s.c.), 60 min and 15 min, respectively, prior to DFP or nerve agents, significantly attenuated AChE inhibition, and prevented myonecrosis and muscle fasciculations as well as other signs of behavioral toxicity. No muscle fasciculations were seen at any time (Gupta and Dettbarn, 1992).

Protection of AChE inhibition was greater when memantine was given prophylactically than therapeutically. It is important to mention that memantine itself does not influence normal activity of AChE in non-OP-treated animals. Although the precise mechanism involved in reduction of AChE inhibition by memantine against nerve agents is yet to be elucidated, spontaneous reactivation as the cause of the remaining high enzyme activity was ruled out since the enzyme activity was determined within a short period of time, i.e. 1 h after acute intoxication with soman, sarin, tabun, VX, or DFP (Gupta *et al.*, 1986, 1987a, b, 1991; Gupta and Dettbarn, 1992), and AChE activity remained low when only atropine sulfate (16 mg/kg, s.c.) was used. Studies by McLean *et al.* (1992) revealed that memantine

did not prevent inhibition of AChE by edrophonium, an anionic site inhibitor, or by decamethonium, a peripheral site inhibitor of AChE. Thus, memantine appears to bind to a different modulatory site to protect this enzyme's activity. The other mechanisms by which memantine might have attenuated AChEI's toxicity may include:

- reduced reflex excitability of both flexors and extensors (Wand *et al.*, 1977)
- reduced high frequency of repetitive activation of peripheral nerves by reducing the permeability of Na^+ and Ca^{2+} in axonal membranes (Wesemann and Ekenna, 1982; Wesemann *et al.*, 1983)
- blockage of nicotinic ACh receptor-ion channel complex (Masuo *et al.*, 1986)
- prevention of neural hyperexcitability (McLean *et al.*, 1992)
- central muscle relaxation (Grossman and Jurna, 1977)
- reduced seizures by uncompetitive NMDA receptor blockage (Danysz *et al.*, 1994; Carter, 1995; Parsons *et al.*, 1999)
- prevention of cellular energy depletion (Gupta and Goad, 2000).

Although not all of these mechanisms have yet been investigated in the protection of OP nerve agent-induced myopathy by memantine, it appears that memantine provides protection by multiple mechanisms. It needs to be mentioned that no significant change occurred in AChE activity in skeletal muscles of OP-untreated rats receiving memantine and atropine sulfate. Prophylactic administration of memantine and atropine sulfate also blocked the AChEI-induced increase in levels of citrulline and F_2-isoprostanes, markers of NO synthesis and lipid peroxidation, respectively. Memantine has the advantage of providing prophylactic benefits without producing sedation or any other side effects. Thus, memantine may prove to be a superior drug to many other agents.

C. Anticonvulsants and Anesthetics

Prevention or treatment of myopathy becomes difficult since individual AChEIs differ in their major sites of action. As mentioned earlier, soman-produced muscle hyperactivity generated mainly in the CNS, while DFP-induced hyperactivity arose approximately equally from the CNS and the peripheral nerve and NMJ. Clinton *et al.* (1988) reported that in the case of both soman (90 µg/kg, s.c.) and DFP (1.5 mg/kg, s.c.) poisoning, ketamine (25 mg/kg, s.c.) reduced centrally generated motor activity, while atropine methyl nitrate (16 mg/kg, s.c.) and sodium phenytoin (15 mg/kg, i.v.) had no significant effect. It was suggested that the effectiveness of ketamine may alter patterns of neuronal firing by reducing high-frequency neuronal discharges that are characteristic of seizures (McLean and MacDonald, 1986). Ketamine is known to act on sodium channels by producing a dose-dependent reduction of inward sodium current in a manner similar to that of local anesthetics, with a resultant suppression of high-frequency neuronal bursts, which are manifested as excess muscle activity seen with OP poisoning. Since ketamine and drugs in its class also have specific anticholinergic effects at both nicotinic and muscarinic (more potent at nicotinic than at muscarinic) receptors (Kloog *et al.*, 1977), it may be a direct antagonist in the CNS against the cholinergic activation caused by AChEIs (Gupta *et al.*, 1987a, b). Additionally, ketamine can inhibit excitation–contraction coupling within the muscle by altering Na^+ conductance at the muscle membrane (Marwah, 1980a, b).

Clinton *et al.* (1988) demonstrated that the anticonvulsant phenytoin did not reduce motor activity of central origin induced by either soman or DFP. Phenytoin suppresses sustained high-frequency neuronal firing through a membrane potential-dependent blockade of Na^+ channels with resulting inhibition of nonsynaptic events involved in epileptogenesis (MacDonald and McLean, 1986; McLean and MacDonald, 1986; Yaari *et al.*, 1986). The failure of phenytoin to control the seizures induced by AChEIs suggests that they may not result from the same mechanisms of epileptogenesis as seizures produced by maximal electrical shock, against which phenytoin is most effective.

Conventional anticonvulsant compounds have been reported to provide limited protection against nerve agent-induced seizures and muscle necrosis when given therapeutically (Lipp, 1972; Clinton *et al.*, 1988). Sedation, tolerance, and abuse potential, however, limit prophylactic use of benzodiazepine compounds.

In animal models, clonidine has also been reported to prevent nerve agent-induced seizures (Buccafusco *et al.*, 1988). Prophylactic use of clonidine may be limited by the marked ataxia and sedation produced by this drug in effective concentrations.

D. Antioxidants and Spin-Trapping Agents

During hyperactivity of the muscles by anti-ChE toxicity, excessive amounts of ROS/RNS can be generated and exceed the capacity of the muscle defense system, thus producing oxidative stress. Excessively generated ROS/RNS can cause muscle injury by reacting with cellular components, such as membrane phospholipids, mitochondrial enzymes/proteins and nucleic acids. There are several points in excitotoxicity mechanisms where antioxidants can intercept, but prevention of the generation of ROS and their scavenging are of special interest. An antioxidant such as the lipid-soluble vitamin E (α-tocopherol) is an excellent blocker of ROS production as it extracts hydrogen and interrupts lipid peroxidation. Vitamin E mainly acts as a chain-breaking antioxidant and radical scavenger, protecting cell membranes against oxidative damage. There are reports demonstrating that vitamin E concentrates in the mitochondria, the major site for the generation of ROS as well as energy metabolites. Therefore, vitamin E regulates ROS production, maintains

oxidative phosphorylation in mitochondria, and accelerates restitution of high-energy metabolites.

Electron spin resonance (ESR) spectroscopy using spin traps allows direct identification and characterization of ROS. A synthetic spin-trapping agent such as phenyl-*N*-*tert*-butylnitrone (PBN) is capable of scavenging many types of free radicals. This compound is widely used to trap ROS in a variety of physical, chemical, and biological studies using electron magnetic resonance spectrometry. PBN is known to be concentrated in the mitochondria, where it reacts with ROS and forms stable adducts, and thereby maintains normal levels of energy metabolites. In addition, PBN has other pharmacological actions, such as: (1) reversible Ca^{2+} channel blockade (Anderson *et al.*, 1993), (2) a direct reversible interaction with AChE against phosphorylation by DFP (Zivin *et al.*, 1999; Milatovic *et al.*, 2000a, b), (3) protection of COX activity (Milatovic *et al.*, 2001), and (4) induction of hypothermia (Pazos *et al.*, 1999).

Pretreatment with PBN (75, 150, or 300 mg/kg, i.p.) 30 min prior to DFP injection (1.7 mg/kg, s.c.) in a dose-dependent manner protected AChE activity from inhibition, and prevented muscles from undergoing necrosis and rats from fasciculations (Milatovic *et al.*, 2000a, b). The protective mechanism of PBN is thought to be due to its ROS/RNS scavenging property. Treatment with PBN 20 min after DFP exposure neither prevented fasciculations nor protected AChE activity. While the role of PBN as an antioxidant is well established, its prophylactic effect against excitotoxicity induced by an AChEI is due to its protection of AChE from critical inhibition (Milatovic *et al.*, 2000a, b). Unlike PBN, vitamin E neither prevented DFP-induced muscle fasciculations nor protected AChE. Like vitamin E, PBN also concentrates in the mitochondria, thereby it can regulate ROS production, maintain oxidative phosphorylation, and accelerate restitution of high-energy metabolites.

E. Creatine and Phosphocreatine

Inhibition of AChE leads to unremitting stimulation of muscles, which in turn, causes marked depletion of high-energy phosphates (HEP), such as ATP and phosphocreatine (PCr) and their major metabolites (Gupta and Dettbarn, 1987). If this stimulation is sufficiently low in intensity or brief in duration, cellular recovery will ensue without lasting consequences. If, however, intense cholinergic stimulation is allowed to persist, a self-reinforcing cycle of cellular damage is set into motion. Phosphocreatine (PCr) is the immediate precursor of ATP and in a reversible Lohman reaction where PCr is synthesized only from ATP, the decline in PCr is always greater than in ATP. ATP depletion for several hours to approximately 30–40% of normal levels leads to a fall in the mitochondrial membrane potential that is associated with: (1) reduced energy production (due to decrease in complex I and complex IV activities), (2) impaired cellular calcium sequestration, (3) activation of protease/caspases, (4) activation of phospholipases, (5) activation of nitric oxide synthase (NOS), and (6) excessive generation of ROS/RNS. Several of these steps are associated with exacerbation and propagation of the initial depletion of ATP; most notable are the decreases in complex I and complex IV activities, the impairment of mitochondrial calcium metabolism that regulates ATP production even in the face of a constant supply of substrates, and the generation of nitric oxide, which binds reversibly to cytochrome c oxidase (COX) in competition with oxygen, with subsequent sensitization to hypoxia. During the course of the excitatory process, such as muscle hyperactivity, a high rate of ATP consumption coupled with the inhibition of oxidative phosphorylation compromises the cell's ability to maintain its energy levels, and excessive amounts of ROS and RNS are generated. Thus, the combination of impaired synthesis of ATP with its greater utilization during muscle hyperactivity appears to result in a significant depletion of ATP.

Since the major energy sources are ATP and PCr, an increase of both compounds in muscles through administration of creatine appears to sustain ATP levels under stress conditions. This is supported by the findings that rats pretreated intravenously with PCr showed reduced muscle necrosis otherwise seen following DFP treatment. PCr did not attenuate the DFP-induced muscle fasciculations generating the necrosis (Clinton and Dettbarn, 1987).

XI. CONCLUDING REMARKS AND FUTURE DIRECTION

Skeletal muscles are the target for a variety of chemicals, especially OP nerve agents. These agents modulate structural and functional properties of the muscles, and the toxic effects can vary from minor chest pain, muscle cramps in the legs to complete paralysis or even death. Both *in vivo* and *in vitro* data strongly implicate that although OP nerve agents initially exert acute toxicity by excitotoxicity involving the cholinergic system, additional noncholinergic mechanisms that contribute to these effects include glutamatergic system and excess generation of free radicals (ROS/RNS) and alterations in antioxidant and the scavenging system, causing oxidative stress, lipid peroxidation, high-energy phosphates depletion, and muscle necrosis. Wide variations exist between slow and fast fiber-containing muscles and they further appear to respond differently to each OP nerve agent. Available sensitive biomarkers are indicative of cytotoxicity and mitochondrial toxicity. Myopathy induced by AChE inhibiting OP nerve agents is a serious concern, since it is untreatable in most circumstances. Instead, prevention seems a better option. More potent AChE reactivators need to be developed for better therapeutic efficacy against OP nerve agent-induced myopathy.

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CHAPTER 36

Reproductive Toxicity and Endocrine Disruption of Potential Chemical Warfare Agents

TIMOTHY J. EVANS

I. INTRODUCTION

Reproduction is a critical biological process in all living systems and is required for species survival. The immediate, short-term, toxicological concerns regarding chemical warfare and acts of agricultural and industrial terrorism are concerned primarily with human and animal incapacitation and mortality. Logically, most of the currently available literature on these aspects of warfare and terrorism focuses on these immediate adverse health effects and their sequelae. However, the entire scope of the effects of these toxic insults can involve severe emotional and financial distress, as well as diminished prognosis for long-term survival of exposed human and animal populations, arising from toxicant-induced abortions, congenital defects, and infertility. It is therefore important that individuals working in human and veterinary medicine, the military, public health, government regulatory agencies, industry and agriculture, as well as public policy makers, be familiar with some of the potential adverse reproductive effects of these types of toxicants on exposed humans and animals. This chapter will review what is currently understood about the potential effects of chemical warfare agents, nuclear fallout, and hazardous industrial and agricultural wastes on human and animal reproductive function.

For the purposes of this chapter, the term ''reproduction'' will be used primarily in reference to vertebrate species of animals (especially mammals) and will be inclusive of ''development'' (Figure 36.1), which is sometimes treated as a separate topic in toxicology texts. This particular chapter emphasizes what is currently known about the adverse effects of known chemical warfare agents and selected environmental contaminants on male and female reproductive function, as well as xenobiotic-induced effects on the growth, maturation, and sexual differentiation of the embryo and fetus. Endocrine disruption is an extremely common mechanism of action for xenobiotics associated with impaired reproductive function and will be discussed along with reproductive toxicity in this chapter. Efforts will be made to clarify the currently used terminology related to these topics and to provide the reader with a brief description of proposed mechanisms of action and observed reproductive outcomes associated with selected toxicants which might be relevant to chemical warfare and/or acts of terrorism.

Unfortunately, although there is relative lack of information documenting long-term, reproductive effects of the types of toxicants covered in this book, space constraints still limit the amount of information which can be presented in this chapter. There are a number of recently published textbooks and book chapters which cover some of these and related subjects in greater detail and provide information which is complementary to what is presented in this chapter (Capen, 2008; Evans, 2007; Foster and Gray, 2008; Golub, 2006a, b; Gupta, 2006, 2007c; Naz, 2005; Rogers and Kavlock, 2008; Romano *et al.*, 2008; Senger, 2003). This is especially true with respect to normal reproductive anatomy and function (Evans, 2007; Senger, 2003), and the reader is directed to these publications and the other references cited in this chapter in order to gain additional insight into specific areas of reproductive function and toxicology.

It is important that the reader understand that the areas of toxicology involving the long-term effects of chemical warfare agents and environmental contaminants, reproductive toxicity, in general, and endocrine disruption, in particular, are in continual flux. New data and exceptions to ''classical'' mechanisms of action are being reported on a regular basis, and there continues to be ongoing debate about the effects of chronic low-level exposures to toxicants available for or arising from military and terrorist activities and various aspects of normal, as well as xenobiotic-induced abnormal reproductive function. Every effort has been made to accurately represent what is currently understood about the topics of discussion in this chapter. Controversial topics or those currently still subject to debate within the scientific community have been noted wherever possible.

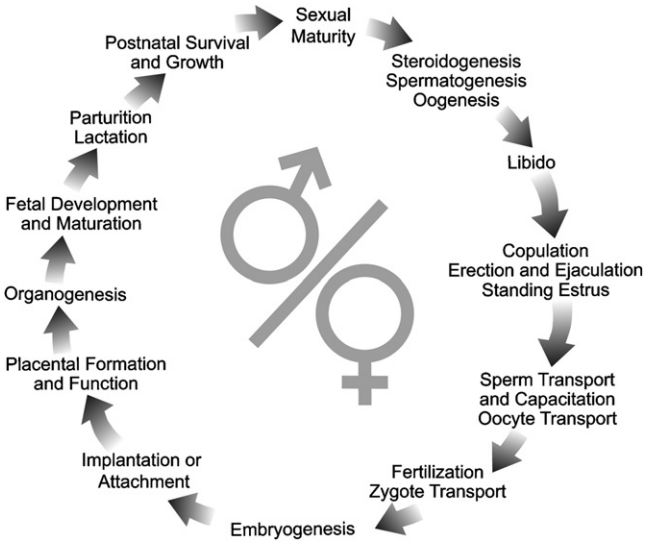

FIGURE 36.1. The multiple steps involved in reproductive development and function in both males and females are shown schematically to illustrate the complexity of reproduction in mammalian species and to demonstrate the various stages in the reproductive process which can be targeted for toxic insult. This figure was adapted, with permission, from Ellington, J.E., Wilker, C.E. (2006). *Small Animal Toxicology*, 2nd edition (M.E. Peterson, P.A. Talcott, eds). Elsevier Saunders, St Louis (modifications and artwork courtesy of Don Connor).

II. IMPORTANT DEFINITIONS AND CONCEPTS

A. Chemical Warfare Agents

For the purposes of this chapter, ''chemical warfare agent'' (CWA) will be used as a fairly comprehensive term to refer to a diverse group of toxicants commonly discussed within the context of chemical incapacitation for crowd control during riots or death or incapacitation associated with military use or terrorism. The lacrimatory and irritant riot control agents will include α-chlorbenzylidene malonitrile (CS), dibenz (b,f)-1:4 oxazepine (CR), ω-chloroacetophenone (CN) and oleoresin of capsicum (OC pepper spray) (Salem *et al.*, 2008a). The CWAs currently of greatest interest and for which the greatest amount of data have been gathered are arsenicals, chlorine gas, phosgene and phosgene oxime, sulfur mustard, ricin, hydrogen cyanide and cyanide-related compounds, and organophosphate nerve agents (Kikilo *et al.*, 2008; Wismer, 2007).

B. Environmental Contaminants Associated with Industrial or Agricultural Terrorism

For the sake of completeness, this chapter will also discuss the adverse reproductive effects of potential toxicants, such as ionizing radiation, pesticides and other organic environmental contaminants, as well as heavy metals, which are known to adversely affect reproductive function. While all of these potential toxicants have been or are currently being released into the environment in generally low concentrations as a consequence of normal industrial and agricultural activities, the results of accidents, such as those which occurred in Chernobyl, in the former Soviet Union (ionizing radiation), Bhopal, India (methyl isocyanate or MIC), Seveso, Italy, and Times Beach, Missouri (dioxins), and Minamata, Japan, and Basra, Iraq (methyl mercury), underscore the potential impact that large-scale, industrial or agricultural terrorism-related releases of these contaminants could have on populations of humans and animals. In fact, these environmental contaminants have the potential to become ''low-tech'' CWAs in the hands of terrorists and less sophisticated military organizations.

C. Reproduction

As represented in Figure 36.1, reproduction in humans and domestic, wild, and laboratory vertebrate animals encompasses the wide range of physiological processes and associated behaviors and anatomical structures involved in the production of the next generation and the survival of a given species of animal (Evans, 2007; Senger, 2003). The physiological processes involved in reproduction generally include the following: (1) gametogenesis (production of sperm or ova) and the pre- and peri-pubertal changes leading up to its onset; (2) release of gametes [i.e. sperm transport and maturation, penile erection and ejaculation of sperm (mammals), copulation between a male and a female of the same species (several vertebrate classes) and ovulation of oocytes]; (3) formation of the zygote (i.e. sperm storage, capacitation, and other processes leading to fertilization, or union, of a single sperm with an egg); (4) embryonic and fetal development during the incubation process in egg-bearing vertebrates or, especially in the case of mammals, during pregnancy (gestation) [i.e. activities related to the initiation and progression of zygote cleavage, blastocyst formation, separation of the germ layers, placentation (mammalian species), neurulation and organogenesis (including sexual differentiation)]; (5) ''birth'' of a single or multiple offspring (hatching in oviparous vertebrates); and, finally, in mammalian species, (6) the initiation and maintenance of milk production (lactation) for the postpartum nutrition of offspring (Evans, 2007).

D. Reproductive Toxicity

For the purposes of this chapter, ''reproductive toxicity'' will refer to any manifestations of xenobiotic exposure, including ''endocrine disruption'' (see discussion below), reflecting adverse effects on any of the physiological processes and associated behaviors and/or anatomical structures involved in animal reproduction or development (Figure 36.1). This is a fairly broad definition which encompasses developmental toxicity, as well as any toxic

effects of post-pubertal exposures to xenobiotics on either male or female reproduction. ''Developmental toxicity'' refers to any adverse effect on the developing organism associated with either pre-conception parental exposures to toxicants or post-conception xenobiotic exposures to the embryo, fetus, or pre-pubertal offspring, and adverse effects associated with developmental toxicity of xenobiotics might not necessarily be observed until after the affected individuals have reached sexual maturity (Eaton and Klaassen, 2001; Evans, 2007; Hodgson *et al.*, 2000).

1. Teratogenesis

The term ''teratogenesis'' is derived from the Greek word for monster (*teras*) and is a form of developmental toxicity (Rogers and Kavlock, 2008). ''Teratogenesis'' refers specifically to developmental defects induced by toxicant exposures occurring between conception and birth (Eaton and Klaassen, 2001; Evans, 2007; Hodgson *et al.*, 2000; Rogers and Kavlock, 2008). The types of abnormalities that are typically associated with teratogenesis include embryonic or fetal death; morphological, functional and/or neurobehavioral abnormalities; and decreased growth rate and/or birth weight (Evans, 2007; Rogers and Kavlock, 2008).

With respect to teratogenesis, there are six basic tenets of teratology, first defined by J.G. Wilson in 1959, which need to be kept in mind whenever gestational exposure to a teratogenic xenobiotic is suspected or when a chemical is being evaluated for its teratogenic potential (Evans, 2007; Wilson, 1977):

1. Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which it interacts with environmental factors.
2. Susceptibility to teratogenic agents varies with the developmental stage at the time of exposure.
3. Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate abnormal embryogenesis.
4. The final manifestations of abnormal development are death, malformation, growth retardation, functional disorder.
5. The access of adverse environmental influences to developing tissues depends on the nature of the influences (agent).
6. Manifestations of deviant development increase in degree as dosage increases from no effect to the totally lethal level.

2. Mechanisms of Reproductive Toxicity and Teratogenesis

In general, normal reproduction and development require rapidly replicating and differentiating cells undergoing mitosis and, within the gonads, meiosis. There are a wide range of specific mechanisms of action by which xenobiotics can adversely affect reproductive function, including embryonic and fetal development. Many of these mechanisms are the same as those for toxicants affecting other organ systems and essentially involve some sort of toxicant-induced interference with the cell cycle, cellular dysregulation, and alterations in cellular maintenance which, when possible, the body attempts to repair, either successfully or unsuccessfully (Gregus, 2008). Oxidative damage and interference with normal enzymatic reactions are two common mechanisms by which xenobiotics and, especially, some CWAs (Smith *et al.*, 2008) can cause the dysregulation and altered maintenance of cells within various organs and tissues.

Teratogenesis can be associated with each of the following mechanisms of action: (1) excessive cell death; (2) interference with apoptosis; (3) reduced cellular proliferation rate; (4) failed interactions between cells; (5) impaired morphogenetic movements; (6) reduced synthesis of components essential for growth and development; (7) mechanical disruption; and (8) alterations in pH (Evans, 2007; Hood, 2006; Hood *et al.*, 2002). Some teratogens are capable of more than one mechanism of action, and it is important to keep in mind that the observed developmental abnormalities associated with exposure to any given teratogen will in large part be dependent on the timing of the exposure to that xenobiotic during gestation. Familiarity with the timing of important developmental events in species of interest is critical in the diagnosis and prevention of teratogenesis, as well as the design of experiments investigating the teratogenic potential of different chemicals in animal models (Evans, 2007).

Normal reproduction and development require signaling within and between a variety of diverse organs, and, in sexual reproduction and mammalian pregnancy, critical communication even takes place between distinctly different organisms (i.e. male and female and mother and offspring, respectively) (Evans, 2007). It should be remembered that premature parturition or abortion can be induced by any circumstances which cause fetal or, potentially, maternal stress and initiate the cascade of endocrine and neural signaling events which would normally lead to parturition. Any sublethal intoxication or emotionally traumatic event in a pregnant woman or animal has the potential to threaten fetal survival.

The dependency of reproductive function on signaling pathways inclusive of gene transcription makes this physiological process especially prone to adverse effects associated with xenobiotic-induced disruption of or interference with cell-to-cell, organ-to-organ and/or even animal-to-animal communication. Many of the mechanisms which interfere in some way with physiological signaling activity can be classified as forms of ''endocrine disruption'' (see discussion below), but there is a great deal of overlap between the various different mechanisms for reproductive toxicity. The level of exposure to a particular toxicant is an important determinant of what toxic effects are observed,

and xenobiotics which ''disrupt'' endocrine pathways can do so without interactions with endogenous receptors, using mechanisms of action which can cause other forms of toxic insult at various dosages.

3. Reproductive Toxicants

Any xenobiotic associated with adverse effects on development of male or female reproductive function can be classified as a ''reproductive toxicant'' (Evans, 2007; Rogers and Kavlock, 2008). Even chemicals adversely affecting animal well-being have a potential negative impact on development and reproductive function. This chapter will attempt to focus on toxicants which are available for or could arise from military and terrorist activities and specific mechanisms of actions which have a direct effect upon the male and/or female reproductive tract or which target normal embryonic and/or fetal growth and maturation (Evans, 2007).

4. Teratogens

The subclass of reproductive toxicants capable of inducing teratogenesis is referred to as ''teratogens''. Some teratogenic chemicals induce their adverse effects indirectly on the fetus by altering maternal synthesis of essential nutrients or by other mechanisms which do not require their transport across the placenta. However, many teratogens directly affect fetal development by crossing the ''placental barrier'' and entering the fetal circulation. The passage of nutrients, hormones, and other endogenous, as well as exogenous, substances across the placenta has been traditionally thought of by some references as primarily a function of the intimacy (i.e. number of tissue layers) between the maternal and fetal circulations, especially with respect to maternal immunoglobulins which cross some types of placentation but not others (Evans, 2007). While it is true that placental characteristics, such as thickness, surface area, carrier systems, and lipid-protein characteristics, can influence the passage of xenobiotics across the placenta and that the placenta is generally impermeable to chemicals with molecular weights greater than 1,000 Da, most xenobiotics have molecular weights less than 500 Da and cross the placenta by simple diffusion (Foster and Gray, 2008). It is currently thought that a potential teratogen's molecular size, degree of ionization, protein binding, and lipid solubility are the most important determinants of that chemical's ability to move from the maternal circulation across the placenta into the fetal circulation (Evans, 2007; Foster and Gray, 2008; Rozman and Klaassen, 2001; Senger, 2003). Some toxic xenobiotics can be actively transported by mechanisms intended for structurally similar endogenous molecules (Rozman and Klaassen, 2001), and there is some experimental evidence to suggest that transplacental transport of lead can mimic that of calcium (Evans, 2007; Evans *et al.*, 2003).

5. Endocrine Disruption

''Endocrine disruption'' is a developing, multidisciplinary area of research, involving aspects of both toxicology and endocrinology (McLachlan, 2001) and is a potential mechanism of action for many toxicants, especially those affecting reproduction. This term has been defined in a variety of different ways, depending on the circumstances and the intended audience. Endocrine disruption can also be defined fairly narrowly with respect to toxicant origin (synthetic versus naturally occurring); source or site of toxicant exposure (exposure from warfare- or terrorism-related activities versus exposure from environmental contamination); xenobiotic mechanism of action (receptor agonism and/or antagonism versus other mechanisms independent of direct interactions between xenobiotics and receptors); and/or the timing of exposure (prenatal versus postnatal exposures) (Evans, 2007; Krimsky, 2000, 2001). However, the definition of endocrine disruption used in this and another book chapter previously written by the author (Evans, 2007) will be fairly broad and will encompass the effects of any synthetic or naturally occurring xenobiotic which can affect the endocrine system of exposed individuals (i.e. the balance of normal hormonal functions) and, as a result of exposure, cause physiological alterations (Evans, 2007; Hodgson *et al.*, 2000; Keith, 1997). Within the broad scope of this definition, reproduction, including prenatal and pre-pubertal development, certainly would be expected to be one of the physiological functions most profoundly affected by chemicals associated with chemical warfare or environmental contamination capable of endocrine disruption; however, adverse effects on other, nonreproductive endocrine systems can also be associated with exposures to xenobiotics (Evans, 2007; Guillette, 2006). Thyroid function, glucocorticoid metabolism, and other endocrine as well as enzymatic factors associated with adipogenesis have recently been shown to be susceptible to interference by several different classes of chemical compounds (Capen, 2008; Cooke and Naz, 2005; Evans, 2007; Guillette, 2006; Grün and Blumberg, 2006; Newbold *et al.*, 2005, 2006).

6. Mechanisms of Endocrine Disruption

Although the imitation and/or inhibition of the actions of androgens and, especially, estrogens by xenobiotics is what was first referred to as endocrine disruption, both the multidisciplinary area of study and mechanism of action generally referred to as endocrine disruption have evolved over the years to encompass a wide range of specific mechanisms of action which can ultimately result in adverse effects on invertebrate and/or vertebrate animals (Evans, 2007; McLachlan, 2001). Endocrine disruption involves many mechanisms of action which can ultimately result in adverse effects on animal species. The mechanisms of action involved in endocrine disruption can include effects which are mediated directly by interactions between the xenobiotic and an endogenous hormone receptor (i.e. the

xenobiotic functions as a ligand for an endogenous receptor and a receptor–ligand complex is formed), as well as those adverse effects which alter hormonal functions without direct interactions between the toxicant and an endogenous receptor (Capen, 2008; Evans, 2007; Keith, 1997). It should also be noted that a given xenobiotic can potentially disrupt the normal balance of hormonal function by more than one mechanism which is independent of direct interactions between the toxicant and an endogenous hormone receptor (Evans, 2007).

''Classic'' endocrine disruption can involve imitation or mimicry of the interactions between cellular receptors and endogenous hormones (i.e. receptor agonism) and/or a blockade or inhibition of the formation of receptor–hormone complexes (i.e. receptor antagonism) (Evans, 2007; McLachlan, 2001), and both genomic and non-genomic physiological responses can be affected by this mimicry or blockade of endogenous hormone receptor-mediated activity (Evans, 2007; Thomas and Khan, 2005). Endocrine disruption can also be mediated by the complex interactions between the endogenous aryl hydrocarbon receptor (AhR) and its major agonists, which are xenobiotics belonging to the class of environmental contaminants referred to collectively as ''halogenated'' or ''polyhalogenated aromatic hydrocarbons'' (HAHs or PAHs, respectively) (Evans, 2007; Safe, 2005).

Endocrine disruption which is independent of interactions between xenobiotics and endogenous hormone receptors can occur in a variety of different ways, including alterations in the number of hormone receptor sites (up- or down-regulation) or direct or indirect hormone modifications which alter hormonal function (Evans, 2007; Keith, 1997). Xenobiotics can change the rate of synthesis or destruction of endogenous hormones and can alter how hormones are stored, how they are released into and/or transported within the circulation, or even how they are eventually cleared from the body (Capen, 2008; Evans, 2007; Keith, 1997; Sikka *et al.*, 2005). Any xenobiotic toxic to hormone-producing organs or tissues (e.g. testis and ovary) also has the potential to decrease hormone synthesis and thereby indirectly cause endocrine disruption (Devine and Hoyer, 2005; Evans, 2007).

In addition to the aforementioned mechanisms of endocrine disruption, there has recently been increasing interest in the association between prenatal exposures to some hormonally active toxicants and the postnatal development of neoplasia (cancer) involving the reproductive tract, as well as the occurrence of transgenerational or vertically transmitted adverse reproductive effects (Crews and McLachlan, 2006). Either genetic mutations [i.e. alterations in the genotype or deoxyribonucleic acid (DNA) sequence] or epigenetic changes, such as DNA methylation of CpG nucleotides in the promoter regions of genes, which are heritable but nongenetic modifications in the properties of a cell (inherited phenotypic alteration without genotypic change), are possible explanations for these phenomena (Crews and McLachlan, 2006; Evans, 2007; McLachlan, 2001). Patterns of DNA methylation are generally established during development at the gastrulation stage (i.e. lineage-specific pattern in somatic cells) and after sex determination (i.e. germ line-specific lineage pattern in the gonad), and DNA methylation can facilitate genomic imprinting, which results in the expression of the allele from only one parent (i.e. monoallelic expression) (Anway and Skinner, 2006; McLachlan, 2001).

7. Endocrine Disrupting Chemicals, Endocrine Disruptors, and Hormonally Active Agents

Any reproductive toxicant capable of endocrine disruption can also be considered an endocrine disrupting chemical (EDC) or an endocrine disruptor. Another term frequently used with respect to endocrine disruption, especially regarding xenobiotics which interact with endogenous hormone receptors, is hormonally active agent (HAA). In most instances, ''endocrine disrupting chemical'', ''endocrine disruptor'', or ''hormonally active agent'' can be used interchangeably to discuss the actions of a given xenobiotic (Evans, 2007).

III. THE REPRODUCTIVE TOXICITY OF SELECTED TOXICANTS

It should be remembered that the use of CWAs will invariably be associated with wars, acts of terrorism, revolutions, and civil unrest, all of which do not occur in a vacuum and will be concurrent with emotional stress, famine, and other conditions leading to reproductive failure. As a result, information is relatively lacking with respect to specific, adverse reproductive effects, as well as endocrine disruption, related to acute and, particularly, low-level, chronic exposures to many potential CWAs. In addition, much of the information which does exist regarding the long-term reproductive and teratogenic effects of these types of weapons is, unfortunately, somewhat contradictory. While it is possible that exposures to some CWAs might not generally be associated with serious, adverse reproductive effects, the discrepancies between the results of different studies are probably, in part, due to variability in individual responses to specific toxicants and the complexity of analyses of human epidemiological data. In addition, differences in dosing regimens, routes of exposure, and the animal models utilized for *in vivo* studies can confound comparisons of experimental results. The relative insensitivity of many reproductive endpoints might also contribute to the impression that some chemical weapons do not adversely affect reproductive function.

The embryo and fetus, without a developed blood–brain barrier and with only rudimentary DNA repair mechanisms and hepatic detoxifying and metabolizing capabilities, are especially susceptible, as compared to adults, to the adverse effects of low-level exposures to xenobiotics (Evans, 2007;

Newbold *et al.*, 2006). There has recently been increasing concern within the regulatory, public health, and scientific communities about the effects of prenatal exposures to potential reproductive toxicants, especially those capable of endocrine disruption, on humans and animals. In order to increase our understanding of the long-term, adverse reproductive effects associated with warfare and acts of terrorism, public knowledge of the use of CWAs or awareness of massive releases of reproductive toxicants associated with confirmed acts of terrorism should result in increased surveillance for phenotypic abnormalities in the most susceptible populations of humans and animals exposed during embryonic, fetal, and early postnatal development. Based on these epidemiological observations, carefully designed experiments mimicking ''real life'' exposures can be performed in multiple laboratory animal species, using sensitive biomarkers of toxic insult to reproductive development and function, to further elucidate the adverse reproductive effects of xenobiotic exposures likely to be associated with military and/or terrorist activities.

Our relative lack of understanding of the effects of CWAs and other xenobiotics on reproductive function can also be attributed to the complexity of the entire reproductive process and the mechanisms by which it is regulated. It should be evident from Figure 36.1 that maximum reproductive efficiency, including normal embryonic and fetal development, is dependent on the structural and functional integrity of multiple organs and tissues, as well as various physiological processes and signaling pathways within and (with respect to sexual reproduction and pregnancy) between individuals. Putative reproductive toxicants can affect one or several different steps in the reproductive process, depending on the physiological timing, duration, and level of exposure (Evans, 2007; Foster and Gray, 2008).

In ''real life'', humans and animals can be exposed to some toxicants both pre- and postnatally. Many organic xenobiotics have the potential to bioaccumulate within exposed individuals, possibly affecting future generations by way of genetic and epigenetic effects. However, reproductive endpoints, such as conception rates and sperm counts, are relatively insensitive, and subtle, toxicant-induced changes in reproductive efficiency can be overlooked or missed (Evans, 2007).

Much of the evidence for the adverse reproductive effects of selected toxicants will be based on cases involving wildlife exposures to environmental contaminants or on the experimental results of research exposing laboratory animals to large, pharmacological doses of potential toxicants. When available, data will be presented from accidental or intentional human and domestic animal exposures to toxicants associated with riot control and chemical warfare or with environmental catastrophes where incidences of infertility, abortion, and teratogenesis have been traced over the course of a number of years.

From an epidemiological perspective, it can be extremely challenging to determine the exact cause of reproductive abnormalities in humans and animals. Questions will often remain as to whether the observed poor reproductive outcomes associated with acute exposures to toxicants are due to direct effects of these chemicals on reproductive function or are secondary to toxicant-induced systemic disease and its accompanying stress (e.g. abortions and preterm births in intoxicated, pregnant women or animals). There are multiple factors, including exposures to mixtures of toxicants and other concurrently occurring causes of reproductive failure, which need to be taken into consideration in heterogeneous populations where exposures to toxic agents are not uniform between individuals. This is especially true in instances when there is a significant time interval between exposure to potential toxicants and the observed reproductive outcomes.

A. The Reproductive Toxicity of Riot Control Agents

The major lacrimatory and irritant riot control agents include α-chlorbenzylidene malonitrile (CS), dibenz (b,f)-1:4 oxazepine (CR), ω-chloroacetophenone (CN), and oleoresin of capsicum (OC pepper spray) (Salem *et al.*, 2008a). Exposure of pregnant women or animals to these compounds could be expected to be associated with maternal and/or fetal stress, which could potentially lead to the induction of premature parturition (Evans, 2007). Although the riot control agents CS and CN are both alkylating agents, with at least the potential to adversely affect embryonic and fetal development, neither of these chemicals has yet been found to be embryotoxic or teratogenic (Salem *et al.*, 2008a; Sanford, 1976). Limited studies performed with laboratory animals suggest that CR is neither embryotoxic nor teratogenic and that OC, with the possible exception of a slightly reduced crown–rump length, is not associated with any adverse effects on reproductive function (Salem *et al.*, 2008a).

B. The Reproductive Toxicity of CWAs

Given current global politics and the ''War on Terrorism'', pertinent information regarding many newer potential CWAs is very likely to be unavailable for public review. In order to be as complete as possible, some of the more historical and currently available CWAs will be discussed in the context of their primary, immediate, adverse effects on humans and animals. By organizing this discussion in that manner, the potential adverse reproductive effects of ''new'' CWAs can be anticipated based on the similarities of those toxicants to existing chemical weapons. For simplicity, CWAs will be broadly classified as vesicants (i.e. blistering agents), inhibitors of protein synthesis (e.g. ricin), inhibitors of cellular respiration or ''blood agents''

(e.g. hydrogen cyanide and cyanide-related compounds), and nerve agents (i.e. organophosphate compounds).

1. Vesicants

a. Arsenicals

While arsenicals will be discussed in this chapter with respect to their use in chemical warfare, the metalloid element arsenic, which is classified as a carcinogen, is also a potential environmental contaminant. Arsenicals continue to have important industrial and agricultural uses, and arsenic-containing feed additives, pesticides, and wood preservatives are all still readily available. In general, arsenic binds to sulfhydryl groups, with the activities of thiol-containing enzymes, including those involved in cellular energy production, frequently being adversely affected (Wismer, 2007). Increased capillary permeability is also associated with many acute intoxications involving arsenic.

It is important when discussing the toxicity of arsenicals to distinguish between the effects of organic and inorganic forms of arsenic. Several organic arsenicals, including Lewisite [dichloro (2-chlorovinyl) arsine or Agent L], diphenylcyanoarsine, and diphenylchloroarsine, have been used as vesicants and systemic toxicants (Ishii *et al.*, 2004; Wismer, 2007). Like other arsenicals, Lewisite has been associated with fetal death in laboratory animals; however, Lewisite is reported not to be teratogenic (Wismer, 2007). In contrast, a degradation product of diphenylcyanoarsine and diphenylchloroarsine, diphenylarsinic acid, has recently been associated with abnormal brain development in humans (Ishii *et al.*, 2004). Inorganic forms of arsenic, particularly arsenite and arsenate, have been associated with neoplasia, estrogenic activity, and testicular and ovarian toxicity, as well congenital neural tube, skeletal, and gonadal abnormalities, in laboratory rodents (Golub, 2006b). Epidemiological evidence in human populations has suggested that acute arsenic exposures with sublethal, maternal toxicity, and subchronic exposures to elevated arsenic concentrations in drinking water have both been associated with increased incidences of abortions, stillbirths, and pre-term deliveries (Golub, 2006b).

b. Chlorine Gas

Chlorine (Cl_2) is one of the more commonly produced chemicals in the USA, and chlorine gas is a potent oxidant which is very irritating and, potentially, corrosive (Kikilo *et al.*, 2008; Smith *et al.*, 2008; Wismer, 2007). Chlorine gas is used as a pulmonary and choking agent, and exposure is frequently associated moderate to severe, painful irritation of the eyes and respiratory tract (Wismer, 2007). Such stressful, sublethal exposures in late-gestational women or animals might be expected to be associated with the induction of premature parturition and, possibly, abortion. Oxidative stress can definitely have adverse effects on reproductive function, but the chronic disease usually associated with chlorine gas exposure is primarily related to the ocular and respiratory systems (Smith *et al.*, 2008). The limited information available on the reproductive effects of chlorine gas indicates that it is teratogenic (Wismer, 2007).

c. Phosgene and Phosgene Oxime

The most important industrial use of phosgene (Agent CG or carbonyl chloride) is in the production of isocyanates (Kikilo *et al.*, 2008), and MIC exposure is discussed with respect to its accidental release in Bhopal, India. Phosgene is classified as a choking agent, and it acylates sulfhydryl, amine, and hydroxyl groups. Phosgene oxime (Agent CX), a halogenated oxime, is a nonpersistent, chemical blistering agent, which, like phosgene, interacts with sulfhydryl and amine groups (Wismer, 2007). Other than possible adverse reproductive outcomes related to maternal and/or fetal stress or increased dermal absorption of concurrently used CWAs, phosgene oxime is unlikely to have direct adverse effects on reproductive function.

d. Sulfur Mustard

Sulfur mustard [*bis*-(2-chloroethyl) sulfide; mustard gas, Agents HD, H or HS] and analogs, such as 2-chloroethyl ethyl sulfide (CEES), are vesicants which can damage cells by alkylation of macromolecules [i.e. DNA, ribonucleic acid (RNA), and proteins], oxidative stress, glutathione depletion, and inflammation (Dacre and Goldman, 1996; Smith *et al.*, 2008; Watson and Griffin, 1992; Wismer, 2007). Similar to ionizing radiation and a variety of other radiomimetic alkylating agents, such as busulfan, cyclophosphamide and nitrogen mustard, sulfur mustard and its analogs can target rapidly dividing cells in multiple organs, including the testes and ovaries, as well as the developing embryo and fetus (Foster and Gray, 2008; Hurst and Smith, 2008; Rogers and Kavlock, 2008; Wismer, 2007). Consistent with DNA alkylation, as well as, possibly, other mechanisms of action, men exposed to sulfur mustard gas have been reported to have lower sperm counts and testosterone concentrations for several weeks following respiratory exposure, and Iraqi use of mustard gas has been associated with alterations in the infant sex ratio and an increase in some birth defects in children (Azizi *et al.*, 1995; Pour-Jafari, 1994; Wismer, 2007).

However, despite the epidemiological and laboratory animal evidence indicating that sulfur mustard is a teratogen and reproductive toxicant in humans and animals (Azizi *et al.*, 1995; Pour-Jafari, 1994; Wismer, 2007), contradictory research data involving several different routes of exposure have suggested no adverse sulfur mustard-related reproductive effects, especially at levels of exposure not associated with maternal intoxication (Dacre and Goldman, 1996; Watson and Griffin, 1992; Wismer, 2007). While, based on its mechanisms of action, it appears that exposures to sulfur mustard should be associated with several potential adverse reproductive outcomes, including maternal and/or fetal stress-induced premature parturition, additional mustard gas

inhalation experiments should be performed in rodent and nonrodent animal models to confirm this suspicion.

2. Inhibitors of Protein Synthesis

a. Ricin

The seeds of the ubiquitous castor bean plant (*Ricinus communis*) contain high concentrations of a highly toxic, relatively stable, heterodimeric, glycoprotein toxin, ricin, which is a type 2 ribosome-inactivating protein (RIP) (Burrows and Tyrl, 2001; Millard and LeClaire, 2008; Salem *et al.*, 2008b). The inhibition of protein synthesis by ricin and related type 2 RIPs has been associated with endothelial toxicity, and, depending on the route of exposure, severe gastrointestinal or respiratory disease and death (Millard and LeClaire, 2008). While it would be anticipated that acute, sublethal ricin intoxication would be associated with abortion or pre-term delivery secondary to maternal and/or fetal stress, ricin has also been shown to have direct adverse effects on reproductive function in female rabbits, causing abortion and inhibiting ovulation and implantation in this species (Salhab *et al.*, 1999). Because of the availability of the raw ingredients and the relative ease of its extraction, ricin has the potential to be a ''low-tech'' alternative for terrorist attacks targeting public water supplies (Salem *et al.*, 2008b). Additional research is needed in order to have a better idea of the adverse reproductive effects which would be anticipated with sublethal and/or chronic exposures to ricin and related toxins (Millard and LeClaire, 2008).

3. Inhibitors of Cellular Respiration (''Blood Agents'')

a. Hydrogen Cyanide and Cyanide-Related Compounds

With respect to their use in chemical warfare and, most likely, acts of terrorism, hydrogen cyanide [Agent AC, hydrocyanic acid (liquid form), or prussic acid], cyanogen halides and other cyanide-related compounds are frequently described as a ''blood agents'' (Kikilo *et al.*, 2008; Wismer, 2007). The major mechanism of action associated with acute cyanide intoxication is the formation of a stable complex with the ferric iron (Fe^{3+}) in cytochrome oxidases, resulting in cytotoxic hypoxia from the inhibition of cellular respiration, oxygen utilization and energy production (Ballantyne and Salem, 2008; Wismer, 2007). As a result of the rapid lethality of this class of compounds, very few studies have been conducted to investigate the adverse reproductive effects of hydrogen cyanide and cyanide-related compounds (e.g. cyanogen halides, cyanides, and nitriles). However, there is evidence to suggest that cyanide exposure in laboratory animals and livestock exposures to plants containing cyanogenic glycosides can be associated with embryonic and fetal death, as well as teratogenesis (Ballantyne and Salem, 2008; Burrows and Tyrl, 2001; Wismer, 2007). In addition, it would be anticipated that sublethal cyanide-induced hypoxia could cause enough maternal and/or fetal stress to result in abortion or pre-term deliveries.

4. Nerve Agents

a. Organophosphate Nerve Agents

The development of easily disseminated nerve agents as chemical weapons has been of interest to both military strategists and terrorist organizations because of the ability of these chemicals to rapidly incapacitate and kill opposing forces, as well as civilian populations. The major chemical nerve agents, tabun (Agent GA), sarin (Agent GB), soman (Agent GD), cyclosarin (Agent GF), and Agent VX, are extremely toxic, and even very brief exposures to these nerve agents can be lethal (Kikilo *et al.*, 2008; Watson *et al.*, 2006; Wismer, 2007). These chemicals are classified as organophosphorus, organophosphate, or ''OP'' compounds, and their mechanism of action involves the competitive and irreversible inhibition of acetylcholinesterase (AChE) (Gupta, 2007a; Watson *et al.*, 2006; Wismer, 2007). Commonly used OP and carbamate pesticides also inhibit AChE, and these chemicals, although generally less potent than those designed for use as CWAs, usually have longer lasting effects and the potential to be used as low-tech chemical weapons.

There have been some discrepancies between the results of various studies evaluating the reproductive toxicity of different OP compounds. While decreased libido has been observed in men following acute exposures to both OP nerve agents and insecticides, this ''reproductive'' effect is most likely related to the neurobehavioral effects, such as post-traumatic stress disorder, associated with acute exposures to these chemicals (McDonough and Romano, 2008). Some nerve agents have been associated with post-implantation morbidity and mortality in laboratory animals (Wismer, 2007), but rats and rabbits exposed to soman did not apparently experience fetal toxicity or prenatal mortality, even with maternal illness (Wismer, 2007). Similarly, low-level exposures to other nerve agents, as well as some insecticides, have not consistently resulted in impaired fertility and developmental abnormalities (McDonough and Romano, 2008). However, adverse reproductive effects have recently been reported by several authors in association with OP insecticide exposures (Joshi *et al.*, 2007; Peiris-John and Wiskremasinghe, 2008; Sikka and Gurbuz, 2006). Chlorpyrifos exposure has resulted in decreased sperm counts and testosterone concentrations, as well as testicular degeneration, in laboratory animals (Joshi *et al.*, 2007). Similar abnormalities have also been observed in humans following low-level exposures to OP insecticides (Peiris-John and Wiskremasinghe, 2008; Sikka and Gurbuz, 2006). It has been reported that OP insecticides can cause disturbances in the feedback loops within the hypothalamic–pituitary–adrenal axis, thereby affecting the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Sikka and Gurbuz, 2006), and some members of this class of pesticides have even been reported to have antiandrogenic activity

(Kitamura *et al.*, 2006). In addition, exposures to OP insecticides can cause oxidative stress leading to increased apoptosis within the testes (Sikka and Gurbuz, 2006).

As mentioned previously, maternal and/or fetal stress associated with sublethal exposures to OP insecticides could lead to an increased incidence of abortions or pre-term births in intoxicated pregnant women and animals. Because of lower levels of detoxifying enzymes (i.e. paraoxonase), the fetus appears to be more susceptible to OP intoxication than adults, and developmental neurotoxicity and growth retardation have been associated with low-level, prenatal exposures of humans to OP insecticides (Desaiah, 1998; Eskanazi *et al.*, 2008; Peiris-John and Wickremasinghe, 2008). In addition to the maternal and fetal effects, OP insecticides can also have direct toxic effects on the placenta, possibly involving (depending on the species) AChE inhibition within the placental cholinergic system (Pelkonen *et al.*, 2006).

Since the effects of OP insecticides are generally longer lasting and more diverse than those of OP nerve agents, extrapolations between OP insecticides and nerve agents need to take into consideration the toxicokinetic and toxicodynamic differences between these two classes of OP compounds (McDonough and Romano, 2008). Given their frequent occupational use and the duration of the toxic effects of OP insecticides, it is probably more likely that adverse reproductive outcomes will be investigated and observed with exposures to these compounds than with exposures to nerve agents specifically designed for immediate incapacitation without environmental persistence. Depending on the circumstances, acute or low-level exposures to OP nerve agents might not be associated with overt effects on reproductive function, especially when direct, toxicant-induced effects on reproductive performance are of secondary importance to neurological and/or psychological concerns and the reproductive endpoints being assessed are relatively insensitive. Duration, route, and amount (i.e. dose) of nerve agent exposure, the developmental period during which exposure occurs, and the reproductive endpoints and animal species being evaluated are factors which will need to be taken into consideration in the design of future studies investigating the direct effects of nerve agents on fertility and embryonic and fetal development.

5. The Reproductive Toxicity of Environmental Contaminants Resulting from Acts of Terrorism

Not all acts of chemical warfare will necessarily involve weapons specifically designed for that purpose. Especially in instances of terrorism or military attacks involving nongovernmental militias or governments with limited weapons resources, chemical warfare can be low tech and make use of toxic chemicals in commercially available products or those hazardous materials present at manufacturing, processing, or storage facilities. Ionizing radiation can be released into the environment through strategic military use of nuclear weapons or from nuclear accidents involving municipal power plants, nuclear-powered aircraft carriers, and submarines or the explosion of misplaced, misused, or stolen nuclear ''weapons of mass destruction''. In addition, many potential reproductive toxicants, including pesticides, are routinely used in various manufacturing and agricultural processes. Acts of terrorism or military strikes on industrial or agricultural complexes have the potential to greatly increase the exposure of humans and animals to a large number of different toxicants in the air, water, soil, and food chain.

Depending on the circumstances, both short- and long-term (i.e. immediate and delayed) morbidity and mortality can be observed in environmental disasters. In these types of catastrophic events, illness and death can be due to the direct effects of toxicants, or they can arise secondarily from other factors associated with the environmental release of xenobiotics. Related conditions, such as famine, can accompany disasters and negatively impact reproductive function in humans and animals. The very real or, in some cases, imagined or exaggerated threats to human and animal welfare from environmental contaminations can be associated with instances of mass fear, panic, and emotional incapacitation. These psychological stresses can affect reproductive function and, especially, gestational length and fetal survival in humans and animals. Emotional responses are naturally heightened with toxic exposures involving pregnant women and children.

6. Ionizing Radiation

Ionizing radiation can target rapidly dividing cells in multiple organs, including the testes and ovaries, as well as the developing embryo and fetus (Cockerham *et al.*, 2008; Foster and Gray, 2008; Rogers and Kavlock, 2008). Exposure of males to ionizing radiation can result in diminished spermatogenesis and testosterone production by the testes, with increased secretion of LH and FSH by the anterior pituitary (Cockerham *et al.*, 2008). Consistent with these effects, Ukrainian workers involved in the cleanup of radioactive materials after the Chernobyl nuclear accident had increased ultramorphological sperm abnormalities (Cockerham *et al.*, 2008; Fischbein *et al.*, 1997). Similar to the radiation-induced endocrine effects observed in the testes, ovarian steroid production is reduced by exposure to ionizing radiation (Cockerham *et al.*, 2008). Depending on the timing and dose of the radiation exposure, ionizing radiation can cause pubertal failure, ovarian failure, or premature menopause in women. Clusters of Down syndrome in Belarus 9 months following the explosion at the Chernobyl nuclear power plant suggest a radiosensitive phase of oogenesis in mammals around the time of ovulation and conception (Zatsepin *et al.*, 2007). High radiation exposure in late-gestational women or pregnant animals has the potential to cause abortion or pre-term births associated with maternal and/or fetal radiation sickness and stress. Depending on the stage of development and the dose of radiation, exposure of the conceptus, embryo, or fetus to

ionizing radiation can result in lethality or morphologic abnormalities (Cockerham *et al.*, 2008), and observations in humans and animals after the Chernobyl incident are consistent with these developmental effects (Østerås *et al.*, 2007; Peterka *et al.*, 2007). In addition, anxiety associated with exposures of pregnant women to ionizing radiation from the Chernobyl nuclear accident reportedly led to increased incidences of induced abortions in several European countries, even in instances where the exposure was minimal (Cordero, 1993).

7. Pesticides and Other Organic Contaminants

Pesticides and other organic contaminants are ubiquitous in both industrial and agricultural settings. Acts of terrorism have the potential to increase the exposure of humans and animals to these types of xenobiotics in the environment by targeting industrial and agricultural complexes. The massive release of pesticides, in particular, has the potential to be a readily available means of inciting fear and inducing morbidity and mortality in humans and animals. In fact, as mentioned previously, carbamate and OP insecticides have the same basic mechanism of action as the previously discussed AChE-inhibiting nerve agents, and MIC, an intermediate in the production of carbamate insecticides, contains a cyanide moiety.

There have been many, well-documented instances of reproductive abnormalities in species of wildlife living in environments contaminated by a wide range of industrial and/or agricultural chemicals (Evans, 2007; Guillette, 2006; Hess and Iguchi, 2002; Jobling and Tyler, 2006; MacLaclan, 2001; MacLaclan *et al.*, 2006). Wildlife populations are very likely sentinels for exposure to reproductive toxicants because of the contamination of the aquatic habitats in which many of them live and the bioaccumulation of some organic chemicals in predators (Hess and Iguchi, 2002). There is also recent evidence to suggest that domestic animals can act as potential sentinels for human exposure to endocrine disruptors and that hyperthyroidism in cats might be associated with exposure to polybrominated diphenyl ethers (PBDEs) (Dye *et al.*, 2007).

Based on the observations of reproductive toxicity (including endocrine disruption) in wildlife and domestic animals, as well as ongoing concerns about reproductive dysgenesis in human populations and the observed effects of industrial accidents involving MIC and dioxins, there has been increasing interest in the effects of prenatal exposures of humans to suspected endocrine disruptors and other reproductive toxicants. However, when impaired reproductive function is discovered in adults, it is difficult to comment with complete certainty on the relative contributions of pre- versus postnatal exposures to reproductive toxicants. There is a wide array of pesticides and other organic environmental contaminants which have the potential to adversely impact reproductive function. Specific epidemiological or laboratory studies suggesting adverse reproductive effects of exposures to these xenobiotics will be discussed with respect to observed abnormalities in male and female reproductive function, as well as embryonic and fetal development.

a. Adverse Effects of Pesticides and Other Organic Contaminants on Male Reproductive Function

''Androgenization'' or a state of indeterminate sexual development encompasses both feminization and demasculinization in males and, similar to the testicular dysgenesis syndrome (TDS) described in humans, has been observed in populations of various vertebrates, including fish, amphibians, reptiles, birds, and mammals (Edwards *et al.*, 2006; Evans, 2007). Adult and immature amphibians exposed to the herbicide atrazine and hatchling, juvenile, and adult male alligators originating from a lake previously contaminated with DDT and other persistent, bioaccumulated pesticides have been reported to exhibit varying patterns of androgenization (Evans, 2007; Hayes *et al.*, 2006; Milnes *et al.*, 2006). Although still somewhat controversial, there is evidence to support the observation that sperm counts in men within some industrialized regions of the world have been decreasing over the last several decades (Jørgensen *et al.*, 2006; Skakkebæk *et al.*, 2006; Swan *et al.*, 2000). The findings of epidemiological studies have suggested a relationship between decreased anogenital distance and prenatal exposures of male infants and phthalates used as plasticizers, as well as a correlation between reduced semen quality in men within certain regions of the USA and the metabolites of several economically important herbicides (Swan *et al.*, 2003a, b, 2005). In addition, a recently completed epidemiological study in Italy has demonstrated a significant relationship between postnatal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and abnormal semen and endocrine parameters in men (Mocarelli *et al.*, 2008).

The dicarboximide fungicides, vinclozolin and procymidone, and/or their metabolites inhibit the binding of androgens to nuclear androgen receptors and can demasculinize and feminize the prenatally exposed male fetus or induce important alterations in pre- or peripubertally exposed offspring (Evans, 2007; Gray *et al.*, 2006; Monosson *et al.*, 1999). While still subject to debate among scientists, vinclozolin has also been reported to be capable of inducing epigenetic modifications which facilitate the occurrence of transgenerational or vertically transmitted reproductive abnormalities (Anway *et al.*, 2005; Anway and Skinner, 2006). Linuron, p,p′-DDE, prochloraz, PBDEs, and selected OPs can function as androgen receptor antagonists (Gray *et al.*, 2006; Kitamura *et al.*, 2006), and AhR-mediated effects of TCDD can interfere with the biosynthesis of testosterone and disrupt testosterone signal transduction pathways (Jana *et al.*, 1999; Mocarelli *et al.*, 2008; Sikka *et al.*, 2005).

The testes have xenobiotic biotransformation capabilities within both Leydig and Sertoli cells (Thomas and Thomas, 2001). While many toxicants and/or their metabolites are capable of producing relatively nonspecific effects, such as

oxidative stress, there are a number of pesticides and other organic compounds which target specific cell populations within the testes. Several toxicants targeting Sertoli cells, including diethylhexyl phthalate (DEHP) and 2,5-hexanedione (a metabolite of *n*-hexane), have age- and species-specific effects (Creasy and Foster, 2002; Foster and Gray, 2008; Thomas and Thomas, 2001). Tri-*o*-cresyl phosphate (TOCP), an industrial chemical used in lacquers and varnishes and associated with some organophosphate insecticides, inhibits LH-induced steroidogenesis in the Leydig cells but, after Leydig cell-mediated conversion to its active metabolite, causes morphological abnormalities in Sertoli cells (Creasy and Foster, 2002; Evans, 2007; Thomas and Thomas, 2001).

As mentioned previously with regard to sulfur mustard, a variety of radiomimetic alkylating agents, including busulfan, cyclophosphamide and nitrogen mustard, can target rapidly dividing mitotic or meiotic germ cells in the testes. In some instances, xenobiotics can target a specific population of germ cell precursors, such as spermatogonia spermatocytes or round or elongate spermatids (Creasy and Foster, 2002; Evans, 2007; Foster and Gray, 2008). TCDD appears to adversely affect several populations of spermatozoal precursors and alters the sex ratio in favor of female offspring (i.e. decreased viability of Y chromosome-bearing sperm) (Foster and Gray, 2008; Ishihara *et al.*, 2007; Mocarelli *et al.*, 2008; Thomas and Thomas, 2001).

b. Adverse Effects of Pesticides and Other Organic Contaminants on Female Reproductive Function

A wide range of agricultural and industrial chemicals have estrogenic and/or antiestrogenic activities (Evans, 2007), and some of the synthetic xenobiotics most commonly discussed with respect to these activities include DDT, polychlorinated biphenyls (PCBs), and TCDD (MacLachlan, 2001). Effluents from industrial and agricultural activities have been shown to have androgenic activities and are associated with masculinization of female fish (Evans, 2007; Gray *et al.*, 2006; Orlando *et al.*, 2004).

The effects of toxicants on specific cell types within the ovaries are not as well understood as they are in the testes (Thomas and Thomas, 2001). Many female reproductive toxicants do not actually target particular cell lines but, rather, disrupt the endocrine milieu of the tubular genitalia or cause changes in ovarian structures secondary to alterations in the hypothalamic–pituitary–gonadal axis (Yuan and Foley, 2002). Like the testes, the ovaries also have some xenobiotic biotransformation capabilities, and oxidative damage can adversely affect ovarian structure and function (Thomas and Thomas, 2001; Yuan and Foley, 2002). Phthalates and TCDD can delay or decrease ovulations, and, like sulfur mustard and ionizing radiation, some of the alkylating agents reported to adversely affect rapidly dividing germ cells within the testes can also adversely affect primordial follicles within the ovary (Devine and Hoyer, 2005; Thomas and Thomas, 2001). Several PAHs [i.e. BaP, 3-methylcholanthrene (3-MC) and DMBA] and 1,3-butadiene appear to target oocytes in preantral follicles, and DMBA can adversely affect antral follicular development (Devine and Hoyer, 2005; Evans, 2007).

c. Adverse Effects of Pesticides and Other Organic Contaminants on Embryonic/Fetal Development

As has been emphasized previously, the developing fetus undergoing phenotypic sexual differentiation is particularly susceptible to the adverse effects of various agonists and antagonists of estrogen and androgen receptors (Evans, 2007; Hess and Iguchi, 2002). A large number of xenobiotics, including many pesticides (e.g. carbamates, OPs, organochlorines, and pyrethroids) and other potential organic environmental contaminants, have been recognized as potential teratogens in humans and animals (Desaiah, 1998; Evans, 2007; Rogers and Kavlock, 2008). Alkylating agents with radiomimetic activity, such as busulfan, cyclophosphamide, and nitrogen mustard, cause teratogenesis by targeting rapidly replicating cells (Rogers and Kavlock, 2008). TCDD has been found to be teratogenic both in laboratory animals exposed experimentally (Aragon *et al.*, 2008; Kransler *et al.*, 2007) and in humans exposed following an accidental release in Seveso, Italy, in 1976 (Alaluusua *et al.*, 2004). Accidental exposure to MIC in Bhopal, India, in 1984 resulted in a significant increase in spontaneous abortions and neonatal mortality in humans (Varma, 1987; Varma and Mulay, 2006), and these epidemiological data were corroborated by the results of a subsequent rodent experiment (Varma, 1987). In addition, some xenobiotics can cause fetal death and abortion by having direct toxic effects on the placenta, rather than on the fetus itself (Gupta, 2007c; Pelkonen *et al.*, 2006).

8. Heavy Metals

Heavy metals are routinely used in various manufacturing processes and are contained within many products commonly used by humans. Acts of terrorism have the potential to increase the environmental exposure of humans and animals to heavy metals by targeting industrial complexes and sewage treatment facilities. Because a number of heavy metals have the potential to affect different stages of reproductive function by different mechanisms of action, the adverse effects of metals on male and female reproduction and embryonic and fetal development will be discussed separately.

a. Adverse Effects of Heavy Metals on Male Reproductive Function

Excessive cobalt can potentially interfere with normal spermatogenesis, and even generalized hypoxia related to increased blood viscosity which affects the testes (Evans, 2007; Thomas, 1995). Chromium and vanadium have also been associated with adverse reproductive effects, and cisplatinum exposure has been associated with the death of spermatocytes and spermatids, as well as disruption of

Sertoli cell tight junctions (Evans, 2007; Thomas, 1995; Thomas and Thomas, 2001). Exposure of male laboratory animals to organotin compounds has been associated with reduced testicular size, alterations in testicular morphology, and impaired spermatogenesis (Ema and Hirose, 2006; Evans, 2007).

Lead and cadmium are ubiquitous heavy metals and have both been associated with testicular toxicity and impaired fertility in a number of species. Divalent lead is known to interact with physiological processes involving calcium and generally has an affinity for sulfhydryl groups (Evans, 2007). Lead is reported to be toxic to germ cells as well as Leydig cells and can suppress anterior pituitary secretion of LH and FSH (Evans, 2007; Thomas and Thomas, 2001). Lead also appears to be able to adversely affect the ability of spermatozoa to fertilize ova; however, this effect, like others associated with lead exposure, appears to be dependent on age and individual variations in susceptibility, adaptation, and reversibility (Evans, 2007; Sokol, 2006). Like lead, cadmium is thought to adversely affect male reproduction by inhibition of spermiation, as well as by interactions with the hypothalamic–pituitary–gonadal axis and adverse effects on the endothelium of the testicular and epididymal vasculature (Akinloye *et al.*, 2006; Creasy and Foster, 2002; Evans, 2007; Thomas, 1995). Cadmium can also alter the junctional complexes between adjacent Sertoli cells and disrupt the integrity of the blood–testis barrier (Akinloye *et al.*, 2006; Evans, 2007; Thomas and Thomas, 2001).

b. Adverse Effects of Heavy Metals on Female Reproductive Function

The ovaries do not appear to be as sensitive to the toxic effects of heavy metals as do the testes (Evans, 2007; Thomas, 1995). The neuroendocrine function of the hypothalamic–pituitary–gonadal axis appears to be targeted by lead in the female, as well as in the male (Evans, 2007). Anterior pituitary release of FSH and LH and ovarian steroidogenesis can be inhibited by cadmium (Evans, 2007; Hoyer, 2006; Thomas, 1995). Exposure of female laboratory animals to organotin compounds has been associated with reductions in follicular development and size, as well as the formation of corpora lutea, and it has recently been suggested that tributyltin and cadmium might also have estrogenic activities (Ema and Hirose, 2006; Evans, 2007; Golub, 2006b).

c. Adverse Effects of Heavy Metals on Embryonic/Fetal Development

Several heavy metals have been identified as teratogens and possible abortifacients in humans and animals, and the adverse effects of prenatal lead exposure on the developing nervous systems of both human and laboratory animal species have been well documented (Evans *et al.*, 2003; Rice, 1998; Rogers and Kavlock, 2008). Prenatal exposure to organotins has been associated with pregnancy loss and impaired ossification in rodents (Ema and Hirose, 2006). The outcomes of an industrial accident in Japan and misuse of contaminated grain in Basra, Iraq, clearly demonstrated the developmental neurotoxicity of organic mercury (i.e. methyl mercury and related compounds) in humans and animals (Chang and Guo, 1998; Cordero, 1993; Golub, 2006b). Other heavy metals, including cadmium, have been associated with placental toxicity, as well as developmental neurotoxicity (Gupta, 2007b; Hastings and Miller, 1998), and it has been recently reported that cadmium and other metals or metalloids might also have estrogenic effects (Golub, 2006b).

IV. CONCLUDING REMARKS AND FUTURE DIRECTION

Reproduction is a critical biological process, required for financially viable livestock production, as well as long-term survival of human and animal populations. Toxicant-induced abortions, congenital defects and infertility can have devastating effects on humans, domestic animals, and wildlife species. There is growing global concern about all of the potential adverse effects, including those on reproduction, of exposures to CWAs, and other xenobiotics resulting from military or terrorist activities. The information presented in this chapter was intended to familiarize the reader with terminology and concepts pertinent to reproductive toxicity, including endocrine disruption, and to provide an overview of what is understood about the adverse reproductive effects of selected toxicants. It is hoped that the information and references provided in this chapter will assist readers in making informed decisions in their interpretation of experimental or epidemiological data and their preparation for future experiments or clinical investigations.

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CHAPTER 37

Liver Toxicity of Chemical Warfare Agents

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I. INTRODUCTION

Xenobiotic-induced liver injury has become the most frequent cause of acute liver failure in humans in the USA and around the world, exceeding all other causes combined (Watkins and Seef, 2006). Owing to its detoxification mechanisms, the liver protects the individual against xenobiotic-induced injury. Certainly, the liver toxicity caused by chemical warfare agents is a potential area of concern.

Chemical-induced liver injury is encountered in a variety of circumstances. Some natural toxins such as the peptides of *Amanita phalloides*, the pyrrolizidine alkaloids, the toxin of the cycad nut, and other plant toxins are hazards posed by the environment. Some mycotoxins are ingested unknowingly because of feed contamination due to climatic conditions favorable to fungal growth. Other circumstances of exposure to hepatotoxins include contamination of water supply with cyanobacterial toxins, which led to the tragic death of 60 patients in a hemodialysis clinic in Brazil in 1996 (Jochimsen *et al.*, 1998).

Research in the last decade has focused on elucidating different mechanisms for chemical-induced liver injury. Investigators have attempted to understand the basis for hepatic injury. The goal of this chapter is to provide a basic understanding of liver pathophysiology and to introduce the general concepts of liver injury. The chapter also describes few examples of chemical warfare agents that can inflict liver damage.

II. STRUCTURAL ORGANIZATION OF THE LIVER

Hepatic lobule and hepatic acinus are relatively well-accepted models to describe the structure and functional aspects of the liver. Histologically, the hepatic lobule is a hexagonal region of the liver parenchyma around the central vein. Typically, six portal triads, consisting of branches from the portal vein and hepatic artery as well as bile ductules, border the edge of the lobule. Cords of hepatocytes are arranged radially around the central vein and blood sinusoids form between them. The hepatic parenchyma is divided into three zones based on the proximity to the central vein. The area closest to the central vein is termed centrilobular, the area near the portal triads is periportal, and the area between the centrilobular and periportal parenchyma is termed midzonal.

Alternatively, hepatic acinus is defined as the structural and functional unit in the liver based on the hepatic microcirculation. In simple terms, the hepatic acinus is defined as a parenchymal mass organized around the portal triad. Within the acinus, blood drains from the portal area via the sinusoids into the central hepatic vein. The acinus is arbitrarily divided into zone 1 which corresponds to the periportal zone of the hepatic lobule, zone 2 which corresponds to the midzonal parenchyma, and zone 3 which corresponds to the centrilobular zone. The majority of blood supply to the liver is from the portal vein. Approximately 60 to 80% of the blood originates from branches of the portal vein and supplies nutrients and toxins from the gastrointestinal tract while 20 to 40% of the blood originates from the hepatic artery supplying oxygen (Treinen-Moslen, 2001). The blood from the portal vein and hepatic artery is mixed in the penetrating vessels which then enter the sinusoids. Blood flows sequentially through zone 1, zone 2, and zone 3 before draining via the central vein. Because of this preferential blood circulation, hepatocytes in zone 1 receive blood that is 9 to 13% oxygenated whereas zone 3 is relatively hypoxic, the blood is 4 to 5% oxygenated and nutrient depleted (Treinen-Moslen, 2001).

In addition to hepatic parenchymal cells which are hepatocytes, hepatocyte stem cells, termed oval cells, are reported to be located in the canals of Hering where bile canaliculi from the hepatic cords converge on bile ductules of the portal triad. It is postulated that new hepatocytes travel down hepatic cords to replace the aging and damaged zone 3 hepatocytes.

Sinusoids lined by specialized endothelium are blood channels located between hepatocyte cords. The endothelial lining of the sinusoids is discontinuous and has fenestrae to facilitate movement of fluid and molecules less than 259 kDa (Treinen-Moslen, 2001; Plumlee, 2004; Watkins, 1999). This material enters the space of Disse, which is located between the endothelium and the hepatocytes. Within the space of Disse, hepatocytes contact free and protein-bound molecules which may be absorbed by diffusion or active transport.

A. Hepatic Functional Capacity

The liver contributes to a plethora of functions. Liver filters the blood drained from the gastrointestinal tract via the portal vein for xenobiotics, endotoxins, ammonia, and other bacteria-derived products (Treinen-Moslen, 2001; Plumlee, 2004). The liver is directly involved in glucose homestasis (Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004), cholesterol synthesis and uptake (Treinen-Moslen, 2001), synthesis of proteins such as clotting factors, albumin, and very low density lipoprotein (Treinen-Moslen, 2001; Plumlee, 2004), storage of glycogen, lipids, minerals, and vitamins (Plumlee, 2004), metabolism and excretion of hemoglobin breakdown products (Plumlee, 2004), steroid hormones (Brown, 2001), and drug metabolites.

There is remarkable regiospecificity and metabolic diversity of the hepatic zones to accommodate its numerous functions. In addition to differences in oxygen gradients (zone 3 hepatocytes are oxygen depleted compared to zone 1 hepatocytes), hepatocytes of zone 3 in particular are rich in drug-metabolizing enzymes. Zone 3 hepatocytes are involved in glycolysis and lipogenesis (Plumlee, 2004) and zone 1 hepatocytes are mitochondria rich (Treinen-Moslen, 2001; Plumlee, 2004). Functions of zone 1 hepatocytes include bile salt extraction, fatty acid oxidation, gluconeogenesis, and protein synthesis (Treinen-Moslen, 2001; Plumlee, 2004; Piñeiro-Carrero and Piñeiro, 2004). Zone 1 hepatocytes have the highest levels of glutathione (Treinen-Moslen, 2001).

Bile secretion is a major function of the liver. Bile is composed of bile salts, bilirubin, glutathione, phospholipids, cholesterol, proteins, organic anions, metals, and conjugated xenobiotics (Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004). Bile salts and bilirubin enter bile canaliculi via active transport through hepatocyte membranes. Canaliculi are dynamic structures located between hepatocytes and formed by hepatocyte membranes (Treinen-Moslen, 2001; Plumlee, 2004). Energy-dependent transport exists for certain hormones, drugs, and other xenobiotics. These include a group of multiple drug resistance p-glycoproteins that transport lipophilic cationic drugs, estrogens, phospholipids, and canalicular multiple organic anion transporters involved in movement of molecules conjugated to glutathione, glucuronide, and sulfate. Metal and mineral transport is important for mineral homeostasis and occurs through facilitated diffusion and receptor mediated endocytosis across the sinusoidal membrane. Lysosomes are involved in storage and export of metals and minerals into canaliculi.

Canaliculi enter canals of Hering in the portal triad and lead to intrahepatic bile ducts which coalesce to form the hepatic bile duct. The bile duct empties the bile into the gall bladder which then is released into the duodenum. Bile that is excreted into the small intestine enhances nutrient uptake, protects enterocytes from oxidation, and facilitates excretion of xenobiotics and endogenous waste in the feces (Treinen-Moslen, 2001).

B. Hepatic Cellular Components

In addition to the hepatocytes and the hepatocytic stem cells (oval cells) which are parenchymal in origin there are four types of nonparenchymal cells present within the liver. The nonparenchymal cells include the endothelial cells lining the sinusoids, bile duct epithelium, Kupffer cells, which are resident macrophages, the Stellate cells, also called Ito cells or fat storing cells, and the pit cells or large granular lymphocytes. In the rat, hepatocytes represent about 60% of the total cell number and 80% of hepatic tissue volume. Nonparenchymal cells in the rat are estimated to constitute about 30% of total cellular population, but comprise only 6–7% of tissue volume due to their small size relative to hepatocytes (Dahm and Jones, 1996).

Kupffer cells represent 80% of the fixed macrophages in the body. These cells are mostly located within the sinusoidal lumina in close association with endothelial cells. Kupffer cells function as phagocytes, ingesting foreign material which may arrive through the portal circulation (Treinen-Moslen, 2001; Plumlee, 2004) as well as apoptotic or necrotic hepatocytes. Kupffer cells have other immune functions in that they act as antigen presenting cells and secrete various cytokines. Kupffer cells may store minerals and are also involved in the pathogenesis of a variety of liver diseases induced by toxins such as ethanol (Laskin, 1990; Thurman *et al.*, 1998).

Stellate cells are located within the sinusoids and store fat and vitamin A (Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004; Plumlee, 2004). In the event of liver injury, stellate cells may become activated to a myofibroblast-like phenotype (Plumlee, 2004; Maddrey, 2005). Activated stellate cells produce collagen and play a role in the pathogenesis of hepatic fibrosis.

Pit cells are natural killer cells which have antineoplastic actions (Treinen-Moslen, 2001; Plumlee, 2004). They are also involved in granuloma formation (Plumlee, 2004).

III. FACTORS INFLUENCING HEPATIC TOXICITY

A. Preferential Hepatic Uptake

As mentioned above, the liver has a dual blood supply. The hepatic artery delivers material from the systemic circulation and the portal blood flow delivers directly from the gastrointestinal system. The portal system is involved in the ''first pass effect'', where the nutrients and xenobiotics that are absorbed from the stomach and intestines are filtered through the liver before reaching the systemic circulation (Treinen-Moslen, 2001). The space of Disse allows close contact between circulating plasma, plasma proteins, and hepatocytes, allowing for rapid diffusion of lipophilic compounds across the hepatocyte membrane. Some compounds are specifically taken up by sinusoidal transporters, including

phalloidin from several species of mushrooms in the genus *Amanita*, microcystin produced by the cyanobacteria *Microcystis aeruginosa*, and bile acids.

Liver cells have the potential to accumulate high levels of metals and vitamins, which can lead to toxic injury. Excessive vitamin A storage in stellate cells acutely leads to activation and proliferation of these cells (Treinen-Moslen, 2001), while chronic high levels can lead to hepatic fibrosis and portal hypertension, precipitating increased fibrosis (Piñeiro-Carrero and Piñeiro, 2004; Zimmerman, 1999; Maddrey, 2005). The liver is also responsible for iron homeostasis. There is a receptor mediated uptake of iron from the sinusoids and sequestration in storage proteins such as ferritin. High levels of iron cause lipid peroxidation of zone 1 hepatocytes (Treinen-Moslen, 2001). A common example of hepatic accumulation of metals is copper mediated liver toxicity noted in certain breeds of dogs (Bedlington terriers, Dobermans, and Dalmatians) where copper is stored within lysosomes of hepatocytes resulting in progressive accumulation of copper resulting in liver necrosis (Rolfe and Twedt, 1995).

B. Xenobiotic Metabolic Bioactivation

Most xenobiotic agents absorbed by the small intestine are highly lipophilic. Renal excretion is the primary mechanism of xenobiotic removal, but kidney excretion of lipophilic compounds, which are frequently protein bound in the circulation, is poor (Sturgill and Lambert, 1997; Watkins, 1999; Dahm, 1996). Such lipophilic compounds must be metabolized to increase their water solubility for excretion (Sturgill and Lambert, 1997; Zimmerman, 1999; Dahm and Jones, 1996). Microsomal enzymes within the liver add functional groups or conjugate xenobiotics to water-soluble molecules to facilitate excretion. While these reactions often function in the detoxification of compounds, there is significant potential for toxification (Zimmerman, 1999). Examples of phase I reactions include oxidation, reduction, and hydrolysis. Phase I enzymes, which are predominantly located in zone 3 of the hepatic lobule, may produce reactive metabolites.

Many hepatic enzymes are present in the smooth endoplasmic reticulum of the hepatocyte. When liver tissue is homogenized, the endoplasmic reticulum breaks down into small vesicles known as microsomes, thus these enzymes are termed microsomal enzymes. As a rule, microsomal enzymes require oxygen and NADPH to function (Brown, 2001; Dahm and Jones, 1996). Most phase I enzymes contain heme, giving them a red coloration, and they absorb light at a wavelength of 450 nm. Most cytochrome P450s act as mixed function oxidases (MFOs). Genes for cytochrome P450s are highly conserved in mammals. There are three gene families, CYP1, CYP2, and CYP3, and more than 36 cytochrome P450 isoenzymes have been identified in animals (Dahm and Jones, 1996; Watkins, 1999).

Oxidation is the major phase I reaction produced by the group of cytochrome P450s. Important substrates for CYP450s include steroid hormones and lipid-soluble drugs (Brown, 2001). Oxidative reactions frequently lead to the formation of highly reactive epoxides. These toxic metabolites are usually detoxified rapidly by phase II conjugation or other mechanisms, such as microsomal epoxide hydrolases (Piñeiro-Carrero and Piñeiro, 2004; Watkins, 1999).

Non-cytochrome P450 enzymes may also be involved in oxidative reactions. One such enzyme is alcohol dehydrogenase whose substrates include vitamin A, ethanol, and ethylene glycol. Aldehyde dehydrogenase is another enzyme. Most reduction reactions also involve microsomal enzymes, with the exception of ketone reduction. Nitro compounds are reduced to amines and volatile anesthetics undergo dehalogenation by microsomal enzymes. Hydrolysis reactions are involved in metabolism of compounds with amide bonds or ester linkages, as in the conversion of aspirin to salicylate (Brown, 2001).

C. Phase II/Conjugation Reactions

Phase II enzymes may be cytosolic or microsomal (Brown, 2001; Dahm and Jones, 1996). Phase II enzymes are predominantly involved in conjugating phase I metabolites or xenobiotics with functional groups. Phase II metabolites are rarely reactive, but there are a few exceptions, such as the glucuronide of the nonsteroidal anti-inflammatory drug diclofenac and the glutathione conjugate of α-naphthothiourea (ANTU). Phase II enzymes conjugate a polar group to the substrate at a hydroxyl group, carboxyl group, amino group, or sulfhydryl group produced through the actions of phase I microsomal enzymes. Polar molecules that are added to the substrate include glucuronic acid, sulfate derived from sulfuric acid ester, acetate, glutathione, methyl groups derived from methionine, and amino acids such as glycine and cysteine. These polar groups significantly increase water solubility of the substrate facilitating rapid renal or biliary excretion.

Glucuronidation is the most common phase II reaction in humans, though it is deficient in the neonate (Sturgill and Lambert, 1997; Piñeiro-Carrero and Piñeiro, 2004; Brown, 2001). Substrates for glucuronidation usually include steroid hormones, thyroxin, and bilirubin as well as many drugs, including salicylates and acetaminophen. Glucuronyl transferases are microsomal enzymes that catalyze the transfer of glucuronide from uridine 5′-diphosphate (UDP) (Watkins, 1999). UDP may be depleted in patients overdosed with acetaminophen or other drugs that undergo this detoxification pathway. Products of glucuronidation may be excreted in the bile or urine. Those excreted in the bile may undergo hydrolysis in the intestine, which leads to reabsorption of the parent compound in a phenomenon called enterohepatic cycling (Brown, 2001). Similar to CYP 450 enzymes, some agents can also induce glucuronyl transferases, such as phenobarbital (Sturgill and Lambert, 1997).

Sulfation is the primary conjugation reaction for substrates with phenol groups or aliphatic alcohols (Sturgill

and Lambert, 1997; Brown, 2001). These reactions are catalyzed by sulfotransferases in the cytoplasm. Agents that undergo sulfation include acetaminophen, morphine, ascorbic acid, and endogenous compounds like chondriotin, heparin, and some steroids. The pool of available sulfates may become saturated in drug overdoses.

Drugs with amine and hydrazine groups may be conjugated to acetate (Sturgill and Lambert, 1997). Sulfonamides often undergo acetylation (Brown, 2001). *N*-acetyltransferase is an enzyme in the cytoplasm involved in acetylation reactions.

Glutathione (GSH) and cysteine both have sulfhydryl groups which readily bind many phase I metabolites (Brown, 2001). GSH is a free radical scavenger that prevents membrane damage from reactive metabolites. These reactions may be spontaneous or catalyzed by GSH peroxidases, selenium-dependent enzymes. Because these enzymes are cytosolic, damaged membrane phospholipids must be released by phospholipase A2 for detoxification. GSH is also involved in reduction and recycling of other antioxidants such as vitamins E and C (Dahm and Jones, 1996). When oxidized, GSH forms a dimer which must be reduced by GSH reductases, NADPH-dependent enzymes. GSH may be depleted in the overdosed patient or due to fasting (Sturgill and Lambert, 1997; Piñeiro-Carrero and Piñeiro, 2004; Dahm and Jones,1996). *N*-acetylcysteine is frequently used to replenish GSH.

D. Phase III Reactions

In addition to phase I and II biotransformation enzymes, studies suggest the involvement of hepatic transporter systems involved in drug efflux from hepatocytes as a means for the liver to rid itself of foreign chemicals. These are termed phase III transporter systems. Several transporter families which mediate uptake of chemicals into liver and excretion of chemicals from liver into blood and/or bile have been cloned and identified. In general, the organic anion transporting polypeptide family (Oatps) along with organic cation transporter 1 (Oct1) and organic anion transporter 2 mediate uptake of a large number of xenobiotics from blood into liver. Conversely, multidrug resistance proteins (Mdrs), multidrug resistance associated proteins (Mrps), and breast cancer resistance protein (Bcrp) mediate efflux of xenobiotics from liver into bile or blood (Klaassen and Slitt, 2005).

E. Pathologic Manifestations of Hepatic Injury

1. Hepatic Steatosis/Fatty Liver

Hepatic steatosis is the accumulation of fat droplets within the hepatocytes. Steatosis is usually a common response noted with a variety of liver toxicants and represents a potentially reversible injury to hepatocytes (Treinen-Moslen, 2001). Grossly, the affected liver will be swollen with rounded edges, friable, and light brown to yellow in color. Compounds that produce prominent steatosis include the antiepileptic drug valproic acid and the antiviral agent fialuridine. Other toxins that may cause hepatic steatosis include aflatoxin and white or yellow phosphorus. Although steatosis has been considered benign and reversible, there are recent reports that suggest the progression of the steatosis stage to steatohepatitis, fibrosis, and cirrhosis (Ramaiah *et al.*, 2004). Recently, there is a syndrome noted in obese individuals, who are often type 2 diabetics, called nonalcoholic fatty liver disease (NAFLD) where hepatocytes are markedly steatotic and there is a marked inflammatory component (Diehl, 2005). Other disorders that result in fatty livers include hepatotoxic chemicals such as thioacetamide, ethanol, and carbon tetrachloride. It should be noted, however, that several endocrine abnormalities result in steatosis, thus assigning the cause to a specific etiology should be done with caution.

Steatosis is termed microvesicular if the fat droplets are small and do not completely displace the nucleus. Microvesicular steatosis likely indicates a slow lipid accumulation (Plumlee, 2004; Bastianello, 1987) and may indicate a deficiency in mitochondrial β-oxidation of fatty acids. It is a relatively severe form of steatosis and has been associated with some toxins including aflatoxin in primates and dogs (Zimmerman, 1999; Bastianello, 1987) and valproic acid in humans (Sturgill and Lambert, 1997; Zimmerman, 1999). In contrast, macrovesicular steatosis describes hepatocytes containing large, usually single fat droplets that displace the hepatocyte nucleus to the periphery of the cytoplasm. Macrovesicular steatosis indicates an imbalance between fatty acid uptake and secretion of very low density lipoproteins. This may be due to increased triglyceride mobilization, decreased fatty acid oxidation, decreased synthesis of very low density lipoproteins, or other metabolic anomalies (Plumlee, 2004; Treinen-Moslen, 2001; Sturgill and Lambert, 1997; Zimmerman, 1999).

2. Steatohepatitis

Steatohepatitis is the accumulation of lipids and the presence of inflammatory cells within hepatic parenchyma. Steatohepatitis is usually the next stage of steatosis if untreated (Bautista, 2002; French, 2003; Lieber, 1994). The inflammatory cells are usually neutrophils and mononuclear leukocytes. Conditions usually associated with steatohepatitis are alcoholic liver disease, NAFLD, and endotoxemia secondary to intestinal disease. Any toxic compounds that cause steatosis can also result in steatohepatitis if the condition is left untreated. Steatohepatitis may progress to fibrosis/cirrhosis and hepatocellular carcinoma if the inciting cause is not removed or treated (Diehl, 2002).

3. Apoptosis Versus Necrosis

Two forms of cell death are described within hepatocytes, apoptosis and necrosis. As with other organs, apoptosis is often called ‘‘programmed cell death’’ and is a normal physiologic process. Individual cells are affected (Dahm and Jones, 1996), cell death is not associated with

inflammation, and normal architecture of the hepatic parenchyma is maintained allowing regeneration (Treinen-Moslen, 2001; Piñeiro-Carrero and Piñeiro, 2004). Apoptotic cells undergo cell shrinkage and nuclear condensation and pyknosis, but mitochondrial function (Piñeiro-Carrero and Piñeiro, 2004) is maintained and the cell membrane remains intact (Zimmerman, 1999). Apoptotic cells are occasionally seen in the centrilobular area but are rapidly phagocytosed by macrophages and other hepatocytes (Plumlee, 2004). Apoptosis may be induced by xenobiotics due to oxidative stress (Piñeiro-Carrero and Piñeiro, 2004), decrease in apoptotic suppressors, or enhanced expression of apoptosis genes (Dahm and Jones, 1996).

Necrosis is the predominant form of cell death in most hepatotoxic insults. The term ''necrotic'' is used to describe ''dead and dying'' cells which are often identified by homogeneous eosinophilic cytoplasm on H&E stained liver sections with variable loss of nuclear and cellular detail. Degenerative changes to the hepatocyte may precede necrosis. During necrosis, cells lose osmotic homeostasis and there is swelling of hepatocytes as observed microscopically and organelles on an ultrastructural basis (Treinen-Moslen, 2001; Dahm and Jones, 1996). Energy production fails due to loss of calcium homeostasis (Dahm and Jones, 1996; Zimmerman, 1999). Eventually there is rupture of the cell membrane and leakage of cell contents.

Necrosis is often initiated by damage to membranes, either the plasma membrane of the cell or the membranes of organelles, particularly the mitochondria (Zimmerman, 1999). Cell membrane damage is often caused by membrane phospholipid peroxidation. Plasma membrane damage interferes with ion regulation, calcium homeostasis, energy production, and decrease in the ability of that organelle to sequester calcium. Inhibition of protein synthesis is an alternate mechanism that may cause cell necrosis. Toxins that act in this way include phalloidin and related mushroom toxins, which inhibit the action of RNA polymerase and therefore mRNA synthesis (Piñeiro-Carrero and Piñeiro, 2004).

4. Hepatic Pigment Accumulation

Various substances may accumulate within hepatocytes or Kupffer cells. These substances may be visible by microscopy as pigment. Occasionally, these pigments lend a grossly visible tint to the liver. Bile pigment may accumulate in canaliculi and bile ducts, particularly in zone 3, leading to a yellow to green color (Plumlee 2004; Zimmerman, 1999). Iron in the form of hemosiderin is stored in the liver as a yellow–brown pigment which may be visualized using Pearl's Prussian blue. Copper may be yellow–brown and is visualized using Rhodenase. Lipofuscin may be present within hepatocytes as a senile change. This yellow–brown pigment represents lipid accumulation within lysosomes.

5. Hepatic Cholestasis

Cholestasis may be transient or chronic (Treinen-Moslen, 2001) and may be subdivided into canalicular cholestasis and cholangiodestructive cholestasis. Canalicular cholestasis can be produced by drugs/chemicals that damage the bile canalicular structures and function. A key component of bile secretion involves several ATP-dependent export pumps such as the canalicular bile salt transporter that moves bile salts and other transporters that export bile constituents from the hepatocyte cytoplasm to the lumen of the canaliculus. Some of the drugs bind these transporter molecules resulting in the arrest of bile formation or movement within the lumen of the canalicular system (Klaassen and Slitt, 2005). Secondary bile injury can result if there is cholestasis due to the detergent action of bile salts on the biliary epithelium or hepatocytes in areas of cholestasis. Cholestasis can occur simply as a result of physical obstruction of canaliculi within the liver parenchyma (intrahepatic) or outside the liver (extrahepatic). Causes of cholestasis may include hepatobiliary tumors, endotoxemia, hepatocyte swelling, and intraductal crystals such as calcium salts of plant saponins. Disruption of actin filaments within the hepatocyte may cause cholestasis by preventing the normal pulsatile contractions that move bile through the canalicular system to the bile ducts. Drugs that bind to actin filaments such as phalloidin, those that affect cytoskeletal assembly such as microcystin, and those that affect calcium homeostasis and cellular energy production can generate this type of energy.

Cholangiodestructive cholestasis is caused by bile duct obstruction which may be intrahepatic or extrahepatic. Bile duct injury may lead to sloughing of epithelial cells into the lumen, cell edema, and inflammation, which may contribute to obstruction (Treinen-Moslen, 2001; Plumlee, 2004). Chronic lesions associated with cholangiodestructive cholestasis typically include bile duct proliferation and periductular fibrosis. Vanishing bile duct syndrome, characterized by a loss of bile ducts, has been seen in chronic cholestatic disease in humans (Zimmerman, 1999; Treinen-Moslen, 2001) and has been produced experimentally in dogs (Uchida, 1989).

6. Hepatic Fibrosis/Cirrhosis

Fibrosis usually results from chronic inflammation which can be the result of continuous exposure to a variety of hepatotoxic chemicals such as organic arsenicals, vinyl chloride, or high doses of vitamin A (Zimmerman, 1999), chronic ethanol ingestion and nonalcoholic fatty liver disease. Fibrosis usually occurs around the portal area, in the space of Disse, and around the central veins. This results in loss of liver architecture and function. The hepatocytes are replaced with fibrous material and thus there is hepatocyte loss. Periportal fibrosis may lead to portal hypertension.

7. Cirrhosis

Hepatic cirrhosis is typically the end stage of liver disease. Cirrhosis describes an irreversible change (Treinen-Moslen, 2001) characterized by accumulation of excessive collagen deposition in the form of bridging fibrosis which disrupts the hepatic architecture. Cirrhosis may be micronodular or macronodular depending on the amount of fibrosis and tissue regeneration. Liver transplantation is the only solution to restore adequate liver function in human medicine.

8. Patho-Mechanisms of Hepatic Injury

Mechanisms of liver injury have been divided into two categories, intrinsic and idiosyncratic. Intrinsic injury may lead to steatosis, necrosis, cholestasis, or a mixed form of damage, often with minimal inflammation (Sturgill and Lambert, 1997). Intrinsic liver injury is a predictable, reproducible, dose-dependent reaction to a toxicant (Sturgill and Lambert, 1997; Zimmerman, 1999; Piñeiro-Carrero and Piñeiro, 2004; Dahm and Jones, 1996). A threshold dose exists for xenobiotics causing intrinsic liver injury. There is commonly a predictable latent period between the time of exposure and clinical evidence of liver injury. This type of liver injury accounts for the vast majority toxic liver injury and is often caused by reactive products of xenobiotic metabolism, most commonly electrophiles and free radicals. A few drugs cause intrinsic liver injury without bioactivation. An abbreviated summary of mechanisms of intrinsic liver injury is illustrated in Figure 37.1.

Idiosyncratic responses are, by contrast, unpredictable responses to a drug or other toxicant. They are rare, non-dose dependent, and often associated with extrahepatic changes (Sturgill and Lambert, 1997; Piñeiro-Carrero and Piñeiro, 2004; Zimmerman, 1999; Shenton, 2004). Idiosyncratic drug reactions often occur after sensitization followed by reexposure to a drug. There is a usually a delay of 1–5 weeks, and occasionally several months, between the time of the first dosing and the time clinical signs become evident, but onset is expedited with rechallenge (Watkins, 1999; Sturgill and Lambert, 1997; Dahm and Jones, 1996). Hepatic changes associated with idiosyncratic drug reactions include necrosis and cholestasis, or both, and there is often an inflammatory response involving macrophages and eosinophils. Extrahepatic clinical signs may include pyrexia, rash, and peripheral eosinophilia. Some idiosyncratic drug reactions resemble serum sickness. Some of the mechanisms of liver injury are described in more detail below.

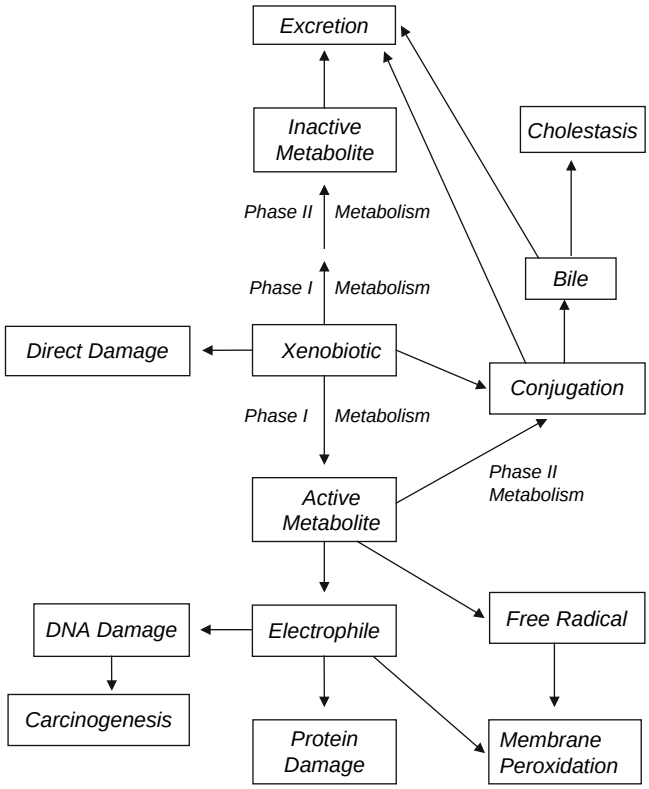

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FIGURE 37.1. Multiple metabolic pathways involved in the mediation of hepatic injury for any compound. The liver is central to xenobiotic (and some endogenous compounds) metabolism which produces water-soluble products amenable to urinary or biliary excretion. Some compounds undergo metabolic activation to produce free radicals, electrophiles, or other toxic products that may induce hepatic injury.

F. Oxidative Stress and Free Radicals with Classic Examples

Free radicals are generated from within hepatocytes in several ways, such as ionizing radiation, oxidative metabolism by cytochrome P450, reduction and oxidation (redox) reactions that occur during normal metabolism, transition metals such as iron and copper, and from nitric oxide generated by variety of inflammatory cells. The reactive species generated result in lipid peroxidation of membranes, oxidative modification of proteins, and lesions within DNA (Crawford, 1999).

Free radicals have unpaired electrons, making them highly reactive. They may be formed by one electron oxidation or reduction reactions, leading to cationic or anionic radicals, respectively (Dahm and Jones, 1996). Alternately, hemolytic bond scission leads to neutral radical formation. Oxygen free radicals result from metabolic processes, leukocytic respiratory burst, or to the effects of ionizing radiation. Hydroxyl radicals, superoxide radicals, and hydrogen peroxide are major reactive oxygen species. The free radical nitric oxide ($NO^{\cdot}$), an important cell signaling agent released by leukocytes, may react with superoxide to form peroxynitrite.

Free radicals cause peroxidation of phospholipids within the plasma membrane of the cell as well as the membranes of the mitochondria and endoplasmic reticulum. The

radicals act by removing a proton (H^+) from a methylene carbon within a polyunsaturated fatty acid, forming a lipid free radical. This lipid free radical may then abstract a proton from a neighboring polyunsaturated fatty acid, generating more lipid free radicals. It is estimated that this can occur four to ten times per initiation. Effects of lipid peroxy radicals on the cell membrane include increased permeability, decreased fluidity, and inactivation of membrane proteins (Dahm and Jones, 1996). Additionally, mitochondrial membranes lose polarity (Watkins, 1999).

Lipid peroxy radicals can react with metal ions stored within the hepatocyte, generating more lipid radicals. It is estimated that propagation by this mechanism can occur in four to ten steps per initiation (Dahm and Jones, 1996). The most frequent mechanism of free radical production leading to hepatocellular injury involves phase I metabolism of xenobiotics and the cytochrome P450 system. Phase I metabolism may lead to bioactivation of the substrate to a high energy reactive intermediate molecule in preparation for phase II conjugation reactions. However, in circumstances such as overdosage, phase I products may accumulate. Lesions produced by these compounds are mostly centrilobular because the cytochrome P450s responsible for metabolism are mostly situated in the centrilobular areas of the liver.

The classic examples of this process are cell death resulting from carbon tetrachloride and acetaminophen toxicosis (Figure 37.2). Acetaminophen has a hydroxyl group that can undergo immediate phase II conjugation reactions. Indeed, at therapeutic doses 90% of this substrate undergoes glucuronide or sulfate conjugation in humans (Sturgill and Lambert, 1997; Court, 1997). These are major metabolic pathways in most species, but glucuronyl transferase deficiency in cats in part explains the sensitivity of felines to this drug.

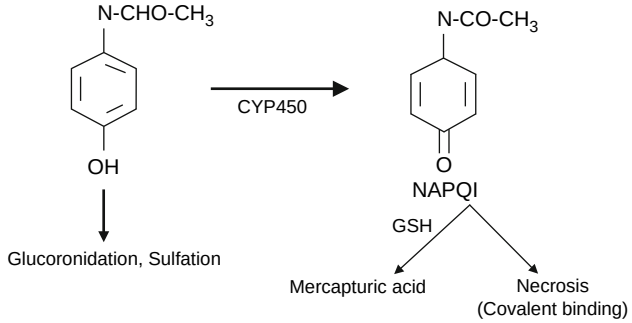

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FIGURE 37.2. Metabolism and mechanism of acetaminophen toxicity. Bioactivation of acetaminophen by P450 enzymes results in the formation of the reactive intermediate (NAPQI) which forms covalent protein adducts with glutathione which is then converted to mercapturic acid. When the amount of the reactive metabolite formed exceeds the glutathione available for binding, the excess metabolite binds to tissue molecules resulting in centrilobular hepatic necrosis.

Acetaminophen in itself is not considered toxic. Cellular injury is caused by the unstable metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI; Figure 37.2). Under normal conditions in humans, 5% of a dose of acetaminophen is oxidized to NAPQI, which is rapidly neutralized by conjugation with glutathione (Sturgill and Lambert, 1997; Maddrey, 2005; Figure 37.2). Toxic levels of NAPQI may accumulate under certain conditions, as when large amounts of substrate are available for metabolism due to either large ingestions or inadequate glucuronidation. Metabolism of acetaminophen to NAPQI is increased in individuals who regularly consume alcohol or take medications that induce microsomal enzymes, e.g. antiepileptic therapy. The hepatic pool of glutathione becomes overwhelmed and depleted permitting the accumulation of NAPQI. Possible additional risk factors that lower the threshold for hepatotoxicity have been identified and include fasting and malnutrition, which deplete glutathione reserves (Treinen-Moslen, 2001; Sturgill and Lambert, 1997; Piñeiro-Carrero and Piñeiro, 2004; Dahm and Jones, 1996).

The reaction that produces NAPQI generates superoxide anions as a by-product. Interactions of NAPQI with other cellular molecules also generate reactive oxygen species, leading to oxidative stress on the hepatocyte (Zimmerman, 1999; Dahm and Jones, 1996). The role of calcium and Kupffer cell activation have been implicated as contributing factors for acetaminophen-induced liver injury by producing reactive nitrogen species (Treinen-Moslen, 2001).

NAPQI also acts as an electrophile, targeting the mitochondria in particular. This reactive metabolite forms covalent adducts with cellular molecules, particularly proteins with thiol groups. Other targets besides mitochondrial proteins include plasma membrane proteins involved in calcium homeostasis and adenine nucleotides (Dahm and Jones, 1996; Sturgill and Lambert, 1997).

G. Disruption of Calcium Homeostasis

Calcium ions (Ca^{2+}) are important for the mediation of hepatic injury. Cytosolic free calcium is maintained at relatively low concentrations compared to the extracellular levels. The majority of intracellular calcium is sequestered within the mitochondria and endoplasmic reticulum. Membrane associated calcium and magnesium ATPases are responsible for maintaining the calcium gradient (Farrell *et al.*, 1990). Significant and persistent increases in the intracellular calcium result from nonspecific increases in permeability of the plasma membrane, mitochondrial membranes, and membranes of the smooth endoplasmic reticulum. Calcium pumps in the mitochondrial membrane require NADPH, thus depletion of available NADPH can cause calcium release from mitochondria (Cullen, 2005).

Elevated cytoplasmic calcium activates a variety of enzymes with membrane damaging effects. The major enzymes that are involved in activation by calcium include

ATPases, phospholipases, proteases, and endonucleases. Thus increased calcium causes increased mitochondrial permeability and induction of apoptosis and necrosis. Calcium is required for maintenance and function of the cytoskeleton as well (Delgado-Coello *et al.*, 2006; Dahm and Jones, 1996).

Although cell injury results in increased calcium, which causes a variety of damaging effects, the cause and effect relationship of calcium in cell damage is not known. The chemicals that cause liver damage by this mechanism include quinines, peroxides, acetaminophen, iron, and cadmium.

H. Inhibition of Mitochondrial Function

Mitochondria function in the production of energy, in the form of ATP, for the cell by oxidative phosphorylation. Hepatocytes are highly metabolically active and require a continuous supply of ATP. Hepatocytes active in detoxification or replacement of damaged tissue have higher ATP requirements still (Dahm and Jones, 1996). Compounds that may disrupt mitochondrial oxidative phosphorylation include bile acids and amiodarone. Mitochondria are also critical to modulation of cell redox status, osmotic regulation, pH control, and cytosolic calcium homeostasis and cell signaling. Mitochondria are important targets for virtually all types of injurious stimuli, including hypoxia and toxins. Mitochondria are targeted by oxidants, electrophiles, lipophilic cations, and weak acids. Damage is often precipitated by increases in cytosolic calcium.

Hepatic injury is frequently accompanied by morphological changes in mitochondria. Mitochondrial changes evident as structural abnormalities include greatly increased size and the development of crystalline inclusions. These changes are usually regarded as pathologic, reflecting as either a protective or degenerative response to injury. Mitochondrial damage may result in the formation of high conductance channels, the so-called mitochondrial permeability transition, in the inner mitochondrial membrane. This is an irreversible change and, because membrane potential is critical for mitochondrial oxidative phosphorylation, constitutes a deathblow to the cell.

Oxidative phosphorylation produces reactive oxygen species (Watkins, 1999). These are deactivated by antioxidants present within the mitochondrion. Glutathione is present within mitochondria as a scavenger for peroxides and electrophiles. Synthesis of glutathione requires ATP and takes place outside of the mitochondrion. Greater than 90% depletion in glutathione reserves decreases the ability of the mitochondrion to detoxify reactive oxygen species produced by oxidative phosphorylation. Glutathione *S*-transferase, the enzyme required for recycling of glutathione, may become overwhelmed by toxicants and reactive metabolites (Dahm and Jones, 1996).

Xenobiotics may cause cell death by their effects on mitochondrial DNA. Some antiviral dideoxynucleoside analogs can disrupt mitochondrial DNA synthesis through the inhibition of DNA polymerase gamma, leading to depletion of mitochondria and consequent hepatocyte death.

Chemicals that damage mitochondrial structure, enzymes, or DNA synthesis can disrupt beta oxidation of lipids and oxidative energy production within hepatocytes. Prolonged interruption of beta oxidation leads to microvesicular steatosis which can progress to macrovesicular steatosis. This sequence of events has been noted with alcoholic and nonalcoholic steatohepatitis. The role of mitochondria has been extensively studied with nonalcoholic fatty liver disease, a major issue in human medicine. Alcoholic steatosis and other forms of hepatic steatosis have been linked to impairment of ATP homeostasis and mitochondrial abnormalities have been reported in a growing body of literature.

There are several drugs that inhibit beta oxidation of fatty acids in mitochondria leading to lipid accumulation, such as aspirin, valproic acid, and tetracyclines.

I. Disruption of Cytoskeleton

Changes in intracellular calcium homeostasis produced by active metabolites of xenobiotics may cause disruption of the dynamic cytoskeleton. There are a few toxins that cause disruption of the cytoskeleton through mechanisms independent of biotransformation. Microcystin is one of these toxins. Microcystin is produced by the cyanobacterium *Microcystis aeruginosa*. Similar toxins are produced by other species of cyanobacteria. The hepatocyte is the specific target of microcystin, which enters the cell through a bile-acid transporter. Microcystin covalently binds to serine/threonine protein phosphatase, leading to the hyperphosphorylation of cytoskeletal proteins and deformation of the cytoskeleton (Treinen-Moslen, 2001).

Phalloidin and related toxins found in some mushrooms, including *Amanita phalloides*, act by binding tightly to actin filaments and preventing cytoskeletal disassembly (Treinen-Moslen, 2001).

1. Cholestatic Mechanisms

Sinusoidal transporters and canalicular transporters are involved in the movement of bile salts from the sinusoids into the canaliculi. Within the hepatocyte, transcytosis is mediated by cytoskeletal transport mechanisms. Bile is moved within the canaliculi through actions of the hepatocyte cytoskeleton causing contraction of the canalicular lumina (Treinen-Moslen, 2001). Xenobiotics acting on any of the above systems may influence bile transport and secretion.

Most chemicals that cause cholestasis are excreted in the bile, including the mycotoxin sporodesmin which concentrates 100-fold in the bile (Treinen-Moslen, 2001).

α-Naphthothiourea (ANIT) is a hepatotoxicant that damages bile duct epithelium and hepatocytes. The drug is used experimentally in rodents as a model of intrahepatic

cholestasis. A single dose of ANTU induces acute cholangeitis; prolonged exposure causes bile duct hyperplasia and biliary fibrosis. Although the biochemical and histological features of ANTU toxicity are well documented, the mechanism by which ANTU causes liver injury remains uncertain. *In vivo*, ANTU does not cause liver damage until it appears in bile (Jean and Roth, 1995).

This drug is initially detoxified in hepatocytes by conjugation with glutathione. ANTU–glutathione complexes are secreted into bile, but they are unstable and rapidly dissociate. This exposes biliary cells to high concentrations of the parent compound, which presumably causes direct cytotoxicity. The reappearance of ANTU in bile also leads to enterohepatic cycling, reuptake of the drugs in the intestine, and repetitive rounds of glutathione conjugation and secretion. This not only delays elimination of the drug but depletes glutathione progressively from hepatocytes and leads to hepatocellular damage. In addition, ANTU is known to cause hepatotoxicity by neutrophil and platelet dependent mechanisms (Jean and Roth, 1995).

2. Idiosyncratic Reactions

Many idiosyncratic drug reactions are believed to be immune mediated. Neoantigens may result from adducts formed from the interaction of reactive drug metabolites with cellular proteins. These neoantigens may be processed by Kupffer cells or other antigen presenting cells, transported to the cell surface, and presented as antigens. Cell and antibody mediated immune response may cause severe liver damage. Various drugs are believed to cause immune mediated idiosyncratic reactions in humans, including halothane, diclofenac, phenytoin, and sulfonamides (Zimmerman, 1999; Treinen-Moslen, 2001; Sturgill and Lambert, 1997; Watkins, 1999).

Liver injury can be a result of both direct cytotoxicity and antibody dependent cellular toxicity. Alcoholic liver disease is another example of possible immune mediated damage. Acetaldehyde, produced by metabolism of ethanol, forms adducts with hepatic proteins similar to halothane, resulting in higher antibody titers to which some of the liver damage following ethanol ingestion may be attributed (Ramaiah *et al.*, 2004). However, the role of immune mediated liver damage following ethanol ingestion is minimal compared to other known mechanisms of alcohol liver damage.

IV. WARFARE AGENTS AFFECTING LIVER

A. Fungal and Plant Toxins

1. Microcystins

The microcystins are hepatotoxic products of freshwater blooms of cyanobacteria of *Microcystis*, *Anabena,* and *Oscillatoria* species (Chen *et al.*, 1993; Luu *et al.*, 1993), with *Microcystis aeruginosa* being the most common. Nearly 60 microcystin cyclic hepatopeptides have been identified of which microcystin-LR, also known as the fast death factor, is the most common, and the toxin of choice to be weaponized (Craig *et al.*, 1993; Rinehart *et. al.*, 1994). This toxin has been reported to be responsible for the deaths of wild animals and agricultural livestock (Carmichael, 1988). A potential threat to the health of humans has been recognized in countries where water supplies are contaminated with cyanobacteria (Yu, 1989). In 1996, microcystin-LR was also implicated in the death of 50 Brazilian dialysis patients (Jochimsen *et al.*, 1998).

This potent mammalian liver toxin (Carmicheal, 1988) acts by altering the hepatocyte cytoskeleton actin filaments leading to disruption of the structural integrity of the sinusoids. This causes massive necrosis in the liver leading to cell death. The toxic effects of microcystin-LR have been reported due to the presence of 3-amino-9-methoxy-10-phenyl-2,6,8 trimethyl deca,4,6 dienoic acid (ADDA), which is believed to be essential for its functioning. In addition, microcystins are known to be potent and specific inhibitors of catalytic subunits of protein phosphatases-1 and 2A (Cohen and Cohen, 1989; Yoshizawa *et al.*, 1991; Honkanen *et al.*, 1990), and activate the enzyme phosphorylase b. Microcystin administered intraperitoneally in mice caused disruption of bile flow in <10 min, and death within a few hours due to hypovolemic shock induced by interstitial hemorrhage following liver necrosis (Carmicheal, 1988). Death was also reported within hours of administration of aerosol (LD_{50}, 67 μg/kg body weight) in mice. In humans, microcystin ingestion leads to diarrhea, vomiting, weakness, and pallor, with death occurring in a few hours if a lethal dose is taken.

2. Aflatoxins

Aflatoxins were first isolated more than 40 years ago after the outbreak of disease and deaths in turkeys. These toxins are produced as secondary metabolites by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, at temperatures between 24 and 35°C and humidity exceeding 7% (Williams *et al.*, 2004). These fungi are known to infect a variety of crops like peanuts, walnuts, pecans, pistachios, and corn.

Aflatoxins are bisfuran polycyclic compounds, and depending on the characteristic blue or green fluorescence produced under ultraviolet light, these compounds are known as aflatoxin B_1, B_2, G_1, and G_2, respectively. Although these compounds have been recognized as potent mutagens and carcinogens, they are still not as toxic as botulinum or ricin. Before the Gulf War, aflatoxins were not recognized as biological warfare agents; however, they were weaponized by Iraq for missile delivery, and at present they can be considered as agents for biological warfare (Marshall, 1997; Zilinskas, 1997).

Of the several aflatoxins, aflatoxin B_1 (AFB_1) is the most prevalent and the most potent toxin. AFB_1 is converted to an unstable metabolite, the 8,9-epoxide, is highly electrophilic, and forms covalent adducts with RNA, DNA, and proteins

(Roebuck and Maxuitenko, 1994). In addition, aflatoxin M_1, a metabolite of AFB_1, has been found in the milk of cows fed AFB_1.

AFB_1 is acutely toxic in all species, with an LD_{50} ranging from 0.5 mg/kg for the duckling to 60 mg/kg for the mouse (Wogan, 1973). Acute exposure to AFB_1 has been reported to cause hepatic lesions with edema, bililary proliferation, and parenchymal cell necrosis. In addition, aflatoxin B_1 poisoning has been reported to cause jaundice, rapidly developing ascites and portal hypertension with high mortality resulting from massive gastrointestinal bleeding. In children, aflatoxin produces a condition called Reye syndrome resulting in disturbed consciousness, fever, convulsions, and vomiting (Palmgren and Ciegler, 1983).

3. Ricin

Ricin, a potent plant toxin, is extracted from the seeds of castor plants (*Ricinus communis*), and has been considered a warfare agent since World War I. Although ricin is not as toxic as botulinum or anthrax, easy availability and lack of specific antidote make it a weapon of choice. Being a ribosome inactivating protein, a single molecule of ricin reaching the cytosol can kill that cell as a consequence of protein synthesis inhibition (Eiklid *et al.*, 1980). Human exposure can be through ingestion and inhalation; however, compared to oral exposure, inhalation of ricin has been reported to be more harmful. In humans the lethal dose has been reported to be 5–10 μg/kg body weight.

In addition to pulmonary toxicity, ricin has been reported to be a major hepatotoxicant (Kumar *et al.*, 2003). Studies by Muldoon *et al.* (1992) reported that humans exposed to ricin experience increased hepatic transaminase and lactate dehydrogenase activities. Furthermore, mice treated with ricin experience significant oxidative stress, resulting in hepatic glutathione depletion and lipid peroxidation (Muldoon and Stohs, 1991). Elevation of lipid peroxidation is reported to originate mainly from the damaged Kupffer cells, which are believed to be the target of this toxin in the liver (Skilleter, 1981). The high sensitivity of Kupffer cells has been ascribed to the ability of ricin to bind to the mannose receptors present on these cells (Skilleter, 1981; Magnusson, 1993).

B. Bacterial (Anthrax)

Anthrax is a disease caused by the spores of the bacterium *Bacillus anthracis*, and is a recognized biological warfare agent. In the USA (2001), anthrax was deliberately spread through the postal system by sending letters with powder containing anthrax. This resulted in 22 cases of anthrax infection. Inhalation and cutaneous exposure are the main routes of infection. Recent studies have revealed that laboratory animals infected with anthrax have developed significant liver injury. Studies by Moayeri *et al.* (2003) reported that mice infected with *B. anthracis* developed extensive liver necrosis in addition to pleural edema. Liver lesions in these animals ranged from small hemorrhagic infarcts to large areas of centrilobular coagulative necrosis. A significant increase in alanine aminotransferase and aspartate aminotransferase was also observed in these animals. Liver dysfunction was also indicated by decreased serum albumin levels in these animals in response to toxin over time.

V. CONCLUDING REMARKS AND FUTURE DIRECTION

In spite of significant advances in our knowledge of the mechanisms of liver damage, scientists seem to have a long way to go before all the mechanisms of toxicity in liver for a given chemical are completely established. In the meantime, new platforms (such as multiplex, multi color flow cytometry, meso-scale discovery) and new ''omics approaches'' have provided added tools for researchers to obtain breakthroughs in the area of liver toxicity. Clearly, current reports on the number of warfare agents targeting the liver are limited and with further research and case studies, these numbers will continue to grow. With the changing political global climate and the potential for chemical warfare agents, target organ toxicities, especially the liver, will likely gain attention.

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CHAPTER 38

Renal Toxicity

MANU SEBASTIAN

I. INTRODUCTION

The kidneys are the primary organ responsible for water and electrolyte homeostasis, thereby playing an important role in total body homeostasis. The kidneys help to maintain this homeostasis by performing functions which include excretion of metabolic waste products and foreign chemicals, regulation of water and electrolyte balances, regulation of body fluid osmolality and electrolyte concentrations, regulation of arterial pressure, regulation of acid–base balance, secretion, metabolism, and excretion of hormones and gluconeogenesis. A toxic insult to the kidneys can disrupt any or all of these functions. This chapter describes detailed toxicity of chemicals that target the renal system.

II. RENAL ANATOMY AND PHYSIOLOGY

Kidneys in mammals are paired organs that lie ventral and adjacent to the lumbar vertebrae. The medial side of each kidney contains an indented region called the hilum, through which pass the renal artery and vein, lymphatics, nerve supply, and ureter, which carries the final urine from the kidneys to the bladder. The kidney is surrounded by a tough fibrous capsule that protects its inner structures. If the kidney is dissected from top to bottom, the two major regions that can be visualized are the outer cortex and the inner region referred to as the medulla. The cortico-medullary ratio is approximately 1:2 or 1:3 in domestic animals. The medulla is divided into multiple cone-shaped masses of tissues called renal pyramids. The base of each pyramid originates at the border between the cortex and medulla and terminates in the papilla which projects into the renal pelvis, a funnel-shaped continuation of the upper end of the ureter. Kidneys of small mammals such as rat, mouse, and rabbit have a single papilla and are called unipapillate. Large mammals, including humans, have more than one papilla per kidney and so are called multipapillate. Cynomolgus monkey kidney is unipapillate with fusion of renal pyramids into relatively short crest-like papilla. Human kidney has no external lobulation but bovine kidney has external lobulation. With regard to anatomy and physiology, pig kidney is similar to human kidney. The outer border of the pelvis is divided into open-ended pouches called major calyces that extend downward and into minor calyces, which collect urine from the tubules of each papilla. The walls of the calyces, pelvis, and ureter contain contractile elements that propel the urine toward the bladder (Ellenport, 1975).

The nephron is the functional unit of the kidneys. Each human kidney contains approximately one million nephrons and each is capable of forming urine. The nephron consists of a tuft of glomerular capillaries called the glomerulus, through which large amounts of fluid are filtered from the blood, and a long tubule, in which the filtered fluid is converted into urine. The glomerulus, which is the filtering unit of the kidneys, is made up of a network of branching and anatomizing capillaries, which have high hydrostatic pressure compared to other capillaries in the body. These capillaries are composed of endothelial cells that have attenuated and fenestrated cytoplasm which allow the passage of molecules. Those compounds with a molecular weight of about 60,000 daltons pass through the glomeruli into the tubular lumen to be excreted with urine. The glomerular capillaries are covered by epithelial cells and the total glomerulus is encased in Bowman's capsule with a space, which is called the Bowman's space. Fluid filtered from the glomerular capillaries flows into the Bowman's space and then into the proximal tubule. The fluid collected in the proximal tubule flows into the loop of Henle, which dips into the renal medulla. Each loop is made up of an ascending and descending limb. The walls of the descending limb and the lower end of the ascending limb are very thin and are called the thin segment of the loop of Henle. After the ascending limb of the loop has returned partway back to the cortex, its walls become much thicker and it is called the thick segment of the ascending limb. Approximately 25% of the filtered Na^+ and K^+ and 20% of the filtered water are reabsorbed by these segments of the loop of Henle.

The glomerular filtration rate is regulated by the macula densa, which is composed of specialized cells located between the end of the thick ascending limb and the early distal tubule, with close proximity to the afferent arteriole. The function of the macula densa is to prevent massive losses of fluid/electrolytes in impaired tubular resorption. After passing this area, the fluids enter the distal tubule, which lies in the renal cortex and moves to the connecting tubule, leading to the cortical collecting tubule. The initial eight to ten collecting ducts join to form a single, larger

collecting duct that runs downwards into the medulla and becomes the medullary collecting duct. The collecting ducts merge to form progressively larger ducts that eventually empty into the renal pelvis through the tips of renal papillae.

Blood flow to the two kidneys is approximately 22–25% of the cardiac output. The kidneys are supplied by the renal artery which enters the kidneys through the hilum and then branches progressively to form the interlobar arteries, arcuate arteries, interlobular arteries (also called radial arteries), and afferent arterioles, which lead to the glomerular capillaries. The distal ends of each glomerulus coalesce to form the efferent arteriole, which leads to a secondary capillary network, the peritubular capillaries which surround the renal tubules. The cortex receives approximately 90% of the blood flow compared to the medulla or papillae so blood-borne toxic molecules reaching the kidneys have a more toxic effect on the cortex, as compared to the medulla or renal papillae. The interstitial space is occupied by the fenestrated peritubular capillaries and a small number of fibroblast-like cells. Increase in thickness of interstitial space in pathological conditions is due to edema, proliferation of fibrous tissue, or infiltration of inflammatory cells (Guyton and Hall, 2006).

A. Biotransformation and Excretion

Gastrointestinal tract, lungs, skin, and other special routes of administration (e.g. intravenous) are the route of entry of xenobiotics into the body which then reach the blood and are eventually distributed throughout the body. The rate of distribution of xenobiotics or their metabolites to an organ or tissue depends on the blood flow and the rate of diffusion out of the primary capillary bed into the cells of a particular organ. The kidneys, being an organ which receives 25% of the cardiac output, are exposed to several xenobiotics and their metabolites. Kidneys, along with liver, are the two important organs in the body which concentrate toxicants. A classic example is metallothionein, a protein which binds to cadmium and zinc with high affinity in the kidneys and liver (Rozman and Klaassen, 2001). Toxic effects of xenobiotics on an organ are by the action of the parent compound or through the metabolites produced as a result of bioactivation via metabolism (biotransformation). Thus, toxicants can induce nephrotoxicity by the parent compound (e.g. gentamicin), the biotransformed molecules in other organs like the liver (e.g. hepatic biotransformation of haloalkene) and by biotransformation in the kidneys (e.g. chloroform). Biotransformation reactions are catalyzed by Phase I and Phase II enzymes which convert the parent compounds to more polar and hydrophilic metabolites, and are thereby excreted more rapidly or converted to reactive chemical species which can induce toxicity. The important Phase I enzymes present in the kidneys are cytochrome P450, prostaglandin synthase, and the reductases. Although cytochrome P450 is present in the kidneys, the concentration is only approximately 10% of that in liver. The highest concentration of cytochrome P450 is present in the renal cortex (the majority are in the proximal tubular epithelial cells) with small amounts in the medulla. The compounds which are biotransformed by cytochrome P450 in the kidneys are acetaminophen (acute overdosage), cephaloridine, chloroform, and ochratoxin (partially). Prostaglandin synthase, another Phase I enzyme, is found primarily in the medullary interstitial and collecting duct cells. Phase I enzymes capable of catalyzing a reduction reaction (reductases) are also present in the kidneys and are involved in the reduction of the 5-nitro group in 5-nitrofurans to reactive chemical species, leading to nephrotoxicity. The important Phase II enzymes present in the kidneys are UDP-glucuronosyltransferases (UGT), sulfotransferases, and glutathione-*S*-transferases. The liver possesses the highest UGT concentration. UGT may contribute to acute or chronic toxicity associated with clofibrate, diclofenac (an NSAID), and other NSAIDs. The substrates for glutathione-*S*-transferases include haloalkenes, acrolein, etc. (Rankin and Valentovic, 2004).

The kidneys are one of the most important organs for the excretion of xenobiotics and their metabolites. The different mechanisms by which the toxicants are removed by the kidneys are glomerular filtration, tubular excretion by passive diffusion, and active tubular secretion. The xenobiotics that enter the blood reach the kidneys, are filtered through the large pores of the glomerular capillaries (70 nm size), pass the tubular lumen, and eventually are excreted through the urine. The removal of toxicants through the kidney tubule is mainly dependent on the water solubility of the toxicant. Those xenobiotics which are biotransformed to more water-soluble products (more polar) are excreted with the urine, while those toxicants with high lipid solubility (high lipid/water partition coefficient) are reabsorbed across the tubular cells into the blood stream. Toxicants can be excreted by passive diffusion through the tubules. The flow of urine is important for maintenance of the concentration gradient necessary for this process. Toxicants are also excreted into the urine by an active transport process with secretion by a transporter. These transporters are localized in the proximal tubules. The important transporters localized on the basolateral membranes of the proximal tubular cells are organic anion transporter (OAT), organic cation transporter (OCT), and a few members of the organic anion transporting polypeptides (OATP). These transporters are involved in the uptake of organic acids (OAT) and a number of cations (OCT) into the renal tubular cells. Multi-resistant drug protein (Mrp) found on the luminal brush border of the tubular epithelium moves xenobiotics from the proximal tubule cells into the lumen. The organic anion transporting polypeptides (OATP) and peptide transporter (Pept) are involved in the reabsorption of chemicals into the proximal tubular cells. A classic example of the action of active transport is the difference in susceptibility of cephaloridine in adult and newborn animals. In newborn animals the active uptake of cephaloridine is not well developed; hence

it is not absorbed and concentrated in the tubular cells that result in toxicity (Rozman and Klaassen, 2001). Thus tubules play an important role in the excretion of toxicants, and a toxic insult to these cells results from accumulation of toxicants in the body.

The toxic effect of many xenobiotic compounds in the kidneys is specific to the anatomical locations, i.e. proximal tubules, glomeruli, medulla/papilla, or loop of Henle. The site-selective injury observed in humans when exposed to many compounds is similar to that in domestic animals. The cause of these site-specific target injuries is multifactorial and may include detoxification mechanisms, regenerative ability, difference in blood flow, and transport and accumulation of the chemicals and their metabolites.

The proximal tubule is the specific target for several antibiotics, mycotoxins, certain antineoplastic drugs, and heavy metals. The proximal tubule can be divided into three discrete segments. The S1 segment (pars convoluta) is the initial portion of the proximal convoluted tubule; S2 is the transition between the pars convoluta and the pars recta, which consists of the end of the convoluted segment and initial portion of the straight segment; and S3 (pars recta) consists of the distal portion of the proximal segment (Figure 38.1) (Berl and Bonventure, 1998). The proximal convoluted tubule is the most common site of toxin-induced injury and there are multiple reasons for this site-specific action. These reasons include cytochrome P450 and cysteine conjugate β-lyase activity localization (those agents requiring bioactivation by these enzymes induce toxicity in this region, e.g. chloroform); selective accumulation of xenobiotics due to a relatively lose epithelium compared to the distal tubules which allow compounds to access the cells easily; increased transport of several molecules like organic anions, cations, and heavy metals resulting in increased accumulation in this area; and increased susceptibility of proximal convoluted tubular epithelial cells to ischemic injury. Compared to the proximal tubules, distal tubules are less affected by toxicant injury (Schnellmann, 2001).

The medulla/papillae are the target site for analgesics like phenylbutazone in horses. The loop of Henle is the target for fluoride ions and the glomeruli for immune complexes. The other anatomical location which is commonly affected by toxicant injury is the renal papillae. A classic example is

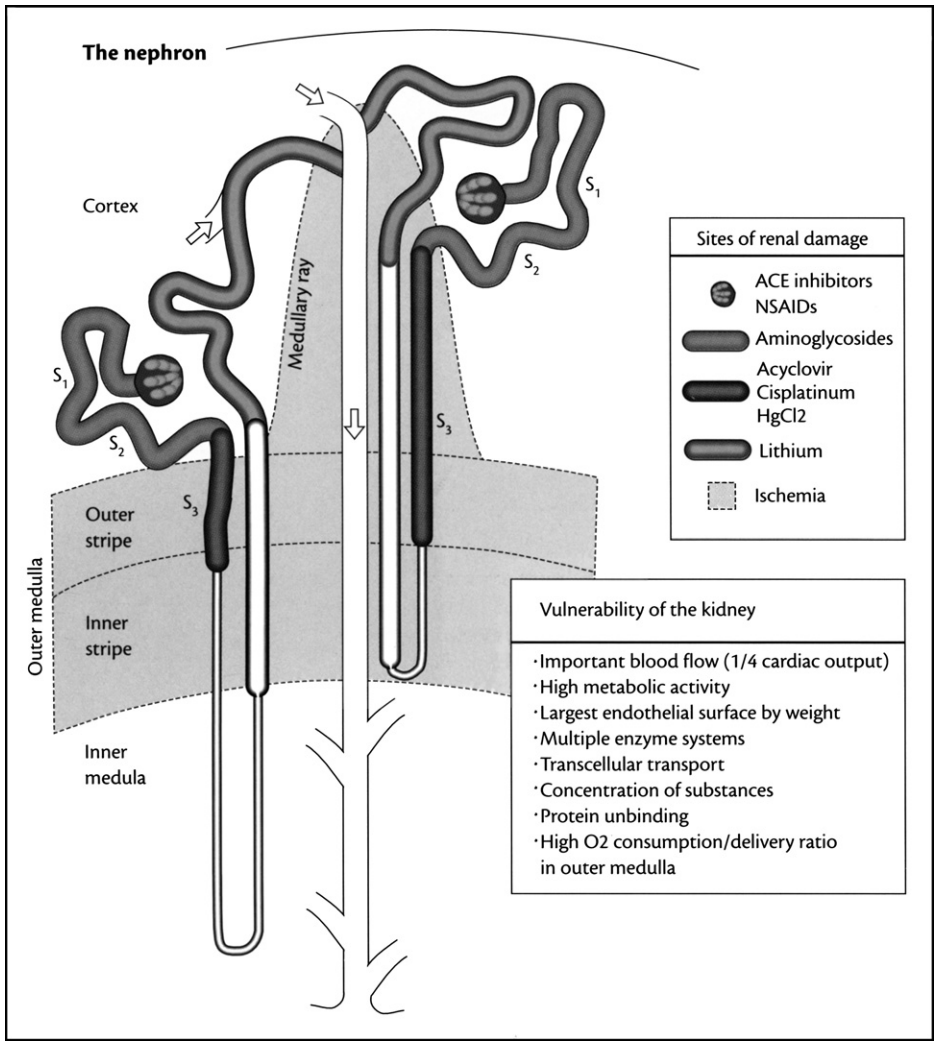

Figure 38.1. Sites of renal damage, including factors that contribute to the kidney's susceptibility to damage. ACE – angiotensin converting enzyme; NSAID – nonsteroidal anti-inflammatory drugs; $HgCl_2$ – mercuric chloride (adapted from *Color Atlas of the Diseases of the Kidney*, Vol. 1, Berl, T., Bonventre, J.V., 1998, with permission).

necrosis from analgesics in horses. The important factors for this site-specific injury are due to a high concentration of prostaglandin H synthase activity in this area (they metabolize analgesics to reactive intermediate compounds which bind to cellular macromolecules), high papillary concentration of toxicants, and inhibition of the vasodilatory prostaglandins, which result in ischemia to this area (Schnellmann, 1998).

III. TOXIC EFFECTS ON THE KIDNEY

A. Acute Renal Failure

Toxicants and toxins can produce their effects on the different anatomical locations of the kidneys by affecting the tubules, glomeruli, ducts, vessels, and interstitium. The majority of toxins manifest their effects by acute renal failure. The most important manifestation of acute renal failure is the decrease in glomerular filtration rate (GFR) which leads to an excess of urea or other nonprotein nitrogenous components in the blood (azotemia). The decrease in GFR can be due to pre-renal, renal, or post-renal causes. Renal toxicity generally leads to renal or sometimes post-renal azotemia. Post-renal azotemia is usually observed in conditions of obstruction to renal outflow or post-renal leakage. The toxic effects of chemicals on the kidneys result in reduced GFR, leading to azotemia. The different mechanisms by which toxins/chemicals induce renal failure include: (1) tubular epithelial damage, leading to formation of tubular casts and tubular blockage, (2) tubular epithelial necrosis, which leads to reabsorption of glomerular filtrate, (3) renal vasoconstriction, which leads to hypoxia and necrosis, and (4) changes in the glomerular ultrafiltration barrier. The majority of chemicals induce renal failure by direct action on the tubules.

The ultrastructural changes which lead to acute renal failure are due to the loss of cell-to-cell adhesion and cell-to-matrix adhesion which eventually results in loss of tubular integrity.

The clinical signs associated with acute renal failure are nausea, vomiting, gastrointestinal bleeding, esophagitis, gastritis, and colitis, which are all associated with azotemia. When azotemia is associated with a constellation of clinical signs and symptoms and biochemical abnormalities, it is called uremia. There is also secondary involvement of the gastrointestinal tract (uremic gastroenteritis), peripheral nerves (peripheral neuropathy), and sometimes the heart (uremic fibrinous pericarditis).

B. Chronic Renal Failure

Chronic renal failure is associated with long-term exposure to toxins and is mostly related to the secondary pathological changes triggered by the initial injury. These secondary changes are compensatory mechanisms to maintain the function of the whole kidney, but they eventually result in reduced glomerular function and tubular and interstitial changes. Once chronic changes are initiated, they progresses to an end stage kidney.

Clinical signs of chronic renal failure primarily include: (1) edema due to reduced renal perfusion leading to stimulation of the renin–angiotensin system, which stimulates aldosterone secretion leading to retention of sodium and water, (2) hypocalcemia with compensatory parathyroid activity and osteodystrophy, and (3) reduced red blood cell counts due to decreased synthesis of erythropoietin as a result of damage to the juxtaglomerular cells.

C. Pathological Correlates

The kidneys have extensive reserve capacity and hence renal diseases may be present with or without any clinical signs or clinicopathological abnormalities. Renal failure occurs or is manifest after substantial loss of the functional unit, the nephron. Renal failure represents clinical signs or laboratory abnormalities as a result of reduced renal function. Hence, measurement of renal function will aid in diagnosing a toxic insult to the renal tubules or glomeruli. The various renal functions, which can be measured to evaluate a toxic insult, include urine concentration, blood urea nitrogen (BUN), urea excretion, serum creatinine, uric acid (in birds), glomerular filtration rate, urine electrolyte clearance ratios, and changes in blood levels of calcium, phosphorus, magnesium, sodium, and potassium.

Urine analysis is one of the important methodologies to evaluate renal function and to interpret injury to the various anatomical components of the kidneys. Urine analysis involves multiple physical and chemical parameters as well as sediment analysis. The different physical characters which are evaluated to assess kidney function include volume, color, transparency, and odor. The chemical characters evaluated include ketones, protein, glucose, bilirubin, blood, urobilinogen, nitrate, and enzymes. The different sediments which aid in evaluating a toxic insult to the urinary system include epithelial cells, erythrocytes, leukocytes, casts, and crystals. The important abnormal sediments which help in diagnosing an acute failure include renal epithelial cells, pigments casts (myoglobin, hemoglobin), and crystalluria (uric acid).

Other parameters which can be measured to evaluate the function of the kidneys include urine specific gravity, BUN, serum creatinine, uric acid excretion (in birds), glomerular filtration rate (GFR), and levels of calcium and phosphorus in the serum. A reduced urine-specific gravity, increased BUN (azotemia), increased serum creatinine concentration, hyperuricemia (in birds), altered GFR, and serum calcium and phosphorus levels are all indicators of renal damage. Because of the high reserve capacity of the kidneys, many of these alterations are observed only when more than two-thirds of the nephrons are damaged (Gregory, 2003).

The gross findings in renal damage are not that evident in acute damage and may include swollen cut sections, pale renal parenchyma, and reddish discoloration. Mineralization and crystal deposits will be manifest by linear pale streaks in the cortex and medulla with a gritty consistency (mineral). Papillary necrosis is characterized by pale discoloration in the papilla and renal crest. The gross observations observed in chronic renal failure are reduced size, irregular subscapular surface, hard to section, and gritty consistency. The important histopathological observations associated with renal damage are necrosis and degeneration of tubular lining epithelium (mostly in the proximal convoluted renal tubular epithelium), regeneration, casts (protein, degenerate cells, globin) in the lumen, mineralization of the basement membranes of the tubules, glomeruli tufts, damaged capsule and blood vessels, necrosis of the renal papillae, necrosis of the glomerular matrix with hemorrhage, and increased cellularity of the glomerular tufts (proliferative glomerulonephritis) (Confer and Panciera, 2001; Sebastian *et al.*, 2007).

1. Drugs

a. Aminoglycosides

The most important toxic effects manifested by aminoglycosides are nephrotoxicity, ototoxicity, and neuromuscular blockade. Aminoglycosides accumulate in the proximal tubular epithelial cells where they are sequestered in the lysosomes and interact with ribosomes and mitochondria, resulting in cellular damage. Gentamicin and several other aminoglycosides have been associated with nephrotoxicity leading to acute renal failure in humans. Aminoglycosides account for approximately half of the drug-induced cases of nephrotoxicity in human patients. The precise mechanism remains unknown; however, studies have shown strong evidence related to the generation of several reactive oxygen species which cause renal failure. Gentamicin toxicity has been reported in dogs, where the kidneys appear pale. Histopathological changes include tubular epithelial necrosis with regeneration and mineralization. Predominantly, the proximal tubules are affected. Lesions will progress from hyaline droplet degeneration with dilated lumen to necrosis of the renal tubular lining epithelium (Sprangler *et al.*, 1980).

Experimental exposure of neomycin in calves has reported both nephrotoxicity and ototocity (demonstrated clinically). The clinical pathological observations included granular casts in urine, proteinuria and low specific gravity, azotemia, decreased creatinine clearance, polyuria, and polydipsia. The histopathological findings included renal tubular epithelial degeneration and necrosis (Crowell *et al.*, 1981).

Beta-lactam antibiotics (pencillins, cephalosporins, and carbapenems) are excreted by the kidneys and hence the kidneys are targets of toxicity. These drugs can cause interstitial nephritis, tubular necrosis, and allergic angitis. Beta-lactams behave like haptens which are then immunologically processed leading to an immunologically mediated drug injury, primarily an acute interstitial nephritis characterized by the presence of edema and focal or diffuse infiltration of inflammatory cells, frequently eosinophils, lymphocytes, and plasma cells in the renal interstitium. A definitive diagnosis is made by renal biopsy, which will reveal normal glomeruli and a patchy, but usually heavy, interstitial infiltrate with lymphocytes, plasma cells, and eosinophils (Linton *et al.*, 1980). Tubular necrosis is observed when the drug is administered at high doses.

b. Amphotericin B

Amphotericin B is an antifungal agent and a polyene macrolide class of antibiotics. Nephrotoxicity is the common adverse effect associated with amphotericin B therapy, which can lead to impairment of both glomerular and tubular function. Nephrotoxicity is caused by acute renal vasoconstriction, which leads to damage of the distal tubular epithelium. The incidence of toxicity is high and acute renal failure is common. Clinical manifestations include azotemia, renal tubular acidosis, decreased concentrating ability of the kidneys, hypokalemia, and hypomagnesemia (Fanos and Cataldi, 2000). Dogs and cats are the most commonly affected species, with more susceptibility in cats. The common histopathological findings include renal tubular necrosis, dilatation of the tubules and mineralization with mild lesions in the interstitium and glomeruli (Rubin *et al.*, 1989).

c. Cisplatin

Cisplatin is an antineoplastic drug which causes toxic changes in the kidneys, intestines, and bone marrow. Cisplatin or its metabolites are eliminated through the kidneys and hence cause renal toxicity. The highest concentration of cisplatin is observed in the cells of the S3 segment (pars recta) of the proximal tubule and this is the area of the nephron which is most susceptible to cisplatin-induced toxicity (Lieberthal and Levine, 1996). Acute renal failure is the most important toxic effect of cisplatin treatment in humans. The toxic effect on the tubular epithelial cells leading to acute tubular necrosis is believed to occur via the formation of DNA adducts and the induction of oxidative stress, which results in genotoxicity and necrosis. The pathological findings associated with cisplatin treatment in dogs include mild renal tubular atrophy and tubular necrosis (Choie *et al.*, 1981; Forrester *et al.*, 1993).

d. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) and Analgesics

NSAIDs are the most widely used analgesics in human and veterinary medicine, and all have some toxic potential. NSAIDs cause an acute reduction in renal function, which leads to nephrotoxic acute renal failure. The abnormalities related to renal function associated with NSAID

nephrotoxicity are fluid and electrolyte disturbances, acute deterioration of renal function, nephrotic syndrome with interstitial nephritis, and papillary necrosis. NSAIDs cause toxicity of the gastrointestinal tract, kidneys, and liver. NSAIDs cause renal toxicity mainly by the inhibition of prostaglandin synthesis and renal blood flow, resulting in renal papillary necrosis. Hepatic toxicity is mostly attributed to the idiosyncratic reaction.

The common species of animals affected by NSAIDs are dogs, cats, and horses. Horses, especially when dehydrated, are susceptible to phenylbutazone toxicity and the target organ is the kidneys. A common gross observation in experimental studies conducted in horses dosed with phenylbutazone and deprived of water was a yellow–green radial streak in the renal papillae. Microscopic observations include sloughing of the renal pelvic epithelium, including the terminal lining of collective ducts with coagulation necrosis of the underlying papillary interstitium, mineralization of the calyx, necrotic papillary interstitium containing nuclear debris, and hemorrhage and dilatation of collecting ducts and cortical tubules above the area of necrotic papillae. Other lesions observed in experimental studies of horses include ulceration and erosions of the glandular portion of the stomach, submucosal edema of the small intestine, erosions and ulcers of the large colon, and ulceration of the gastric mucosa and tongue. Pathological findings in experimental studies of horses exposed to flunixin meglumine (aminonicotinic acid) were glandular mucosal erosion and ulceration of the stomach (Gunson and Soma, 1983; MacAllistar *et al.*, 1993). Renal tubular or papillary necrosis is also reported in a limited number of canine cases (Jones *et al.*, 1992).

Large doses of acetaminophen can cause renal and hepatic toxicity in rats and mice. Toxicity is characterized by renal tubular necrosis in the proximal tubules (Schnellmann, 2001). Acetaminophen toxicity has also been reported in humans. Generally, toxicosis is a result of large overdoses which result in proximal tubular necrosis. Aspirin, ibuprofen, and acetaminophen are the important analgesics, which are reported to cause toxicosis in veterinary medicine. Renal lesions including renal tubular necrosis and papillary necrosis have been reported in dogs.

Recently, a case of vulture mortality was reported from the Indian subcontinent related to NSAID toxicity. Diclofenac is a widely available veterinary NSAID in the Indian subcontinent, used in domestic livestock. Vultures were exposed to the drug when they consumed carcasses of cattle that were treated with diclofenac shortly before death. Experimental studies of this drug in vultures showed marked nephrotoxicity. The gross observations were primarily deposits of urate on the surface of internal organs related to renal failure. Histopathological findings were acute necrosis of the proximal renal tubular epithelium with minimal inflammatory response and deposits of urate crystals (Oaks *et al.*, 2004; Meteyer *et al.*, 2005).

e. Sulfonamides

Renal toxicity associated with sulfonamides is rare as most of the current pharmaceutical preparations are relatively highly soluble at the pH normally occurring in the kidneys. Toxicity occurs due to very high overdosage. Sulfonamides can cause both acute and chronic toxic effects. Animals with renal toxicity will show elevated levels of BUN and creatinine. In some animals, sulfonamide crystals will be observed in the renal pelvis as a gross finding and kidneys may be gritty in texture when cut. Histopathological observation is primarily renal tubular epithelial degeneration and necrosis due to the direct action of the crystals. These crystals are visible with polarized light only if the kidneys are processed to prevent dissolution (Jones *et al.*, 1996).

f. Tetracyclines

Tetracyclines have been reported to cause toxicity and kidneys are the primary organs. Renal tubular necrosis is the common pathological finding. Animals with renal toxicity will show oliguria, marked azotemia, moderate proteinuria, and tubular casts in urinary sediment with an inability to concentrate urine (Lairmore *et al.*, 1984).

g. Vitamin K_3 (Menadione Sodium Sulfate)

Toxicity is commonly reported in horses. In experimental studies, the gross lesion observed was enlargement and paleness of the kidneys. The microscopic lesions included diffuse or multifocal tubular necrosis and dilation with proteinaceous and cellular debris from red blood cells, neutrophils and casts, diffuse interstitial edema, and lymphocytic infiltration regeneration of the tubular epithelium. In animals that survived for 3 months, renal tubules showed degeneration, necrosis, and dilation. In chronic renal failure, following vitamin K_3 administration, the kidneys were reduced in size, the capsule adherent, and pale streaks of connective tissue were very apparent. Microscopic findings included connective tissue proliferation with mononuclear cell infiltration and moderate hypercellularity of glomeruli with few sclerotic glomeruli. The tubules were dilated but the lining epithelium appeared normal with mineral, cellular, and proteinaceous casts seen in many tubules (Rebhun *et al.*, 1984).

h. Cholecalciferol/Vitamin D_3

Vitamin D_3 toxicity in veterinary medicine is by overdosage of vitamin supplements or exposure to rodenticide. The common species affected are dogs and cats, but any species may be affected. The common clinical pathological finding is rapid increase in plasma phosphorus in acute cases, followed by an increase in plasma calcium levels. Histological findings include mineralization in multiple organs including the kidneys, lungs, myocardium, stomach, and vessels. In kidneys, mineralization of arteries as well as mineral deposition in the tubular basement membranes is observed. Diagnosis is by chemical evaluation of serum, plasma, bile,

urine, and kidneys for PTH/25-hydroxycholicalceferol and correlating pathological findings (Gunther *et al.*, 1988; Fooshee and Forrester, 1990; Talcott *et al.*, 1991). Differential diagnosis includes ethylene glycol toxicity, hypercalcemia of malignancy, chronic renal failure, and other causes of hypercalcemia.

i. Cyclosporine

Cyclosporine is a macrolide antibiotic and has been used as an immunosuppressive agent. Cyclosporine can cause both renal and nonrenal toxicity. Clinically renal toxicity consists of four discrete syndromes which include acute reversible renal functional impairment, delayed renal allograft function, acute vasculopathy, and chronic nephropathy with interstitial fibrosis. Proximal tubular epithelium is uniquely sensitive to the toxic effect. The toxic effect is characterized by isometric cytoplasmic vacuolations (several small equally sized vacuoles in cytoplasm), necrosis with or without subsequent mineralization, inclusion bodies (giant mitochondria), and giant lysosomes. Acute vasculopathy consists of vacuolization of the arteriolar smooth muscles and endothelial cells leading to necrosis. In some cases, thrombotic microangiopathy develops, characterized by thrombosis of the renal microvasculature. Long-term treatment with cyclosporine results in chronic nephropathy with interstitial fibrosis (Charney *et al.*, 2004).

j. Furazolidone

Furazolidone toxicity has been reported in goats. The gross observations included congestion in brain, liver, and kidneys. The histopathological findings included degenerative changes in the renal tubules, hepatocyte necrosis, and degeneration in the centrilobular areas (Ali *et al.*, 1984).

k. Radiocontrast agents

Radiographic contrast agent administration can lead to acute renal failure in humans due to acute decline in renal function. Patients with nephropathy associated with radiographic compounds have an acute rise in serum creatinine anywhere from 24 to 48 h after the contrast study. In most cases renal failure is nonoliguric. Urine examination often reveals granular casts, tubular epithelial cells, and minimal proteinuria, but in many cases may be entirely bland. Nephrotoxicity appears to be the result of a synergistic combination of direct renal tubular epithelial cell toxicity and renal medullary ischemia. The diagnosis can be made based on the typical course of events following administration of contrast agent along with excluding other nephrotoxins (Murphy *et al.*, 2000).

l. Statins

HMG-CoA reductase inhibitors (statins) are the most common therapeutic medication used in patients with elevated low-density lipoprotein cholesterol. Significant side effects associated with statins are infrequent. Myalgia and arthralgia are common (1–7%) but frank rhabdomyolysis is rare and is generally associated with concurrent fibrate use. The specific mechanism of toxicity is still unknown and is related to the interruption of a wide variety of metabolic functions. Renal damage associated with statins is generally due to associated rhabdomyolysis. Findings indicating acute tubular toxicity have been reported, evident by irregular vacuolated renal tubular cells, loss of tubular cells with denudation of tubular basement membranes, as well as tubular cell casts seen both on histology and in the urine (Zyl-Smit *et al.*, 2004).

m. Dietary Supplements and Alternative Medicinal Agents

Herbs, nonherbal supplements, and vitamins used in complementary and alternative medicine have been reported to cause nephrotoxicity. Chromium picolinate sold as a nutritional supplement to prevent or treat chromium deficiency has been reported to cause tubular necrosis and interstitial nephritis. Creatine supplements are used to increase high-intensity athletic performance. Although uncommon, these supplements have been reported to cause renal tubular necrosis and interstitial nephritis. L-lysine used as an immunoboosting agent in alternative medicine has been reported to cause Fanconi syndrome and tubulointerstitial nephritis. Several cases of toxicity associated with high-dose administration of vitamin C have been reported, leading to extensive oxalate deposition in renal tubules. Nephrolithiasis has been reported associated with consumption of ephedra-containing dietary supplements. *Glycyrrhiza glabra* (licorice) associated renal toxicity secondary to hypokalemia has been reported. *Hedeoma pulegioides* (pennyroyal) can lead to renal dysfunction and toxicity along with hepatic toxicity. *Thevetia peruviana* (yellow oleander) has been reported to cause renal tubular necrosis along with hepatic failure leading to death.

Several adulterants added to nonherbal supplements, vitamins, and herbal medicine preparations can cause renal dysfunction and renal failure. Aristolochic acid is used as a herbal remedy for weight loss and has been reported to cause ''Chinese herb nephropathy'' characterized by extensive interstitial fibrosis with tubular atrophy and loss. Herbal medicine preparations produced in South Asia contain potentially harmful levels of lead, mercury, and/or arsenic which can lead to renal toxicity.

Several NSAIDs such as ibuprofen, phenylbutazone, and mefenamic acid have been reported to be a contaminant in several alternative medicine preparations and these compounds cause renal toxicity as described before (Saper *et al.*, 2004; Gabardi *et al.*, 2007).

2. Metals

The main targets of heavy metal toxicity are the kidneys and central nervous system. In humans, occupational or environmental exposure of inorganic heavy metals is known to be nephrotoxic at relatively high levels of exposure, with numerous reports of tubulointerstitial nephritis possibly

leading to renal failure. Low environmental levels of these heavy metals can lead to early signs of renal dysfunction, apparent by decreased GFR (lead) or increased urinary loss of tubular enzymes (cadmium).

a. Mercury

Fungicides, preservatives, and fixatives are the main sources of mercury toxicosis. Often, toxicosis is related to accidental ingestion of obsolete mercury products. The lesions of mercury toxicosis are distributed in the gastrointestinal tract, kidneys, and sometimes in the brain (alkyl organic mercury). Inorganic mercurial salts accumulate in the renal cortex and are excreted primarily in the urine. Inorganic mercurial salts cause direct tissue necrosis and renal tubular epithelial necrosis by two mechanisms. The mercuric ion binds covalently with sulfur and inhibits sulfhydryl-containing enzymes in microsomes and mitochondria. The mercurial salts may bind to protein as mercaptides. Death occurs in acute toxicity and oliguria, and azotemia is observed in animals surviving acute exposure (Osweiler, 1996). Chronic exposure to inorganic mercury leads to an immune mediated membranous glomerulonephritis. Histopathological findings in the kidneys are primarily renal tubular degeneration and necrosis, especially in the proximal tubular epithelium. In chronic exposure, glomerulonephritis is observed due to antigen–antibody complex deposition. Diagnosis and confirmation can be made by analytical estimation in the kidneys (Jones *et al.*, 1996).

b. Cadmium

Cadmium toxicity in humans is caused by smoking, inhalation of dust and fumes, and by contaminated food. In animals, cadmium exposure is mostly by ingestion of cadmium–nickel batteries. Cadmium is concentrated in the renal tubules and to a lesser degree in the hepatocytes. Cadmium induces metallothionein (low molecular weight cysteine-rich metal binding proteins) production and forms a cadmium–metallothionein complex. This complex is filtered freely by the glomerulus and is reabsorbed by the proximal tubules. Inside the tubules, renal metallothionein is formed and once the renal metallothionein pool is saturated, free cadmium ions initiate renal tubular injury. Cadmium produces proximal tubular injury leading to increased excretion of glucose amino acids, calcium, and cellular elements. Over the course of time, injury progresses to a chronic interstitial nephritis (Osweiler, 1996; Schnellmann, 2001).

c. Arsenic

Arsenic toxicity from drinking water is a major public health concern in many countries throughout the world. If exposure is to the inorganic form, the kidneys are the target organ due to its involvement in *in vivo* biotransformation and elimination. Very few clinical cases of toxicity are reported in humans. Most clinical cases of toxicosis are reported in animals, especially cattle and dogs, by accidental exposure, but all species of domestic animals can be affected. Trivalent arsenicals inhibit cellular respiration and cause capillary dilatation and degeneration. Pentavalent inorganic arsenicals cause uncoupling of oxidative phosphorylation, leading to cellular energy deficiency. In acute toxicosis, the pathological changes are mostly limited to the gastrointestinal tract. The systemic toxicity occurring in severe acute arsenic poisoning may be accompanied by acute tubular necrosis and acute renal failure. Subacute toxicity is by exposure to low doses for several days. In subacute cases the kidneys are affected, leading to proximal tubular necrosis. The clinical signs associated with subacute toxicity are oliguria and proteinuria, followed by polyuria. Watery diarrhea exhibited in acute poisoning will also be observed. Death results due to dehydration, acidosis, and azotemia (Osweiler, 1996). Histopathological lesions consist of dilation of the vessels, submucosal congestion and edema, necrosis of the intestinal epithelium, renal tubular necrosis and fatty degeneration of the hepatocytes (Jones *et al.*, 1996). Diagnosis is by correlating the case history, pathological findings, and analytical estimation of arsenic in liver, kidneys, and other tissues (hair). Chronic arsenic ingestion is strongly associated with an increased risk of skin cancer, and may cause cancers of the lung, liver, bladder, kidneys, and colon. Chronic inhalation of arsenicals has been closely linked with lung cancer. The specific mechanism of arsenic-related carcinogenicity is unknown. Although carcinogenicity of arsenic in humans has been established, no animal model has yet been developed.

d. Copper

Acute copper toxicosis results from accidental ingestion or administration of copper containing formulations, such as anthelmintics, feed additives, foot baths used for livestock, and pesticides, including fungicides and algaecides at a toxic dose. Copper toxicosis is a problem frequently observed in sheep, as they are sensitive to excessive copper accumulation. Sheep accumulate copper in the liver in proportion to intake, where there is a slow build-up of the copper and then a sudden release, resulting in copper in the circulation. This excess copper oxidizes the erythrocyte membrane, resulting in intravascular hemolysis. Clinical signs in sheep are due to acute anemia and include weakness, anorexia, fever, pale mucous membranes, and dyspnea. Generally, the kidneys have a dark red or bluish black color (gun metal kidneys) and the liver will be pale yellow and friable. Microscopic findings include necrosis of renal tubular epithelium and hemoglobin casts (Osweiler, 1996; Maiorka *et al.*, 1998). Diagnosis is by correlating liver copper values with pathological findings and case history.

e. Fluoride

Fluoride toxicosis reported primarily in animals can be acute or chronic. Acute toxicosis is by exposure to insecticides/rodenticides and volcanic dust. Fluoride is metabolized via renal excretion and is preferentially deposited in

bones and teeth. Acute fluoride intoxication produces clinical signs and lesions of gastroenteritis and renal tubular necrosis. In a study conducted in sheep exposed to fluoroacetate, the microscopic findings in acute toxicosis included degeneration and necrosis of myocardial fibers. In a study conducted in sheep poisoned by sodium fluoride, the pathological findings included necrosis of the proximal tubular epithelial cell, necrotizing ruminitis, reticulitis, and abomasitis (Shupe, 1980; O'Hara *et al.*, 1982; Maylin *et al.*, 1987).

f. Lead

The main sources of lead exposure are paints, water, food, dust, soil, kitchen utensils, and leaded gasoline. The majority of cases of lead poisoning are due to oral ingestion and absorption through the gut. Lead poisoning in adults occurs more frequently during exposure in the workplace. Lead toxicosis affects hemoglobin synthesis by inhibiting the enzyme's delta-aminolevulinic acid synthetase and ferrochelatase, resulting in anemia. Clinical signs include indications of central nervous system involvement, anorexia, anemia, and proteinuria. Clinical pathological findings, suggestive of lead toxicosis, are basophilic stippling of erythrocytes and metarubricytosis with minimal polychromasia. Anemia may be microcytic hypochromic to normocytic normochromic (Prescott, 1983). The potential toxic effect of lead on the kidneys ranges from reversible proximal tubular dysfunction and ultrastructural changes, to chronic nephropathy. Subclinical lead poisoning in humans affects the functions of the proximal tubular lining cells and leads to renal insufficiency. Characteristics of early or acute nephropathy include dysfunction of the proximal tubules, manifesting as aminoaciduria, glycosuria, and phosphaturia with hypophosphatemia and increased sodium and decreased uric acid excretion. These effects are reversible. Characteristics of chronic lead nephropathy include progressive interstitial fibrosis, a reduction in the glomerular filtration rate, and azotemia. These effects are irreversible. The acute form of nephropathy is most frequently reported in children, while the chronic form is mainly reported in adults (Papanikolaou *et al.*, 2005). In chronic cases, the kidneys' histopathological changes are characterized by degeneration and necrosis of the renal tubular epithelium which may show regeneration. Degenerative changes of the renal epithelial cells and hepatocytes have been reported in chronic lead poisoning in dogs. Intranuclear inclusions may be visible in the renal tubular epithelial cells, but their absence does not rule out lead toxicosis (Jones *et al.*, 1996; Osweiler, 1996).

In birds, especially water fowl, lead toxicosis has been reported and lead shot may be found in the gizzard. The birds are generally emaciated with extensive muscle wasting and atrophy of the breast muscles. Changes in the kidneys are primarily renal tubular degeneration or necrosis (with or without intranuclear inclusions) (Locke and Thomas, 1996). Diagnosis is by analytical estimation and history of exposure. Liver and kidneys are the preferred samples for analysis of lead.

3. Mycotoxins

a. Citrinin and Ochratoxin

Ochratoxins are mycotoxins produced by the toxic strains of *Pencillium* and *Aspergillus* spp., mostly due to improper storage conditions of grain, peanuts, dried fruits, grapes/raisins, cheese, coffee, etc. They are also found in meat. Two different ochratoxins have been isolated and characterized (ochratoxin A and B). Of the two, ochratoxin A is the most frequently encountered and the most toxic. The subchronic and chronic effects of this toxin are of more concern than the acute toxicity. Exposure to ochratoxin A in humans has been linked to the development of Balkan endemic nephropathy (BEN). BEN is a disease that occurs in some areas of Bosnia and Herzegovina, Bulgaria, Croatia, Romania, Serbia, and Montenegro. BEN occurred in a limited geographical distribution of farming households with a high mortality from uremia. The first outbreak of the disease in endemic areas occurred simultaneously in the early 1950s, although there are indications that sporadic cases were seen previously. Due to its carcinogenic properties ochratoxin has been classified as a possible carcinogen in humans (Fuchs and Peraica, 2005).

Pigs are the most common species to be affected by ochratoxicosis. Clinical pathological changes include increased BUN values, reduced creatinine clearance, and urine specific gravity. The gross pathological changes observed in experimental studies of pigs include enteritis, pale tan discoloration of liver, edema, and hyperemia of the mesenteric and other lymph nodes. The microscopic findings are mostly confined to the kidneys and gastrointestinal tract, and include necrosis of the renal tubular epithelium most frequently affecting the proximal convoluted tubules, dilatation of the tubules, necrotizing enteritis in multiple locations, fatty degeneration of liver, and focal necrosis of lymphocytes in the germinal center of the lymph nodes (Szczech *et al.*, 1973a; Zomborszky-Kovacs *et al.*, 2002). The renal pathological changes observed in experimental studies of dogs are similar to those observed in the porcine studies and include necrosis and desquamation, predominantly in the proximal convoluted tubules, and eosinophilic granular casts in proximal and distal convoluted tubules (Szczech *et al.*, 1973b; Szczech, 1975). Ochratoxicosis has also been reported commonly in avian species. Experimental studies in young broilers with ochratoxin A show renal edema and tubular necrosis (Wyatt *et al.*, 1975; Duff *et al.*, 1987).

Citrinin is a benzopyran metabolite produced by the toxic strains of *Pencillium ochraceus* and *Aspergillus verrucosum*. Some hepatotoxic effects have been reported for citrinin but the lethal effects are largely due to the nephrotoxicity. The specific mechanism of action in the kidneys is not known, but it leads to acute tubular necrosis. In pigs, rats, and rabbits, the proximal segments are affected. Histopathological findings in different species of animals

include necrosis and desquamation of the proximal renal epithelium, basement membrane thickening, tubule dilatation and proliferation of cells in the interstitium (O'Brien and Dietrich, 2004). Experimental studies show that citrinin induces renal damage in turkeys and ducklings, along with hepatic degeneration and lymphoid necrosis (Mehdi *et al.*, 1984). For further details on the toxicity of ochratoxin and citrinin, readers are referred to a recent publication elsewhere (Gupta, 2007).

b. Fumonisins

Fumonisins are mycotoxins produced by the fungus *Fusarium moniliforme*, primarily in corn. The common species affected are equine and swine, and the target organs are brain and liver. In rats and rabbits, the kidneys are the target organ. Histopathological changes primarily affect the renal tubular epithelium at the junction of cortex and medulla and include prominent apoptosis of epithelial cells of the proximal convoluted tubules (Bucci *et al.*, 1998; Zamborsky-Kouacs *et al*. 2002).

c. Oosporein

Oosporein, which is produced primarily by *Oospora colorans* and *Caetomium trilaterale*, is observed in feed stuffs, cereals and peanuts. It has been reported to be toxic in poultry resulting in nephrotoxicity, and visceral and articular gout. The pathological observations in oosporein toxicity include necrosis of the tubular epithelial cells in the proximal tubules with basophilic casts, hyaline casts in the distal tubules with fibrosis and interstitial pyogranulomatous inflammation, urate deposits in various tissues, and proventricular enlargement with mucosal necrosis (Pegram and Wyatt, 1981; Brown *et al.*, 1987).

4. Pesticides

Pesticide toxicity is common in humans as well as in animals. The common pesticides that cause nephrotoxicity include paraquat, diquat, and zinc phosphide.

a. Paraquat and Diquat

Paraquat is a widely used bipyridyl broad-spectrum herbicide. The most common route of intoxication is ingestion. Among domestic animals, toxic exposure has been reported in cattle, sheep, horses, pigs, poultry, and dogs. Paraquat primarily affects the lungs. Kidney and liver may also be affected and the histopathological change is renal tubular epithelial degeneration. Diquat toxicity results in intracerebral hemorrhage and acute renal failure (Nagata *et al.*, 1992). Diagnosis is made by correlating history of exposure with pathological findings.

b. Zinc Phosphide

Zinc toxicosis results from ingestion of bait containing this compound or by eating tissues of zinc phosphide-poisoned animals. All domestic animals can be affected and reports are common in dogs. Zinc phosphide and aluminum phosphide both release phosphine gas when they come in contact with the acidic contents of the stomach. Both phosphine and intact zinc phosphide are absorbed from the gastrointestinal (GI) tract. The phosphine is believed to cause the majority of acute signs, while the intact phosphide may cause hepatic and renal damage later. Histopathological findings in the kidneys include renal tubular degeneration and necrosis (Casteel and Bailey, 1986). Diagnosis is by analysis of stomach contents, vomitus, liver, and kidneys for phosphine gas. Samples must be submitted frozen in airtight containers for analysis of phosphine.

5. Miscellaneous Agents

a. Ethylene Glycol

Ethylene glycol (EG) is found in many agents, including antifreeze, brake fluids, and industrial solvents. Ingestion of ethylene glycol by humans is uncommon, but when ingested, it can cause serious poisoning. It is readily absorbed from the GI tract and the maximal blood concentration is reached within 1–4 h. The half-life is 3–8 h. The estimated lethal dose of 100% EG is approximately 1.4 ml/kg. Ethylene glycol has a low toxicity, but it is biotransformed in the liver by alcohol dehydrogenase into four organic acids: glycoaldehyde, glycolic acid, glyoxylic acid, and oxalic acid. These metabolites cause central nervous system depression, and cardiopulmonary and renal failure. The rate-limiting step in the metabolism of ethylene glycol is the conversion of glycolic acid to glyoxylic acid. This results in an accumulation of glycolic acid in the blood. Glycolic acid causes severe acidosis, and oxalate is precipitated as calcium oxalate in the kidneys and other tissues. Histological examination of the kidneys reveals marked necrosis of the tubular epithelium with deposition of retractile oxalate crystals in the tubules (Figure 38.2) and collecting ducts. Although the intratubular crystals may be partly responsible

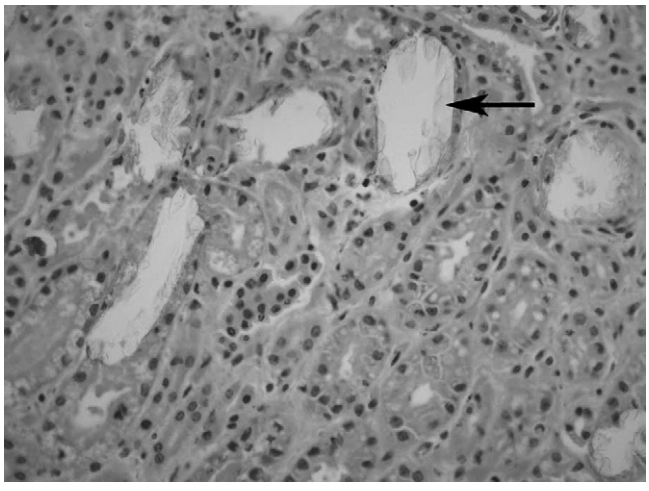

FIGURE 38.2. Ethylene glycol poisoning, canine kidney; tubules are lined by degenerate epithelium and multiple tubules contain crystalline material. Arrows show crystals in tubules. H & E stain ×40.

for renal failure, the action of toxic metabolites on the tubular epithelium is also thought to be important. In chronic cases, there will be atrophy of the tubules with interstitial fibrosis (Leth and Gregersen, 2005).

In animals, EG toxicity is common and is caused by consumption of antifreeze solution which contains up to 95% EG. Mostly cats and dogs are the affected species but there are rare reports of intoxication in other species. The common clinical and pathological findings in cats and dogs include neutrophilia, lymphopenia, azotemia, hyperphosphatemia, hypocalcemia, hyperglycemia, and decreased whole blood bicarbonate. The common findings in urine analysis include proteinuria, glucosuria, hematuria, calcium oxalate and hippurate crystalluria, and the presence of renal epithelial cells, white blood cells, and granular and cellular casts in the urine sediment.

Generally, the kidneys are firm with pale streaks at the corticomedullary junction. In some cases pulmonary edema and hyperemia of the gastric and intestinal mucosa are observed. The microscopic findings include marked renal interstitial fibrosis (in long-time survivors), mild lymphocytic infiltration in the interstitium, atrophy, degeneration and necrosis of the renal tubular epithelium with birefringent crystals, and mineralization and glomerular atrophy in some cases (Jones *et al.*, 1996). The antemortem diagnosis is by clinical pathological evaluation (increased anion and osmolal gaps, suggestive), colorimetric test kit, and GC assay in serum for EG and glycolic acid. Postmortem diagnosis is by pathological evaluation or chemical analysis of the kidneys.

b. Diethylene Glycol

Diethylene glycol (DEG) is a sweet-tasting, colorless, nonodoriferous, hygroscopic liquid commonly used in the commercial preparation of antifreeze, brake fluid, cigarettes, and some dyes. It is an excellent solvent for many relatively insoluble substances. DEG is also commonly used in human medications, such as acetaminophen and sulfanilamide, either intentionally or accidentally leading to episodes of human poisoning, several being fatal. DEG contaminated a shipment of glycerin imported to Haiti from China through Europe. This glycerin was used in numerous locally manufactured liquid pharmaceutical products, including acetaminophen syrup, and consumption of this product led to numerous cases of renal failure associated fatality (O'Brien *et al.*, 1998). Additionally, it has been used as an illegal adulterant in wine. Because of its toxicity, DEG is not allowed in food and drugs. DEG affects the CNS, heart, respiratory system, liver, pancreas, and kidneys. Biochemical changes associated with toxicity include elevated hepatic enzyme levels, as well as increased serum BUN and creatinine levels. The clinical presentation is characterized by a nonspecific febrile prodromal illness, and within 2 weeks followed by anuric renal failure, pancreatitis, hepatitis, and neurologic dysfunction, progressing to coma. Histopathology of kidney tissue demonstrates acute tubular necrosis with regeneration and interstitial edema. DEG poisoning shows extensive tubular necrosis; however, the oxalate deposition is of EG poisoning, which causes renal and cerebral damage, and has not been observed in DEG poisoning. Similar to EG, DEG is not toxic but its metabolites are. Recently, an episode of DEG associated toxicity in cough syrup medication was reported from Panama, resulting in a mortality of more than 100 people.

6. Pet Food-Related Toxicity by Melamine and Cyanuric Acid

Since March 2007, several deaths were reported in dogs and cats associated with contamination of melamine and cyanuric acid in wheat gluten and rice protein (imported from China) used for manufacturing pet food in the USA. Melamine was identified in pet food and in the urine and kidneys of the cats that consumed the pet food. Analysis of the crystals revealed that they were composed of approximately 70% cyanuric acid and 30% melamine and were extremely insoluble. Significant clinical pathologic findings included azotemia and hyperphosphatemia, consistent with acute renal failure. In some cases, leukocytosis was observed. The majority of cases had oliguria or anuria and death was due to acute renal failure. No significant gross observations were noticed except in some cases which included gastric ulcer and green–yellow fluid with green crystals within the renal pelvis. Histopathological observations included degeneration and occasional necrosis of renal tubular epithelial cells with numerous crystals (Figure 38.3) evenly distributed throughout the cortex and medulla, within renal tubules and collecting ducts. Under polarized

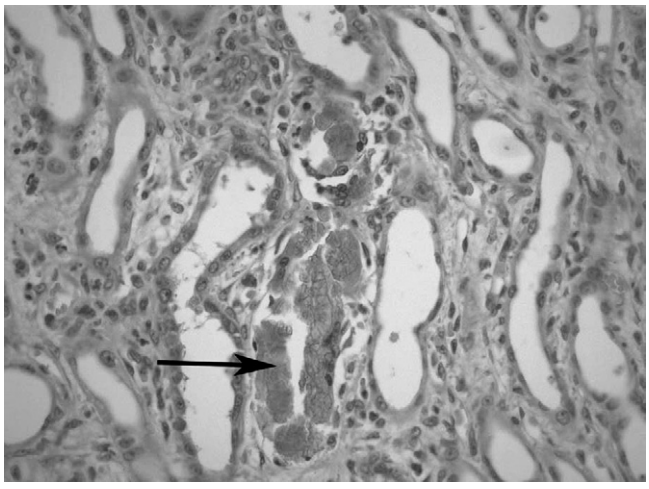

FIGURE 38.3. Melamine cyanuric acid poisoning, feline kidney; tubules are lined by degenerate, necrotic and flattened epithelium and contain crystalline material. Arrow shows crystalline material arranged in concentric pattern. H & E stain ×40.

light, these crystals showed birefringence. Many of them were round, pale brown, and appeared to have a rough surface as a result of smaller crystalline structures being arranged radially and more randomly within the entire birefringent crystal. In some areas, these crystal structures were arranged in concentric circles. The crystals were present within renal tubular epithelial cells and in the lumens of tubules, filling the lumen and at times distending the tubules. The melamine-containing crystals do not stain with Alizarin Red S or Von Kossa but do stain with Oil Red O. Calcium phosphate and calcium oxalate crystals do not stain with Oil Red O, which helps in the differential diagnosis of these crystals from crystals associated with EG toxicity (Thompson *et al.*, 2008).

7. Snake Bite

The majority of poisonous snakes in the USA are of the Crotalidae family, which includes rattle snakes, water moccasins, and copperheads. Crotalid snakes produce venoms which are mostly necrolytic and hemolyzing. Hemorrhagins in Crotalidae venom are toxic to the vessels and hence cause hemorrhage and edema at the wound site, in addition to systemic hemorrhage and shock. Marked anemia is observed due to hemolysis and extravasation. Disseminated intravascular coagulation is observed in some cases. The common initial clinical pathological changes include echinocytosis, thrombocytopenia, leukocytosis, and prolonged activated clotting time. Generally, edema and erythema, along with fang marks (Crotalidae family), may be observed at the site of bite, although it is difficult to identify due to the thick hair coat in animals. The major route of venom excretion is through the kidneys and hence kidney failure is observed in many cases of crotalid snake bites, characterized by renal tubular necrosis with protein casts, hemorrhage, and necrosis in the glomeruli (mesangiolysis). Mesangial proliferative glomerulonephritis is observed in patients who survive (Dickinson *et al.*, 1996; Sitprija and Chaiyabutr, 1999; Hacket *et al.*, 2002).

8. Raisins

Grape and raisin ingestion has been reported to cause renal toxicity in dogs. The specific mechanism of action is not known. Clinical and pathological findings include hypercalcemia, hyperphosphatemia, increased BUN, and serum creatinine concentrations, indicating renal damage.

Gross lesions are absent and microscopic findings include degeneration and necrosis of proximal renal tubular epithelium with intact basement membrane, regeneration of the tubular epithelium and mineralization. The tubules contain granular and protein casts. In some animals, fibrinous arteritis of the large colon was also observed (Eubig *et al.*, 2005; Morrow *et al.*, 2005).

IV. CONCLUDING REMARKS AND FUTURE DIRECTION

The primary function of the kidneys is to rid the body of waste materials that are either ingested or produced by metabolism, and to control the volume and composition of the body fluids. The toxins absorbed by the different routes are biotransformed and enter the blood. They are then eliminated through the urine, feces, and air. Since the kidneys receive approximately a quarter of the cardiac output, it is an important organ for the exposure of toxicants and their metabolites. Recent incidents of pet food toxicity and diethylene glycol toxicity indicate that contamination of animal and human food and drugs, respectively, can lead to severe mortality and morbidity as a result of renal toxicity.

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CHAPTER 39

Ocular Toxicity of Sulfur Mustard

MARION K. GORDON, ROBERT W. ENZENAUER, AND MICHAEL C. BABIN

I. INTRODUCTION

The eye is the most sensitive organ to sulfur mustard [bis (2-chloroethyl) sulfide; HD; SM – in this chapter we refer to sulfur mustard as SM]. Although the skin's response to this vesicant is blistering, the cornea experiences microbullae formation without fully fledged blistering. Corneal SM exposure causes injuries that range from mild conjunctivitis to advanced corneal disease. As a warfare agent, SM is extremely effective because even mild ocular exposure causes some visual incapacitation and a panic and fear of blindness whose psychological impact cannot be underestimated. As for clinical symptoms, extensive military data have documented that the effects of sulfur mustard vary widely according to the exposure time and concentration of the vesicant, as well as individual susceptibility (Derby, 1919, 1920; Ireland, 1926). SM used by the Germans during WWI caused thousands of cases of eye damage (Whiting *et al.*, 1940), and during the Iran–Iraq war (1985–1988), nearly 10% of SM gas casualties exhibited severe ocular damage (Safarinejad *et al.*, 2001; Ghassemi-Broumand *et al.*, 2004; Etezad-Razavi, 2006). Soldiers with mild to moderate injuries are somewhat visually disabled for approximately 10 days with conjunctivitis, photophobia, and perhaps corneal edema (Whiting *et al.*, 1940). Severe ocular SM exposure is often repaired much more slowly, requiring months to heal (Derby, 1919). In some reported cases healing never occurred and permanent visual impairment resulted. Also, a devastating discovery was the potential for a completely healed cornea to experience a recurrence of injury years after the initial exposure (Whiting *et al.*, 1940; Duke-Elder and MacFaul, 1972; Pleyer *et al.*, 1999; Vidan *et al.*, 2002; Ghassemi-Broumand *et al.*, 2004; Javadi *et al.*, 2005, 2007; Etezad-Razavi *et al.*, 2006). The reasons for recurrence are not yet understood. This chapter describes the consequences of SM exposure to the eyes, and what has been learned in the last 100 years about treatments for exposure.

II. BACKGROUND

A. Historical Perspective

World War I (WWI) heralded the use of military offensive chemical weapons as we know them today. Medical officer Major S.J.M. Auld, MC, was a witness to the first military gas attack early in WWI, and after his service on the front, he served as a member of the British Military Mission to the USA as part of the Liaison Effort between the defense organizations of the USA and her allies. While the war still raged, he reported on the effects of mustard gas in his textbook *Gas and Flame in Modern Warfare*. According to Dr Auld, the effects of mustard gas

> are not transitory and a man put out of action by mustard gas is going to be a casualty for several weeks and perhaps longer. Mustard gas principally affects the eyes and the lungs, but in a very strong vapor or in contact with any of the actual liquid from the shell a man's skin may be burned very severely – even through his clothes. More attention has been turned to this blistering effect of the gas than to anything else, but as a matter of fact the blistering is of secondary importance and in itself does not result in the loss of many men to the line … The chief effects of the mustard gas are on the eyes and lungs. The first thing you notice is the smell – which is slightly of garlic or mustard – and irritation of the nose and throat. Neither effect is enough to make you feel gassed, and the chief symptoms develop at a later time. When the gas is strong it is apt to cause sickness and sometimes actual vomiting. Later on, the eyes inflame and get very sore, the lids swell and blister, but no permanent injury to the eyes takes place, though the victim may be temporarily blinded. The effects developed in the lungs are equally painful and consist of severe inflammation and bronchitis, which may take some time to get better and if not well looked after may develop into pneumonia. (Auld, 1918)

Data collected later in WWI and throughout the rest of the 20th century indicate that, if Auld had seen more cases, he would have concluded that permanent injury to the eye is certainly possible. No one at the time he was reporting his observations could have foreseen the delayed recurrence of eye and skin injuries, or the downstream neoplastic consequences of exposure to SM. The clinical effects from mustard exposure are related to the concentration and duration of the mustard gas vapor (Derby, 1919; Sidell *et al.*, 1997). Since the vesicating gases persist for a long time, they are effective in extremely low concentrations, provided the victim remains in the contaminated area long enough. As mentioned, severe exposures often take many months to heal, and permanent damage causing blindness is a possible sequela.

B. Pathogenesis

Several components of the eye are affected by SM exposure: the outer structures are the cornea (transparent area over the pupil), the sclera (the white of the eye), and the limbal region, which joins the two. The limbus contains the corneal stem cells. In addition, there is the eyelid (i.e. the palpebra), with its outer skin layer facing the world and its inner skin positioned directly over the eye. The conjunctiva is the transparent moist mucous membrane that lines the inner surfaces of the eyelids and covers the sclera, but not the cornea. The portion of the conjunctiva that covers the rounded surface of the eye is also called the bulbar conjunctiva. The cornea is highly innervated, so that a speck of dust feels like a boulder in the eye. As shown in Figure 39.1, the cornea has three cell layers: the epithelium faces the exterior; the stroma contains the fibroblastic-like keratocytes; and the thin diameter, evenly spaced collagen fibrils. These fibrils are unidirectional in mat-like layers, and in each layer the fibrils are at about 90 degree angles to the fibrils in the layer above and the layer below (Coulombre and Coulombre, 1958); the innermost corneal cell layer is the endothelium, which faces the anterior chamber. The endothelium pumps water out of the cornea, keeping it condensed and relatively dehydrated, which is a necessity for transparency (Oyster, 1999). Swollen edematous corneas are cloudy. Mild to moderate SM exposures generally affect only the epithelial layer of the cornea, while severe ones may involve all three cell layers.

Ocular manifestations of SM exposure are generally separated into early signs and symptoms, and late signs and symptoms. Physicians and researchers use scoring systems for how severe the typical signs and symptoms are. One such scoring system for the cornea is reported in Table 39.1. It is also important to follow the progression of healing of the symptoms. Table 39.2 gives an example of animal data showing the temporal changes occurring with regard to a symptom that is both early and late, i.e. corneal thickening. A severe exposure to rabbit eyes, such as the one shown from this experiment, would likely cause months of vision impairment in humans. As seen in Table 39.2, the effect on the corneas is worst at 1 week after exposure, but even 16 weeks after exposure the cornea is still almost twice as thick as an untreated, healthy cornea. Clearly this is a severe exposure, where the endothelium has been affected, since the cornea is unable to regain its dehydrated, condensed transparent phenotype even after 4 months.

Ophthalmologists Sir Stewart Duke-Elder and P.A. MacFaul summarized the clinical course of ocular lesions as being divided into five stages: (1) a latent period, usually of 6–8 h, after minimal initial irritation; (2) a primary edematous reaction with swelling and cellular infiltration of the cornea; (3) clinical improvement after about a week with diminution of the edema; (4) progressive vascularization of the cornea, associated with a secondary edema and a recurrence of the symptoms – a phase which tended to quieten after several weeks; and (5) recurrent and persistent ulceration in the cornea after a latent period varying from 10 to 25 years (i.e. delayed mustard gas keratitis) (Duke-Elder and MacFaul, 1972). Mild exposures may only reach stage 1. Only a small percentage of severe exposures ever reach stage 5.

A 1919 clinical summation reported the first manifestations of a severe SM exposure as being pain and irritation to the eyes. Depending on the concentration of SM, this can

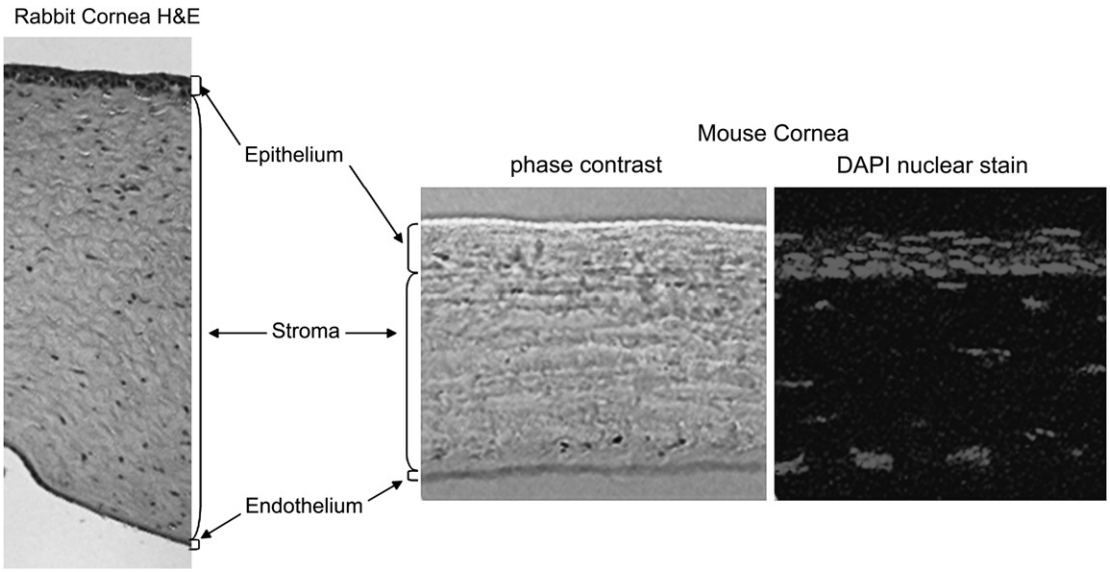

FIGURE 39.1. The three cell layers of the cornea. Shown on the left is a frozen section of an adult rabbit cornea stained with hematoxylin and eosin. On the right is a frozen section of a 6 week old mouse cornea. The mouse cornea is not fully mature until 8 weeks after birth. The left half visualizes the cornea by phase contrast microscopy. The right half shows the nuclei by DAPI staining. The density of the cells in the epithelial layer, and the paucity of keratocytes in the stromal layer are clearly seen with the nuclear stain.

TABLE 39.1. A typical scoring system for defining the degree of corneal stromal injury after SM exposure

Clinical parameter	Grade	Loss of corneal transparency	Assessment
Corneal stromal injury (opacity)	0	None	Not applicable
	1	Minimal	Slight loss in transparency
	2	Moderate	All anterior segment structures are easily discernible on examination
	3	Severe	Some anterior segment structures are not discernible on examination
	4	Diffuse	All anterior segment structures are not discernible on examination
	5	Diffuse	All anterior segment structures are not discernible on examination and perforation of the cornea has occurred

begin to be felt as early as 2–6 h after exposure. This progresses to photophobia, lacrimation, and blurred vision. The conjunctiva and eyelid become edematous, and the patient experiences blepharospasm (uncontrollable blinking) and pain (Derby, 1919). Anterior chamber inflammatory reaction is common. After several hours, the corneal epithelium begins to slough (Solberg *et al.*, 1997). All this would take the patient only through stage 2 of Duke-Elder and MacFaul's clinical course.

Most descriptions of the degree of ocular injury in humans characterize the phenotypes into three classes, each being a variant of mild, moderate, or severe. Such descriptions were developed in WWI and presented to the scientific community in an effort to understand the overall impact of mustard exposure. In March 1918, it was reported to the French Ophthalmological Society that there were three severities of SM injury, identifying them as: (1) benign, representing 10–15% of cases, with a duration of 10 to 15 days; (2) medium, about 80% of cases, with a duration of 5 to 6 weeks; and (3) severe, 3–5% of cases, where the victim experiences marked general symptoms, often developing a bronchopneumonia in addition to the ocular effects. George S. Derby, a Boston ophthalmologist, reported that this was not the experience of the British and American doctors. They saw a much, much larger percentage of mild cases, which recovered in 10–14 days (Derby, 1919). More than 20 years later, a discussion on gas injuries to the eye, held in January 1940 by the Section of Ophthalmology at the Royal Society of Medicine, agreed with the American and British views about the percentages of eye exposures that fit each class of severity. These discussants summarized the injuries from 1917 seen at the 83rd General Hospital in Boulogne, and believed the largest percentage (75%) of cases were mild. Recovery was rapid, and the soldiers were usually sent back to duty in 1–4 weeks. The moderately severe cases (15%) took about 4–6 weeks or longer to heal, with conjunctival chemosis (edema) subsiding fairly quickly. Those severely exposed developed corneal changes which resolved in longer periods of time. Of those, only a very small minority sustained permanent impairment or total loss of vision (Whiting *et al.*, 1940).

Scoring systems have elucidated the general patterns of pathogenesis for each classification of exposure. With mild exposures, the patients recover within a few days, with minimal lid erythema, moderate conjunctival changes, and minimal corneal involvement (Solberg *et al.*, 1997). A moderate exposure may first feel like a dry sensation with photophobia and severe eye pain. Chemosis and congestion of the conjunctiva are observed, as well as corneal epithelial edema and punctuate erosions. The corneal epithelium usually is repaired in 4–5 days, while the entire area takes 6 weeks or longer to heal (Solberg *et al.*, 1997). The symptoms of a severe exposure go beyond those of a moderate exposure by including blepharospasm, reduced vision, and severe pain. The limbal vasculature can be affected. Eyelids become red and swollen; there is significant chemosis and conjunctival congestion, with areas of ischemia and necrosis. There may also be iridocyclitis. The irregularities in the cornea give it an orange peel appearance. Erosions may manifest, and the epithelium may slough. Epithelial defects are prone to infection, especially to *Pseudomonas aeruginosa*. The conjunctival epithelium also necroses, with edema of the subconjunctival tissues, hemorrhaging, and leukocyte infiltration. The depth of the

TABLE 39.2. Relative index values for corneal thickness measured by ultrasonic pachymetry over time[a]

Group	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 16
HD positive control	1.0	2.4	2.3	2.3	2.3	2.2	1.9	1.8

[a]Data summarized from Figure 1 in Babin *et al.* (2004)
All corneal thicknesses are normalized relative values compared to the week 0 corneal thickness, which was assigned the thickness value of 1

necrosis in the conjunctival epithelium is much less than that in the eyelid epidermis. This difference is because the hair follicles of the eyelid can collect the SM, while the conjunctiva is a moist mucosal tissue with a protective tear film. Overall, healing after a severe exposure is often suboptimal, with vascularization and corneal cicatrization (healing with scar formation) and visual impairment (Derby, 1919; Warthin and Weller, 1919; Solberg *et al.*, 1997; Javadi *et al.*, 2005, 2007).

The pattern of injury in the human eye is analogous to that observed in animal studies. In severe SM injury, there is often full thickness corneal lesions (i.e. involving the stroma and the endothelium as well as the epithelium), and iritis, which starts to improve within 1–2 weeks. There can also be avascular necrosis in the limbus, which may diminish the number of stem cells available for repairing the corneal epithelium. A few weeks later, neovascular elements can arise from the healthy limbus and invade the corneal stroma (Petrali *et al.*, 2000; Babin *et al.*, 2004; Amir *et al.*, 2000). White opacities may occur as the vessels degenerate (Borak and Sidell, 1992). English-born ophthalmologist Dr Ida Mann found that in rabbits, about 8 weeks after a severe exposure to SM, the vessels of neovascularized corneas also regressed, leaving behind cholesterol crystals deposits. As the cholesterol worked its way to the corneal surface it caused ulcerations, leaving pits in the cornea. Mann believed that surfacing of cholesterol was the basis of delayed keratitis (Mann and Pullinger, 1942a). Mann wrote extensively on delayed keratitis, suggesting that, in her opinion, it should be called delayed ulceration (Mann and Pullinger, 1942a; Mann, 1944).

Because of the good correlation of pathology between human and animal eye exposures, animal studies offer great hope for identifying countermeasures to sulfur mustard, and for furthering the arsenal of drugs used to combat the effects of exposure.

C. Delayed Effects

Severe corneal wounds may resolve after several months, or may remain a chronic problem for years. In a small percentage (0.5%) of severely injured patients, delayed ulcerative keratopathy develops decades after SM exposure (Heckford, 1937; DeCourcy, 1943; Atkinson, 1947; Blodi, 1953, 1971; Duke-Elder and MacFaul, 1972; Dahl *et al.*, 1985; Solberg *et al.*, 1997; Javadi *et al.*, 2005, 2007). Delayed keratopathy is one of the more perplexing late sequelae of sulfur mustard ocular injury because casualties, sent home from the hospital as ''cured'' through months of treatment, became symptomatic again 25 or more years later. Photophobia, tearing, and visual deterioration were often the start of this delayed onset effect, followed by pain. Vascularization and corneal ulcerations sometimes occurred, and crystalline deposits appeared (Solberg *et al.*, 1997; Javadi *et al.*, 2005). Recurrence and remission of this phenomenon occur unpredictably. The pathogenesis is not yet understood. Mann's explanation of surfacing cholesterol may be the cause. An alternative explanation may come from the fact that some of the Iranian soldiers who experienced this delayed phenomenon showed a mixed inflammatory response without specific features when their conjunctival and corneal tissues were taken for analysis (Javadi *et al.*, 2005).

III. OCULAR TOXICITY AND TOXICOKINETICS

A. Toxicity

The toxicity associated with SM is quite profound. The Army's Chemical Defense Equipment Process Action Team estimated in 1994 that a 900 mg-min/m^3 SM vapor exposure would be lethal in 2–10 min, based on animal studies (National Research Council Review, 1997). Fortunately, in the battlefield, lethality has been limited. Only 1–3% of exposed soldiers died from SM exposure after WWI, and mortality mostly was not a direct consequence of SM, but rather the indirect effect of secondary respiratory infections. The 1999 Material Safety Data Sheet, put out by the US Army Soldier and Biological Chemical Command, USA Edgewood Chemical Biological Center, has estimated the LD_{50} of a skin exposure to sulfur mustard as 100 mg/kg. This roughly translates into as little as 7 ml of neat SM (i.e. 8.9 g) spread over the skin resulting in the death of a 80 kg adult (Department of the Army, MSDS, 1999). The cornea, of course, is more sensitive than the skin. Below we review three chief toxic effects of severe SM exposure to the cornea.

1. Corneal Opacity

An increase in hydration, separation in the collagen fibril lattice, disarrangement of the collagen fibrils, vascularization, and surface irregularities all reduce corneal transparency (Slatter, 1990; Oyster, 1999). Most, if not all, of these factors are generally present with a clinically significant challenge of SM, causing opacity. Scoring systems similar to that in Table 39.1 have been devised to quantitate the degree of opacity secondary to corneal injury (for example, see Conquet *et al.*, 1977). By using measurements such as those shown in Table 39.1 to grade SM injury in animal studies, the effect of countermeasures against the vesicant can be evaluated and compared over extended time periods.

2. Neovascularization

Neovascularization (NV) is the formation of new blood vessels from existing vessels. Figure 39.2 shows neovascularization that has developed in a rabbit cornea after a mustard exposure. Normally the cornea is avascular, and thus, to become vascularized, vessels in the periphery (the limbus and beyond) must respond to signals from the

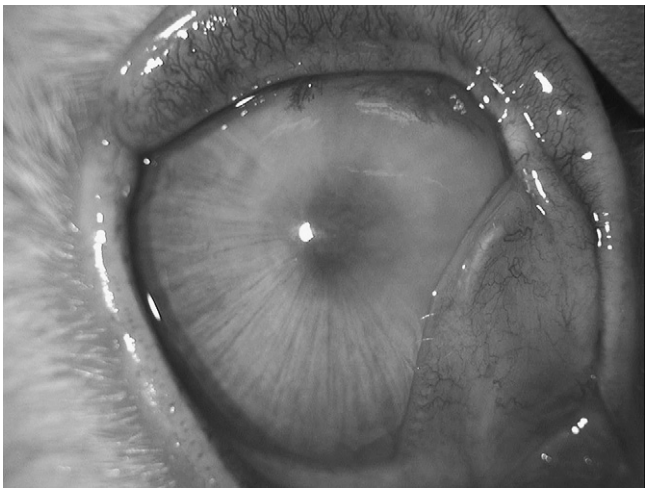

FIGURE 39.2. Typical presentation of neovascularization occurring after a neat liquid SM challenge. The NV was first noticed a couple weeks after challenge and continued to progress over time. Note the severe corneal scarring and adnexial involvement. (Courtesy of Dr John Schlager, unpublished data)

cornea. To avoid vessel ingrowth, the uninjured cornea maintains its avascularity with a carefully regulated balance of angiogenic factors and anti-angiogenic factors. The presence of angiogenic factors is essential, since these have multiple functions and are used for nonangiogenic purposes by corneal cells. Thus, their levels of activity are strictly regulated so that angiogenesis is not a favored process. However, many of the angiogenic factors (i.e. vascular endothelial growth factor, fibroblast growth factor, matrix metalloproteinases) become dysregulated or deregulated when an injured cornea mounts an inflammatory response (Chang *et al.*, 2001). NV is a very serious consequence of corneal injury and a particularly devastating sequela in moderate to severe SM exposure. The prevention, control, and elimination of NV has been, and still is, a very active area of ocular research (Dorrell *et al.*, 2007a, b; Kvanta, 2006; Lee *et al.*, 1998). As a major complication of sulfur mustard injury, NV may promote corneal scaring, edema, lipid deposition, and inflammation. Besides the obvious effect of loss of visual acuity, NV can also increase the risk of corneal graft failure (Chang *et al.*, 2001). A number of scoring systems, ranging from simple observations to elaborate image analysis techniques, have been used to quantitate the amount of corneal neovascularization present after injury (Conrad *et al.*, 1994; Proia *et al.*, 1988; Klotz *et al.*, 2000; Galardy *et al.*, 1994; Miyamoto *et al.*, 1999; Yamada *et al.*, 1999; Babin *et al.*, 2004).

3. Corneal Perforation

One of the worst consequences of SM exposure is corneal perforation. This effect can occur many weeks after exposure, and animal data are beginning to suggest that treatments for SM injury may, in themselves, contribute to toxicity. In an ongoing study, rabbits exposed to the same damaging dose of SM, then treated with prednisolone acetate over the course of 1 month with a decreasing steroid dose administration each week, had revealed no corneal perforations (John Schlager and Michael Babin, unpublished data). These data are discussed further in the treatment section below, and they strongly suggest that the toxicity and response sensitivity of any treatment must be considered in addition to the toxicity of the mustard when prescribing therapeutics for patients.

B. Human Exposure Levels Affecting the Eye

The eye, being the most sensitive organ to SM, shows irritation at concentrations ten times lower than that needed to irritate the airways (Smith and Dunn, 1991). An early experiment to show the level of toxicity of SM to the eyes was conducted on humans in 1919. Using a 10,000 liter exposure chamber, C.I. Reed of the Medical Division of the Chemical Warfare Service, United States Army, exposed individuals to exact concentrations of SM to determine what would be the smallest exposure amount that would cause ocular symptoms. To calculate what that dose might be, Reed used all the data available at the time. A lethal dose for animals was exposure to approximately 8 μg/l for 8 h. The skin of a human exposed to 2.5–5 μg/l for 0.5–1 h (i.e. 75–300 μg-min/l) showed cutaneous erythema several hours after exposure. The dose for the eyes had to be lower since it had been observed on the battlefield and in laboratory animal experiments that the eyes were more sensitive than the skin. From dog eye exposures, a 2 h challenge with 1 μg/l caused definite conjunctivitis many hours later, but a 1 h exposure did not (i.e. a 120 μg-min/l caused symptoms vs a 60 μg-min/l exposure that did not). Putting together these pieces of information, Reed reasoned that, from the available data, a 1 μg/l SM exposure for 1–2 h (60–120 μg-min/l) should not seriously affect a human, even though 8–10 h of this exposure would likely be incapacitating. Therefore, using himself as the first test subject, he exposed himself to a concentration he believed would cause only slight effects: he aspirated 12 mg SM into the 10,000 liter chamber and exposed himself for 45 min (1.2 μg/l for 45 min = 54 μg-min/l). Twelve hours after exposure Reed experienced severe blepharospasm and photophobia, as well as pronounced tearing. The severe conjuctival injection that developed persisted for 6 days, then lessened, but was still visible after a month. Overall, Reed was very surprised to find that the human eye was so much more sensitive than the dog eye. His eyes took 3 months to heal fully. In addition, Reed experienced unexpected cutaneous effects. These manifested 2 days after exposure and led to exfoliation of the skin from his trunk and face. His nasopharynx mucous membranes were also considerably exfoliated. Fortunately, he experienced no pulmonary symptoms. Reed reported that for 2 weeks he endured severe pain and tenderness around the eyes, nose, and mouth and his trunk was excruciatingly itchy.

Still on track to define the minimum concentration of SM that affects the eyes, Reed identified 13 human subjects whose skin sensitivity had already been tested and quantitated. These subjects were outfitted with a respirator canister, a nose clip, and a goggle so that the ungoggled eye would be exposed to 1.1 μg/l SM for times from 10 to 25 min (i.e. 11–27.5 μg-min/l), and the goggled eye would serve as a control. While doing these exposure experiments, he also aspirated air samples from the chamber to measure exactly the SM concentration in the air within, and found that it was about one-half (i.e. 0.48–0.58 μg/l) of what was injected into the chamber. After exposure, eight of the 13 subjects developed conjunctivitis with varying degrees of other subjective eye symptoms. Three of these eight suffered from diffuse skin erythema and itching for several days post-exposure. Reed concluded from this work that SM at 0.5 μg/l of air in less than 1 h (in actuality 10–25 min) would cause the first clinical symptoms of mild conjunctivitis. Unfortunately for Reed, he experienced recurrences of the itchy skin and tenderness around the eyes at various intervals over the course of the following year (Reed, 1920).

Battlefield air concentrations of mustard vapor in World War I were estimated to be in the range 19–33 mg/m^3 (i.e. 19–33 μg/l), and soldiers were exposed for various lengths of time (Ashkenazi *et al.*, 1991; Thorpe and Whiteley, 1939). At these concentrations, unshielded eyes exposed for only a few minutes would incur irreversible corneal damage. Exposure levels causing injury have been graded as outlined below (Ashkenazi *et al.*, 1991; Solberg *et al.*, 1997; Dahl *et al.*, 1985).

Mild (12–70 mg-min/m^3. This equals 12–70 μg-min/l – compare this with the 54 μg-min/l Reed used on himself, and the 11–27.5 μg-min/l used on his test subjects.)
Moderate (100–200 mg-min/m^3)
Severe (>200 mg-min/m^3)

The lethal concentration for humans, estimated from animal data, is considered to be 1,800–9,000 mg-min/m^3, i.e. 900 mg/m^3 for 2–10 min, as mentioned earlier (National Research Council Review, 1997). The LD_{50} is 100 mg/kg and the LCt_{50} is 1,500 mg-min/m^3 (LCt_{50} is the concentration multiplied by minutes of exposure that is lethal to 50% of those exposed) (Sidell *et al.*, 1997).

The toxicity of both neat and vapor exposures of SM to the eye is highly predictable. After a time period of no symptoms, lacrimation begins and quickly progresses to conjunctivitis, pain, blepharospasm, and photophobia. Corneal edema follows rapidly as a result of the loss of the epithelium, which allows water to enter the stroma from the precorneal tear film (Slatter, 1990). By 24 h after exposure, it is not unusual to see a 100 to 300% increase in corneal thickness evident by a substantial increase in opacification. Fluorescein staining at 24 h will reveal ulcers that may cover a majority of the corneal surface. By 48 h, evidence of healing is seen, and by 96 h, it is not unusual to have minimal staining. Fluorescein stainings of SM exposed rabbit corneas are shown in Figure 39.3.

C. Animal Toxicity Studies

The use of animals in experimental SM research dates back to the late 1800s. Theodor Leber, considered the founder of ophthalmic research (he also identified a congenital amaurosis that bears his name), exposed a single rabbit to a pure substance, SM, that his colleague, Victor Meyer, had prepared. The result was development of conjunctival lesions (Leber, 1891). In 1926, reports appeared describing animals exposed to liquid and vapor forms of SM, which were followed for up to 7 weeks to document the course of the resultant injury. Along with the gross visual documentation of the lesions, the histopathologies were also reported (Ireland, 1926).

Today, the overwhelming majority of animal ocular toxicity studies are performed in the rabbit model, and the study of SM is no exception. New Zealand white rabbits have been used extensively with both liquid SM and vapor exposures (Amir *et al.*, 2000, 2003; Bossone *et al.*, 2002; Vidan *et al.*, 2002; Babin *et al.*, 2004). Other animal models have been employed, including those using bovine and rat corneas. Many articles appear on these in the *Bulletin of Johns Hopkins Hospital*, Vol. 82, 1948. Individual articles from this volume are cited in the mechanism of action section.

The distribution kinetics of SM in the NZW rabbit eye have been determined using labeled SM, and show that the cornea has a greater affinity for the vesicant than other parts of the eye. Following the application of a 0.5 mg dose of liquid ^{14}C-SM, eyes were enucleated at 1, 6, and 24 h. This dose causes severe injury. After 1 h, only about 2% of the radioactive compound was recovered in ocular tissues. The cornea was found to contain the highest concentration of radioactivity, followed by the tarsal section of the eyelid, the aqueous humor, the nictitating membrane and the frontal sclera, including the conjunctiva (Amir *et al.*, 2003). This study correlated nicely with one published in 1946 that employed ^{35}S-labeled sulfur mustard in a 5 min vapor exposure. Enucleation after the challenge showed the highest concentration of label in the cornea, with lesser amounts in the iris, lens, and conjunctiva. If enucleation was delayed for 7 days after exposure, the SM was still found to be located primarily in the cornea (Axelrod and Hamilton, 1947).

Once the toxic effects of SM have resulted in epithelial loss, the progression of corneal edema is rapid and persistent for weeks. As noted in Table 39.2, 1 week after exposure to a 0.8 μl (1 mg) challenge, the average thickness of the rabbit corneas increased almost 2.5 times and remained at more than twice the thickness of the control contralateral naïve eye for more than 5 weeks. By 6 weeks, although not as thick, most of the exposed corneas were still above the baseline thickness values of a typical cornea (Babin *et al.*, 2004)

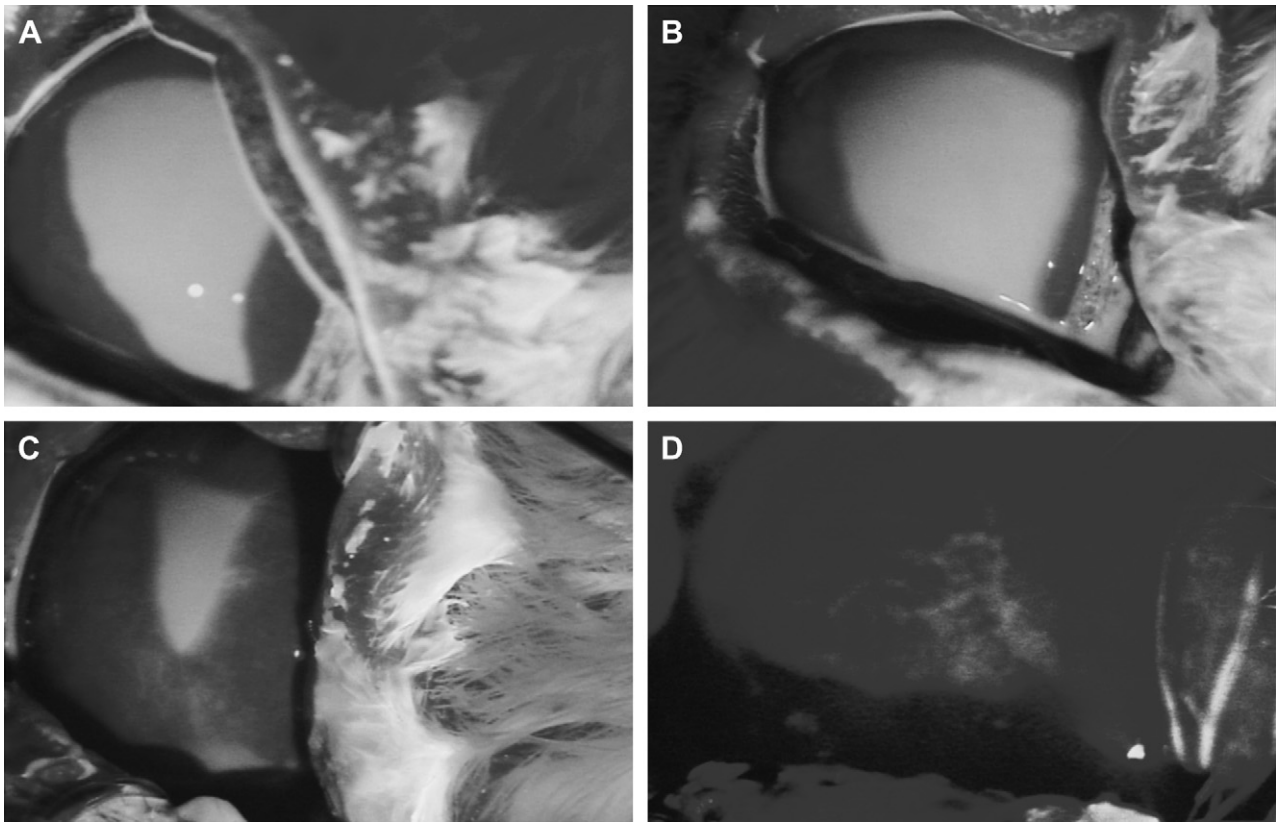

FIGURE 39.3. Fluorescein staining of rabbit cornea after sulfur mustard injury showing the extent of injury. Panel A, early damage, 24 h after challenge. The remaining panels show the progression of re-epithelialization over time: Panels B, C, and D: 48 h, 72 h, and 96 h, respectively, after SM exposure. The fluorescein penetrates into the underlying corneal stoma staining it a bright green color, which looks white or light gray in the figure.

suggesting the endothelial layer was still unable to pump water properly from the stroma. Data on neovascularization of the cornea, as well as on the long-term clinical response to SM, demonstrate that both animals and humans have a degree of individual variability in their response to SM (Kadar *et al.*, 2001; Amir *et al.*, 2004; Babin *et al.*, 2005). Reed may have suffered so badly after his self-exposure to SM because of individual variability. He may have been more sensitive to SM's effects than the majority of people.

Electron microscopy has been used to examine the toxic effects of SM exposure, viewing the cells at high magnifications. By 24 h after exposure to a 0.4 µl (0.5 mg) dose of neat SM, rabbit corneal epithelial cells showed progressive degenerative changes, including pyknosis, necrosis, and the sloughing of the epithelium. The cornea epithelium consists of 5–7 layers of cells. Sloughing occurred to cells in the superficial layers of the epithelium, as well as to the basal cells just above the basement membrane. The pathogenesis of the detachment was the result of degenerative acantholysis (i.e. epithelial dissociation) of the basal cells and the disruption of basal cell hemidesmosomes and anchoring filaments (discussed later in the mechanism of action section). Edema with the presence of inflammatory cells, mainly neutrophils and mast cells, were consistently found 24 h after exposure with this dose (Petrali *et al.*, 1997, 2000).

Toxicity studies using SM vapor have been performed on multiple animal species and documented as early as 1926 (Ireland, 1926). Unlike liquid SM exposures, vapor challenges allow the researcher greater control for restricting the mustard injury to the cornea with minimal or no damage to the eyelid and conjunctival tissues. By adjusting the size of the delivery apparatus, the entire structure of the eye can also be exposed. For example, glass goggles have been used to deliver SM vapor to the eyes of unanesthetized rabbits in a controlled environment (Amir *et al.*, 2000). Both 2 and 4 min exposures to 370 and 420 µg/l of SM have been used to produce the severe clinical lesions seen with 0.5–1 mg doses of liquid agent (Kadar *et al.*, 2001, 2005).

Because of the highly toxic and reactive nature of the vesicant, the dosing of SM in research animals can be problematic. The application technique, the dose of SM delivered, and the contact time will greatly influence the severity of the resulting lesion and affect toxicity calculations. Multiple methods for administering the minute quantities of liquid SM needed for challenge have been used. In 1926 a method for using a ''fine pipette'' to deliver ''uniform minute droplets'' of 0.5 mg SM to the cornea of

rabbits was described in the *Medical Aspects of Gas Warfare* (Ireland, 1926). In 1940 and 1942, Ida Mann and B.D. Pullinger published descriptions of a technique in which a 0.25 mm rounded-tip glass rod was successfully used to deliver multiple spots of SM to the central cornea and other regions of the rabbit eye (Mann and Pullinger, 1940, 1942b). More recently, a Gilson ''Pipetman'' P-2 pipetter (Rainin Instrument Company, Inc.) has been used to deliver a 1 mg dose to NZW rabbits (Babin *et al.*, 2004). Currently, a 10 µl Hamilton Microliter syringe attached to a Hamilton PB600 Repeating Dispenser is being tested for delivery of a 0.4 µl neat SM dose to rabbit corneas (Michael Babin, unpublished results). Any device selected to deliver liquid SM to the cornea should be adequately characterized. The precision and accuracy of the device should be determined prior to its use, and these must be within acceptable standards if one is to properly assess the toxicity of a delivered dose of SM.

IV. MECHANISM OF ACTION

As part of the world war II (WWII) war effort, investigators at the Wilmer Institute in Baltimore, including Jonas Friedenwald, Alan C. Woods, Alfred Maumenee, Roy Scholz, Heinz Herrmann, Fay Hickman, Albert Snell, Jr., Wilhelm Buschke, William F. Hughes, and others, attempted to go beyond clinical and histopathology studies of SM exposure toward understanding the mechanisms by which SM caused corneal damage. In putting together Volume 82 of the *Bulletin of the Johns Hopkins Hospital* (1948), Friedenwald concluded from their research that

> the clinically and histopathologically recognizable changes which follow chemical damage of the tissue are only remotely and indirectly connected with the initial chemical injury … the clinical and histologic reactions disclosed by routine study are, in the main, the consequences of cellular death. Once the cells are dead, the reactions have a monotonous uniformity.

Friedenwald ultimately expressed some disappointment that, after years of effort put in by the team, so much was still unknown by the late 1940s. However, several facts had been elaborated and published in the volume: threshold doses of mustard cause a transitory inhibition of mitotic activity of the corneal epithelium, and this appeared to be the most sensitive index of threshold effects. The healing of small wounds by migratory action of the cells was not disturbed during the period of mitotic inhibition (Friedenwald *et al.*, 1948a). The electrical resistance of the cornea is not affected for several hours after exposure (Friedenwald *et al.*, 1948b). Moderate doses caused epithelial nuclear fragmentation and nuclear fragility (Maumenee and Scholz, 1948; Friedenwald and Buschke, 1948). Nuclear fragmentation, however, was not the mode of death for the stromal cells. Instead, death occurred as a consequence of the keratocytes swelling and bursting. Following massive exposures, all types of corneal cells underwent pycnosis (Friedenwald, 1948). The loosening of the corneal epithelium from the stroma was also investigated (Maumenee and Scholz, 1948; Herrmann and Hickman, 1948), and indicated that the cohesion between the two layers appeared to result from the surface having protein-lipoid characteristics, disruptable by proteases such as trypsin, or the aliphatic butyl and amyl alcohols. It was concluded that the loosening between the cell layers was similar to vesication of the skin. It was also deduced that loosening involved an oxidative step because the epithelium did not separate from the stroma when cultured corneas were incubated under anaerobic conditions (Herrmann and Hickman, 1948). Immune reactions against corneal proteins were postulated to be responsible for the long-term damage observed (Maumenee and Scholz, 1948). By the end of WWII, other groups had also confirmed that SM targeted the basal epithelial cell nuclei (Gilman and Phillips, 1946; Biesele *et al.*, 1950; Mehtab, 1953).

SM gas is lipophilic, and has affinity for the lipid layer of the tear film. Because it is also electrophilic it is likely that it easily partitions into the aqueous and mucin-rich layers of the tear film to access the corneal epithelium. Thus, it is expected that the moist nature of the ocular surface is the reason why the eye is the most sensitive organ to the vesicant (Hughes, 1942; Dahl *et al.*, 1985; Solberg *et al.*, 1997; Safarinejad *et al.*, 2001). The moistness of the cornea is both good and bad: on the one hand it helps wash away the agent, but on the other hand, the layers of the tear film probably facilitate concentration of the agent at the corneal surface. Once there, sulfur mustard penetrates the cornea readily. The half-life of mustard in the cornea was found to be 3 min at 38°C and 13 min at 24°C (Kinsey and Grant, 1946).

Unfortunately, the exact mechanisms of action of SM still remain elusive. In the last 30 years, most of the data concerning the mechanism of action of sulfur mustard are derived from nonocular studies, but these are still applicable to the eye. Sulfur mustard combines with proteins, RNA, DNA, and components of the cell membrane. The modifications of DNA result in strand breaks and interstrand crosslinks (Roberts *et al.*, 1968). One possible mechanism for how the vesicant alters proliferation, transcription, and translation can be deduced from sodium chloride extracts of SM-exposed lymphocytes, where it has been found that some of the histones that package the DNA were soluble. This was elucidated by finding that the extracts, 4–24 h after SM exposure, contained three proteins that were significantly increased in amounts compared to control extracts. One protein had sustained alterations. Sulfhydryl reducing agents did not attenuate the increased levels of these proteins, nor did preincubation of the cells with cycloheximide (to inhibit protein synthesis). Microsequencing identified the altered protein as a cleavage product of histone H2B. The others were identified as intact histone H2B and histone H3. Thus, SM may disrupt the nucleosome structure by making the core

histones abnormally disengage from, or be proteolytically cleaved from, the nucleosomes (Millard *et al.*, 1994).

The highly reactive alkylating properties of SM contribute significantly to toxicity, and alkylation suggests mechanisms of action for SM that align with the ultimate pathology of SM exposure. One hypothesis for how SM acts involves poly (ADP-ribose) polymerase (PADPRP). The proposed mechanism of action starts with irreversible alkylation of purines (adenine and guanine), which causes depurination of the DNA, leading to cleavage by apurinic endonucleases. These DNA breaks activate the enzyme PADPRP, which requires nicotinamide adenine dinucleotide (NAD^+) as a substrate. Thus, the cellular levels of NAD^+ are lowered. The consequence of reducing NAD^+ levels is inhibition of glycolysis, which causes glucose-6-phosphate to accumulate. In response, the $NADP^+$-dependent hexose monophosphate shunt pathway is stimulated. This pathway induces the secretion of proteases that lead to cell pathology (Dixon and Needham, 1946; Papirmeister *et al.*, 1984, 1985; Gross *et al.*, 1985; Meier *et al.*, 1987; Mol *et al.*, 1989; Smith *et al.*, 1990; Petrali *et al.*, 1990; Yourick *et al.*, 1991; Rosenthal *et al.*, 2000). Some data have indicated that the decrease in NAD^+ leads to cell death (Meier *et al.*, 1987), although this idea has been disputed (Mol *et al.*, 1991).

Friedenwald found extracellular glutathione (GSH), a free radical scavenger, is depleted in response to SM exposure (Friedenwald *et al.*, 1948b). More recent data have generated another major hypothesis, derived from the post-1948 observation that mustard also reacts with intracellular GSH, thereby depleting it. A consequence of this rapid inactivation of sulfhydryl groups is loss of protection against oxygen-derived free radicals, specifically those causing lipid peroxidation. Through some intermediate enzymatic effects, intracellular calcium levels increase, causing cell death through the activation of proteases and phospholipases that break down membranes and the cytoskeleton, as well as activation of endonucleases that can degrade DNA (Orrenius and Nicotera, 1987; Miccadei *et al.*, 1988).

The testing of a similar hypothesis further delineates how the reduction of glutathione levels may lead to calcium mediated toxicity. With a Ca^{2+}-specific fluorescent probe, intracellular levels of calcium were found to increase, and were significantly elevated at 6 h after SM exposure. High levels of intracellular calcium enhance phospholipase A2 activity. The activity of this enzyme in response to SM was elucidated by following the increasing levels of arachidonic acid (AA). The use of AA levels as a measure of phospholipase A2 activity was justified by the fact that inhibitors of phospholipase A2 prevented release of AA from the cells. Thus, it was found that after SM exposure AA levels being released from the cell membrane were increased, implicating activation of phospholipase A2 (Orrenius and Nicotera, 1987; Elsayed *et al.*, 1989; Ray *et al.*, 1995). Released AA would decrease membrane fluidity, cause membrane damage, and result in cell death (Ray *et al.*, 1995). Glutathione consumption after SM exposure may also induce free radical-mediated oxidative stress (Elsayed *et al.*, 1992). Free radical formation resulting from inhibition of respiratory enzymes has also been hypothesized as a mechanism of SM-induced injury (Aasted *et al.*, 1987).

While alkylation of DNA is likely to play a key role in SM toxicity, one cannot underestimate the potential problems resulting from concomitant alkylation of the amino and carboxyl groups of proteins. SM affects the basal epithelial cell-specific intermediate filaments, an important component of the basal cell's cytoskeletal structure. Epidermal keratinocytes in culture treated for 5 min with 400 μM SM showed a 30% decrease in their K14 intermediate filaments after 1 h, and a 79% decrease in K14 after 2 h. K5 was decreased about 29% after 1 h, but not further unless the amount of SM was increased. It was suggested that the decrease in levels of K14 may disrupt the stability or assembly of the K14 intermediate filament polymers, thus disrupting the integrity of the cytoskeletal connection to the hemidesmosomes, which in turn tack the epithelial cells to their basement membrane (Werrlein and Madren-Whalley, 2000).

The proteins most obviously adversely affected are, indeed, those involved in the strong adhesion at the border between the epithelial and stromal layers, i.e. the basement membrane zone (Smith *et al.*, 1998), and it is unknown why this is so. The loss of the epithelial–stromal adhesion is dramatic, initiating blistering of the skin. The cornea does not blister. Although the microbullae that form between the corneal epithelium and stroma in response to SM exposure seem less dramatic, they clearly impair corneal integrity. Figure 39.4 shows some of the

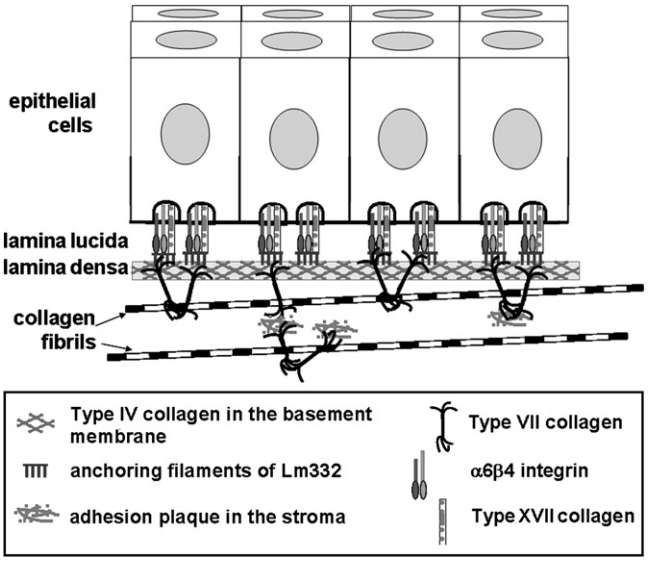

FIGURE 39.4. The epithelial–stromal border and molecular components of the anchoring complex. For simplicity, the plectin and BP230 components of the hemisdesmosome have been omitted from the diagram. Transmembranous collagen XVII and α6β4 integrin are localized to the hemidesmosome (the half circle at the base of the epithelial cells), with their extracellular domains reaching into the lamina lucida. These bind with laminin 332, which in turn binds with type VII collagen in the stroma.

components involved in the ''anchoring complex'', the structure which ''rivets'' the basal epithelial layer to the uppermost region of the stroma, i.e. Bowman's layer.

This structure is also sometimes referred to as the adhesion complex, or the attachment complex. It is composed of three multicomponent units. One unit is the electron-dense hemidesmosome. The hemidesmosome is indirectly connected to the nuclear membrane via the previously mentioned K5/K14 intermediate filaments. The hemidesmosome is comprised of plectin, BP230, and two transmembranous molecules: type XVII collagen and α6β4 integrin (Gipson *et al.*, 1989; Gerecke *et al.*, 1994). The transmembranous molecules attach to the basement membrane, and thus the basement membrane is indirectly connected to the nucleus through the hemidesmosomes and the K5/K14 intermediate filament cytoskeleton. The hemidesmosomal integrin subunits α6 and β4 were examined after SM exposure of skin, and showed a 25% decrease in the α6 integrin subunit and a 18.9% decrease in the β4 subunit (Zhang and Montiero-Riviere, 1997). The identity between hemidesmosomal components in skin and cornea suggests that, in cornea too, integrin uncoupling is another mechanism of action by which SM damages the epithelial–stromal junction.

The other two units of the anchoring complex are on the extracellular side of the basal epithelial cell surface. These are the anchoring filaments and the anchoring fibrils. The anchoring filaments are composed of laminin 332 (formerly Lm 5), and they bind to the transmembranous hemidesmosomal components, collagen XVII, and α6β4 integrin on one end, and the anchoring fibrils on the other. The anchoring fibrils are composed of type VII collagen, and are situated in the region at the base of the basement membrane, extending into the stroma. The adhesion complex is analogous to ''spot welds'' in the epithelial basement membrane, which, in regions outside of the anchoring complex, is composed of type IV collagen, laminins (other than laminin 332), and heparin sulfate proteoglycans. The interfacial matrix attachment between the basement membrane and the stroma is weak without the anchoring complexes. Thus, the complexes are necessary for corneal integrity, and a reduction in any of the components involved cannot help but compromise the integrity of adhesion between the epithelial and stromal cell layers. The SM-induced split between layers occurs above the level of type IV collagen in the basement membrane (Mol *et al.*, 1991; Petrali *et al.*, 1992). Shown in Figure 39.5 are electron micrographs of corneas, untreated or treated with half mustard (i.e. CEES).

Comparing the panels in Figure 39.5, it can be seen that the last connections to break between the epithelium and the stroma are those of the adhesion complex. The half mustard, being not as strong as SM, causes separations between the epithelial and stroma layers in between the anchoring complexes, but is not strong enough in the conditions used to disrupt the anchoring complexes themselves.

Experimental evidence suggests that, after SM exposure, alkylation and crosslinking of matrix molecules at the epidermal–dermal junction could adversely affect the ability of the epithelium to tack down once again to the

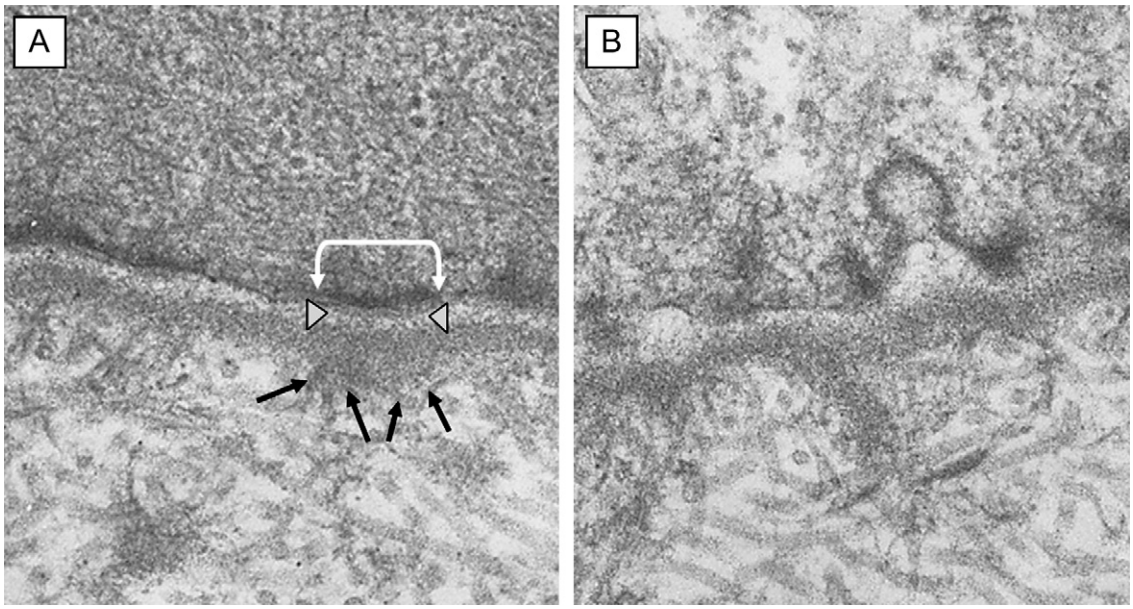

FIGURE 39.5. Electron micrographs of the rabbit corneal epithelial–stromal border. Panel A, the three structural components of the anchoring complex are shown. The hemidesmosomes are the flattened electron-dense plaques at the epithelial surface. One is indicated by an inverted white ''U'' shape with arrow ends. The anchoring filaments appear as threads in the laminina lucida. The anchoring filaments under the indicated hemidesmosome are flanked by arrowheads. A selection of the anchoring fibrils in the stroma is indicated by black arrows. Panel B, a micrograph of the epithelial–stromal junction 24 h after a 2 h exposure to 200 nmole CEES. Local separations between the epithelial and stromal layers are seen between the anchoring complexes.

dermal layer. Due to the conservation between components of the epidermal–dermal junction and the corneal epithelial–stromal junction, results from the skin apply to the cornea. *In vitro*, ^{14}C-labeled SM has been shown to alkylate laminin 111 (formerly known as Ae.B1e.B2e. laminin or Lm 1), laminin 311 (formerly known as K.B1e.B2e. laminin or Lm 6), fibronectin, and heparin sulfate proteoglycan. Not only were the molecules alkylated, they were also crosslinked via an unidentified mechanism by SM. As a consequence, fewer human skin keratinocytes were found to adhere to tissue culture dishes coated with SM treated-laminin or SM-treated fibronectin. After a 20 h incubation, ~60% fewer cells bound to SM-treated laminin than untreated laminin, and ~35% fewer cells adhered to SM-treated fibronectin compared to untreated fibronectin (Zhang *et al.*, 1995). Further studies in the mouse ear model found that, after SM exposure, most of the collagen XVII, fibronectin, laminin, and collagen IV were localized to the dermal side of the blister plane (Monteiro-Riviere *et al.*, 1999). Crosslinking of these components at this site could interfere with reattachment of the epithelium. Again, because of the close identity of components in the corneal epithelial–stromal junction with that of the epidermal–dermal junction, it is likely that these components will also be found on the stromal side of SM-induced microbullae in the cornea.

In addition to damage to the anchoring complex, one must consider that SM may adversely impact the basement membrane proper, enhancing the separation between layers. As shown in Figure 39.5, by the focal detachment of the epithelium from the stroma in regions between anchoring complexes, clearly, the basement membrane area is compromised by mustard exposure (Smith *et al.*, 1998). The gelatinases, matrix metalloproteinases-2 and -9 (abbreviated MMP-2 and MMP-9), which degrade basement membrane components and play important roles in inflammatory responses, were shown to be elevated eight-fold 24 h after SM exposure of the anterior mouse ear, as compared to untreated control ears (Shakarjian *et al.*, 2006). Activity was equally high after 7 days. Since activity assays cannot differentiate between MMP-2 and MMP-9, mRNA and protein levels were assessed, and demonstrated that MMP-9, but not MMP-2 expression, was induced by sulfur mustard exposure – Westerns indicated a nine-fold increase in MMP-9 by 7 days after exposure. Further support for this assertion is the fact that MMP-9 deficient mice are resistant to an experimentally induced form of the blistering disease bullous pemphigoid (Liu *et al.*, 1998). The two pieces of evidence together suggest the following: MMP-9 activity is turned on to remodel a wound, leaving a provisional extracellular matrix that favors cell migration and facilitates healing. This provisional matrix will ultimately be replaced by the normal matrix typical of mature, healthy tissue. SM exposure interferes with MMP-9 being appropriately turned off. The enzyme's activation may prevent reestablishment of the anchoring complexes. Thus the extracellular matrix of the basement membrane does not proceed past the provisional matrix stage (Gordon, Gerecke, Casillas and Babin, unpublished). Finally, inflammatory cell infiltration, which may occur about a day after exposure, may also play a role in delaying SM wound healing (Maumenee and Schloz, 1948). The sheer number of potential target areas in a multilayered, multicell organ that may be affected by sulfur mustard make the task of determining SM's exact mechanism of action quite difficult.

V. RISK ASSESSMENT

Assessing the risk of consequences after a corneal exposure to SM is not easy, so here we will discuss what little information is available. The cornea is composed of a lipophilic epithelium, hydrophilic stroma, and a lipophilic endothelium. Because sulfur mustard penetrates the cornea so rapidly (within about 3–5 min), if detoxification measures are available, they must be employed immediately if there is even a remote hope of minimizing ocular damage. Of course, such measures are only useful if one knows one has been exposed to sulfur mustard. Most people who have been exposed are unaware of their situation for quite some time, until symptoms manifest, but by this time, the damage is already on an irreversible course. They will incur damage, and their only recourse will be treating symptoms. Several factors determine the type and severity of injury: the SM quantity, the time the agent is in contact with the tissue, the form (solid, liquid, vapor) of SM employed, and which tissue is affected (i.e. cornea, lung, or skin).

Although eye exposures may be avoided by use of a gas mask, the mask itself presents a risk to soldiers in danger of being exposed to SM. First, it is cumbersome to carry and uncomfortable to wear, especially in desert climates. Second, as in WWI, today's wearer of the mask still has difficulty breathing through it. Third, the ability to make decisions about topographical movements may be impaired during a chemical attack since the mask impedes vision. Most importantly, the problems with the mask make the soldier think it is best not to wear it unless he is exposed, but the agent cannot usually be sensed immediately upon exposure, potentially delaying the use of the mask until injury is unavoidable.

The major long-term health risk to the eye after SM exposure is the recurrent keratitis mentioned earlier, which can take decades to manifest. Other long-term risks, such as cancer, do not involve the eye. The National Research Council Subcommittee on Chronic Reference Doses for Selected Chemical-Warfare Agents, with Oak Ridge National Laboratory and other experts, has assessed for the Army the overall cancer risk from sulfur mustard exposure. By analyzing the physical and chemical properties, the environmental fate, animal and human toxicity data, and carcinogenicity, 9.5 mg/kg per day had been proposed as the carcinogenic potency of sulfur mustard. A lower value of 1.6 mg/kg per day has been more recently considered (Crossgrove, 1999).

While the risk assessment for an accidental SM exposure is relatively low, such exposures have occurred all too frequently. For example, although chemical agents were not used during WWII, on July 1, 1942, a site-decontamination demonstration involving the use of mustard gas was staged for the members of the Civil Defense Services. This resulted in premature functioning of the demonstration weapon and accidental skin exposure to at least a dozen spectators (Grant and Ritchie, 1942). In December 1943 the USS *John Harvey* was secretly carrying a large number of mustard bombs. The ship was attacked while docked at Bari, Italy, resulting in 617 US mustard casualties (83 fatal) from shells exploded in the water and from the smoke. An undetermined number of Italian civilians were also casualties of the smoke (Alexander, 1947; Infield, 1971, 1988). Additionally, since mustard was introduced, there have been a number of nonbattlefield exposures. After WWII, large stockpiles of mustard were removed from Germany and dumped into the Baltic Sea by allied forces. In 1984, at least 23 fisherman showed varying severities of SM exposure after dredging up these same munitions (Wulf *et al.*, 1985). A half-century after WWII, a souvenir collector was injured after unearthing a buried artillery shell (Ruhl *et al.*, 1994). In China's Jilin Province, two children found a shell in July 2004 that had been left behind by the Japanese Army at the close of WWII. The shell leaked mustard, injuring their legs and hands (*Japanese Times*, 2004).

The USA has disposed of stockpiles of sulfur mustard at sea. Fisherman or boaters who come across discarded canisters, leaky from sitting in salt water, unknowingly have become exposed. In treating these exposures, if the vesicant is pervasive, it is important to decontaminate in a pre-hospital setting, and have health care providers wear appropriate personal care protection. Latex gloves are not sufficient – butyl rubber is needed. Health care workers do not need to fear the patient's blisters, since these do not contain the vesicating agent.

How dangerous are the disposed SM stockpiles? In earlier years, good records were not kept about the locations of sulfur mustard disposal sites, and thus there are ongoing efforts to find these. In 2001, the Army identified three chemical warfare material disposal sites near Hawaii, which contained sulfur mustard discarded between 1925 and 1948. The danger posed appears to be within acceptable risk levels for the following reasons: sulfur mustard hydrolyzes to thiodiglycol and HCl in seawater at approximately the same rate as it dissolves. A toxic hydrolysis intermediate, β-chloroethyl hydroxyethyl sulfide, can be produced, which also has the same hydrolysis rate as sulfur mustard. Thus, at any given time, only a few ppm of toxic mustards would likely be present in seawater surrounding submerged leaking containers of the material. However, a hard polymer layer can develop at the interface of the mustard and seawater, which protects and shields the mustard from hydrolysis. This would allow bulk mustard to potentially remain stable underwater for years. Overall, once the mustard dissolves, the rapid hydrolysis to generate innocuous products would yield very small concentrations of hazardous materials. Any SM leaking from a canister would undergo rapid hydrolysis and be diluted by diffusion facilitated by currents. The canisters are outside of regular shipping lanes, away from fishing grounds, submarine operating areas, and ocean cables (Department of the Army Information Paper, 2006). Thus, it seems likely that, for these underwater disposals, a small leak would cause little danger to humans.

VI. TREATMENTS

Our goal in this section is to report treatments that have evolved over the last 100 years, starting with what was found useful in WWI, followed by what was recommended during WWII, assuming sulfur mustard might be used, and moving on to what has been recommended in the last 25 years. Finally, we will end with what is being learned about potential countermeasures from animal studies. Again, we mention that the effect of mustard is nearly instantaneous (Sorsby, 1939), and nothing can be done to prevent the damage from exposure. For 70 years we have known that even immediate irrigation makes no difference to the subsequent injury (Juler, 1939; Poole, 1939a, b, 1940; Barrett, 1940). Yet this fact goes against what feels to be common sense, and because something must be done to help, irrigation is always recommended as a first treatment. If nothing else, this makes the patient feel that he is receiving medical attention to cope with a frightening health crisis.

A. WWI

Soldiers in WWI endured many degrees of severity of SM exposure. In their 1919 text *The Medical Aspects of Mustard Gas Poisoning*, Warthin and Weller summarized their treatment recommendations based on experience gained in the war:

> The use of any method of treatment which brings pressure upon the lids and eyeball such as tight bandaging or heavy compresses is absolutely contraindicated. For the mild cases, frequent irrigation with saturated boracic acid, the use of lightweight boracic acid compresses, hot vapor baths, and protection of the eyes by darkening of the room, or by a light gauze bandage or by the use of smoked goggles are recommended. Special care must be taken to prevent the gluing together of the eyelids by accumulation of the exudates. Should this occur the exudates should be softened and washed away with boracic acid with care to avoid forcible separation of the lids. The use of argyrols, silvol, etc., is in our opinion undesirable. The use of cocaine is considered unwise. For the severe forms the use of a 1 per cent Dakin's solution is found to be too irritating. We therefore recommend the employment of the chlorcosane solution of dichloramine-T in a strength of 0.5 to 1 per cent, or even

> stronger. We further advise that in all cases of exposure to mustard gas this solution be used as an immediate irrigant for its prophylactic effects, followed in the milder cases of injury by boracic acid irrigation and in the severe cases by repeated irrigation with the dichloramine-T solution for the prevention of secondary infection. For the treatment of the refractive errors and more permanent disturbances of vision the patient must be referred to a competent ophthalmologist (Warthin and Weller, 1919).

Sir William Lister, an experienced ophthalmologist in WWI with extensive experience in the treatment of the early stages of gassed eyes, stressed the psychological value of separating the eyelids, and of thus convincing the patient that his sight had not been irreparably damaged (Barrett, 1940). Following irrigation, a drop of oil was recommended. Liquid albolene was the least irritating. In those cases where secondary infection developed, a weak antiseptic solution was believed to be therapeutic, most often using argyrols or protargol. Atropine was recommended to be continued as long as any corneal involvement continued, and a shade or dark glasses for symptomatic relief was beneficial (Derby, 1919).

B. WWII

Mustard was not used in WWII, but the fear of its use was pervasive, and medical teams procured information about how to treat potential victims of exposure. One such piece of information was provided by the Ministry of Health in 1941 in a memorandum for medical practitioners, published in the *British Medical Journal*. It reminded medical personnel that ''Treatment of the eyes, if affected, should always be given preference over treatment of the skin'' (Ministry of Health memorandum, 1941). Unfortunately, despite the intervening years between the wars, only small advances had been made in treating SM ocular injuries. Irrigation, of course, was the first order of treatment, although damage would already be in process. Experiments using trephinated buttons of bovine corneas, performed in 1942, but published in 1946, verified this once again. At best, a therapeutic agent had 3–5 min to neutralize SM after an eye exposure (Kinsey and Grant, 1946), and people could not recognize they were gassed until about an hour or more after they were exposed.

As in WWI, WWII recommendations aimed at the relief of symptoms, the control of the lesion, and the prevention of secondary infections and complications. The patient was to be reassured he would not be blinded by the medical practitioner opening his eyelids. Bandages were not to be used. Symptomatic treatment of conjunctivitis was to be treated with irrigation, often with sodium bicarbonate solution (Sorsby, 1939; Cowell, 1939; Bickerton, 1940). Caster oil drops or liquid paraffin drops were thought to be helpful. First-aid treatment with cod-liver oil was felt to do no harm and had been found to give immediate pain relief. It also smoothed corneal roughness and eased lacrimation in patients with delayed keratitis or ulceration (Stevenson, 1939, 1940). Atropine drops were also used when the cornea was affected (Sorsby, 1939). These recommendations were not vastly different from the treatment used in WWI.

Small advances in treatment options were made. One improvement was an improvised eye irrigator, suggested by a military ophthalmologist (Stallard, 1940). As physicians planned for possible emergency care of old or middle-aged persons in air-raids, Dr Agnes Estcourt-Oswald recommended treatment with homatropine rather than atropine to minimize the danger of precipitating or increasing glaucoma (Estcourt-Oswald, 1939). Sulfanilamide preparations (albucid) were used for secondary infection of the conjunctiva, but were not really proven to be efficacious (Juler and Whiting, 1941a, b).

Among the many patients cared for by Dr Ida Mann, treatment with contact lenses resulted in improvement in visual acuity in at least half of them because the smooth glass corrected for the irregular and uneven corneal surface that was a consequence of mustard injury (Mann, 1944). This was a most significant contribution, since severe scarring from mustard could result in total blindness.

C. Post-WWII

The life story of Canadian Lieutenant Eddie Baker demonstrates a heartwarming example of progress. Baker was blinded in WWI by sniper fire. After the war, he devoted himself to improving the lives of blinded veterans. He raised the funds used to start the Canadian National Institute for the Blind (CNIB) and, later, was involved in the development of eye banks. The downstream effect of his efforts was the first cornea transplant in Canada, performed in 1956 on a WWI veteran, 40 years after he was blinded by gas (Wilton, 1996). While this is wonderful medical progress, it cannot undo the fact that there is still no antidote against SM.

Today's political climate and the estimation that at least a dozen countries have mustard in their current arsenals (Sidell *et al.*, 1997) means that the search for countermeasures against SM must not stop. Egypt had been suspected of using mustard against Yemen in the mid-1960s (Balali-Mood *et al.*, 1993). Thousands of Iranian soldiers were exposed to mustard during the Iran–Iraq conflict between 1983 and 1988 (Balali-Mood and Hefazi, 2006). In 1988, Saddam Hussein used mustard and nerve agents on his own people in the village of Halabja, causing the death of 5,000 people within less than 10 min, and maiming many thousands more. After terrorist attacks became a worldwide concern, an ''Expert Group on the Management of Chemical Casualties Caused by Terrorist Activity'' was formed by the Department of Health of Great Britain, and was chaired by Professor P.G. Blain, CBE. In October 2003, they published recommendations for treatment of SM exposure. A summary of these is: irrigate immediately to decontaminate and minimize corneal damage with large

quantities of clean water or isotonic fluids such as saline; anesthetic drops can be used during irrigation; reassure patients with eye exposure; give systemic analgesics and dark glasses; petroleum jelly can be used to prevent lid adhesion; bandages should be avoided (Department of Health, UK report, 2003). These guidelines are again essentially the same as treatments recommended in WWI. However, also added is that steroids may be used if tapered off with time, since their use may impair epithelial regeneration and collagen repair. Vitamin C may help since it is an antioxidant and may prevent damage by free radicals released at the time of injury. Acetylcysteine may be helpful since it contributes to the crosslinking of new collagen and inhibition of collagenases released at the time of tissue damage. Antibiotics may be used to prevent secondary infection if there is loss of the corneal epithelium. It seems likely that several of these recommendations are based on the panel members having familiarized themselves with the results of animal studies, such as those that implicate *N*-acetyl-cysteine in decreasing the inflammatory response and improving outcome (Anderson *et al.*, 2000).

The UK Department of Heath report also includes information on how to assess the extent of SM injury, along with providing recommendations for which types of injury require hospital admission. For example, early assessment of injuries should include visual acuity, reactivity of the pupil, extent of burns or necrosis to the eyelids, epithelial loss in the conjunctiva and limbus with necrosis and ischemia, corneal epithelial loss, edema, and stromal opacity, fibrin deposition, uveitis, iris atonicity, hemorrhage, lens opacity, lens debris and leakage. The extent of the corneal chemical injury should be graded to decide the next course of action. A grade 1 injury, which would have a good prognosis, could show epithelial damage with ischemia. A grade 2 injury, also with a good prognosis, would include corneal haze, but with iris details visible to the examiner. Only one-third or less of the limbus should be ischemic for assigning grade 2. A grade 3 injury would have a guarded prognosis, and would involve total loss of the epithelium, with stromal haze and obscured iris detail. One-third to one-half of the limbus may be ischemic. Grade 4 would represent a poor prognosis, and would be characterized by corneal opacity, with a pupil and iris that is not visible. One half of the limbus would potentially be ischemic. Intraocular damage, especially if accompanied by a raised intraocular pressure, indicates a poor prognosis. Admission to hospital is advised if there are grade 3 or 4 changes. In the long term, severe cases may require amniotic membrane transplantation and limbal stem cell allografts (Department of Health, UK Report, 2003).

D. Animal Studies

There is much research being done to identify countermeasures against mustards. The Department of Defense has traditionally funded this research in the USA, but currently NIH is contributing through a program called CounterACT. It will probably be easiest to identify treatment drugs for skin, and hardest to identify ones for lung. Therapeutics effective for SM exposures of skin may be useful for the eye, but will likely need modifications, since they may be irritating to the cornea. Vanilloids, for example, hold promise for skin (Casbohm *et al.*, 2004) but would need chemical alterations to avoid corneal irritancy while maintaining efficacy. In the next few paragraphs we will review some of the most promising therapies for mustard exposure.

Mustard exposure causes depletion of ATP in cells. Better outcomes after exposure have been demonstrated when mitochondrial substrates were provided to offset this depletion. CEES-exposed rabbit corneas have been treated after exposure with a mix of taurine, pyruvate, α-keto glutarate, and pantothenic acid. Analysis showed reduced necrosis of the cornea. Electron microscopic and other analyses showed protection against membrane damage and oxidative damage (Varma *et al.*, 1998a, b).

Anti-inflammatory drugs show promise as SM countermeasures. Dexamycine® (0.1% dexamethasone sodium phosphate and 0.5% neomycin sulfate) ophthalmic solution was used to treat NZW female rabbits after a 2 min SM vapor challenge at 342 µg/l. Two treatment regimens, one started 1 h after challenge, the other at 6 h after, were administered 3 times/day for 2 weeks. The animals were observed over the course of 3 months. The data demonstrated that neovascularization was delayed or prevented in the groups receiving Dexamycine, independent of the start time of the treatment, as compared to the group exposed to SM without a countermeasure (Kadar *et al.*, 1996).

A follow-up study used Dexamycine and Voltaren® Ophtha (diclofenac), a nonsteroidal anti-inflammatory drug, after a 2 min 390–420 µg/l SM vapor challenge to rabbit eyes. The Dexamycine and Voltaren were administered separately, or in combination, 4 times/day for 2 weeks, starting 1 h after exposure. Rabbits were clinically observed for up to 3 months. Rabbits receiving the anti-inflammatory drugs had a much improved clinical assessment score, a reduction in protein and prostaglandin E in the anterior chamber, and a reduction in corneal edema and infiltrate during the acute phase after challenge. Histological examination at 48 h revealed minimal inflammatory infiltrate present in the corneal tissue of the drug-treated groups. Dexamycine was found to be more effective than Voltaren and the combination was more effective than either drug given alone. It was also demonstrated that Dexamycine administered at a high dose for 1 day, or at a low dose for 5 days was effective in improving the outcome, compared to SM-exposed animals receiving no Dexamycine. Treatments of drug started 15 min after challenge produced similar results to treatments started 1 h after challenge. Unfortunately, none of the Dexamycine treatments reduced the occurrence of corneal erosions. These were the same as in SM exposed animals without subsequent drug treatment (Amir *et al.*, 2002).

Anti-inflammatory drugs were also used on the eyes of rabbits challenged with a 1.02 mg droplet of neat sulfur mustard. Ten minutes after exposure, the group whose eyes were to receive subsequent treatment were dosed with a 1% ophthalmic suspension of prednisolone acetate. After 2 h, the prednisolone suspension was discontinued, and a sub-Tenon's injection containing 20 mg triamcinolone and 50 mg cefazolin was immediately administered. Corneal thickness, stromal injury, neovascularization, eyelid notching and chemosis were recorded weekly for 6 consecutive weeks, and then again on week 16 after the challenge. The majority of the drug treated group had a significant reduction in corneal thickness at 2, 3, and 4 weeks, compared to the rabbits exposed to SM without post-exposure treatment. For the other parameters, significant evidence of a protective effect due to treatment was seen at 4, 5, and 6 weeks after exposure. In addition, corneal stromal injury was reduced at 2 and 3 weeks, and eyelid injury (notching) at 2 weeks. By week 3, all control SM exposed animals developed neovascularization, in contrast to one of seven animals that received post-exposure anti-inflammatory drugs. By week 6, all SM exposed animals still exhibited neovascularization, while only two of the seven drug-treated animals showed this phenotype. This study strongly suggests once again that anti-inflammatory drugs are effective in treating severe corneal injuries in the first couple of months after SM exposure (Babin *et al.*, 2004).

One of the worst consequences of SM exposure is corneal perforation. This effect can occur many weeks after exposure, and animal data are beginning to suggest that the toxicity of steroids may play a role in its occurrence. In a study of 15 rabbits challenged with 0.8 µl (1.02 mg) of liquid neat SM and treated with 20 mg of triamcinolone by sub-Tenons injection, four rabbits (27%) had lesions severe enough to cause a perforation of the cornea by 16 weeks (Babin *et al.*, 2004). When challenged at half this dose of SM (i.e. 0.51 mg) and subsequently treated with prednisolone acetate three times/day for 3 weeks, 60% of the rabbits developed corneal perforation (Bossone *et al.*, 2002). In an ongoing study, after a neat SM challenge, rabbits treated with decreasing doses of prednisolone acetate over a 1 month period were devoid of cornea perforations (John J. Schlager, Air Force Research Laboratory, and Michael C. Babin, Battelle Biomedical Research Center, unpublished data). These results suggest that the use of corticosteroids during the acute injury phase following SM exposure should be closely monitored and should follow the common practice of reducing the dose and frequency over time.

The explosion of science in the latter half of the 20th century, elucidating components of the adhesion complex between the epithelial and stromal layers of the cornea, suggest the basement membrane zone may become a target area for therapy in the future. Indeed, protease inhibitors of enzymes that remodel the basement membrane show promise as countermeasures. Using rabbits for an ocular SM vapor challenge, the matrix metalloprotease inhibitors ilomostat and doxycycline, and the serine protease inhibitor alpha-1 protease inhibitor, were administered 15 min after challenge, either alone or in combination. Groups of six rabbits were used, and drugs were delivered four times/day for 6 days of each week, over the course of 8 weeks in total. Analysis extended beyond the 8 week treatment: eyes were evaluated weekly for 12 weeks following exposure. In the 4 days after exposure, daily assessments were performed. During the first 2 weeks after challenge, the ilomostat and doxycycline groups showed better healing compared to the alpha-1 protease inhibitor animals, and all three drug treatment groups showed a significant reduction in lesion size compared to the SM exposure control group. Furthermore, ilomostat scored better than doxycycline in improving acute phase healing parameters. In the later phase of injury, after 2 weeks, both matrix metalloproteinase inhibitors significantly reduced corneal neovascularization. Surprisingly, the addition of alpha-1 protease inhibitor to either ilomostat or doxycycline negatively affected the outcome. Significant reductions in treatment efficacy were seen (Amir *et al.*, 2000, 2004).

Compared to the amount of research that has been invested in the study of SM skin injury, the eye has received relatively little attention during the last 20–30 years. The eye is, however, a target tissue that has a high potential for successful intervention against SM. There has been some use of corneal transplantation after mustard gas injury; however, there is a high risk for transplant rejection due to inflammation and vascularization of the cornea. Despite this, keratoplasty has been used successfully in some blinded Iraq–Iran conflict veterans (Richter *et al.*, 2006). It is likely to be one of the more successful techniques to restore vision to severely exposed individuals, especially if the limbus is also transplanted. This is needed to provide an additional supply of corneal epithelial stem cells, which become depleted over the years of trying to heal the SM latent wounds.

VII. CONCLUDING REMARKS AND FUTURE DIRECTION

From the 1920s until his death in the 1950s, Jonas Friedenwald, MD treated patients with eye problems and managed a varied research career touching on many vision-related areas. He concluded that

> the possibility of obtaining a completely effective detoxifying agent for mustard is rather poor, because no substance has been found which will remove mustard from its combination with proteins without destroying the proteins. Consequently, it can be hoped only that an antidote will neutralize the mustard which remains on the surface of the tissues but has not yet reacted with them when the antidote is applied. There is a brief interval between 2 and 5 minutes after exposure during which a penetrating decontaminant might be expected to be beneficial.

Since Friedenwald wrote this there is still no antidote and still no curative therapies, but there have been enormous strides generally in molecular biology, cell biology, drug development, and medicine which improve the chances of identifying suitable therapies. With the research being done today toward understanding the molecular events induced by sulfur mustard, causing eye, skin and lung injuries, there is much hope that effective post-exposure treatments will be found.

It is clear that more information is needed on the mechanisms of action of sulfur mustard, since elucidating these is currently providing the clues for identifying therapies. In the future, the cause(s) of delayed wound healing needs to be illuminated, in order that reagents or drugs to offset this effect can be developed. Perhaps this may be accomplished by researching the nature of the epithelial–stromal junction in a different manner. Research in the wound healing area, identifying individual steps in the process, induces many researchers who work on SM to ask why mustard wounds heal more slowly than other types of burn wounds. Our laboratories consider the provisional wound bed matrix to be important. For example, SPARC is a component present in the provisional wound extracellular matrix. Mice with a targeted null mutation in the SPARC gene show faster wound healing (Bradshaw *et al.*, 2002). Thus, we are asking whether SPARC is overexpressed, or aberrantly persists, in SM-induced wounds, slowing healing (Gordon, Gerecke, Chang, Casillas, and Babin, unpublished data). In addition, research in the field of wound healing suggests that we should examine the expression of components that replace the provisional matrix, i.e. the components of the final mature extracellular matrix typical of healthy, healed tissue. It is possible we may be able to improve healing of SM wounds if we find ways to promote their expression.

Ideally, the future will hold some prophylactic treatments that prevent recurrences such as corneal erosion. Perhaps this will be accomplished by mildly modulating the immune system for an extended period of time after injury. Finally, it would be beneficial to identify genetic alterations that might be used to monitor the chance of developing cancers from the SM exposure. In the mechanism of action section, we stressed that the sheer number of potential targets of SM in an organ with multiple cell layers and extracellular matrices, such as the cornea, make the task of determining the vesicant's mechanism of action very difficult. While this is undoubtedly true, there is a positive way to view the situation: the large number of potential targets provides a large number of potential intervention sites. As we improve our understanding of the mechanisms of action with animal studies, we increase our opportunities for identifying and developing successful therapeutic countermeasures.

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CHAPTER 40

Immunotoxicity

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I. INTRODUCTION

Immunotoxicity is defined as adverse effects on the functioning of both local and systemic immune systems that result from exposure to toxic substances including chemical warfare agents. Observations in humans and animal studies have clearly demonstrated that a number of environmental and industrial chemicals can adversely affect the immune system. Alteration in the immune system may result in either immunosuppression or exaggerated immune reaction. Immunosuppression may lead to the increased incidence or severity of infectious diseases or cancer, since the immune system's ability to respond adequately to invading agents is suppressed. Toxic agent-induced immunostimulation can cause autoimmune diseases, in which healthy tissue is attacked by an immune system that fails to differentiate self-antigens from foreign antigens. For example, the pesticide dieldrin induces an autoimmune response against red blood cells, resulting in hemolytic anemia. Immunotoxicology deals with the effects of toxic substances and explores the mechanisms underlying these effects in a biological system.

Although immunotoxicology is a relatively new field, a considerable amount of data has accumulated during the past few years on immunotoxicity of certain xenobiotics. The majority of the research thus far carried out has been on environmental contaminants. Thus, from the defense point of view considerable work is required to investigate the immunotoxicity of several chemicals and some bacterial and fungal toxins which may be potential chemical warfare agents. Furthermore, there are several chemicals used in the defense industry to which the defense industrial workers may be constantly exposed. These chemicals, following low-level exposure to humans and animals, may cause immunological alterations. Thus immunotoxicity studies on such chemicals are being conducted to understand the potential risks of such exposure on the host's defense as well as the cellular and molecular mechanism of such immunomodulatory action.

A chemical warfare agent (CWA) is a substance which is intended for use in military operations to kill, seriously injure, or incapacitate people because of its toxicological effects. Although CWAs have been widely condemned since their first use on a massive scale during World War I, they have been used in many conflicts during the 20th century. As chemical weapons are cheap, relatively easy to produce and can result in mass casualties, they will continue to be used in future wars and terrorist attacks.

Although most of the compounds of CWAs are not persistent in the environment, repeated exposure and persistence of some of the compounds result in immunotoxicity. This chapter describes the immunotoxicity of CWAs and gives an insight into the probable mechanisms of such effects.

II. THE IMMUNE SYSTEM

The immune system is composed of several organs, cells, and noncellular components which act in an interrelated manner to protect the host against foreign organisms and chemical substances. The immune system participates in the mechanisms responsible for the maintenance of homeostasis and an altered immune system reflects the adverse changes in both internal and external microenvironments. The immune system protects organisms against pathogens or other innocuous substances like pollens, chemicals, indoor molds, potential food allergens, and environmental agents, and acts as layered defenses of increasing specificity. Most simply, physical barriers (e.g. skin) prevent pathogens and xenobiotics from entering the organism. If they breach these barriers, the innate immune system provides an immediate but nonspecific response. However, if pathogens successfully evade the innate response, there is a third layer of protection, i.e. the adaptive immune system, which is activated by the innate response. Here, the immune system adapts during an infection to improve its recognition of the pathogen and its response is then retained after the pathogen or xenobiotic has been eliminated. This immunological memory allows the adaptive immune system to respond faster with a stronger attack each time the same insult is encountered (Kindt *et al.*, 2007).

The immune system protects organisms from infection with layered defenses of increasing specificity. The layered defense includes mechanical, chemical, and biological barriers which protect organisms from toxic substances. Skin, a mechanical barrier, acts as the first line of defense against infection. In the lungs, coughing and sneezing mechanically eject pathogens and other irritants from the

respiratory tract while mucus secreted by the respiratory and gastrointestinal tract traps and entangles microorganisms and other toxins (Boyton and Openshaw, 2002). Chemical barriers also protect against infection. The skin and respiratory tract secrete antimicrobial peptides such as the β-defensins. Enzymes such as lysozyme and phospholipase A2 in saliva, tears, and breast milk are also antibacterials (Moreau *et al.*, 2001; Hankiewicz and Swierczek, 1974). In the stomach, gastric acid and proteases serve as powerful chemical defenses against ingested pathogens.

A. The Innate Immune System

The innate immune system defends the host from infection and toxicants, in a nonspecific manner. This means that the cells of the innate system recognize, and respond in a generic way, but do not confer long-lasting or protective immunity to the host. The innate immune response was initially dismissed by the immunologist as it was thought to provide a temporary holding of the situation until a more effective and specific adaptative immune response develops. But it has now been clear that it plays an important role as a dominant system of host defense in most organisms (Litman *et al.*, 2005). The major function of the innate immune system is to recruit immune cells to sites of infection and inflammation. Inflammation is one of the first responses of the immune system to infection or irritation through the production of cytokines. These cytokines released by injured cells serve to establish a physical barrier against the spread of infection. Several chemical factors are produced during inflammation, e.g. histamine, bradykinin, serotonin, leukotrienes, and prostaglandins, which sensitize pain receptors, cause vasodilation of the blood vessels, and attract phagocytes. The inflammatory response is characterized by the redness, heat, swelling, pain, and possible dysfunction of the organs or tissues involved. The fluid exudate contains the mediators for four proteolytic enzyme cascades: the complement system, the coagulation system, the fibrinolytic system, and the kinin system. The exudate is carried by lymphatics to lymphoid tissue, where the product of foreign organism can initiate an immune response.

The activation of the complement cascade helps to identify the invading substance, activate cells, and promote clearance of dead cells by specialized white blood cells. The cascade is composed of nine major components, designated C1 to C9, which are plasma proteins synthesized in the liver, primarily by hepatocytes. These proteins work together to trigger the recruitment of inflammatory cells. One of the main events is the splitting of the C3, which gives rise to various peptides. One of them, C3a (anaphylatoxin), can stimulate mast cells to secrete chemical mediators and another, C3b (opsonin), can attach to the surface of a foreign body and facilitates its ingestion by white blood cells. C5 is a powerful chemotactic of white cells and causes release of mediators from mast cells. Later components from C5 to C9 assemble in a sequence at the surface of bacteria/xenobiotics and lead to their lysis, ridding the body of neutralized antigen–antibody complexes. The main events of this system can also be directly initiated by the principal enzymes of the coagulation and fibrinolytic cascade, thrombin and plasmin, and by enzymes released from white blood cells. Further, an innate immune system leads to the activation of an adaptive immune system.

B. The Adaptive Immune System

The adaptive immune system is composed of highly specialized, systemic cells and processes that eliminate pathogenic challenges and provide the ability to recognize and mount stronger attacks each time the same pathogen is encountered. Antigen specificity requires the recognition of specific ''nonself'' antigens during a process called antigen presentation. The ability to mount these immune responses is maintained in the body by ''memory cells''. The cells of the adaptive immune system are special types of leukocytes, B cells and T cells, which constitue about 20–40% of white blood cells (WBCs). The peripheral blood contains 20–50% of circulating lymphocytes and the rest move within the lymphatic system (Kindt *et al.*, 2007). B cells and T cells are derived from the same pluripotential hematopoietic stem cells in the bone marrow, and are indistinguishable from one another until after they are activated. B cells play a large role in the *humoral immune response*, whereas T cells are intimately involved in *cell-mediated immune responses*. B cells derive their name from the bursa of Fabricius, an organ unique to birds, where the cells were first found to develop. However, in nearly all other vertebrates, B cells (and T cells) are produced by stem cells in the bone marrow (Kindt *et al.*, 2007). T cells are named after thymus where they develop and through which they pass. In humans, approximately 1–2% of the lymphocyte pool recirculates each hour to optimize the opportunities for antigen-specific lymphocytes to find their specific antigen within the secondary lymphoid tissues. Both B cells and T cells carry receptor molecules that recognize specific targets.

T cells express a unique antigen-binding molecule, the T cell receptor (TCR), on their membrane. There are two well-defined subpopulations of T cell: T helper (T_H) and T cytotoxic (T_C) cells. They can be distinguished from one another by the presence of either CD4 or CD8 membrane glycoproteins on their surfaces. T cells displaying CD4 generally function as T_H cells whereas those displaying CD8 function as T_C cells. T cells recognize a ''nonself'' target, such as a pathogen, only after antigens have been processed and presented in combination with a ''self'' receptor called a major histocompatibility complex (MHC) molecule. T_C cells only recognize antigens coupled to class I MHC molecules, while T_H cells only recognize antigens coupled to class II MHC molecules.

B cells are the major cells involved in the creation of antibodies that circulate in blood plasma and lymph, known as humoral immunity. Like the T cell receptor, B cells

express a unique B cell receptor (BCR), in this case an immobilized antibody molecule. The BCR recognizes and binds to only one particular antigen. A critical difference between B cells and T cells is how each cell ''sees'' an antigen. T cells recognize their cognate antigen in a processed form – as a peptide in the context of an MHC molecule – while B cells recognize antigens in their native form. Once a B cell encounters its cognate (or specific) antigen [and receives additional signals from a helper T cell (predominantly Th2 type)], it further differentiates into an effector cell, known as a plasma cell.

Plasma cells are short-lived cells (2–3 days) which secrete antibodies that circulate in blood plasma and lymph, and are responsible for humoral immunity. Antibodies (or immunoglobulin, Ig) are large Y-shaped proteins used by the immune system to identify and neutralize foreign objects. In mammals there are five types of antibody: IgA, IgD, IgE, IgG, and IgM. Differing in biological properties, each has evolved to handle different kinds of antigens. These antibodies bind to antigens, making them easier targets for phagocytes, and trigger the complement cascade. About 10% of plasma cells will survive to become long-lived antigen-specific memory B cells (Lu and Kacew, 2002). Already primed to produce specific antibodies, these cells can be called upon to respond quickly if the same foreign body reinfects the host. This is called ''adaptive immunity'' because it occurs during the lifetime of an individual as an adaptation to infection with that pathogen and prepares the immune system for future challenges.

A number of animal models have been developed and validated to detect the chemical-induced direct immunotoxicity. Several compounds, including certain drugs, have been shown in this way to cause immunosuppression or skin allergic responses. In this chapter, the various mechanisms of immunotoxicity are discussed by which a compound affects different cell types and interferes with immune responses, ultimately leading to immunotoxicity as well as sensitizing capacity.

III. TARGETS OF IMMUNOTOXICITY

A. Effects on Precursor Stem Cells

The bone marrow is an organ with precursor stem cells that are responsible for synthesizing peripheral leukocytes. All leukocyte lineages originate from these stem cells, but once distinct subsets of leukocytes are established, their dependence on replenishment from the bone marrow differs vastly. The turnover of neutrophils is very rapid, i.e. more than 10^8 neutrophils enter and leave the circulation in a normal adult daily so there is dependence on new formation in the bone marrow. In contrast, macrophages are long-lived and have little dependence on new formation of precursor cells. The adaptive immune system, comprising antigen-specific T and B lymphocytes, is almost completely established around puberty and is therefore essentially bone marrow independent in the adult.

As a consequence of their high proliferation rate, stem cells in the bone marrow are likely to be extremely vulnerable to cytostatic drugs and chemicals like CWAs. Lineages like neutrophils with rapid turnover will be most vulnerable and will be affected first by such treatments/exposures. After prolonged exposure, macrophages and T or B cells of the adaptive immune system are also suppressed.

B. Effects on Maturation of Lymphocytes

T lymphocytes mature in the thymus by a very complex selection process that takes place under the influence of the thymic microenvironment and ultimately generates an antigen-specific, host-tolerant population of mature T cells. This process involves cellular proliferation, gene rearrangement, apoptotic cell death, receptor up- and down-regulation and antigen-presentation processes, and is very vulnerable to a number of chemicals. Drugs may target different stages of T cell differentiation like naïve T cells, proliferating and differentiating thymocytes, antigen-presenting thymic epithelial cells and dendritic cells, cell death processes, etc. (Vos *et al.*, 1999). In general, immunosuppressive drugs may cause a depletion of peripheral T cells, particularly after prolonged treatment and during early stages of life when thymus activity is high and important in establishing a mature T cell population. In addition, suppression of T cells may result in suppression of the adaptive immune system by affecting the maturation of B cells and thus antibody level.

C. Effects on Initiation of Immune Responses

The innate and adaptive immune systems act together to eliminate invading pathogens. Ideally, T cells tailor the responses to neutralise invaders with minimal damage to the host. The recognition of autoantigens is maintained by the two distinct signals that govern lymphocyte activation. One is the specific recognition of antigen via clonally distributed antigen receptors and the other is antigen nonspecific co-stimulation or ''help'' and involves interactions of various adhesive and signaling molecules expressed in response to tissue damage, linking initiation of immune responses to situations of acute ''danger'' for the host (Vos *et al.*, 1999). This helps to aim immune responses at potentially dangerous microorganisms (nonself), while minimizing deleterious reactions to the host (self). Xenobiotics, however, can interfere with the initiation of immune responses if they act as antigens, by forming haptens or by releasing previously hidden self-antigens. They may also trigger an inflammatory response, or disturb T–B cell cooperation.

CWAs with large molecular weight can function as antigens and become targets of specific immune responses themselves. This is particularly relevant for foreign protein

pharmaceuticals, as these can activate both T and B lymphocytes. The resulting immune responses may lead to formation of antibodies, and induce specific memory which can lead to allergic responses to the drug. Immunotoxic effects may occur after repeated treatment with the same CWA. However, low molecular weight CWAs cannot function as antigens, because they are too small to be detected by T cells. Reactive chemicals that bind to proteins, however, can function as haptens and become immunogenic if epitopes derived from them prime T cells, which in turn provide co-stimulation for hapten-specific B cells. This effect is responsible for allergic responses to many new (neo) epitopes formed by chemical haptens.

Modification of autoantigens can also lead to autoimmune responses to unmodified self-epitopes. Haptenated autoantigens can be recognized and internalized by antigen presenting cells. These cells subsequently present a mixture of neo- and self-epitopes complexed to distinct class II major histocompatibility (MHC-II) molecules on their surface and neospecific T cells. Th cells provide signals for the B cell. This leads to production of either antihapten or anti-self antibodies depending on the exact specificity of the B cell. Moreover, once these B cells are activated, they can stimulate autoreactive Th cells recognizing unmodified self-epitopes. This process is called epitope (determinant) spreading and causes the diversification of adaptive immune responses. For example, injection of mercury salts initially induces response directed only to unidentified chemically created neoepitopes, but after 3–4 weeks include reactivity to unmodified self-epitopes. Thus the allergic response may gradually culminate as autoimmune responses reflecting the relative antigenicity of the neo- and self-epitopes involved (Lu and Kacew, 2002).

IV. EXPOSITION OF AUTOANTIGENS AND INTERFERENCE WITH CO-STIMULATORY SIGNALS

Self-tolerance involves specific recognition of autoantigen leading to selective inactivation of autoreactive lymphocytes at birth, but tolerance is not established for (epitopes of) autoantigens that are normally not available for immune recognition. Pharmaceuticals can expose such sequestered epitopes by disrupting barriers between the antigen and the immune system (i.e. blood–brain barrier, blood–testis barrier, cell membranes). Tissue damage, cell death, and protein denaturation induced by chemicals can largely increase the chances of such (epitopes of) autoantigens for immune recognition. Antigen recognition followed by co-stimulation of signaling molecules leads to activation of lymphocytes and initiation of immune responses. Many xenobiotics have the inherent capacity to induce or inhibit this co-stimulation due to their intrinsic adjuvant activity.

V. INDUCTION OF INFLAMMATION AND NONCOGNATE T–B COOPERATION

Cytotoxic chemicals or their reactive metabolites can induce tissue damage which results in release of proinflammatory cytokines like tumor necrosis factor α (TNFα), interleukin-1 (IL-1), and IL-6, and attracts inflammatory cells like granulocytes and macrophages. Cytokines produced during this inflammatory response activate antigen-presenting cells and accumulation of tissue debris. The epitopes of antigens on debris provide co-stimulation for Th cells, which lead to the initiation of an adaptive immune response. Reactive xenobiotics may also stimulate adaptive immune responses by disturbing the normal cooperation of Th and B cells. Normally, B cells receive stimulation from Th cells that recognize (epitopes of) the same antigen. However, when Th cells respond to nonself-epitopes on B cells, such B cells may be noncognately stimulated by the Th cell. This occurs during graft-versus-host responses following bone marrow transplantation, when Th cells of the host recognize nonself-epitopes on B cells of the graft and vice versa. This leads to T and B cell activation and results in production of autoantibodies to distinct autoantigens like DNA, nucleoli, nuclear proteins, erythrocytes, and basal membranes. Drug/chemical-related lupus is characterized by a similar spectrum of autoantibodies, and noncognate – graft-versus-host-like – T–B cooperation is therefore suggested to be one of the underlying mechanisms.

VI. REGULATION OF THE IMMUNE RESPONSE

The type of immune response elicited in response to a foreign pathogen or allergen is the result of a complex interplay of cytokines produced by macrophages, dendritic cells, mast cells, granulocytes, and lymphocytes. Immunotoxic chemicals that somehow influence the immune system can lead to either immunosuppression or immune exaggeration, i.e. hypersensitivity and autoimmunity. Hypersensitivity is an immune response that damages the body's own tissues. Hypersensitivity reactions require a pre-sensitized (immune) state of the host. They are divided into four classes (Type I–IV) based on the mechanisms involved and the time course of the hypersensitive reaction. Type I hypersensitivity is an immediate or anaphylactic reaction, often associated with allergy. Symptoms can range from mild discomfort to death. Type I hypersensitivity is mediated by IgE released from mast cells and basophils. Type II hypersensitivity occurs when antibodies bind to antigens on the patient's own cells, marking them for destruction. This is also called antibody-dependent (or cytotoxic) hypersensitivity, and is mediated by IgG and IgM antibodies. Immune complexes (aggregations of antigens, complement proteins, and IgG and IgM antibodies) deposited in various tissues trigger Type III hypersensitivity reactions. Type IV hypersensitivity (also

known as cell-mediated or delayed type hypersensitivity) usually takes between 2 and 3 days to develop. Type IV reactions are involved in many autoimmune and infectious diseases, but may also involve contact dermatitis (poison ivy). These reactions are mediated by T cells, monocytes, and macrophages. Actual development of clinical symptoms is influenced by the route and duration of exposure, the dosage of the pharmaceutical, and by immunogenetic (MHC haplotype, Th1-type versus Th2-type responders) and pharmacogenetic (acetylator phenotype, sulfoxidizer, Ah receptor, etc.) predisposition of the exposed individual. Moreover, atopic individuals that tend to mount Th2 immune responses are more susceptible to anaphylaxis triggered by an IgE response to chemical haptens than typical Th1 responders. Genetic variation in metabolism of pharmaceuticals is important as it determines the formation and clearance of immunotoxic metabolites. The slow acetylating phenotype, for instance, predisposes for drug-related lupus because reactive intermediates of phase I metabolism have an increased opportunity to bind proteins as they are only slowly conjugated.

Immune dysregulation can also be in the form of immune suppression and both innate and adaptive arms of the immune systems play crucial roles. A wide variety of physiological, pharmacological, and environmental factors can exert a negative influence on the immune system and sometimes result in immunotoxicity. Recent experimental data have shown that emotional and environmental stressors influence the functioning of the immune system and this is reflected in the various markers of specific immunity (Ray *et al.*, 1991; Koner *et al.*, 1998). Such experimental stressors consistently suppressed both humoral and cell mediated immune responses in experimental animals. Both antibody forming cell counts and antibody titer were lowered and a neuroendocrine–immune axis concept was proposed. Similar attenuations in cell mediated immune responses were also seen after such stressors and DTH responses, leukocyte/macrophage migration indices and also cytokine profiles (both Th1 and Th2 dependent). Further analysis of the mechanisms involved indicated that CNS mediated changes could have contributed to this immunotoxicity. Depletion or antagonism of brain dopamine aggravated emotional stress-induced immune suppression, whereas psychoactive drugs like benzodiazepines and opioids prevented this response (Ray *et al.*, 1992; Puri *et al.*, 1994). In another set of experiments, rats exposed to several environmental pollutants like DDT showed graded degrees of immune suppression and immunotoxicity, when the exposure lasted for a reasonably long period of time. Gradual accumulation in the various body tissues resulted in a variety of untoward effects in the immune system, which was particularly susceptible to such xenobiotic-induced damage (Banerji *et al.*, 1996; Koner *et al.*, 1998). Both humoral and cell mediated immune response were affected depending on the quantum and duration of exposure to these xenobiotics. Further, a combination of emotional stress and xenobiotic exposure had additive effects on the immunotoxicity parameters studied (Banerjee *et al.*, 1997). Recent studies revealed that such emotional stress and xenobiotic-induced immunotoxicity was accompanied by derangements in oxidative stress parameters, such as enhancements in MDA levels and lowering of GSH/SOD levels in the blood (Koner *et al.*, 1997; Ray and Gulati, 2007; Gulati *et al.*, 2007).

VII. IMMUNOTOXICITY OF CHEMICAL WARFARE AGENTS

A chemical warfare agent (CWA) is a substance which is intended for use in military operations to kill, seriously injure, or incapacitate people because of the severe pathophysiological changes induced by them in various body systems. A United Nations report from 1969 defines chemical warfare agents as ''chemical substances, whether gaseous, liquid or solid, which might be employed because of their direct toxic effects on man, animals and plants''. However, the Chemical Weapons Convention defines chemical weapons as including not only toxic chemicals but also ammunition and equipment for their dispersal. Toxic chemicals are stated to be ''any chemical which, through its chemical effect on living processes, may cause death, temporary loss of performance, or permanent injury to people and animals''. Normally, they are either liquids or solids.

Chemical agents have been used in war since time immemorial. In 600 BC Helleborus roots were used successfully by the Athenians to contaminate water supplies during the siege of Kirrha. Spartans ignited pitch and sulfur to create toxic fumes during the Peloponnesian War in 429 BC. The uses of CWAs in battlefields reached a peak during World War I and the French were the first to use ethylbromoacetate. It was followed by *o*-dianisidine chlorosulphonate, chloroacetate, chlorine, phosgene, hydrogen cyanide, diphenylchloroarsine, ethyl- and methyldichloroarsine, and sulfur mustard resulting in nearly 90,000 deaths and over 1.3 million casualties (Eckert, 1991). CWAs were most brutally used by the Germans in the gas chambers for mass genocide of Jews during World War II, and have been used intermittently both in war, as in the Iraq–Iran War, as well as in terrorist attacks in the Japanese subway stations. It is estimated that nearly 100,000 US troops may have been exposed to CWAs during operation Desert Storm (Chauhan *et al.*, 2008).

CWAs have been widely condemned since they were first used on a massive scale during World War I. However, they are still stockpiled and used in many countries as they are cheap and relatively easy to produce, and can cause mass casualties. Although the blood agent CK is extremely volatile and undergoes rapid hydrolysis, the degradation of three types of vesicant CWAs, the sulfur mustards, nitrogen mustards, and Lewisite, results in persistent products. For

example, sulfonium ion aggregates formed during hydrolysis may be persistent and may retain vesicant properties. The nerve agents include the V agent VX as well as three G agents (tabun, sarin, and soman). VX gives rise to two hydrolysis products of possible concern: EA 4196, which is persistent, and EA 2192, which is highly toxic and is possibly persistent under certain limited conditions (Small, 1984). Thus, their long-term persistence in the body may lead to alterations in the immune system of the exposed population.

CWAs can be classified in many different ways. There are, for example, volatile substances, which mainly contaminate the air, or persistent substances, which are nonvolatile and therefore mainly cover surfaces. CWAs mainly used against people may also be divided into lethal and incapacitating categories. A substance is classified as an incapacitating agent if less than 1/100 of the lethal dose causes incapacitation, e.g. through nausea or visual problems. The limit between lethal and incapacitating substances is not absolute but refers to a statistical average. Chemical warfare agents are generally classified according to their principal target organs.

1. **Organophosphate (OP) nerve agents.** These agents are extremely toxic compounds that work by interfering with the nervous system, and include soman, sarin, cyclosarin, tabun, and VX.
2. **Blister agents/vesicants.** These compounds severely blister the eyes, respiratory tract and skin on exposure, and include nitrogen mustard, sulfur mustard, Lewisite, etc.
3. **Choking agents.** These agents cause severe irritation primarily affecting the respiratory tract, and include phosgene, ammonia, methyl bromide, methyl isocyanate, etc.
4. **Blood agents.** These agents are absorbed into the blood and interfere with the oxygen carrying capacity, e.g. arsine, cyanides, carbon monoxide, etc.

Very few studies have been conducted to explore the immunomodulation and immunotoxic potential of CWAs, and there is little evidence that these drugs are associated with such undesirable, immunologically significant effects. The reason may be due to confounding factors such as stress, nutritional status, lifestyle, co-medication, and genetics (Vos *et al.*, 1999). The exposure to CWA can result in immunodepressed conditions on the one hand and to allergic and autoimmune diseases on the other. Few conventional compounds have been shown to induce unexpected enhancement of immune competence. However, introduction of biotechnologically manufactured agents like cytokines has been shown to induce unwanted immunostimulation. Drug-induced hypersensitivity reactions and autoimmune disorders are a major concern, whereas some of these chemicals also result in immunosuppression. In particular, impaired activity of the first line of defense of the natural immune system can have disastrous consequences. These are generally not influenced by the genetic predisposition of the exposed individual, but on actual outbreak of infections and the general immune status prior to exposition. This explains why immunosupressive xenobiotics are most likely to have clinical consequences in immunocompromised individuals such as young children, the elderly, and transplant recipients.

A. Nerve Agents

Nerve agents are highly toxic organophosphorus compounds (OPs) which represent potential threats to both military and civilian populations, as evidenced in recent terroristic attacks in Japan (Ohtomi *et al.*, 1996). Commonly known as nerve agents or nerve gases, these are the deadliest of CWAs. These agents have both chemical names as well as two-letter NATO codes. These are categorized as G series agents: GA (tabun), GB (sarin), GD (soman), GF (cyclosarin), and V series agents: VE, VG, VM, and VX, the letter ''G'' representing the country of origin ''Germany'' and letter ''V'' possibly denoting ''Venomous''. Their initial effects occur within 1–10 min of exposure followed by death within 15–30 min for sarin, soman, and VX, and within 30–60 min for tabun. The ease and low cost of production make sarin gas a tool of mass destruction in the hands of terrorist groups and rogue nations. While people in the immediate vicinity of a sarin attack may receive neurotoxic doses, people remote from the vicinity are likely to receive subclinical exposures.

Short- and long-term health effects from exposure to OP nerve agents and insecticide nerve agents are compiled on the basis of scientific literature published on health effects in humans and animal studies. Four distinct health effects are identified: acute cholinergic toxicity; OP-induced delayed neuropathy (OPIDN); subtle long-term neuropsychological and neurophysiological effects; and a reversible muscular weakness called ''intermediate syndrome''. Each effect has data suggesting threshold exposure levels below which it is unlikely to be clinically detectable. High-level exposure results in definitive cholinergic poisoning; intermediate-level threshold cholinergic effects include miosis, rhinorrhea or clinically measurable depression of cholinesterase; and low-level exposure results in no immediate clinical signs or symptoms. Threshold exposure levels for known long-term effects from OP nerve agent are at or above intermediate-level exposure (Brown and Brix, 1998). However, subclinical doses of sarin cause subtle changes in the brain, and subclinical exposure to sarin has been proposed as an etiology to the Gulf War Syndrome.

The wide use of cholinesterase inhibitors in various spheres of human activities and the risk of acute and chronic intoxications associated with this process prompted investigation of the role of acetylcholinesterase (AChE) and nonspecific esterases in the immunotropic effects of these chemicals. They irreversibly bind to AChE that normally catalyzes the hydrolysis of acetylcholine (ACh) at the

cholinergic synapses and neuromuscular junctions (NMJs). The inhibition of degradation results in accumulation of ACh in the cholinergic synapses, causes the overstimulation of peripheral as well as central cholinergic nervous systems, and is clinically manifested as acute cholinergic crisis (convulsions, respiratory failure, and/or death) (Marrs, 1993; Taylor, 2006).

1. Immunotoxicity

Kalra *et al.* (2002) suggested that low doses of sarin are highly immunosuppressive, and suppress glucocorticoid production. The effects of sarin exposure on the immune system are attenuated by ganglionic blockers and decreased glucocorticoid level may be a biomarker for cholinergic toxicity. In addition, nerve agents cause the activation of multiple noncholinergic neurotransmitter systems in the central nervous system (CNS) thus resulting in mutagenic, stressogenic, immunotoxic, hepatotoxic, membrane, and hematotoxic effects (Bajgar, 1992). The CNS and the immune system communicate bidirectionally, and cholinergic agents modulate the immune system. The ability of OP compounds to induce an alteration of the immune system was primarily demonstrated in animals or humans exposed to OP insecticides (OPIs). The results provide evidence that, especially neutrophil function, natural killer cell, cytotoxic T cell and humoral immune functions, and spontaneous as well as mitogen-induced lymphocyte proliferation, are altered in animals or humans exposed to OP compounds (Casale *et al.*, 1984; Hermanowitz and Kossman, 1984; Li *et al.*, 2002; Newcombe and Esa, 1992). In addition, a decreased number of cells in the spleen and thymus (Ladics *et al.*, 1994), an inhibition of chemotaxis in neutrophils (Ward, 1968), inhibition of monocyte accessory functions or inhibition of interleukin-2 production (Casale *et al.*, 1993; Pruett and Chambers, 1988) were reported following the exposure to OPs, at relatively high toxic doses.

Lee *et al.* (1979) were the first to draw attention to the possible effects of OPs on human leukocyte function. They demonstrated that lymphocyte proliferation to phytohemaglutinin *in vitro* was decreased in the presence of OPs. Although most of the studies described the results of OPI exposure, there are studies about the immunotoxic effects of highly toxic nerve agents and their by-products. Marked impairment in neutrophil chemotaxis and neutrophil adhesion and a reduction in the natural killer cell and cytotoxic T cell function were observed in workers exposed to OPIs and by-products of sarin (Hermanowitz and Kossman, 1984; Newcombe and Esa, 1992; Li *et al.*, 2002). Kant *et al.* (1991) documented a decrease in the weight of thymus, an important immune organ in severely affected soman survivors, but other tests of immune function did not show differences between control and soman-exposed rats. Samnaliev *et al.* (1996) described a decrease in the number of plaque forming cells in soman-exposed rats after the administration of sheep red blood cells as an antigen. However, Johnson *et al.* (2002) demonstrated that OP-induced modulation of immune functions can involve not only their suppression but also their activation. Similar activation of some immune functions involving ''acute phase response'' such as an increase in the synthesis of acute phase proteins, increase in release of histamine from basophile leukocytes and activation of macrophages were observed following the exposure to soman (Sevaljevic *et al.*, 1992; Newball *et al.*, 1986). Although most of the studies dealt with exposure to high doses, Kassa *et al.* (2004) confirmed that not only symptomatic but also asymptomatic doses of nerve agent sarin were able to modify various immune functions. The proportion of T lymphocytes was found to be decreased, while the B cell levels were raised. However, sarin significantly suppressed nonspecific *in vitro* stimulated proliferation of both T and B cells, which suggests that it can also block normal immune response to infection. While the lymphocyte mediated immunity is rather suppressed, the peritoneal as well as alveolar macrophages and NK cells were activated after exposure to both levels of sarin, which was explained to be the result of compensatory reactions of immune functions rather than the result of direct effects of inhalation.

Immunosuppression may result from direct action of acetylcholine upon the immune system or it may be secondary to the toxic chemical stress associated with cholinergic poisoning (Pruett *et al.*, 1992). Further, immunomodulation at low levels seems to be very complex and it is suggested that there are probably other protein targets very sensitive to some anticholinesterases including nerve agents. However, the function of these protein targets is not yet known (Ray, 1998). Some immune functions are probably stimulated due to the development of ''acute phase response'' generally characterized for inflammatory reaction of OP-exposed organism (Sevaljevic *et al.*, 1989, 1992). Other immune functions are suppressed due to immunotoxicity of OP compounds. Although these findings are difficult to extrapolate directly to low-level exposures to nerve agents, they indicate that subtle alteration of immune system could also occur in humans at exposure levels which do not cause any clinical manifestation. Post-intoxication immunodeficiency can promote infectious complications and diseases.

It has been shown that T lymphocytes have AChE located on the plasma membrane, while B cells are esterase negative (Szelenyi *et al.*, 1982). Thus, AChE inhibition by toxic agents in sublethal doses may play an important role in immunodeficiency following exposure to nerve gases. Zabrodskii *et al.* (2003) showed inhibition of AChE in T cells and the decrease in the number of esterase-positive T lymphocytes (and, to a certain extent, in monocytes and macrophages) directly correlated with suppression of T cell-dependent antibody production and to the degree of DTH reduction, on exposure to dimethyl dichlorovinyl phosphate, sarin, VX, lewisite, tetraethyl lead, and dichloroethane. This presumably involves the loss of some functions by T

lymphocytes (e.g. by Th1 cells), which leads to attenuation of T-dependent immune reactions. This can be explained by excessive acetylcholine (ACh) stimulation of muscarinic and nicotinic receptors present on T lymphocytes, as a result of which the optimal cAMP to cGMP ratio in immunocytes, essential for their proliferation and differentiation, is distorted (Richman and Arnason, 1979). Thus, the anticholinesterase effect of lewisite, TEL, and DCE may be one of the important mechanisms in the formation of T cell mediated immunodeficiency.

B. Blister or Vesicant Agents

These agents act on skin and other epithelial tissues and severely blister the eyes, respiratory tract, and internal organs, and also destroy different substances within cells of living tissue. The symptoms are variable depending upon the compound and the sensitivity of the individual. Acute mortality is low; however, they can incapacitate the enemy and overload the already burdened health care services during war time. Some of these agents are HD (sulfur mustard), HN (nitrogen mustard), L (Lewisite), and CX (phosgene oximine).

Sulfur mustard (SM) was the most widely used chemical warfare agent (CWA) in the Iran–Iraq War, resulting in over 100,000 chemical casualties between 1980 and 1988. It acts as an alkylating agent with long-term toxic effects on several body organs, mainly the skin, eyes, and respiratory system (Willems, 1989). The extent of tissue injury depends on the duration and intensity of exposure. When absorbed in large amounts, SM can damage rapidly proliferating cells of bone marrow and may cause severe suppression of the immune system (Willems, 1989).

1. Immunotoxicity

Evidence that SM causes immunosuppression in humans has emerged from several lines of investigation. The earliest evidence came from clinical observations of humans directly exposed to sulfur mustard during World War I, who showed significant changes (quantitative and qualitative) in the circulating elements of the immune system. Stewart (1918) studied ten fatal cases of mustard poisoning and observed striking depression of bone marrow production of white blood cells. Among the sulfur mustard casualties during the Iran–Iraq conflict leukopenia accompanied by total bone marrow aplasia and extensive losses of myeloid stem cells was the most common finding (Balali-Mood, 1984; Eisenmenger *et al.*, 1991). These findings provide further evidence of an association between suppression of immunologic functions and an increased incidence of infectious disease.

SM has been widely used during Iran–Iraq conflict and there are many reports of influence of SM on the respiratory system, gastrointestinal system, and endocrine system as well as the immune system (Balali-Mood, 1984; Balali-Mood and Farhoodi 1990; Emad and Razaian 1997; Sasser *et al.*, 1996; Budiansky, 1984). The influence of SM on the immune system has been the subject of many researchers since 1919 (Krumbhaar and Krumbhaar, 1919; Hektoen and Corper 1921). Early investigations on SM casualties during the Iran–Iraq War showed decreased immunoresponsiveness, expressed as leukopenia, lymphopenia, and neutropenia, as well as hypoplasia and atrophy of the bone marrow (Willems, 1989; Tabarestani *et al.*, 1990; Balali-Mood *et al.*, 1991). Chronic exposure to SM has been associated with the impairment of NK cells among workers of poison gas factories in Japan (Yokogama, 1993). Similarly, cell mediated immunity was found to be suppressed following mustard gas exposure (Zandieh *et al.*, 1990).

Leukopenia has been the first manifestation to appear within the first days of post-exposure. Thrombocytopenia and anemia followed later if the patients survived (white blood cells of some patients dropped to less than 1,000 per cm^3). Although most of these patients suffered skin burns, clinicians reported cases that had minor skin lesions and yet developed leukopenia. Bone marrow biopsies revealed hypocellular marrow and cellular atrophy involving all elements (Willems, 1989). Studies on the status of immunocompetent cells in the blood of patients exposed to sulfur mustard showed that T cell and monocyte counts dropped in 54% and 65% of the patients, respectively, from day 1 and up to 7th week post-exposure (Hassan and Ebtekar, 2002). Eosinophil counts dropped in 35% and neutrophil numbers in 60% of the patients. B lymphocyte counts were normal up to 7th week (Manesh, 1986). The majority of the patients showed increased levels of IgG and IgM during the 1st week, but the percentage decreased over the next 6 months. The percentage of patients with increased levels of C3, C4, and CH50 was somewhat higher than of healthy controls during the 1st week and up to 6th month (Tabarestani *et al.*, 1990) and remained higher 3 years post-exposure especially in the severely affected group. Eight years after exposure there was a significant increase in the number of atypical leukocytes (such as myelocytes). The severely affected group presented with significantly lower CD56 NK as well as CD4 and CD8 counts compared with healthy controls (Yokogama, 1993). Hassan and Ebtekar (2002) reported that there was no major difference between the severely affected patients and healthy controls concerning CD19 B cells, CD14 monocytes, and CD15 granulocytes. The moderately and mildly affected patients did not significantly differ in their leukocyte subset counts from the control group 8 years after exposure (Mahmoudi *et al.*, 2005). Follow-up studies on the clinical conditions of exposed Iranian victims still show that they suffer from three major problems: recurrent infection, septicemia and death, respiratory difficulties and lung fibrosis, as well as a high incidence of malignancies, septicemia, and death.

Hassan and Ebtekar (2002) suggested that patients with moderate clinical manifestations may be experiencing a shift from Th1 to Th2 cytokine patterns since leukocyte cultures from this patient group showed a decrease in IFN-γ

levels. When absorbed in large amounts, SM can damage rapidly proliferating cells of bone marrow and may cause severe suppression of the immune system (Sasser *et al.*, 1996). Moreover, this alkylating agent has been reported to produce short- and long-term suppression of antibody production in both animals and humans. It also affects complement system factors C3 and C4. Incidences of acute myelocytic and lymphocytic leukemia are reported to be 18 and 12 times higher in patients exposed to SM, in comparison with the normal group (Zakeripanah, 1991). Willems (1989) reported that exposure to SM could result in the impairment of human immune function, especially in the number of B and T lymphocytes. Hence, SM is still a potential threat to the world and effective therapeutic measures must be taken for the relief of the victims of this incapacitating agent. Ghotbi and Hassan (2002) showed that the percentage of NK cells, playing an important role in cellular immunity, was significantly lower in severe patients than in the control group. Studies on animal models have shown that alkylating agents such as SM mainly affect B cells, which is why hypogammaglobulinemia is one of the main features in animal models, whereas studies on human cases, following a treatment with cytotoxic drugs, suggest that low-dose exposure to alkylating agents impairs cellular immunity and high-dose exposure to such agents impairs both cellular and humoral responses (Marzban, 1989; Malaekeh *et al.*, 1991). There are reports suggesting that sulfur mustard can produce toxicity through the formation of reactive electrophobic intermediates, which in turn covalently modify nucleophilic groups in biomolecules such as DNA, RNA, and protein (Malaekeh *et al.*, 1991), resulting in disruption of cell function, especially cell division (Crathorn and Robert, 1966). As a result, these agents are particularly toxic to rapidly proliferating cells including neoplastic, lymphoid, and bone marrow cells. Mahmoudi *et al.* (2005) reported higher IgM levels after 16 to 20 years of exposure to SM, compared to the control group. A significant decrease in the number of NK cells in severe patients is probably due to the destructive effect of this alkylating agent on NK cell precursors in bone marrow. However, activity of NK cells was found to be noticeably above normal which possibly compensates for the reduction in the number of these cells.

Recently, Korkmaz *et al.* (2006) explained the toxicodynamics of sulfur mustards in three steps: (1) binding to cell surface receptors; (2) activation of ROS and RNS leading to peroxynitrite ($OONO^-$) production, and (3) $OONO^-$-induced damage to lipds, proteins, and DNA, leading to polyadenosine diphosphate ribose (PARP) activation. This could provide a lead for devising strategies for protection against/treatment of mustard toxicity.

In conclusion, the results suggest that exposure to SM causes a higher risk of opportunistic infections, septicemia, and death following severe suppression of the immune system especially in the case of lesions and blisters produced by these agents. As alkylating agents, they form covalent linkages with biologically important molecules, resulting in disruption of cell function, especially cell division. As a result, these agents are particularly toxic to rapidly proliferating cells including neoplastic, lymphoid, and bone marrow cells. However, there is still a paucity of information regarding the long-term immunosuppressive properties of SM in the setting of battlefield exposure to this agent.

C. Choking Agents

Choking agents act on the pulmonary system causing severe irritation and swelling of the nose, throat, and lungs, e.g. CG (phosgene), DP (diphosgene), chlorine, and PS (chloropicrin). These inhalational agents damage the respiratory tract and cause severe pulmonary edema in about 4 h, leading to death. The effects are variable, rapid, or delayed depending on the specific agent (Gift *et al.*, 2008).

Phosgene was first used as a chemical weapon in World War I by Germany and later as offensive capability by French, American, and British forces. In this conflict, phosgene was often combined with chlorine in liquid-filled shells, so it was difficult to state the number of casualties and deaths attributable solely to phosgene. In military publications, it has been referred to as a choking agent, pulmonary agent, or irritant gas. Since World War I, phosgene has rarely been used by traditional militaries, but the extremist cult Aum Shinrikyo used this agent in an attack against the Japanese journalist Shouko Egawa in 1994. Nowadays, phosgene is primarily used in the polyurethane industry for the production of polymeric isocyanates (USEPA, 1986). Phosgene is also used in the polycarbonate industry and in the manufacture of carbamates and related pesticides, dyes, pharmaceuticals, and isocyanates.

As mentioned earlier, the primary exposure route for phosgene is by inhalation. Suspected sources of atmospheric phosgene are fugitive emissions, thermal decomposition of chlorinated hydrocarbons, and photo-oxidation of chloroethylenes. Individuals are most likely to be exposed to phosgene in the workplace during its manufacture, handling, and use (USEPA, 1986). Phosgene is extremely toxic by acute (short-term) inhalation exposure. Severe respiratory effects, including pulmonary edema, pulmonary emphysema, and death have been reported in humans. Severe ocular irritation and dermal burns may result following eye or skin exposure. Chronic inhalation exposure to phosgene has been shown to result in some tolerance to the acute effects noted in humans, but may also cause irreversible pulmonary changes of emphysema and fibrosis (US Department of Health and Human Services, 1993).

Primarily because of phosgene's early use as a war gas, many exposure studies have been performed over the past 100 years to examine the effects and mode of action of phosgene following a single, acute (less than 24 h) exposure. Many studies have examined the effects of acute phosgene

exposure in animals but the human data are limited to case studies following accidental exposures.

Most studies were performed in rodents and dogs, with exposure concentrations ranging between 0.5 and 40 ppm (2–160 mg/m^3) and duration intervals ranging from 5 min to 8 h. Acute exposure studies in animals suggest that rodent species may be more susceptible to the edematous effects of phosgene acute exposure than larger species with lower respiratory volumes per body weight such as dogs and humans (Pauluhn, 2006; Pauluhn *et al.*, 2007).

Pauluhn *et al.* (2007) reported that acute phosgene exposure results in increased lung lavage protein, phospholipid content, enzyme levels, number of inflammatory cells, and lethality (LC_{50}). Rats seem to be able to survive approximately three-fold higher levels of lung edema than humans (100-fold versus 30-fold), thus rat responses in short- and long-term assays may still be relevant to humans even if it is ultimately shown that rats produce higher levels of edema following acute phosgene exposure.

1. Immunotoxicity

Acute exposure to phosgene has been shown to result in immunosuppression in animals, as evidenced by an increased susceptibility to *in vivo* bacterial and tumor cell infections (Selgrade *et al.*, 1989) and viral infection (Ehrlich and Burleson, 1991) as well as a decreased *in vitro* virus-killing and T cell response (Burleson and Keyes, 1989). Selgrade *et al.* (1989) reported that a single 4 h exposure to phosgene concentrations as low as 0.025 ppm significantly enhanced mortality due to streptococcal infection in mice. Furthermore, when the exposure time was increased from 4 to 8 h, a significant increase in susceptibility to streptococcus was seen at an exposure concentration of 0.01 ppm.

Selgrade *et al.* (1995) administered *Streptococcus zooepidemicus* bacteria via an aerosol spray to the lungs of male Fischer 344 rats immediately after phosgene exposure and measured the subsequent clearance of bacteria. They also evaluated the immune response, as measured by an increase in the percentage of polymorphonuclear leukocytes (PMN), in lung lavage fluid of uninfected rats similarly exposed to phosgene. This experiment showed that all phosgene concentrations from 0.1 to 0.5 ppm impaired resistance to bacterial infection and that the immune response is stimulated by phosgene exposure. After 4 weeks following exposure, bacterial resistance as well as immune response returned to normal.

Yang *et al.* (1995) also reported a decrease in bacterial clearance in the lungs at 24 h after infection following a single 6 h exposure to phosgene concentrations of 0.1 and 0.2 ppm. In comparison with single exposures, the multiple daily exposures extending to 4 and 12 weeks in the Selgrade *et al.* (1995) report showed a slight enhancement of effect in the 0.1 ppm group at 24 h post-infection, but no ''adaptation'', or lessening of the effect. Yang *et al.* (1995) found that if the bacteria are administered 18 h after single phosgene exposures rather than immediately, the clearance is normal which indicates that recovery from the toxic effect of phosgene is rapid.

When inhaled, phosgene either is rapidly hydrolyzed to HCl and CO_2 and exhaled (Schneider and Diller, 1989; Diller, 1985) or penetrates deep into the lungs and is eliminated by rapid reactions with nucleophilic constituents of the alveolar region (Pauluhn *et al.*, 2007). As phosgene is electrophilic, it reacts with a wide variety of nucleophiles, including primary and secondary amines, hydroxy groups, and thiols. In addition, it also reacts with macromolecules, such as enzymes, proteins, or other polar phospholipids, resulting in a marked depletion of glutathione (Sciuto *et al.*, 1996) and forms covalent adducts that can interfere with molecular functions. Phosgene interacts with biological molecules through two primary reactions: hydrolysis to hydrochloric acid and acylation reactions. Although the hydrolysis reaction does not contribute much to its clinical effects, the acylation reaction is mainly responsible for the irritant effects on mucous membranes. The acylation reactions occur between highly electrophilic carbon molecules in phosgene and amino, hydroxyl, and sulfhydryl groups on biological molecules. These reactions can result in membrane structural changes, protein denaturation, and depletion of lung glutathione. Acylation reactions with phosphatidylcholine are particularly important as it is a major constituent of pulmonary surfactant and lung tissue membranes. Exposure to phosgene has been shown to increases the alveolar leukotrienes, which are thought to be important mediators of phosgene toxicity to the alveolar–capillary interface. Phosgene exposure also increases lipid peroxidation and free radical formation. These processes may lead to increased arachidonic acid release and leukotriene production. Proinflammatory cytokines, such as interleukin-6, are also found to be substantially higher 4–8 h after phosgene exposure. In addition, studies have shown that post-exposure phosphodiesterase activity increases, leading to decreased levels of cyclic AMP. Normal cAMP levels are believed to be important for maintenance of tight junctions between pulmonary endothelial cells and thus for prevention of vascular leakage into the interstitium. Oxygenation and ventilation both suffer, and breathing is dramatically increased.

Schneider and Diller (1989) and Diller (1985) reported that inhalation of phosgene at high concentrations results in a sequence of events, including an initial bioprotective phase, a symptom-free latent period, and a terminal phase characterized by pulmonary edema. The first is an immediate irritant reaction likely caused by the hydrolysis of phosgene to hydrochloric acid on mucous membranes, which results in conjunctivitis, lacrimation, and oropharyngeal burning sensations. This symptom complex occurs only in the presence of high-concentration (>3–4 ppm) exposures but does not have any prognostic value for the timing and severity of later respiratory symptoms. The most important finding to identify during this stage is a laryngeal

irritant reaction causing laryngospasm, which may lead to sudden death. The irritant symptoms last only a few minutes and then resolve as long as further exposure to phosgene ceases.

The second phase, when clinical signs and symptoms are generally lacking, may last for several hours after phosgene exposure. The duration of the latent phase is an extremely important prognostic factor for the severity of the ensuing pulmonary edema. Patients with a latent phase of less than 4 h have a poor prognosis. Increased physical activity may shorten the duration of the latent phase and worsen the overall clinical course. Unfortunately, there are no reliable physical examination findings during the latent phase to predict its duration. However, histologic examination reveals the beginnings of an edematous swelling, with exudation of blood plasma into the pulmonary interstitium and alveoli. This may result in damage to the alveolar type I cells and a rise in hematocrit. The length of this phase varies inversely with the inhaled dose. The third clinical phase peaks approximately 24 h after an acute exposure and if lethality does not occur, recedes over the next 3–5 days. In the third clinical phase of phosgene toxicity, the accumulating fluid in the lung results in edema. Oxygenation and ventilation both suffer, and the breathing is dramatically increased. Often positive end expiratory pressure (PEEP) is required to stent open alveoli that would otherwise collapse and result in significant ventilation/perfusion (V/Q) mismatch. This hyperventilation causes the protein-rich fluid to take on a frothy consistency. A severe edema may result in an increased concentration of hemoglobin in the blood and congestion of the alveolar capillaries.

Increased levels of protein in bronchoalveolar lavage have been shown to be among the most sensitive endpoints characterizing the early, acute effects of phosgene exposure, and are rapidly reduced after the cessation of exposure (Sciuto, 1998; Schiuto *et al.*, 2003). With continuous, chronic, low-level phosgene exposure, there may be transition of edema to persistent cellular inflammation leading to the synthesis of abnormal Type I collagen and pulmonary fibrosis. An increased synthesis of Type I relative to Type III collagen can lead to chronic fibrosis (Pauluhn *et al.*, 2007). Surfactant lipids are important for maintaining alveolar stability and for preventing pulmonary edema. Pauluhn *et al.* (2007) reported that the induction of surfactant abnormalities following phosgene exposures is a key pathophysiological event leading to pulmonary edema and chronic cellular inflammation, leading to the stimulation of fibroblasts and the synthesis of ‘‘abnormal’’ collagen in pulmonary fibrosis. As discussed earlier, a breach in the chemical layer of defense followed by pulmonary edema may lead to a cascade of other immunological responses/reactions. There are limited studies, in both humans and experimental animals, to evaluate immunotoxicity of chronic low-level environmental exposures to phosgene. The lack of studies examining the effects in humans or laboratory animals from chronic exposure to phosgene is a concern and the sequela of effects leading to phosgene-induced pulmonary fibrosis is not well understood.

D. Blood Agents

Agents like SA (arsine), cyanide, and carbon monoxide are absorbed into the blood and affect its oxygen carrying capacity, and are thus termed blood agents. They are highly volatile and rapidly acting, and produce seizures, respiratory failure, and cardiac arrest. Hydrogen cyanide has been known as a potent toxicant for over 200 years. It was used as a chemical warfare agent during World War I by France. Although it is highly volatile (and was later considered ‘‘militarily useless’’ because of its volatility), no deaths from its military use during World War I were ever reported. After World War II, the importance of hydrogen cyanide as a chemical warfare agent diminished rapidly, primarily as a result of the rise of nerve agents. Although reduced in importance, there are some reports of hydrogen cyanide being used as a war gas by Vietnamese forces in Thailand territories and during the Iran–Iraq War in the 1980s (Sidell, 1992).

Hydrogen cyanide can be detoxified rapidly by humans. It is very volatile and massive amounts of the gas are needed for it to be effective as a chemical warfare agent. Cyanide is primarily an environmental contaminant of industrial processes. It is used in the metal-processing industry for electroplating, heat treating, and metal polishing and can be found in waste waters from many mining operations that use cyanide compounds in the extraction of metals, such as gold and silver, from ore.

The acute toxicity of cyanide has been well documented in humans and experimental animals. Symptoms of toxicity in humans include headache, breathlessness, weakness, palpitations, nausea, giddiness, and tremors (Gupta *et al.*, 1979). Depending on the degree of intoxication, symptoms may include ‘‘metallic’’ taste, anxiety and/or confusion, headache, vertigo, hyperpnea followed by dyspnea, convulsions, cyanosis, respiratory arrest, bradycardia, and cardiac arrest. Death results from respiratory arrest (Berlin, 1977). Onset is usually rapid. Effects on inhalation of lethal amounts may be observed within 15 s, with death occurring in less than 10 min. Hydrogen cyanide should be suspected in terrorist incidents involving prompt fatalities, especially when the characteristic symptoms of nerve agent intoxication are absent. Chronic exposure to low-level cyanide can result in neuropathies, goiter, and diabetes. Cyanide and derivatives prevent the cells of the body from using oxygen. Cyanide acts by binding to mitochondrial cytochrome oxidase, blocking electron transport, thus inhibiting enzymes in the cytochrome oxidase chain and in turn blocking oxygen use in metabolizing cells and preventing the use of oxygen in cellular metabolism. These chemicals are highly toxic to cells and in high doses may result in death. Cyanide is more harmful to the heart and brain as these organs require large amounts of oxygen.

1. Immunotoxicity

There are very few reports on immunotoxicity of the compound; however, acrylonitrile (vinyl cyanide, VCN), an environmental pollutant which is metabolized to cyanide, has been shown to be an animal and human carcinogen particularly for the gastrointestinal tract (Mostafa *et al.*, 1999; National Toxicology Program Technical Report Series, 2001). Earlier Hamada *et al.* (1998) evaluated the systemic and/or local immunotoxic potential of VCN and demonstrated that VCN induces immunosuppression as evident by a decrease in the plaque forming cell (PFC) response to SRBCs (sheep red blood cells), a marked depletion of spleen lymphocyte subsets, as well as bacterial translocation of the normal flora leading to brachial lymph node abscess. These results suggested that VCN has a profound immunosuppressive effect which could be a contributing factor in its gastrointestinal tract carcinogenicity.

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

The immune system is extremely vulnerable to the action of xenobiotics for several reasons. The immune response is associated with rapidly multiplying cells and synthesis of regulatory/effector molecules and the immune system works as an amplifier for this integrated information network. Immunologic tissue damage can result from activation of the cellular and biochemical systems of the host. The interactions of an antigen with a specific antibody or with effector lymphocytes trigger the sequence of humoral and cellular events to produce the pathophysiologic effects that lead to tissue injury or disease. Stem cells often appear to be sensitive targets for therapeutic and environmental toxicants, most likely because of their rapid proliferation. Xenobiotics or various drugs that are toxic to the myelocytes of the bone marrow can cause profound immunosuppression due to loss of stem cells.

Humans are now under sustained and increasing pressure of xenobiotics exposure. Xenobiotics can stimulate the immune system as antigens by provoking a substantial immune response. Even mild disturbances of this network could result in detrimental health effects. The influence of the xenobiotics on the immune system is either suppressive or enhancing. The former leads into immunosuppression with consequent increased susceptibility to infection and cancer. The latter is associated with the development of autoimmune reactivity such as delayed hypersensitivity, atopy, systemic or organ-specific immunopathology, and granulomas formation. It is likely that overall immunosuppressive effects of xenobiotics are caused by the interference with cellular proliferation and differentiation, downregulation of the cytokine signaling, and enhanced apoptosis of immune cells. In contrast, autoimmune reactions are induced by abnormal activation of immune cells followed by dysregulated production of cytokines resulting in harmful inflammatory response.

The field of immunotoxicology is new but developing rapidly. Attempts must be made to conduct basic research to address the cellular and molecular mechanism of immunomodulatory action of various xenobiotics. The newly emerging technologies such as genomics, proteomics, and bioinformatics will be certainly helpful to investigate the interactions between the immune system and xenobiotics in their full complexities. Toxic compounds may be antigenic or act as haptens and can evoke an antibody response. If these antibodies bind to the determinant on the parent molecule which is responsible for causing toxicity, then it can lead to the biological inactivation of the parent molecule and thereby prevent toxicity. This may constitute an immunological antidote approach to neutralize the toxicity of certain compounds. Thus, passive administration of the antibodies may be used to prevent the toxic effects of the specific compound and this approach may be useful in biological or chemical warfare to protect against the toxicity of known chemicals or toxins. The antibodies can also be used to protect industrial workers against the toxic effects of known chemicals or gases during accidental exposure. Although this assumption seems logical, it will involve elaborate and time-consuming research to identify the site of the parent molecule responsible for causing toxicity, to chemically link the molecule with a large protein molecule which should be immunogenic but not toxic, and to screen various antibodies raised for their capacity to prevent the toxicity of the compound.

In view of the current global scenario, it appears that CWAs are likely to be used in different types of warfare, and it is unlikely their usage will cease in the near future. CWAs for warfare and other related activities are here to stay. These agents are not only inexpensive but easy to disseminate with the help of unsophisticated devices. Hence the medical profession should assemble on a common platform through globally recognized organizations like the WHO and put in efforts to monitor, research, and study the scientific and medical aspects of CWAs in the interest of humankind. Guidelines should be regularly updated on the prevention and management of CWA-induced insults and thereby aim to reduce morbidity and mortality. Nations worldwide should ensure that adequate supplies of antidotes (wherever available), protective equipment, and decontamination devices are available in adequate quantities and at all times. The need of the hour is a multisectorial approach involving health, defense, agriculture, and environmental specialists, with clearly defined roles of each, for establishing and maintaining effective, robust, and sustainable strategies to countermeasure this threatening situation.

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CHAPTER 41

Dermal Toxicity of Sulfur Mustard

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I. INTRODUCTION

Skin integrity is in part determined by the interaction of a number of proteins that form a continuum of molecules linking together to ensure the epidermis and dermis are tightly attached to one another. These macromolecules include the keratins that form the intermediate filaments in the cytoplasm of keratinocytes, integrins which are made of two subunits and found in the hemidesmosomes of the basal keratinocyte membrane, and a variety of laminins, collagens, and their receptors, situated both in the basement membrane that separates the epidermis from the dermis, and in the dermis itself. Any chemical agent that disrupts this continuum of linking proteins will result in a breach in skin integrity and ensuing histopathology (Uitto *et al.*, 2007). One such compound is the alkylating agent sulfur mustard [bis(2-chloroethyl) sulfide; HD; SM], which causes detachment of the epidermis from the dermis. (This chapter uses the abbreviation SM for sulfur mustard since in civilian dermatological research HD is the abbreviation for the hemidesmosome, an important morphological structure found in the cell membrane of keratinocytes.) Skin exposure to SM starts a complex series of events with a host of normal skin responses to wounding which interact with, influence, and regulate each other to result in cutaneous toxicity. Various mediators of injury that regulate inflammation, immune responses, cell death, and a number of signaling pathways have been implicated in the process. This chapter describes our current knowledge of the cutaneous actions of SM, discussing the basic mechanism of action and mediators involved, to provide for the reader a comprehensive understanding of the histopathology of SM-exposed skin. The injury process is described and SM-induced injury is compared to other types of wound injury. Additionally, various SM-injury models are described and potential therapeutic countermeasures discussed.

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II. BACKGROUND

A. Military Use

Sulfur mustard, a vesicant or blistering agent, has been used intermittently as a chemical warfare agent since 1917 when Germany first used it as a weapon against British soldiers in Ypres, Belgium. Subsequently in the 1930s it was reported to be used by Italy against Abyssinia (Ethiopia), Poland against Germany, and Japan against the Chinese. During the 1960s SM was allegedly used by Egypt against Yemen. The last well-documented use in the 1980s was by Iraq against Iran and the Kurds (Papirmeister *et al.*, 1991a).

B. Wound Repair

Even repair of the simplest skin wound requires the coordination of a number of physiological processes and events. The tissue injury causes blood vessel disruption and an activation of the inflammatory pathway that releases a battery of mediators. Re-epithelialization requires activation of additional pathways and the initiation of specific events at specific times, such as extracellular matrix remodeling and the proliferation and migration of keratinocytes. If these events are not precisely coordinated, the end result can be delayed healing and/or scar formation. Since the repair process is complicated, a review of the wound healing events in dermal injury might be helpful. There are at least three phases to wound healing following dermal injury (Gurtner *et al.*, 2008). (1) Inflammation (first 24–48 h). This phase occurs early and involves activation of the coagulation cascade, inflammatory pathways, and immune system. These biological processes involve an attempt to maintain homeostasis by preventing blood and fluid loss, removing dead tissue, and preventing infection. (2) Wound repair phase (2–10 days after injury). This phase involves the proliferation and migration of several cell types. Keratinocytes migrate over the injured dermis, endothelial cells proliferate to form new blood vessels, and fibroblasts differentiate into myofibroblasts in order to initiate wound contraction. (3) Remodeling phase (2–3 weeks post-injury). This phase results in the final wound repair. All the activated cellular processes turn off. Many cell types undergo apoptosis or leave the wound site.

The extracellular matrix is remodeled and resumes normal functions.

These same basic phases are applicable to the injury caused by SM, but the pathology frequently is more severe than other types of dermal wounds and the basic timing of the various phases may be extended in SM injury (Papirmeister *et al.*, 1991a; Dacre and Goldman, 1996; Balali-Mood and Hefazi, 2006). Normal clothing provides little protection to the skin from a liquid or vapor exposure to SM. Once in contact with the skin, the lipophilic properties of SM allow it to rapidly penetrate the epidermal barrier, while its high reactivity and bifunctional nature lead to alkylation of a wide range of molecules (Figure 41.1). The ultimate injury caused by SM is highly dependent on the dose and length of exposure to the agent resulting in a wide range of histopathology. Erythema, resembling sunburn, may be the mildest and earliest form of skin injury (Figure 41.2), occurring 2 to 48 h post-SM exposure (USAMRICD, 2007). The erythema may be accompanied by pruritus or burning pain and small vesicles may eventually develop which coalesce to form bullae. Fluid-filled blisters increase in size, their color ranging from yellowish to tan. The fluid itself does not contain active alkylating agent, nor is it a vesicant. When SM is applied as a liquid, the exposure concentration is even higher than a vapor application and severe lesions are more likely. The resultant wounds are prone to secondary infections and may cause chronic ulcers that are resistant to wound repair. There may be permanent pigmentation changes in the skin after exposure to SM. Clearly, the effects of SM on skin are complex and involve many systems and pathways. The following sections attempt to address the mechanism of action of SM and the systems and pathways involved in an organized way.

III. PATHOGENESIS

The bulk of what is known about human skin injury from SM has been gathered from victims of the military use of the compound (Balali-Mood and Hefazi, 2006; Hefazi *et al.*, 2006; Requena *et al.*, 1988). Nevertheless, there are several reports where the cutaneous vesicating effects of SM were studied experimentally in human subjects (Papirmeister *et al.*, 1991a, b; Dacre and Goldman, 1996). In one comparative study (Daily *et al.*, 1994) 12 volunteers had forearm vapor cup applications of SM, trifunctional nitrogen mustard (HN3), and lewisite. The conclusions were

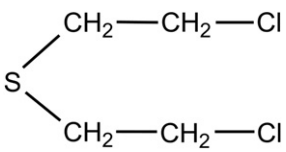

FIGURE 41.1. Chemical structure of sulfur mustard [bis(2-chloroethyl) sulfide].

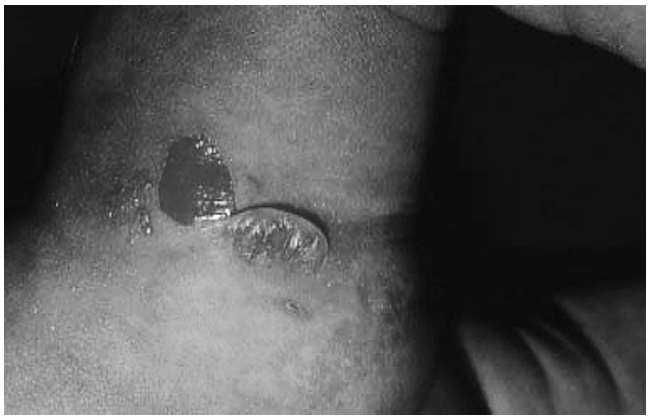

FIGURE 41.2. Fluid-filled bullous of patient with epidermolysis bullosa caused by truncated gamma-2 polypeptide chain of laminin-332 (formerly laminin 5, a heterotrimeric glycoprotein).

that SM exposure caused the largest vesicles formed, the earliest peak vesication, the longest healing time, and the most severe final scar. Lewisite was the second most potent agent and HN3 the least potent, although the HN3 lesions had the most edema of the three vesicants examined. The results confirmed the previously published studies which followed the development of skin pathogenesis after topical exposure to SM.

Clinical injury by SM has been extensively reviewed and will be summarized here. Sulfur mustard injury to human skin begins almost immediately after exposure when the highly reactive bifunctional SM directly alkylates resident proteins. However, recognizable skin pathology does not usually occur for several hours to a day after exposure. The very first physical sign of SM exposure is usually erythema which may or may not be associated with itching (Papirmeister *et al.*, 1991a). This is often followed later by distinct fluid-filled blisters that break open and become covered with a scab. Many researchers have noted that the blisters resemble those formed by epidermolysis bullosa (EB), a genetic or acquired skin pathology that results in a separation of the epidermis from the dermis (Figure 41.2). In both SM exposure and EB, there may be multiple rounds of blistering and healing in an individual (Balali-Mood and Hefazi, 2005; Pillay, 2008). In fact some of the therapeutic agents for EB are being tested as potential medical countermeasures against SM injury. These will be addressed later.

A. Cytotoxicity of Sulfur Mustard

While stable in lipophilic solvents, SM has a half-life of only 24 min at room temperature in aqueous physiological solutions since it rapidly reacts with water to form thiodiglycol and HCl. This rapid activation of SM in an aqueous environment also allows it to react with small molecules of biological interest, as well as proteins, carbohydrates, lipids, RNA, and DNA (Bartlett and Swain, 1949; Debouzy *et al.*, 2002; Noort *et al.*, 2002; Papirmeister *et al.*, 1991c).

The initial reaction involves the formation of a cyclic ethylene sulfonium ion which readily targets reactive groups on skin components, including sulfhydryls, phosphates, ring nitrogens, and carboxyl groups. As a bifunctional alkylating agent, SM forms monofunctional adducts and intra- and intermolecular crosslinks. Since it is so reactive and indiscriminate in its molecular targets, SM affects many pathways and is cytotoxic on many levels. There are at least three independent mechanisms of cytotoxicity that have been proposed and each will be addressed in separate sections.

1. Alkylation of DNA/Poly(ADP-Ribose) Polymerase (PARP) Activation

One of the major targets of SM alkylation is DNA which it can form both monofunctional adducts and bifunctional crosslinks (Papirmeister *et al.*, 1991c). Complementary DNA strand and intrastrand crosslinks occur after SM exposure (Walker, 1971). The crosslinking of cellular DNA underscores the importance of DNA repair pathways in preventing cytotoxicity of SM-exposed cells. These repair pathways include both the nucleotide and base excision pathways (Matijasevic and Volkert, 2007).

DNA alkylation can also lead to single and double strand DNA breaks. These breaks trigger activation of a family of nuclear repair enzymes called poly(ADP-ribose) polymerases (PARP) (Papirmeister *et al.*, 1985; Shall and de Murcia, 2000). While low levels of PARP activation may signal repair, excessive activity can deplete cells of PARP's major substrate, NAD^+. Depletion of NAD^+ in turn inhibits ATP production, which is essential for metabolism (Martens and Smith, 2008). Apoptosis or necrosis may result, depending upon level of ATP depletion, cell type and other factors (Rosenthal *et al.*, 2001). Preventing the depletion of NAD^+ by interfering with PARP activation has been the rationale for testing PARP inhibitors as therapeutic countermeasures (Debiak *et al.*, 2008). To date, the literature suggests this strategy has been unsuccessful *in vivo* (Casillas *et al.*, 2000b).

2. Reactions with Glutathione/Lipid Peroxidation

Another hypothesis explaining the alkylation effects of SM is that it reacts with the free radical scavenger glutathione (GSH). GSH is able to neutralize free radicals in the cytoplasm that might be harmful to the cytosol of cells, including free radicals that are by-products of oxidative phosphorylation. Glutathione peroxidase catalyzes the reaction of reduced glutathione with hydrogen peroxide. Depletion of intracellular GSH allows the accumulation of oxidants such as H_2O_2 which become abundant and actively contribute to lipid peroxidation and other types of cellular damage. Dermatotoxic agents such as ultraviolet radiation (UVA) and psoralens have been shown to deplete intracellular glutathione, resulting in cellular toxicity (Wheeler *et al.*, 1986). Furthermore, depletion of intracellular reduced glutathione by buthionine sulfoxamine sensitizes cells treated *in vitro* with 10 μM SM (Gross *et al.*, 1993). In other tissues, SM exposure has been shown to reduce glutathione peroxidase and glutathione reductase activity, potentially contributing to the depletion of reduced glutathione (Husain *et al.*, 1996; Jafari, 2007). In the skin, glutathione peroxidase gene expression is increased following SM treatment in the mouse ear vesicant model, suggesting that this enzyme is important in reducing oxidative stress due to SM exposure (Buxton *et al.*, 2001).

3. Reactions with Glutathione/Calcium Homeostasis

Depletion of GSH also affects calcium homeostasis; treatment of primary human epidermal keratinocytes with buthionine sulfoxamine depressed the level of reduced glutathione but increased intracellular Ca^{2+} (Ray *et al.*, 1993). Neuroblastoma cells treated *in vitro* with 0.3 mM SM maintained high cell viability for nearly 9 h, which then decreased with time. The decrease in cell viability was prefaced by an increase in free intracellular calcium that occurred between 2 and 6 h post-exposure (Ray *et al.*, 1995). Intracellular free calcium is a well-recognized marker of cell stress (Ruff and Dillman, 2007). Induction of intracellular calcium is thought to contribute to apoptosis induced by SM exposure in keratinocytes *in vitro* (Rosenthal *et al.*, 2003). Despite these findings, Sawyer's group has shown that the sensitivity of primary human keratinocytes to SM was unaffected when calcium was reduced using chelators or increased using the membrane ionophore ionomycin (Sawyer and Hamilton, 2000). These findings suggest that although calcium levels are significantly altered by SM treatment, modulation of calcium alone is neither necessary nor sufficient for SM-induced apoptosis.

B. Inflammation

Inflammation is likely one of the major driving forces of the skin pathology caused by SM exposure. The contribution of cytokines to the inflammatory events is well established and their signaling pathways are important targets of medical intervention for potential countermeasures of vesicant injury. The literature linking inflammation and wounding is so extensive as to fill a book by itself. This section will therefore only present an overview of the inflammatory response to dermal skin injury. In short, cytokine signaling involves several Janus kinase (Jak)-signal transducer and activator of transcription (Stat) pathways (O'Shea and Murray, 2008). These pathways are the major regulators of all the cell types involved in inflammation and they can be either positive or negative mediators to the inflammatory process. Both pro-inflammatory and anti-inflammatory events are under the influence of the Jak-Stat pathways and hundreds of cytokine receptors are regulated via these pathways including more than 40 type I and type II cytokine receptors, and receptors for interleukins, interferons, growth stimulating factors, leptin, erythropoietin, and many more.

As for vesicant-injured skin, the inflammatory response appears to be biphasic. This is based upon our years of experience in addition to others who reported that inflammation played a minor role in the initial events of SM-induced cutaneous injury, but had much greater importance at later stages (Papirmeister *et al.*, 1991c). In a more recent examination, Cowan and Broomfield (1993) argued that inflammation may be more significantly involved in the vesication event than previously believed, with inflammatory cells and mediators contributing directly to the formation of the primary lesion.

Numerous accounts, from both *in vivo* and *in vitro* studies, have now documented a rise in a number of inflammatory cytokines in response to SM. Applications of SM in the mouse ear vesicant model (MEVM) have resulted in induction of IL-1β, IL-6, TNFα and GM-CSF within 6 h (Sabourin *et al.*, 2000; Wormser *et al.*, 2005). While IL-1α was not detected in these studies, its inducibility by SM vapor was confirmed in experiments in which the backs of hairless mice were exposed (Ricketts *et al.*, 2000). Further, relative mRNA levels of IL-1β, IL-6, IL-8, and TNFα have been demonstrated to significantly rise within 24 h of SM vapor treatment in full thickness skin biopsies from weanling pigs (Sabourin *et al.*, 2002). Studies with cultured human keratinocytes have shown that these cells respond directly to SM with the production of cytokines. Interleukins-1β, -6, -8, and TNFα were detectable in culture media from these cells 24 h after treatment with 100–300 μM SM (Arroyo *et al.*, 2000). Cultured skin fibroblasts have also been shown to express TNFα in response to SM. The chemotactic activity of keratinocyte-derived IL-8 could initiate the transmigration of circulating granulocytes into SM exposed tissues where they could contribute to the primary lesion. Other cytokines may be involved in priming and activation of the recruited immune cells. While cytokine induction may occur via several means, there is now ample evidence that the inflammatory transcription factor NF-κB is one such pathway activated by SM (Atkins *et al.*, 2000; Minsavage and Dillman Iii, 2007; Rebholz *et al.*, 2008). In fact, enhanced synthesis of the aforementioned cyokines in resident skin cells may occur as a result of this NF-κB activation (Ghosh *et al.*, 1998).

Additional inflammatory mediators have been detected in cutaneous tissues as a consequence of SM treatment. These include free arachidonic acid (Lefkowitz and Smith, 2002) and its cyclooxygenase (Dachir *et al.*, 2004; Rikimaru *et al.*, 1991) and lipoxygenase products (Tanaka *et al.*, 1997). Furthermore, the increased capillary permeability observed would allow a variety of circulating inflammatory participants, such as complement components, kininogens, etc., to enter the dermal interstitium (Rikimaru *et al.*, 1991). Clearly, vesicant injury involves a host of inflammatory mediators similar to those seen in other types of wounds, where individual cytokines have been singled out as potential therapeutic targets.

C. Protease Activation

While there may be protease involvement in the initial SM injury, most studies have viewed the involvement of serine and matrix metalloproteases (MMPs) in SM injury as a downstream event since MMPs in particular regulate various inflammatory and repair processes (Parks *et al.*, 2004). The regulatory role confounds the potential use of MMP inhibitors as therapeutic agents because they play a beneficial role in wound repair and skin degradation. Increased protease activity following SM exposure has been reported *in vitro* in human peripheral blood lymphocytes (PBL) (Cowan *et al.*, 1993) and human epidermal keratinocytes (Smith *et al.*, 1991), *ex vivo* in rabbit skin organ cultures (Higuchi *et al.*, 1988; Woessner *et al.*, 1990) and human skin explants (Lindsay and Rice, 1996; Rikimaru *et al.*, 1991), *in vivo* in hairless guinea pig skin (Cowan *et al.*, 1993, 1994; Kam *et al.*, 1997), and *in vivo* in the mouse ear (Powers *et al.*, 2000) and hairless mouse (Casillas *et al.*, 2000a). The continued assessment of proteolytic activity in animal models is useful for characterizing specific proteases important to SM injury and for identifying effective protease inhibitors with therapeutic use in reducing or eliminating tissue injury caused by SM cutaneous exposure. The relationship between SM-increased protease activity and the subsequent vesication (blistering) that occurs in SM lesions remains unclear, but emerging literature implicating protease involvement is consistent with the known ability of proteases to degrade basement membrane components *in vitro* and *in vivo*. Inhibition of these proteases should reduce the extent of the injury, promote a more rapid recovery, and provide a useful adjunct to other therapeutic strategies aimed at preventing SM-induced degenerative pathophysiological events (Cowan and Broomfield, 1993).

D. Immune Response

Vesicant wounds undoubtedly initiate an immune response due to the nature of the wounds. This response begins almost immediately when the initial events promote capillary permeability, and the interstitium becomes inundated with circulatory components. And the wound site itself is subject to secondary infections by pathogens that reach the open wound area. These pathogens may be responsible for the large number of infiltrating cells that are seen in the mouse ear vesicant model 7 days post-SM exposure (Figure 41.3).

E. Apoptosis

Mechanisms underlying SM-induced apoptosis have been carefully explored using primary cultures of human keratinocytes. Treatment of keratinocytes with 100–300 μM SM resulted in activation of both caspase 8, which initiates the Fas-dependent death receptor pathway, and caspase 9,

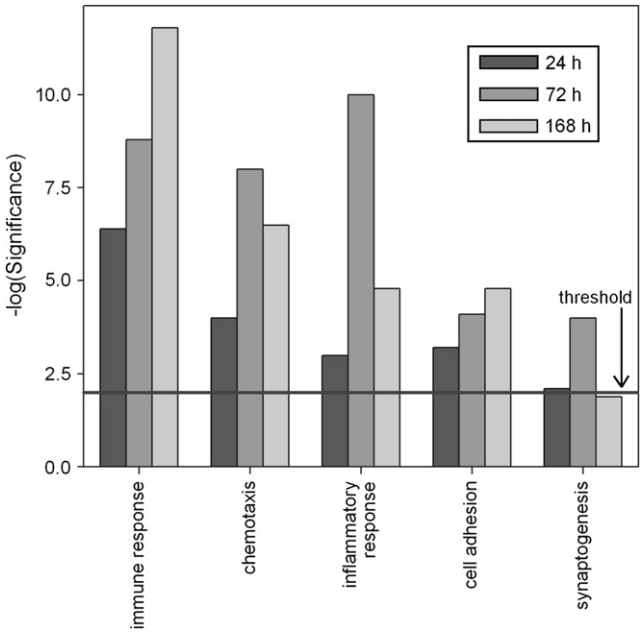

FIGURE 41.3. Results of biological processes sorting for microarray data from three different timepoints after sulfur mustard exposure using the mouse ear vesicant model. The timepoints were 24, 72, and 168 h post-exposure. The bars represent positive fold change increases over the carrier solvent alone samples. The immune response genes were within the top three biological processes activated for all the timepoints studied.

which initiates the mitochondrial apoptotic pathway (Rosenthal *et al.*, 2003). Fas and Fas ligand (FasL) were up-regulated in a concentration-dependent manner by SM leading to activation of caspase 3, the central executioner protease. Transfection of immortalized keratinocytes with a dominant negative Fas activated death domain (FADD) resulted in a blunted caspase response to SM. Microvesication and tissue injury produced *in vivo* by SM exposure of transfected cells after grafting onto athymic nude mice was also reduced by this treatment.

Changes in intracellular calcium levels are known to activate the mitochondrial pathway of apoptosis. A key regulator to Ca^{2+}-dependent proteins is calmodulin. SM has been shown to cause a time-dependent induction of calmodulin in keratinocytes (Simbulan-Rosenthal *et al.*, 2006). Moreover, depletion of calmodulin using antisense probes attenuated SM-induced activation of caspases involved in the mitochondrial pathway of apoptosis. Both antisense and pharmacological inhibition of calmodulin prevented SM-induced nuclear fragmentation in the keratinocytes. Bad, a proapoptotic Bcl-2 family member present in an inactive phosphorylated form in viable cells, was also activated by SM. Furthermore, cyclosporine A, a selective inhibitor of calcineurin, a Bad phosphatase, inhibited SM-induced keratinocyte apoptosis. These results suggest that calcium-dependent activation of Bad may be a mechanism by which SM induces apoptosis in keratinocytes.

One form of cellular demise common to epithelial cells is detachment-initiated apoptosis, also referred to as anoikis (Frisch and Francis, 1994). Epidermal keratinocytes rely on signals derived from the surrounding extracellular matrix (ECM) for survival. It is possible that loss of these signals plays a role in SM-induced epidermal cell injury, and that cell detachment from the basal lamina precedes cytotoxicity. Several lines of evidence support this possibility. First, SM can alter the dynamics of cytosolic proteins that exert control over the attachment of cells to the basement membrane. For example, SM can modify intracellular actin microfilaments and keratin intermediate filaments known to be important in maintaining epithelial cell connections with the basal lamina. Thus, Hinshaw *et al.* (1999) reported that SM causes changes in the actin microfilament architecture and morphology of human keratinocytes within 3 h of exposure. This was associated with a significant decrease in keratinocyte adherence without evidence of cytotoxicity. In addition, Werrlein and Madren-Whalley (2000) found that SM caused rapid, significant decreases in immunodetection of keratins 5 and 14, an intermediate filament pair found in undifferentiated keratinocytes. In both *in vivo* (Gunhan *et al.*, 2004) and *in vitro* studies with human keratinocytes (Dillman *et al.*, 2003), and with purified proteins (Hess and FitzGerald, 2007), keratins 5 and 14 have been found to be alkylated by SM as well as nitrogen mustard (mechlorethamine, HN2) and 2-chloroethyl ethyl sulfide (CEES), the monofunctional analog of SM. Sites of alkylation may be similar to dominantly acting mutations in keratins 5 and 14 that are known to be responsible for the human blistering disorder, epidermolysis bullosa simplex, in which, like SM-induced blistering, basal epidermal cells are targeted (Fuchs, 1997). The keratin cytoskeleton of basal keratinocytes links to the hemidesmosome and makes connections, through plectin, with the β4 cytoplasmic tail of integrin α6β4, thereby strengthening adhesion to the basement membrane via laminin-332 (Giancotti and Tarone, 2003). Alkylation of keratins 5 and 14 could cause aggregation and loss of function of the intermediate filament network and serve as a prelude to basal cell separation from the basement membrane.

In addition to its actions on epidermal cells, SM can directly alkylate extracellular matrix proteins in the skin, a process that can also alter the ability of basal keratinocytes to maintain vital connections with the basement membrane. In support of this idea, Gentilhomme *et al.* (1998) showed that SM treatment of human dermal equivalents reduced the ability of naïve keratinocytes to deposit laminin at the dermal–epidermal interface. In addition, Zhang *et al.* (1995a, b) found that treatment of fibronectin and laminin with SM interfered with the ability of human epidermal keratinocytes to adhere to these matrix proteins. This inhibitory action was determined to be alkylation-dependent, because it could be prevented by co-treatment with SM scavengers. Sulfur mustard and nitrogen mustard also reduce cell and tissue immunoreactivity for laminin-332, as well as integrin α6β4

and collagen XVII (also known as bullous pemphigoid antigen), two hemidesmosomal components that are critical for keratinocyte adherence (Kan *et al.*, 2003; Smith *et al.*, 1997b, 1998; Werrlein and Madren-Whalley, 2000; Zhang and Monteiro-Riviere, 1997). Interestingly, each of these proteins, like keratins, have been implicated in human blistering disorders involving separation of the epidermis at the dermal–epidermal junction (Pulkkinen and Uitto, 1998; Yancey, 2005). These findings suggest that SM can alter the interaction of basal cells with matrix proteins critical for basement membrane detachment. The alteration may occur by reducing protein functionality, decreasing protein expression, or increasing protein degradation. By whichever mechanism, a negative alteration in these critical anchoring components could lead to basal cell detachment and initiate anoikis.

F. Signal Transduction Pathways

Sulfur mustard exposure induces the activation of many molecular signaling pathways (reviewed in Ruff and Dillman, 2007). These pathways mediate many responses including inflammation, cell proliferation, cell differentiation, and apoptosis. Some that involve inflammation include the transcription factor NF-κB and the p38 MAP kinase. NF-κB is a transcription factor that is induced within 2–4 h after SM exposure (Minsavage and Dillman Iii, 2007). Dillman has suggested that this delayed induction is due to a nontraditional pathway of stimulation, whereby p90RSK phosphorylates IκB or the p65 subunit of NF-κB (Ruff and Dillman, 2007). The MAP kinase p38 is activated in response to damaging stimuli including heat, ultraviolet radiation, and pro-inflammatory stimuli. Dillman's group and others have noted an increase in activation of p38 by phosphorylation and demonstrated that inhibition of p38 phosphorylation resulted in a decrease in SM-induced pro-inflammatory cytokine production *in vitro* (Kehe *et al.*, 2008). However, the importance of NF-κB or p38 activation in mediating SM cutaneous injury has not been demonstrated *in vivo*. Also, while important for understanding SM-mediated toxicity, these pathways are difficult to target pharmacologically.

IV. MODELS OF DERMAL SULFUR MUSTARD EXPOSURE

A. Introduction

Although there has been considerable investigation of SM therapies since the publication of Bruno Papirmeister's landmark compilation of *Medical Defense Against Mustard Gas* nearly 20 years ago, no suitable treatment for SM exposure to the skin has been developed (Papirmeister *et al.*, 1991b). Since that time, there has been an increased focus on civilian research as the perceived risk of a terrorist-driven exposure to civilian populations has been recognized. The field has dramatically changed direction from strategies and therapies based on protection from SM exposure to therapies designed to subvert the blistering process and increase the rate of wound healing. New pharmaceutical strategies will be increasingly focused on combination therapies that target multiple processes in blister formation and the wound healing process, together with increased recognition of the importance of pharmaceutics.

Although many advances have been made in the production of skin barrier protection or post-exposure skin decontaminants [such as skin exposure reduction paste against chemical warfare agents (SERPACWA) and reactive skin decontamination lotion (RSDL), respectively], drug countermeasures against vesicants remain a subject of intense investigation. The primary treatment strategy following exposure to vesicants such as mustard gas is decontamination. However, given the high reactivity of SM, there is a very short timeframe (3 to 5 min) in which decontamination can be effectively accomplished (reviewed in Vogt *et al.*, 1984; Wormser, 1991); and some studies suggest that an extractable reservoir persists for much longer. Furthermore, because SM exposure does not cause immediate pain or noticeable effects, exposure is often not recognized until the effective window for decontamination has passed. Therefore, much research has focused on the identification of treatments that can be performed after exposure has occurred. These treatments can be directed at any stage during the progression of injury resulting from vesicant exposure, including before or after blister formation. This chapter focuses on the progress that has occurred since Papirmeister's publication (Papirmeister *et al.*, 1991b).

B. Model Systems for Screening Sulfur Mustard

The lack of effective *in vitro* and *in vivo* models for SM skin injury provides an ongoing challenge. No definitive animal model produces macroblister formation like that which occurs in humans, the major clinical target for SM treatments (extensively reviewed in Papirmeister *et al.*, 1991b). Furthermore, experimental animal models have different skin characteristics, including a reduced barrier for chemical penetration as compared to human skin (Bartek *et al.*, 1972). The reduced barrier results in greater systemic toxicity in animals, complicating other measures of cutaneous injury. Despite these difficulties, animal models have displayed great utility, and surrogate endpoints such as microblister formation or edema are acceptable biomarkers for measuring efficacy of candidate compounds.

A systematic characterization of animal models including the euthymic hairless guinea pig (HGP), weanling pig (WP), the mouse ear vesicant model (MEVM), and the hairless mouse (HM) showed that SM induced subepidermal blister formation and epidermal cell death in all models tested (Smith *et al.*, 1997a). Hairless mice are useful models of

human skin; the absence of hair on the skin and increased skin thickness reduce the rapid penetration of toxicants (Walter and DeQuoy, 1980). First characterized in 1967, the hairless guinea pig has been the most widely used model for vesicant exposure (Cowan *et al.*, 1993, 1994; Graham *et al.*, 1994; Kam *et al.*, 1997; Kim *et al.*, 1996; Marlow *et al.*, 1990a, b; Mershon *et al.*, 1990; Millard *et al.*, 1997; Smith *et al.*, 1995; Yourick *et al.*, 1991, 1992, 1993, 1995). Like human skin, SM-treated hairless guinea pigs experience defects in epithelial cell regeneration, basement membrane modifications, and increased necrosis and apoptosis, making them a good model for therapeutic testing (Dachir *et al.*, 2006; Kan *et al.*, 2003).

The mouse ear vesicant model (MEVM) is performed by application of vesicant to the inner (medial) side of the ear (Casillas *et al.*, 1997). Edema can be quantified by measuring 8 mm diameter ear punch biopsy weights. Microscopic investigation shows clear histopathologic changes that include edema, epidermal–dermal separation, and necrosis. These effects occur in a dose-dependent manner (Figure 41.4). This model has been used to screen many compounds and remains the most cost-effective live animal screen available (Babin *et al.*, 1999, 2003; Casillas *et al.*, 2000b; Inman *et al.*, 1999; Kiser *et al.*, 2005; Sabourin *et al.*, 2004). Furthermore, the benefits of a fully sequenced mouse genome and wide range of antibodies available make this model superior. Systemic effects do occur, as physiological and toxicological effects are detected in the contralateral (untreated) ear of animals (Babin *et al.*, 2000).

Weanling pigs (WP) have also been used extensively for SM-induced skin injury (Graham *et al.*, 1999, 2000; Logan *et al.*, 2000). Erythema was found to peak at 24 h after a 15-min vapor exposure, with maximal edema occurring at 48 h post-exposure (Graham *et al.*, 1999; Smith *et al.*, 1996, 1997a). The dermal–epidermal junction was also damaged at 48 h, with laminin 5 (renamed laminin 332) showing a progressive decrease in protein expression following SM injury (Smith *et al.*, 1997b). Furthermore, inflammatory markers including IL-8, IL-6, IL-1β, and MMP-9 are induced within 72 h of treatment with SM (Sabourin *et al.*, 2002). The weanling pig is also used as a model of wound debridement to enhance repair (Dalton *et al.*, 2008b; Graham *et al.*, 2002a, b, 2006; Reid *et al.*, 2007, 2008b), as discussed in greater detail later in this chapter.

The isolated perfused porcine skin flap (IPPSF) model is an effective *in vitro* model for SM exposure, characterized extensively by Monteiro-Riviere's laboratory (Monteiro-Riviere *et al.*, 1990). Skin flaps consist of isolated, perfused skin with intact dermis and epidermis maintained by microcirculation in a system that mimics normal blood flow. Unlike other animal models, gross blisters are obtained, with blister-induced epidermal–dermal separation occurring at the upper lamina lucida (Monteiro-Riviere and Inman, 1993, 1995, 1997; Riviere *et al.*, 1995). This model has allowed for several interesting experiments which could not have otherwise been performed. For example, the flow rate and composition of perfusing media can be altered: higher flow rate is associated with increased blister formation, whereas increased glucose in the perfusion medium has been shown to block the formation of microvesicles and blisters (Riviere *et al.*, 1997). Whether increased glucose can protect against blister development in live animals has not been tested. No reports using this model have been published since 2004.

In addition to cell lines, artificial human skin has been used to test the effects of SM (Petrali *et al.*, 1993). Human skin equivalent (HSE), commercially available as EpiDerm, is a fully differentiated artificial human skin with both a dermis and an epidermis (Monteiro-Riviere *et al.*, 1997). Full thickness models (EipDerm-FT) have been evaluated for their potential use in SM models as well (Hayden *et al.*, 2005; Paromov *et al.*, 2008). The latter model has been successfully applied for screening of antioxidant molecules in the treatment of SM injury (Paromov *et al.*, 2008). A lack of knowledge regarding the identity of important biochemical

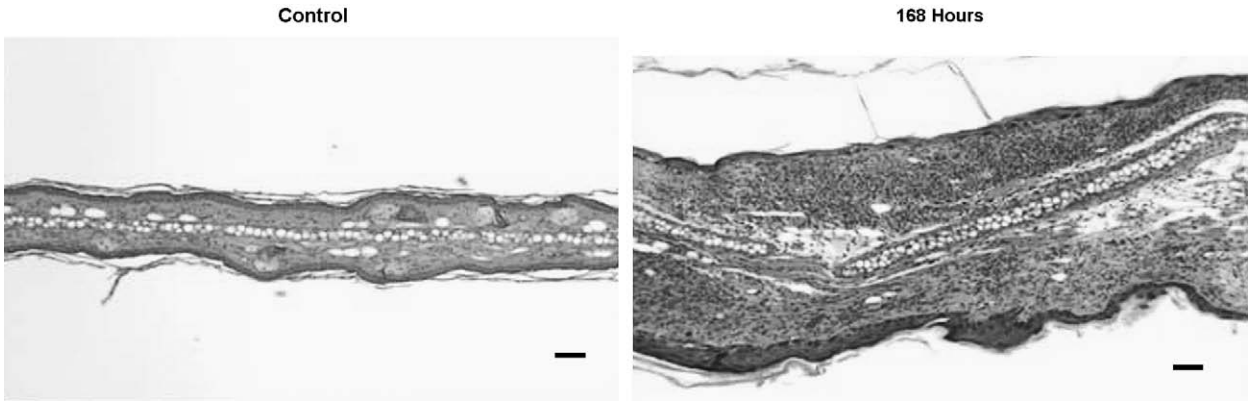

FIGURE 41.4. Biphasic inflammatory response in the MEVM: hematoxylin and eosin stained paraffin sections of mouse ears with (right, 168 h post-exposure) and without (left, carrier solvent, alone). Note the edema in the treated ear. The 168 h post-SM sample had a very large inflammatory cell infiltration (purple nuclei of thousands of infiltrating cells is apparent).

targets of SM has largely prevented the development of *in vitro* biochemical assays that might be used in rapid screening of chemical libraries.

C. Decontamination

If accomplished early, skin decontamination can be an effective means of reducing injury from SM exposure (Sidell *et al.*, 1997). Most SM decontamination strategies focus on its removal with solvents or adsorption of SM to inert substances. Even simple techniques such as washing with soap and water are surprisingly effective (Aasted *et al.*, 1987). Washing with many other substances has been suggested, including kerosene, oil, gasoline, surgical spirits, and neutral sodium hypochlorite (Chiesman, 1944; Gold *et al.*, 1993; Jelenko, 1974; Sollmann, 1919; Wormser *et al.*, 2002). Exposure to mustard liquid can also be treated with the use of binding agents, of which Fuller's earth is the best example. Fuller's earth is clay-rich soil commonly used to purify hydrophobic materials, including oils and greases (Chilcott *et al.*, 2001). In SM injury, it is thought to function by binding in a near-irreversible manner to SM, preventing its absorption, association, and reaction with skin components. Fuller's earth is the standard to which other decontamination agents are tested; modifications of this compound and other related clay-like materials may be more efficacious (Joiner *et al.*, 1987; Lyle *et al.*, 1984, 1986). However, it is difficult to contain and uncomfortable to apply. Other items, including flour and talcum powder, were shown to be effective in reducing the progression of injury (Van Hooidonk *et al.*, 1983). However, decontamination is ineffective in cases of vapor exposures to SM, which occur over a longer time period (McNamara, 1960). These results suggest that nearly anything that could adsorb a hydrophobic product would be effective in reducing SM injury.

Another strategy for decontamination is active chemical neutralization, whereby the SM undergoes a chemical reaction to prevent its reaction with outside components. There have already been successes with this type of approach, with the best example being reactive skin decontamination lotion (RSDL), a product approved for military use by the USA and a number of other nations (FDA, 2003). RSDL has been demonstrated to be as effective as Fuller's earth in its decontamination properties (Taysse *et al.*, 2007). The first generation of this product was composed of a solution of potassium 2,3-butanedione monooximate and its free oxime, diacetyl monooxime, in a mixture of monomethyl esters of polyethylene glycol. The solvent portion of the lotion is designed to solubilize chemical weapons away from the skin, while the oxime component readily reacts with mustards and with nerve agents, producing less toxic products (Sawyer *et al.*, 1991a, b). In domestic pigs, treatment with RSDL 5 min after exposure to SM resulted in significantly less injury observed 3 days post-exposure (Taysse *et al.*, 2007).

Other approaches to chemical neutralization include the use of sodium thiosulfate as a reducing agent to function by reacting with activated SM (Bonadonna and Karnofsky, 1965; Hatiboglu, 1960; Owens and Hatiboglu, 1961), reviewed in McKinley *et al.* (1982). Original studies (WW1-1970) focused on its use as an injectable drug to prevent the bone marrow suppression seen with exposure to SM and related mustard agents. Systemic administration of this and other antioxidant molecules such as *N*-acetyl cysteine is discussed later in this chapter. Another thiol, 2,3-dimercaptopropane sulfonic acid (DMPS), showed protection against injury in mice exposed to SM vapor (Pant *et al.*, 2000).

Decontamination can also be accomplished by the application of creams containing fluorinated crosslinker monomers (Liu *et al.*, 1999). Deactivation occurs when substances within the cream actively react with and decontaminate the agents. Care should be taken with administration of these creams, since they can trap chemical agents on the skin and prevent natural off-gassing. This may result in enhanced injury if inadvertently applied after exposure to a chemical agent. In some cases, perfluorinated creams have caused an 18-fold reduction in the rate of skin absorption (Chilcott *et al.*, 2002).

After exposure to SM, a significant fraction of the agent remains unreacted in the skin, in what is often described as a skin reservoir. This reservoir is thought to contribute to ongoing injury by continually releasing SM, allowing further damage to occur. Indeed, recent studies demonstrated that application of ^{14}C-labeled SM was extractable from an *in vitro* pig skin model for up to 6 h (Dalton *et al.*, 2004; Hattersley *et al.*, 2006). Future work might focus on targeting this reservoir to prevent ongoing damage; no therapies are currently available for this purpose. Although still present in unreacted form, it seems that SM in this reservoir cannot be removed from the skin through the methods discussed above.

D. Treatment of Blisters

The prolonged pathology observed in SM injury suggests two pathological mechanisms: an ongoing toxicity that contributes to a step-wise progression culminating in blister formation, and a healing process that has been subverted. This is quite unlike the wound healing of thermal injuries. Aspiration (removal of the fluid within the blister) and deroofing (removal of the epidermal layer that constitutes the roof of the blister) are the main courses of action taken for larger, coalesced blisters, in order to promote the healing process (reviewed in Graham *et al.*, 2005).

More recently, work has focused on the use of physical debridement of tissues, the surgical removal of tissue beyond the epidermal (roof) layer, to enhance the rate of wound healing (Graham *et al.*, 2005) (Table 41.1). The slow rate of healing suggests that SM-modified proteins or other cellular components are preventing the normal healing process (Eldad *et al.*, 1998). SM-mediated crosslinking of structural

TABLE 41.1. Comparison of dermabrasion (debridement) strategies for enhancing the rate of SM-induced skin injury repair

Therapy	Time of administration	Agent route	Species/system	Efficacy	Reference
Reactive Skin Decontamination Lotion (RSDL)	Pretreatment	Topical	*In vitro,* human skin	18-fold reduction in skin absorption rate, may partially extract SM from the skin reservoir	Chilcott *et al.* (2002)
Dermabrasion	96 h post	Vapor, skin	Yucatan pigs (miniature, larger white)	Accelerated ($\leq$3 $\times$) wound healing	Rice *et al.* (2000)
Debridement, CO_2 laser	6, 24, or 48 h post	Vapor, skin, 15 min	WP	3-fold fewer wounds. Improved histological skin structure	Graham *et al.* (1997)
Debridement, CO_2 laser	48 h post	Topical, liquid, 2 h	WP	Improved histological skin structure	Graham and Braue (2000)
Debridement, xeroform petrolatum	48 h post	Topical, liquid, 2 h	WP	Skin elasticity similar to sham (non-HD-treated) pig skin	Graham *et al.* (2006)
Debridement, Scarlet Red Ointment	48 h post	Topical, liquid, 2 h	WP	Skin elasticity similar to sham (non-HD-treated) pig skin	Graham *et al.* (2006)
Debridement, surgical tangential excision	48 h post	Topical, liquid, 2 h	WP	Mild improvement in healing	Graham and Braue (2000)
Debridement, surgical tangential excision and skin grafting	48 h post	Topical, liquid, 2 h	WP	Improved histological skin structure	Graham and Braue (2000)
Debridement, Versajet	48, 72, and 96 h post	Not specified	WP	No improvement at day 14	Dalton *et al.* (2008)
Debridement, Compound W	48, 72, and 96 h post	Not specified	WP	No improvement at day 14	Dalton *et al.* (2008)
Debridement, Collagenase Santyl	48, 72, and 96 h post	Not specified	WP	No improvement at day 14	Dalton *et al.* (2008)

[a] WP – wearling pig

proteins such as laminins may not be easily repaired and may contribute to a delayed wound healing response (Zhang *et al.*, 1994). This could occur by promoting prolonged inflammation or by preventing the normal wound closure event that occurs as dermal cells migrate across and repair the blister area. Early studies demonstrated that SM-induced lesions in the skin of weanling pigs or Yucatan miniature pigs underwent faster wound healing when treating lesions by debridement after exposure to SM, whether the debridement was performed by CO_2 laser or surgically (Graham *et al.*, 1997, 2002b; Rice *et al.*, 2000). Furthermore, when combined with skin grafting, debridement promotes wound repair (Graham *et al.*, 2002a; Rice, 2003). Further investigations into different types of debridement in conjunction with other therapies are underway (Dalton *et al.*, 2008a; Evison *et al.*, 2006).

V. THERAPEUTICS

A. Antioxidants

Chemical scavengers may be used to inactivate free radical forms of SM or the oxygen or nitrogen radicals thought to be formed as a consequence of SM activation. If given early enough after SM exposure, such scavengers might directly deactivate SM and perhaps reduce the reservoir of SM present in the skin. However, scavengers seem to have efficacy beyond the lifetime of SM itself in the skin, suggesting that other physiological mechanisms such as inflammation and oxidative stress might be reduced by these agents (reviewed in Papirmeister *et al.*, 1991b). Scavengers have mostly been tested for their ability to reduce leukopenia and death due to the systemic effects of mustard exposure, rather than the reduction of skin injury (reviewed in Papirmeister *et al.*, 1991b). Although most work has focused on scavengers as therapeutics in pulmonary exposures where ongoing oxidative stress contributes to toxic outcomes such as pulmonary fibrosis or chronic obstructive pulmonary disease, there has been limited research on their efficacy in treating cutaneous injury.

Sodium thiosulfate, a potent antioxidant and scavenger, has been shown to be effective in reducing leukopenia and platelet depression when given systemically in treatment for nitrogen mustard, particularly when given prior to exposure (Bonadonna and Karnofsky, 1965; McKinley *et al.*, 1982; Owens and Hatiboglu, 1961). In the IPPSF model, perfusion with sodium thiosulfate modestly reduced microvesicle formation and attenuated the vascular response (Zhang *et al.*, 1995b). However, it has limited efficacy against skin injury (Vojvodic *et al.*, 1985; Zhang *et al.*, 1995a, b).

Glutathione depletion has been shown to occur in several tissues and cell lines in response to mustard treatment (Kulkarni *et al.*, 2006; Omaye *et al.*, 1991; Ray *et al.*, 1995). Depletion of glutathione by buthionine sulfoximine treatment of isolated human leukocytes increased their sensitivity to SM toxicity (Gross *et al.*, 1993). Given the critical role of glutathione in maintaining the intracellular reducing state of the cell, restoration or pretreatment with glutathione may protect against SM toxicity. Indeed, pretreatment of the basal epidermal keratinocyte cell line SVK-14, the upper respiratory tract cell line BEAS-2B, or the lower airway type II epithelial cell line A549 with glutathione was shown to provide resistance to SM toxicity (Andrew and Lindsay, 1998; Lindsay and Hambrook, 1998; C.N. Smith *et al.*, 1997). Similar results were seen with other human cell lines, including G361, SVK14, HaCaT, and NCTC human skin cells (Simpson and Lindsay, 2005). Also, stimulation of glutathione concentration by pretreatment with glutathione itself or the cysteine precursor 10 mM L-oxothiazolidine-4-carboxylate was shown to be protective against SM *in vitro* (Amir *et al.*, 1998; Gross *et al.*, 1993). However, these early successes did not translate effectively to animal model systems. Reduced glutathione (400 mg/kg) given once before and twice after SM did not protect mice from SM toxicity (Kumar *et al.*, 2001). Because glutathione would be difficult to administer cutaneously, it is an unlikely therapeutic agent for the skin.

Cysteine is an amino acid with a reduced sulfur group that acts as an antioxidant. Cysteine residues are selectively alkylated in proteins by SM and the bifunctional nature of SM allows chemical crosslinking (Byrne *et al.*, 1996). Early studies with nitrogen mustard in mice demonstrated that pretreatment with cysteine protected against toxicity (Contractor, 1963). However, microvesicle formation and dark basal cell formation were not protected in the IPPSF model (Zhang *et al.*, 1995b). *N*-acetyl-L-cysteine (NAC) acts both as a scavenger and as an inducer of glutathione synthesis, restoring the normal reducing status of the cell. Because SM lowers intracellular-reduced glutathione, its restoration may contribute to increased tissue survival and repair. In addition, pretreatment with NAC may elevate glutathione levels above normal and offer protection against low concentrations of SM (Atkins *et al.*, 2000). *In vitro*, pretreatment with NAC protected peripheral blood lymphocytes from 10 μM SM (Gross *et al.*, 1993). Endothelial cells pretreated with NAC were resistant to loss of cell adherence and rounding following exposure to 250 μM SM (Dabrowska *et al.*, 1996). Liposomes containing NAC increased the viability of HaCaT keratinocytes in an *in vitro* study if given simultaneously with CEES (Paromov *et al.*, 2008). Although antioxidants have proven to be efficacious if given prior to SM exposure, their ability to ameliorate skin damage or enhance wound repair has not been shown.

B. Proteolytic Inhibitors

Proteases play a critical role in wound repair and remodeling, and therefore are likely important targets for the enhancement of wound repair (Mohan *et al.*, 2002). In particular, damage to the basement membrane by MMPs is

thought to be one mechanism responsible for dermal–epidermal separation seen in SM-induced blister formation. This could occur by up-regulation of MMP expression, reduced competition for MMPs by adhesion molecules, or both (Danne *et al.*, 2001; Mol, 1999; Shakarjian *et al.*, 2006). MMP-9 expression is up-regulated in WP skin (Sabourin *et al.*, 2002) and the MEVM following SM exposure (Gerecke *et al.*, 2004). Inhibition of proteases might therefore ameliorate damage caused by SM to structural components of the skin (Table 41.2). Explant cultures of SM-treated human skin cotreated with Ilomastat showed no epidermal–dermal separation (Schultz *et al.*, 2004). Sulfur mustard-induced MMP-9 mRNA in the MEVM was partially inhibited by pretreatment with the MMP-9 inhibitor GM 1489 (Gerecke *et al.*, 2005). The antibiotic doxycycline is also an MMP inhibitor that was shown to attenuate SM-induced pulmonary injury (Guignabert *et al.*, 2005). However, doxycycline does not protect HaCaT viability; cells lose adherence and undergo apoptosis (Lindsay *et al.*, 2007).

Microvesication of human skin *in vitro* was inhibited by treatment with MMP inhibitors such as Illomastat, but HGP skin had no decrease in microvesication or necrosis following repeated treatments with Ilomastat (Mol and Van den Berg, 2006). Mol and Van den Berg suggest that this may be due to lack of delivery of the MMP inhibitor to the site of action. Although MMP inhibitors may reduce the ongoing injury caused by up-regulation of MMP-9, most inhibitors have difficulty penetrating the skin. The application of pharmaceutical concepts may aid in the development of better delivery systems that would enhance the efficacy of this class of drugs.

C. Steroids, Corticosteroids, and Glucocorticoids

Steroids have shown some efficacy in reducing inflammation and blister formation in response to SM or genetic blistering diseases such as bullous pemphigoid (Di Zenzo *et al.*, 2007). Early studies showed that several types of corticosteroids were effective in reducing edema induced by SM (Dannenberg and Vogt, 1981). Glucocorticoids were shown to be effective in reducing edema in the initial phase of injury, but did not affect the overall rate of healing (Vogt *et al.*, 1984). Hydrocortisone given systemically or topically 2 h prior to HD administration resulted in a reduction in ear inflammation in the MEVM (Babin *et al.*, 2000; Casillas *et al.*, 2000b). Steroids given after SM exposure also enhance wound healing in a Yorkshire pig model of SM injury (Reid *et al.*, 2008a). Dexamethasone in combination with the nonsteroidal anti-inflammatory diclofenac produced more than a 60% reduction in edema in mouse ears treated with SM (Dachir *et al.*, 2004). However, reduction of edema, while important, did not necessarily correlate with a reduction in the progression of injury as seen in later in this work. Studies using newer steroids such as clobetasol showed improved healing, lower

TABLE 41.2. Summary of the effects of protease inhibitors on SM toxicity

Therapy	Time of administration	Therapy route	Therapy concentration	Agent route	Species/system	Efficacy	Reference
Doxycycline	Cotreatment, then post-treatment	In media	500 µM	In media	HaCaT cells, *in vitro*	Reduced cellular detachment, but did not prevent apoptosis	Lindsay *et al.* (2007)
Doxycycline	1 h post	In media	100 µM	In media, 200 µM	HEK keratinocytes, *in vitro*	Reduction of SM-induced IL-8 production	Nicholson *et al.* (2004)
Doxycycline	Cotreatment	Topical	90 µM	Topical	*In vitro* human skin explants	No effect on SM-induced dermal–epidermal separation	Schultz *et al.* (2004)
GM 1489[a]	Pretreatment 15 min	Topical	20 µl of 25 mM	Liquid	Mouse	Reduced expression of MMP-9 mRNA	Gerecke *et al.* (2005)
Ilomastat	Pretreatment 15 min	Topical	20 µl of 25 mM	Liquid	Mouse	No effect	Gerecke *et al.* (2004)
Povidone iodine	15 m and 24 h post	Topical	40 mg 10% povidone iodine	HN2, 0.5 mg	Haired guinea pig	>80% reduction in MMP-2 and MMP-9 activity in skin	Wormser *et al.* (2002)
Iodine	20 m post	Topical	1% w/v	Liquid, 1.27 mg	Pig	No effect	Margulis *et al.* (2007)

[a]GM 1489 – N-[(2R)-2,4-methylpentanoyl]-L-tryptophan-(S)-methyl-benzylamide

severity of basal cell necrosis, and less inflammation (Reid *et al.*, 2008a). Co-treatment of steroids with nonsteroidal anti-inflammatory drugs (NSAIDs) shows greater promise: co-treatment with diclofenac and tacrolimus in the hairless guinea pig exposed to SM vapor showed less erythema, reduced lesion area, and fewer lesions (Dachir *et al.*, 2008). A similar combination of steroid (Adexone) and NSAID (Voltaren) applied to mouse ears treated with SM led to reduced inflammation, less edema, reduced area of clinical damage, and reduced damage to epithelial cells (Dachir *et al.*, 2004).

D. Nonsteroidal Anti-Inflammatory Drugs

Several studies have demonstrated that NSAIDs given systemically or topically offer protection against continued SM-mediated toxicity. This suggests that inflammation is a key component of SM-induced injury as it is with other cutaneous injuries, such as those induced by ultraviolet radiation (Yourick *et al.*, 1995). These results suggest that inflammation is involved in the ongoing pathology of SM-induced injury, and that anti-inflammatory drugs should be considered as part of a drug cocktail for treatment of SM injury. Early studies with indomethacin in the HGP model showed that oral pretreatment could attenuate erythema and cutaneous injury (Yourick *et al.*, 1995). Partial protection by indomethacin against microvesicle formation was found in the IPPSF model (Zhang *et al.*, 1995b). Significant reductions in SM-induced early edema were found when indomethacin was administered from 24 h prior to exposure to 24 h after exposure (Babin *et al.*, 2000) or when given 20 min post SM challenge (Kiser *et al.*, 2001). Topical indomethacin given 2 h prior to SM exposure in the MEVM protected against early (24 h) but not late (72 h) edema (Casillas *et al.*, 2000b).

Early studies using the MEVM showed that post-treatment with NSAIDs, particularly in combination with steroids, could diminish SM-induced inflammation at early time points, although later effects were not measured (Dachir *et al.*, 2002). As mentioned above, Voltaren, an NSAID, given in combination with the steroid Adexone, reduced skin injury (Dachir *et al.*, 2004). More recently, bifunctional compounds containing NSAIDs (Ibuprofen or Diclofenac) tethered to pyridostigmine, an acetylcholinesterase inhibitor, were somewhat effective against SM dermal toxicity (Amitai *et al.*, 2005). This combination was effective in the MEVM in reducing subepidermal blistering (Amitai *et al.*, 2006). In fact, subepidermal blistering in the MEVM was reduced by these tethered pharmaceuticals (Amitai *et al.*, 2006).

E. TRPV1 Ligands

An emerging hypothesis suggests a neurological component to SM toxicity in the skin. Nonmyelinated sensory C-fibers arising from the dorsal route ganglion transmit sensory information from the skin to the central nervous system in response to noxious stimuli, such as pain and heat. These fibers function as dual sensory efferents and release nociceptive and inflammatory neuropeptides such as substance P, peripherally (Szallasi and Blumberg, 1999; Szolcsanyi, 2004). Agents such as capsaicin, the active ingredient in hot peppers, produce analgesia by binding as an agonist to the transient receptor potential V1 channel (TRPV1) (Szolcsanyi, 2004). Capsaicin rapidly produces desensitization and interferes with the release of neuropeptides from sensory fibers (Campbell *et al.*, 1993). Moreover, TRPV1 are expressed on cells in a number of nonneuronal cutaneous tissues, including keratinocytes and mast cells (Li *et al.*, 2007). Capsaicin and its structural analogs, known collectively as vanilloids, have been shown to have anti-inflammatory activity, as demonstrated by inhibition of edema, mast cell degranulation, and leukocyte migration (Brand *et al.*, 1990; Bunker *et al.*, 1991). Pretreatment of skin with vanilloids prior to SM exposure was shown to significantly reduce edema formation (Babin *et al.*, 2000, 2003; Casillas *et al.*, 2000b; Sabourin *et al.*, 2003). One such vanilloid, olvanil, is a highly lipophilic analog of capsaicin, and has been shown to reduce SM-induced histological damage and edema as well as cytokine and chemokine mRNA induction (Casillas *et al.*, 2000b; Sabourin *et al.*, 2003). Analogs octyl homovanillamide and heptyl isovanillamide were shown to display similar protective activities against SM (Casbohm *et al.*, 2004). There appears to be much promise in following these leads and more closely examining the role of neurogenic inflammation and neuropeptides in the cutaneous damage induced by SM.

F. Temperature

Anecdotal and scientific evidence has shown that exposure to SM in hot environments causes more extreme damage, but little work had been performed on the effect of cooling in the prevention of injury until recently. Human skin keratinocytes treated with SM and cultured at 25°C had less injury after 24 h than keratinocytes grown at 37°C (Sawyer and Risk, 1999). Similarly, hairless guinea pigs with skin exposed to SM had less injury after 72 h if treated for 4.5 h post-exposure with cold (Sawyer and Risk, 1999). Other studies showed that anesthetized swine skin exposed to mild cooling (15°C) for 2–4 h following SM exposure had significantly less injury progression after 7 days (Sawyer *et al.*, 2002). However, later studies demonstrated that this effect was temporary, slowing the rate of injury progression rather than reducing overall injury. Indeed, tissue and animal studies showed that temperature-mediated inhibition of injury was reversible upon return of the tissue to normal body temperature (37°C) (Risk *et al.*, 2001). Sawyer *et al.* (2002) have suggested that cooling might therefore be used as a temporary measure that ‘‘increases the therapeutic window in which other medical countermeasures are useful (Nelson and Sawyer, 2006; Sawyer and Nelson, 2008).’’

VI. CONCLUDING REMARKS AND FUTURE DIRECTION

Countermeasure research to date has focused primarily on the development of therapeutics, but little attention has been given to the use of advances in drug delivery, or pharmaceutics. Weak efficacy can sometimes be enhanced by the use of better delivery mechanisms. Drug delivery by encapsulation of active moieties in nanoparticles has been increasingly used for chemotherapeutic agents such as doxorubicin (Haley and Frenkel, 2008). However, with the exception of barrier creams, nanoparticle-mediated cutaneous SM detoxification has largely been limited to decontamination (Braue and Hobson, 2005; Hobson *et al.*, 2002a, b; Prasad *et al.*, 2007a, b; Singh *et al.*, 2008; Stengl *et al.*, 2005). Most studies with creams focus on the use of nanoparticles that increase the rate of deactivation of the chemical agent (Koper *et al.*, 1999). Liposomes used as delivery devices for hydrophilic or hydrophobic antioxidants have shown enhanced drug delivery efficacy using *in vitro* models (Paromov *et al.*, 2008). NAC-containing liposomes have been used to treat CEES-induced lung injury, suggesting that encapsulated scavengers may be effective at restoring intracellular reducing agents following SM exposure (Hoesel *et al.*, 2008). Formulations that allow effective dermal delivery may enable the development of scavengers, antioxidants, or protease inhibitors for SM treatment. NAC-containing liposomes tested on an *in vitro* model of the skin (Epiderm) maintained cell viability if given concomitantly with SM; whether post-treatment was efficacious is unknown (Paromov *et al.*, 2008). These studies demonstrate that advances in pharmaceutics may dramatically improve the efficacy of previously discarded therapies.

Effective management of SM-exposed patients is likely to include combination therapies that act at the same stage or at different stages of the SM injury and recovery process to enhance overall efficacy. Dermabrasion has been shown to be effective in conjunction with split-thickness wound grafting (Graham *et al.*, 2002a). Other studies have shown increased efficacy when NSAIDs are given in conjunction with steroids (Dachir *et al.*, 2002, 2004, 2008). It is therefore likely that several therapies with limited efficacy could have an additive or synergistic effect for enhancement of SM-mediated injury healing.

Perhaps the largest problem inhibiting the development of effective preventive and therapy-based countermeasures is our continued lack of understanding of which of SM's many actions are critical events leading to SM injury. The major difficulty of SM is that although a large number of biochemical pathways are altered, the identification of pathways with pharmacological significance has yet to be clearly established. Previous studies suggested that the PARP pathway, calcium mobilization, and intracellular pH are affected in SM-mediated injury, but therapies designed against these pathways have not demonstrated effectiveness beyond *in vitro* approaches or have not shown feasibility for development as drugs. It is likely that these pathways, while important, are not the initiating events of toxicity, and therefore targeting these pathways might have limited effectiveness. Indeed, only anti-inflammatory drugs seem to be moderately effective in the treatment of SM injury. If SM is to be effectively treated, more research into the basic mechanisms of SM injury needs to be performed. As Brimfield suggests, ''the search for the primary biochemical lesion is still underway (Brimfield, 2004)''. Given the complex progression of SM injury, multiple interventions used in combination against various components of the progression of injury are likely to be required to deliver the best result. Recent major advances, such as the discovery that cooling of target tissues can slow the rate of injury, are likely to play an important role in treatment strategies (Risk *et al.*, 2001).

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CHAPTER 42

Excitotoxicity, Oxidative Stress, and Neuronal Injury

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I. INTRODUCTION

Exposure to anticholinesterase agents, organophosphates (OPs), and carbamates in the form of insecticides and chemical warfare agents affects or threatens much of the world's population. At present, more than 100 different OPs are used as insecticides worldwide (Kwong, 2002). The advantage of a lower environmental stability compared to organochlorine pesticides and high effectiveness against different insect species is accompanied by the disadvantage of high mammalian toxicity (Lotti, 2001). The widespread use and easy accessibility to these compounds result in a huge number of intoxications and several hundred thousand fatalities annually (Gunnell and Eddleston, 2003). Other derivatives of phosphoric acid, warfare nerve agents, are considered to be the most toxic compounds among all chemical weapons. Devastating effects of these agents have been demonstrated during the Iraqi conflict with Iranian troops and Kurdish civilians, as well as the terrorist attack on the Tokyo underground train system in 1995, resulting in over 5,500 casualties (Black *et al.*, 1994; Nozaki *et al.*, 1995; Nagao *et al.*, 1997). Terrorist attacks involving warfare nerve agents, weapons of mass destruction, or other industrial chemicals present worldwide security threats and health concerns. Therefore, anticholinesterase agents represent a significant potential threat not only in military arenas, but also to the general civilian population.

The nerve agents were originally synthesized during the 1930s in Germany in attempts to achieve more efficient pesticides based on OP compounds. Tabun (GA; ethyl *N*-dimethyl-phosphoramidocyanidate) was the first compound synthesized, followed by sarin (GB; isopropyl methylphosphonofluoridate), soman (GD; 1,2,2-trimethypropyl-methyl-phosphonofluoride), and VX [*S*-(2-diisopropyl-aminoethyl) *O*-ethyl methyl phosphonothiolate]. They differ from each other by the type of chemical group replacing the [$\cdot OH^-$] radical on the acid–base structure (Figure 42.1).

They also differ from one another in their potency and volatility, as well as in their ability to cross the blood–brain barrier (BBB) and to exert central nervous system (CNS) toxicity.

Pharmacologically, all these compounds are acetylcholinesterase (AChE) inhibitors. Their acute symptoms are attributed to accumulation of acetylcholine (ACh), thus exhibiting cholinergic toxicity. Phosphorylation of the esteratic site of the enzyme (Taylor, 1990) diminishes its capacity to catalyze its endogenous substrate ACh. Most of the OP compounds combine with the AChE only at the esteratic sites and the stability of the bond depends upon the structure of the compound attached. OP compounds containing larger alkyl groups may hinder cleavage, leaving the phosphorylated AChE inactivated almost indefinitely, so that normal activity recurs only upon the synthesis of a new enzyme. This process is known as ''aging'' and its time course varies depending on the nerve agent. Consequently, the hydrolysis of ACh is prevented, leading to accumulation of ACh in the synaptic cleft and overstimulation followed by desensitization of muscarinic and nicotinic ACh receptors. The constantly activated nicotinic cholinergic receptors generate involuntary skeletal muscle contraction, followed by complete depolarization block, the clinical manifestation of which is flaccid paralysis. In a manner similar to the events in the peripheral nervous system, the accumulation of ACh in CNS nerve endings causes anxiety, disorientation and general convulsions, followed by loss of consciousness and respiratory arrest. Anticholinesterase agent-induced ACh accumulation at the muscarinic sites also enhances the activity of various secretory glands, leading to excessive salivation, lacrimation, bronchorrhea, diarrhea, and sweating. The severity of poisoning by nerve agents varies from mild cases (mild dyspnea, blurred vision, and glandular hypersecretion) to more severe poisoning, which is characterized by severe dyspnea, skeletal muscle fasciculation, convulsions, and unconsciousness, which occurs soon after an intense exposure of only a few minutes (Goldfrank *et al.*, 1982; Weinbroum, 2005). Thus, dependent upon the degree of AChE inhibition, cholinergic stimulation may also lead to respiratory failure, coma, and death.

Exposure to these agents can also lead to long-term neurological impairments, including: (1) a delayed intermediate syndrome affecting muscles, which occurs 24–96 h post-exposure; and (2) subtle, long-term neurological

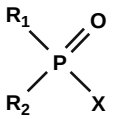


FIGURE 42.1. General structural formula of organophosphates: R1 and R2 are alkyl-, alkoxy-, or amido groups; X is the acyl residue (labile fluorine-, cyano-, substituted, or branched aliphatic, aromatic, or heterocyclic groups).

effects, which may last months or even years. Chronic OP induced neuropsychiatric disorder (COPIND) (Jamal, 1997) with symptoms of anxiety and depression; memory and attention deficit have also been described in workers exposed to OP compounds. In addition, dystonic reactions, schizophrenia, cog-wheel rigidity, choreoathetosis, and electroencephalographical changes have been reported with high-dose exposures. These extrapyramidal symptoms are thought to be due to the inhibition of AChE in the human extrapyramidal area. Psychosis, delirium, aggression, hallucination, and depression may also be seen during recovery from the cholinergic syndrome. Commercial sprayers of insecticides exhibit high level of anxiety (Levin *et al.*, 1976) and other types of delayed neurobehavioral effects are seen among people exposed to low doses of OP compounds for prolonged periods. Notably, clinical features of psychological syndromes occurring after chronic exposure to OP compounds exhibit many similarities to chronic fatigue syndrome (Behan, 1996).

Convulsions are a major sign of OP nerve agent poisoning (Misulis *et al.*, 1987). OP-induced seizures rapidly progress to status epilepticus, which leads to profound structural brain damage (Lemercier *et al.*, 1983; McLeod, 1985). It has been hypothesized that several neurotransmitter systems become involved sequentially in the initiation and maintenance of seizures elicited by OP compounds (McDonough and Shih, 1997). The progression of events includes initial high cholinergic activity followed by a transitional phase of cholinergic and glutamatergic hyperactivity and finally after about 40 min following the onset of seizures a predominantly glutamatergic phase (McDonough and Shih, 1997). Glutamate is therefore a major mediator of central neurotoxicity associated with exposure to OP nerve agents.

II. EXCITOTOXICITY AND NEURODEGENERATION

Previous studies have emphasized the role of glutamate receptors in the propagation and maintenance of OP-induced seizures (Shih *et al.*, 1990; Lallement *et al.*, 1991a, b, 1992; Sparenborg *et al.*, 1992; McDonough and Shih, 1993; De Groot *et al.*, 2001). OP-induced sustained seizure activity is thought to result in the release of excessive amounts of glutamate (Choi, 1998; Danysz *et al.*, 1995; Faden and Salzman, 1992). It has been shown that soman-induced seizures produce an increase in extracellular glutamate in the pyriform cortex (Wade *et al.*, 1987) and cornu ammonis (CA) region of hippocampus (Lallement *et al.*, 1992), followed by activation of *N*-methyl-D-aspartate (NMDA) glutamate receptors in the CA1 region. Moreover, glutamate stimulates the continuous release of ACh (Anderson *et al.*, 1994), contributing to further excitatory stimulation, prolongation of the seizures and thus like a brushfire it perpetuates excitotoxic neurodegeneration in vulnerable brain regions (Wade *et al.*, 1987; Lallement *et al.*, 1991a, 1992). Microdialysis studies reported an immediate increase in extracellular glutamate concentrations in the septum, pyriform cortex, hippocampal regions, and amygdale following soman-triggered seizures (Lallement *et al.*, 1991a, b; Wade *et al.*, 1987). Importantly, blocking specific glutamate receptors reduces neuropathogenic responses, including nerve agent toxicity (Sheardown *et al.*, 1990; Sparenborg *et al.*, 1992; Deshpande *et al.*, 1995).

Excitotoxic injury caused by increased levels of glutamate causes cognitive dysfunction (Phillips *et al.*, 1998; O'Dell *et al.*, 2000; Faden *et al.*, 2001). Interestingly, studies show sublethal nerve agent exposure can lead to memory and attention deficits that are controlled by the hippocampus (Hatta *et al.*, 1996; Nishiwaki *et al.*, 2001; Miyaki *et al.*, 2005). High concentrations of NMDA and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors, which play a key role in hippocampal-mediated learning and memory (Izquierdo and Medina, 1997), also make the hippocampus highly vulnerable to glutamate-induced excitotoxic injury following nerve agent poisoning (Shih *et al.*, 1990; Lallement *et al.*, 1991a, b, 1992; Sparenborg *et al.*, 1992; McDonough and Shih, 1993; De Groot *et al.*, 2001). Increased synaptic glutamate concentrations following OP exposure also alter glutamate receptor expression (Piehl *et al.*, 1995; Kreutz *et al.*, 1998; Cebers *et al.*, 2001) and perturb NMDA receptor subunit distribution, thus changing the overall physiology of the receptor and the functionality of the hippocampus (Cebers *et al.*, 1999). Many models of cognitive dysfunction have shown altered glutamate receptor expression (Luthi-Carter *et al.*, 2003; Mishizen-Eberz *et al.*, 2004) and NMDA subunit ratio rearrangement (Mikuni *et al.*, 1999), suggesting these mechanisms may also contribute to cognitive dysfunction following sublethal nerve agent exposure.

The role of glutamate receptors in mediating seizure-induced brain damage was first shown in the 1980s when kainic acid (KA) was found to damage limbic structures by inducing limbic epilepsy in rats (Ben-Ari *et al.*, 1980). Kainate is a rigid analog of glutamate, the principal excitatory neurotransmitter in the central nervous system (CNS), and it is a very potent stimulant of a subset of the ligand-gated ion channel, called KA receptors (Milatovic *et al.*, 2005b). Activation of the KA subtype of ionotropic glutamate receptors results in sustained epileptic activity

in the hippocampus, followed by a selective pattern of neuropathology that is similar to human temporal lobe epilepsy (Ben-Ari, 1985; Ben-Ari and Cossart, 2000; Schwob *et al.*, 1980). Kainate administration and intense seizure activity associated with status epilepticus is sufficient to induce degeneration of hippocampal CA neurons, and hyperexcitability of surviving hippocampal CA neurons (Dudek *et al.*, 1994; Ben-Ari, 1985, 2001; Dong *et al.*, 2003, Zaja-Milatovic *et al.*, 2008) (Figure 42.2).

Results from studies with kainate and other glutamate analogs led to the hypothesis that excessive amounts of glutamate are released during limbic seizures following OP exposure. Cellular events involved in this neurotoxicity are first marked by acute neuronal swelling resulting from Na^+ influx through NMDA receptor ion channel, followed by a secondary influx of Ca^{2+} ions and water into affected neurons. Whereas neurons are rapidly damaged when loaded with Ca^{2+} ions through NMDA receptors, a similar Ca^{2+} load incurred through other non-NMDA receptors (Bouchelouche *et al.*, 1989) and alternative influx pathways, such as voltage-sensitive Ca^{2+} channels, is innocuous (Tymianski *et al.*, 1993; Sattler *et al.*, 1998). In addition, alterations in Ca^{2+} and glutamate induce other biochemical mechanisms, which further compromise cellular viability. For example, increased Ca^{2+} is also induced by activating voltage-dependent Ca^{2+} channels (VDCC) and NMDA activates the syntheses of nitric oxide (NO) (Garthwaite *et al.*, 1989), a molecule believed to be toxic to neurons and to participate in the formation of seizure activity and neurodegeneration following nerve agent intoxication (Lallement *et al.*, 1996). Accordingly, antagonists of NMDA receptors or VDCC are effective in protecting CNS neurons against glutamate-mediated neuronal excitotoxicity (Weiss *et al.*, 1990; Mattson, 2003).

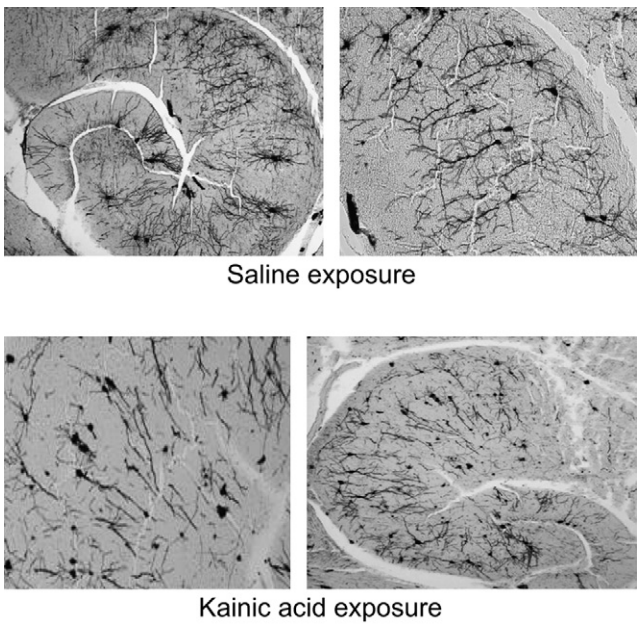


FIGURE 42.2. Photomicrographs of mouse hippocampi with pyramidal neurons from the CA1 hippocampal area of brains 1 h after saline (control) and kainic acid (KA, 1 nmol/5 µl, i.c.v.) injections. Treatment with KA induced degeneration of hippocampal dendritic system and a decrease in total length of dendrite and spine density of hippocampal pyramidal neurons.

III. OXIDATIVE INJURY

The brain is considered to be exceedingly sensitive to oxidative stress because of its: (1) great consumption of O_2, glucose, and energy, (2) large amounts of peroxidizable fatty acids, (3) relatively low levels of antioxidants, and (4) ease of peroxidation of peroxidizable fatty acids (Floyd, 1997; Simonian and Coyle, 1996). During normal physiological conditions, reactive oxygen species (ROS) are generated at a low rate and are efficiently removed by scavenger and antioxidant systems. However, in the pathophysiological conditions, such as seizures, OPs or carbamates, acetylcholinesterase inhibitors (AChEI) induced a high rate of ATP consumption accompanied by increased generation of ROS. Previous studies have supported a role for oxidative stress and excessive generation of ROS and reactive nitrogen species (RNS) in anticholinesterase-induced neurotoxicity (Dettbarn *et al.*, 2001; Gupta *et al.*, 2001a, b, 2007; Milatovic *et al.*, 2005a). During sustained seizures the flow of oxygen to the brain is greatly increased at a time when the use of ATP is greater than the rate of its generation. This metabolic stress results in a markedly increased ROS generation. A greatly increased rate of ROS production, overwhelming the capacity of inherent cellular defense systems, results in attack on the mitochondria and cell membranes, leading to peroxidation of lipids, cell lesions and, in turn, cell death (Sjodin *et al.*, 1990).

Neurons in particular are dependent upon mitochondrial energy production. This high utilization of energy, coupled with the inhibition of oxidative phosphorylation, compromises the cell's ability to maintain its energy and antioxidant levels, causing excessive production of ROS/RNS, which leads to neuronal damage (Dettbarn *et al.*, 2001; Gupta *et al.*, 2001a, b). The most consistent pathological findings in acute experiments with anticholinesterase agents include degeneration and cell death in the pyriform cortex, amygdala, hippocampus (where the CA1 region is preferentially damaged), dorsal thalamus, and cerebral cortex. The early morphological changes in AChEI-induced status epilepticus (SE) include dendritic swelling of pyramidal neurons in the CA1 region of the hippocampus (Carpentier *et al.*, 1991).

Accordingly, the hypothesis concerning OP-induced neuronal oxidative injury is that overstimulation of glutamatergic receptors results in sustained epileptic activity in the hippocampus, followed by a typical pattern of neuropathologic changes predominantly in the pyramidal neurons. Cell damage is thought to result from intense transient influx of calcium leading to mitochondrial

functional impairments characterized by activation of the permeability transition pores in the inner mitochondrial membrane, cytochrome c release, depletion of ATP, and simultaneous formation of ROS (Heinemann *et al.*, 2002; Cadenas and Davies, 2000; Patel, 2002; Nicholls and Ward, 2000; Nicholls *et al.*, 2003). In addition, increase in cytoplasmic calcium ions triggers intracellular cascades through stimulation of enzymes, including proteases, phospholipase A_2, and nitric oxide synthase, which also lead to increased levels of free radical species and oxidative stress (Lafon-Cazal *et al.*, 1993; Farooqui *et al.*, 2001). Since free radicals are direct inhibitors of the mitochondrial respiratory chain, ROS generation perpetuates a reinforcing cycle, leading to extensive lipid peroxidation and oxidative cell damage (Cock *et al.*, 2002; Cadenas and Davies, 2000).

Two radicals that play predominant roles as initiators of lipid peroxidation are the hydroxyl radical (OH^-) and the peroxynitrite radical ($OONO^-$). The superoxide anion radical (O_2^-), which is generated during the electron transport process in mitochondria, is involved in the generation of both OH^- and $OONO^-$. Superoxide dismutase (MnSOD and Cu/ZnSOD) converts O_2 to hydrogen peroxide (H_2O_2), which is then converted to OH^- via the Fenton reaction, catalyzed by Fe^{2+}, Cu^+, or Mn^{2+}. $OONO^-$ is generated from the interaction of NO with O_2^-. A major stimulus for NO production is elevation of intracellular Ca^{2+}, which binds to calmodulin, resulting in the activation of NO synthase. Peroxynitrite is a powerful oxidant exhibiting a wide array of tissue damaging effects ranging from lipid peroxidation, inactivation of enzymes and ion channels via protein oxidation and nitration to inhibition of mitochondrial respiration (Virag *et al.*, 2003). Peroxynitrite, which dissipates during oxidation (Wang *et al.*, 2003), has also been found to nitrate as well as oxidize adenine, guanine, and xantine nucleosides (Sodum and Fiala, 2001). Low concentrations of peroxynitrite trigger apoptotic death, whereas higher concentrations induce necrosis with cellular energetics (ATP and NAD) serving as a switch between the models of cell death. Thus, increased ROS and RNS production has been directly linked to oxidation of proteins, DNA, and lipids, which may cause injury or induce a variety of cellular responses through the generation of secondary metabolic reactive species (Figure 42.3).

IV. LIPID PEROXIDATION AND *IN VIVO* MARKERS OF OXIDATIVE DAMAGE

Due to a high concentration of substrate polyunsaturated fatty acids (PUFAs) in cells, lipid peroxidation is a major outcome of free radical-mediated injury (Montine *et al.*, 2002a, b). A critically important aspect of lipid peroxidation

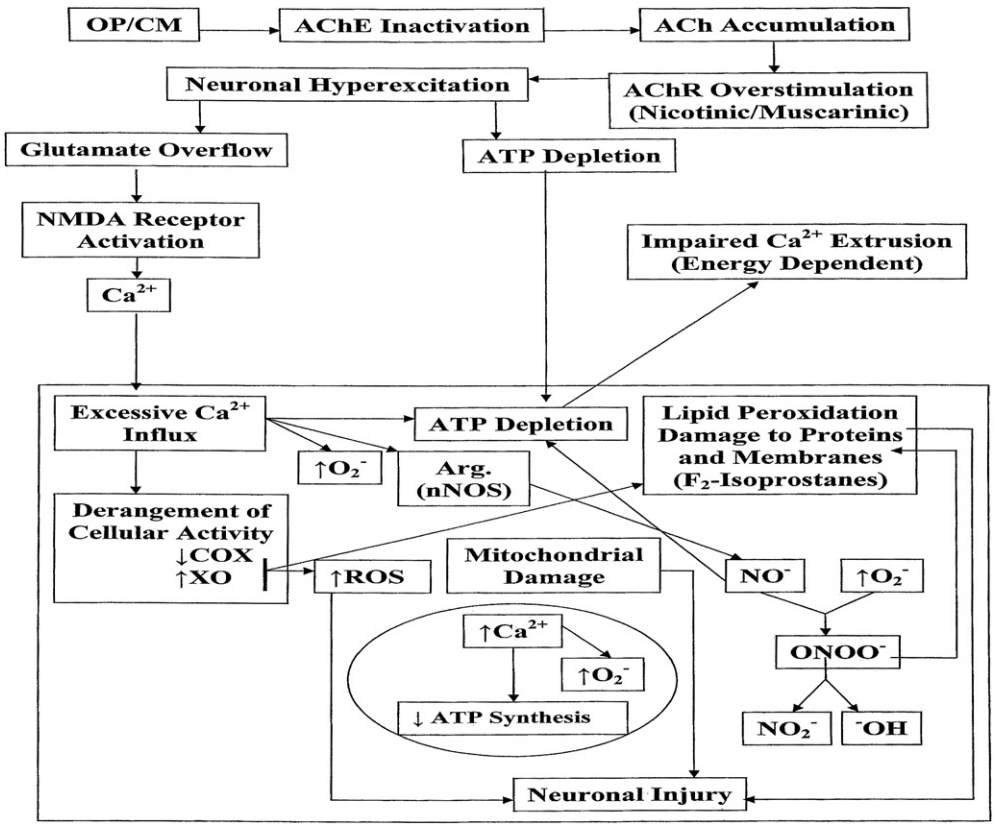


FIGURE 42.3. A schematic diagram showing possible mechanisms involved in an anticholinesterase-induced neuronal injury or death by excessive production of ROS/RNS leading to lipid peroxidation. See text for the details.

is that it will proceed until the oxidizable substrate is consumed or termination occurs, making this fundamentally different from many other forms of free radical injury in that the self-sustaining nature of the process may entail extensive tissue damage (Porter *et al.*, 1995). Decreased membrane fluidity following lipid peroxidation makes it easier for phospholipids to exchange between the two halves of the bilayer, increase the leakiness of the membrane to substances that do not normally cross it other than through specific channels (e.g. K^+, Ca^{2+}), and damage membrane proteins, inactivating receptors, enzymes, and ion channels (Halliwel and Gutteridge, 2006; Halliwell, 2007). Increases in Ca^{2+} induced by oxidative stress can activate phospholipase A_2, which releases arachidonic acid (AA) from membrane phospholipids. The free AA can then both undergo lipid peroxidation (Farooqui *et al.*, 2001) and act as a substrate for eicosanoid synthesis. Increased prostaglandin synthesis is immediately linked to lipid peroxidation, because low levels of peroxides accelerate cyclooxygenase action on PUFAs (Smith, 2005). Phospholipase A_2 can also cleave oxidized AA residues from membranes.

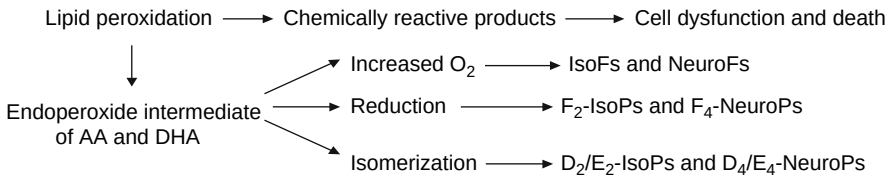
The use of reactive products of lipid peroxidation as *in vivo* biomarkers is limited because of their chemical instability and rapid and extensive metabolism (Gutteridge and Halliwell, 1990; Moore and Roberts, 1998). For these reasons, other more stable lipid products of oxidative damage have generated intense interest in recent years as *in vivo* markers of oxidative damage. These compounds include the F_2-isoprostanes (F_2-IsoPs), isofurans (IsoFs), and F_4-neuroprostanes (F_4-NeuroPs) (Morrow *et al.*, 1990; Fessel *et al.*, 2002). F_2-IsoPs are formed by peroxidation of AA. Three major structural isomers of IsoPs are formed from a common intermediate: F-ring IsoPs formed by reduction and D/E-ring IsoPs formed by isomerization (Morrow *et al.*, 1998). The ratio of F-ring to D/E-ring compounds reflects the reducing environment in which F_2-IsoPs form, with greater reducing equivalents favoring a higher ratio of F- to D/E-ring compounds (Morrow *et al.*, 1998). More recently, it has been shown that in the presence of increased oxygen tension in the microenvironment in which lipid peroxidation occurs, an additional oxygen insertion step may take place (Fessel *et al.*, 2002). This step diverts the IsoP pathway to form tetrahydrofuran ring-containing compounds termed IsoFs, which are functional markers of lipid peroxidation under conditions of increased oxygen tension.

The measurement of F_2-IsoPs is a method that has been extensively replicated as an efficient means of quantifying free radical damage in *in vivo* models associated with neurodegenerative diseases, including Alzheimer's disease (Montine *et al.*, 1999), inflammation (Milatovic *et al.*, 2003, 2004), and excitotoxicity (Milatovic *et al.*, 2005b). Since AA present throughout the brain and in different cells in the brain at roughly equal concentrations, F_2-IsoPs reflects damage to brain tissue but not necessarily to neurons.

Similar studies of lipid peroxidation products have been performed for other substrate lipids. Of particular interest are oxidation products of docosahexaenoic acid (DHA), which have been termed F_4-neuroprostanes (F_4-NeuroPs) (Roberts *et al.*, 1998). In contrast to AA, which is evenly distributed in all cell types in all tissues, DHA is highly concentrated in neuronal membranes (Salem *et al.*, 1986; Montine *et al.*, 2004). Thus, determination of F_4-NeuroPs permits the specific quantification of oxidative damage to neuronal membranes *in vivo* (Montine *et al.*, 2004). In fact, to our knowledge, F_4-NeuroPs are the only quantitative *in vivo* marker of oxidative damage that is selective for neurons.

V. HIGH-ENERGY PHOSPHATES

Inhibition of AChE leads to unremitting stimulation of nervous tissue and muscle, which, in turn, causes depletion of high-energy phosphates (HEP), ATP, and phosphocreatine (PCr) (Dettbarn *et al.*, 2001). If this stimulation is sufficiently low in intensity or brief in duration, cellular recovery will ensue without lasting consequences. If, however, intense cholinergic stimulation is allowed to persist, a self-reinforcing cycle of cellular damage is set into motion. ATP depletion for several hours to approximately 30–40% of normal levels leads to a fall in the mitochondrial membrane potential that is associated with: (1) reduced energy production (due to decrease in complex I and complex IV activities), (2) impaired cellular calcium sequestration, (3) activation of protease/caspases, (4) activation of phospholipases, (5) activation of nitric oxide synthase (NOS), and (6) excessive generation of ROS (Milatovic *et al.*, 2006). Several of these steps are associated with exacerbation and propagation of the initial depletion of ATP; most notable are the decreases in complex I and IV activities, the impairment of mitochondrial calcium



metabolism that regulates ATP production even in the face of a constant supply of substrates, and the generation of nitric oxide, which binds reversibly to cytochrome c oxidase (COx) in competition with oxygen, with subsequent sensitization to hypoxia. COx is the terminal complex in the mitochondrial respiratory chain, which generates ATP by oxidative phosphorylation, involving the reduction of O_2 to H_2O by the sequential addition of four electrons and four H^+. Electron leakage occurs from the electron transport chain, which produces the superoxide anion radical (O_2^-) and H_2O_2. Under normal conditions, COx catalyzes more than 90% of the oxygen consumption in the cells. The chance of intermediate products, such as O_2^- and H_2O_2 and hydroxyl (OH^-) radical, escaping is small under conditions where COx remains active. During the hyperactivity of brain or muscle, the activity of COx is reduced (Milatovic *et al.*, 2001), leading to an increased electron flow within the electron transport chain, thereby increasing ROS generation, oxidative damage to mitochondrial membranes, and vulnerability to excitotoxic impairment (Soussi *et al.*, 1989; Gollnick *et al.*, 1990; Bose *et al.*, 1992; Bondy and Lee, 1993; Yang and Dettbarn, 1998). We have previously established that several of these key events occur within 1 h of anticholinesterase treatment (Gupta *et al.*, 2000). DFP-induced seizures markedly lowered the cellular ATP and PCr levels in discrete brain regions (Gupta *et al.*, 2001a, b, c) and significantly reduced COx activity (Milatovic *et al.*, 2001).

VI. NITRIC OXIDE (NO/NOS)

Another important free radical related to lipid peroxidation is NO. NO is synthesized by neuronal cells from L-arginine by NO synthases (NOS), with the accompanying release of citrulline (Wendland *et al.*, 1994; Burette *et al.*, 2002). There are three different isoenzymes of NOS, neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). A major stimulus for NO production is elevation in intracellular Ca^{2+}, which binds to calmodulin, resulting in the activation of constitutive NOS (nNOS and eNOS). NOS activity increases during anticholinesterase- and KA-induced excitotoxicity (Parathath *et al.*, 2006; Gupta *et al.*, 2007; Zaja-Milatovic *et al.*, 2008). It has been suggested that NO is involved in glutamate receptor-mediated neurotoxicity by decreasing intracellular ATP levels. There are two possible mechanisms responsible for energy depletion caused by NO in neuronal cells: one is the prolonged activation of poly-(ADP ribose) polymerase (PARP), which can be activated by NO (Zhang *et al.*, 1994), leading to depletion of ATP. The other mechanism is the inhibition of the mitochondrial complexes, leading to diminished ATP production. Many reports establish that NO impairs mitochondrial/cellular respiration and other functions by inhibiting the activities of several key enzymes, particularly COx, thereby causing ATP depletion (Yang and Dettbarn, 1998; Milatovic *et al.*, 2001; Gupta *et al.*, 2001a, b; Dettbarn *et al.*, 2001). NO was also reported to inhibit complexes II and III (Bolanos *et al.*, 1994), as well as complex IV (Lizasoain *et al.*, 1996) in neuron-derived mitochondria and neuronal energy production in cultured hippocampal neurons (Brorson *et al.*, 1999), leading to rapid ATP depletion. In addition, increased production of NO in the presence of the superoxide anion radical may generate peroxynitrite radical (Montine *et al.*, 2002c; Milatovic *et al.*, 2002), a potent inducer of lipid peroxidation.

VII. DENDRITIC INJURY

In addition to formation of ROS, RNS and depletion of HEP, seizures may cause brain injury via a number of mechanisms, potentially contributing to neurological and cognitive deficit alterations. The hippocampus and its pyramidal neurons are particularly susceptible to excitotoxicity, resulting in the selective deterioration of synapses and cytoskeletal damage. Although seizures can induce neuronal death, they may also have ‘‘nonlethal’’ pathophysiological effects on neuronal structure and function. Dendritic spines represent the structural sites of contact for the majority of excitatory, glutamatergic synaptic inputs onto neurons and are strongly implicated in mechanisms of synaptic plasticity and learning. NMDA and other glutamate receptor subtypes are clustered in dendritic spines (Rao and Craig, 1997; O’Brien *et al.*, 1998), which serve as integrative units in synaptic circuitry and participate in synaptic plasticity (Yuste and Denk, 1995). The accumulation of glutamate receptor clusters in spines is governed by excitatory synaptic activity, and increases when activity is suppressed (Rao and Craig, 1997; O’Brien *et al.*, 1998). Conversely, excitotoxicity produces a rapid and profound loss of dendritic spines in cultured neurons (Halpain *et al.*, 1998), mimicking the loss in dendritic spine synapses in neurological conditions including epilepsy, aging, and schizophrenia (Jeffrey *et al.*, 1997; Jiang *et al.*, 1998). This suggests that receptor localization at synapses might be critical to excitotoxicity, and govern neuronal vulnerability to excitotoxicity.

A number of studies demonstrate a loss of dendritic spines in pathophysiological specimens from animal seizure models (Olney *et al.*, 1986), suggesting that seizures can cause dendritic injury. It has also been shown that overstimulation and altered expression of glutamate receptor following anticholinesterase exposure are associated with synaptic architecture dysfunction and cellular deterioration in the brain, especially in the hippocampus (Johnson *et al.*, 2008). Thus, excitotoxic levels of glutamate following soman exposure are thought to be involved in the dendritic and synaptic damage as an early toxicological response that leads to neuronal dysfunction and memory impairment (Carpentier *et al.*, 1991).

VIII. ANTICHOLINESTERASE-INDUCED SEIZURES, OXIDATIVE INJURY, AND NEURODEGENERATION

Lipid peroxidation, mitochondrial dysfunction, reduced neuronal energy levels, and reduced COx activity support the contention that anticholinesterases, such as diisopropylphosphorofluoridate (DFP) and carbofuran (CF), cause neuronal injury by excessive formation of ROS (Yang and Dettbarn, 1998; Jeyarasasingam *et al.*, 2000; Gupta *et al.*, 2001a, b; Milatovic *et al.*, 2000a, b, 2001, 2005a). Additionally, our recent studies showed that seizure-induced cerebral oxidative damage in adult animals is accompanied by alterations in integrity of the hippocampal CA1 dendritic system (Gupta *et al.*, 2007; Zaja-Milatovic *et al.*, 2008).

A single injection of DFP (1.5 mg/kg, s.c.) or another AChE inhibitor, carbofuran (CF, 1.5 mg/kg, s.c.), produces toxic signs in rats, including salivation, tremors, wet dog shakes, fasciculations, and mild to moderate seizures with rearing and rolling over, with progression to severe seizures within 7–15 min (Milatovic *et al.*, 2006; Gupta *et al.*, 2007). Signs of maximal intensity such as severe muscle fasciculations, seizures, and convulsions develop within 15–30 min and last for more than 2 h before tapering off. By 24 h, animals are free of toxic signs. The observed signs are typical of anticholinesterase toxicity and reveal the involvement of both the central as well as the peripheral nervous systems (Gupta *et al.*, 2001a, b; Milatovic *et al.*, 2005a). Analysis of brains from saline-treated control rats revealed regional variability in brain AChE activity (cortex, 222.0 ± 10.7; amygdala, 529.2 ± 10.29; and hippocampus 301.2 ± 9.5 µmol/g wet weights). A single acute dose of DFP (1.5 mg/kg, s.c.) suppressed AChE activity to less than 20% in all brain regions compared to control 60 min following the exposure. Similarly, 60 min after a single acute dose of CF (1.5 mg/kg, s.c.), AChE was markedly depressed (% remaining activity: cortex, 10.02 ± 1.04%; amygdala, 18.18 ± 1.48%; and hippocampus, 12.75 ± 0.74%) (Gupta *et al.*, 2007). At the time of high AChE inhibition and resultant severe seizure activity, significant increase in biomarkers of global free radical damage (F_2-IsoPs) and the selective peroxidation biomarker of neuronal membranes

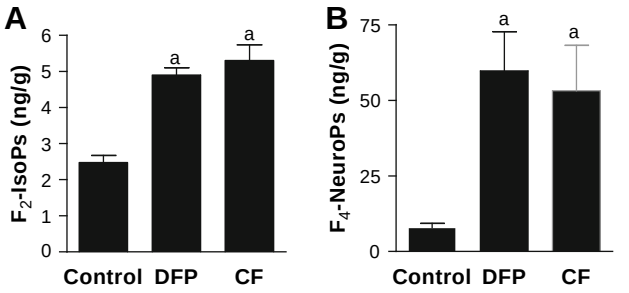


FIGURE 42.4. Effect of DFP (1.5 mg/kg, s.c.) and carbofuran (CF, 1.5 mg/kg, s.c.) on F2-IsoPs (A) and F4-NeuroPs (B) levels in rat brain. Values are mean ± SEM ($n = 4$–6). [a]Significant difference between control and DFP- or CF-treated rats ($p < 0.05$).

(F_4-NeuroPs) were seen in the brain of DFP and carbofuran exposed animals.

While two-fold elevations are seen in F_2-IsoPs levels, F_4-NeuroPs levels are more than five-fold higher compared to controls (Figure 42.4). The results confirm the presence of oxidative damage in cerebrum as a novel aspect of anticholinesterase toxicity. The selective increase in F_4-NeuroPs indicates that neurons are specifically targeted by this mechanism.

DFP and exposure also caused marked elevation in brain citrulline levels (an indicator of NO/NOS activity) (Gupta *et al.*, 2001b, 2007). Control levels of citrulline are similar in the hippocampus (247.90 ± 4.10 nmol/g) and the amygdala (293.20 ± 6.90 nmol/g) (Gupta *et al.*, 2007). Within 5 min of CF injection, the citrulline levels were elevated more than two-fold in the investigated brain areas. Within 15 min of CF treatment, the levels of citrulline were significantly higher in both brain regions and were maximally elevated at 30 min post-injection (three- to four-fold). They remained elevated up to 60 min, but returned to control levels when measured 24 h later (Figure 42.5). Similar response was seen following DFP exposure.

Many reports provide evidence that NO impairs mitochondrial/cellular respiration and other functions by inhibiting the activities of several key enzymes, particularly COx, thereby causing ATP depletion (Yang and Dettbarn, 1998; Milatovic *et al.*, 2001; Gupta *et al.*, 2001a, b; Dettbarn *et al.*, 2001). Results from our experiments also showed that

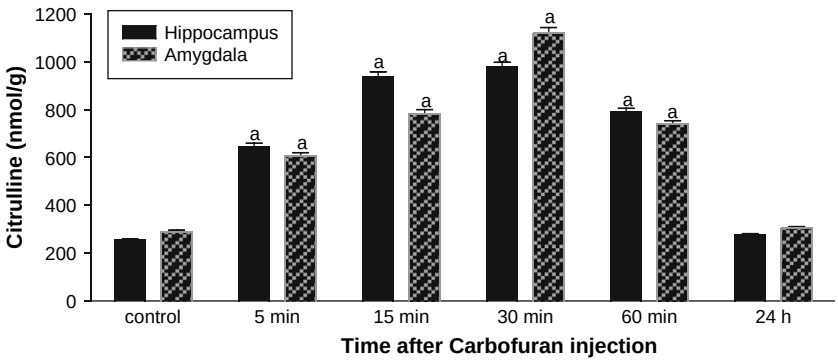


FIGURE 42.5. Citrulline levels in brain regions of rats intoxicated with an acute dose of carbofuran (1.5 mg/kg, s.c.). Values of citrulline are presented as mean ± SEM ($n = 4$–6). [a]Significant difference between values from control and carbofuran-treated rats ($p < 0.05$).

1 h after DFP (1.5 mg/kg, s.c.) or CF (1.5 mg/kg, s.c.) treatment, the levels of ATP and PCr were significantly reduced in the hippocampus and amygdala. (Figure 42.6). With either DFP or CF treatment, the reduction in ATP and PCr levels was similar in the amygdala and hippocampus. During the course of these excitatory processes, a high rate of ATP consumption, coupled with the inhibition of oxidative phosphorylation, compromises the cell's ability to maintain its energy levels, and excessive amounts of ROS and RNS may be generated. Thus, the combination of impaired synthesis of ATP with its greater utilization during brain hyperactivity appears to result in a significant depletion of ATP. Three days after anticholinesterase treatment, significant recovery of ATP and PCr is observed in discrete brain regions (Figure 42.6). The rapid decrease in energy metabolites at the onset of seizures indicates early onset of mitochondrial dysfunction, in turn, further increasing ROS production and neuronal injury.

An important question that emerged from previous studies is whether brain hyperactivity, such as seizures, first generates increases in ROS and then causes a decrease in HEPs. The findings revealed that within 5–15 min after carbofuran injection (the time required for onset and development of clinical signs), NO levels increased more than five- to six-fold in the cortex and more than two- to three-fold in the amygdala and hippocampus. The maximum increase in NO occurred at 30 min post-injection in all three brain regions. The data also revealed that maximum decline in HEPs occurred 1 h after carbofuran injection. This is in agreement with our previous data showing a rapid and significant increase in NO precedes increases in lipid peroxidation, mitochondrial dysfunction, loss of energy metabolites, as well as a reduction of COx activity, and an increase in xanthine oxidase (Dettbarn *et al.*, 2006). Therefore, the findings suggest that in the case of carbofuran, the increase in ROS preceded the decrease in HEPs.

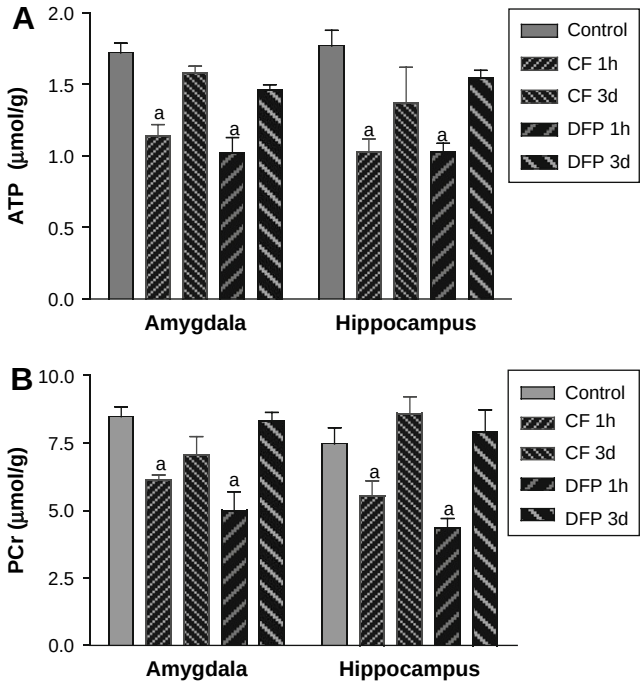


FIGURE 42.6. Levels of high-energy phosphates, ATP (A) and PCr (B) in amygdala and hippocampus of rats intoxicated with an acute dose of carbofuran (CF, 1.25 mg/kg, s.c.) or diisopropyl phosphorofluoridate (DFP, 1.25 mg/kg, s.c.). Rats were sacrificed 1 hour (1 h) or 3 days (3 d) after CF or DFP injection. Values of ATP and PCr are presented as means ± SEM ($n = 4$–6). [a]Significant difference between values from control rats and DFP- or CF-treated rats ($p < 0.05$).

Seizures, convulsions, and CNS lesions are typical results of systemic application of sublethal doses of anticholinesterase agents (Sparenborg *et al.*, 1992). The most consistent pathological findings in acute experiments include degeneration and cell death in the pyriform cortex, amygdala, hippocampus (where the CA1 region is preferentially destroyed), dorsal thalamus, and cerebral cortex. The early morphological changes in AChEI-induced status epilepticus (SE) include dendritic swelling of pyramidal neurons in the CA1 region of the hippocampus (Carpentier *et al.*, 1991). Therefore, we have investigated whether seizure-induced cerebral oxidative damage in adult rats is accompanied by alterations in the integrity of the hippocampal CA1 dendritic system. Our results showed that anticholinesterase-induced early increases in biomarkers of global free radical damage (F_2-IsoPs) and the selective peroxidation biomarker of neuronal membranes (F_4-NeuroPs) was accompanied by dendritic degeneration of pyramidal neurons in the CA1 hippocampal area. Anticholinesterase-induced brain hyperactivity targeted the dendritic system with profound degeneration of spines and regression of dendrites, as evaluated by Golgi impregnation and Neurolucida-assisted morphometry (Figure 42.7).

Rats injected with DFP show a significant decrease in total dendritic length and spine density compared to pyramidal neuron from the hippocampal CA1 area of control rats (Figure 42.7). Taken together with the biochemical data presented above, our results suggest that oxidative damage that selectively targets cerebral neurons is a hitherto unrecognized aspect of anticholinesterase toxicity. Results also revealed that anticholinesterase exposure is associated with oxidative and nitrosative stress, alteration in energy metabolism and consequent degeneration of pyramidal neurons from the CA1 hippocampal region of rat brain. Ultimately, the additive or synergistic mechanisms of cellular disruption caused by anticholinesterase agents lead to cellular dysfunction and neurodegeneration.

IX. OXIDATIVE DAMAGE AND DENDRITIC DEGENERATION FOLLOWING KAINIC ACID-INDUCED EXCITOTOXICITY

Since excessive presynaptic release of glutamate and activation of NMDA and non-NMDA receptors have

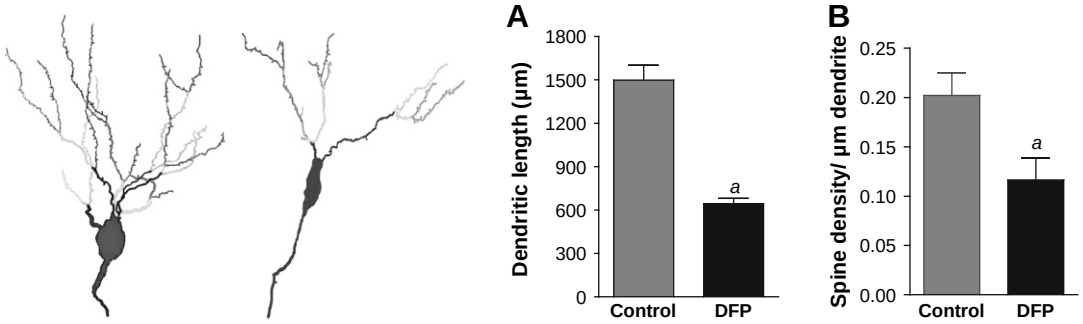


FIGURE 42.7. Morphology and quantitative determination of dendritic length (A) and spine density (B) of hippocampal pyramidal neurons from CA1 sector of rats treated with saline (control) or DFP (1.5 mg/kg, s.c.) and sacrificed 1 h after the treatment. Four to six Golgi-impregnated dorsal hippocampal CA1 neurons were selected and spines counted by using the Neurolucida system. [a]Significant difference between control and DFP-treated rats ($p < 0.05$). Treatment with DFP induced degeneration of the hippocampal dendritic system and a decrease in total length of dendrite and spine density of hippocampal pyramidal neurons. Tracing and counting are done using the Neurolucida system at 100× under oil immersion (MicroBrightField, VT).

a significant role in anticholinesterase-induced neurotoxicity, we have investigated the role of glutamatergic excitation, oxidative injury, and neurodegeneration in the model of KA excitotoxicity. We have used intracerebroventricular (i.c.v.) injection of KA, known as an experimental model for investigation of cerebral vulnerability, particularly during acute brain disorders (Ben-Ari, 1985) and status epilepticus (Ben-Ari and Cossart, 2000; Schwob *et al.*, 1980). The study was designed to investigate whether F_2-IsoPs and F_4-NeuroPs formation correlated with the vulnerability of pyramidal neurons in the CA1 hippocampal area following KA-induced excitotoxicity. Our results showed that i.c.v. KA-induced early increase in biomarkers of oxidative damage, F_2-IsoPs and F_4-NeuroPs was accompanied by dendritic degeneration of pyramidal neurons in the CA1 hippocampal area.

Time-course changes in biomarkers of oxidative damage in the rat model of anticholinesterase-induced seizures showed that the highest increase in F_2-IsoPs was evaluated at 1 h after the injection of anticholinesterase agent or 40 min after the beginning of seizure symptoms (Gupta *et al.*, 2007). In the model of KA-induced excitotoxicity the earliest time point evaluated was 30 min since seizures start immediately after the i.c.v. KA injection. Elevated levels of these *in vivo* markers of oxidative damage are in agreement with our previous findings (Montine *et al.*, 2002c; Milatovic *et al.*, 2005b; Gupta *et al.*, 2007), as well as those of others (Patel *et al.*, 2001), and indicate that KA injection leads to profound cerebral and neuronal oxidative damage in mice.

Our results also showed that the transient rise in F_2-IsoPs and F_4-NeuroPs is accompanied by rapid evolution of dendritic abnormalities, apparent in significant decrease in dendritic length and spine density of pyramidal neurons as early as 30 min post-KA injection. However, the recovery in both oxidative damage biomarkers at 60 min following the injection was not paralleled by the rescue of damaged neurons from the CA1 hippocampal area. Extended seizure activity (60 min) induced the same level of dendritic length and spine density decrease when compared to 30 min following KA injection (Table 42.1). Together, these data suggest that both oxidative stress and neurodegeneration occur as an early response to seizures, but do not establish whether oxidative stress is a cause or

TABLE 42.1. Cerebral concentrations of F2-IsoPs and F4-NeuroPs and dendritic degeneration of hippocampal pyramidal neurons following KA-induced seizures in mice

	F_2-IsoPs (ng/g)	F_4-NeuroPs (ng/g)	Dendritic length (µm)	Spine density (number/100 µm dendrite)
Control	3.07 ± 0.05	13.89 ± 0.58	1032.10 ± 61.41	16.45 ± 0.55
KA 30 min	4.81 ± 0.19[a]	34.27 ± 2.71[a]	363.44 ± 20.78[a]	8.81 ± 0.55[a]
KA 60 min	3.40 ± 0.18	18.55 ± 1.26	425.71 ± 23.04[a]	7.44 ± 0.56[a]

Data from KA-exposed mice were collected 30 or 60 min post-injection

[a]One-way ANOVA showed $p < 0.0001$ for each endpoint. Bonferroni's multiple comparison test showed significant difference ($p < 0.001$) compared to vehicle-injected control

an effect of seizure-induced CA1 cell damage. Neuronal damage processes triggered by sustained seizure activity may occur as a continuum, last longer than formation of oxidative lipids and, although not evident by the markers, may already be in progress when the peak increases in F_2-IsoPs and F_4-NeuroPs occur. Thus, we investigated dynamic changes in lipid peroxidation and dendritic structures immediately after seizures, but future studies over the longer period should be able to determine the long-term time course of these spine and dendritic changes. It is very likely that the spine loss seen in our study is the initial phase of more chronic spine loss and progressive neurodegeneration reported in other studies (Muller *et al.*, 1993; Multani *et al.*, 1994; Isokawa and Levesque, 1991; Jiang *et al.*, 1998; Zeng *et al.*, 2007).

In vivo data have also established that KA induced a significant increase (more than two-fold) in citrulline concentrations 30 min following the injection (Zaja-Milatovic *et al.*, 2008). Although we did not determine whether increased citrulline originated from a combination of NOS isozymes or one in particular, our data are in agreement with the results from the models of anticholinesterase toxicity and activated innate immunity (Gupta *et al.*, 2007; Milatovic *et al.*, 2003, 2004) and indicate that a subset of NOS activity also contributes to cerebral oxidative damage in the model of KA-induced excitotoxicity.

X. SUPPRESSION OF SEIZURE-INDUCED OXIDATIVE INJURY AND NEURODEGENERATION

A. Antioxidants

Antioxidants (e.g. vitamins, glutathione, selenium, zinc, creatine, and arginine) and antioxidant enzymes (e.g. superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase) exert synergistic actions in scavenging free radicals. A large body of literature supports the notion that antioxidants play an important role in preventing many human diseases (e.g. cancer, atherosclerosis, stroke, rheumatoid arthritis, and neurodegeneration) (Fang *et al.*, 2002). Vitamin E has been recognized as one of the most important antioxidants. Vitamin E inhibits ROS-inducing generation of lipid peroxyl radicals, thereby protecting cells from peroxidation of PUFA in membrane phospholipids, from oxidative damage of cellular proteins and DNA, and from membrane degeneration (Topinka *et al.*, 1989). Vitamin E mainly acts as a chain breaking antioxidant and radical scavenger, protecting cell membranes against oxidative damage (VanAcker *et al.*, 1993). In addition, vitamin E regulates ROS production (Chow *et al.*, 1999), maintains oxidative phosphorylation in mitochondria, and accelerates restitution of high-energy metabolites (Punz *et al.*, 1998; Kotegawa *et al.*, 1993). Decreased levels of vitamin E in response to hyperoxia or treatment with convulsants reported in recent studies (Mori *et al.*, 2004; Onodera *et al.*, 2003; Rauca *et al.*, 2004), suggest that vitamin E in the brain is consumed to prevent oxidative damage. Vitamin E also prevented metasystox (OP insecticide)-induced changes in lipase activity and lipid peroxidation in the brain and spinal cord of rats (Tayyaba and Hasan, 1985).

A synthetic spin trapping agent such as phenyl-*N*-*tert*-butylnitrone (PBN) is also capable of scavenging many types of free radicals. This compound is widely used to trap ROS in a variety of physical, chemical, and biological studies using electron paramagnetic resonance spectrometry. PBN is known to be concentrated in the mitochondria, where it reacts with ROS and forms stable adducts, and thereby maintains normal levels of energy metabolites. Numerous *in vitro* and *in vivo* experiments have shown the beneficial effects of PBN on the prevention of neuronal degeneration. Protective effects are described in experimental models of brain ischemia/reperfusion (Phillis and Clough-Helfman, 1990; Carney and Floyd, 1991; Gido *et al.*, 1997; Fetcher *et al.*, 1997), excitotoxicity (Cheng and Sun, 1994; Lancelot *et al.*, 1997; Milatovic *et al.*, 2002), inhibition of nitric oxide synthase induction (Krishna *et al.*, 1996; Miyajima and Kotake, 1995), and in different models of seizures (He *et al.*, 1997; Thomas *et al.*, 1997). Additional findings also corroborate that PBN effectively prevents neurodegeneration in Parkinson's disease (Fallon *et al.*, 1997; Frederiksson *et al.*, 1997; Sack *et al.*, 1996), Alzheimer's disease and anticholinesterase neurotoxicity (Sack *et al.*, 1996; Gupta *et al.*, 2001a, b). Thus, PBN has been proven to rescue neurons in multiple experimental injury models. Other pharmacological properties of spin trapping agents have been described that could influence the outcome of oxidant injury. These have been described for PBN as reversible Ca^{2+} channel blockade in vascular muscle causing vasodilatation (Anderson *et al.*, 1993), direct effect on striatal function, including inhibition of excitation–contraction coupling (Andersen *et al.*, 1996), and induction of hypothermia (Pazos *et al.*, 1999).

Previous studies have shown that antioxidant pretreatment suppressed DFP- or CF-induced alterations in HEP, their metabolites and citrulline levels, supporting the possibility that increased generation of ROS/RNS contributes to depletion of energy phosphates (Gupta *et al.*, 2001a, b). PBN or vitamin E treatment alone did not alter the levels of high-energy phosphates, their major metabolites, or citrulline in any of the brain regions. Vitamin E pretreatment suppressed the depletion of HEP and their metabolites and increased citrulline levels without preventing seizures (Gupta *et al.*, 2001a). The protective efficacy provided by vitamin E against DFP- or carbofuran-induced changes in energy metabolites was of varying degrees in different brain regions and could partly be due to pharmacokinetic variables involved in attaining different levels of vitamin E in different brain regions. However, PBN pretreatment, 1 h before anticholinesterase agent, protected mitochondria, maintained cellular level of

high-energy metabolites, but also prevented DFP- or CF-induced convulsions and seizures (Gupta *et al.*, 2001a, b). This could primarily be due to a protective interaction of PBN with AChE, sufficient to protect a critical fraction of AChE against phosphorylation by DFP or carbamylation by CF (Zivin *et al.*, 1999; Milatovic *et al.*, 2000a, b). We have also shown that AChE inhibitor-induced increases in NO (citrulline) were significantly prevented by PBN as well as by vitamin E (Gupta *et al.*, 2001a). There is evidence that suggests that PBN inhibits the induction of inducible NOS (iNOS) by reducing the expression of iNOS protein (decrease in mRNA expression), thus preventing the over-production of NO (Miyajima and Kotake, 1995, 1997).

The efficacy of the spin trapping agent PBN and the antioxidant vitamin E was tested to suppress increase in NO and lipid peroxidation and prevent neurodegeneration of pyramidal neurons in the CA1 hippocampal area in the model of KA-induced excitotoxicity. Vitamin E or PBN alone did not alter basal citrulline and F_4-NeuroPs levels or dendritic arborization. However, vitamin E and PBN suppressed KA-induced increases in citrulline and cerebral and neuronal markers of oxidative damage, F_2-IsoPs and F_4-NeuroPs, respectively (Figures 42.8 and 42.9).

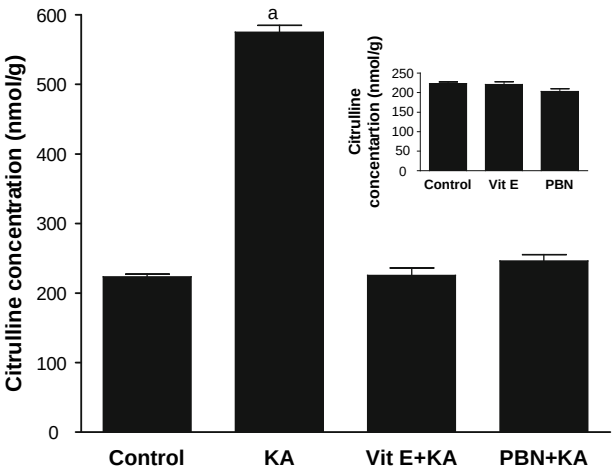


FIGURE 42.9. Ipsilateral cerebral citrulline concentrations following i.c.v. KA with or without vitamin E (Vit E) or *N-tert*-butyl-α-phenylnitrone (PBN) pretreatment. Brains from mice exposed to KA were collected 30 min post-injections ($n \geq 5$ for each group). One-way ANOVA had $p < 0.001$ with Bonferroni's multiple comparison tests significant for KA vs control, Vit E + KA, or PBN + KA treatment.

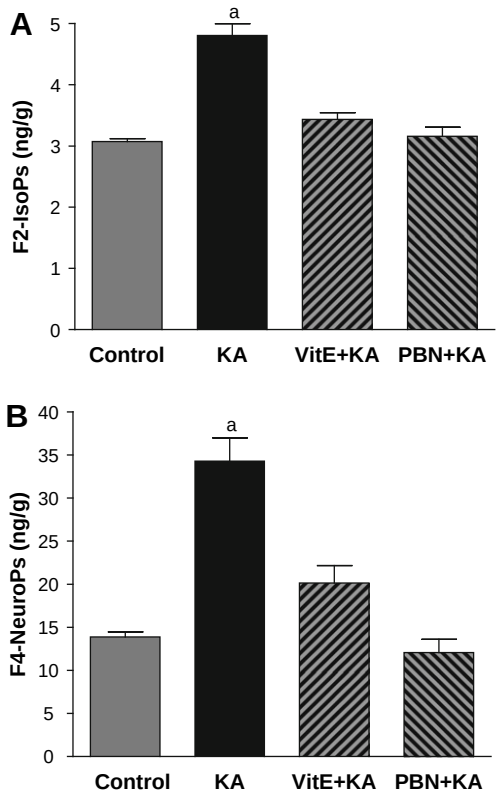


FIGURE 42.8. Ipsilateral cerebral F2-IsoPs (A) and F4-NeuroPs (B) concentrations following i.c.v. KA with or without vitamin E (Vit E) or *N-tert*-butyl-α-phenylnitrone (PBN) pretreatment. Brains from mice exposed to KA were collected 30 min post-injections ($n \geq 5$ for each group). One-way ANOVA had $p < 0.0001$ with Bonferroni's multiple comparison tests significant for KA vs control, Vit E + KA or PBN + KA treatment.

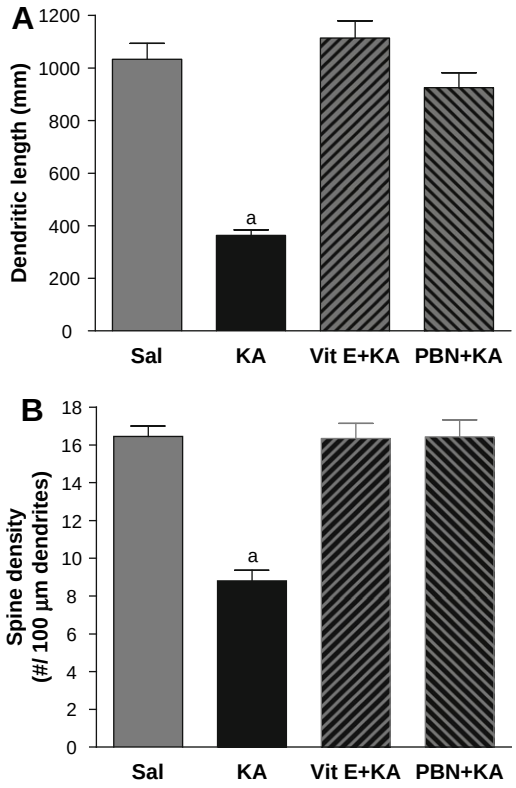


FIGURE 42.10. Dendritic length (A) and spine density (B) of pyramidal neurons from the CA1 hippocampal area of mice following i.c.v. KA with or without vitamin E (Vit E) or *N-tert*-butyl-α-phenylnitrone (PBN) pretreatment. Brains from mice exposed to KA were collected 30 min post-injections ($n \geq 5$ for each group). One-way ANOVA had $p < 0.001$ with Bonferroni's multiple comparison tests significant for KA vs control, Vit E + KA or PBN + KA treatment.

Importantly, vitamin E and PBN completely suppressed both reduction in dendrite length and reduction in spine density of pyramidal neurons from the CA hippocampal area from KA-exposed mice (Figure 42.10).

A close concordance was found between these results showing that protection of the cerebrum from neuronal oxidative damage also protected hippocampal CA1 pyramidal neurons from dendritic degeneration. These agents did not alter kainite-induced seizure severity, indicating that the protective effect of vitamin E and PBN is most likely mediated by scavenging ROS, preventing lipid peroxidation and consequent neuronal damage, and not by a specific effect on seizures *per se*. Furthermore, since antioxidants minimize lipid peroxidation following an increase in α-tocopherol and PBN, then a parallel reduction in neuronal damage provides strong evidence that oxidative stress and lipid peroxidation in a causal way mediate seizures and the corresponding injury. One limitation to the potential therapeutic application is that the drugs, to be effective, need to be administered prophylactically before the onset of seizures. Future research should address the efficacy of these agents when administered at higher concentrations either during or possibly even after seizures, in preventing seizure-induced oxidative and dendritic changes and potentially reducing resultant neurocognitive deficits.

XI. NMDA RECEPTOR ANTAGONIST (MEMANTINE)

As excitotoxicity-induced neuronal damage in the model of anticholinesterase-induced seizures is explained by excessive release of glutamate that activates both NMDA and non-NMDA posynaptic receptors, antagonism of the excitotoxicity mechanism may protect the CNS from the deleterious effects of anticholinesterase agents. Several NMDA receptor antagonists have been shown to exert anticonvulsant effects against nerve agent-induced seizures when administered as a pretreatment or after the seizure has been initiated, usually terminating the convulsions after an initial period of epileptical activity (Shih, 1990; Sparenborg *et al.*, 1992). NMDA receptor antagonists do not modify the events responsible for the early phase of the seizure, but block the subsequent recruitment of glutamate receptor activation and hence the maintenance of seizure activity and irreversible functional and structural brain damage.

Among promising candidates as antidotes against CNS intoxication by OP nerve agents, memantine (MEM) has been shown to pose both anti-excitotoxic and anti-epileptic properties. Memantine is an uncompetitive NMDA receptor antagonist, clinically used for the treatment of Alzheimer's disease, Parkinson's disease and spasticity, in the absence of serious side effects (Ozsuer *et al.*, 2005; Lipton, 2005). From a series of rat *in vivo* experiments, it is evident that pre-administration of memantine significantly protects AChE activity from inhibition caused by AChE inhibitors, including OP and CM insecticides and OP nerve agents (Gupta and Kadel, 1990; Gupta and Dettbarn, 1992; McLean *et al.*, 1992; Gupta and Dekundy, 2005). By now, it is well established that memantine exerts various pharmacological effects by multiple pharmacological mechanisms: (1) blockage of nicotinic acetylcholine receptor–ion channel complex (Masuo *et al.*, 1986), (2) reduced reflex excitability of both flexors and extensors (Wand *et al.*, 1997), (3) prevention of neural hyperexcitability (McLean *et al.*, 1992), (4) central muscle relaxation (Grossman and Jurna, 1997), and (5) prevention of AChE inhibitor-mediated energy loss from muscle cells (Milatovic *et al.*, 2005a). Memantine is also able to prevent the pathogenic calcium influx caused by continuous mild activation by low-level glutamate. On the other hand, memantine allows the physiological activation of the NMDA channels by high concentrations of glutamate, a phenomenon necessary for synaptic plasticity underlying normal learning and memory (Parsons *et al.*, 1999).

In recent studies it has also been demonstrated that memantine treatment significantly reduces lipid peroxidation (Figure 42.11), alterations in citrulline and HEP

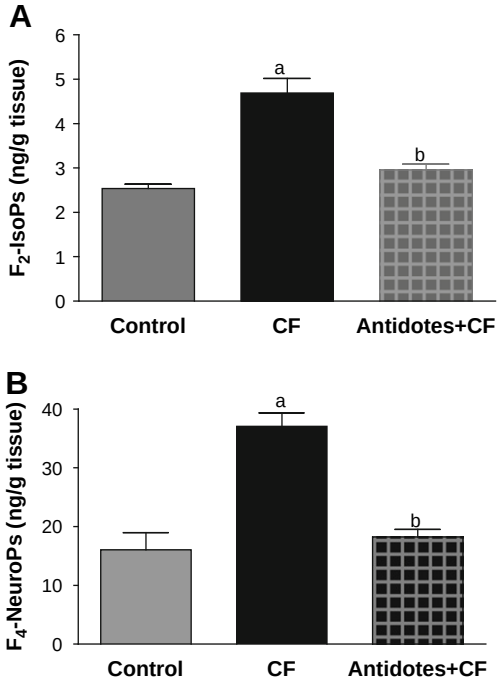


FIGURE 42.11. Cerebral F2-IsoPs (A) and F4-NeuroPs (B) concentrations following carbofuran (CF, 1.5 mg/kg, s.c.) with or without antidote (memantine, 18 mg/kg, and atropine, 16 mg/kg, given prophylactically, 60 min and 15 min, respectively, before CF administration) pretreatment. Brains from rats exposed to CF were collected 60 min post-injections ($n \geq 5$ for each group). One-way ANOVA had $p < 0.0001$ with Bonferroni's multiple comparison tests significant for CF vs control (a), and for CF vs antidote + CF (b).

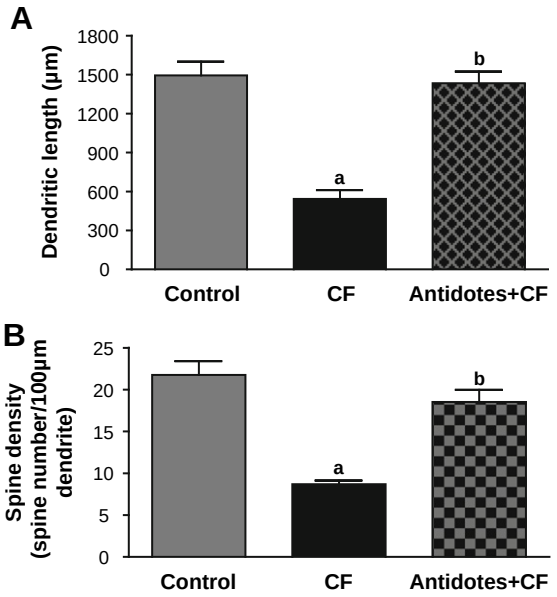


FIGURE 42.12. Dendritic length (A) and spine density (B) of pyramidal neurons from the CA1 hippocampal area of mice following carbofuran (CF, 1.5 mg/kg, s.c.) with or without antidote (memantine, 18 mg/kg and atropine, 16 mg/kg given prophylactically, 60 min and 15 min, respectively, before CF administration) pretreatment. Brains from rats exposed to CF were collected 60 min post-injections ($n \geq 5$ for each group). One-way ANOVA had $p < 0.0001$ with Bonferroni's multiple comparison tests significant for CF vs control (a), and for CF vs antidote + CF (b).

levels in muscles and brain of rats intoxicated with carbofuran (Milatovic *et al.*, 2005a; Gupta *et al.*, 2007).

No significant alterations in biomarkers of neuronal damage, citrulline, HEP, and their metabolite levels were seen in any of the brain regions receiving MEM and atropine. In addition, MEM and ATS exposure did not induce any alteration in neuronal morphometry but when given as pretreatment, provided protection against carbofuran-induced morphometric changes in hippocampal neurons (Figure 42.12). Memantine, in combination with atropine, completely suppressed both reduction in dendrite length and reduction in spine density of pyramidal neurons from the CA hippocampal area from CF-exposed rats (Figure 42.12).

In conclusion, the data demonstrated that synergistic mechanisms of cellular disruption caused by anticholinesterase agents led to cellular dysfunction and neurodegeneration. It has also been demonstrated that preventing CF-induced neuronal hyperactivity by pretreatment with MEM and atropine blocks pathways associated with oxidative damage in rat brain. The documented ability of MEM therapy to reduce free radical generation and lipid peroxidation, prevent HEPs and attenuate the morphological injury provides further support for the role of ROS and RNS in anticholinesterase-induced seizures.

XII. CONCLUDING REMARKS AND FUTURE DIRECTION

Exposure to OP nerve agents induces seizures, rapidly progressing to status epilepticus and profound structural brain damage. The progression of events includes initial high cholinergic activity followed by activation of glutamatergic neurons as a result of release of glutamate. Moreover, glutamate stimulates the continuous release of ACh, contributing to further excitatory stimulation, prolongation of the seizures, and excitotoxic neurodegeneration in vulnerable brain areas. The ensuing neuronal damage is thought to result from intense transient influx of calcium leading to mitochondrial functional impairment, cytochrome c inactivation, depletion of ATP, simultaneous formation of free radical species, and oxidative stress. Therefore, control of excitotoxicity and oxidative stress, better understanding of the mechanisms of noncholinergic mediated activities, and pathways that protect or promote neuronal survival are essential for development of efficacious treatments and preventive therapies associated with OP exposures.

We have explored mechanisms associated with OP-induced neurotoxicity by probing their effects on oxidative stress and associated dendritic degeneration of pyramidal neurons in the CA1 hippocampal area. We have also investigated different pathways to attenuate biomarkers of oxidative damage associated with anticholinesterase exposure and the extent to which such attenuation is accompanied by rescue from neurodegeneration. Results from our studies suggest that vitamin E, PBN, and memantine efficiently suppress oxidative injury. Future studies should be directed at deciphering the mechanisms of protection, addressing the ability of these agents to attenuate OP neurotoxicity via radical scavenging, AChE inhibition, and/or glutamate antagonism. Additional studies should also determine whether a combination of these treatments improve the therapeutic index against OP poisoning (compared to administration of each alone). Complementary studies should also investigate not only the prophylactic, but also therapeutic effects of these neuroprotectants. Successful identification of safe and effective neuroprotectants that suppress noncholinergic activities associated with anticholinesterase exposure will provide new pharmacological modalities to protect and treat both the acute and delayed effects of nerve agent poisoning.

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CHAPTER 43

Neuropathologic Effects of Chemical Warfare Agents

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I. INTRODUCTION

Nerve agents exert their effects through binding and irreversible inactivation of acetylcholinesterase (AChE), the enzyme that hydrolyzes acetylcholine, leading to a toxic accumulation of acetylcholine at nicotinic (skeletal muscle and preganglionic autonomic) receptors, muscarinic (mainly postganglionic parasympathetic) receptors, and central nervous system synapses. Exposure to symptomatic doses of these agents leads to characteristic neuropathologic effects that have been examined in a variety of animal models. In addition, it is becoming increasingly recognized that even lower levels of exposure to these agents, even in the absence of seizures or other severe manifestations of acute toxicity, may be associated with more subtle forms of injury to nervous tissue. Historically, this has been of concern in organophosphate and carbamate insecticide exposure among farm workers; however, it is likely that use of nerve agents in civilian populations or against military personnel would result in a range of exposures that stem from both the proximity of various groups to the site of deployment and the persistence of residues of some agents in the environment. This chapter describes the neuropathologic consequences of exposure to agents, and touches upon underlying mechanisms that may mediate their effects in nervous tissues.

II. BACKGROUND

Common nerve agents developed for chemical warfare purposes include the G-series (so named because they were first developed by German scientists in the mid-1930s) and V-series (a designation of more ambiguous origin) weapons. The G-series includes tabun (GA), sarin (GB), soman (GD), and cyclosarin (GF); the V-series includes VX, first synthesized by the British in 1954, which is approximately an order of magnitude more potent than agents of the G-series. The G-series compounds are volatile liquids at room temperature, are soluble in both fat and water, and are absorbed readily through the eyes, respiratory tract, and skin. V-series agents are viscous and toxic mainly via skin exposure, and therefore pose a lower inhalation hazard than the G-agents. However, their persistence in the environment has implications for chronic exposures, including low-level exposures that may also lead to more subtle toxicities.

Nerve agents are organic esters of phosphorus-based acid derivatives that are potent inhibitors of AChE. A large toxicological and medical literature has been produced since their development, and it has been helpful to classify exposure-associated health effects in terms of four general clinical classifications that have been described elsewhere in the literature (Brown and Brix, 1998), and are summarized as 1 through 4 below. To these, I have added a fifth, for which I have finally presented a brief rationale below, and will deal with in more detail subsequently. These are:

1. Acute cholinergic effects that occur within minutes to hours, which may be lethal or lead to at least some degree of recovery.
2. Long-term neurological and neuropsychological effects that sometimes follow recovery from acute toxicity, and which may persist for years.
3. A delayed peripheral polyneuropathy, known as organophosphate-induced delayed neurotoxicity (OPIDN), from which recovery may be poor.
4. A delayed syndrome of muscle weakness (the so-called ''intermediate syndrome'') that occurs within days of recovery from severe acute effects and is typically reversible.
5. In addition, new literature supports the hypothesis that even asymptomatic exposures to these agents, especially if these occur over a prolonged period of time, may lead to effects on nervous tissue, the clinical consequences of which are as yet poorly understood. This would be an obvious concern in the event that populations would be exposed to nerve agents, as unrecognized exposure and repeated low-dose exposure during the course of inappropriately conducted decontamination procedures could lead to these effects. Furthermore, the possibilities of additional susceptibilities of pregnant women and children to low doses of nerve agents in these circumstances are areas of concern that we know very little about.

Nonneurologic effects of nerve agent exposure have also been reported and include psychiatric and cardiac disease (Brown and Brix, 1998). However, this chapter will focus on known neuropathologic effects, which are highly correlated with the cognitive effects of nerve agent exposure (Myhrer *et al.*, 2005). For the purpose of organization of this chapter, the clinical classification of exposure effects presented above will be used as an approximate scaffold for the description of lesions, predominantly in the central nervous system, that have been encountered in approximately equivalent stages of exposure in animal models. In addition, the limited amount of data regarding lesions encountered in humans who have been exposed to these agents will be described.

III. ACUTE EFFECTS OF SYMPTOMATIC EXPOSURE

A. Ischemic/Hypoxic Injury

A large number of studies using experimental animals, including rodents, cats, and nonhuman primates, have been carried out to characterize the neurobehavioral effects of nerve agent exposure and their histopathologic correlates in brain tissue (Baze, 1993; Carpentier *et al.*, 1990; Churchill *et al.*, 1985; Hymowitz *et al.*, 1990; Kadar *et al.*, 1995; Lemercier *et al.*, 1983; Petras, 1981, 1994; Singer *et al.*, 1987). Acute exposure to nerve agents is associated with a range of clinical symptoms, varying from abnormal movements and salivation, to limb tremor and muscle fasciculations, to convulsions. In general, axonal degeneration was present in the cerebrum of all animals with convulsions and in the majority of animals with tremor and fasciculations. In a variety of animal and especially rodent models, animals that survived seizures tended to manifest extensive bilateral brain neuronal necrosis that affected predominantly the forebrain, thalamus, tegmentum, and spinal cord. In monkeys, the striatum, globus pallidus, substantia nigra, amygdala, hippocampus, and optic pathway including the lateral geniculate bodies, pretectum, and superior colliculi also sustained injury proceeding to necrosis.

Acute injury may be attributable to several mechanisms. Ischemic hypoxia may derive from respiratory insufficiency during prolonged seizures, and evidence of cellular ischemia is present in brains of exposed animals. This consists of shrinkage of the cell soma and proximal dendrites, cytoplasmic microvacuolation due to mitochondrial swelling, dispersion of Nissel substance (cytoplasmic RNA), increased cytoplasmic eosinophilia, nuclear changes including displacement of the nucleus to an eccentric position in the neuron, shrinkage, and darkening. Generally, these nerve agent-associated lesions were described as being indistinguishable from those associated with brain ischemia or anoxia (Brierley *et al.*, 1973; Brown and Brierley, 1966, 1972; McLeod *et al.*, 1984).

Subsequent data argued against the hypothesis that hypoxic injury, at least in isolation, primarily accounts for the patterns of injury observed with acute nerve agent-induced brain injury. Although the presence of prolonged seizure activity is highly correlated with nerve-agent associated brain damage (Carpentier *et al.*, 2000; Lemercier *et al.*, 1983; McDonough and Shih, 1997), the changes in blood or brain oxygenation preceding or during nerve agent-induced seizures are minimal (Carpentier *et al.*, 1990; Clement and Lee, 1980; Goldman *et al.*, 1993; Lynch *et al.*, 1985), and in rat experimental models, increases in regional blood flow and glucose uptake are actually observed (Goldman *et al.*, 1993; Maxwell *et al.*, 1987; McDonough *et al.*, 1983; Miller and Medina, 1986; Pazdernik *et al.*, 1985; Samson *et al.*, 1985; Scremin *et al.*, 1991; Shih and Scremin, 1992). In addition, hippocampal slices exposed in culture to soman at levels that elicited spontaneous epileptiform activity were found to have earliest morphologic changes that recapitulated those seen in soman-treated intact animals; these included nuclear indentations and a decrease in nuclear size (Sikora-VanMeter *et al.*, 1985, 1987). These findings differed from the dilated endoplasmic reticulum and mitochondrial alterations observed in hippocampal slices maintained in hypoxic conditions, and argued that some other aspect of seizure activity, apart from hypoxic injury, contributes mechanistically to at least part of the damage to cerebrum associated with nerve agent exposure.

B. Nerve Agent-Associated Excitotoxic Injury

Within minutes after exposure to nerve agents, there is a marked decrease in AChE activity and associated rise in ACh. Earliest seizure activity begins in the absence of other significant neurotransmitter alterations and is prevented by anticholinergic drugs. These observations suggest that seizure-associated neuropathologic findings that occur upon nerve agent exposure are caused primarily by a mechanism of cholinergic toxicity. However, if seizures progress untreated, other neurotransmitter systems display secondary alterations, and the involvement of these has been invoked in models of injury to cerebrum that involve mechanisms of ‘‘excitotoxic’’ injury. Specifically, these refer to the involvement of the excitatory amino acid transmitter glutamate, which increases intracellular calcium mobilization. In excitotoxicity, overstimulation of glutamatergic synapses leads to marked neuronal calcium dyshomeostasis that in turn leads to neuronal injury (Aarts and Tymianski, 2004; Arundine and Tymianski, 2003; Bittigau and Ikonomidou, 1997; Sattler and Tymianski, 2000). Excitotoxic injury contributes to neuronal pathology in a variety of conditions including epilepsy, stroke, traumatic brain injury, multiple sclerosis, and an assortment of neurodegenerative diseases (Corona *et al.*, 2007; Estrada Sánchez *et al.*, 2008; Fujikawa, 2005; Gonsette, 2008; Hazell, 2007;

Hynd *et al.*, 2004; Jennings *et al.*, 2008; Meldrum, 1991, 1993; Sheldon and Robinson, 2007; Ting *et al.*, 2007).

Protein expression changes support the involvement of glutamate receptors in nerve agent exposure. Hippocampal lysates from soman-exposed guinea pigs were found not to differ in their content of neurofilament or synaptophysin, but showed significant increases in subunits of a variety of glutamate and *N*-methyl-D-aspartate (NMDA) receptors (Johnson *et al.*, 2008) that mediate excitatory neurotransmission. Experiments in which nerve agents were injected directly into various brain sites established that the areas most sensitive to seizure induction lie generally in the ventrolateral aspects of the forebrain that contain high concentrations of AChE and ACh (McDonough *et al.*, 1987, 1993). Following activation of these areas, seizures can propagate to sites distant from the area of injection, and these may also demonstrate morphologic evidence of neuronal injury. Importantly, pretreatment or early post-exposure treatment of experimental animals with anticonvulsants, for example with the benzodiazepine diazepam, blocks nerve agent-associated seizures which in turn prevents or diminishes neuropathologic effects (Clement and Broxup, 1993; Hayward *et al.*, 1990; Martin *et al.*, 1985; McDonough *et al.*, 1989, 1995; Philippens *et al.*, 1992). This occurs in the absence of a direct effect on cholinergic processes. These observations, taken together, suggest strongly that excitotoxic mechanisms contribute largely to the structural changes observed in cerebrum upon nerve agent exposure. Abrogation of these mechanisms by anticholinergic drugs within 20–40 min of the onset of seizures is sufficient in most cases to significantly diminish neuropathologic lesions (McDonough and Shih, 1997). The failure of anticholinergic drugs to prevent nerve agent-associated pathologic changes after this time period has been attributed to the recruitment and dominance of non-cholinergic mechanisms of excitotocity and perhaps to secondary loss of the integrity of the blood–brain barrier (McDonough and Shih, 1997).

C. Additional Acute Effects of Nerve Agents on Brain Tissue

Literature on excitoxicity suggests a variety of subsequent mechanisms by which calcium dyshomeostasis leads to neuronal dysfunction and injury. It is conceivable that pathologic effects of nerve agents could also arise via additional, as yet incompletely characterized, mechanisms that are independent of excitotoxicity, or that involve a variety of self-reinforcing cycles of brain tissue injury. A recent ''toxicogenomic'' study of mRNA expression changes following acute sarin exposure provided evidence for increased free radical stress and neuroinflammation in cerebrum, as well as anticipatable changes in the expression of genes involved in calcium flux and binding, neurotransmission, synaptic activity, and glial activation (Damodaran *et al.*, 2006). Roles for oxidative injury and neuroinflammation have been hypothesized in a host of other neurologic conditions, including neurodegenerative diseases (Carnevale *et al.*, 2007; DeLegge and Smoke, 2008; Klegeris *et al.*, 2007; Reynolds *et al.*, 2007; Shi and Gibson, 2007; Tansey *et al.*, 2008), and the demonstration of their involvement in nerve agent-associated neuropathologic changes in brain may suggest additional prophylactic or therapeutic opportunities that are being explored in other neurologic diseases. Hence, the possibility of modulation of the effects of AChE inhibitors on brain tissue by antioxidant and anti-inflammatory drugs has been the subject of a number of recent investigations (Dettbarn *et al.*, 2001; Gupta *et al.*, 2000, 2001a, b; Klaidman *et al.*, 2003; Milatovic *et al.*, 2006).

The morphologic manifestations of immune activation upon exposure of soman-treated rats include a rapid increase in glial fibrillary acidic protein staining, a marker of gliosis, as well as subsequent (1 to 4 hours post-exposure) microglial activation in susceptible areas including the piriform cortex and hippocampus (Zimmer *et al.*, 1997). This is accompanied by increased expression of central neuroinflammatory genes (Williams *et al.*, 2003; Levy *et al.*, 2004; Chapman *et al.*, 2006; Dhote *et al.*, 2007). These observations have led to the development of a novel class of anti-inflammatory, anticholinergic drugs including AChE reactivators that may have utility in nerve agent poisoning (Amitai *et al.*, 2005). In a rat model of AChE inhibitor intoxication, markers of reactive oxygen species (ROS) and reactive nitrogen species (RNS) were found to be elevated in susceptible regions of brain within 15 min to 1 h of exposure (Gupta *et al.*, 2007); this was accompanied by dendritic damage to the hippocampus, a sensitive morphologic marker of neuronal injury (Gupta *et al.*, 2007; see also Figure 43.1). Consistent with an excitotoxic contribution to neurotoxicity, the NMDA receptor antagonist memantine, in combination with the anticholinergic drug atropine, prevented increases in ROS and RNS as well as dendritic damage (Gupta *et al.*, 2007). These findings, taken together, suggest that novel combination therapies that target a variety of processes of nerve agent toxicity may offer enhanced protection, or at minimum may offer a degree of protection if post-exposure treatment is delayed past the point at which anticholinergic drugs have lost much of their utility.

IV. PROLONGED EFFECTS OF SYMPTOMATIC EXPOSURE

A number of studies describe the long-term effects of nerve agent exposure in cerebrum (Collombet *et al.*, 2005a, 2006; Grauer *et al.*, 2008; Kadar *et al.*, 1992; Lemercier *et al.*, 1983; McDonough *et al.*, 1998). Inflammatory changes are pronounced, and may occur in two phases, namely an initial response to cholinergic cell death, as well as a second, delayed inflammatory process that begins approximately 1 month after exposure and persists for at least several months (Grauer *et al.*, 2008). Morphologically, reactive changes

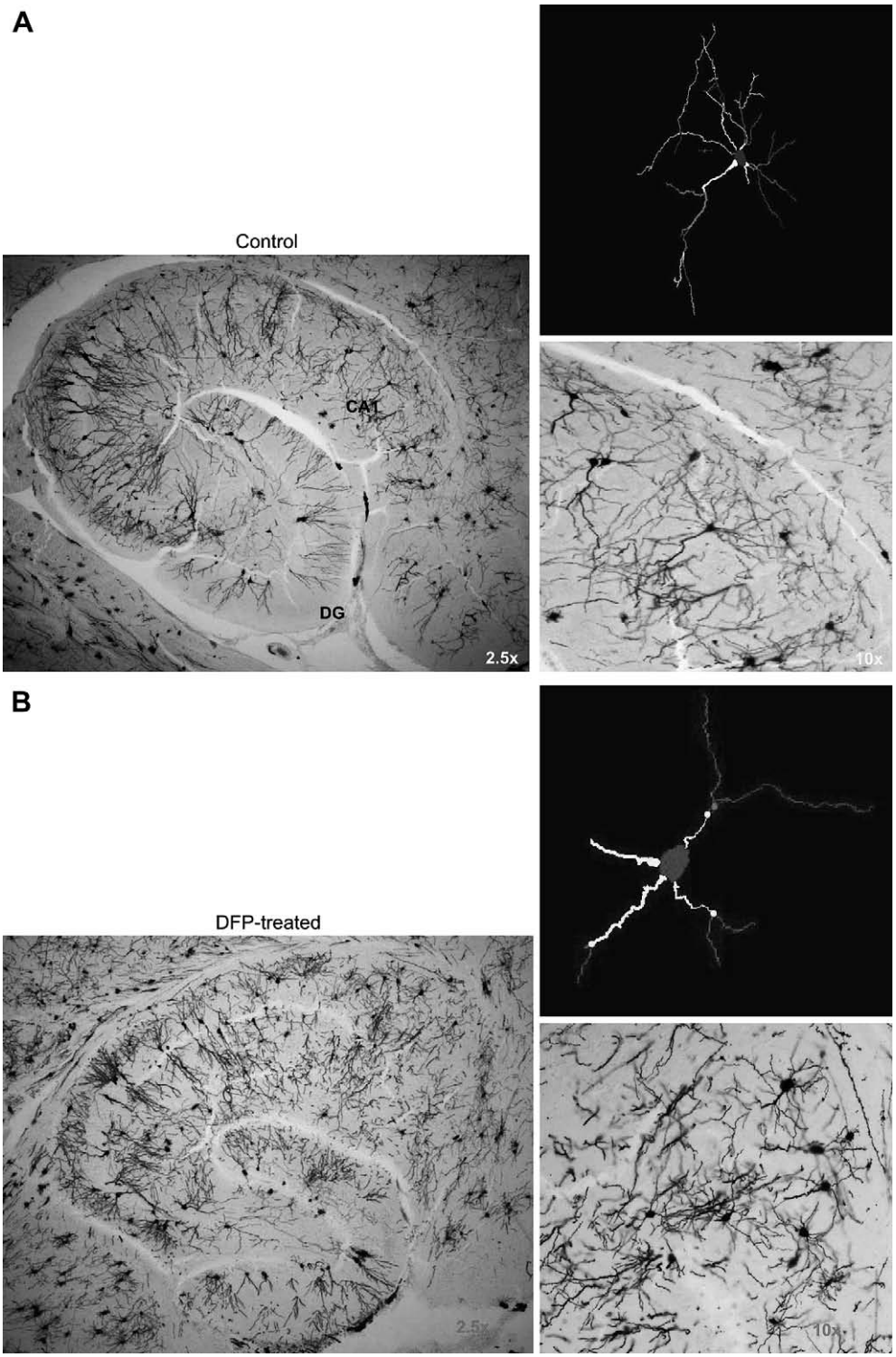

FIGURE 43.1. Photomicrographs of rat hippocampi with pyramidal neurons and their tracings from CA1 hippocampal area (DG, dentate gyrus) of rat brains 1 h after saline (control, panel A) or diisopropyl phosphorofluoridate (DFP, 1.5 mg/kg, s.c.) (panel B) injections. DFP acts as a potent acetylcholinesterase inhibitor with a structure similar to that of organophosphate nerve agents. A single injection of DFP produces toxic signs in rats, including salivation, tremors, fasciculations, and seizures within 15 min that last for more than 2 h before tapering off. The observed signs are typical of anticholinesterase toxicity and reveal the involvement of both the central and the peripheral nervous systems.

Morphological correlates of anticholinesterase exposure were investigated using the structural integrity of the CA1 dendritic system, the neuronal compartment most sensitive to both age-related and disease-related degeneration (Uylings and de Brabander, 2002), as an experimental endpoint. Golgi impregnation of 50 μm thick rat brain sections from paraffin-embedded blocks was carried out according to

that may be seen with many etiologies of brain tissue injury may be observed if survival is sufficiently prolonged after exposure. These include subpial astrogliosis with hyperplastic, swollen astroctyes, as well as reactive microgliosis with microglial lipid accumulation. Focal calcification of brain parenchyma may also be observed, also with localized associated gliosis.

Other long-term changes may be more particular to nerve agent exposure. After acute soman exposure in mice, neuronal degeneration in the hippocampus begins within 1 day. After initial neuronal losses, a subpopulation of degenerating neurons persists for approximately 30 to 90 days before many of these are lost as well (Collombet *et al.*, 2006). These losses are accompanied by a dramatic decline in spatial memory, with a slight recovery at 90 days that may reflect partial neuronal degeneration (Collombet *et al.*, 2006). Similar changes were described in the amygdala (Collombet *et al.*, 2008).

Neurogenesis has also been described in cerebrum in response to focal or global ischemia and epilepsy-associated neurotoxicity (Dong *et al.*, 2003; Nakatomi *et al.*, 2002; Scharfman *et al.*, 2000; Schmidt and Reymann, 2002). After soman-induced toxicity, neural progenitor cells in the subgranular zone of the dentate gyrus and the subventricular zone of the cerebrum expand (Collombet *et al.*, 2005a) and migrate to sites of brain injury (Collombet *et al.*, 2005b), where they engraft and differentiate into neurons and astrocytes within approximately 34 days after exposure (Collombet *et al.*, 2005b). Whether neurotrophic treatments that have been proposed and are under investigation in models of neurodegenerative diseases (Blesch, 2006; Counts and Mufson, 2005; Hennigan *et al.*, 2007; McGeer and McGeer, 2005; Mocchetti *et al.*, 2008; Schulte-Herbrüggen *et al.*, 2008) may have a place in the therapy of nerve agent exposure remains currently a speculative matter, but one that surely warrants investigation.

The only published study of the brain structural effects of nerve agent exposure in a human population focused on long-term changes induced in victims of the 1995 Tokyo subway sarin attack (Yamasue *et al.*, 2007). In this event, which resulted in 12 deaths, approximately 5,500 victims were exposed to sarin, and essentially all patients exhibited typical symptoms of acute intoxication (Lee, 2003; Ohbu *et al.*, 1997). A variety of somatic, ocular, and cognitive symptoms were reported to persist among victims even 5 years after the attack (Kawana *et al.*, 2001). Magnetic resonance imaging revealed decreased regional brain volumes in the insular cortex, neighboring white matter, and hippocampus of victims (Yamasue *et al.*, 2007); reduced regional white matter volume was significantly correlated with decreases in serum cholinesterase levels and with victim symptoms (Yamasue *et al.*, 2007). Although these findings are compatible with sites of susceptibility reported in the nonhuman primate studies cited above, the findings reported were from purely imaging studies, and the precise histopathologic correlates of these findings remain unknown.

V. ORGANOPHOSPHATE-INDUCED DELAYED NEUROTOXICITY (OPIDN)

OPIDN is defined as a delayed onset central and peripheral distal sensorimotor polyneuropathy caused by exposure to nerve agents (Brown and Brix, 1998), typically within 1 to 2 weeks, and less than 4 weeks, after exposure (Johnson, 1980). Symptoms attributable to effects on sensory (numbness, tingling, pain) and motor (fatigue, weakness, and paralysis) targets are present and display a typical axonal length-associated pattern (e.g. predominantly lower extremities, with upper extremities affected at higher agent exposure). No treatment exists, and recovery is slow and rarely complete.

OPIDN has been attributable to inhibition of neuropathy target esterase (NTE), rather than AChE, as inhibition of AChE is not necessary for the development of OPIDN (Jamal, 1997; Pope *et al.*, 1993; Wu and Casida, 1996). Hence, since nerve agents target AChE much more than NTE (Gordon *et al.*, 1983), the degree to which OPIDN would emerge as a significant consequence of nerve agent exposure alone is questionable; rather, doses that could potentially lead to OPIDN would, as a practical matter, cause lethal acute anti-AChE effects first. In fact, unlike many other organophosphates, VX has not been shown to induce OPIDN and is reported to be at least 1,000 times less effective than sarin in inhibiting NTE (Gordon *et al.*, 1983; Vranken *et al.*, 1982). However, several therapies, such

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the manufacturer's specifications (FD Rapid GolgiStain Kit). Golgi-impregnated pyramidal neurons with no breaks in staining along the dendrites from CA1 sector of the hippocampus were selected and spines counted according to the methods by Leuner *et al.* (2003). Tracing and counting were carried out with a Neurolucida system at ×100 objective magnification under oil immersion (MicroBrightField, VT).

In each panel, the lower left view is a low (2.5× original objective) magnification view of the hippocampus, the lower right view shows dendritic structure at higher magnification (10× original objective), and the upper right view shows a Neurolucida tracing of dendritic structure, in which colors indicate the degree of dendritic branching (yellow = 1°, red = 2°, purple = 3°, green = 4°, turquoise = 5°, gray = 6°).

Anticholinesterase-induced seizure is accompanied by rapid evolution of dendritic abnormalities, apparent in a significant decrease in dendritic length and spine density of pyramidal neurons as early as 1 h post-DFP. Dendritic degeneration seen in this model of DFP-induced seizures is similar to neurodegeneration of pyramidal neurons in CA1 hippocampal area in mouse models of kainic acid-induced excitotoxicity (Zaja-Milatovic *et al.*, 2008) and activated innate immunity (Milatovic *et al.*, 2003). (Courtesy of Dr Dejan Milatovic, Department of Pediatrics, Vanderbilt University School of Medicine)

as the anticonvulsant agent diazepam, that are used to minimize or prevent injury to the central nervous effects of nerve agents have fewer significant effects in the periphery and specifically no effects on the targeting of NTE at peripheral sites. Therefore it is conceivable that OPIDN could be a significant effect in treated survivors of nerve agent exposure. Indeed, exposure of humans to OP insecticides has led in some instances to OPIDN even in the absence of symptoms associated with acute toxicity (Barns, 1975). Experimentally, OPIDN has been induced in hens treated with subsymptomatic doses of OPs, if the time of dose administration is not prolonged (Barns, 1975).

Morphologically, OPIDN is manifested as classic neuropathologic lesions associated with Wallerian-type, or ''dying-back'' degeneration of axons, with secondary demyelination. Briefly, this begins with swelling of the axolemma, the membrane of the axon, and is followed by granular disintegration of intraaxonal organelles and cytoskeleton, including the microtubular network. Schwann cells react rapidly, stopping synthesis of new myelin initially, and clearing degenerating myelin debris; they are aided in this by subsequently recruited macrophages. In the recovery phase, which may be initiated within days after injury, Schwann cells proliferate and release neurotrophic factors which encourage the growth of proximal axonal sprouts from intact axons. This pattern is a nonspecific response to axonal injury and in the absence of a history of exposure to nerve agents, the clinical and neuropathologic differential diagnosis is fairly broad, and includes amyotrophic lateral sclerosis as well as a variety of toxic neuropathies attributable to heavy metal, solvent, and other exposures (Barrett and Oehme, 1985; Longstreth, 1994). Hence, the history of nerve agent exposure is critical in establishing the etiology of clinical neuropathy.

VI. ORGANOPHOSPHATE-ASSOCIATED MUSCLE WEAKNESS

The ''intermediate syndrome'' refers to a delayed syndrome of muscle weakness that occurs shortly after recovery from severe acute effects of OP exposure and is typically associated with full recovery within approximately 4 weeks (Marrs, 1993; De Bleecker, 2006). Although details of the mechanism by which this occurs are not known, it is believed to be attributable to accumulation of ACh at the neuromuscular junction (Karalliedde and Henry, 1993). Consistent with this, morphologically, one finds a necrotizing myopathy that appears somewhat localized to regions of nerve endplate regions (Ariens *et al.*, 1969; Fenichel *et al.*, 1972, 1974; Good *et al.*, 1993; Kawabuchi *et al.*, 1976). Ultrastructural studies reveal vacuolated myofibers in association with nerve terminals containing swollen mitochondria (Hudson *et al.*, 1978; Laskowski *et al.*, 1975, 1977; Meshul *et al.*, 1985; Petras, 1981, 1994). In these studies, a large degree of variability both between experimentally treated animals and within affected muscles was observed; hence, it is clear that unknown additional factors mediate susceptibility. Separation of nerve terminals from the muscle surface was attributable to progressive myofiber vacuolar swelling and insertion of Schwann cell or macrophage processes into the region. This appears likely to be incompatible with effective neurotransmission at this site, and the reversibility of this process may account for the transient nature of OP-associated muscle weakness.

VII. EFFECTS OF SUBSYMPTOMATIC EXPOSURE TO NERVE AGENTS

The classic neuropathologic lesions of nerve agent exposure in cerebrum, as described above, are highly associated with the induction of clinically and electrographically apparent seizure activity. Recently, however, concern has been raised about even low levels of exposure to nerve agents that may not be sufficient to result in symptoms at the time of exposure, but may nevertheless lead to clinical symptoms or neuropathologic effects after long delays, perhaps in the context of chronic or repeated exposures. One scenario in which this might occur invokes an urban exposure to an agent such as VX followed by incomplete decontamination with prolonged evaporative or other contact with persistently contaminated surfaces (Bloch-Shilderman *et al.*, 2008). In addition, practical experience with nerve agent exposure has shown that most episodes involve small or asymptomatic doses, for example to emergency and medical treatment personnel, as opposed to acute symptomatic toxicity (Gray *et al.*, 1999; Levin and Rodnitzky, 1976; Morita *et al.*, 1995).

Concern about the effects of low doses of nerve agents is founded in part on observations in populations, such as agricultural workers, with chronic occupational exposure to OP compounds, such as pesticides. Many reports relate neurologic changes to these exposures (Kamel *et al.*, 2005, 2007; Pilkington *et al.*, 2001; Rohlman *et al.*, 2005; Roldán-Tapia *et al.*, 2005, 2006; Rothlein *et al.*, 2006; Salvi *et al.*, 2003; Wesseling *et al.*, 2002), and relevant biochemical alterations have been described in these subjects, including changes in antioxidant status (López *et al.*, 2007). Other investigators have shown biochemical changes including muscarinic receptor decreases and mRNA cytokine expression changes in affected cerebrum in animal models (Henderson *et al.*, 2002). In a rat model of subsymptomatic exposure, repeated exposure to sarin at a dose that induced a 30% inhibition of erythrocyte AChE led eventually to ataxic gait, increased stereotyped behavior, and increased central nervous system excitability following administration of the convulsive drug pentamethylenetetrazol (Kassa *et al.*, 2001). Repeated low-dose sarin exposure also led to a disrupted electroencephalographic sleep pattern (Shih *et al.*, 2006) and decreased locomotor activity and cognitive performance tests in rats and mice (Baille *et al.*, 2001; Nieminen *et al.*, 1990; Russell *et al.*, 1986; Sirkka *et al.*, 1990).

Interestingly, some endpoints, such as temporal perception, were initially impaired by low-dose soman treatment, but led to tolerance later on, whereas others, such as nociceptive sensory and perceptual thresholds, became and remained elevated throughout the course of treatment (Russell *et al.*, 1986). In nonhuman primates treated with subtoxic doses of sarin (Burchfiel *et al.*, 1976) as well as humans with symptomatic but limited exposure to nerve agents (Duffy *et al.*, 1979; Metcalf and Holmes, 1969; Wadia *et al.*, 1974), electroencephalographic changes were significant and persisted many months after exposure.

In no study of subsymptomatic nerve agent exposure were significant neuropathologic lesions, as determined by standard histopathologic techniques, found to be associated with behavioral or biochemical endpoints. However, it remains to be determined whether more sensitive morphologic endpoints, such as hippocampal dendritic structure as illustrated in Figure 43.1, are altered by exposures of this nature. This having been said, the data reviewed here illustrate the difficulty of determining ''safe'' levels of nerve agent exposure in human populations.

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

The neuropathologic effects of chemical warfare agents, particularly OP nerve agents, have been characterized and related to a variety of central and peripheral clinical syndromes. The most prominent of these are neuronal losses at characteristic sites in cerebrum that follow and correlate with the presence of nerve agent-induced seizure activity, with accompanying glial reaction and a subsequent, incomplete neuroregenerative response. Recent progress in our understanding of excitotoxic, neuroinflammatory, and oxidative processes that may contribute to the development of neurotoxicity provides a conceptual link to other neurologic diseases. Therapies that prove successful in preventing or treating these diseases might also be translated into neuroprotective strategies that might be useful in populations exposed to chemical warfare agents. Conversely, if novel, effective treatments for nerve agent exposure can be developed, we should be mindful of the possible value of their application to a host of neurological illnesses, especially age-related neurodegenerative diseases that are becoming increasingly prevalent in Western societies.

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CHAPTER 44

Molecular and Transcriptional Responses to Sarin Exposure

T.V. DAMODARAN

I. INTRODUCTION

Chemical warfare related agents such as cyclosarin, sarin, soman, tabun, VR, and VX have become the focus of attention of both the international scientific community and the public ever since the end of Gulf War in 1991 (Golomb, 2008), followed by the tragic nerve agent exposure-related deaths and sicknesses in Tokyo in 1995. As a result of the September 11, 2001 terrorist attacks on the World Trade Center, and the ensuing possibility of unexpected war and terrorist attack scenarios that may involve chemical agents, there is a general heightened awareness of the necessity for more detailed studies on the nature of biological effects of these agents, and to improve diagnostic, preventive, and treatment aspects of toxic chemical exposure.

Sarin (*O*-isopropyl methylphosphonofluoridate; GB) is an organophosphorus (OP) ester that belongs to a group of highly toxic nerve agents. Many individuals have been suspected of exposure to various levels of sarin during the Gulf War (Golomb, 2008) as well as the Tokyo subway attacks (Miyaki *et al.*, 2005). Clinical symptoms such as arthralgia, weakness, fatigue, headache, memory loss, and increased susceptibility to infections were recorded in these individuals. There have been speculations about the low level of sarin exposure affecting distinct regions of the brain (Damodaran *et al.*, 2002a). High levels of exposure result in seizures, which increase the release of glutamate and cause toxicity to the surrounding cells (Solberg and Belkin, 1997). However, very little is known about the genomic and related nongenomic effects of sarin exposures at different dosage levels leading to pathological outcome.

II. BACKGROUND

One of the major mechanisms known for sarin-induced toxicity is the irreversible inhibition of the enzyme acetylcholinesterase (AChE), resulting in central accumulation of acetylcholine (ACh) and overexcitation of cholinergic neurons in sarin exposure related neurotoxicity. Besides this, a range of noncholinergic effects have been observed (Ray, 1998). Cholinergic components including ACh, choline acetyltransferase (ChAT), AChE, vesicle acetylcholine transporter (VAChT), muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively) have been identified in numerous nonneuronal cell and tissue types (Kawashima and Fujii, 2008) and thus make the biological response to sarin exposure into a very complex phenomenon. Given the complex levels of physiological alterations, one can expect global changes in RNA and protein species of different genes. Hence, gene expression approaches and other relevant molecular assays will help to identify key pathways that are triggered immediately and showed sustained expression over a long period of time, thus giving a mechanistic view on the possible mode of initiation, modification, persistence or disappearance of distinct molecular lesions that may translate into cellular/tissue level pathology leading to clinically and behaviorally observable disease/syndromic features (Damodaran *et al.*, 2006a).

A. Gene Expression as a Tool of Toxicity Testing

The idea of using gene expression as a tool in toxicology is based on the known fact that all toxic responses require modulation of expression of several key genes involved in various biological pathways to some degree and that no gene is regulated in isolation, even in the simplest biological system. Gene expression studies can be useful for the following reasons: (1) the construction of a large database of gene expression information linked to toxic endpoints, (2) more detailed understanding of the molecular mechanisms of compound toxicity, (3) identifying expression signatures that distinguish toxic effects of closely related chemicals, (4) identification and monitoring of different doses of suspected toxic chemical exposures (such as low vs high level), (5) differentiating acute vs chronic exposures and different combinations of both types, (6) identifying different chemicals in scenarios of suspected exposure to unknown chemical mixtures, (7) therapy or antidote response and effectiveness, and (8) long-term monitoring for any unknown biological effects. The strategic application of information

obtained by the above-mentioned approaches should result in the development of both more rapid, mechanism-based diagnostic screens for toxicity and novel therapeutic targets. Thus the development and use of gene expression-based biomarkers in toxicology are becoming widespread, specifically in the areas of exposure monitoring, and the determination of response and susceptibility to toxins.

B. Application of Genomics Technology for Studying Gene Expression

Two general approaches of gene expression related to toxicology and safety assessment have evolved, either of which can be deployed depending on the nature of the investigation being carried out.

The first (candidate gene or deductive) approach is to use either a single gene or arrays with discrete, often limited number of known (candidates for disease pathology or monitoring either one or several clinical and treatment endpoints related to toxic exposure) genes that have been selected on a rational basis as being associated with one or more toxic endpoints. The advantage here is that the methodology is relatively simple, and data analysis is considerably easier and restricted to those genes that are likely to be ''interesting'' and relevant to the toxicological problem in question. A large body of data has been published regarding expression profiles of few candidate genes for many toxic substances such as sarin and similar chemicals. Very often, the data obtained by this approach can be categorized as ''hypothesis testing'' – for example, the hypothesis that a subacute dose (0.5 LD_{50}) of sarin may affect neuronal and astroglial cells was tested by studying the expression of neuron-specific alpha tubulin (Damodaran *et al.*, 2002b), astrocyte-specific glial fibrillary acetic protein (GFAP), and vimentin (Damodaran *et al.*, 2002a). These time course studies confirmed the hypothesis by yielding expression data indicative of differential (various regions of the brain) and temporal (different days after exposure) modulation of all of these genes. Furthermore, another hypothesis that differential and temporal expression of AChE genes may explain the various molecular, cellular, and behavioral aspects of sarin exposure was tested by this approach (Damodaran *et al.*, 2003) in a time course study which also provided valuable data that confirmed biochemical, cell biological, and behavioral data obtained earlier (Jones *et al.*, 2000; Abou-Donia *et al.*, 2002; Abdel-Rahman *et al.*, 2002).

The second (global gene expression profiling or ''inductive'') approach is to use gene arrays with as many genes as possible, both characterized and otherwise, to maximize the amount of information obtainable from a single experiment. This scenario is applicable when novel genetic markers associated with a particular toxicity are sought or when a gene regulation ''fingerprint'' associated with a particular compound or toxic endpoint is to be generated. Moreover, identification of several novel pathways and gene clusters by this approach makes it a much more attractive option. The amount of data obtained by this approach is enormous and very often requires complex statistical and bioinformatics tools. Other disadvantages include situations where the interaction of cells and tissues with certain compounds such as xenobiotics will result in changes in patterns of gene expression, not all of which will be of significance in terms of toxicity. The data obtained by this approach are generally ''hypothesis generating'' for the most part, while they can be used to verify already known facts or hypotheses. For example, the hypothesis that early molecular events after sarin exposure define long-term pathology was studied by global gene expression approaches (Damodaran *et al.*, 2006a, b). Enormous amounts of data obtained by this study not only verified already known facts and verified the above-mentioned hypothesis, but also generated more hypotheses for future testing. For example, in a recent study, it was shown that signature expression profiles found in lung cancer patients with and without long-term exposure to pesticides were significantly different, thus paving the way to develop novel approaches to classify the patients based on this molecular profile, identify therapeutic targets, and monitor efficacy of treatment (Potti *et al.*, 2005). Hence, ultimately the decision to select an approach depends on the short- and long-term objectives in the effort to elucidate the toxicity of chemicals under study. Very often, both approaches are used simultaneously for better and faster understanding of the biological phenomena associated with toxicity of the chemical being studied.

1. Current Methodologies Available for Studying Gene Expression

Several methods have been developed to screen cells/tissues for differentially expressed genes, such as screening of differential cDNA libraries (Dworkin and David, 1980), screening of subtractive cDNA libraries (Sargentg, 1998), screening by differential display by PCR (Liang and Pardee, 1992), and sequence-based serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995). While these procedures are suitable for identifying few-fold or greater changes in gene expression (i.e. when genes are either turned off or on), they are inadequate for detecting subtle changes in gene expression. High-density cDNA arrays allow one to rapidly screen cells/tissues for differential gene expression as well as quantify changes as small as 0.5-fold or less with statistically significant levels of accuracy (Damodaran *et al.*, 2006b). Recent assessment of the relative impact of experimental treatment and platform on measured expression found that biological treatment had a far greater impact on measured expression than did platform for more than 90% of genes, and thus confirmed that global approaches in gene expression studies are reliable and promising (Larkin *et al.*, 2005).

2. Latest Genomic Technologies that Hold Promise for Future Investigations

Although most of the gene expression studies on sarin and related compounds address the changes in transcriptomes,

there have been recent developments in microarray technology such as: (1) global analysis of changes at DNA level such as deletions, inversions, duplications, and translocations in monogenic diseases, complex diseases, and various types of cancers, possibly due to exposure to chemical and physical agents, using comparative genomic hybridization (CGH) methodology, (2) genotyping single nucleotide polymorphism (SNP) or other point mutations as a factor in susceptibility to certain disease development and/or as an adverse or protective effect against certain chemical exposures or drug treatment, (3) level of methylation changes due to chemical exposure or other disease states using global epigenetic profiling, and (4) microRNA expression profiling as an adjunct technology to address specific effects of chemical exposure on molecular regulation of differentiation and proliferation-related changes in various target tissues. It is noteworthy to mention here that there are studies using sister chromatid exchange (SCE) analysis of peripheral blood lymphocytes from victims of sarin exposure from the Tokyo train terrorist attack (Li *et al.*, 2000) and scoring of various cytogenetic anomalies such as mitotic chromosome and chromatid breaks, gaps, and other anomalies of anaphase such as lagging of chromosomes have been employed in studies of *in vitro* peripheral blood culture from humans exposed to pesticides (Das *et al.*, 2006) to assess genomic effects on somatic cells. Furthermore, the use of multicolor fluorescence *in situ* hybridization (FISH) on germ cells (sperms) of individuals exposed to OPs was also attempted (Recio *et al.*, 2001). There is an excellent Prospectus for identifying subtle DNA level changes which could be either megabase deletions of large contiguous regions of genome or minor deletions of a few kilobases of genomic regions of instability and/or recombination due to exposure to warfare chemicals by CGH technology. This also corresponds to the identification of the modifying effect of inherited and acquired interactions between AChE and paraoxonase1 (PON1) polymorphisms modulating AChE and plasma paraoxonase enzyme activities both within and between genotypes on the susceptibility of individuals, in their response to OPs exposure warrants, such as genotype studies and other SNP-based screenings at population level (Bryk *et al.*, 2005; Sirivarasai *et al.*, 2007). This kind of population data will be of great advantage in situations where there is a need to screen soldiers, medical personnel, and members of other support organizations during warfare or a terrorist attack for assessing their increased or decreased susceptibilities to warfare agents. The modifying effects of methylation and its importance in transmitting abnormally methylated alleles to future generations are also gaining widespread attention. Besides these latest developments, other established and emerging technologies such as proteomics and global analysis of post-translation changes (such as phosphorylation), respectively, may address several biological end points relevant to sarin-induced toxicity, its treatment and management as well as long-term monitoring. Tissue arrays and high throughput validation (such as high content confocal analysis) methodologies related to genomic technologies make it a very promising future for prompt and precise detection of toxic exposure that can lead to better treatment options, thus reducing overall mortality and morbidity associated with any such scenarios.

III. SARIN-INDUCED CLINICAL CHANGES AS A FUNCTION OF CELLULAR AND MOLECULAR ALTERATIONS

A. Acute Sarin Exposure and Clinical and Molecular Symptoms

A high dose of acute exposure results in marked muscle fasciculation, tachycardia, high blood pressure (nicotinic responses), sneezing, rhinorrhea, miosis, reduced consciousness, respiratory compromise, seizures, and flaccid paralysis (Yokoyama *et al.*, 1998). High-dose acute exposure targets several pathways such as cholinergic, cyclic nucleotide signaling, catecholaminergic signaling, GABAergic signaling, glutamate and aspartate signaling, nitric oxide signaling, and purinergic, serotogenic, as well as cholinergic signaling, within the first 15 min to 2 h of sarin exposure (Damodaran *et al.*, 2006a, b). Other signal transduction pathways such as calcium channels and binding proteins, ligand-gated ion channels, neurotransmission and neurotransmitter transporters, neuropeptides, and their receptors are also altered. Inflammation-related pathways such as TNF pathways, chemokines and their receptors, and cytokines are also altered within 15 min to 2 h after sarin exposure (Damodaran *et al.*, 2006a).

B. Sarin-Induced Delayed Neurotoxicity

High doses of sarin exposure can lead to delayed onset of ataxia, accompanied by wallerian-type degeneration of the axon and myelin of the central nervous system (CNS) and peripheral nervous system (PNS), also known as both organophosphorus ester-induced delayed neurotoxicity and OPIDN (Smith *et al.*, 1930; Abou-Donia, 1990). After the Tokyo subway incident, a 51-year-old man who survived the initial acute toxicity later died (after 15 months), with neurological deficits and histopathological lesions consistent with OPIDN (Himura *et al.*, 1998). This observation strongly suggests that humans are more sensitive to OPIDN caused by sarin than the hen (a commonly used experimental model organism to study OPIDN), as it required 26–28 daily doses of sarin LD_{50} (25 μg/kg, i.m.) to produce OPIDN in this species (Davies and Holland, 1972). It has been shown that the anomalous increase in phosphorylation and altered expression of mRNA and proteins of cytoskeletal genes is the central mechanism in the pathogenesis of OPIDN in model systems (Abou-Donia, 1995; Gupta *et al.*,

1998, 1999, 2000a, b, 2001; Gupta and Abou-Donia, 1999; Damodaran and Abou-Donia, 2000; Xie *et al.*, 2001). Sustained hyperphosphorylation of cytoskeletal proteins is not only a feature of OPIDN, but also a mediator of axonal dysfunction that perturbs the dynamics of the cytoskeleton. There are several kinases [such as protein kinase A (PKA)] associated with cytoskeletal proteins [such as neural filaments (NFs), tau, tubulin] shown to be altered in OPIDN (Gupta and Abou-Donia, 1999). Abnormal axonal transport is one of the main reasons for the degeneration of axons in OPIDN. Microtubules form the base for transport of axonal constituents, which are essential for the maintenance of axons. Microtubules interact with microfilaments, neurofilaments, kinesin, dynein, and microtubule-associated proteins (e.g. MAP-1, MAP-2, tau). Altered mRNA expression of several major cytoskeletal genes such as beta tubulin (Tubb1), Nestin (Nes) at immediate early (15 min), and neurofilament heavy (NEFH), GFAP at early (2 h), as well as MAP2 (microtubule associated protein 2) at late (3 months) time points after sarin exposure in rats indicates the existence of OPIDN-related molecular changes (Damodaran *et al.*, 2006a, b). Similar changes were noted in studies on hens showing clinical and histopathological features typical of OPIDN (Gupta *et al.*, 1999; Damodaran and Abou-Donia, 2000).

C. Sarin-Induced Chronic Neurotoxicity

The category is postulated as organophosphorus-induced chronic neurotoxicity (OPICN) which is characterized as long-term neurological deficits accompanied by brain neuronal cell death (Abou-Donia, 2003). Exposure to large toxic or small subclinical doses of OPs can result in such syndrome. Some of the Tokyo subway incident's victims developed long-term, chronic neurotoxicity characterized by central nervous system deficits and neurobehavioral impairments (Masuda *et al.*, 1995). Rescue workers and some victims who failed to show any early symptoms of neurotoxicity exhibited a chronic decline in memory 3 years and 9 months after the Tokyo incident (Yokoyama *et al.*,1998). Several genes (Bax, Bok, Bcl2l1, Casp6, Bcl-X, Dedd) related to cell death phenomena showed alterations at various time points after sarin exposure (Damodaran *et al.*, 2006a, b). Differential alteration of mRNA expression of AChE in rat brain regions exposed to sarin has been reported (Damodaran *et al.*, 2003) and is postulated to be related to the cell death observed in earlier studies on the brain of sarin-treated rats (Kadar *et al.*, 1995; Abdel-Rahman *et al.*, 2002).

D. Sarin-Induced Molecular Changes

Because of the complexity of the nervous system and the multiplicity of neurotoxic effects (sensory, motor, cognitive, etc.), it is apparent that several mechanisms of neurotoxicity exist; some may be specific to the nervous system, while others may be specific to other target organs and represent general cytotoxicity processes. Some of the major mechanisms include:

1. direct effect on a particular step in cell signaling and associated gene expression (e.g. protein kinases)
2. effect on second messenger responses activated by an endogenous compound (e.g. a neurotransmitter-induced activation of phospholipid hydrolysis) and related gene expression
3. indirect effect due to other toxic actions (e.g. induction of oxidative stress) and related gene expression.

The complexity of the pathways altered by sarin can be very well understood by the schematic pathway diagram (Figures 44.1–44.3).

1. Sarin-Induced Changes in Cell Signaling

Cell signaling is the fundamental process by which specific information is transferred from the cell surface to the cytosol and ultimately to the nucleus, leading to changes in gene expression. Since these chains of biochemical and molecular steps control the normal function of each cell, any disruption of these processes will have a significant impact on cell physiology. The phosphoinositide/calcium/PKC system, with a focus on the cholinergic system, plays a prominent role in several CNS- and PNS-related functions. ACh, by activating muscarinic receptors, may play a fundamental role in the regulation of synaptogenesis, neurocytomorphogensis, and glial cell proliferation (Costa, 1993) and exposure to sarin may alter its effect on the above-mentioned biological aspects. In astrocytes, ACh stimulates the metabolism of phosphoinositides and the hydrolysis of phosphatidylcholine increases intracellular calcium levels. These effects linked to muscarinic receptor stimulation may be modulated by sarin exposure. Other pathways important for glial cell proliferation (e.g. the phosphoinositide 3-kinase, MAP kinase, etc.) may be activated by ACh in these cells and may represent significant targets for sarin's action.

Neurotransmitters, hormones, and growth factors serve as first messengers to transfer information from one cell to another by binding to specific cell membrane receptors. This interaction results in activation or inhibition of specific enzymes and/or opening of ion channels, which leads to changes in intracellular metabolism and, in turn, to a variety of effects, including activation of protein kinases and transcription factors. These intracellular pathways can be activated by totally different receptors and are very interactive, or crosstalking, so they can control and modulate each other. The principal systems that link membrane receptors to second-messenger systems are represented by G-protein coupled receptors (GPCRs) and by receptors that possess intrinsic tyrosine kinase activity (Costa, 1998).

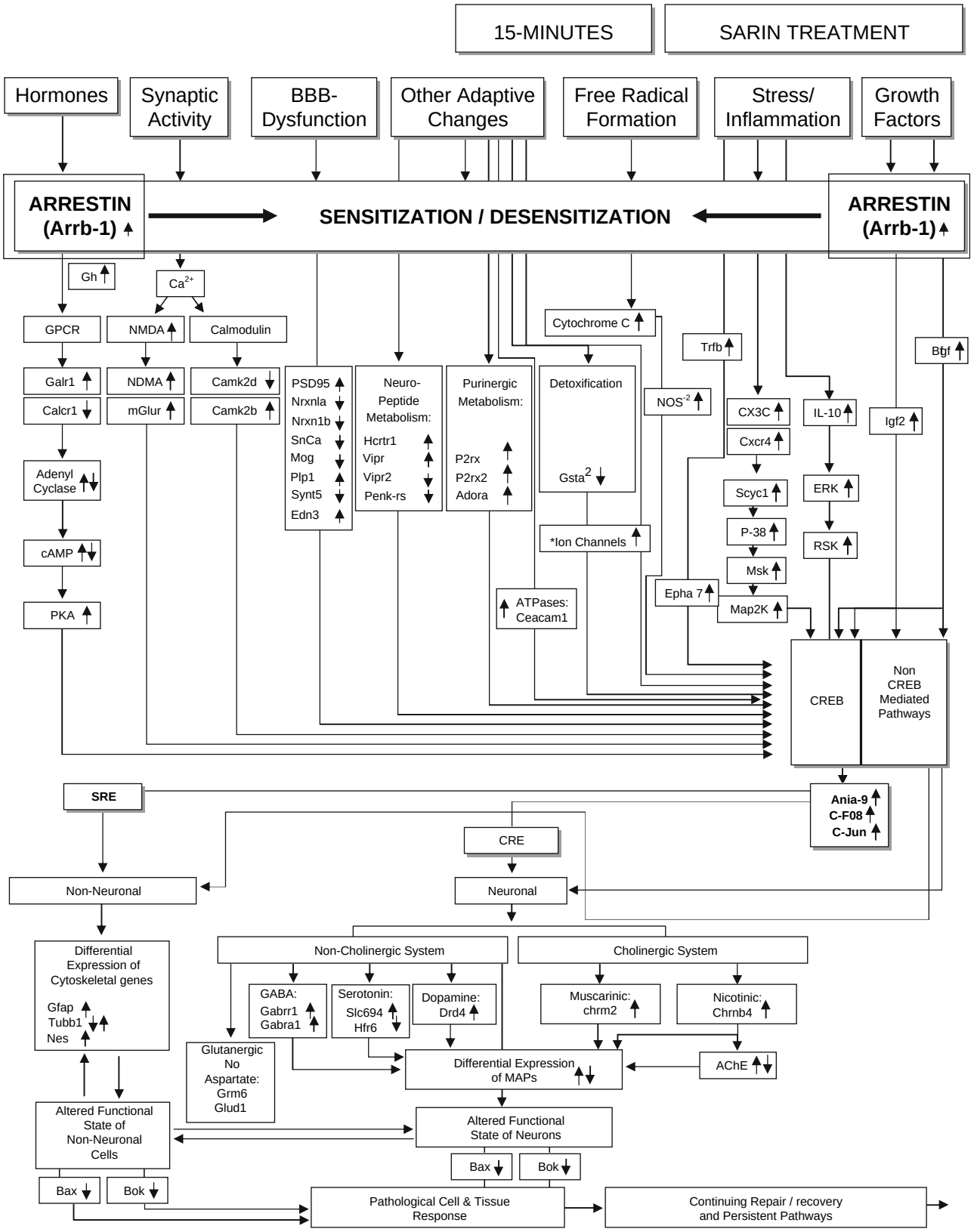

FIGURE 44.1. Similar phenomena as shown in Figure 44.1, at 2 h post-sarin exposure (0.5 LD_{50}) in rat brain, to much lesser complexity, as indicated by the fewer number of genes (pathways) altered. Major upstream events include beta arrestin/GPCR pathways followed by CREB/non-CREB pathways. In spite of the same dose (0.5 LD_{50}) used in both, 15 min (Figure 44.1) and 2 h (Figure 44.2), there are far fewer genes altered at 2 h, which strongly suggests initiation of prompt and effective recovery, repair, and adaptive processes early on. This figure is reproduced with permission from Springer Pvt Ltd.

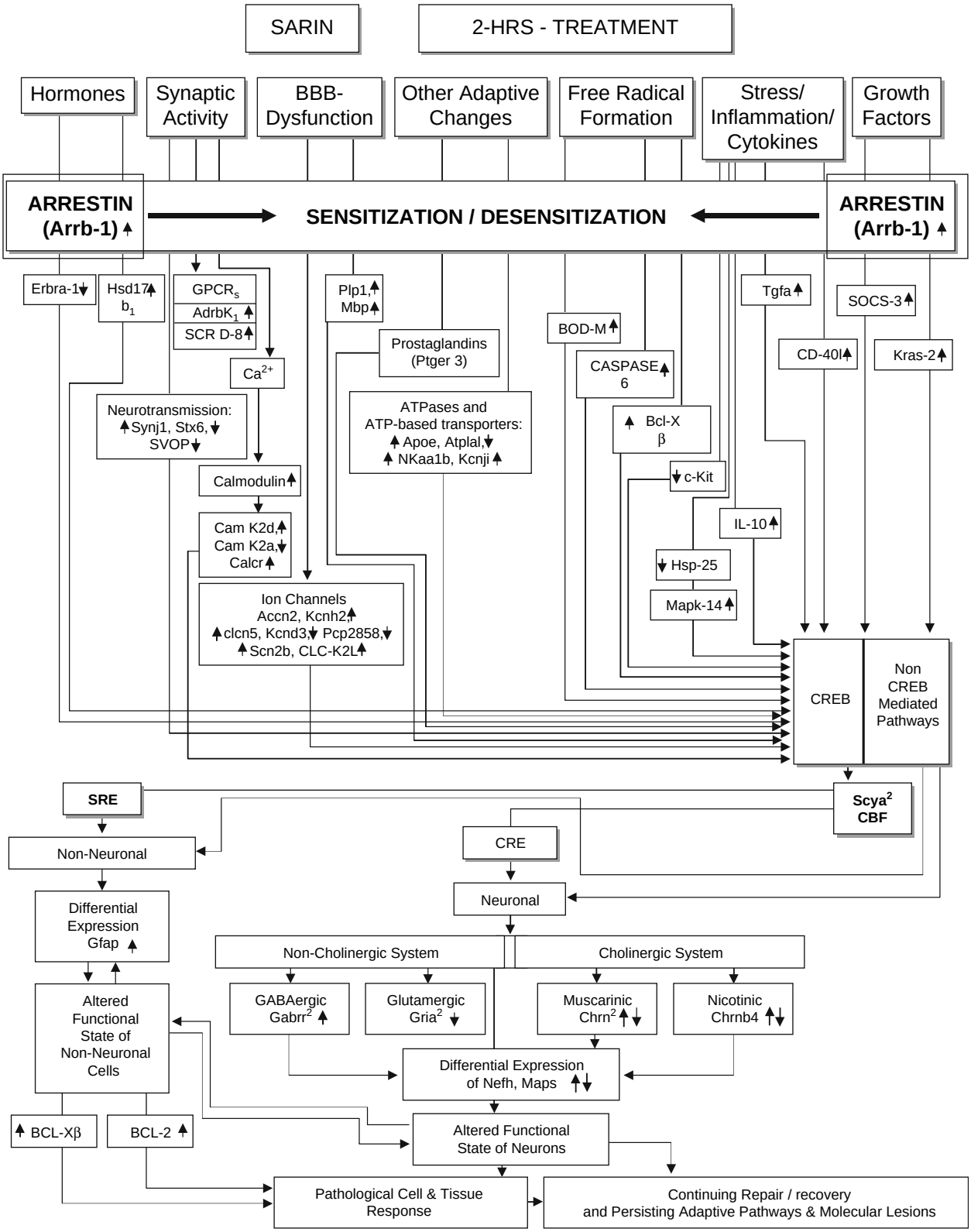

FIGURE 44.2. The complex gene expression changes initiated by sarin exposure (0.5 LD_{50}) in rat brain. Various factors such as hormones, altered synaptic activity, BBB dysfunction, free radical formation, stress/inflammation, alteration in the level of growth factors, and other adaptive pathways are found to be triggered by these changes. Major upstream events are all mediated through altered sensitization/desensitization mechanisms of beta arrestin/GPCR pathways. Another major upstream event which is downstream to beta arrestin/GPCR is the CREB (phosphorylated and/or unphosphorylated) and non-CREB mediated pathways. The signals processed at these two levels cause further changes in cholinergic and noncholinergic pathways in neuronal and nonneuronal cells. Thus, there are alterations in several mechanisms related to cell and tissue homeostasis, repair, and recovery pathways, thus leading to several adaptive changes. This figure is reproduced with permission from Elsevier Pvt Ltd.

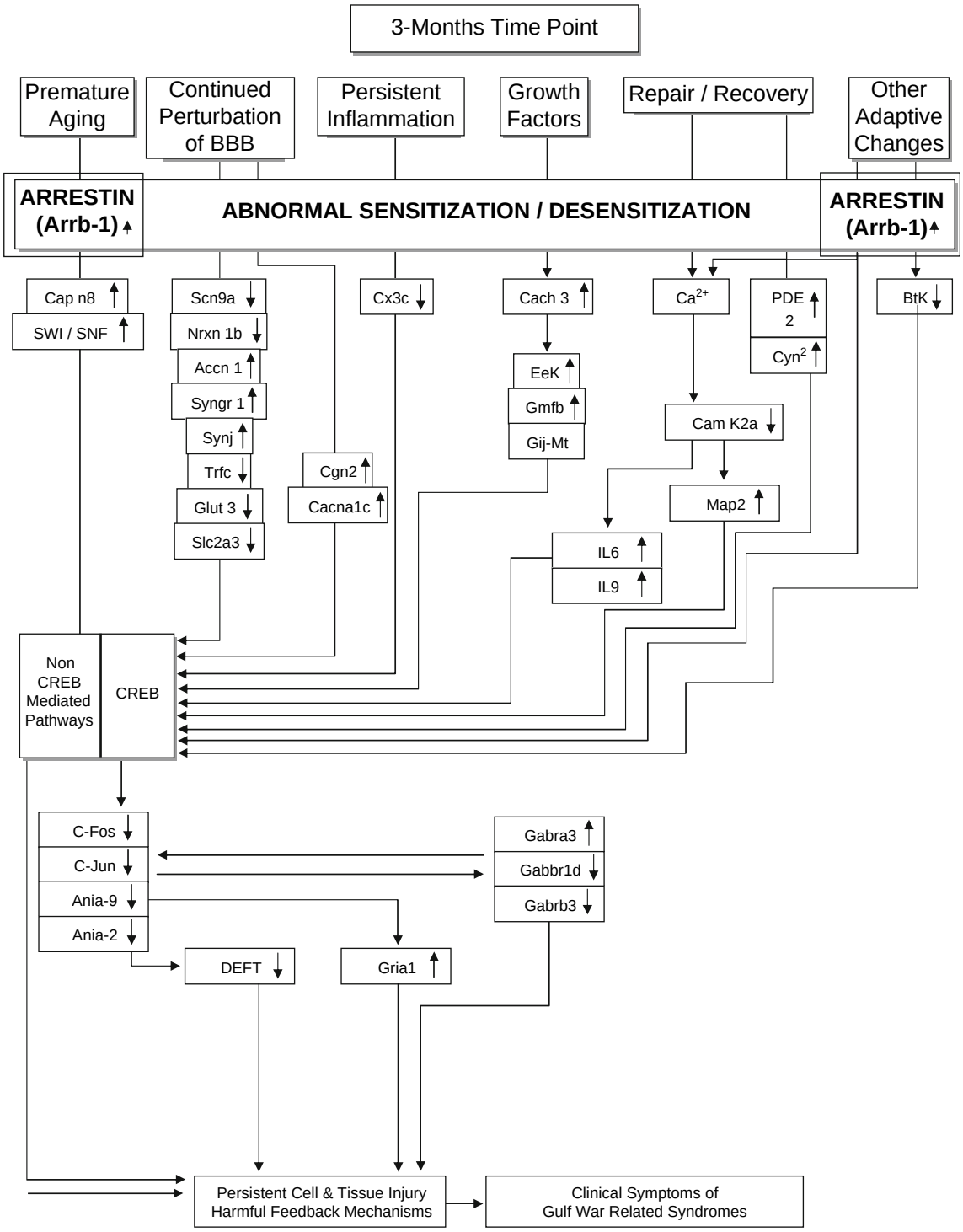

FIGURE 44.3. Pathways (genes) triggered at 3 months post-sarin exposure ($1 \times LD_{50}$) in rat brain. Various factors such as abnormal or premature aging process, continued BBB perturbations, persistent inflammation, and other adaptive, repair, and recovery pathways are found to be altered by upstream events such as beta arrestin/GPCR. This is followed by CREB/non-CREB pathways leading to changes in other downstream mechanisms of cell and tissue damage which lead to observable clinical symptoms as noted in Gulf War Syndrome patients and Japan subway terrorist incident-related victims. This figure is reproduced with permission from Elsevier Pvt Ltd.

2. Sarin-Induced Changes in G-Protein Coupled Receptors (GPCR) and Arrestin Mediated Pathways

G-protein coupled receptors (GPCRs) are one of the largest gene families of signaling proteins. Residing in the plasma membrane with seven transmembrane domains, GPCRs respond to extracellular stimuli that include catecholamine neurotransmitters, neuropeptides, larger protein hormones, lipids, nucleotides, and other biological molecules. They activate cellular responses through a variety of second messenger cascades (such as PKA and PKC signaling pathways). These receptors provide rapid responses to a variety of stimuli, and are often rapidly attenuated in their signaling. Failure to attenuate GPCR signaling can have dramatic consequences. One method to attenuate GPCR signaling is by removal of the stimulus from the extracellular fluid. At the synapse, removal of neurotransmitter or peptide signaling molecules is accomplished by either reuptake or degradation. ACh is removed from synapses through degradation by the enzyme AChE. Inhibition of AChE results in prolonged signaling at the neuromuscular junction (NMJ), and uncontrollable spasms in humans caused by sarin and other OPs. Transporters for serotonin, dopamine, GABA, and noradrenaline remove these neurotransmitters from the synapse to terminate signaling. The attenuation of GPCR signaling is receptor desensitization, in which receptors are modified to terminate signal transduction, even if the stimulus is still present.

The overexpression of either arrestin 1 or arrestin 2 has been demonstrated to augment the desensitization and internalization of several GPCRs (Pippig *et al.*, 1993; Oakley *et al.*, 1999). Thus, overexpression of arrestin at 15 min, 2 h, and 3 months post-sarin exposure, along with continued overexpression of several other genes belonging to several classes (Camk Ib, Camk2d, SCR D-8, M2-AchR; GALR1; CRLR) at 15 min, 2 h or 3 months, clearly indicate that sarin-induced hyperphosphorylation and related molecular changes persist until a later time point, thus causing defects in the tissue repair process (Damodaran *et al.*, 2006a, b).

3. Sarin-Induced Immediate Early Genes (IEGs)

In sarin-induced Ania-9 (activity and neurotransmitter-induced early gene 9) at 15 min post-exposure, the levels were down-regulated to 42% of the control value at 3 months post-exposure. Other genes such as c-fos, c-jun, and Ania-2 were also severely down-regulated at 3 months (Damodaran *et al.*, 2006a). Scya2 (immediate early serum responsive JE) was the only gene induced (285% of control levels) at 2 h post-sarin exposure (Damodaran *et al.*, 2006b). It is a well-known fact that ITFs (immediately induced transcription factors) and IEGs are among the most rapidly degraded of all proteins suggesting breakdown by the calpain and ubiquitin systems operating through lysosomes and proteosomes (Jariel-Encontre, 1997). Thus, down-regulation of these IEGs at a late time point can be caused by (1) successful regeneration, or, if this cannot be achieved, (2) a resting atrophic state, or (3) cell death (Damodaran *et al.*, 2006a, b).

4. Cholinergic System as the Primary Target of Sarin-Induced Toxicity

Sarin, like other OPs, elicit its toxic effects by irreversibly inhibiting AChE in the central cholinergic system, and excessive stimulation of postsynaptic cholinergic receptors. The overstimulation of the central cholinergic system is followed by the activation of other neurotransmitter systems including glutamate receptors leading to an increase in extracellular levels of the excitatory amino acid glutamate (McDonough and Shih, 1997). Numerous studies have shown that excessive accumulation of ACh leads to activation of ligand-gated ion channels, nicotinic ACh receptors (nAChRs), and muscarinic ACh receptors (mAChRs), which activate diverse kinds of cellular response by distinct signaling mechanisms (Abou-Donia *et al.*, 2002; Damodaran *et al.*, 2006a). Immediate and overexpression of the M2 receptor (214%) of the control value (100%) confirms biochemical data from several studies, showing consistent alterations in receptor density at 1, 3, 6, 15, and 20 h post-treatment (Abou-Donia *et al.*, 2002; Damodaran *et al.*, 2006a, b). nAChRs are essential for neuromuscular signaling, apoptotic signaling, neuroprotection, and synapse formation. The induced levels of nAChRs (150%) probably indicate its role in all of the above-mentioned capacities (Damodaran *et al.*, 2006a).

It is a well-known phenomenon that the cholinergic system interacts with various CNS neurotransmitters including serotonin (Quirion *et al.*, 1985), dopamine (Lehmann and Langer, 1982), catecholamines (Mason and Fibiger, 1979), GABA (Scatton and Bartholini, 1980), and neuropeptides (Lamour and Epelbaum, 1988). The cholinergic neurotransmitter system also controls cerebral vasculature (Armstrong, 1986). Modified gene expression of genes belonging to various systems such as serotonin [Slc6a4 (141%), Htr6 (47%)], dopamine [Drd4 (225%)], GABA [Gabbr-1 (179%), Gabra1 (130%)], and neuropeptides [Vipr (219%), Edn3 (236%), Hcrtr1 (139%)] from the control value (100%) confirms such an active interaction (Damodaran *et al.*, 2006a, b).

In addition, neurons express two pools of AChE: active and inactive (Choi *et al.*, 1998). It is not known how these two pools of AChE are regulated post-translationally.

Moreover, Kaufer *et al.* (1999) suggested that in addition to ACh, an autologous feedback response could regulate transcriptional elevation from the AChE gene through AChE-antiChE complexes on signaling intracellular pathways. Excess muscarinic activation induced either by agonist application or by inhibition of the AChE results in long-lasting modifications of gene expression and protein levels of key cholinergic proteins (Meshorer and Soreq, 2006). Specifically, such changes involve transcriptional accentuation as well as shifted alternative splicing of AChE

pre-mRNA transcripts. This yields elevated levels of the stress-induced ''readthrough'' AChE-R mRNA transcript, translated into soluble AChE-R monomers rather than the primary ''synaptic'' AChE-S membrane-altered tetramers (Meshorer and Soreq, 2006). These two AChE variants can both hydrolyze ACh with similar efficiency, yet their different C-terminal sequences affect their subcellular localization as well as their protein partners (Meshorer and Soreq, 2006). Thus, multilevel changes initiated by sarin exposure on the cholinergic system have a major effect on several downstream processes of cell metabolism.

5. Sarin-Induced Changes in Protein Kinase C (PKC) Pathway

The protein kinase C (PKC) signaling pathway has been associated with modulation of *N*-methyl-D-aspartate (NMDA) receptor activity, motor behavior, learning, and memory, all of which are severely impaired in intoxication with sarin and similar OPs. There was a reduction in the immunoreactivity levels of betaII-PKC and Zeta-PKC in the frontal cortex (up to 24 h), and in the striatum (up to 5 days) post-sarin exposure, in contrast to the increase in the immunoreactivity of both enzymes in the hippocampus or thalamus, following a $1\times LD_{50}$ exposure to sarin. These observations suggest a role for both conventional and atypical PKC isozymes in OP-induced neuropathy in the rat and further support their role in cell death (Bloch-Shilderman *et al.*, 2005).

6. Sarin-Induced Alterations in Gene Expression Related to Electrophysiological Activities of CNS

Abnormal electrophysiological recordings following a single large dose or repeated subclinical dose of sarin in rhesus monkeys (Burchfiel *et al.*, 1976; Burchfiel and Duffy, 1982) and neuropathy in rats (Kadar *et al.*, 1995) have been reported. High concentrations of OPs have been reported to affect excitable membranes directly, by blocking peripheral nerve conduction (Woodin and Wieneke, 1970). A very large group of ion channel-related genes, belonging to almost every possible type (sodium, potassium, chloride, and proton-gated) such as Scn4a (338%), Kcnma1 (331%), Kcnj3 (199%), Clns1a (177%), Clcn5 (146%), Kcnd (138%), and Accn1 (129%), were induced to various degrees from the control levels (100%) as well as down-regulation of Kcnn2 to 64%, and clearly support severely altered electrophysiology at 15 min post-exposure (Damodaran *et al.*, 2006a). Similarly, several genes encoding mRNA for ion channels remained induced although to a lesser extent at 2 h after sarin exposure (Damodaran *et al.*, 2006b). The list includes Clcn5 (155%), Scn2b (138%), Cncg (136%), Clc-k21 (130%), Accn2 (122%), Kcnh2 (113%). However, the overall trend seems to be that of returning to control levels. Two genes, namely Kcnd3 (76%) and Pcp2858 (72%), showed down-regulation. On the other hand, there were only two genes (Accn1: 130% and Scn9a: 47%) showing altered expression at 3 months (Damodaran *et al.*, 2006a). The fact that two genes such as voltage-gated sodium channel (Scn9a) and neuronal degenerin channel MDEG (Accn1) showed persisting alterations at 3 months clearly indicates that sarin-induced alterations in the electrophysiological changes initiated at an early time point were minimized, but not completely normalized in rat animal models. Some degeneration and other problems related to memory and other defects in sarin-exposed GWS-related clinical features could be due to such a phenomenon. In a similar way, the highest expression of Ecto-ATPase at 15 min (Damodaran *et al.*, 2006a) and the persistence of altered levels of several other ATPases and ATP-based transporters at a 2 h time point (Damodaran *et al.*, 2006b) as well as at 3 months (other transporters like transferrin and neuron glucose transporter) clearly support the idea that perturbations in electrophysiological parameters of CNS persisted for a long time after the initial acute sarin exposure event (Damodaran *et al.*, 2006a, b).

7. Sarin-Induced Calcium Channels and Binding Proteins

Ca^{2+} is involved in the control of neuronal membrane excitability, neurosecretion, synaptic plasticity, gene expression, and programmed cell death. In physiological conditions, neuronal stimulation induces transient increases in the $[Ca^{2+}]$. Basal Ca^{2+} levels in the cytoplasm are principally maintained by efflux of Ca^{2+} through a membrane-associated Ca^{2+}-ATPase and also through a membrane Na/Ca^{2+} exchanger. In addition, cytosolic Ca^{2+} concentration is controlled by active Ca^{2+} sequestration into intracellular stores (endoplasmic reticulum and mitochondria) and by Ca^{2+} binding to intracellular proteins. An increase in the cytosolic concentration of Ca^{2+} can be the result of either influx of extracellular Ca^{2+} or the release of Ca^{2+} from internal stores. Ca^{2+} can enter the neuron through two major classes of Ca^{2+} channels, such as voltage-sensitive Ca^{2+} channels (VSCCs), which are sensitive to alteration in membrane potential and receptor-operated Ca^{2+} channels (ROCCs).

A sustained increase in cytosolic Ca^{2+} concentration, different from the rapid and transient changes occurring in physiological conditions, is invariably associated with neural damage (Nicotera, 1992). Sarin may induce alterations in the physical integrity of the plasma membrane, mitochondrial impairment, and consequent ATP depletion (Komulainen and Bondy, 1988). In addition, $[Ca^{2+}]$ overload is postulated to play an important role in hypoxic–ischemic brain damage, as a consequence of excitatory amino acid excessive stimulation and enhanced Ca^{2+} influx through membrane channels (Kristian and Siesjo, 1996). Moderate induction of CamK1b, Cacna1g, and down-regulation of Cacna1d and Camk2d were noted at 15 min post-sarin exposure (Damodaran *et al.*, 2006a). Induction of several genes (Calcr: 207%; Camk2d: 159%;

Calm: 132%; S100b, calcium binding protein: 130%) and down-regulation of CamK2a (56%) was found at 2 h post-sarin exposure (Damodaran *et al.*, 2006b). Induction of CaCh3 (neuroendocrine calcium channel alpha 1) to 220% and Cacna1c (L-type VDCC alpha unit) to 149%, accompanied by down-regulation of Camk2a (21% of the control levels) and Atp2a3 (Ca^{2+} transport ATPase 2) to 79%, suggest expression changes persisted at 3 months post-sarin exposure (Damodaran *et al.*, 2006a).

8. CaMKII as the Key Molecule in the Development of Neurotoxicity

The processing of a Ca^{2+} signal requires its union with specific intracellular proteins. Calmodulin is a major Ca^{2+} binding protein in the brain, where it modulates numerous Ca^{2+}-dependent enzymes and participates in relevant cellular functions. Among the different calmodulin-binding proteins, the Ca^{2+}/calmodulin-dependent protein kinase II and the phosphatase calcineurin are especially important in the brain because of their abundance and their participation in numerous neuronal functions. Studies on the acute effects of sarin on microsomal and cytosolic components of the calmodulin system in the rat striatum indicated CaM/calmodulin levels in both cell fractions were significantly increased by sarin (Hoskins *et al.*, 1986). Decreased cyclic AMP-PDE and adenylate cyclase activities and cyclic GMP-PDE and guanylate cyclase activities were noted after sarin exposure. Sarin administration caused significant increases in microsomal protein kinase activity (Hoskins *et al.*, 1986). Recent gene expression studies also confirmed the prominent role of the CAMK system by the altered mRNA levels of CaMK 2 alpha, CaMK 2 delta, calmodulin, and CaMK1. Persistent down-regulation of CamK2a at early (2 h) and late (3 months) time points clearly indicates its important role in sarin-induced neurotoxicity (Damodaran *et al.*, 2006a, b).

9. Sarin-Induced Blood–Brain Barrier (BBB) Damage and Related Alterations in Gene Expression

The entry of molecules to the CNS is regulated by the BBB, which is the interface between blood and the CNS, and this entry is based on the size, charge, hydrophobicity, and/or affinity to carriers. MOG (myelin oligodendrocyte glycoprotein), PSD-95, and neurexin were also altered, indicating disturbances to the BBB-related function. Large doses of sarin caused BBB damage and BBB disruption plays an important role in sarin-induced cell death (Abdel-Rahman *et al.*, 2002; Damodaran *et al.*, 2003). Kinase cascades such as CaMKII and MAP kinase kinase are altered in cell types with compromised BBB, within 15 min, which probably received transducing signals due to changes seen in several upstream molecules (Damodaran *et al.*, 2006a, b). Tyrosine phosphorylation is the next logical step, which may increase tight junction permeability (Staddon *et al.*, 1995). Up-regulation of tyrosine kinase receptor EHK-3, altered levels of transporters such as sodium-dependent neurotransmitter transporter, and ATP-based transporters were suggested to support such a possibility (Damodaran *et al.*, 2006a). A primary mediator of injury-induced BBB disruption is nitric oxide (NO). Induction of nitric oxide synthase along with changes in EDN 3 expression clearly support such a scenario (Damodaran *et al.*, 2006a). Agents that regulate vasoactive processes, such as vasoactive intestinal receptor-1 (Vipr 1) and 2 (Vipr 2), may be involved in the biochemical opening of the BBB (Damodaran *et al.*, 2006a), as other vasoactive genes such as bradykinin and angiostensin have been shown to (Black, 1995).

Other changes noted in the NMDA receptor function-related genes such as PSD-95 as well as PSD-95/SAP-90-associated protein were up-regulated at 15 min post-sarin exposure, along with NMDA and NO synthase levels (Damodaran *et al.*, 2006a). PSD-95 participates in the anchoring of the NMDA receptor and interacts with neuronal NO synthase at the postsynaptic membrane, and thereby plays a fundamental role in synaptic transmission and memory formation (Kim *et al.*, 2002) about the role of NOS/NMDA mediated apoptosis in DFP (a structural analog of sarin)-treated rat CNS. Altered levels may explain an early abnormal event that may initiate a complex process leading to memory loss observed in sarin-exposed rats (Kassa *et al.*, 2001). Besides, persistence of altered expression of BBB-related molecules such as neurrein-1 adds support to the important role of BBB damage in initiating and amplifying neurotoxicity-related phenomena.

10. Effect of Sarin on Hormones and Growth Factors

Hormones play an important role in the cholinergic neurotransmission at pre- and postsynaptic levels in the basal forebrain. The neurobiological mechanisms that underlie these effects are not fully understood, but most likely reflect effects of hormones on the survival, connectivity, and function of specific neural systems in the hippocampus and may trigger a variety of intracellular signals (Foster, 2005). All of the growth factor-related genes such as rat growth hormone, basic fibroblast growth factor, and insulin-like growth factor-II were found to be induced at 15 min, while at 3 months growth factors related to glial function (Gmfb) were induced along with eek (eph and elk-related kinase) and growth inhibitory factor-metallothionein homolog (Gif-Mt) (Damodaran *et al.*, 2006a, b). C-erb-A-Thyroid hormone receptor and 17 betahydroxysteroid dehydrogenase showed down- and up-regulation, respectively, 2 h after sarin exposure (Damodaran *et al.*, 2006b). DFP (structural analog to sarin) has been shown to reduce serum prolactin, thyropton, luteinizing hormone, and growth hormone, and increase adenocorticotropin and corticosterone in rats and it was concluded that cholinesterase inhibition evokes a multiplicity of effects on anterior pituitary function (Smallridge *et al.*, 1991). A similar mechanism of action can be expected for the effect of sarin exposure on

endocrine systems. A statistically significant correlation between body weight loss, plasma cholinesterase levels, and hormonal control of hydration levels in sarin-dosed animals established that the satiety center controlling the hypothalamus and CNS might be affected by sarin treatment (Young *et al.*, 2001).

It is known that cells that are stimulated by bFGF also showed robust expression of nestin, which plays a role in the neuronal glial differentiation of bipotential cells (Cameron *et al.*, 1998). Both nestin and bFGF are induced in the sarin-treated CNS at 15 min, thus confirming their protective role either inducing the proliferation or increasing the differentiation of bipotential cells into neurons or glia as and when required at distinct regions of damaged CNS (Damodaran *et al.*, 2006a, b).

11. Effect of Sarin on CREB Pathway

CAMP-response element binding protein (CREB) is a member of a large family of structurally related transcription factors which has the transcriptional control of numerous genes, many of which are rapidly expressed in response to an elevation of cytoplasmic cAMP or Ca^{2+} levels. The persistent phosphorylation of CREB in the CNS, in response to OPs like diisopropyl-phosphorofluoridate (DFP), has been shown to be involved in the initiation of delayed neurotoxicity (Damodaran *et al.*, 2002c). There are numerous adaptive changes that can be broadly divided into (a) cell survival and (b) cell death that may be mediated through CREB pathways. Altered gene expression for several genes (Camk2, Pka, c-Fos, c-Jun) was shown in DFP-treated hen CNS (Gupta *et al.*, 1997, 1998, 2000a, b; Gupta and Abou-Donia, 1999; Damodaran *et al.*, 2003). Similarly, expression profiles for several genes (Cam2a, Camk2d, Calm, Cbf-C, and Scya2) were also shown to be altered in sarin-treated rats (Damodaran *et al.*, 2006a, b). Transducing signals of several other kinases also converge on the CREB pathway leading to the activation of several downstream processes. Activation of c-Jun N-terminal kinase (JNK) and slight activation of mitogen-activated protein kinase (MAPK) in the cytosol fraction of the brain homogenate was observed after exposure to sarin-like organophosphate BIMP (bis isopropylmethyl phosphonate), which increased tyrosine phosphorylation of several proteins in the cytosol fraction (Niijima *et al.*, 2000). Altered expression of Camk2a, Camk2d, Calm, JNKs, and MAPKs by sarin exposure clearly supports such a phenomenon mediated through CREB pathways.

12. Sarin-Induced Alterations in Gene Expressions Related to Astroglial Marker Genes

Astrocyte activation or reactive gliosis involves proliferation, recruitment to the site of injury (Walton *et al.*, 1999), and release of numerous cytotoxic agents including proteolytic enzymes, cytokines, complement proteins, reactive oxygen intermediates, NMDA-like toxins, and nitric oxide (Weldon *et al.*, 1998). Sarin exposure activated astrocytes and resulted in the altered mRNA expression profile of GFAP and vimentin in a time course study (Damodaran *et al.*, 2002b). Global gene expression studies showed an alteration in the expression levels of S-100 beta, an astrocyte-specific Ca^{2+}-binding protein (neurotropic factor), at 2 h post-treatment, thereby confirming the fact that sarin-induced calcium changes resulted in alterations in several downstream molecules. The alteration in S-100 levels also indicates changes taking place in Schwann cells of the CNS. In rats, S-100 beta has been shown to present in about 35% of the neurons (Landgrebe *et al.*, 2000). Hence up-regulation at 2 h probably indicates that both neurons and glial cells are affected by sarin treatment. Another gene of special interest induced by sarin is apolipoprotein E (apoE), which may play a role in various CNS disorders, and its altered levels indicate modified astrocyte and/or neuronal response and functions (Damodaran *et al.*, 2006b). Altered expression of apoE has been associated with chronic reactive gliosis (Martins *et al.*, 2001) and is also suggested to be neuroprotective (Boschert *et al.*, 1999). ApoE has been shown to stimulate the transcriptional activity of CREB by activating the extracellular signal-regulated kinase (ERK) cascade in rat primary hippocampal neurons (Ohkubo *et al.*, 2001).

13. Gender-Based Differences in Gene Transcript Expression

Overall, female rats were more sensitive to sarin vapor toxicity than male rats over the range of exposure concentration and duration studied (Mioduszewski *et al.*, 2002). In a recent global gene expression study, male and female Sprague-Dawley rats exposed to low-level doses (0.010 to 0.033 mg/m^3) of the aerosolized sarin via whole body inhalation for 4 h showed differential gene expression response between male and female rats (Sekowski *et al.*, 2002). Many of the altered genes participate in cellular processes critical to detoxification pathways and neurological homeostasis which may reflect the difference in the sensitivities as demonstrated by the differences in the miosis EC_{50} levels. Transcripts of UDG glycolase (Uracil DNA glycosylase) were shown to be significantly up-regulated at the highest dose point (0.033 mg/m^3) for females but not for males. UDG glycolase is involved in base excision repair (Scharer and Jiricny, 2001). The transcripts for the gene Cyp2A and other Cyp2 family members also showed differential expression pattern. While the expression pattern of all Cyp2 family members in females was not altered by any of the doses of sarin tested, the Cyp2A family of enzymes is variably, but significantly influenced by the doses of sarin tested in males. Furthermore, it appears that the constitutive levels of these enzymes are higher in males than in females. It is well known that the Cyp2 family of enzymes is involved with primary metabolism and bioconversation of many toxicants (Sekowski *et al.*, 2002).

DNA polymerase alpha was up-regulated following low-level sarin exposure. Since mammalian DNA replication (and possibly DNA repair) mechanisms utilize DNA

polymerase alpha, the DNA replication machinery may be turned on or up-regulated to synthesize DNA and/or repair lesions in the DNA (Sekowski *et al.*, 2002). Sarin exposure also down-regulated endothelin-1, which plays a critical role in vascular tension, as it interacts with the sympathetic nervous system and the renin–angiotensin system (Sekowski *et al.*, 2002).

14. Sarin-Induced Changes in Cytokine Profile

Sarin exposure resulted in prolonged central neuro-inflammatory processes in rat brain tissues (Damodaran *et al.*, 2006a, b; Chapman *et al.*, 2006). Gene expression analysis at various time points (15 min, 2 h, and 3 months) in sarin-treated rats indicated up-regulation of 1L-10 at early time points, while IL6 and IL9 were noted at late (3 months) time points (Damodaran *et al.*, 2006a, b). Biochemical evaluation of rat brain tissues revealed a significant increase in the level of the proinflammatory peptides starting at 2 h and peaking at 2–24 h following sarin exposure (Chapman *et al.*, 2006). Hippocampal values of IL1-beta increased one-fold at 2 h and nine-fold at 8 h. PGE2 (prostagladin E2) levels in the hippocampus increased up to four-fold to six-fold at 2 and 8 h. In addition, a second increase in inflammatory markers was observed 30 days following sarin exposure only in the rats with 30 min of prolonged seizures. Marked histological damage to the brain was demonstrated following 30 min of seizure activity, consisting of severe damage to the hippocampus, piriform cortex, and some thalamic nuclei as compared to rats with 5 min seizures.

Subacute doses of sarin on wistar rats led to suppression of cellular and humoral immune reactions and to a decrease in blood concentrations of cytokines (IL-2, IL-4, IFN-gamma), with a reduction of the IFN-gamma/IL-4 and IL-2/IL-4 ratios, which attests to a more pronounced decrease in Thy1 lymphocyte function in comparison with Th2 cells (Zabrodskii *et al.*, 2007). Grauer *et al.* (2008) studied the long-term neuronal and behavioral deficits after a single whole body exposure to sarin vapor. Neuronal inflammation was demonstrated by a 20-fold increase in prostaglandin (PGE2) levels 24 h following exposure that markedly decreased 6 days later. An additional, delayed increase in PGE2 was detected at 1 month and continued to increase for up to 6 months post-exposure. Glial activation following neural damage was demonstrated by an elevated level of peripheral benzodiazepine receptors (PBR) seen in the brain 4 and 6 months after exposure. Six week, 4 and 6 month post-exposure behavioral evaluations were performed. In the open field, sarin-exposed rats showed a significant increase in overall activity with no habituation over days. In the working memory paradigm in the water maze, these same rats showed impaired working and reference memory processes with no recovery. Thus, the data suggest long-lasting impairment of brain functions in sarin-exposed animals. The PGE2-dependent, ERK (extracellular signal-regulated kinase), 1/2-regulated, microglia–neuron signaling pathway may mediate the interaction between microglia and neurons in pain maintenance after injury (Zhao *et al.*, 2007) in sarin-exposed animals.

15. Sarin-Induced Oxidative Stress and Cell Death/Neuroprotection-Related Gene Expression

Oxidative stress mediated free radical generation and alterations in the anti-oxidative scavenging system have been implicated in OP-induced neurotoxicity (Abu-Qare and Abou-Donia, 2001; Abu-Qare *et al.*, 2001). Alteration in the levels of brain Acyl-CoA synthetase and leptin accompanied by alterations in the mRNA levels of key mitochondria associated proteins such as Bax (apoptosis inducer) and BOK (BCL-2-related ovarian killer protein) in sarin-exposed rats clearly supports oxidative stress-induced cell death (Damodaran *et al.*, 2006a). Induction of both proapoptotic (Bcl2l11, caspase 6) and antiapoptotic (Bcl-X) genes, besides suppression of p21, suggests complex cell death/protection-related mechanisms operating early on. Moreover, persistent alteration in the cell death-related molecule DEFT at 3 months adds support to the notion that oxidative stress-related changes can cause long-term complications in cell physiology and pathology (Damodaran *et al.*, 2006a, b).

Reactive oxygen species (ROS) have been shown to initiate a number of signaling events that lead to endothelial cell ‘‘activation’’ and up-regulation of cell adhesion molecules and chemoattractants. Strong induction of tumor necrosis factor (TNF)-beta along with down-regulation of CX3C at 15 min and further down-regulation at 3 months post-sarin exposure strongly suggest such a mechanism (Damodaran *et al.*, 2006a). Many cell adhesion and cytoskeleton and BBB-related molecules (neurexin 1-beta; neurexin 1-alpha, PSD-95/SAP90-associated protein 4), and cytoskeletal genes (nestin, beta tubulin) showed prominent alterations in mRNA expression (Damodaran *et al.*, 2006a, b).

Lipid peroxidation has been shown to be present in the CNS of rats treated with sarin (Abou-Donia *et al.*, 2002). Altered expression levels of lipophilin supports to the notion of sarin exposure initiated lipid peroxidation as an important by-product of oxidative stress. The glutathione system is an important protective mechanism responsible for removing H_2O_2 and maintaining protein thiols in their appropriate redox state in the cytosol and mitochondria for minimizing oxidative damage. Altered expression of glutathione, dopamine (Drd4), and GABA (Gabbr-1, Gabra1, Gabra3, Gabbr-1d, Gabrb3)-related genes may potentiate the cell death and injury to these populations (Damodaran *et al.*, 2006a).

16. Effect of Sarin on DNA and Protein Content

Kassa *et al.* (2000) showed that not only symptomatic level 3 but also asymptomatic levels 1 and 2 of sarin were able to significantly decrease the incorporation of radiolabeled thymidine without changing total concentrations of DNA or

protein at 3 months following exposure. On the other hand, the significant decrease in total contents of DNA and protein in liver without the changes in the incorporation of tritiated thymidine was noted in liver 6 months following sarin exposure. No significant changes in the metabolism of DNA and protein were observed at 12 months following sarin exposure. Thus, not only clinically manifested intoxication but also low-level, asymptomatic exposure to sarin altered nucleic acids and protein metabolism, several months following exposure.

Repeated low-dose exposure to sarin produced a dose-dependent response in leukocytes at 0 and 3 days post-exposure. There was a significant increase in all measures of DNA fragmentation at 0.2 and 0.4 LD_{50}, but not at 0.1 LD_{50}. There was no significant increase in DNA fragmentation in any of the groups at 17 days post-exposure. Sarin did not produce systematic dose-dependent response in parietal cortex at any of the time points. However, significant increases in DNA fragmentation at 0.1 and 0.4 LD_{50} were observed at 0 and 3 days post-exposure and levels were back at control level by day 17 (Dave *et al.*, 2007). Analysis of phosphor-carrying metabolites of sarin and its by-products by Li *et al.* (2000) in urine samples from the victims of the Tokyo train terrorist attack suggested that they were exposed not only to sarin, but also to by-products generated during sarin synthesis, i.e. diisopropyl methylphosphonate (DIMP) and diethyl methylphosphonate (DEMP). Sister chromatid exchange (SCE) was significantly higher in the victims than in a control group. Both DIMP and DEMP significantly inhibited NK and CTL activity in a dose-dependent manner. The inhibition induced by DIMP was stronger than that by DEMP. The effect of DIMP and DEMP on the splenic NK activity of mice was stronger than on the splenic CTL activity, and the human lymphocyte is more sensitive to DIMP and DEMP than the splenocytes of mice.

17. Effect of Sarin on Immune System

There is increasing evidence that the immune, endocrine, and nervous systems communicate with each other through hormones, neurotransmitters, and cytokines (Tracey, 2005). Immune function is not only regulated by the cytokine system, it is also under the control of an independent lymphoid cholinergic system (Kawashima and Fujii, 2008). Immunosuppressive effects of sarin are mediated through the central and peripheral mechanisms (Sopori *et al.*, 1998). Interestingly, serum corticosterone levels of the sarin-treated animals were dramatically lower than the control animals (Kalra *et al.*, 2002). Subclinical doses (0.2 and 0.4 mg/m^3) inhibited the anti-sheep red blood cell antibody-forming cell (AFC) response of spleen cells without affecting the distribution of lymphocyte subpopulations in the spleen (Kalra *et al.*, 2002). Moreover, sarin suppressed T cell receptor (TCR) antibody-induced T cell proliferation and the rise in the intracellular calcium following TCR ligation. These concentrations of sarin altered regional but not total brain acetylcholinesterase activity (Kalra *et al.*, 2002). Pretreatment of animals with the ganglionic blocker chlorisondamine abrogated the inhibitory effects of sarin on spleen cell proliferation in response to Con A and anti-TCR antibodies. These results suggest that effects of sarin on T cell responsiveness are mediated via the autonomic nervous system and are independent of the HPA (hypothalamus–pituitary–adrenal axis). Furthermore, sarin-induced inhibition of T cell dependent functions such as the AFC response and the TCR mediated rise in intracellular Ca^{2+} is lost 2 weeks after sarin exposure. Low-level (single or repeated) inhalation exposure of sarin on inbred BALB/c mice to evaluate the effect of sarin on immune functions indicated that sarin is able to alter the reaction of the immune system at 1 week after exposure to sarin (Kassa *et al.*, 2004). While the number of CD3 cells in the lung was significantly decreased, a slight increase in CD19 cells was observed especially in the lungs after a single sarin inhalation exposure. Lymphoproliferation was significantly decreased regardless of the mitogen and sarin concentration used and the number of low-level sarin exposures. The ability of peritoneal and alveolar macrophages to phagocytose the microbes was also decreased regardless of the number of low-level sarin exposures. The production of N-oxides by peritoneal macrophages was decreased following a single low-level sarin exposure but increased following repeated low-level sarin exposure. However, the change in the production of N-oxides that reflects a bactericidal activity of peritoneal macrophages was not significant. The natural killer activity was significantly higher in the case of inhalation exposure of mice to low concentrations of sarin regardless of the number of exposures. Generally, repeated exposure to low concentrations of sarin does not increase alteration of immune functions compared to the single low-level sarin exposure, with the exception of phagocyte activity of alveolar macrophages and natural killer cell activity.

Sarin exposure also up-regulated the mRNA expression of proinflammatory cytokines in the lung, which is associated with the activation of NFκB in bronchoalveolar lavage cells (Pena-Philippides *et al.*, 2007). These effects were lost within 2 weeks of sarin inhalation. While the effects of sarin on T cell function were on the autonomic nervous system (ANS), the decreased corticosterone levels by sarin might result from its effect on the HPA axis. Sarin induces lung inflammation resulting from neutrophilic and eosinophilic infiltration (Levy *et al.*, 2004; Pant *et al.*, 1993). Subclinical doses of sarin did not cause signs of lung inflammation, but left molecular imprints of lung inflammation. Sarin promotes the nuclear translocation of NFκB in BAL cells. Therefore, increased gene expression of IL-1, TNF-alpha, and IL-6 observed in BAL cells from sarin-treated animals may result through activation of NFκB. In higher doses, sarin may elevate the transcription, as observed by others (Levy *et al.*, 2004; Pant *et al.*, 1993; Damodaran *et al.*, 2006a).

18. Studies on the Long-Term Effect of Sarin Exposure to Human Subjects Support Data from Animal Models

Golomb (2008) summarized the findings from several epidemiological studies, and suggested that AChE inhibition by various OP nerve agents and other OP and carbamate pesticides could be linked to Gulf War Syndrome-related illness. Thirty-eight victims of the Tokyo subway sarin attack exhibited smaller than normal regional brain volumes (of the matched controls) in the insular cortex and neighboring white matter, as well as in the hippocampus, all of whom had been treated in an emergency department for sarin exposure in a long-term study, using T1-weighted and diffusion tensor magnetic resonance imaging (DTT), the voxel-based morphometry.

Furthermore, an extensively lower than normal fractional anisotropy was also noted in the victims. The reduced regional white matter volume correlated with decreased serum cholinesterase levels and with the severity of chronic somatic complaints related to interoceptive awareness (Yamasue *et al.*, 2007). Similarly, another study of Gulf War veterans showed subtle but persistent central nervous pathology such as reduced white matter and increased right and left lateral ventricle with higher levels of exposure to sarin and cyclosarin (Heaton *et al.*, 2007). Moreover, electrophysiological deficits of controlled attention, accompanied by lower P300 amplitude and smaller anterior cingulated cortex volume, were observed in patients (victims of the Tokyo subway sarin attack), and suggested a link to brain morphological changes (Araki *et al.*, 2005). Similarly, the patients from subway sarin attack also performed significantly less well in the psychomotor function test (tapping) than the referent group. There were dose-dependent effects for these observations and thus these results indicate a chronic decline of psychomotor function and memory function 7 years after sarin exposure (Miyaki *et a*l., 2005). Investigations on the neurobehavioral task performance of Gulf War veterans categorized as having received high, moderate, or low-to-no exposure dose levels to sarin and cyclosarin indicated that sarin and cyclosarin exposure was significantly associated with less proficient neurobehavioral functioning on tasks involving the psychomotor dexterity and visuospatial abilities 4–5 years after exposure (Proctor *et al.*, 2006). All these observations from human studies strongly correlate with the data from gene expression and other molecular studies in animal models.

19. Sarin-Induced Altered Expression Profile Provides Biomarker Panels and Therapeutic Targets

Molecules that show persistent altered expression over a period of several time points strongly suggest that they play important roles in preserving, amplifying, and transmitting the altered expression as and when needed, which could be either degenerative or regenerative in nature. There are several molecules such as Arrb-1, Nrxn-1b, Nos-2a, Ania-9, PDE2, Gabab-1d, CX3C, CamK2a, Camk2d, Clcn5, IL-10, c-Kit, and Plp1 that showed such a pattern (Damodaran *et al.*, 2006a, b). Beta-arrestin (Arrb-1) maintained its altered state at three different time points such as immediate early (15 min), early (2 h), and late (3 months), thus confirming persistent pathological changes due to altered signal transduction pathways mediated through beta arrestin. Continued down-regulated mRNA levels of neurexin (Nrxn-1b) and persistent up-regulation of Nos2a clearly support an idea that BBB-related perturbations were still persistent at 3 months after the treatment, and might lead to persistent cell death in susceptible brain regions. Immediate early up-regulation of Ania-9 at 15 min followed by significant down-regulation at 3 months strongly indicates either successful regeneration or a continuing cell death/atropic state. Initial down-regulation at 15 min, followed by up-regulation of PDE 2 at 3 months, probably indicates recovering cyclic nucleotide metabolism at 3 months from its sarin-induced suppression. Initial up-regulation (15 min) followed by severe down-regulation of GABAB-1d (3 months) confirms the persistent defective GABAerginic metabolism. Induction at early time points followed by down-regulation at late time points makes CX3C one of the potential biomarker molecules. Clcn5 showed persistent alterations at both immediate early (15 min) and early (2 h) suggesting altered ion-channel-related functions. Persistent overexpression of IL-10 at early time points may indicate an underlying anti-inflammatory response. Continued alteration of kinase pathways was suggested by the persistent alterations in the levels of Camk2a, Camk2d, and c-Kit. Hence early events such as BBB damage (neurein 1-beta, Plp1, Nos2a), IEGs alteration (Ania-9, probably c-Fos and cJun and other Ania members), modified GPCR mediated signaling pathways (mediated by beta arrestin), altered cyclic nucleotide metabolism (PDE 2), and inflammation and injury-related changes (CX3C), probably play major roles along with other known mechanisms as shown in Figures 44.1–44.3. These persistently altered genes can become candidate biomarkers to monitor sarin exposure as well as to develop therapeutic targets. For example, the Camk2 family of genes can be a good therapeutic target of intervention, as it seems to be central to the upstream and downstream signal transduction processes. Large amounts of data are already available for Camk2 regarding its structure, expression, inhibitors, and potential target genes (pathways). Similarly, AChE is a good candidate for such intervention, as already several studies attempted for that purpose show promising results at least at the level of ''proof of hypothesis'' (Curtis *et al.*, 2005, 2008; Li *et al.*, 2006). Time course studies on both Camk2 and AChE have shown that they are differentially altered in various regions of the brain (Gupta *et al.*, 1998; Damodaran *et al.*, 2003, 2006a). Beta arrestin can be a good candidate for monitoring any interventions, as its expression was consistently up-regulated. Different doses of sarin exposure can be identified by monitoring abundant yet

dose-dependent expression of selected genes, representative of various subregions (such as cerebellum, hippocampus, etc.) and cell types (neuronal, astroglial, microglial, oligodendrocytes, endothelial, etc.) which can be identified by further testing on several published genes (Damodaran *et al.*, 2006a, b). Other approaches include measuring the levels of tyrosine adducts which are relatively long-lived and are not degraded by oxime therapy, after exposure to sarin and other warfare chemicals (Williams *et al.*, 2007).

IV. CONCLUDING REMARKS AND FUTURE DIRECTION

Sarin exposure results in significant levels of transcriptional changes (either activation or suppression) of several genes (pathways) in the brain of animal models like the rat, mouse, and, potentially, humans. Global and candidate gene expression studies in model systems yielded data that confirm the toxic effect of sarin exposure on different cell types of the central nervous system such as neuronal, astroglial, microglial, and endothelial as well as oligodendrocytes. Sarin exposure also causes damage to structural components of the brain such as BBB at gross level as well as to the cytoskeletal framework at the molecular level as indicated by the altered expression data and histopathological data specific to such structures. There seem to be gender-specific gene expression profiles as a response to sarin exposure in rats, suggesting higher susceptibility in females than in males. The changes indicate that persistence of both altered degenerative and regenerative-related gene expression patterns activated early on may contribute to the level of neurodegeneration and neuropathology at a later time point. Sarin exposure also affected the molecular profiles of immune and endocrine systems besides the nervous system, as it is a well-known fact that they communicate with each other through cytokines, hormones, and neurotransmitters. Exposure to sarin can initiate (1) acute response-related clinical changes, (2) OPIDN, and (3) OPICN. Altered profiles of gene expression, protein expression, hormonal and other biochemical parameters in response to sarin exposure confirm the highly toxic nature of the nerve agent leading to significant amounts of nervous system pathology and end organ system pathology. Various neurological abnormalities observed in the victims of sarin exposures may be due to the above-mentioned molecular changes. These data thus provide a wealth of novel insights into the mechanisms of toxicity, so that newer approaches can be initiated in the diagnosis, prevention, and treatment aspects of toxic exposures to warfare chemicals like sarin.

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CHAPTER 45

The Effects of Organophosphates in the Early Stages of Human Muscle Regeneration

TOMAZ MARS, KATARINA MIS, SERGEJ PIRKMAJER, AND ZORAN GRUBIC

I. INTRODUCTION

The clinical condition of organophosphate (OP) poisoning is complex. Increased acetylcholine (ACh) concentration in the cholinergic synapses due to acetylcholinesterase (AChE) inhibition leads to altered signaling in these synapses causing various pathological effects. Particularly threatening among these effects is hypoxia which develops as a result of impaired exchange of gases in the lungs combined with bradycardia. In addition, cholinesterases are not the only targets of OPs, and various intracellular mechanisms are modified due to direct or indirect OP actions (Jett and Lein, 2006). DNA microarray analysis of cells treated with OP diazinon disclosed overwhelming modulation (up- or down-regulation) of a wide range of genes (Mankame *et al.*, 2006). Although still poorly understood, these noncholinergic OP actions may directly or indirectly modify the functioning of the complex intracellular mechanisms and in this way contribute to the effects of OP poisoning.

Typical of OP poisoning are myopathies (Preusser, 1967; Wecker *et al.*, 1978). Their pathophysiology is complex and is a combination of muscular hyperactivity and consequent alterations in oxidative processes in the muscle fibers. During sustained seizures and fasciculations, the flow of oxygen through the muscle is greatly increased at a time when the use of ATP is greater than the rate of its generation. Under such conditions production of reactive oxygen species (ROS) is greatly increased and exceeds the protective capacity of the cellular defense system so that their damaging effects result in muscle injury (Dettbarn *et al.*, 2001, 2006; Gupta *et al.*, 2002).

Skeletal muscle responds to injury by the activation of a regeneration process (Bischoff, 1979). As described in more detail below, muscle regeneration is a repeated embryonic development of the skeletal muscle only that in this case it starts from the dormant mononucleated satellite cells located between the basal lamina and cell membrane of the adult skeletal muscle fiber. The final outcome after the muscle damage therefore depends not only on the extent of the damage itself but also on the efficiency of the regeneration process that begins in the injured muscle. Especially at high OP intakes, typically found in suicide attempts, poisoning is often complicated by respiratory failure combined with metabolic and respiratory acidosis (Emerson *et al.*, 1999). These patients must be treated in intensive care units and often require mechanical ventilation. Such critically ill patients frequently develop skeletal muscle dysfunction (de Letter *et al.*, 2001; Bolton, 2005), which often persists even after hospital discharge. Insufficient muscle regeneration might be an important contributing factor in such myopathies (Prelovsek *et al.*, 2006) and it is therefore important to know whether OPs in anyway interfere with the mechanisms involved in this process. This aspect of OP poisoning has not been approached yet in human muscle and in this chapter we provide evidence that the mechanisms underlying early stages of human muscle regeneration are indeed affected by OPs.

The most important step in the process of muscle regeneration is myoblast proliferation which decisively determines the mass of regenerated muscle material. Our investigations on the effects of OPs on muscle regeneration were therefore focused on these early precursors of muscle fibers. All experiments were carried out on the *in vitro* model in which the process of human muscle regeneration is genuinely reproduced. We describe various OP influences on the early precursors of muscle development and discuss their potential effects on muscle regeneration. Since AChE is the best established target of OPs we also approached the question of role and expression of this enzyme in the mononuclear myoblasts and studied the effects of OPs on its expression. Diisopropylphosphorofluoridate (DFP) was used throughout our studies.

II. REGENERATION PROCESS IN THE HUMAN SKELETAL MUSCLE

The process of human muscle regeneration is just a repeated process of muscle embryonic development (Figure 45.1A). The earliest myogenic precursors in this development are mononuclear myoblasts. These still monunucleated but

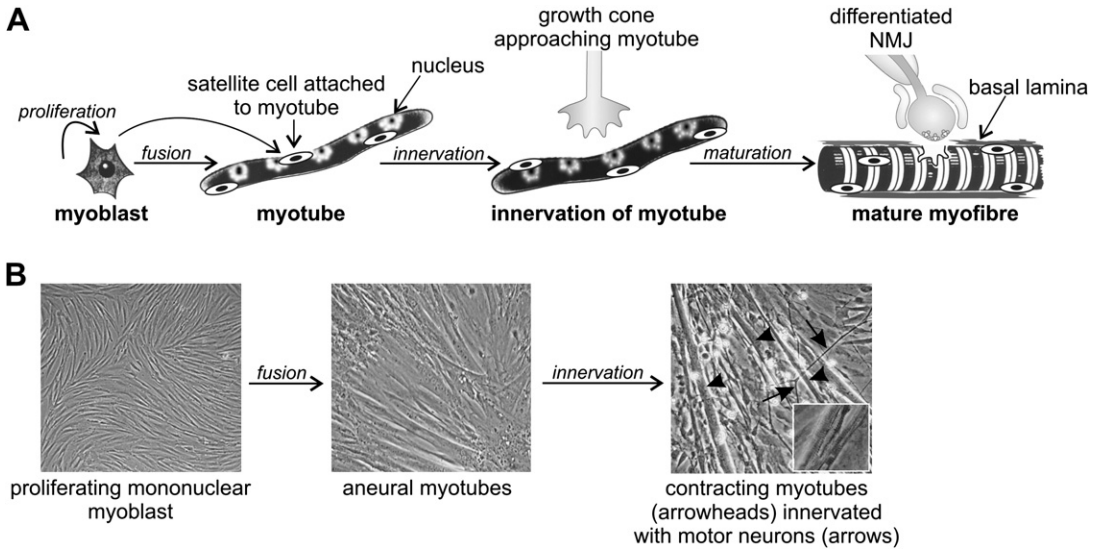

FIGURE 45.1. (A) Schematic presentation of the stages of muscle development and (B) reproduction of these stages under *in vitro* conditions. A: Stages of muscle development from mononuclear myoblast to the mature innervated myofiber. Nuclei, which are still centrally located in myotubes, move to the periphery in the mature fiber as a result of the synthesis of contractile elements in the sarcoplasm. Mature fibers contract and exhibit cross-striations. Only mononuclear myoblasts still have the capacity to divide and proliferate. Some of them do not fuse and become entrapped as mononuclear satellite cells under the basal lamina of the mature fiber. B: Reproduction of this process in the experimental model of the *in vitro* innervated human muscle; axons are labeled by arrows and functionally innervated myotubes by arrowheads. Cross-striations could be seen in the innervated myotubes at higher magnification (insert).

already committed cells proliferate several times in order to reach sufficient density before they fuse into multinucleated myotubes. Once formed, myotubes enter into the long-term and complex process of muscle differentiation which results in the development of fully functional mature myofibers. An important step in the transition from myotube to myofiber is its innervation and formation of the complex structure of the neuromuscular junction (Emerson and Hauschka, 2004).

All these steps are repeated in the regeneration process except that mononuclear myoblasts are derived from the satellite cells. These primordial cells failed to enter the developmental and differentiation process during the embryonic myogenesis but retained the capacity to enter the myogenetic process when activated by the muscle damage. As such they serve as mononuclear muscle precursors of the *de novo* formed muscle fibers during muscle regeneration (Figure 45.1A).

All stages of muscle regeneration can be reproduced in the experimental model of the *in vitro* innervated human muscle (Figure 45.1B). This system was first described in the 1980s (Kobayashi and Askanas, 1985; Askanas *et al.*, 1987) and then further characterized in studies in various laboratories, including ours. In this experimental model satellite cells are released by trypsinization from small pieces of adult human muscle routinely removed at the orthopedic operations of the equinovarus. These cells are the source of mononucleated myoblasts which then proliferate and at certain density start to fuse and form multinucleated myotubes. At that stage an explant of the rat embryonic spinal cord is placed on a monolayer of muscle cells. After 6 to 10 days in co-cultures motor neurons functionally innervate myotubes which then start contracting. These co-cultures are long-lived and contract for up to 6 months. Morphology of the neuromuscular junctions does not differ from that observed *in vivo* (Askanas *et al.*, 1987) and glial cells in the embryonic spinal cord explant also recapitulate temporally regulated developmental steps as reported in the *in vivo* studies (Mars *et al.*, 2001). For the details of the preparation and various applications of this model see also Grubic *et al.* (1995), Mars *et al.*, (2003) and Jevsek *et al.* (2004).

III. THE EFFECTS OF DFP ON THE REGENERATION PROCESS IN THE HUMAN SKELETAL MUSCLE

A. The Effect of DFP on the IL-6 Secretion from the Mononuclear Myoblasts and Myotubes

In our previous study we found that cultured human myoblasts, fusing myoblasts and myotubes constitutively secrete interleukin-6 (IL-6) (Prelovsek *et al.*, 2006). This cytokine is known as the major cytokine released from the skeletal muscle under various conditions (Febbraio and Pedersen, 2005). We also found that IL-6 secretion is stimulated by the major proinflammatory agents like tumor necrosis factor (TNF)-α and endotoxin lipopolysaccharide (LPS). Since IL-6 is a potent stimulator of myoblast

proliferation (Austin *et al.*, 1991; Austin and Burgess, 1992; Cantini *et al.*, 1995; de Letter *et al.*, 2001; Baeza-Raja and Munoz-Canoves, 2004; Serrano *et al.*, 2008) it is assumed that the physiological meaning of this response is a promotion of muscle regeneration so that the myopathy due to septic conditions is at least to some extent compensated by the muscle regeneration.

As mentioned above severe OP poisoning is often complicated by the critical illness which is very often accompanied by the so-called critical illness myopathy. Such a condition is even more likely after OP poisoning which specifically causes myopathy (Dettbarn *et al.*, 2006). It is therefore important to find out whether OP intoxication affects IL-6 signaling and consequently muscle regeneration. The effects of OP on the IL-6 secretion from human myoblasts can be expected since it has already been reported that OPs drastically interfere with cytokine signaling in mouse immune system (Alluwaimi and Hussein, 2007).

Here we show that IL-6 secretion from the human myoblasts is indeed significantly inhibited by DFP (Figure 45.2). The decrease in IL-6 secretion of about 50% in myoblasts might be important in reducing the efficiency of the muscle regeneration process in the damaged muscle. The mechanism underlying this effect of DFP remains to be established and is probably complex. In our culture medium DFP is hydrolyzed quickly and becomes practically inactive in less than an hour (Worek *et al.*, 2004). Its effects are therefore not continuous; they are probably induced shortly after DFP addition and then last beyond the period during which DFP is still active.

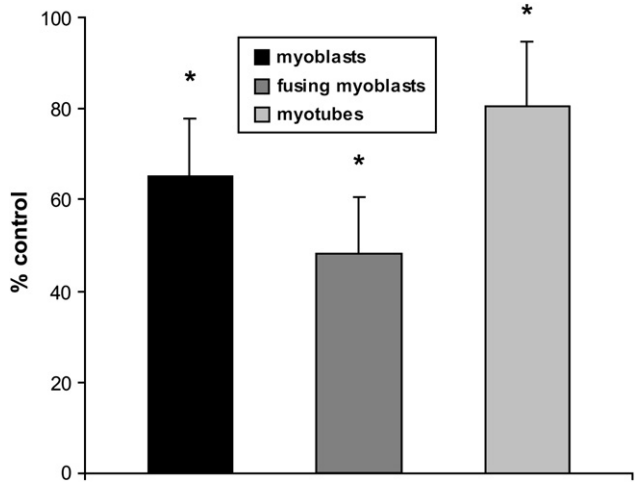

FIGURE 45.2. IL-6 secretion from myoblasts, fusing myoblasts, and myotubes. Cultured human myoblasts, fusing myoblasts, and myotubes were added DFP at the 10^{-5} M concentration. The secreted IL-6, calculated per 100,000 nuclei, was determined 24 h later with ELISA kit (Endogen, Rockford, IL, USA). *Significant difference (Student *t*-test, $p < 0.05$; $n = 3$) between control and treated cultures was observed at all stages studied but was most prominent in the myoblasts.

B. Heat-Shock Proteins in the Human Myoblasts and Myotubes after Treatment with DFP

One of the most prominent cellular responses to stress is a rapid change in gene expression to yield a family of highly conserved proteins known as heat shock proteins (HSPs) (Welch, 1992; Morimoto, 1993; Kiang and Tsokos, 1998; Kregel, 2002). They are cytoprotective due to their chaperone functions in protein folding and protein degradation. Various physical, chemical, biological and environmental stress factors including xenobiotics can induce this response (Wu and Tanguay, 2006). As it was reported that OP pesticides are among inductors of HSP response *in vivo* and *in vitro* (Bagchi *et al.*, 1996) we tested whether DFP can induce such cytoprotection in the precursors of muscle regeneration. We studied the levels of two HSPs: HSP 27 and HSP 70, which are typically induced by stress factors. The level of stress factor of HSP 27 but not of HSP 70 was slightly but significantly increased in the DFP-treated human myoblasts (Figure 45.3), which is in accord with the reported selectivity of HSP induction in various tissues (Wu and Tanguay, 2006). The HSP response to DFP becomes more prominent in the later, myotube stages, when HSP 70 also becomes significantly increased (Figure 45.3). This pattern of effects is just the opposite of that observed for the decrease in IL-6 secretion (Figure 45.2) suggesting that these two effects are not related. From the standpoint of muscle regeneration it remains to be investigated whether the observed HSP response to DFP in any way protects proliferative myoblast potential which is hampered by DFP-induced reduced IL-6 secretion. These results are again indicative for the wide spectrum of effects that OPs induce by direct action on muscle tissue.

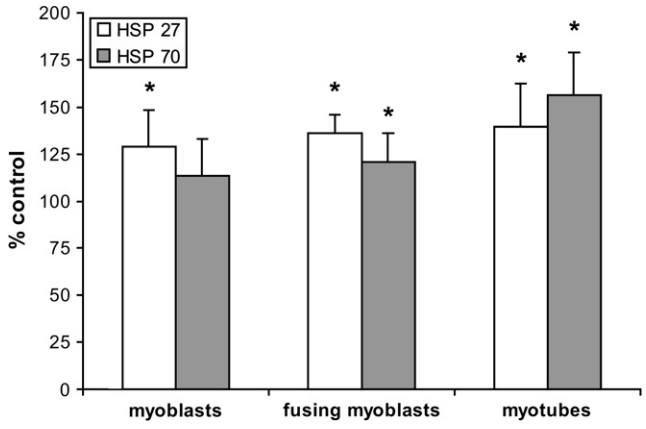

FIGURE 45.3. The effects of DFP on the HSP 27 and HSP 70 levels in myoblast, myoblasts in fusion, and myotube cultures. Levels of HSP 27 and 70 were determined 24 h after addition of DFP at the 10^{-5} M concentration. They were quantitated by Western blot with Chemi Genius BioImaging System (Syngen, Cambridge, UK). *Significant difference (Student *t*-test, $p < 0.05$; $n = 4$) between control and DFP-treated cultures was observed in all determinations except for HSP 70 in myoblasts.

C. Response of Human Myoblasts to Hypoxia

Severe OP poisoning can result in respiratory failure with arterial oxygen partial pressures below 50–60 mm Hg (6.65–7.99 kPa) (Tsao *et al.*, 1990). In such circumstances peripheral tissues, including skeletal muscle, become extremely hypoxic which might have an important effect on the cellular precursors of human muscle regeneration. As known from the extensive studies in other systems, central to the cellular response to hypoxia is hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor, consisting of an oxygen-regulated α-subunit (HIF-1α) and an oxygen-independent β-subunit (HIF-1β). HIF-1α, which is normally almost undetectable due to its continuous degradation in the ubiquitin–proteosome pathway, rapidly accumulates in hypoxia and translocates to the nucleus where it dimerizes with HIF-1β to form a functional transcription factor that controls expression of hundreds of genes (Manalo *et al.*, 2005; Semenza, 2007a). Through its effects on gene expression HIF-1 not only increases oxygen delivery to hypoxic tissues by stimulating erythropoiesis and angiogenesis but also promotes cell survival by redirecting cellular energy metabolism towards glycolysis (Pasteur effect) (Seagroves *et al.*, 2001; Semenza, 2007b). Due to its central role in response to hypoxia, we tested whether such adaptation also takes place in human myoblasts *in vitro*. We exposed human myoblasts to acute hypoxia (1% O_2, 4 h) and found a 6.2-fold increase in HIF-1α level compared to normoxic control (Figure 45.4). This means that human myoblasts respond to hypoxia in a similar fashion to other cell types. Investigations are under way in our laboratory with the aim of finding out whether myoblast response by increased HIF-1α leads to modifications in IL-6 secretion and in this way also in muscle regeneration.

D. AChE Expression in the Normal and DFP-Treated Myoblasts, Myotubes, and Innervated Myotubes

Unlike most of the contractile and synaptic muscle proteins which could first be detected in muscle after fusion, AChE is expressed already at the myoblast stage (Tennyson *et al.*, 1971; Grubic *et al.*, 1995). Its role at this earliest stage of muscle ontogenesis is not known. Since no other component of the cholinergic system is present in myoblasts, only some noncholinergic or noncatalytic AChE role(s) (Meshorer and Soreq, 2006) can be considered. We approached this question by selective elimination of AChE expression in cultured human myoblasts by siRNA. Although the AChE level (determined on the basis of catalytic activity) fell to about 50% of control after siRNA treatment (Figure 45.5), we could not detect any functional or morphological changes in myoblast cultures in comparison to control. The addition of DFP to the siRNA-treated myoblasts, which practically blocked all AChE catalytic activity, also resulted in no visible changes in myoblast cultures. Obviously, binding of DFP to AChE does not change or modify the function (if any) of AChE in the human myoblasts.

Since *de novo* AChE synthesis is an important contributor to the recovery of this enzyme in the skeletal muscle after OP poisoning (Grubic *et al.*, 1981) we wanted a more detailed insight into this process in our experimental model of human muscle. We followed and compared the levels of AChE mRNA and AChE activity in the siRNA-treated myoblasts and in this way estimated the temporal relationship between AChE expression at the mRNA and mature protein level. We then followed AChE mRNA expression at the various developmental stages of muscle ontogenesis. We also tested whether, due to diverse DFP effects, AChE mRNA levels are in any way influenced by DFP treatment at these stages. Since AChE is polymorphic and alternative splicing of primary transcript gives rise to three AChE mRNA species: tailed (T), hydrophobic (H), and read-through (R), which encode differently targeted AChE catalytic subunits (Massoulie, 2002), we followed these mRNA species separately in these experiments.

Three stages could be distinguished in a 6-day period in which we followed AChE activity/AChE mRNA ratio in the siRNA-treated myoblasts. In the first 10 h after siRNA treatment AChE mRNA already fell to about 60% of control level, while AChE activity remained practically unchanged. In the second stage (hours 10 to 50) AChE mRNA fell to

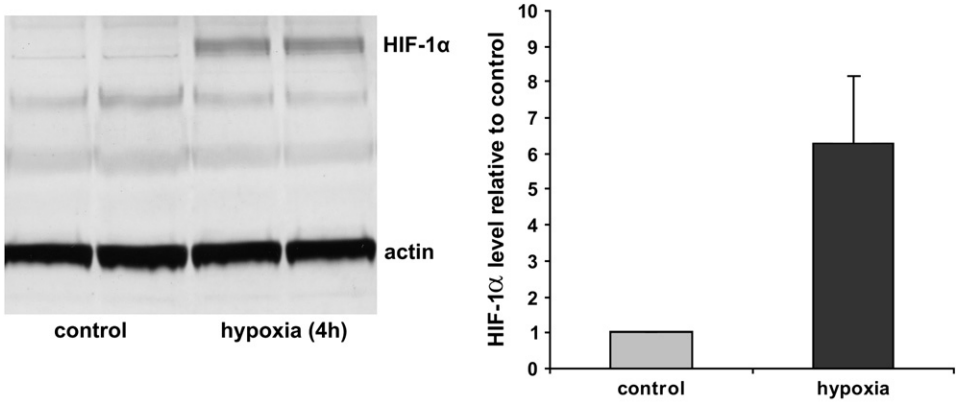

FIGURE 45.4. The effect of acute hypoxia on HIF-1α expression in human myoblasts. Human myoblasts were exposed to 1% O_2 for 4 h. A representative Western blot is shown on the left. Relative expression level of HIF-1α (three independent experiments) is shown on the right (arbitrary units, control = 1) (Student *t*-test, $p = 0.004$). Quantification was performed with Chemi Genius Bio-Imaging System (Syngen, Cambridge, UK).

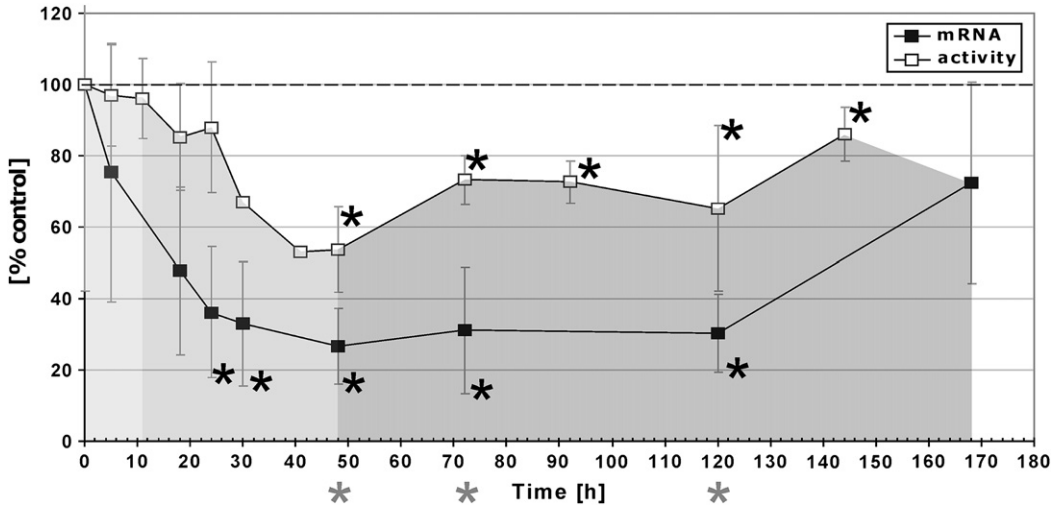

FIGURE 45.5. Relative changes of AChE-T mRNA and AChE activity in lysates of human myoblasts during the first week following siRNA application. Cultures of control and treated myoblasts were prepared from the satellite cells of the same donor and were processed in parallel. Gene silencing was achieved by lipofection of siRNA (Dharmacon); for details see Mis *et al.* (2006). Q-PCR with TaqMan chemistry was employed. AChE mRNA levels were standardized to GAPDH mRNA. AChE activity was expressed per 10,000 cells the number of which was determined with the help of Hoechst 33258 nuclear staining. Each point represents mean of three to seven measurements in each of which we determined the AChE-T mRNA or AChE activity ratio between control and siRNA-treated levels (% control). The statistically significant (Student *t*-test, $p < 0.05$) differences relative to the starting point are marked by black *. The statistically significant (Student *t*-test, $p < 0.05$) % differences between AChE activity levels determined by Ellman technique and mRNA levels at the same time point are marked by gray *. Three shades of gray correspond to the three stages discussed in the text.

about 30% of control. AChE activity followed this fall and reached its lowest level of about 50% of control 50 h after siRNA treatment reflecting AChE turnover. From hour 50 onward a new, more or less constant relationship between AChE and its mRNA was established.

Of the three mRNA species, AChE-H and AChE-T mRNA followed practically the same pattern of expression after siRNA treatment, while AChE-R mRNA behaved in a completely different and less reproducible manner (Figure 45.6). In comparison to the other two mRNA

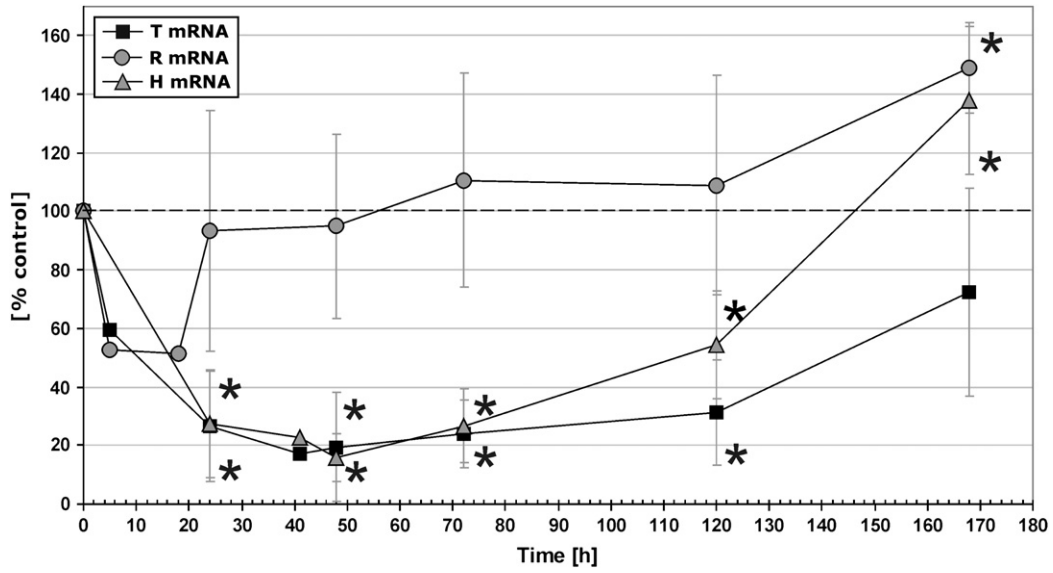

FIGURE 45.6. Relative changes of AChE-H, -R and -T mRNAs in human myoblasts during the first week following siRNA application. Q-PCR with TaqMan chemistry was employed (standardized to GAPDH mRNA). Each point represents the mean of five separate siRNA experiments. In absolute terms the levels of AChE-T mRNA were about 50 times higher than the levels of H and R (see Figure 45.7). The differences regarding starting point (*) were statistically significant (Student *t*-test, $p < 0.05$).

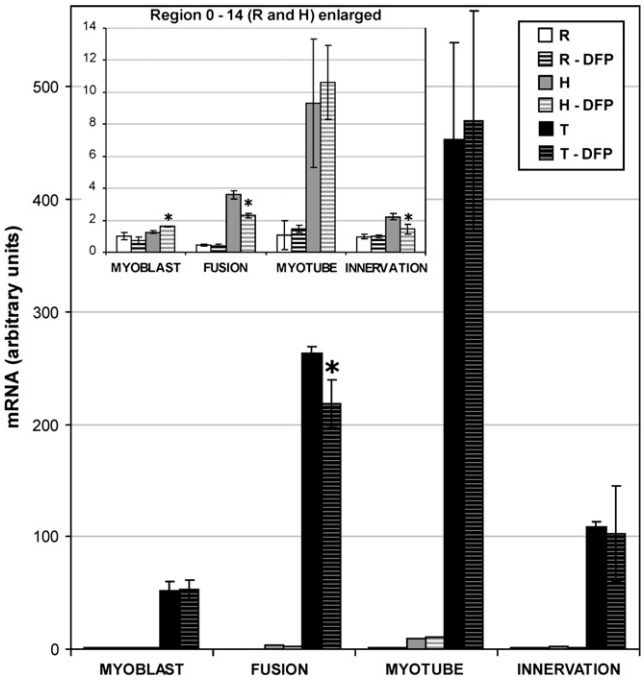

FIGURE 45.7. The levels of R, H, and T AChE mRNA species at four differentiation stages of cultured human muscle in the control and DFP-treated cultures. AChE mRNA levels were measured 24 h after addition of 10^{-5} M DFP by Q-PCR with TaqMan chemistry and GAPDH mRNA as a standard. Top left: Enlarged 0–14 region of the *y* axis showing R and H AChE mRNA species. Each bar represents mean of three measurements. *Significant difference (Student *t*-test, $p < 0.05$) between control and DFP-treated cultures.

species, which fell to about 20% of control after siRNA treatment, AChE-R mRNA never fell below 50% of control. It quickly recovered and then increased so that 7 days after siRNA treatment its level was almost 1.5 times higher than in control. On the 7th day but not earlier we observed similar overshoot also for AChE-H but never for AChE-T. At present we have no explanation for the different expression pattern of R-mRNA species in comparison to the other two. Increased AChE-R mRNA levels have been reported in mouse brain after exposure to stress (Nijholt *et al.*, 2004) but under *in vivo* conditions external factors not present in our culture could be responsible for changed expression of this mRNA. There have been several reports from the H. Soreq group in which the AChE-R variant was specifically induced by various stressors (Cohen *et al.*, 2003; Grisaru *et al.*, 2006; Ofek *et al.*, 2007; Evron *et al.*, 2007; Shapira-Lichter *et al.*, 2008) but we do not know whether our results are related to these findings. In any case R-species represent only a very small fraction of AChE mRNA (Figure 45.7) and it is not known whether it is translated.

All three AChE mRNA species could be detected at all developmental stages. Patterns of their expression were developmental stage dependent, but at all stages T-species was highly predominant (Figure 45.7). No significant changes in the levels of any of the three AChE mRNA species could be observed at any of the developmental stages of human muscle after treatment with DFP (Figure 45.7), suggesting that OPs have no direct influence on AChE expression in human muscle during the regeneration process.

IV. CONCLUDING REMARKS AND FUTURE DIRECTION

It has been known for some time that myopathies are one of the typical consequences of OP poisoning (Dettbarn *et al.*, 2001, 2006) which might be additionally complicated by reduced efficiency of muscle regeneration. According to the present studies various intracellular mechanisms are involved in the response to DFP in the precursors of muscle regeneration. The findings revealed significantly reduced IL-6 secretion, increased levels of HSPs and also increased levels of HIF-1α as a response to secondary hypoxia. Future research will be devoted to the better understanding of molecular mechanisms, which will be reflected in the observed changes and their relationships with OP poisoning.

Acknowledgments

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CHAPTER 46

Organophosphate Intoxication: Molecular Consequences, Mechanisms and Solutions

BRIAN C. GEYER, TAMA EVRON, HERMONA SOREQ, AND TSAFRIR S. MOR

I. INTRODUCTION

Our understanding of the essential role of acetylcholinesterase (AChE) in the neuromuscular junction (NMJ) predicts severe consequences following loss of function by its irreversible inhibition (Silman and Sussman, 2005; Soreq and Seidman, 2001). Through the use and misuse of nerve agents, pesticides, and therapeutic anticholinesterases we learned about the acute and life-threatening manifestations of organophosphate (OP) toxicity observed at all levels, from dysregulated molecular interactions to severe and potentially fatal clinical presentations. The immediate negative physiological consequences associated with critical deficiency in acetylcholine (ACh) hydrolysis in the NMJ and central nervous system are readily explained by the well-characterized role of AChE in cholinergic signal termination. Yet loss of homeostasis can likewise be a result of overcompensation and overproduction of AChE associated with many disease states and indeed with long-term outcomes of sublethal OP exposure. Supporting this notion are the delineated manifestations with span motor, inflammatory, and cognitive effects (Meshorer and Soreq, 2006). Importantly, biological monitoring of cholinergic homeostasis depends on appropriate substrate release, ACh receptor function, AChE isoform selection, and localization.

II. HUMAN CHOLINESTERASES: THE COMMON CORE

The vital role of the acetylcholine-hydrolyzing enzyme AChE in terminating cholinergic neurotransmission has been recognized for almost as many years as ACh has been recognized as a neurotransmitter (Dale, 1914a, b). For nearly as long, AChE research was intimately linked to the study of its inhibitors [Loewi *et al.*, 1926, cited in Loewi's Nobel lecture (http://nobelprize.org/nobel_prizes/medicine/laureates/1936/loewi-lecture.html) and by Dale, 1962]. The vulnerability of cholinesterases to certain natural compounds promoted the discovery and synthesis of new inhibitors for use as pesticides, therapeutics, and, unfortunately, as chemical warfare agents (Gupta, 2006). These inhibitors helped to elucidate the enzymatic mechanism of action and this knowledge, in turn, was used to design even more potent or more specific anticholinesterases.

Seven decades of research led to a widely accepted view of cholinesterases as ''a family of enzymes that hydrolyze choline esters'' (Small *et al.*, 1996). Uncertainty existed, perhaps, only for the mammalian AChE paralog butyrylcholinesterase (BChE): ''the function of [BChE] is unknown, while the function of AChE is to terminate nerve impulse transmission at cholinergic synapses'' (McTiernan *et al.*, 1987). By the early 1990s, this functional definition of AChE and BChE was well complemented by a molecular one of serine hydrolases belonging to the α/β fold superfamily. This resulted from cloning of cholinesterase genes from invertebrates (e.g. Hall and Spierer, 1986) and vertebrates including humans (e.g. McTiernan *et al.*, 1987; Prody *et al.*, 1987; Schumacher *et al.*, 1986; Soreq *et al.*, 1990) and the consequent elucidation of the three-dimensional structure of cholinesterases (Sussman *et al.*, 1991).

The three-dimensional crystal structure of human synaptic AChE (Kryger *et al.*, 2000) reveals a globular core, penetrated by a narrow groove (the ''gorge'') at the bottom of which lies the active site. Several functional subsites in the active site gorge were identified (numbering according to the human AChE sequence – see Soreq *et al.*, 1990). These include (a) the ''catalytic triad'', Ser-203, Glu-334 and His-447, which transiently binds the acyl moiety of the substrate, (b) the ''acyl pocket'', and (c) the ''hydrophobic subsite'', which accommodates the alcohol moiety of the tetrahedral transition state. Another important component is (d) the ''oxyanion hole'', which stabilizes the transition state by accommodation of the negatively charged carbonyl oxygen. In addition to the invariant catalytic triad, there exists (e) a secondary substrate-binding site, referred to as the ''peripheral anionic site'' (PAS). This site is involved in both modifying catalytic activity (substrate inhibition) and mediating many inhibitor interactions of AChE. In addition, this site is involved in some nonhydrolytic functions of AChE (Inestrosa *et al.*, 1996). At the enzymatic level, AChE has

been characterized by its substrate specificity, which is essentially limited to ACh (Radic *et al.*, 1997), and its mode of interaction with selective inhibitors (Ariel *et al.*, 1998). The broader catalytic spectrum of BChE and its preference for longer chain substrates (Kaplan *et al.*, 2001; Loewenstein *et al.*, 1993) can be explained by its acyl binding pocket that is significantly bigger than that of AChE (Ngamelue *et al.*, 2007; Nicolet *et al.*, 2003).

Cholinesterases are heavily glycosylated. The three glycosylation sites on human AChE are all occupied (Kronman *et al.*, 2000) and at least eight of the nine potential sites on human BChE are occupied as well (Kolarich *et al.*, 2008). Glycosylation is variable and depends on (among other factors) cell type (Moral-Naranjo *et al.*, 1997; Saxena *et al.*, 1997b) and physiological state (e.g. Saez-Valero *et al.*, 2003; Silveyra *et al.*, 2006). Additional, isoform-dependent, post-translational modifications include oligomerization, proteolytic processing, and (for one of the C-terminal variants) attachment to a glycosylphosphatidylinositol (GPI) anchor.

The ''classic'' view of choinesterases as ~70 kDa cholinolytic glycoproteins whose sole function is to terminate cholinergic signals at the synapse has been repeatedly challenged by observations that were incongruent with it and suggested a far more interestingly complex picture of multiple molecular forms with distinct temporal and spatial expression patterns. Moreover, the various protein products of the *ACHE* locus (and BChE) play surprisingly diverse cholinergic and noncholinergic functions in health and disease (Appleyard, 1992; Greenfield, 1984, 1996; Layer, 1996; Silman and Sussman, 2005; Small *et al.*, 1996; Soreq and Seidman, 2001).

III. HUMAN CHOLINESTERASES: MYRIAD OF MOLECULAR FORMS

Humans possess two cholinesterase genes at two separate loci: *BCHE* (3q26, Allderdice *et al.*, 1991) and *ACHE* (7q22, Ehrlich *et al.*, 1992), respectively, encoding BChE and the plethora of AChE protein isoforms. The latter are translated from several mRNA variants arising, as described below, through highly regulated transcriptional and post-transcriptional processes.

Alternative splicing at the 3′-end of the *ACHE* pre-mRNA yields three different mRNA transcripts (Figure 46.1), which encode proteins with distinct carboxyl termini (Soreq and Seidman, 2001). The synaptic form, AChE-S (also known as T for ''tailed''), is generated by splicing of exon 4 to exon 6. This is the dominant transcript in most tissues. AChE-S mRNA translation gives rise to a 40 amino acid C-terminal extension of the core AChE. This C-terminal peptide contains cysteine, which allows the formation of dimers and tetramers. These are able to further bind a cholinesterase-specific collagen tail (ColQ) unique to AChE in the NMJ (Massoulie, 2002) or a proline-rich membrane anchor (PRiMA) in the central nervous system (Perrier *et al.*, 2002). Within the NMJ, interactions between the ColQ tails of two or three different tetrameric units and the two structural subunits generate an ''asymmetric'' AChE form and anchor it to the synapse (Massoulie, 2002).

A second transcript, AChE-E (also known as H for ''hydrophobic''), includes exon 5. Dimerization of AChE-E occurs through a cysteine residue in position 8 of the 43 residue-long C-terminal domain, which is cleaved after amino acid 14 (557 from the N-terminus). The newly

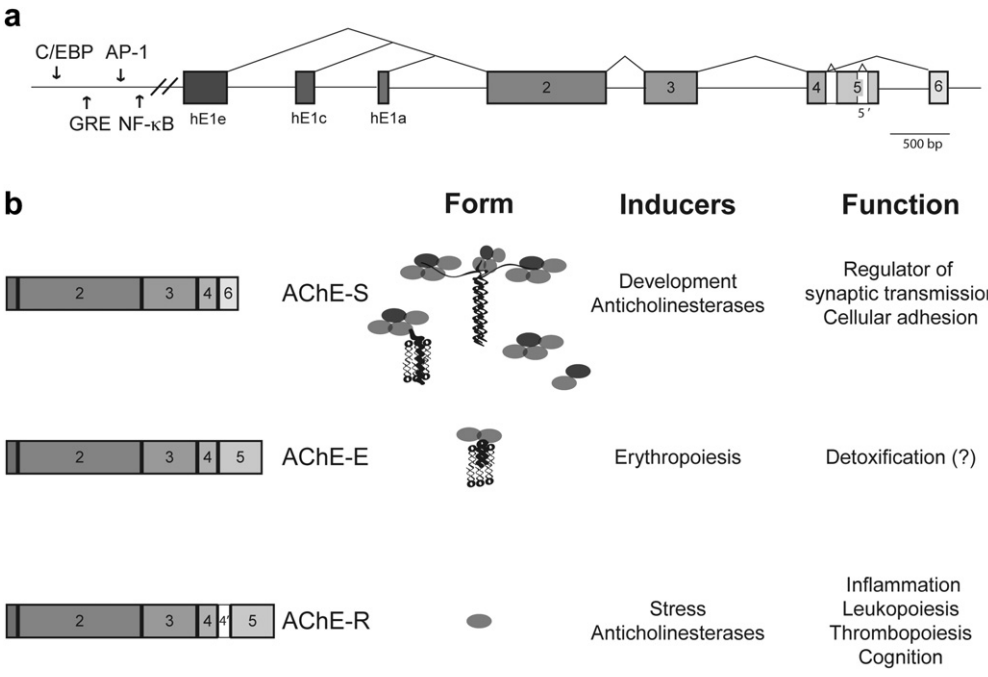

FIGURE 46.1. Regulation at the human AChE locus. (a) The human AChE gene contains several putative upstream enhancers, including the inflammation-associated C/EBP, AP-1 and NF-κB, and the stress-associated glucocorticoid response element (GRE). (b) Diverse cellular mechanisms regulate alternative splicing, yielding three C-terminal isoforms with diverse physiological activities.

generated C-terminus is GPI-anchored to the erythrocytic membrane.

A third form, the ''readthrough'' AChE-R, is encoded by a transcript containing pseudointron 4. This variant accumulates under various stressful insults through a feedback response that activates AChE mRNA transcription and shifts splicing in brain (Kaufer *et al.*, 1998; Meshorer *et al.*, 2002; Meshorer and Soreq, 2006), muscle (Lev-Lehman *et al.*, 2000), spermatocytes (Mor *et al.*, 2001a, 2008), hematopoietic cells (Gilboa-Geffen *et al.*, 2007; Grisaru *et al.*, 2006), and intestine (Shapira *et al.*, 2000). AChE-R has a shorter C-terminal peptide: 26 and 30 amino acids long in human and mouse, respectively. This C-terminus lacks a cysteine residue and hence AChE-R remains monomeric and soluble (Soreq and Seidman, 2001). Although the protein products of these mRNAs differ in their C-terminal peptide sequence and capacity to oligomerize, all of the molecular forms of AChE carry an identical active site and display similar catalytic properties (Schwarz *et al.*, 1995). Importantly, the distinct C-termini dictate dissimilar biological roles and noncatalytic properties.

The 5′-end of the AChE pre-mRNA is subject to alternate promoter usage, together yielding five and three alternative transcripts in mice and humans, respectively (Figure 46.1; Meshorer *et al.*, 2004). The alternative 5′- and 3′-end significantly increase the combinatorial complexity of AChE. Most of the identified 5′ exons produce alternative untranslated regions. However, at least one of those exons in humans is translated to a protein, with an extended N-terminus (N-AChE; Meshorer *et al.*, 2004). Based on the combinations of the 3′ and 5′ alternative transcripts, N-AChE-E, N-AChE-R, N-AChE-S might all exist (Meshorer and Soreq, 2006). Since N-AChE was shown to associate with the plasma membrane of blood cells (Meshorer *et al.*, 2004), it might have the capacity to dock the soluble AChE-R to synaptic membranes, without ColQ or PRiMA.

IV. REGULATION OF AChE GENE EXPRESSION

Regulation of AChE is primarily at the transcriptional and post-transcriptional levels (Soreq and Seidman, 2001). Transcriptional control of the *ACHE* gene is principally regulated by two proximal promoters, one that is clearly dominant and the other an alternative promoter (Atanasova *et al.*, 1999; Li *et al.*, 1993). Additionally, enhancers are present distally and within the first intron (Camp *et al.*, 2008; Chan *et al.*, 1999; Shapira *et al.*, 2000) as well as proximally to the four alternative first exons (Meshorer and Soreq, 2006). The upstream region of the *ACHE* locus contains numerous putative transcription-factor binding sites, including those which potentially bind NF-κB, AP-1 as well as a glucocorticoid response element (GRE) binding site. An increase in *ACHE* transcription is noted as a consequence of various stimuli as diverse as differentiation (Rotundo, 1990), vitamin D exposure (Grisaru *et al.*, 1999a), anticholinesterase exposure (Darreh-Shori *et al.*, 2004; Kaufer *et al.*, 1998), and transiently reduced levels of AChE (Galyam *et al.*, 2001). Decreases in AChE transcription are seen in mice deficient in the neural growth factor TrkA (Schober *et al.*, 1997). Additionally, the effects of AChE overproduction can be attenuated by activity dependent, secondary feedback responses in the nervous system (Soreq and Seidman, 2001). This can be seen in AChE overexpressing transgenic mice where increased hydrolysis of ACh is at least partially counterbalanced by a parallel increase in ACh synthesis (Erb *et al.*, 2001).

In vertebrate skeletal muscle, the collagen-tailed AChE-S variant is primarily detectable under normal physiological conditions (Li *et al.*, 1991, 1993). Muscle-specific expression of AChE is believed to be controlled by the transcription factors MyoD and myocyte enhancer factor-2 acting on the regulatory region located within the first intron (Camp *et al.*, 2008). This region is exceptionally well conserved between higher vertebrates, suggesting a generalization of this phenomenon. Negative regulation of AChE expression has been shown via a calcitonin-related gene polypeptide (CGRP)-dependent mechanism. CGRP is secreted from motor neurons and binds to the calcitonin receptor located on the skeletal muscle membrane (Rossi *et al.*, 2003). Receptor activation results in increased intracellular cAMP, and subsequent attenuation of AChE transcription through a cAMP-response element in the AChE promoter. It has more recently been shown that CGRP also reduces synthesis of the proline-rich membrane anchor region (PRiMA) of skeletal muscle AChE, and that overexpression of PRiMA itself can drive AChE transcription (Xie *et al.*, 2007).

V. LOCATION AND FUNCTION OF AChE IN THE NEUROMUSCULAR JUNCTION

AChE is found in the neuromuscular junction (NMJ) anchored to the cell membrane and is spatially related to the nicotinic ACh receptor (nAChR; Jasmin *et al.*, 1993). This specific localization is conferred by an N-box motif located within a 500nt region of the first intron (Chan *et al.*, 1999). Within the muscle, axons branch to innervate multiple muscle fibers, each fiber receiving a single synaptic input. The post-synaptic membrane of the skeletal muscle forms deep invaginations which are called junctional folds. nAChR molecules are concentrated in the peaks of the folds ($\sim$104/μm^2), close to the neuronal pre-synaptic active zone enriched in ACh-releasing vesicles (Sanes, 1997). nAChRs belong to the superfamily of ligand-gated ion channels. Each AChR molecule is a pentamer composed of four different subunits with the stoichiometry of $\alpha 2\beta\delta\gamma$ (in the adult) or $\alpha 2\beta\delta\varepsilon$ (in embryos). The synaptic cleft is filled by

the basal lamina of the NMJ, highly enriched in AChE (~2,500 sites/μm^2; Anglister, 1991; Anglister *et al.*, 1998; Massoulie *et al.*, 1993). Depolarization of the nerve terminal by nerve impulse is followed by an influx of calcium ions into the terminal via voltage-gated calcium channels. This influx results in exocytosis of many synaptic vesicles and the release of a corresponding number of ACh quanta into the synaptic space. Binding of two ACh molecules to the nAChR multisubunit complex opens the AChR channel, allowing the inward flux of cations along their electrochemical gradient and producing endplate potential (EPP). If this depolarization exceeds the threshold required to open the voltage-gated sodium channels at the depths of the folds, an action potential is generated, leading to muscle contraction. The evoked responses that can be recorded from the surface of the muscle following nerve stimulation are called a compound muscle action potential (CMAP). The CMAP represents the cumulative action potentials of all muscle fibers activated by nerve stimulation. Hydrolysis of ACh by AChE prevents free diffusion of the transmitter within the synapse and additional binding events. Part of the resulting choline pool is recycled with the aid of a high affinity transporter. These molecules enter the pre-synaptic nerve terminal and are being used as substrate by choline acetyltransferase (ChAT) to generate ACh.

VI. BUTYRYLCHOLINESTERASE AS THE INHERENT PROTECTOR OF AChE

When examining OP effects on the NMJ, one must first consider the various layers of inherent protection afforded by other OP-scavenging molecules. Like AChE, BChE can hydrolyze ACh, albeit with considerably slower kinetics. BChE binds most anticholinesterases that threaten synaptic AChE, making it a natural scavenger of OP nerve agents and pesticides (Ashani, 2000). The natural role of BChE is believed to be detoxification due to its ability to hydrolyze many naturally occurring and synthetic compounds such as succinylcholine, cocaine, and aspirin (Masson *et al.*, 1998). Providing an evolutionary justification, many plant alkaloids can inhibit both AChE and BChE (Fletcher *et al.*, 2004; Mukherjee *et al.*, 2007). Interestingly, increases in serum BChE are seen in such hypertriglyceridemic conditions as diabetes and obesity, and insulin or bacterial endotoxin are both sufficient to induce BChE production (Ofek *et al.*, 2007; Randell *et al.*, 2005). While BChE is a promiscuous enzyme with multiple functions, those subjects carrying mutations rendering BChE inactive possess a normal phenotype in the absence of anticholinesterase or succinylcholine challenge (Chatonnet and Lockridge, 1989). Additionally, BChE is highly polymorphic, further underscoring the dispensable nature of this protein in humans (Primo-Parmo *et al.*, 1996). Parallel roles can be ascribed to erythrocytic AChE-E (Salmon *et al.*, 1999; Simone *et al.*, 1994).

VII. ACUTE TOXICITY PRODUCES SHORT-TERM LOSS OF FUNCTION

A. Introduction

Anticholinesterases can be commonly encountered as industrial pesticides, weapons of war, Alzheimer's medications as well as the natural toxins of many organisms including fungi, plants, and animals. Exposure to clinically relevant doses of anticholinesterases results in immediate and multisystem physiological disturbances that underscore the broad anatomical distribution of the mammalian cholinergic system. The body adapts to the insult and attempts to compensate for the cholinergic dysregulation by inhibitor–enzyme interactions, NMJ remodeling, and changes in circulating cytokine profiles. This is the acute phase of anticholinesterase intoxication, encompassing the vast majority of the work in this field, which has resulted in a nearly complete and predictable clinical picture. One area of recent research intensity involves the delineation of a mechanism for immunosuppression in the context of excessive cholinergic stimulation (Nizri *et al.*, 2006; Oke and Tracey, 2008; Pavlov *et al.*, 2003; Van Westerloo *et al.*, 2005). We now possess a framework for understanding the immunomodulation that has been periodically reported in the literature and which enhances our understanding of cholinergic biology and suggests novel therapeutic interventions that may yield positive outcomes in the clinic.

B. Gross Clinical Presentation

Unregulated overstimulation of nicotinic and muscarinic ACh receptors through inhibition of AChE and subsequent ACh accumulation initiates a variety of detrimental processes that can culminate in severe consequences for the exposed patient (Brown and Brix, 1998; Marrs, 1993; Weinbroum, 2005; Yanagisawa *et al.*, 2006). These range, acutely, from excessive glandular secretions and headache to respiratory depression and death. Chronic manifestations in patients include NMJ dysmorphology, inflammation, and behavioral and cognitive deficits. Clinically, cholinergic dysregulation is most often found secondary to accidental or intentional ingestion of a variety of agents from anticholinesterase pesticides, nerve agents, and Alzheimer's therapeutics to cholinergic agonists like nicotine. The resulting pathology is referred to as a ''cholinergic crisis''. Diagnosis of anticholinesterase toxicity is made by patient history and clinical presentation, with confirmation by measurement of the degree of inhibition of erythrocyte AChE activity (Nozaki *et al.*, 1997).

Existing treatment protocols emphasize three complementary approaches: antagonize the muscarinic ACh receptor, reactivate endogenous AChE with oxime therapy, and manage the severe toxicity symptomatically (Lee, 2003). Atropine is the drug of choice for mitigating the synaptic ACh accumulation, and is titrated until the patient

is breathing comfortably. Airway management may require endotracheal intubation; however, succinylcholine is contraindicated as it is metabolized by BChE (Li *et al.*, 2008). Diazepam is used to manage the seizures that are seen with anticholinesterase toxicity. While these approaches have proven value in reducing mortality, they are unable to prevent the debilitating acute morbidity or long-term consequences that are associated with anticholinesterase poisoning (Huang *et al.*, 2007).

In 1877 (Taylor, 1996), prior to the discovery of ACh as a neurotransmitter in the brain in 1914 (Dale, 1914a, b), physostigmine (eserine) – a carbamate extracted from the seeds of *Physostigma venenosum* – became the first cholinesterase inhibitor used therapeutically to control increased ocular pressure, caused by wide angle glaucoma. In 1993, tacrine became the first ChE inhibitor approved in the USA for the treatment of Alzheimer's disease (Giacobini, 1998). More recently, second generation anti-ChEs such as rivastigmine, donepezil, and eptastigmine have shown higher efficacy, lower toxicity, and easier administration (Ringman and Cummings, 2006). In the Far East, plant-originated anti-ChEs such as Huperzine have been used for thousands of years in the treatment of aging-induced memory impairments (Haviv *et al.*, 2007).

In the peripheral nervous system, anti-ChEs elevate ACh levels at the NMJ, ameliorating the damaged cholinergic balance and transmission. Neostigmine and physostigmine were also the first effective treatments for myasthenia gravis (MG). Since physostigmine is capable of crossing the blood–brain barrier (BBB), neostigmine was the preferred theraputic. Pyridostigmine bromide (Mestinon™) became available in the 1950s as a longer-lasing agent, active for 3–6 h with fewer side effects and the ease of oral administration. Longer-lasting agents were not used since they accumulated to overdose levels. A similar problem was reported when OP inhibitors were tested. Importantly, anti-ChE therapy can cause a variety of side effects, including gastrointestinal problems, salivation, sweating, and cardiac and hypotension effects (Fisher, 2000).

C. Molecular Mechanisms of Cholinesterase Inhibition

The therapeutic/toxic action of AChE inhibitors is targeted at the well-known hydrolytic activity of AChE. Such inhibitors can bind to the catalytic site at the bottom of the gorge (Sussman *et al.*, 1991), the peripheral site (Silman and Sussman, 2005), or both. AChE inhibition leads to the accumulation of free ACh, which further leads to uncontrolled cholinergic neurotransmission. Since the cholinergic balance depends on well-regulated release and hydrolysis of ACh, excessive inhibition of AChE may lead to overstimulation and damaged cholinergic neurotransmission (Soreq and Seidman, 2001), which in high doses may be fatal.

Different types of AChE inhibitors exist. The carbamate group of anti-AChEs includes physostigmine (which can penetrate the BBB) and pyridostigmine (unable to cross the BBB under normal conditions); both harbor a reactive carbamate group. Similar to ACh, they undergo acylation (carbamylation) to form a carbamyl–enzyme intermediate, but the subsequent hydrolysis rate of this hemi-substrate is very slow, occupying the enzyme carbamylated for a considerably long time and competing with the natural substrate (Conner *et al.*, 1996). Carbamylation of AChE with this type of carbamate is often termed irreversible inhibition, but since some carbamylated ChEs can undergo rapid reactivation, carbamates have been incorrectly considered as reversible inhibitors for many years (Feldman, 1999b). A second group of irreversible inhibitors includes the OPs. OPs are esters of phosphoric, phosphonic, and phosphinic acids, with no free hydroxyl group on the phosphorus atom (Feldman, 1999a). Similar to carbamates, they react with ChEs and other serine esterases due to the structural resemblance of their substrates. However, phosphorylated enzyme is reactivated considerably slower than the carbamylated enzyme and certain OPs can further undergo dealkylation following their interaction with the enzyme and completely block its reactivation (''aging''; Feldman, 1999a; Taylor, 1996). Both carbamates and OPs are used in agriculture as pesticides and therapeutically to elevate ACh levels. The high toxicity of OPs further led to their use as chemical warfare agents (e.g. OP nerve agents, soman, sarin, cyclosarin, tabun, and VX), calling for development of therapeutic agents to prevent their lethal effects (Cowan *et al.*, 2004; Newmark, 2004; Raveh *et al.*, 1993).

D. AChE Dynamics at the NMJ

Inhibition of AChE at the NMJ results in persistent cholinergic transmission that must be rapidly corrected by modifications in the signaling environment. This occurs primarily by rapid internalization of the AChR with homeostasis being restored through the simultaneous process of AChE replacement (Krejci *et al.*, 2006). OP treatment has been shown to inactivate the AChR within seconds of application, validating the concept of receptor-level compensations (Katz *et al.*, 1997). In an elegant experimental series, a one-time blockade of AChE resulted in a 54% internalization of the AChR at the NMJ within 3 days, presumably in an attempt to mitigate the increased synaptic ACh. The degree of AChE inhibition appears to be tightly linked to AChE replacement and remodeling, with chronic blockade producing a $t_{1/2}$ of 12 h. One-time inhibition of NMJ AChE results in a biphasic $t_{1/2}$, which is increased initially to 3 days and subsequently to 12 days. Interestingly, one-time inhibition of AChE results in a 1:1 ratio of replaced:internalized AChE. However, chronic exposure decreases this ratio significantly, presumably due to cellular toxicity. Importantly, almost one-third of the initially inhibited AChE may be present weeks later, implying that there may be two pools of AChE at the

NMJ, with the remaining pool possibly representing ColQ-linked AChE.

AChE-S location in the NMJ basal lamina has been well established (Anglister *et al.*, 1994). AChE-S, but not AChE-R, accumulates and adheres to the active zone at the NMJ (Harlow *et al.*, 2001; Seidman *et al.*, 1995) and is an essential component of functional NMJs. Compatible with this notion, the loss of the ColQ collagen-like structural subunit linking AChE-S to the NMJ leads to congenital myasthenia (Donger *et al.*, 1998; Ohno *et al.*, 2000), and modified AChE levels were associated with muscle dystrophy in chickens (Silman *et al.*, 1979). However, the inhibitory action of small molecule anti-ChEs exerts complex consequences. For example, AChE inhibitors induce AChE-R overproduction in muscle, have deleterious effects on muscle and NMJ morphology (Lev-Lehman *et al.*, 2000), and modify ACh levels, affecting the inflammatory status (Pollak *et al.*, 2005). When up-regulated, AChE-R is capable of reaching the synaptic cleft and changing the ACh balance there, as well as in the circulation. Together, this implies that AChE-R might participate in acquired or inherited myopathies and that it should serve as a preferred target for therapeutic agents.

E. The Cholinergic Anti-Inflammatory Pathway

The term ''cholinergic anti-inflammatory pathway'' was coined in recognition of the ability of excessive stimulation of nAChRs to prevent the secretion of the pro-inflammatory cytokines TNFα, interleukin (IL)-1β, IL-6, and IL-18, but not the anti-inflammatory cytokine IL-10, from macrophage (Czura and Tracey, 2005; Gallowitsch-Puerta and Tracey, 2005; Tracey, 2002; Tracey *et al.*, 2001). In lipopolysaccharide (LPS)-stimulated human macrophage cultures, nAChR stimulation impairs positive stimulation by pattern-associated molecular patterns (PAMPs) such as bacterial LPS (Borovikova *et al.*, 2000a, b). Vagus nerve secretion of ACh inside the spleen is the principal site of *in vivo* anti-inflammatory action, as splenectomized mice show profoundly reduced pro-inflammatory cytokine levels following LPS administration (Huston *et al.*, 2006). This neuroregulated anti-inflammatory pathway using ACh has been shown to operate through the suppression of NF-κB, much like HPA axis-induced immunosuppression utilizing glucocorticoids (Pavlov *et al.*, 2003). Long ago the deleterious effects of OPs on immune function were demonstrated, most notably affecting humoral immunity (Beaman *et al.*, 1999; Casale *et al.*, 1983, 1984). More recently, OP-mediated suppression of TNF-α, IL-1β, and iNOS has been shown, as well as a confirmation of decreased humoral immunity through suppression of IL-4 (Singh and Jiang, 2003). This, in turn, has led to investigation of OP-mediated suppression of the HPA axis (Pena-Philippides *et al.*, 2007). In addition to OPs, therapeutic anti-cholinesterases have also been shown to exert anti-inflammatory properties (Tyagi *et al.*, 2007). Thus it can be seen that anti-ChEs are valid inducers of the cholinergic anti-inflammatory pathway, presumably through their role in increasing ACh levels.

A reciprocal role to that of ACh is implicated for the soluble AChE-R protein, which should principally reduce ACh levels while inducing pro-inflammatory properties. Indeed, the response to chemical warfare agents such as OP inhibitors of AChE shows interesting parallels with those symptoms caused by the release of cytokines. Accordingly, the nerve agent soman and other anti-AChEs initiate immune responses as well. Soman induces an increase in the pro-inflammatory cytokine IL-1β in rats, including brain where IL-1β is thought to contribute to irreversible brain damage following exposure (Svensson *et al.*, 2001). Moreover, immune stimulation by LPS influences AChE activity in human serum (Cohen *et al.*, 2003; Ofek *et al.*, 2007) and in pro-megakaryocytes (Pick *et al.*, 2006). In light of these reports, and due to the fact that IL-1 causes AChE overproduction in PC12 cells and in the rat cortex (Y. Li *et al.*, 2000a, b), the following relationships can be postulated: IL-1 induction of AChE overexpression suppresses ACh levels, ablating ACh's capacity to attenuate IL-1 production, a cycle that may explain the prolonged anti-AChE effects. Conversely, AChE suppression was shown to suppress IL-1 production in brain and blood, presumably by increasing ACh levels (Pollak *et al.*, 2005). Hence, AChE inhibitors will initially block the inflammatory response due to their capacity to increase ACh levels, but in the long term they will not be able to prevent AChE's noncatalytic effects, such as NMJ proliferation, especially since AChE inhibition induces AChE-R overproduction. Increased inflammatory response is hence predicted to follow the feedback response of AChE-R accumulation (Figure 46.2).

The cholinergic anti-inflammatory pathway has profound implications for excessive nAChR stimulation in the context of an infected host by increasing susceptibility to initial infection or therapeutically reducing the host overreaction seen in sepsis. Because OP levels inducing immunosuppression appear to be lower than those inducing acute cholinergic crisis, it is important to consider treating OP-exposed patients not exhibiting acute cholinergic symptoms in order to reduce the likelihood of opportunistic infections following survivable OP intoxication. A bioscavenger like AChE-R that enhances host immunity may be useful in this situation.

VIII. LONG-TERM EFFECTS OF AChE-R OVERPRODUCTION

A. Introduction

Loss of synaptic cholinergic regulation by AChE inhibition has immediate negative consequences for mammalian physiology. The severe multisystem clinical presentation of anti-ChE intoxication demonstrates the essential and

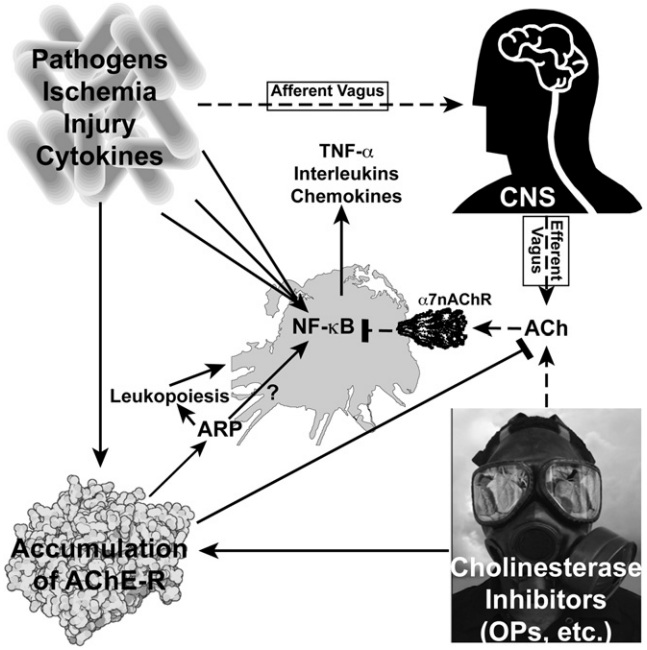

FIGURE 46.2. Cholinergic regulation of inflammation. Upper left corner: Diverse physiological insults converging on the cell are initially attenuated by the cholinergic anti-inflammatory pathway (dashed lines). Long-term cholinergic regulation of inflammation adopts a decidedly pro-inflammatory character constituting a cholinergic pro-inflammatory pathway, principally mediated by AChE-R and the peptide derivative ARP (solid lines). This bivalent perpetuation of inflammation operates through suppression of anti-inflammatory pathways (flat heads) and activation of novel pro-inflammatory pathways (arrow heads). Lower right corner: Anticholinesterases, such as OPs, have similar short- and long-term effects on the immune system.

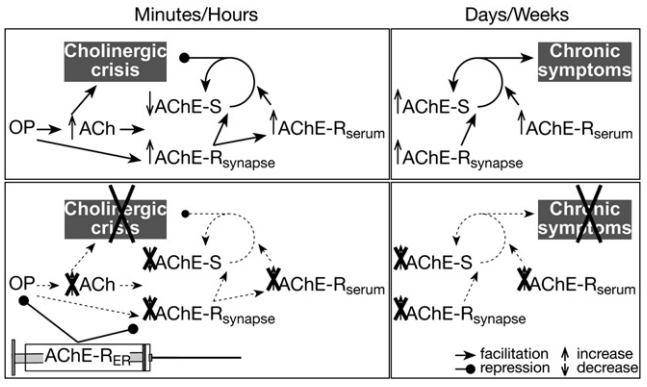

FIGURE 46.3. Model for mechanism of protection from acute and chronic consequences of OP intoxication afforded by plant-derived AChE-R.

ubiquitous nature of the mammalian cholinergic system. Conventional wisdom holds that surviving the acute phase confers a positive prognosis. While this is certainly true for the immediately life-threatening complications of OP intoxication, this dogmatic and historically practiced approach ignores many new advances in the understanding of AChE physiology and molecular biology.

Exposure to anti-ChEs affects both nicotinic and muscarinic cholinergic pathways and results in cholinergic hyperexcitation (e.g. excessive salivation) and neuromuscular malfunctioning (e.g. shortness of breath). Such intoxication, when surviving the acute exposure, has long-term consequences, including OP-induced delayed neuropathy, social/behavioral effects, and delayed muscle weakness (Brown and Brix, 1998). In the brain, effects of OP exposure, like many other stressful insults, are mediated by the accumulation of the early immediate protein c-Fos followed by up-regulation of AChE gene expression (Friedman *et al.*, 1996) and a 3′ alternative splicing shift from AChE-S to AChE-R (Kaufer *et al.*, 1998; Meshorer *et al.*, 2002). This prolonged overexpression of AChE-R causes dramatic deleterious consequences, manifested as degenerated synaptic folds in NMJs, enlarged motor endplates, disorganized muscle fibers and branded terminal nerves (Lev-Lehman *et al.*, 2000), accompanied by physiological malfunctioning (Farchi *et al.*, 2003). Finally, mice overexpressing AChE-R display significant deficits in both normal social behavior and memory (Cohen *et al.*, 2002; Farchi *et al.*, 2007b; Nijholt *et al.*, 2004), compatible with the assumption that AChE-R excess is causally involved with these consequences of anti-ChE exposure.

B. "Non-Classical" Roles of Cholinesterases

In addition to the traditional roles of regulators of cholinergic transmission in the serum and at the synapse, many novel roles have been proposed for ChEs (Soreq and Seidman, 2001). For example, BChE has recently been shown to attenuate amyloid plaque formation and also be involved in neurogenesis (Diamant *et al.*, 2006; Mack and Robitzki, 2000). AChE-E has been shown to degrade heroin into 6-amino morphine, a function that AChE-S cannot perform (Salmon *et al.*, 1999).

The first discovered noncatalytic role of AChE-S was the enhancement of embryonic neurite extension, both in cell cultures (Bigbee *et al.*, 2000; Layer *et al.*, 1993; Small *et al.*, 1995) and in *Xenopus* motor neurons that express human AChE-S (Sternfeld *et al.*, 1998). Lately, many additional roles have been proposed for AChE, apart from its traditional role of terminating cholinergic transmission at the synapse (Silman and Sussman, 2005; Soreq and Seidman, 2001). For example, AChE was suggested to function as an adhesion molecule, due to its sequence homology to ChE-like adhesion molecules such as neuroligin. In that way, it was argued that AChE may be involved in synaptogenesis and synaptic maintenance (Silman and Sussman, 2005). Other noncatalytic roles include accentuation of amyloid plaque formation by AChE-S (Inestrosa *et al.*, 1996; Rees *et al.*, 2003, 2005; Rees and Brimijoin, 2003) and the inverse attenuation of plaque formation by AChE-R (Berson *et al.*, 2008).

Perhaps the most significant advances in understanding nontraditional roles of ChEs have come through the study of AChE-R (Meshorer *et al.*, 2002). First recognized as a stress-induced soluble AChE variant, it was quickly noticed that AChE-R maintained a role as a contributor to numerous important processes from fear to hematopoiesis and inflammation. Specifically, AChE-R has been shown to enhance fear memory and long-term fear potentiation (Birikh *et al.*, 2003; Nijholt *et al.*, 2004). A proliferation function of AChE-R has recently been recognized in hematopoiesis (Pick *et al.*, 2006) and osteogenesis (Grisaru *et al.*, 1999a), while AChE-S was inversely shown to interfere with lymphopoiesis (Perry *et al.*, 2007). Importantly, the cleavable C-terminus peptide of AChE-R, acetylcholinesterase readthrough peptide (ARP), was shown to have a role in hematopietic proliferation and pro-inflammatory cytokine production (Gilboa-Geffen *et al.*, 2007; Deutsch *et al.*, 2002; Grisaru *et al.*, 2006), contextual fear and LTP enhancement (Farchi *et al.*, 2007b; Nijholt *et al.*, 2004), neuronal development, plasticity (Dori and Soreq, 2006), and inflammation-associated neuropathies (Dori *et al.*, 2007).

C. Cholinergic Hyperexcitation and Induction of AChE-R Production

Acute psychological stress and sublethal intoxication with AChE inhibitors both induce cholinergic stimulation, yielding neuronal hyperexcitability (Ennis *et al.*, 1992; Imperato *et al.*, 1991). Immediate reactions to such cholinergic stimulation include a dramatic increase in neuronal c-Fos mRNA (Kaufer *et al.*, 1998; Lev-Lehman *et al.*, 2000). The c-Fos protein further binds and activates the promoters of the ChAT (Bausero *et al.*, 1993), VChAT (Cervini *et al.*, 1995), and ACHE (Meshorer *et al.*, 2004) genes. A consequent decrease in ChAT and VChAT then suppresses ACh synthesis and packaging, while enhanced AChE mRNA synthesis and translation facilitates ACh hydrolysis, together yielding an effective restraint of the hypercholinergic response. The larger AChE activity observed in both brain and NMJ following AChE blockade reflects the induction of a soluble AChE variant, AChE-R (Kaufer *et al.*, 1998). This profound feedback response is largely controlled by the splice factor SC35 (Meshorer *et al.*, 2005a). It serves well in the short term to quickly reduce the cholinergic hyperactivity following an acute insult (Kaufer and Soreq, 1999), but may entail long-term damage. Thus, AChE-R mRNA levels remain elevated for several weeks following a days-long exposure, even to exceedingly low doses of the OP AChE inhibitor diisopropyl fluorophosphate (DFP; Lev-Lehman *et al.*, 2000; Meshorer *et al.*, 2002). In the striatum, exposure to AChE inhibitors predictably increased AChE-R mRNA by three-fold but did not affect AChE activity or total AChE mRNA, possibly suggesting arrested translation or production of inactive AChE-R (Perrier *et al.*, 2005). In the hippocampus, exposure to AChE inhibitors could not be rebalanced by subsequent cholinergic stimulation due to limited ability for additional AChE-R induction (Meshorer *et al.*, 2002). Therefore, repeated cholinergic stimulation by AChE blockade induces long-term hypersensitization with potentially damaging consequences. Supporting this notion, transgenic mice overexpressing AChE-S in the central nervous system possess delayed cognitive and neuroanatomical pathologies (Cohen *et al.*, 2002; Farchi *et al.*, 2003). In humans, traumatic stress with a severe stress response is often followed by long-term pathological changes (McEwen, 1999; Sapolsky *et al.*, 2000). Under extreme conditions, such changes are clinically referred to as the anxiety-associated post-traumatic stress disorder (PTSD; Mezey and Robbins, 2001). Physiological relevance of AChE-R to these increases was reported in healthy human volunteers, where higher blood AChE-R levels occurred in association with the subjects' anxiety (Sklan *et al.*, 2004). It is still unknown whether this stress-induced alternative splicing of AChE occurs as a cause or an outcome of anxiety, or both. However, in either case this phenotype may reflect physiological relevance of the overexpressed AChE-R in reaction to stress. Apart from this apparent association, AChE-R may mediate at least some of the adverse cellular changes associated with delayed stress responses (e.g. spine loss or glial hyperactivation, see Sternfeld *et al.*, 2000). Intriguingly, AChE-R mRNA was found in apical dendrites of neurons from all cortical layers following exposure to cholinesterase inhibitors (Kaufer *et al.*, 1998; Meshorer *et al.*, 2002), suggesting possible local regulation and translation of AChE in the synapse also in noncholinergic neurons.

D. AChE-R Accumulation in NMJs Under Cholinergic Imbalances

Similar to the case in the brain, AChE-R accumulates in neuromuscular tissues under diseases and stress conditions. AChE-R levels rise both in the serum and muscles of experimental autoimmune myasthenia gravis (EAMG) rats and in the serum of MG patients (Brenner *et al.*, 2003; Euron *et al.*, 2005). In the primate spinal cord, AChE-R accumulated under handling stress in a cell size-dependent manner, suggesting that the characteristic response of shift in the alternative splicing of AChE pre-mRNA spans different cell types and is pivotal for controlling motor functions. In the spinal cord, cholinergic interneurons that innervate large motor neurons, but not the motor neurons themselves, respond to stress conditions by elevating AChE-R and by reducing the translocation of AChE-S mRNA into neurites, similarly to AChE-R elevation in mouse tongue muscles following exposure to AChE inhibitors (Lev-Lehman *et al.*, 2000) and compatible with the shift in alternative splicing toward AChE-R and its transport to neural extensions, where it replaces the normally expressed transcript, AChE-S (Farchi *et al.*, 2007a; Meshorer *et al.*, 2002). A rapid and transient elevation in serum AChE-R following stress responses further suggests a mechanism for suppressing

ACh mediated excitation after stress. However, prolonged elevation in AChE-R, for example, following stress and anti-ChE intoxication, may damage cholinergic signaling and exert noncholinergic effects.

E. Noncholinergic Effects of AChE-R Excess in NMJs

A direct role for AChE-R in MG symptoms likely involves several functional levels. The first is the clear cholinergic function of AChE-R in controlling ACh levels at the NMJ. The second is interference with the anti-inflammatory role of ACh, exacerbating the outcome of the autoimmune origin of the disease. Finally, secondary signaling effects that further change the disease progression may involve AChE-R protein–protein interaction with the scaffold protein RACK1 and its target, protein kinase C βII (PKCβII; Birikh *et al.*, 2003; Sklan *et al.*, 2006). These interactions have already been identified within brain neurons with an association to fear-induced behavior (Nijholt *et al.*, 2004) and in hematopoietic cells (Pick *et al.*, 2006) and may be relevant to more tissues, including muscles. Another potential mechanism is based on AChE's sequence homology to cell surface adhesion molecules such as neuroligin (Silman and Sussman, 2005). In brain synapses, AChE-R might compete with neuroligin on interaction with its partners (i.e. neurexin), interfering with and even modifying the post-synaptic signaling pathway. Such interference may serve as a potential mediator of cytoarchitectural changes (Grifman *et al.*, 1998) in central nervous system synapses. Both neuroligin and neurexin are also expressed and function at the NMJ (Johnson *et al.*, 1995; Sons *et al.*, 2006), suggesting parallel relevance to NMJ architecture. Within the NMJ, synaptic differentiation is induced by axon-derived agrin and muscle-derived laminin (Fox and Umemori, 2006). Laminin 1 was suggested as a potential partner for AChE (Johnson and Moore, 2003, 2004; Paraoanu and Layer, 2004) and for AChE-R's C-terminal peptide, ARP (Johnson and Moore, 2007). This may further explain the increase in NMJ density and area following OP intoxication associated with elevated AChE-R (Evron *et al.*, 2007a, b; Lev-Lehman *et al.*, 2000). Furthermore, this morphological effect was partially preventable by either EN101/Monarsen (Lev-Lehman *et al.*, 2000) or by the plant-derived AChE-R_{ER} (Evron *et al.*, 2007a, b). Further experiments are required to elucidate the mechanisms underlying AChE's noncatalytic roles in general and within neuromuscular tissues in particular.

Compatible with previous findings (Perry *et al.*, 2004; Perry and Soreq, 2004; Sklan *et al.*, 2004), AChE-R appears to contribute to the maintenance of cholinergic homeostasis while accentuating neuronal inflammatory reactions. Because AChE-R accumulation reduces ACh levels, suppression of AChE-R overproduction can retrieve the blockade over pro-inflammatory cytokine production, suppressing the inflammatory status in treated subjects.

F. mARP as a Physiological Mediator of Recovery

Exposure to OP inhibitors of AChE leads to short-term severe symptoms, followed by longer-term effects such as memory impairments and muscle fatigue (Rosenstock *et al.*, 1991), and accompanied by AChE-R up-regulation (Kaufer *et al.*, 1998; Lev-Lehman *et al.*, 2000; Salmon *et al.*, 2005). In farmers, exposure to OP insecticides correlates to a higher risk of leukemia (Brown *et al.*, 1990), which was tentatively correlated to AChE-R accumulation (Perry and Soreq, 2004). However, immune stimulation by LPS also influences AChE levels (Cohen *et al.*, 2003; Ofek *et al.*, 2007; Pick *et al.*, 2006). In CD34+ hematopoietic progenitors in culture, cortisol treatment also induced AChE-R accumulation (Grisaru *et al.*, 2001). The up-regulated AChE-R can further induce hematopoietic cellular processes through its noncatalytic properties and the enhanced interaction with RACK1 (Pick *et al.*, 2006). Moreover, AChE-R elevation in the serum of stressed mice was associated with an apparent cleavage of the AChE-R C-terminal peptide, human (h) ARP (Grisaru *et al.*, 2001). A parallel cleavage also occurred in the serum of human volunteers following exposure to LPS (Cohen *et al.*, 2003). Mimicking this phenomenon by *ex vivo* administration of a synthetic human ARP, promoted expansion and differentiation of hematopietic stem cells (Deutsch *et al.*, 2002; Grisaru *et al.*, 2006), accentuated neuronal plasticity (Dori *et al.*, 2005; Dori and Soreq, 2006), facilitated LTP enhancement (Farchi *et al.*, 2007b; Nijholt *et al.*, 2004), and induced inflammation-associated neuropathies (Dori *et al.*, 2007), all known as pivotal elements of organismal reactions to acute stress and exposure to poisonous substances. Intriguingly, RNA-targeted suppression of AChE-R prevented at least some of the described effects of the mouse administered mARP (Deutsch *et al.*, 2002; Grisaru *et al.*, 2001; Nijholt *et al.*, 2004). In cultured CD34+ cells, administered human ARP enhanced AChE gene expression (Grisaru *et al.*, 2006). Importantly, human and mouse ARP differ in their primary amino acid sequences, but share common immunogenic features and cell proliferative function(s) (Dori *et al.*, 2007).

In the brain, mARP is capable of undergoing endocytosis and retrograde transport through a mechanism which was reported to involve the recruitment of RACK1 and PKCβII (Nijholt *et al.*, 2004). In blood cell progenitors, human ARP promoted megakaryocytopoiesis with similar involvement of RACK1 and PKCε (Pick *et al.*, 2006). Systemic administration of mARP to mice following paraoxon and LPS exposures affected multiple levels of body defense. Those included both organismal responses such as changes in body temperature and arrested motor activity and molecular reactions such as modified corticosterone levels and muscle AChE gene expression; and led to a longer-term dramatic increase in motor activities, high above normal values. In contrast to the rapid effect following paraoxon exposure,

mARP administration increased light- and dark-phase motor activities several days post-LPS exposure. This is compatible with the symptoms recorded for AChE-R overexpressing transgenic mice, which presented dramatic activity changes following a circadian shift (Cohen *et al.*, 2002). It is also consistent with the tissue-specific effect on endogenous AChE mRNA and protein production. While in the plasma AChE activity was elevated, mARP reduced total AChE and AChE-S mRNA levels within the muscle. This was compatible with the decrease observed in the muscle AChE protein following treatment with plant-produced recombinant AChE-R_{ER} (Evron *et al.*, 2007a), supporting the hypothesis of auto-regulatory effects of mARP on AChE gene expression in the *in vivo* context as well. Importantly, reduced levels of muscle AChE were associated with mitigated damage in diaphragm NMJs. Altogether, our findings hence raise the possibility of using synthetic ARP as a regulatory element, capable of facilitating recovery and minimizing exposure-induced damage, following chemical stresses.

G. Modified Alternative Splicing Patterns by Spinal Cord Cholinergic Signaling

Modified and aberrant alternative splicing in the nervous system is associated with diseases, aging, and stress (Meshorer and Soreq, 2002; Nissim-Rafinia and Kerem, 2005; Stamm *et al.*, 2005). For example, aberrant splicing of the glutamate transporter gene EAAT2 is associated with ALS (Lin *et al.*, 1998). The detailed mechanisms by which missplicing or modified splicing patterns lead to human diseases are not clear yet, but probably involve multiple factors that participate both in basic splicing (e.g. serine–arginine (SR)-rich proteins, small nuclear ribonucleoproteins (snRNPs); reviewed in Stamm *et al.*, 2005) and in alternative splicing processes. Cholinergic stress responses include a transient increase in ACh levels in the mammalian brain (Masuda *et al.*, 2004), similar to the outcome of exposure to the small molecule inhibitors of AChE. Additionally, AChE alternative splicing following stress leads to a shift from AChE-S transcripts to AChE-R ones. The consequent accumulation of AChE-R then mediates at least part of the cellular changes of delayed stress responses (Meshorer and Soreq, 2006). In the mouse prefrontal cortex, AChE-R accumulation was further associated with parallel increases of the splicing factor SC35 (Meshorer *et al.*, 2005b). SC35 is a member of the serine-arginine (SR) proteins, involved in both constitutive and alternative splicing (Tacke and Manley, 1999). In cell cultures, SC35 shifted the splicing toward the AChE-R variant when cotransfected with the AChE minigene (Meshorer *et al.*, 2005b). In the hematopoietic system, SC35 is essential for the normal development of T lymphocytes (Wang *et al.*, 2001), a process subjected to stress-induced modulation (Gilboa-Geffen *et al.*, 2007; Pick *et al.*, 2004, 2006). Also, cultured cells overexpressing either AChE-R or AChE-S inversely show reduced SC35 levels (Ben-Ari *et al.*, 2006), indicating network contributions to this effect. A direct association between the levels of nuclear SC35 and cytoplasmic AChE-R mRNA was found in interneurons, but not in large motor neurons, further reflecting a cell-size dependent effect for this association (Evron *et al.*, 2005). Together, our observations support the notion of a reciprocal control of the spliceosome function by cholinergic signaling. SC35 is required for the formation of the earliest ATP-dependent splicing complex (Kramer, 1996). It also binds the 5′ and 3′ splice sites during spliceosome assembly. Our findings therefore suggest that cholinergic signaling regulates the splicing and alternative splicing patterns in the spinal cord.

H. AChE-R in Neuromuscular Pathologies

Two weeks following DFP exposure for 4 days, the mouse tongue muscles show a significant increase in AChE catalytic activity (Lev-Lehman *et al.*, 2000). The punctuated expression pattern of NMJ-related AChE-S mRNA remains unchanged, but AChE-R mRNA is significantly induced and exhibits a diffuse, extrajunctional distribution. TgS mice, which also show host AChE-R mRNA overexpression, presented a similar expression pattern (Lev-Lehman *et al.*, 2000). Furthermore, in control animals, histochemical activity staining localized the signal to motor endplates, whereas in TgS mice, the soluble AChE-R presented dispersed staining in muscle fibers, not restricted to the endplate region.

In both TgS mice and strain-matched controls chronically treated with DFP, AChE-R overexpression was accompanied by neuromuscular pathology. This neuropathology was exhibited as a chaotic fiber disorder with severe atrophy and vacuolization, compared to control mice where fiber organization was conspicuous (Lev-Lehman *et al.*, 2000). A significant increase was observed by silver staining in small unbundled neurites, but not in large nerve bundle fibers, both in TgS mice and following chronic DFP treatment compared to strain-matched controls. Corresponding to the previously reported neurite-promoting activity of AChE (Grifman *et al.*, 1998; Sternfeld *et al.*, 1998), these findings indicate axon branching under the influence of AChE-R overexpression and suggest that the affected muscles were subjected to denervation–reinnervation processes. Importantly, nontransgenic mice treated with DFP and TgS mice exhibited a significant increase in the number of endplates in their diaphragm muscle compared to FVB/N controls (Lev-Lehman *et al.*, 2000). These endplates were smaller in diameter; compatible with the assumption that reinnervation occurred. Intact TgS muscles stimulated directly via the phrenic nerve examined *ex vivo* demonstrated rapid fatigue following initial stimulation, accompanied by enlarged decrements and delayed recovery compared to strain-matched controls, which was attributed to both neuronal and muscle impairments (Farchi *et al.*, 2003).

Inherited (Donger *et al.*, 1998; Lindstrom, 1998) or acquired (Engelhardt *et al.*, 1997; Livneh *et al.*, 1988; Schonbeck *et al.*, 1990) interference with ACh mediated neurotransmission in the NMJ causes a variety of myasthenic syndromes, i.e. progressive muscle weakness and fatigue. These impairments of cholinergic neurotransmission further induce neuroanatomical NMJ pathologies characterized by co-deterioration of muscle structure and function. Interestingly, sepsis-mediated critical illness polyneuropathy (CIP; Hund, 2001a) and critical illness myopathy (Hund, 1999) were also shown to be responsible for muscle weakness and atrophy in intensively treated patients. This weakness is mediated by neuropathy and disturbance of neuromuscular transmission as well as disturbance in the function and/or structure of the muscle (Hund, 2001b), suggesting that neuromuscular impairments may also be induced by inflammatory signals.

Ample information suggests the involvement of AChE-R overexpression in neuromuscular failure (Table 46.1). Additionally, TgS transgenic mice overexpressing the AChE-S ''synaptic'' form of human AChE in spinal cord motor neurons displayed progressive neuromotor impairments that were associated with changes both in NMJ ultrastructure (Andres *et al.*, 1997) and in spinal cord synapses (Andres *et al.*, 1998). Among the delayed effects of anti-ChE intoxication are degeneration of synaptic folds, terminal nerve branching, enlargement of motor endplates, and disorganization of muscle fibers (Kawabuchi *et al.*, 1976; Laskowski *et al.*, 1975). The morphogenic effects of transgenic AChE were largely attributed to the deposition and accumulation of neuronal AChE in NMJs (Andres *et al.*, 1998).

IX. PREVENTION OF ANTICHOLINESTERASE TOXICITY: THE ''NEXT GENERATION''

A. Stoichiometric Bioscavengers

Current medical intervention in the case of acute exposure to anticholinesterase agents includes use of the muscarinic receptor antagonist atropine to block overstimulation, and oximes to reactivate the OP-modified AChE (Dunn and Sidell, 1989; Gunderson *et al.*, 1992; Millard and Broomfield, 1995; Schwarz *et al.*, 1995; Taylor, 1996). The reversible carbamate inhibitor, pyridostigmine bromide, is also used for prophylaxis. However, these conventional treatments have limited effectiveness and may have serious short- and long-term side effects (Dunn and Sidell, 1989; Friedman *et al.*, 1996; Maxwell *et al.*, 1993; Schwarz *et al.*, 1995). In fact, the routine treatments, while successfully decreasing anticholinesterase-induced lethality, rarely alleviate post-exposure toxicity and result in significant performance deficits and even permanent brain damage (Castro *et al.*, 1992; Dunn and Sidell, 1989; Leadbeater *et al.*, 1985; Maxwell *et al.*, 1993).

The shortcomings of the pharmacological approach to the problem prompted the development of an alternative approach that makes use of the OP-binding potential of soluble isoforms of AChE and the related enzyme BChE (Ashani, 2000; Saxena *et al.*, 1997a). Administration of exogenous cholinesterases can provide protection against nerve agents, as was demonstrated in a variety of rodent models (Allon *et al.*, 1998; Ashani *et al.*, 1991; Brandeis *et al.*, 1993; Genovese and Doctor, 1995; Maxwell *et al.*, 1993) and in primates (Maxwell *et al.*, 1992; Raveh *et al.*, 1997). The considerable success with plasma-purified butyrylcholinesterase (BChE) as an OP bioscavenger (Ashani, 2000; Doctor and Saxena, 2005) brings to the foreground, nonetheless, the question of the practicality of this therapeutic approach as it depends on the availability of large amounts of these human enzymes, which are required in stoichiometric rather than catalytic quantities. Indeed the 1:1 stoichiometry necessary for OP bioscavenging by ChEs, stemming from the virtual irreversibility of the interaction of the enzymes with the inhibitors, is one of the major limitations of this approach. Co-administration of oximes to enhance regeneration of the bioscavengers is only partially effective (and not effective at all against some nerve agents such as tabun and soman) and the chemistry involved in the process results in highly reactive and toxic phosphyloximes (Caranto *et al.*, 1994; Herkenhoff *et al.*, 2004).

B. Catalytic Bioscavengers

An ideal bioscavenger should be broad-acting, avoid aging, and have sufficient reactivation kinetics, i.e. it should be catalytically active. Several enzymes from diverse organisms [ranging from bacteria (Benning *et al.*, 1994) through invertebrates (Hartleib and Ruterjans, 2001) to vertebrates (Harel *et al.*, 2004)] are known to hydrolyze anti-ChE compounds and despite their genetic and structural diversity are lumped together as OP hydrolases or as phosphotriesterases. Their utility as catalytic bioscavengers of nerve agents and pesticides did not go unnoticed (Masson *et al.*, 1998; Sogorb *et al.*, 2004). The most broadly active members of this artificial grouping are the enzymes from *Pseudomonas diminuta* and *Flavobacterium* sp. (Masson *et al.*, 1998), and their study yielded valuable structural and functional insights (Benning *et al.*, 1994; Gopal *et al.*, 2000; Grimsley *et al.*, 2005), but their utility for treating humans is questionable because of their bacterial origin. On the other hand, mammals have several serum enzymes that reveal low, but significant, hydrolyzing activity toward OPs. These include not only paraoxonase but also members of the α/β fold hydrolase family, particularly BChE and carboxylesterase (CarbE; Li *et al.*, 2005).

Of the three members of the paraoxonase (PON) family, the most studied and hence best known is PON1 (Van Himbergen *et al.*, 2006). The genes encoding for the various PONs are >60% identical and all three are mapped to nearby loci on the long arm of chromosome 7 (Primo-Parmo

TABLE 46.1. AChE-R associated pathologies

Pathology	Cell/animal model	AChE-R involvement/ suggested role	References
CNS			
Alzheimer's disease	*In vitro* SH-SY5Y cells APPsw/AChE-R mice	Suppresses amyloid-beta fibrils formation and toxicity (neuroprotective)	Berson *et al.* (2008)
Alzheimer's disease	Human	Reduced in Alzheimer's brains and CSF. Elevated levels following tacrine and rivastigmine treatments are correlated to improved cognition	Darreh-Shori *et al.* (2004)
Parkinsonism	AChE-R mice	Attenuates disease progression following MPTP exposure (neuroprotective)	Ben-Shaul *et al.* (2006)
Photoreceptor damage	Albino rats	Overexpressed and involved in light-induced photoreceptor damage	Kehat *et al.* (2007)
Anxiety	Human	Inversely correlated with trait anxiety	Sklan *et al.* (2004)
Blood–brain barrier (BBB) disruption	Human	Elevated levels in CSF associated with BBB impairments	Tomkins *et al.* (2001)
PTSD	Mice	Key molecule, elevated in the Ca^{2+}-dependent feedback response to cholinergic insult	Kaufer *et al.* (1998) Zimmerman and Soreq (2006)
Synaptic plasticity (stress associated behaviors)	Mice	Interacts with RACK1 and PKCβ2 and promotes contextual fear, fear-induced conflict behavior, and PKC-augmented LTP in CA1 region	Nijholt *et al.* (2004) Birikh *et al.* (2003)
Neuronal hypersensitivity	PC12 cells Primary cerebellar mouse neurons	AChE-R translocation to neurites under stress modulates hypersensitivity to both anticholinesterases and atropine	Meshorer *et al.* (2002)

Closed head injury	Traumatized mice	Overexpressed in somatosensory cortex following injury. CA3 hippocampal neuron death	Shohami *et al.* (2000)
Stress-related neurodegeneration	AChE-R mice	Protects the hippocampus from stress-related morphologies (neuroprotective)	Sternfeld *et al.* (2000)
Glioblastoma	Human U87MG glioblastoma cells	PKCε-mediated cell proliferation	Perry *et al.* (2004)
Periphery			
Myasthenia gravis (MG)	EAMG rats Human patients	Overexpressed and involved in muscle malfunctioning and disease progression	Brenner *et al.* (2003); Argov *et al*., (2007)
Myopathies	AChE-S mice; OP-treated mice	Promote excessive neurite outgrowth and disorganized muscle fibers	Lev-Lehman *et al.* (2000)
MG-related thymic hyperplasia	Human AChE-R mice	Interferes with thymic differentiation mechanisms toward positive selection of CD4(+)CD8(+) cells	Gilboa-Geffen *et al.* (2007)
Thrombocytopenia	AChE-R mice human CD34+ cells	Promotes thrombopoietic recovery by elevating thrombopoietin levels and platelet counts.	Pick *et al.* (2006)
Impaired sperm quality	AChE-R mice; Human patients with impaired sperm qualities	Interacts with RACK1 and enolase-α and affects both sperm differentiation and motility	Mor *et al.* (2008) Mor *et al.* (2001)

et al., 1996), and, interestingly, only 5.5 Mb away from the *AChE* locus (Sklan *et al.*, 2004). Of the three, PON1 and PON3 are serum enzymes associated with high-density lipoprotein (HDL) (Mackness *et al.*, 1998a; Reddy *et al.*, 2001), whereas PON2 is expressed in many tissues (Mochizuki *et al.*, 1998). The three PONs can hydrolyze a variety of esters and lactones (Jakubowski, 2000), but despite their name, weak OP hydrolyzing activity is associated only with PON1 (Aharoni *et al.*, 2004; Harel *et al.*, 2004). Although the precise physiological roles of PONs remain elusive, epidemio-genomic studies (e.g. Carlson *et al.*, 2006), *in vitro* biochemical experiments (Rosenblat *et al.*, 2006), and *in vivo* experiments with knockout mice (Shih *et al.*, 1998, 2000) build a compelling case for the involvement of at least PON1 in protection from atherosclerosis (Lusis, 2000; Mackness *et al.*, 1998b). At the same time, similar lines of evidence point to a much broader role in protection, especially neuroprotection. PON1, true to its name, is an OP hydrolase, but its hydrolytic capacity is quite promiscuous (La Du *et al.*, 1999). Levels of serum PON activity, on the one hand, correlate well with polymorphisms in the PON1 locus and, on the other hand, correlate with sensitivity to anticholinesterase pesticides (Costa *et al.*, 2003; Furlong *et al.*, 2005, 2006, 2000). Revealingly similar correlation between the PON1 status and susceptibility for disease exists with the incidence of several neurodegenerative diseases such as Alzheimer's disease (Dantoine *et al.*, 2002; Erlich *et al.*, 2006; Janka *et al.*, 2002; Pola *et al.*, 2005), Parkinson's disease (Akhmedova *et al.*, 1999; Benmoyal-Segal *et al.*, 2005; Kondo and Yamamoto, 1998; Taylor *et al.*, 2000), psychiatric disorders (e.g. anxiety), and neurodevelopmental disorders, such as autism (Berkowitz *et al.*, 2004; D'Amelio *et al.*, 2005; Pasca *et al.*, 2006; Serajee *et al.*, 2004). The relevance of AChE (and related proteins) and of AChE inhibitors to these pathologies is well known for some and suspected for others (Birikh *et al.*, 2003; De Jaco *et al.*, 2006; Greenfield and Vaux, 2002; Nijholt *et al.*, 2004; Perry *et al.*, 2001; Toiber and Soreq, 2005). The most compelling evidence for the potential role of PON1 as xenobiotic scavenger comes from the studies conducted with PON1 knockout mice (W.F. Li *et al.*, 2000; Shih *et al.*, 1998).

Based on biochemical data (Aharoni *et al.*, 2004; Josse *et al.*, 1999a, b, c, 2001) but mainly on their 2.2 Å-resolution crystal structure of a recombinant PON1 variant (Harel *et al.*, 2004), Tawfik and co-workers recently offered a model for the architecture and function of the lactonase active site(s) of the PON enzymes (Khersonsky and Tawfik, 2006; Rosenblat *et al.*, 2006). According to the crystal structure (Harel *et al.*, 2004), PON is a six-bladed β-propeller, with two calcium ions located in the central tunnel – the structural Ca^{2+} is buried while the catalytic Ca^{2+} was solvent exposed. Two His residues, the so-called His^{115}-His^{134} dyad, mediate the lactonase (and esterase) activity of PON enzymes: the former acts as general base to activate a water molecule attacking the carbonyl oxygen of the substrate, the latter is a proton relay and the calcium is stabilizing the negatively charged intermediate (Khersonsky and Tawfik, 2006).

While the His dyad model is consistent with other data and nicely explains both lactonase activity and consequently PON1's anti-atherosclerotic function (Rosenblat *et al.*, 2006), it fails to describe the phosphotriesterase activity of the enzyme. Nonetheless, molecular evolution experiments from the Tawfik laboratory (Aharoni *et al.*, 2004), together with site-directed mutagenesis by that group and others (Khersonsky and Tawfik, 2006; Yeung *et al.*, 2004, 2005), indicate that while the lactonase and OP hydrolase active sites are partially overlapping, substrates are oriented differently so as to face different chemically active residues. Several mutations specifically increase the OP hydrolase activity. For example, H134Q increases it three to six-fold relative to WT (Harel *et al.*, 2004; Khersonsky and Tawfik, 2006), the same mutation reduces the lactonase activity to 11% (Khersonsky and Tawfik, 2006) and two other mutations, H134W and H134F, abolish both activities (Yeung *et al.*, 2004). Even more dramatic is the >40-fold increase compared to WT in the OP hydrolysis activity of either V346A or L69V obtained through *in vitro* evolution of the gene (Aharoni *et al.*, 2004). Further work is obviously needed to develop PON1 into a more efficient OP hydrolase.

It is noteworthy that the two enzymes that thus far were shown to provide the best protection, AChE and BChE, belong to the α/β fold hydrolase family. These bind OP anticholinesterases very efficiently but the phosphorylated enzymes fail to reactivate as was described above. However, certain mutants of BChE were shown to reactivate much less slowly, effectively making them OP hydrolases (Broomfield *et al.*, 1999; Lockridge *et al.*, 1997; Millard *et al.*, 1995, 1998). Unfortunately, the process is still very inefficient, especially with the soman-inhibited enzyme, which is rapidly undergoing aging. Mutations that reduce the rate of the dealkylation aging process were reported in both BChE (Millard *et al.*, 1998) and AChE (Maxwell *et al.*, 1999). Although significant improvements were achieved, they are still not sufficient. In this context it is interesting to note that murine BChE, but not its human counterpart, can spontaneously reactivate (Kaliste-Korhonen *et al.*, 1996; Li *et al.*, 2005). Carboxylesterase, CarbE, is another member of the same family, with broader catalytic activities, and sensitive to OPs. CarbE can self-activate, and it is speculated that a naturally occurring His residue within the sequence WI**H**GGGL plays a role in this process. The corresponding sequence of BChE (and AChE) is WI**Y**GGGF. One of the OP-hydrolase enhancing mutations, G117H of BChE, is in the same region (WIYGG**H**F). But, unlike PON1 and the ChE enzymes, CarbE *is not* normally found in human serum (Li *et al.*, 2005). The OP-hydrolyzing activities associated with native CarbEs, murine BChE, and recombinant human BChE raise the option of evolving these enzymes into more efficient phosphortriesterases.

C. Production Systems for OP Bioscavengers

Stoichiometric or catalytic human enzymes intended for use as bioscavengers have to be produced in a eukaryotic system. This is demonstrated by the difficulties of producing human PONs in *Escherichia coli* (Aharoni *et al.*, 2004) which may lead to unfortunate artifacts (see Corrigendum for Harel *et al.*, 2004). Of the candidate bioscavengers, human BChE is the most explored. Several strategies for production of BChE have recently been evaluated, including purification from outdated blood-banked human plasma (Doctor and Saxena, 2005; Grunwald *et al.*, 1997; Lenz *et al.*, 2005) and milk of transgenic goats (Cerasoli *et al.*, 2005). BChE purification from serum (Grunwald *et al.*, 1997) is supply-limited, extremely costly, and carries the risk of human-pathogen contamination in the final product. Similarly, production of recombinant cholinesterases in mammalian cell cultures (e.g. Kronman *et al.*, 1992; Velan *et al.*, 1991) is also confronted with limited scalability, high costs, and risk of pathogen contamination. For AChE-R, mammalian-based production systems seem less promising for large-scale production because of the natural low levels and relative instability of the protein and its cognate mRNA in such systems (Chan *et al.*, 1998; Cohen *et al.*, 2003). Recent reports (Cerasoli *et al.*, 2005) describe the use of transgenic goats expressing human BChE in their milk. The limitations of this technique include low efficiency, high cost, and current lack of a regulatory framework for the production of pharmaceuticals in lower mammalian species (Baldassarre *et al.*, 2004). Additionally, transgenic animals must be consistently maintained, and production and purification should occur continuously.

In the 16 years since the first tentative report of expression of pharmaceutically relevant human proteins in plants (albumin; Sijmons *et al.*, 1990) to the recent USDA approval of the first plant-derived vaccine (Vermij, 2006), the use of plants as a means to produce protein pharmaceuticals (so called ‘‘molecular pharming’’) is slowly coming of age with major scientific strides enabling this novel technology to challenge the conventional fermentation-based technologies. As an alternative to other available technologies for the production of recombinant proteins, transgenic plants and cultured cells thereof offer several unique advantages such as low costs of production and scale-up (and equivalent costs for purification) as well as biosafety (Fischer and Emans, 2000; Mason *et al.*, 2002).

Transgenic plants have been shown by us to be capable of producing AChE functionally equivalent to other sources (Evron *et al.*, 2007a; Fletcher *et al.*, 2004; Geyer *et al.*, 2005, 2007; Mor and Soreq, 2004; Mor *et al.*, 2001b). Plant-produced bioscavengers have key advantages including equivalent quality and improved safety of the product (plants do not contain human pathogens or agents such as prions) as well as significant savings on production costs. We conservatively estimate costs of raw materials containing the target protein to be $20/g of target protein, with purification and regulatory costs which are comparable to other production systems used. Of major importance to pharmacoeconomics, however, is that the increased production of more plant material is scalable by simply planting more seed, and does not require a ‘‘before the market’’ capital investment decision on construction of fermentation facilities. This is particularly relevant for some products such as bioscavengers for which a strategic reserve must be created with the capacity to scale up production if/when an emergency occurs; in the plant-production scenario, advanced research can create the seed supply which can be processed as needed for a drug supply and replenished as needed by simply growing more of the crop. But, the single most important aspect of plant-derived enzyme is the scalable expansion of production that will be suitable for establishment of an industrial scale supply.

D. AChE-R_{ER} Amelioration of Chronic OP Toxicity Effects: Interference with AChE Gene Expression

The long-term effects of sublethal exposure to OPs are muscle fatigue and cognitive decline, including cardiac failure and memory impairments (Rosenstock *et al.*, 1991). Recently, an association was reported between exposure to sublethal doses of sarin and anatomical brain damage, including reduced white matter volume and increased lateral ventricle volumes (Heaton *et al.*, 2007). In a seminal series of experiments recently reported by our groups we have tested the effects of treating mice with plant-derived AChE-R in conjecture with their exposure to OPs (Evron *et al.*, 2007a). Interestingly, by 10 days post-exposure, AChE-R prophylaxis ameliorated the chronic effects of OP toxicity by mitigating the long-term up-regulation of AChE gene expression: it mitigated plasma murine AChE-R levels as well as the NMJ dismorphology. However, in addition to its capacity to restore the cholinergic balance immediately after the acute intoxication, prolonged overproduction of AChE-R associates with overproduction of pro-inflammatory cytokines (Grisaru *et al.*, 2006), behavioral impairments (Birikh *et al.*, 2003), declarative memory loss (Farchi *et al.*, 2007b; Nijholt *et al.*, 2004), muscle malfunctioning (Brenner *et al.*, 2003), and excessive myelopoiesis (Gilboa-Geffen *et al.*, 2007; Pick *et al.*, 2006). Pretreatment with the enzyme would likely cause a transient elevation of inflammation markers (Grisaru *et al.*, 2006). Nevertheless, the relatively rapid clearance of the plant-derived AChE-R_{ER}, which makes it transient, would limit the duration of such effects. Moreover, interference with the overproduction of endogenous AChE-R in the circulation should further limit the duration of such symptoms, avoiding much of the delayed post-poisoning phenotype which is due to exposure-induced AChE-R accumulation. These findings highlight the advantages of plant-produced AChE-R_{ER} for both short- and long-term protection.

By utilizing AChE-R, a known positive modulator of inflammation, we have demonstrated a means for counteracting the immunosuppressive actions of nicotinic AChR stimulation. AChE-R has been shown to induce a pro-inflammatory cytokine response as well as an enhanced cellular response. We demonstrated that the effects of OP-induced immunosupression in skeletal muscle are reversed following treatment with AChE-R. Thus in AChE-R we have produced a therapeutic protein that is capable of preventing both the acute presentation and chronic dysmorphology and immunosuppression of OP toxicity.

The result of the inherent comparison of AChE and BChE that was completed in our laboratory revealed two very adept OP bioscavengers. The major difference noted in this study was an ineffectiveness of oxime-mediated reactivation of BChE relative to the accelerated rates seen with AChE (Geyer, Cerasoli, Lenz, and Mor, unpublished results). In the case of both enzymes, by utilizing purification protocols that were only mildly adapted from those used to purify serum BChE or recombinant ChEs we can assume that any associated purification costs will be similar to those incurred in other systems. Before this project is ready to transition into a clinical study, a further refinement of the PEGylation and other modifications that have been shown to enhance the circulatory profile of these plant-derived ChEs must be undertaken.

E. Selective RNA-Targeted Suppression of AChE Gene Expression

That AChE-R overexpression leads to muscle weakening (e.g. in myasthenic animals; Brenner *et al.*, 2003) tentatively attributed these symptoms to AChE-R accumulation. The effects of excess AChE-R in neuromuscular pathologies could be alleviated by small molecule inhibitors, but their effects were short-lasting. This could be due to the failure of the small molecule inhibitors to distinguish between the different AChE variants. Thus, indiscriminate inhibition of both AChE-S and AChE-R would induce general increases in ACh and subsequent feedback overproduction of yet more AChE-R, exacerbating muscle weakness. Supporting this notion, the effect of such small molecule inhibitors to improve muscle functioning in human MG patients has also been known as short-lasting, to the extent that patients typically require oral drug administration every 4–6 h, and up to every 3 h in more refractory cases (Drachman, 1994; Ropper and Brown, 2005). Antisense oligonucleotide suppression of nascent AChE-R mRNA transcripts emerged as an effective means for challenging the hypothesis that AChE-R was causally involved with these symptoms, as well as a promising therapeutic alternative. Designated AS3 (Galyam *et al.*, 2001; Grifman and Soreq, 1997; Grisaru *et al.*, 1999b), EN101 (Dori *et al.*, 2005), or Monarsen (Argov *et al.*, 2007; Kehat *et al.*, 2007), the AChE mRNA-targeted agent is a 20 residue long antisense molecule, 2′-oxymethylated at its three 3′-terminal positions. It is targeted at exon 2 of AChE mRNA, which is common to the different AChE mRNA splice variants (Meshorer and Soreq, 2006; Soreq and Seidman, 2001). Mouse, rat, and human EN101/Monarsen are each complementary to the corresponding exon 2 sequence in AChE mRNA. Nevertheless, the nascent, relatively unstable AChE-R transcripts (Chan *et al.*, 1998) demonstrated particular sensitivity to the antisense treatment compared to AChE-S mRNA. This was the case in cultured primary brain cells (Meshorer *et al.*, 2002), live mice (Birikh *et al.*, 2003; Cohen *et al.*, 2002; Nijholt *et al.*, 2004), and human-originated cell lines (Grisaru *et al.*, 2001; Perry *et al.*, 2004).

In muscle, AChE's catalytic activity was increased 2 weeks following repeated DFP exposure for 4 consecutive days. This increase reflected the overexpressed AChE-R and could be significantly reduced by systemic antisense treatment. EN101/Monarsen treatment brought down AChE's catalytic activity to the range of control animals (Lev-Lehman *et al.*, 2000). Importantly, this treatment further prevented the increase in NMJ endplate density, supporting the notion that these plastic changes were also attributed to AChE-R. The muscle weakness characteristic of AChE-R excess resembles the reported weakness syndrome that follows continuous or repeated exposure to agricultural anti-AChE pesticides, such as the OP paraoxon (Ray and Richards, 2001) or warfare agents such as those associated with the Gulf War Syndrome (Haley *et al.*, 1999). Thus, excess AChE-R may be pivotal for rapid regaining of homeostasis following OP exposure, but its long-term accumulation entails multiple delayed damages.

The success of EN101/Monarsen treatment in animal models led recently to clinical trials in human myasthenic patients, which are simultaneously run in Israel and the UK. EN101/Monarsen is provided at a daily single orally delivered dose followed by measurable improvement in muscle functioning (Argov *et al.*, 2007). The reported effect lasts over 24 h, with a 100-fold lower required molar dose than that of pyridostigmine.

F. RNA-Targeted Suppression vs Small Molecule AChE Inhibitors

Small molecule AChE inhibitors that are currently used in therapy are unable to distinguish between the different AChE variants, since the different variants all harbor identical active and peripheral anionic sites. In principle, EN101/Monarsen is also designed to bind a common sequence of all of the AChE mRNA variants; however, it was experimentally demonstrated that the nascent transcript of AChE-R is more sensitive to EN101/Monarsen suppression (Grisaru *et al.*, 2001). EN101/Monarsen suppressed AChE-R in EAMG rat muscles as well as in monkey spinal cord neurons, leaving AChE-S almost unaffected (Brenner *et al.*, 2003; Evron *et al.*, 2005). Moreover, anti-AChEs induce a feedback response and up-regulation of the AChE-R variant, suggesting that they would actually exacerbate

AChE-R overexpression in myasthenic muscles, and perhaps explaining their short-lasting effect. In contrast, EN101/Monarsen should not activate this feedback loop, providing yet another added value to its use.

Based on the above considerations, using EN101/Monarsen to enhance NMJ functioning may also be relevant for gaining effective recovery from neuromuscular blockade (NMB), used in the clinic to achieve surgical relaxation or facilitate mechanical ventilation in critically ill patients (Bom *et al.*, 2007). The majority of neuromuscular blocking agents (NBAs) in the clinical practice are competitive antagonists of the post-synaptic nAChR. These mimic the structure of ACh and thus operate as blockers of the corresponding AChR. After terminating the infusion of an NBA, a large portion of the post-synaptic AChR remains bound to the NBA, hence being unavailable for neuromuscular transmission for a long period of time. This results in residual NMB, which may be seen in over 40% of patients following general anesthesia (Debaene *et al.*, 2003). Recovery from NMB is achieved when ACh quanta levels at the synaptic cleft overcome the blocking effect of the NBA. EN101/Monarsen may possibly be used for reversal of NMB; due to its prolonged effect, it may also prevent residual NMB in anesthetized and paralyzed patients. This could be an advantage over current AChE inhibitors in clinical use such as neostigmine or edrophonium, which have relatively short-lived action that can produce recurrence of NMB with hazardous effects on respiration. EN101/Monarsen could potentially become a ''cleaner'' drug with precise effects.

G. Modified Cytokine Expression under RNA-Targeted Suppression of AChE-R

In EAMG animals and MG patients, EN101/Monarsen treatment promoted an improved clinical status (and improved survival of EAMG rats). In Cynomolgus monkeys, systemic EN101/Monarsen administration (either oral or intravenous) caused a reduction in pro-inflammatory cytokines within spinal cord interneurons (Brenner *et al.*, 2003; Euron *et al.*, 2005). These neurons are known to carry the α7 nicotinic ACh receptor (Hellstrom-Lindahl *et al.*, 1998), which in macrophages controls the levels of neuronal pro-inflammatory cytokines such as IL-1β and IL-6 (Wang *et al.*, 2003). Since oligonucleotides are usually unable to cross the BBB (Jaeger and Banks, 2004), it has been considered that the observed anti-inflammatory effects were initiated in the periphery (either in the digestive tract or in the circulation). Importantly, cumulative data show peripheral transduction of altered inflammatory cytokines also beyond the BBB. For example, induction of inflammation by systemic administration of LPS was shown to up-regulate pro-inflammatory cytokines, such as IL-1β within the rodent brain without LPS penetration of the BBB (Pitossi *et al.*, 1997; Quan *et al.*, 1994; Turrin *et al.*, 2001). Furthermore, systemic administration of LPS or IL-1β regulates CNS neuronal function by stimulating the vagal sensory pathways (Ek *et al.*, 1998). Compatible with this hypothesis, systemic EN101/Monarsen suppression of AChE activity in plasma (as was shown in the plasma of treated monkeys) can elevate ACh levels in the periphery, which can reduce the levels of peripheral pro-inflammatory cytokines. This would subsequently reduce the cytokine effects on brain neurons, suppressing the reciprocal production of yet more cytokines in these neurons. This self-propagating pathway may explain the increase of pro-inflammatory cytokines under cholinergic imbalances (e.g. exposure to OPs; Svensson *et al.*, 2001) and points at the neuronal inflammatory response as a body ''self-defense'' machinery protecting it from excessive cholinergic load.

X. CONCLUDING REMARKS AND FUTURE DIRECTION

Throughout this chapter we have explored current thinking and experimental data in their support of the principal roles that cholinesterases play in maintaining synaptic and systemic cholinergic homeostasis. This view puts the various protein members of the cholinesterase family, especially the readthrough variant of AChE, at a crucial and therefore vulnerable intersection between the central and peripheral nervous systems and other body networks such as the hematopoietic and immune systems. We presented an intricate balancing act consisting of transcriptional, post-transcriptional, and post-translational regulation aiming at mitigating the short- and long-term consequences of exposure to chemical stressors that interfere with cholinergic neurotransmission and have system-wide repercussions. Through gain and loss of function experimental approaches and the use of various *in vivo*, *ex vivo* and *in vitro* systems, a more coherent picture is beginning to emerge, a picture that is also suggestive of novel therapeutic strategies relying on enzyme supplementation in combination with RNAi agents to restore a healthy cholinergic status by carefully manipulating AChE levels to ameliorate the chronic effects of OP toxicity.

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CHAPTER 47

Chemical Warfare Agents and Risks to Animal Health

TINA WISMER

I. INTRODUCTION

Animals are susceptible to all four basic types of military agents: choking (chlorine, phosgene), blister (mustard, lewisite, phosgene oxime), blood (cyanide, hydrogen cyanide), and nerve agents (tabun, sarin, soman, VX). They can also be affected by incapacitating agent BZ (3-quinuclidinyl benzylate), riot control agents, ricin, and abrin. Since chemical warfare agents can be deployed by a variety of inexact methods (bomb, spray tanks, rockets, missiles, land mines, and artillery projectiles), domestic and wild animals living in proximity to human populations will be affected (USACHPPM, 2001a). Americans own 62 million dogs and 68 million cats and more than half of American households own at least one pet (Wise *et al.*, 2003). Due to the expansion of suburbia, human–wildlife interactions are also increasing (Daszak *et al.*, 2004). Livestock are very important potential targets of attack as they have secondary ramifications for human health and disruption of the food chain.

The CDC Strategic Planning Working Group, as part of their preparedness plan for possible terrorist attacks using biological or chemical weapons, has called for ''prompt diagnosis of unusual or suspicious health problems in animals'' (Anonymous, 2000a). The CDC recommended establishing ''criteria for investigation and evaluation of suspicious clusters of human and animal disease or injury and triggers for notifying law enforcement of suspected acts of chemical terrorism''. With many of the military agents, there are few initial indicators of a chemical attack. It has been proposed that animals could serve as sentinels for chemical terrorism. These animals would be similar to the canaries used by coal miners in the UK and USA to provide early warning of deadly mine gases (Burrell and Seibert, 1914).

Throughout history, it has been noted that during chemical warfare attacks, animals may also be affected. A newspaper article from World War I gives details of the effects on animals during an unspecified type of gas attack (Anonymous, 1918):

> Results show that horses suffer much from the noxious fumes, and are subsequently thrown into a state of nervous terror on again scenting them. Mules are more inclined to stand their ground, and appear as if trying not to breathe … cats quickly scent the gas, and run about howling. Guinea pigs are first to succumb … Rats and mice emerge from their holds, and are found dead in quantities, which as the soldiers say, is the only advantage of a gas attack by the enemy.

The same account discusses how much these animals meant to the soldiers as they even managed to fashion gas masks for several of these species to protect them during gas attacks (Anonymous, 1918). The soldiers believed that different species of animals may have been more sensitive to certain types of gas attacks than humans:

> … Poultry of all kinds are useful for giving warning, ducks and fowl becoming agitated 10 min or so before the oncoming gas clouds. Many kinds of wild birds are greatly excited, and the usually unruffled owl becomes, as it were, half demented. Only the sparrow seems to disregard the poisonous vapor, and sparrows chirp on where horses are asphyxiated, and bees, butterflies, caterpillars, ants and beetles die off in great numbers. The gas at once kills snakes, and earthworms are found dead in their holes many inches below the ground.

Not much has changed in using animals for sentinels over the years. Crates of rabbits were placed on the cargo deck of ships transporting nerve gases during World War II and crewmen were instructed to watch for sudden animal deaths that could signal a gas release (Brankowitz, 1987). Even today with the development of sophisticated biosensor technology the use of animals as sentinels continues to be explored (Paddle, 1996). After the Aum Shinrikyo sarin attack in Tokyo, Japanese policemen used canaries to serve as a warning of poison gas release (Biema, 1995). The USA also planned to use chickens during the initial invasion of Iraq in 2003 (Ember, 2003). The chickens were to be used as early warnings of nerve gas agents. It was thought that if caged chickens remained alive following a warning alert, it would be safe for soldiers to remove their gas masks. Using live animals would help avoid possible false alarms with ion-mobility spectrometry biosensors. The plan was never implemented as there were no controlled studies showing that chickens were likely to show effects of nerve agents before humans (Garamone, 2003). The EPA is now

considering evidence regarding the use of animals as sentinels for chemical threats (EPA, 2006).

Some of the important chemical warfare agents that may pose risks to animal health are described below. For the information on their mechanism of action, readers are referred to Section II of this book.

II. CHEMICAL WARFARE AGENTS

A. Chlorine Gas

1. Clinical Signs

Chlorine gas is very irritating, or in concentrated amounts, even corrosive. Eyes, skin, nose, throat, and mucous membranes can all be affected. When inhaled or ingested, chlorine combines with tissue water and forms hydrochloric acid, and reactive oxygen species. Oxidation of respiratory epithelium leads to alveolar capillary congestion and accumulation of edematous fluid (Noe, 1963). Death is due to cardiac arrest from hypoxemia secondary to atelectasis, emphysema, and membrane formation (Decker, 1988).

Ocular exposure can result in severe pain and blindness. Erythema and pain are also common after dermal exposure. Both liquid and high concentrations of chlorine gas can cause dermal burns (Raffle *et al.*, 1994). Inhaled chlorine gas causes rhinorrhea, ataxia, syncope, muscle weakness, dyspnea, tachypnea, bronchospasm, and acute lung injury. Inhalation of high concentrations can cause laryngospasm, tachycardia, and hypoxia (Noe, 1963). Cardiovascular collapse and respiratory arrest may develop and lead to rapid death.

Chlorine is teratogenic. One hundred ppm when given to pregnant rats caused both biochemical and metabolic effects in the newborns, while 565 mg/kg given prior to mating was embryotoxic (RTECS, 2008). Carcinogenicity can be seen with chronic exposures (Morris *et al.*, 1992; RTECS, 2008).

2. Kinetics

Respiratory, dermal, and ocular irritation starts immediately. The speed of onset and severity of signs is directly related to the concentration (Bingham *et al.*, 2001). The water solubility of chlorine allows it to have a greater effect on the lower respiratory tract as a large percentage bypasses the upper airways. Acute lung injury peaks in 12 to 24 h. Death usually occurs within 48 h (Decker, 1988).

With mild exposures, signs disappear within 6 h, but can continue for more than 24 h with severe exposures. Exposure to moderate or severe concentrations can result in chronic respiratory dysfunction (Decker, 1988; Schwartz *et al.*, 1990). This can be career or life ending if the affected animal is an equine athlete or a working dog.

3. Decontamination and Treatment

Move animals into fresh air and onto higher ground. Monitor respiratory rates and oxygenation status (SpO_2). If coughing or dyspnea develops, provide supplemental oxygen and ventilation. Bronchodilators should be used to counteract bronchospasm (Guloglu *et al.*, 2002). Sedation and pain control may be needed so the animal can be handled safely.

Corticosteroid use is controversial. Animal models have shown positive results in hastening recovery from severe chlorine gas poisoning; however, administration to humans has not been shown to provide any significant change (Traub *et al.*, 2002). Wang *et al.* (2004) demonstrated that pigs exposed to chlorine gas responded better to a combination of aerosolized bronchodilators and corticosteroids (terbutaline, budesonide) than to either therapy alone. Sheep nebulized with 4% sodium bicarbonate had decreased mortality and improved oxygenation after inhalation of chlorine gas (Chisholm *et al.*, 1989).

For ocular exposures, eyes should be flushed with generous amounts of tepid 0.9% saline or tap water for at least 15 min. After flushing, fluorescein should be used to stain the eyes and check for corneal ulcers (Grant and Schuman, 1993). Bathing with dish soap and water will remove chlorine from the skin and fur/feathers.

Chlorine does not leave an environmental residue, so animals may be returned to affected pastures within hours to days (more quickly in warm environments) (Munro *et al.*, 1999). When entering the area contaminated with chlorine gas, rescuers should wear self-contained breathing apparatus (SCBA) and protective clothing (gloves, gowns, masks) until the gas dissipates. The risk for secondary contamination of rescuers is low. Chlorine gas does not bind to leather or fabrics.

4. Species Susceptibility

Chlorine gas is heavier than air and will settle in low areas. Pets and smaller livestock may be more at risk than humans due to their proximity to the ground. There are no controlled studies that show animals are more sensitive to chlorine gas or that they will develop signs sooner than humans.

B. Phosgene

1. Clinical Signs

Most animal exposures to phosgene are from inhalation, but there can also be dermal exposures to the liquefied material. The severity of pulmonary injury correlates with the concentration and length of exposure, and initial symptoms are not always a good indicator of prognosis (Diller, 1985; Bingham *et al.*, 2001). Due to low water solubility phosgene is able to penetrate deeply into the lungs where it irritates the lower respiratory tract (Franch and Hatch, 1986). When phosgene gas contacts water in the lungs, it forms hydrochloric acid causing cellular injury (Murdoch, 1993). Phosgene also causes protein and lipid denaturation and changes in membrane structure, leading to increased pulmonary vascular permeability (Borak and Diller, 2001). This fluid accumulation in the lung interstitium and alveolae results in gas diffusion abnormalities and pulmonary edema

(Diller, 1985; Ghio *et al.*, 1991). Pulmonary injury is exacerbated by elevated levels of leukotrienes and neutrophil chemotactic agents. The neutrophils release cytokines and other reactive mediators worsening pulmonary injury (Ghio *et al.*, 1991; Sciuto *et al.*, 1995). Localized emphysema and partial atelectasis occur. Death is from anoxia secondary to pulmonary edema (Borak and Diller, 2001; Proctor and Hughes, 2004).

Dyspnea, cough, cyanosis, and hemoptysis can progress to hypoxemia and hypoventilation (Borak and Diller, 2001). Animals may develop secondary organ damage from anoxia. Dogs experience bradycardia followed by tachycardia and progressive hypotension with severe phosgene poisoning (Patt *et al.*, 1946). With concentrations >200 ppm, phosgene can enter the blood and cause hemolysis and coagulopathies (Sciuto *et al.*, 2001). Direct contact with liquid phosgene can cause dermal burns (Proctor and Hughes, 2004). Ocular exposure to both liquid phosgene and highly concentrated phosgene gas can cause severe eye irritation and corneal opacification (Grant and Schumann, 1993; Proctor and Hughes, 2004).

Prognosis is directly related to the severity of pulmonary injury. Animals that survive the first 24 to 48 h still have a guarded prognosis. These animals are more susceptible to infectious agents as they have suppressed natural killer cell activity. Infections may become evident 3 to 5 days after exposure. Animals may develop chronic exercise intolerance and abnormal pulmonary function (Borak and Diller, 2001). Working dogs and equines may no longer be able to fulfill their functions.

2. Kinetics

Respiratory signs develop 2 to 6 h post-exposure in most patients, but can be delayed up to 15 h with exposures to lower concentrations (<3 ppm) (Borak and Diller, 2001). Concentrations of 3 to 5 ppm cause immediate conjunctivitis, rhinitis, pharyngitis, bronchitis, lacrimation, blepharospasm, and upper respiratory tract irritation. Extended (170 min) exposure was fatal (Diller 1985; Proctor and Hughes, 2004). Fifty ppm for 5 min or longer will cause pulmonary edema and rapid death (Borak and Diller, 2001; Chemstar, 1996; RTECS, 2008). If the animal survives, pulmonary edema begins to resolve in 2 to 3 days.

3. Decontamination and Treatment

Move animals to fresh air and higher ground. Bathe animals with soap and water and flush eyes for 15 min with tepid water or 0.9% saline. Animals should be monitored for 24 h for the development of pulmonary edema (Borak and Diller, 2001). If coughing or dyspneic, administer 100% oxygen. Animals may need to be intubated and ventilated. Animals can be nebulized with beta adrenergic agonists to combat bronchospasm. Patients with pulmonary edema should be managed the same as an ARDS (acute respiratory distress syndrome) patient (mechanical ventilation with oxygen and positive end-expiratory pressure).

Rabbits exposed to toxic levels of inhaled phosgene, did not develop noncardiogenic pulmonary edema if given intravenous aminophylline and subcutaneous terbutaline within 10 min of exposure (Kennedy *et al.*, 1989). Intertracheal *N*-acetylcysteine (Mucomyst®) administered to rabbits 45 to 60 min after inhalation of phosgene (1,500 ppm/min) decreased pulmonary edema, production of leukotrienes, and lipid peroxidation, and maintained normal glutathione levels as compared to rabbits exposed to phosgene only (Sciuto *et al.*, 1995). Rat and rabbit studies have shown that ibuprofen also protects against acute lung injury from phosgene (Guo *et al.*, 1990). Oxygen, sodium bicarbonate, and aerosolized surfactant have all been shown to be beneficial in dog experiments (Mautone *et al.*, 1985). Corticosteroids, prostaglandin E1, and atropine may be helpful in reducing phosgene-induced pulmonary edema (Chemstar, 1996). Intravenous fluids should be used for cardiovascular support, but monitor for overhydration. Colloids are preferred over crystalloids as they will remain in the vascular space for a longer period of time. Oxygen supplementation will resolve most of the arrhythmias.

Phosgene is nonpersistent in the environment. Moisture reduces air concentrations (Borak and Diller, 2001). The potential for secondary contamination of rescue personnel is low, but rescuers should wear proper protective clothing. Phosgene gas does not persist in fabric or leather.

4. Species Susceptibility

Phosgene is heavier than air and will settle close to the ground. This can affect species that are low to the ground or that are pastured in low-lying areas. There is no indication that animals are affected before or at lower levels than their human counterparts.

C. Mustard Gas

1. Clinical Signs

Mustard gas is a vesicant that is toxic by all routes of exposure (oral, inhaled, dermal, and ocular) (EPA, 1985b; Lewis, 2000; Pohanish, 2002; Sidell *et al.*, 1997). Mustard causes both localized and systemic cellular damage and tissues with high cell turnover are the most affected (NATO, 1973). Mustard gas can produce erythema, severe pruritus, blistering, ulceration, and necrosis of exposed skin (Borak and Sidell, 1992; Budavari, 2000; Dacre and Goldman, 1996; Pohanish, 2002). Skin lesions are most severe at warm and moist sites (genitalia, perineal regions, groin, skin folds, and axillae), due to the high number of sweat glands in these areas. Amounts as low as 0.02 mg of mustard will cause blisters (Smith *et al.*, 1997).

Ocular exposure can cause pain, lacrimation, corneal ulceration, swelling, blepharospasm, and blindness as the eyes are very sensitive to the effects of mustard gas (Borak and Sidell, 1992; Dacre and Goldman, 1996; Garigan, 1996; NATO, 1973). Pathognomonic signs of mustard gas poisoning include porcelain-white areas in the episcleral

tissues and formation of large, varicose veins (Grant and Schuman, 1993). Conjunctivitis and keratopathy can be seen chronically after exposure (Blodi, 1971).

Inhalation of small amounts of mustard gas produces nasal discharge, sneezing, epistaxis, and coughing within 12 to 24 h of exposure. Higher concentrations or longer exposures can cause pulmonary damage, hypoxia, and respiratory acidosis. Seizures may be seen with extremely acute, high doses (Sidell *et al.*, 1997).

Mustard gas is also a radiomimetic (Sidell *et al.*, 1997). It destroys precursor cells in the bone marrow leading to leukopenia, thrombocytopenia, pancytopenia, and anemia (Borak and Sidell, 1992; Dacre and Goldman, 1996). Infection can be seen secondary to bone marrow damage (Sidell *et al.*, 1997). Bone marrow aplasia and death can be seen in severe cases.

Mustard gas is a possible animal teratogen. Some rat studies show abnormalities of the musculoskeletal system in the offspring of rats orally dosed with mustard gas, but only at doses high enough to be toxic to the dams, while other rat and rabbit studies showed no correlation (Dacre and Goldman, 1996; RTECS, 2008). Injection of mustards both IV and IP into male mice caused inhibition of spermatogenesis, but full recovery was seen within 4 weeks (Dacre and Goldman, 1996). Mustard gas is both a human and animal carcinogen (NTP, 2005). It has been linked to carcinomas in the skin, limbs, lungs, thorax, and leukemia in rats and mice after inhalation and IV exposure (RTECS, 2008).

2. Kinetics

Both liquid and vaporized mustard have rapid skin penetration. The higher the dose, temperature and humidity, the quicker the absorption (NATO, 1973). Mustard is dermally absorbed through hair follicles and sweat glands within minutes. Cellular reactions begin within 1 to 2 min of contact of mustard to skin or mucous membranes, but clinical effects are delayed between 2 and 24 h (Grant and Schuman, 1993; Sidell *et al.*, 1997).

The skin initially pales and then becomes erythematous within a few hours of exposure (Requena *et al.*, 1988). Erythema, blisters, bulla, and small vesicles form over 2 to 24 h. The blisters can progress for several more days. Erythema resolves over 3 to 7 days, while ulcers take 6 to 8 weeks to heal (Garigan, 1996; Sidell *et al.*, 1997). Discoloration (brown or black hyperpigmentation) commonly occurs after resolution of the burns, especially in areas with thinner skin (Requena *et al.*, 1988).

Ocular absorption happens within minutes. With mild ocular exposures, conjunctivitis and lacrimation begin about 4 to 12 h after exposure. Moderate ocular exposures produce conjunctivitis, blepharospasm, lid inflammation, corneal damage, and eyelid edema about 3 to 6 h post-exposure. Severe ocular exposures will lead to marked swelling of lids, corneal ulceration, corneal opacification, severe pain, and miosis in 1 to 2 h (Requena *et al.*, 1988).

Inhalation produces respiratory signs (rhinorrhea, sneezing, epistaxis, and coughing) within 12 to 24 h of exposure. A severe exposure produces a productive cough, tachypnea, pulmonary edema (rare), and pulmonary hemorrhage within 2 to 4 h. Studies in dogs indicate that equilibrium between blood and tissues was achieved within 5 min after inhalation (IARC, 1975). Ingestion of small amounts can cause hypersalivation and vomiting within 24 h, while larger amounts can cause gastrointestinal bleeding (rare) and bloody diarrhea within 3 to 5 days.

Mustard preferentially accumulates in fatty tissue (in decreasing concentration: fat, skin with subcutaneous tissue, brain, kidney, muscle, liver, cerebrospinal fluid, spleen, and lung) (Somani and Babu, 1989). Excretion is through the urine in rabbits, mice, and rats (IARC, 1975). Mustard is excreted over 72 to 96 h after IV administration in rats and mice (Dacre and Goldman, 1996; Maisonneuve *et al.*, 1993). Complete blood count (CBC) changes are not evident for 3 to 5 days post-exposure. Leukopenia usually occurs at day 7 to 10 (Garigan, 1996).

3. Decontamination and Treatment

Animals should be moved into fresh air. Emesis is not recommended and activated charcoal administration after oral ingestion is controversial. Activated charcoal appears to have some beneficial effects if administered within 1 h of ingestion. Sodium thiosulfate (2% solution) given orally may help in cases with oral exposures (Borak and Sidell, 1992; Dacre and Goldman, 1996). Perforation and stricture formation can follow esophageal burns.

For ocular exposures, flush eyes with tepid water for at least 15 min. Follow with 2.5% sodium thiosulfate ophthalmic to help neutralize the mustard. The eyes need to be decontaminated quickly as mustard disappears from the eye very rapidly, and late flushing of the eye generally provides no benefit (Sidell *et al.*, 1997). Topical ophthalmic antibiotics and pain control should be used if corneal lesions are present (Sidell *et al.*, 1997). Corneal transplants may be considered for valuable animals (Blodi, 1971).

Animals need to be bathed with copious amounts of soap and water. If dermal decontamination is not implemented quickly, mustard will react with the skin and cannot be easily removed (Sidell *et al.*, 1997). Sodium thiosulfate (2.5% solution) can be used dermally to neutralize mustard exposures (Garigan, 1996). Animals may also be bathed with dilute (0.5%) hypochlorite solutions (Borak and Sidell, 1992). Monitor for dermal burns. Secondary infection is common. Topical silver sulfadiazine can be applied to all burns and an Elizabethan collar placed to decrease ingestion of the ointment and self trauma. Topically applied dexamethasone and diclofenac reduced inflammation in a mouse model when applied within 4 h (Dachir *et al.*, 2004). All equine and ovine patients should be inoculated with tetanus toxoid. Vaccination of other species should be determined on a case by case basis.

Monitor for respiratory irritation (coughing, dyspnea). If seen, monitor arterial blood gases, and pulse oximetry. Thoracic radiographs may be taken but there can be a lag time of up to 2 days before infiltrates are seen radiographically (Smith, 1999). Nebulization with 2.5% sodium thiosulfate or *N*-acetylcysteine may help neutralize the mustard gas. *N*-acetylcysteine may also be given intravenously as a potential mustard gas antagonist (Garigan, 1996). A loading dose of 140 mg/kg should be given, followed by 40 mg/kg every 4 h for a total of 17 doses (Garigan, 1996). Provide oxygen, ventilation, and inhaled beta agonists if needed. Dexamethasone, promethazine, vitamin E, and heparin have all shown protective effects against mustard gas poisoning in laboratory animals (Requena *et al.*, 1988; Vojvodic *et al.*, 1985).

Serial CBCs with platelets should be monitored for 2 weeks after exposure. Antibiotics should be given if leukopenia develops (Sidell *et al.*, 1997). Mustard can be detected in urine and body tissues for up to 1 week post-exposure using gas chromatography–mass spectrometry (Vycudilik, 1985). This can confirm diagnosis, but is not likely to be of value in the management of the patient.

Mustard is persistent in the environment. Mustard may remain in the environment for up to 1 week in temperate areas. It disappears more quickly in hot climates and in desert conditions; persistence is reduced to about 1 day. Since mustard binds to vegetation for days to weeks, grazing animals need to be kept away from these areas (USACHPPM, 2001b).

Rescue personnel must wear protective clothing, eye protection, and a respirator as the potential for secondary contamination is high (HSDB, 2008). Mustard gas will penetrate wood, leather, rubber, and paints.

4. Species Susceptibility

Animal model studies of skin exposure to nitrogen and sulfur mustard show that these agents cause more severe skin lesions in hairless animals and less in fur-covered species (Smith *et al.*, 1997). Dermal absorption of mustard varies greatly by species. Rats absorb about 75% of a dermal dose through their skin, while only 20% is absorbed through human skin (Hambrook *et al.*, 1992; Smith, 1999). The rat dermal LD_{50} is only 5 mg/kg while the mouse and human dermal LD_{50}s are 92 and 100 mg/kg, respectively (Lewis, 2000; RTECS, 2008). However, with oral dosing, humans appear to be much more susceptible (LD_{50} oral 0.7 mg/kg for humans, 17 mg/kg for rats). Due to these variable results, more studies are needed to determine if rats or mice would make good sentinel animals.

D. Lewisite

1. Clinical Signs

Lewisite is an arsenical compound that acts locally as a vesicant, but also causes systemic effects (HSDB, 2008; Sidell *et al.*, 1997). Lewisite directly affects enzyme systems, resulting in decreased ATP production. Lewisite also causes increased capillary permeability, leading to a significant loss of blood plasma into the extravascular space (''lewisite shock'') and hypotension (Sidell *et al.*, 1997). Lung capillaries are affected not only by inhalation but also by first pass through the lungs following dermal exposure. Pulmonary edema or ARDS can develop (Sidell *et al.*, 1997).

Signs can be seen when lewisite is contacted dermally, orally, ocularly, inhaled, or ingested. Dermal and respiratory exposures are seen most frequently. Lewisite causes dermal, ocular, and respiratory lesions similar to mustard gas. Lewisite is about ten times more volatile than mustard gas (Budavari, 2000).

Exposure to lewisite is very painful. Both the vapor and liquid lewisite can penetrate skin. Reddening of the skin is followed by tissue destruction (EPA, 1985a; Goldman and Dacre, 1989; Pohanish, 2002; Sidell *et al.*, 1997). Amounts as small as 0.5 ml may cause severe systemic effects and 2 ml may be lethal. Severe edema develops secondary to increased capillary permeability. Dermal burns are deeper than those seen with mustard gas and are quicker to appear (Goldman and Dacre, 1989; Sidell *et al.*, 1997).

Ocular exposure causes immediate pain, lacrimation, and blepharospasm. Without rapid decontamination, within 1 min, permanent blindness may occur (EPA, 1985a; Pohanish, 2002). In livestock this is a death sentence as they cannot survive on the range when blind. Droplets as small as 0.001 ml can cause corneal perforation and blindness (Sidell *et al.*, 1997).

Inhalation of vapor causes irritation to the nasal passages, rhinorrhea, and violent sneezing (HSDB, 2008). Coughing and hemoptysis commonly occur (HSDB, 2008; Sidell *et al.*, 1997). After inhalation, dogs developed necrotizing pseudomembranous laryngotracheobronchitis (Goldman and Dacre, 1989). Death can occur within 10 min after inhalation of high concentrations (EPA, 1985a).

Lewisite does not cause damage to the bone marrow or immunosuppression (Sidell *et al.*, 1997). Arrhythmias and renal dysfunction are due to hypovolemia from fluid loss. Lewisite was fetotoxic to rats and rabbits and is a suspected carcinogen (Goldman and Dacre, 1989; RTECS, 2008).

2. Kinetics

Immediate pain occurs upon inhalation, dermal, or ocular contact with lewisite. Skin penetration occurs within 3 to 5 min, especially following liquid exposures (Sidell *et al.*, 1997). The skin becomes red, then gray, within 15 to 30 min after exposure (EPA, 1985a; Goldman and Dacre, 1989; Pohanish, 2002; Sidell *et al.*, 1997). Severe blisters develop within 12 h. The blisters rupture about 48 h after occurrence, with large amounts of fluid seeping from the site. Healing is generally complete within 4 weeks (much faster than with sulfur mustard-induced lesions).

Lewisite has extensive tissue distribution (HSDB, 2008). The highest concentrations were found in the liver, lungs,

and kidneys in rabbits (greater than seven times blood concentration). Arsenic crosses the placenta and is excreted in the milk. Nursing animals may be at risk (Barlow and Sullivan, 1982). Elimination half-life of arsenic in rabbits is 55 to 75 h (HSDB, 2008).

3. Decontamination and Treatment

Remove animal from affected areas. If any coughing or respiratory distress, monitor blood gases and SpO_2. Provide oxygen and assisted ventilation as needed. Nebulized beta agonists and possibly corticosteroids can be used to treat bronchospasm. Monitor electrolytes and PCV as there can be fluid shifts out of the vasculature (Goldfrank *et al.*, 2002). Urine arsenic levels may be measured, but are not clinically useful due to the lag time before results are obtained. Watch for liver and kidney failure.

As lewisite is a vesicant, emesis is not recommended in those species that can vomit (dogs, cats, swine, and ferrets). Dilution with milk or water is recommended. Activated charcoal is not recommended as severe irritation/vesication of the esophagus or gastrointestinal tract is likely to occur. Endoscopy can be performed very carefully to determine the extent of injury. Esophageal perforation and/or stricture formation may occur.

Eyes should be flushed with copious amounts of tepid water for at least 15 min. If 5% BAL (dimercaprol, British Anti-Lewisite) ophthalmic ointment can be applied within 2 min this may prevent a significant reaction. Application up to 30 min after exposure will lessen the ocular reaction but will not prevent all damage (Goldfrank *et al.*, 2002).

Animals should be washed with water and dilute household bleach (5% sodium hypochlorite) as soon as possible. Application of a 5% BAL ointment within 15 min can be effective in diminishing the blistering effects of lewisite (Smith, 1999). Remove BAL ointment with soap and water after 5 min. Leaving the ointment on can cause stinging, itching, or urticaria. Burns should be managed with pain control, antibiotics, and debriding as needed.

A chelator should be given if there is dyspnea, pulmonary edema, or skin burns larger than palm size (Goldfrank *et al.*, 2002). BAL is the traditional arsenic chelator, but it has numerous side effects. The deep intramuscular injections are very painful and BAL can cause hypertension, tachycardia, and vomiting. 2,3-Dimercaptosuccinic acid (DMSA, Succimer®) can also be used to chelate arsenic (Graziano *et al.*, 1978). 2,3-Dimercapto-1-propanesulfonic acid (DMPS) is used in Europe and has been effective in protecting rabbits from the lethal effects of lewisite (Aposhian *et al.*, 1982).

Lewisite remains in the environment for about 24 h and it can react with water to form a solid arsenoxide that also has vesicant properties. Affected areas can be treated with strong alkalis to form less harmful substances. Rescue personnel need to wear protective clothing and masks as the risk for secondary contamination is high. Carcasses should be disposed of properly, either buried deeply (away from water supplies), rendered, or incinerated to insure safety of the food supply.

4. Species Susceptibility

Lewisite has the potential to cause skin lesions in any species but the risk is greatest in hairless animals such as pigs, and decreases in fur-covered species (Smith, 1997). Mice and rats appear to be almost twice as susceptible to dermal lewisite exposures than humans (LD_{50} equals 12, 15, and 30 mg/kg, respectively) (DeRosa *et al.*, 2002; RTECS, 2008; Sidell *et al.*, 1997). More studies are needed to determine if rodents would be good sentinel animals.

E. Phosgene Oxime

1. Clinical Signs

Phosgene oxime in both its liquid and vapor forms causes severe pain and local tissue destruction on contact with skin, eyes, and mucous membranes (Sidell *et al.*, 1997). Signs depend on its route of entry, as phosgene oxime exerts its greatest effects in the first capillary bed it encounters.

Dermal, ocular, and respiratory lesions are similar to those caused by mustard gas. Inhalation and oral absorption may cause respiratory tract irritation, dyspnea, and pulmonary edema. Dermal lesions are erythematous and extremely painful. With ocular exposure to phosgene oxime, very low concentrations can cause lacrimation, inflammation, and temporary blindness, while high concentrations can cause permanent corneal lesions and blindness (Sidell *et al.*, 1997; NATO, 1996; USACHPPM, 2001c). Death is generally due to respiratory arrest.

2. Kinetics

Complete absorption occurs in both dermal and inhalational exposures within seconds (Sidell *et al.*, 1997). Dermal lesions begin to form within seconds; grayish tissue damage may be seen within several minutes and within 1 h the area becomes edematous. Phosgene will spread in sweat and move to other nonexposed areas of the body (DeRosa *et al.*, 2002). Pulmonary edema can be seen on thoracic radiographs within 2 h of high-dose exposure, 4 to 6 h of moderate exposure, and approximately 8 to 24 h after low dose exposure (Sidell *et al.*, 1997). The skin turns brown and blistering occurs the next day. It takes about 3 weeks for desquamation, necrosis, crust formation, and purulent exudate to occur (NATO, 1996; Sidell *et al.*, 1997). Pain can last for several days and healing of dermal lesions can take from 1 month to over a year.

3. Decontamination and Treatment

Animals should be moved into fresh air. Emesis is not recommended due to the irritant and corrosive effects of phosgene oxime. Dilution with milk or water is recommended for oral ingestions. Activated charcoal is not recommended as the primary toxicity is a local corrosive

injury. Sedation and pain control (opioids) may be needed to allow safe decontamination.

Irrigate eyes with tepid water until pH returns to neutral and remains so for 30 min after irrigation is discontinued (Brodovsky *et al.*, 2000). Time until decontamination after ocular exposure is important since phosgene oxime is absorbed within seconds. Corneal ulcers should be treated with atropine ophthalmics to prevent synechiae formation and other ophthalmics to aid in re-epithelialization (Brodovsky *et al.*, 2000; Grant and Schuman, 1993).

The animal should be bathed with copious amounts of water and a mild soap. Phosgene oxime reacts quickly with tissue and decontamination is not expected to be entirely effective after pain has been produced. Sodium hypochlorite (0.5%) or isotonic sodium bicarbonate can help neutralize phosgene oxime that has not yet reacted with tissue. Burns should be managed with topical silver sulfadiazine and systemic antibiotics as needed (Roberts, 1988). Horses and sheep should receive tetanus prophylaxis. Other species should be vaccinated on a case by case basis.

Monitor oxygenation and thoracic radiographs in patients following significant exposures. Administer oxygen, perform endotracheal intubation, and provide assisted ventilation if needed. Nebulized beta adrenergic agonists can help if bronchospasm develops. Administer IV fluids but monitor for overhydration (Goldfrank *et al.*, 2002).

Phosgene oxime is nonpersistent in the environment and it hydrolyzes rapidly in aqueous alkaline solutions. Veterinary personnel and rescuers should wear aprons, rubber gloves, and masks when treating patients to avoid secondary contamination.

4. Species Susceptibility

There are no controlled studies showing that any species of animal would make a good sentinel for phosgene oxime exposure.

F. Cyanide and Hydrogen Cyanide

1. Clinical Signs

Cyanide and hydrogen cyanide (HCN) are classified as blood agents. They cause toxicity by forming a stable complex with ferric iron (Fe^{3+}) in cytochrome oxidase enzymes. This inhibits cellular respiration, oxygen utilization, and ATP production, causing deprivation of oxygen to the body at the cellular level (Way *et al.*, 1988). Both arterial and venous blood appears cherry red due to the accumulation of oxyhemoglobin (Bingham *et al.*, 2001; Lewis, 2000). Unhaired skin may also appear bright pink due to the high concentration of oxyhemoglobin in the venous return (HSDB, 2008). HCN and cyanogen chloride are the volatile, water-soluble, liquid forms of cyanide and are the most likely to be used for terrorism purposes.

Cyanide exposure can cause transient CNS stimulation, followed by syncope, ataxia, dyspnea, seizures, paralysis, apnea, and coma (Hall and Rumack, 1986; Vogel *et al.*, 1981). The odor of bitter almonds may be noted in gastric or ruminal contents and expired breath. Initial tachypnea is followed by respiratory depression. Dyspnea without cyanosis can help with the diagnosis. Chickens develop tachypnea, have rapid eye blinking, hypersalivation, and lethargy (Wiemeyer *et al.*, 1986). Blindness may occur from cyanide-induced damage to the optic nerve and retina (Grant and Schuman, 1993; Vogel *et al.*, 1981). Hypersalivation, vomiting, and abdominal pain may occur after ingestion (Hall and Rumack, 1986; Singh *et al.*, 1989; Vogel *et al.*, 1981). Metabolic and lactic acidosis is commonly seen. Death can occur within minutes.

2. Kinetics

Cyanide and HCN can be absorbed by all routes (inhalation, oral, ocular, and dermal) (Hall and Rumack, 1986). There is rapid diffusion into tissues and cyanide irreversibly binds to its target sites. Cyanide has a wide volume of distribution and will concentrate in red blood cells two to three times greater than in plasma (HSDB, 2008). Cyanide preferentially accumulates in the hypothalamus, with levels about 40% higher compared to other areas of the brain (Borowitz *et al.*, 1994).

Cyanide is metabolized by rhodanese in the liver to thiocyanate (Hall and Rumack, 1986). Thiocyanate is excreted mainly in the urine. Without administration of an antidote, the half-life for the metabolism of cyanide to thiocyanate is 20 min to 1 h (Feldstein and Klendshoj, 1954). Cyanide can be excreted in breast milk (Soto-Blancoand Gorniak, 2003).

3. Decontamination and Treatment

Remove animal from the affected area. Do not induce vomiting due to the rapid progression of the clinical signs and potential for seizures, coma, or apnea. One gram of activated charcoal will bind 35 mg of cyanide and activated charcoal may be beneficial if administered immediately after ingestion (Lambert *et al.*, 1988). Irrigate eyes for at least 15 to 20 min with tepid water. Bathe animals thoroughly with soap and water.

Blood gases and serum electrolytes should be monitored and corrected as needed (Hall and Rumack, 1986; Vogel *et al.*, 1981). Blood cyanide levels can confirm exposure, but due to the time needed to get the results, they are not clinically useful. Provide supplemental oxygen with assisted ventilation as indicated. Animal study results for hyperbaric oxygen therapy have been questionable (Way *et al.*, 1972). Acidosis (pH <7.1) should be corrected with intravenous sodium bicarbonate, but acidosis may not resolve until after the administration of antidotes (Hall and Rumack, 1986). Benzodiazepines or barbiturates can be used to control seizures.

Cyanide toxicosis progresses so rapidly that treatment is rarely administered to animals in time. If the animal is still alive but in respiratory distress or a coma, antidotal agents may still be life saving. The classic treatment for cyanide

intoxication includes several steps. Sodium nitrite is given intravenously over 15 to 20 min (fast administration causes hypotension). Sodium nitrite reacts with hemoglobin in the red blood cells to form methemoglobin. Methemoglobin combines with free cyanide to form cyanomethemoglobin. Sodium thiosulfate is given after sodium nitrite. Sodium thiosulfate supplies sulfur for the rhodanese reaction (Hall and Rumack, 1987). The sulfur reacts with cyanomethemoglobin to form hydrogen thiocyanate which is excreted in the urine.

Hydroxocobalamin (Cyanokit®) has been the chelator of choice in Europe and Australia, and was just approved for use in the USA in 2007. It works by combining with cyanide to form cyanocobalamin (vitamin B_{12}) (Hall and Rumack, 1987). Hydroxocobalamin has been shown to reduce mortality in rats, mice, and Beagle dogs and has the advantage of producing neither methemoglobinemia nor hypotension, as sodium nitrite does (Borron *et al.*, 2006; Hall and Rumack, 1987). Dicobalt-EDTA (Kelocyanor®) and 4-dimethylaminophenol hydrochloride (4-DMAP) are other chelators available in Europe, Israel, and Australia, but not in the USA (Hillman *et al.*, 1974; Weger, 1990).

Other substances that have been tested in the lab on animals include: stroma-free methemoglobin solutions, alpha-ketoglutaric acid, chlorpromazine, hydroxylamine, phenoxybenzamine, centrophenoxine, naloxone, etomidate, para-aminopropiophenone, and calcium channel blockers (Amery *et al.*, 1981; Ashton *et al.*, 1980; Bright and Marrs, 1987; Budavari, 2000; Dubinsky *et al.*, 1984; Johnson *et al.*, 1986; Leung *et al.*, 1984; Ten Eyck *et al.*, 1985; Yamamoto, 1990). The use of these substances has shown positive results, but they have not been tried during actual poisoning situations.

HCN is lighter than air and has a long half-life in air. However, in open spaces, HCN is rapidly dispersed and is diluted to nontoxic concentrations. Cyanide does not bind to soil or plant material, but can mix with water. Contaminated water can be treated with ozone, hydrogen peroxide, or calcium/sodium hypochlorite bleach. Rescue personnel should wear boots, gloves, goggles, full protective clothes, and a self-contained positive pressure breathing apparatus as the potential for secondary contamination is high (AAR, 2000).

4. Species Susceptibility

There are significant interspecies differences in the toxicity of hydrogen cyanide (Sousa, 2003). Dogs appear to be more susceptible than humans to cyanide poisoning. This is thought to be due to lower levels of endogenous rhodanese (hepatic enzyme that catalyzes the sulfuration of cyanide to thiocyanate) (Aminlari and Gilanpour, 1991). Barcroft (1931) exposed both a man and a dog simultaneously to hydrogen cyanide gas. The 70 kg man and 12 kg dog were exposed to HCN concentrations between 500 and 625 ppm in an airtight chamber. The dog became ataxic at 50 s, unconscious at 74 s, and began to seizure at 90 s. At 91 s, the man walked out of the exposure chamber with no symptoms, although over the next 10 min he developed transient nausea and difficulty concentrating (Barcroft, 1931). As HCN is lighter than air, it is probably not that the small dog experienced a higher exposure than the human.

Cats are more resistant to subcutaneous HCN than humans. The LDLo (SQ) for humans is 1 mg/kg, as compared to 11 mg/kg for cats (Sax and Lewis, 1989). The LDLo (oral) for HCN in humans, dogs, and rabbits is comparable (5.7, 4, and 4 mg/kg, respectively) (Sax and Lewis, 1989).

Dogs may be good sentinel animals as they appear to have increased susceptibility relative to humans based on physiological differences. More controlled studies are required, however.

G. Military Nerve Agents

1. Clinical Signs

Military nerve agents are probably the most toxic of the known chemical warfare agents. Military nerve agents are divided into ''G'' (for Germany) agents (sarin, soman, tabun) and ''V'' (for venomous) agents (VX). Nerve agents are extremely dangerous as they are absorbed without producing any irritation or other sensation on the part of the exposed person or animal (HSDB, 2008).

Nerve agents are organophosphates (OPs). Acute exposure to OPs can cause muscarinic, nicotinic, and CNS signs. Muscarinic effects include salivation, lacrimation, urination, dyspnea, diarrhea, emesis (SLUDDE) along with miosis, bradycardia, hypotension, and bronchoconstriction. Nicotinic effects include muscle fasciculations and weakness (including the diaphragm), tachycardia, hypertension, and mydriasis. CNS effects include restlessness, anxiety, seizures, and coma (Garigan, 1996). VX has CNS effects that are unrelated to AChE activity and prolonged effects may be seen following convulsive doses (Young *et al.*, 1999). Death is due to paralysis of the diaphragm, airway obstruction from increased bronchial secretions, or depression of the CNS respiratory center (Garigan, 1996).

The ''G'' agents present a vapor hazard as they are very volatile. VX has a high dermal toxicity, even through intact skin, as the liquid does not evaporate quickly (Berkenstadt *et al.*, 1991; Sidell *et al.*, 1997). VX is 300 times more toxic than tabun on skin. A very small drop on the skin may cause sweating and fasciculations at the site. A larger dermal drop may cause loss of consciousness, seizures, apnea, and flaccid paralysis. Toxicity in descending order, on a per weight basis, is: VX > soman > sarin > tabun (HSDB, 2008).

Delayed peripheral neurotoxicity has been reported in animal studies. Soman produced severe delayed neuropathy in the atropinized hen assay at 1.5 mg/kg (Willems *et al.*,

1984). Sarin, tabun, and VX cause post-implantation mortality and fetotoxicity (HSDB, 2008; RTECS, 2008).

2. Kinetics

Nerve agents can be absorbed by any route (ocular, oral, inhalation, dermal) (RTECS, 2008; HSDB, 2008). Onset of signs and duration of effects depend on the form of nerve gas (vapor, liquid) and the route of exposure. With a vapor exposure and inhalation, local signs of nasal discharge and respiratory noise begin within one to several minutes and signs can last for a few hours (mild exposure) up to 1 to 2 days (severe exposure) (Pfaff, 1998). Inhalation of a large amount of the vapor will result in sudden loss of consciousness, apnea, flaccid paralysis, and seizures within seconds to 2 to 3 min (Sidell *et al.*, 1997). Peak effects are seen within 20 to 30 min and death is usually due to respiratory failure (Berkenstadt *et al.*, 1991).

Liquid nerve agents applied dermally cause local sweating and muscular twitching starting 3 min to 2 h after exposure. Signs last for 3 to 5 days. Following dermal exposure to a large drop clinical effects start within 30 min but with small drops a delay of up to 18 h can be seen (Sidell *et al.*, 1997).

Ocular exposure to vapor causes miosis, conjunctival hyperemia, and eye pain within one to several minutes. Signs can last 2 to 3 days. Liquid tabun penetrates the eye quickly and can result in death nearly as rapidly as an inhalational lethal dose (1 to 10 min) (EPA, 1985c). Ingestion of the liquid causes muscarinic, nicotinic, and CNS signs about 30 min after mild exposures. The signs can last several hours up to 2 to 5 days depending on the amount of exposure.

Volume of distribution is slightly different for each of the nerve agents. Sarin is distributed to the brain, liver, kidney, and plasma of mice (Little *et al.*, 1986). Soman is distributed throughout the mouse brain, with the highest levels found in the hypothalamus (Wolthuis *et al.*, 1986). Tabun is also found in high concentrations in the hypothalamus after IV administration in mice (Hoskins *et al.*, 1986). Soman is unique in that it has apparent storage in body ''depots'' and is released over time. This release can result in eventual death in animals who survive the initial dose of soman (Wolthuis *et al.*, 1986).

Most military nerve agents have rapid ''aging'' of the OP–enzyme complex. After aging the inhibitor–enzyme complex becomes resistant to reactivation (Young *et al.*, 1999). VX and tabun are exceptions to this rule with aging half-lives ($t_{1/2}$) of greater than 40 h (Garigan, 1996). The aging $t_{1/2}$ is only a few minutes for soman and about 5 h for sarin (Garigan, 1996).

Sarin is metabolized to isopropyl methylphosphonic acid (IMPA) and excreted by the kidneys (Little *et al.*, 1986). Approximately 50% of soman is converted to free pinacolyl-methylphosphonic acid within 1 min in mice. The half-life of this metabolite is less than 1 h (Reynolds *et al.*, 1985).

Cholinesterase levels can take weeks to return to normal (Rengstorff, 1985). RBC AChE recovers even more slowly (several days to 4 months) depending on the severity of the depression (Grob, 1956). Pupillary reflexes also remain suppressed for weeks to 1.5 months (Rengstorff, 1985).

3. Decontamination and Treatment

Administer oxygen and remove the animal from the toxic environment. For ocular exposures, flush eyes with copious amounts of tepid 0.9% saline or water for at least 15 min. Wash all animals three times with either soap and water, dilute bleach solution (1:10 with water), ethanol, or a tincture of green soap (Cancio, 1993). Towelettes impregnated with alkaline chloramine and phenol are used by the military (M291 Skin Decontaminating Kit, Rohm and Haas).

Due to the rapid development of signs, emesis is not recommended in oral ingestion. Activated charcoal can be used in both oral and dermal exposures. Seizures can be controlled with diazepam, methocarbamol, or barbiturates as needed. Assisted ventilation may be necessary if signs progress.

Atropine is an antidotal treatment. It is used to reverse the muscarinic signs, but it will not reverse the nicotinic effects (muscular weakness, diaphragmatic weakness, etc.). Atropine blocks the effects of accumulated acetylcholine (ACh) at the synapse and should be continued until the nerve agent is metabolized (Midtling *et al.*, 1985). Overatropinization can cause hyperthermia, tachycardia, agitation, mydriasis, and ileus, which can be life threatening in the horse (Meerstadt, 1982).

Oximes are used to treat the nicotinic signs. Pralidoxime (2-PAM) is the oxime of choice in the USA and it is most effective when administered in the first 1 to 3 h. Due to the quick aging of soman, pralidoxime is rarely given soon enough to be effective (Sidell *et al.*, 1997). 2-PAM can be given up to 48 h after exposure to VX and tabun, due to slow aging (Sidell and Groff, 1974). Other oximes, such as obidoxime dichloride (Toxogonin, LüH-6), are used in other countries (Belgium, Israel, The Netherlands, Scandinavia, Portugal, and Germany). HI-6 is another alternative oxime that has excellent acetylcholinesterase regenerating action with VX, very good action with sarin, good response to soman, but a poor or no response following tabun exposures (Hoffman, 1999).

Autoinjectors (AtroPen®, Mark I®, Combopen MC®) are atropine or atropine and pralidoxime combinations available for human use. They are not used in veterinary medicine as they are not adjustable for different sized patients. Experimental vaccines against nerve agent VX, and monoclonal antibodies which protect against soman, sarin, and tabun toxicity have been produced and are being tested (Dunn and Sidell, 1989; Somani *et al.*, 1992).

AChE activity can be tested in plasma, serum, or whole blood. In most animal species, 80% or more of the total blood AChE activity is in the RBC (as compared to 50% in humans); therefore, whole blood is the preferred sample for

most veterinary diagnostic labs. In general, in the context of OP poisoning, whole blood AChE activity <50% of normal is generally associated with severe symptoms (Midtling *et al.*, 1985). Furthermore, inhibition of RBC AChE is interpreted as: 10–20%, no reliable evidence of exposure; 30–50%, mild poisoning; 50–70%, medium or moderate; and 70–90%, severe intoxication. This decrease is in good agreement with results on humans and animals (Bajgar, 1992).

The ''G'' agents are volatile and evaporate over several hours. They are nonpersistent in the environment (Garigan, 1996). Environmental persistence is estimated to be 0.5 to 1 day for tabun, 1 to 2 days for soman, and 5 days for sarin. VX is an oily liquid that remains in the environment for weeks or longer after being dispersed (Budavari, 2000; Garigan, 1996; Munro *et al.*, 1999; Sidell *et al.*, 1997). Contaminated soil should be treated with alkaline substances (sodium carbonate, sodium bicarbonate, calcium hydroxide, calcium hydroxide, or calcium carbonate) or chlorine compounds (sodium hypochlorite or calcium hypochlorite) (EPA, 1975).

Protective equipment (rubber gowns, aprons, and gloves), along with respiratory protection, must be worn by rescuers and veterinary personnel. Leather and fabrics absorb organophosphates and are extremely difficult to decontaminate. Collars, muzzles, and other items should be incinerated.

4. Species Susceptibility

Nerve agent susceptibility varies widely between species. Animals may be more at risk for greater exposures than nearby human populations. Both the ''G'' and the ''V'' nerve agents are heavier than air, and animals with a lower breathing zone will be more affected (Rabinowitz *et al.*, 2008). Some species differences can make animals more sensitive than humans, while others will make them more resistant. Rats may be less susceptible than humans, since they possess aliesterases (enzymes) that can reduce the toxicity of certain nerve agents such as GA (Fonnum and Sterri, 1981). These enzymes are not present in humans. Nasal breathers, such as rodents, are capable of partially detoxifying nerve agents in the nasal pathways, presumably by hydroxylation and other mechanisms (Garamone, 2003). Carboxylesterase activity in guinea pigs and rabbits may provide protection from soman, when compared to humans (Maxwell *et al.*, 1987).

RBC AChE activity also varies among species. In humans, low RBC AChE activity has been associated with increased susceptibility to nerve agents (Leng and Lewalter, 1999). Pigs, sheep, dogs, rabbits, and cats have less RBC cholinesterase activity than humans (Anonymous, 2000b; Ellin, 1982). This would make these species more sensitive to nerve agents unless they have an alternative system to combat cholinesterase inhibition.

The LCt_{50} (50% lethal concentration via inhalation route) for military personnel for different nerve agents has been estimated from animal studies. Humans appear to be more sensitive to tabun than other animals. The LCt_{50} for tabun is 70 mg.min/m^3 for humans, 320 mg.min/m^3 for dogs, 450 mg.min/m^3 for rats, and 960 mg.min/m^3 for rabbits (Anonymous, 2000a; NRC, 2003; Sidell *et al.*, 1998). Goats are more sensitive than humans to VX. The LCt_{50} for goats is 9.2 mg.min/m^3, while the human LCt_{50} is 15 mg.min/m^3 (Anonymous, 2000a; NRC, 2003; Sidell *et al.*, 1998).

Even though the lethal nerve agent exposure level for animals may be higher than that for humans, it is still possible that animals could exhibit other nonlethal effects sooner and more noticeably than humans. Rabbits develop 90% miosis at a lower inhaled concentration of cyclohexyl sarin when compared to humans (2.71 mg.min/m^3 versus 13.85 mg.min/m^3) (NRC, 2003).

Another way that animals could provide an early warning is via their sense of smell. Most animals have much more sensitive olfactory systems when compared to humans. Animals may be able to be trained to sense low nerve gas concentrations (Dalton, 2003).

It is unknown if animals may have decreased dermal absorption compared to humans due to protective fur or feathers. Comparing dermal exposures in humans and other species also shows differences for the various nerve agents. Mice appear to be more sensitive to dermal sarin than humans, LD_{50}s are 1.08 and 28 mg/kg, respectively (RTECS, 2008; Sidell *et al.*, 1997). The mouse may also be a good sentinel animal for tabun. The dermal LD_{50} for mice is 1 mg/kg, rats 18 mg/kg, and humans 14 mg/kg. Mice may be slightly more resistant to dermal soman, with LD_{50}s of 7.8 mg/kg for mice and 5 mg/kg for humans.

There are a few cases where shared exposures to nerve agents have been reported for both animals and humans. In 1968, there was an accidental release of two different nerve agents (one is thought to have been VX) in Utah. A flock of sheep that was grazing near the base was noted to be acting ''crazy in the head'', and thousands died less than 24 h later (Boffey, 1968). Nearby humans, cattle, dogs, and horses did not develop symptoms. The sheep had severely depressed cholinesterase levels, as did the cattle and horses. Cholinesterase testing of dogs and humans was normal. The sheep may have been more affected due to higher exposures through ingestion of contaminated pasture, or by spending more time in the vicinity of the chemical release.

H. 3-Quinuclidinyl Benzilate (BZ)

1. Clinical Signs

3-Quinuclidinyl benzilate (BZ) is a centrally acting synthetic anticholinergic agent. BZ is used as a hallucinogenic and incapacitating chemical warfare agent. It is about 25 times more potent than atropine and has a very long duration of action. BZ is disseminated as an aerosol, with the primary route of absorption through the respiratory system.

BZ affects both peripheral and central nervous systems. Peripheral anticholinergic effects can be summarized by the mnemonic ''dry as a bone, red as a beet, hotter than Hades and blind as a bat''. BZ inhibits glandular secretions (''dry as a bone'') leading to a dry mouth and foul breath (Holstege, 2006). Cutaneous vasodilation and skin flushing may be noted (''red as a beet'') due to decreased capillary tone. Hyperthermia (''hotter than Hades'') is due to elevated body temperature secondary to inhibition of sweating and inability to dissipate heat. Vision loss (''blind as a bat'') is from a loss of accommodation reflexes and decreased depth of field secondary to ciliary muscle paralysis and mydriasis (Anonymous, 1998; Holstege, 2006). Paralytic ileus is commonly seen as a result of anticholinergic toxicity (Holstege, 2006; Ketchum and Sidell, 1997). This can lead to fatal colic in equids. Urinary retention is also a common anticholinergic effect following exposure to BZ (Holstege, 2006; Ketchum and Sidell, 1997).

CNS signs of disorientation, agitation, tremor, ataxia, stupor, coma, and seizures may occur from inhibition of central muscarinic receptors (Holstege, 2006; Ketchum and Sidell, 1997). It is unknown if animals hallucinate like people, but they do appear distressed. Rhabdomyolysis can be seen secondary to seizures and agitation. If severe, myoglobinuric renal failure could develop (Holstege, 2006).

Other systemic signs may also occur. Sinus tachycardia is common (Anonymous, 1998; Holstege, 2006; Ketchum and Sidell, 1997). Moderate hypertension may occur and tachypnea may be expected following an acute exposure. Nausea and vomiting may also occur.

2. Kinetics

BZ easily crosses the blood–brain barrier leading to mostly CNS effects. Signs are dependent on the dose and time post-exposure. Prolonged effects may occur depending on the dose of BZ absorbed. Tachycardia and dry mouth develop within 15 min to 4 h after exposure (Anonymous, 1998). Peak effects occur at 8 to 10 h (Anonymous, 2001).

BZ accumulates in the cerebrum following an intravenous injection in rats (Sawada *et al.*, 1990). It is highly lipophilic and has a high degree of plasma protein and red cell binding. Steady state was reached in the cortex and caudate within 1 to 5 min. Binding to high-affinity m-AChRs is essentially irreversible for the first 6 h.

BZ is excreted via the kidneys (Holstege, 2006). Only about 3% is excreted unchanged in rat urine (Byrd *et al.*, 1992). The two major metabolites are 3-quinuclidinol and benzilic acid. Without treatment following an incapacitating dose, recovery is gradual, requiring 72 to 96 h (Anonymous, 2001; Ketchum and Sidell, 1997).

3. Decontamination and Treatment

Move into fresh air. Monitor animals for respiratory distress. If cough or dyspnea develops, administer supplemental oxygen. Assisted ventilation may be required. Nebulized beta-adrenergic agonists should be used if bronchospasm develops.

Due to the method of distribution (aerosol) most exposures are expected to be inhalational. However, with animals' tendency to groom, dermal exposures can also become oral exposures. Emesis is not recommended due to potential seizures and coma. Activated charcoal or gastric lavage can be helpful after oral exposures. These procedures can be successful even if delayed, as anticholinergics slow gastrointestinal motility.

Flush eyes with copious amounts of tepid water for at least 15 min. Animals should be bathed with soap and water. Bathing will not only remove the BZ but will also provide external cooling to combat hyperthermia. BZ may be detected in urine, serum, or blood, but there are no rapid tests to diagnose exposure to BZ, so clinical use is minimal.

Benzodiazepines can be used to control agitation. Avoid phenothiazines due to possible hypotension. Animals may need to be heavily sedated so they do not injure themselves or others. Control seizures with a benzodiazepine, phenobarbital, or propofol. Rhabdomyolysis can occur if agitation or seizures are not controlled.

Treat arrhythmias symptomatically (lidocaine, propranolol, etc.). Physostigmine can be used to treat severe arrhythmias but long lasting reversal of anticholinergic toxicity does not occur. Physostigmine is ineffective if given during the first 4 to 6 h following the onset of BZ effects. If physostigmine is discontinued, recovery from BZ may be slightly prolonged (Ketchum and Sidell, 1997).

Monitor electrolytes and renal function tests in symptomatic patients. Administer intravenous fluids to maintain urine output and to protect the kidneys from myoglobinuria. The prognosis is good if animals do not develop rhabdomyolysis or secondary infection. No chronic problems are expected from BZ itself (Holstege, 2006).

BZ is stable and environmentally persistent. ''Off gassing'' may occur from contaminated patients. Goggles and masks should be worn by all personnel until the animal is decontaminated (Anonymous, 1998; Holstege, 2006). Remove contaminated collars, leashes, harnesses, halters, etc., and discard as leather and fabrics absorb BZ.

4. Species Susceptibility

There are no studies demonstrating that animals are more sensitive than humans.

I. Riot Control Agents (Lacrimators)

1. Clinical Signs

Chloroacetophenone (CN, mace, tear gas), chlorobenzylidene malonitrile (CS, Paralyzer®, super tear gas), and oleoresin capsicum (OC, pepper-mace) are lacrimators used in riot control. They are solid chemicals administered as a fine dust or aerosol spray, and not true gases. Exposure to lacrimators causes immediate pain, blepharospasm, lacrimation, rhinorrhea, coughing, and sneezing but usually

no permanent tissue damage (Blain, 2003; Grant and Schuman, 1993). With higher ocular concentrations, chemical burns with keratitis and loss of the corneal epithelium may occur (Hoffman, 1967). A 4% w/v CN product has caused permanent corneal injury and ocular necrosis in animals, but no such injury was seen in a 10% w/v CS product (Gaskins *et al.*, 1972; Grant and Schuman, 1993). Oral ingestion will also cause eye irritation, lacrimation, vomiting, and diarrhea (Blain, 2003). Laryngospasm may occur in some cases due to the irritant effects; it can progress to pulmonary edema, bronchospasm, and bronchopneumonia.

Dermal contact with lacrimators is very painful (Pinkus, 1978). Erythema and blisters are common. The extent of dermal effects depends on the thickness of the stratum corneum, and the extent of exposure (Blain, 2003). High concentrations can cause first and second degree burns of the skin (Hu *et al.*, 1989; Stein and Kirwan, 1964). No teratogenicity or carcinogenicity has been demonstrated in humans or animals (Blain, 2003; Folb and Talmud, 1989; Himsworth *et al.*, 1971; Upshall, 1973).

2. Kinetics

The effects of lacrimators occur very quickly (Beswick, 1983). Pain, salivation, coughing, rhinorrhea, sneezing, and erythema begin within seconds of exposure and can last approximately for an hour (Blain, 2003). Delayed effects may also be seen. Conjunctivitis and ocular edema may be noted from 1 to 2 days following exposure. Laryngospasm and pulmonary edema may occur up to 48 h (usually 12 to 24 h) post-exposure. Bronchospasm may also be delayed up to 48 h post-exposure and become chronic (Folb and Talmud, 1989).

One of the CS metabolites is cyanide, but cyanide toxicosis does not appear to happen with routine use (Cucinell *et al.*, 1971). Gastrointestinal signs resolve over 24 h (Solomon *et al.*, 2003). Erythema disappears over 48 h and coughing may persist for weeks after exposure (Blain, 2003).

3. Decontamination and Treatment

Animals may need to be sedated to be able to treat them safely. Move animals into fresh air and monitor for respiratory distress. If cough or dyspnea develops, monitoring is necessary for oxygenation status. Supplemental oxygen may be needed. Laryngospasm may require intubation to permit adequate ventilation. Inhaled beta-2 agonists (albuterol, salbutamol), corticosteroids, and aminophylline may help reduce bronchospasm (Ballantyne and Swanston, 1978; Folb and Talmud, 1989). Thoracic radiographs should be monitored if pulmonary edema is expected (Stein and Kirwan, 1964).

Flush eyes with copious amounts of tepid 0.9% saline or water for at least 15 min. Diphoterine® solution can be used for decontamination of both eyes and skin after exposure to lacrimators (Viala *et al.*, 2005). Ocular signs resolve approximately 3 to 7 min after decontamination with Diphoterine®.

Animals should be bathed with soap and copious amounts of cold water. Using small amounts of water can actually increase irritation (Lee *et al.*, 1984). Topically applied magnesium hydroxide–aluminum hydroxide–simethicone suspension (Maalox Max®) caused resolution signs within 2 min after application after exposure to OC. If chemical burns develop, clean wounds with a mild disinfectant soap and water. Pain control and antibiotics may be needed. Tetanus toxoid should be given if burns are present. With oral ingestions, antacids may help decrease gastrointestinal signs.

Secondary contamination is common and personnel should wear aprons, rubber gloves, and masks as needed. Contaminated items can be washed in cold water (hot water will cause residual gas to vaporize) with soap or allow nonwashable items to air out for a few days. Most lacrimators dissipate quickly, but CS may be micronized and mixed with an antiagglomerant agent (CS1) which remains active for up to 5 days. A similar formulation mixed with silicone (CS2) remains in the environment for up to 45 days (Hu *et al.*, 1989).

4. Species Susceptibility

There are no controlled studies showing that animals are more sensitive to lacrimators than humans.

J. Ricin and Abrin (Toxalbumins)

1. Clinical Signs

Ricin and abrin are toxalbumins. Toxalbumins are plant lectins with a specific affinity for animal cell receptors. *Ricinus communis* (castor bean plant, castor oil plant, koll, mole bean, moy bean, palma christi) contains ricin. The castor bean plant is grown throughout the USA as an ornamental. The brown and white seeds, which resemble large ticks, contain the ricin. Most animals are exposed to ricin by eating the seeds, but ricin has been used as a chemical warfare agent, a reagent for pepsin and trypsin, an experimental antitumor and immunosuppressive agent, and a commercial mole killer (Budavari, 2000; Hayes, 1982; HSDB, 2008; Sax and Lewis, 1989). Abrin is found in the *Abrus precatorius* plant (Buddhist rosary bead, crab's eyes, Indian bead, Indian licorice seed, jequirity bean, jungle bead, love bean, lucky bean, mienie-mienie, ojo de pajaro, prayer bead, rosary pea, seminole bead, weather plant). This tropical ornamental vine produces colored seeds that are popular for use in jewelry and other decorative items. The seeds come in three different colors: red with a black eye, black with a white eye, and white with a black eye (Niyogi, 1969).

Variable toxicity is seen in cases of oral ingestion. Seeds that are chewed are much more likely to cause clinical signs. Seeds swallowed whole may not cause any problems at all (Kinamore *et al.*, 1980). The amount of toxalbumins in the

seeds will vary by size, weight, moisture content, region, season, and the period of plant growth at the time of harvesting (Audi *et al.*, 2005). The ricin content of castor beans can vary from 1 to 10% (Balint, 1974; Waller and Negi, 1958). The abrin content in *Abrus precatorius* seeds is estimated at 0.15% (Lin *et al.*, 1971). Toxicity and death have occurred with ingestions of one to two chewed beans of either plant. Ricin levels can be measured in plasma and urine but are not clinically useful (Kopferschmitt *et al.*, 1983).

Vomiting, abdominal pain, and bloody diarrhea are the most common signs seen after ingestion (Kopferschmitt *et al.*, 1983; Malizia *et al.*, 1977; Pillay *et al.*, 2005). Toxalbumins cause severe gastrointestinal lesions of the oropharynx, esophagus, and stomach. The lesions are clinically similar to alkaline burns. Fluid losses can lead to dehydration, electrolyte disturbances, hypotension, and tachycardia (Ingle *et al.*, 1966).

Liver damage occurs in toxalbumin toxicosis. Histopathology reveals injury to the smooth endoplasmic reticulum and depletion of liver glycogen (Balint, 1978). The liver enzyme values for ALT, total bilirubin, AST, alkaline phosphatase, and GGT can all be elevated (Niyogi, 1977). Glucose metabolism is affected by ricin. Not only do glycogen stores decrease, but gastrointestinal absorption of glucose decreases and glucose concentrations fall (Lampe, 1976; Malizia *et al.*, 1977). Hypoglycemia is a common finding.

Mild to moderate CNS depression is commonly observed. Seizures are reported more frequently in animals than in people (Frohne and Pfander, 1984; Hart, 1963). While hematuria is commonly reported, the hemagglutination that is seen in animal and laboratory work is almost never seen in actual toxicities (Corwin, 1961; Jelinikova and Vesely, 1960; Malizia *et al.*, 1977; Waller *et al.*, 1966).

Ricin is among the most toxic compounds known when given parenterally. Parenteral toxicity is much greater than oral toxicity. The oral lethal dose of ricin is estimated to be 1 mg/kg (Kopferschmitt *et al.*, 1983). When given by injection, the lethal dose of ricin drops to about 1 µg/kg (Budavari, 2000). With an inhalation exposure, signs seen are cough, dyspnea, arthralgias, fever, and death (Griffiths *et al.*, 2007). Other organ system dysfunctions may not occur (Audi *et al.*, 2005).

2. Kinetics

Gastrointestinal effects usually develop in under 6 h, but vomiting can begin in 1 to 3 h post-ingestion of castor beans (Kopferschmitt *et al.*, 1983; Spyker *et al.*, 1982). In some cases, signs may be delayed for several days. The cytotoxic effects on the liver, CNS, kidney, and adrenal glands may not occur for 2 to 5 days post-exposure. With an inhalation exposure symptoms begin within 8 h. Death occurs about 48 h after a parenteral or oral exposure (Budavari, 2000).

Glycoproteins are large molecules and are poorly absorbed from the gastrointestinal tract. Many cell surfaces contain receptors specific for ricin and the toxin is taken up by active transport. The primary site of distribution of ricin is the liver, spleen, and adrenal cortex in the mouse. A high concentration was also found in the bone marrow (Godal *et al.*, 1984). In rats, abrin is distributed primarily to the liver (12%) and spleen (Lin *et al.*, 1970, 1971).

The metabolism and elimination of toxalbumins is poorly understood. Ricin is eliminated by first order kinetics when injected IV into mice and human cancer patients (Godal *et al.*, 1984). The plasma half-life in humans is about 2 days (Kopferschmitt, 1983).

3. Decontamination and Treatment

There are no specific treatments for toxalbumin exposure. Aggressive decontamination is recommended. With ingestions, emesis should be induced in species that can vomit (dogs, cats, ferrets, and swine), if the animal is not already vomiting. Activated charcoal can bind orally ingested toxalbumins. Monitor asymptomatic animals for 8 h following any exposure to toxalbumins. Monitor for dehydration, electrolyte disturbances, elevated liver enzymes, and hypoglycemia.

Intravenous fluids are very important to maintain normovolemia and urine output. If hematuria is present, consider alkalinization of the urine. A goat anti-ricin polyclonal and a mouse anti-ricin A-chain monoclonal antibody have been tested and shown to neutralize ricin in castor bean extract, but they are not clinically available (Lemley and Wright, 1991; Wannemacher *et al.*, 1991).

If the toxalbumin was distributed by air, move the animal into fresh air and monitor for respiratory distress. If cough or dyspnea develops, administer supplemental oxygen. Wash animals thoroughly with soap and water. Eyes should be flushed with copious amounts of tepid 0.9% saline or water for at least 15 min. Other treatments are the same as for an oral exposure.

4. Species Susceptibility

There are no controlled studies demonstrating that animals are more susceptible to developing acute effects than humans.

III. CONCLUDING REMARKS AND FUTURE DIRECTION

The lack of controlled studies comparing animals and humans needs to be addressed if animals are to be used as sentinels. The existing studies and anecdotal reports do not provide enough convincing evidence (Rabinowitz, 2008). Sentinels would need to demonstrate easily recognizable signs before the emergence of human illness. A good sentinel could have either greater susceptibility to

a particular toxin relative to humans or a shorter latency time from exposure to onset of signs (Cottrell and Morgan, 2003).

With any suspicious outbreaks, the local health department, poison center, law enforcement agency, and US Federal Bureau of Investigations (FBI) should be contacted immediately. Due to the absence of a nationwide surveillance system for animal diseases, the responsibility for detecting possible outbreaks of unusual symptoms in animals will fall over several different groups of people. Farmers, agriculture officials, veterinarians, animal control officers, wildlife rehabilitators, (animal) poison control centers and the lay public (animal owners) may all be involved in detecting outbreaks.

During a chemical warfare incident, the human casualities should be addressed first, followed by the animal casualities. Situations in which herds of livestock or flocks of poultry are affected are going to be much more complex to manage than exposures to house pets. The simple logistics in getting the personnel and equipment to the site of exposure can be daunting, if not impossible. Handling livestock can be dangerous, especially to untrained personnel. The use of antidotes and other pharmaceuticals must be documented in food-producing animals and withdrawal times for meat and milk followed. In many herd situations, humane euthanasia may be the best solution.

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CHAPTER 48

Potential Agents that Can Cause Contamination of Animal Feedstuffs and Terror

R.W. COPPOCK

I. INTRODUCTION

A. Terror Objectives

Terrorism has long been recorded in the chronicles of history. Terror is not new in terms of its objectives. The objective of any act of terrorism, though dangerous or lethal to human and animal life, is to intimidate or coerce a civilian population (Crutchley *et al.*, 2007). Acts of terrorism can create fear and anger among the public, influence the policy of and affect the conduct of a government, and topple political leaders. A key message to the public is that their government(s) is powerless to protect them. New technologies have produced a whole new arsenal of weapons for terrorists.

Feedstuffs and foodstuffs (foodstuffs and food refer to substances consumed by humans and feedstuffs and feeds refer to substances consumed by nonhuman animals) are vulnerable to terrorist attack. A terrorist attack targeted at feedstuffs could have a remarkable impact on the feed and livestock industries. The World Health Organization (WHO, 2002) considers that a local terrorist attack on food could have global impacts. These impacts can largely be due to the public losing faith in the ability of governments to detect harmful adulterants in foodstuffs and feedstuffs and thereby protect health. Specific industries can be targeted with significant economic losses (Crutchley *et al.*, 2007). Terrorist attacks can indirectly affect feedstuffs. For example, fallout from a dirty bomb can contaminate forages, cereals, and other feedstuffs. Humans are exposed by consuming contaminated edible animal products. Companion animals can be exposed through contaminated animal products being incorporated into pet foods. Attacks on companion animal feedstuffs can have a considerable emotional impact on a community.

B. Agricultural Food Ecosystem and Terror

A brief review of the agri-food ecosystem is important in the discussion of terrorist-linked toxicology of animal feedstuffs. Generally, the activities and technologies in the agro-ecosystem are maximized for human control and economic gain. All aspects of food production in the agro-ecosystem, including input chemicals and fertilizers, can be the target for terrorists. Harmful agents and organisms can be added directly or indirectly to feedstuffs. Tragic incidents of food-borne intoxication can occur without malicious intent. The scientific literature has examples wherein xenobiotics in feedstuffs were relayed to humans via contaminated animal products, and resulted in human illness (Curley *et al.*, 1971; Gerstner and Huff, 1977). For example, humans have been poisoned by consumption of methyl mercury-contaminated grain donated for use as seed (Gerstner and Huff, 1977; Al-Damluji, 1976).

1. Upsetting the Margins Between Safe and Unsafe Practices

The production of cereal grains, oilseeds, other commodities, and forage occurs essentially in an artificial ecosystem (Sinha, 1995; Dorea, 2006). Activities in this agro-ecosystem include seeding, growing, harvesting, storing, and transportation of cereal grains, oilseeds, forages, nuts, fruits, raw materials for beverages, and animal bedding (Balazs and Schepers, 2007). The agronomic ecosystem is driven by economic decisions that generally maximize profitability and minimize labor and input costs. This ecosystem is dependent on management that ensures activities are done in a timely manner, and in a manner that ensures the safety of foodstuffs and feedstuffs. The margin between safe and unsafe practices is generally close. The decision-dependent activities include fertilization, weed and other pest control, harvest methods, preservation, and enhancements. Management of the agro-ecosystem is important to prevent feedstuffs being damaged by pests and spoilage while minimizing exposure of animals and humans to toxic substances.

The agro-ecosystem is vulnerable to attacks by terrorists which can narrow the margins between safe and unsafe practices. For example, the tactics used by terrorists can have huge impacts on foodstuffs and feedstuffs and can be as simple as, for example, disrupting the distribution of electrical and fossil fuel energy. For example,

a remarkable reduction in available and affordable energy can immediately alter practices traditionally used to harvest and preserve feedstuffs and increase the risk of growth of toxigenic fungi. Manufacturing of feedstuffs and foodstuffs also occurs within this ecosystem. This agroecosystem is especially vulnerable to persistent toxigenic organisms, and persistent chemicals that are recycled and bioconcentrated.

2. Economics of Chemical Incidents

Examples exist where large outbreaks of animal to human contamination not linked to terror can have a distinct economic impact (Kojima and Fujita, 1973; Kay, 1977; Fries, 1985; Dorea, 2006; Burns, 2007). These incidents have long-term impacts on human health (Kojima and Fujita, 1973; Sweeney and Symanski, 2007). Chemical-induced illness at the local level can have a significant impact on the health system and government agencies (Kojima and Fujita, 1973; Green *et al.*, 1987).

II. MYCOTOXINS AND TOXIGENIC FUNGI

A. Background

The use of mycotoxins as a chemical weapon has a controversial history (Tucker, 2001; Katz and Singer, 2007). Mycotoxins and toxigenic fungi can be used for terrorist attack on both feedstuffs and foodstuffs (Tucker, 2003; Shannon, 2004). Mycotoxicosis as disease entities are not commonly considered in a differential diagnosis by medical professionals in the USA. In addition to low consideration as the etiology of disease, the clinical presentation caused by mycotoxins can easily be diagnosed as being caused by other etiologies (Paterson, 2006). Thus, the delay in the identification of mycotoxins as the causal agent(s) could occur over a considerable period of time. Delay in recognition can increase the numbers of animals and humans exposed.

B. Applications of Biotechnology

It should be assumed that the effective use of mycotoxins as terrorist weapons can be increased with the use of biotechnology. Development of fungi for bioterrorism has been reported to share technologies with the forensic development of fungi for use as weapons (Paterson, 2006). The genetics of mycotoxin production are better understood (Bhatnagar *et al.*, 2006). It is well known that the conditions for mycotoxin production are the presence of toxigenic fungi having favorable growing conditions which include temperature and substrate moisture content. It is possible that aggressive strains of toxigenic fungi can be developed that produce mycotoxins at lower temperatures and moisture levels than would be considered the usual limits for a particular species. Also, fungal varieties can be genetically modified (GM) in a manner that would enhance toxin production by removing the ''restrictors'' of toxin production. The use of toxigenic fungi and mycotoxins in terrorist activities may likely follow the developments in genetic research and biotechnology.

C. Potential Use of Fungal Biocontrol Agents

Fungal biocontrol agents (FBCAs) could be used in terrorist activities. Some FBCAs have been developed using bioselection and some have been developed using genetic modification technologies (Paterson, 2006). A number of the FBCAs produce mycotoxins and known toxigenic fungi have been used to control unwanted vegetation. These actions have occurred, for example, to control the plants in the Erythroxylaceae family (cocca) of plants. If the target had been a crop used for production of substances other than an illicit drug, the act may have been considered bioterrorism or use as a biological weapon.

D. Economics of Mycotoxins as Weapons

The use of toxigenic fungi and mycotoxins for bioterrorism can have economic consequences. Increasing the environmental prevalence of aggressive strains of toxigenic fungi could increase the losses due to mycotoxins from ~$2.5 billion to some substantially higher number (CAST, 2003). These losses may be underestimated because Robens and Cardwell (2003, 2005) projected the loss due to aflatoxins at $1.5 billion/year without including secondary industry and trade losses. International commodity trading can be used as a method of disseminating the spores of GM aggressive toxigenic strains of a particular fungus. Once released into the environment, the GM fungi would be difficult to control.

E. Use of Mycotoxin Contaminated Feedstuffs

Mycotoxin contaminated feedstuffs could be purchased and used as a terrorist tactic in manufacture of a completed feed. For example, horse or swine feeds could be formulated to contain a high level of screenings which are known to contain mycotoxins. Field incidences of mycotoxicoses not linked to terrorist activities have been reported from the use of fumonisin contaminated screenings in formulating horse and swine feeds (Wilson *et al.*, 1990; Osweiler *et al.*, 1992).

F. Residues in Edible Tissues

Food-producing animals can ingest mycotoxin contaminated feedstuffs and mycotoxin residues can be present in edible animal products. The concern for residues of mycotoxins in edible animal products has primarily been focused on residues of aflatoxins (Coppock and Christian, 2007). Residues of some mycotoxins in animal products can be

regulated. Incidents of aflatoxin contaminated feedstuffs being fed to milk-producing animals and subsequent contamination of foodstuffs are a public concern. Bioterrorism using aflatoxins could contaminate a large volume of dairy products with aflatoxin M_1.

G. Mycotoxicology

A discussion of mycotoxicology is beyond the scope of this chapter. For further details on mycotoxicology, the reader is referred to recent publications elsewhere (CAST, 2003; Plumlee, 2004; Gupta, 2007).

III. MICROBIAL TOXINS

A. Botulism Toxin

1. Background

Botulism toxins can be used as a terrorist weapon (Osborne *et al.*, 2007; Adler *et al.*, 2008). This group of toxins can easily be produced at low cost. Exposures to botulism toxins are oral, and these toxins have been formed in feedstuffs, or *in vivo* from toxin production by *Clostridium botulinum* growing in the gut or wounds (Critchley, 1991; Bohnel *et al.*, 2001). The production of botulism toxin is considered to be inexpensive and requires a low level of technology.

Clinical reports have shown that botulism occurs in livestock and some outbreaks can be associated with feedstuff containing animal carcasses (Bienvenu *et al.*, 1990; Kinde *et al.*, 1991; Braun *et al.*, 2005; Otter *et al.*, 2006). Type D botulism has been identified in cattle fed chicken litter, type C has been associated with ensiled chicken litter, and type B has been associated with forages (Long and Tauscher, 2006).

2. Mechanism of Action

Botulism neurotoxins bind with synaptic vesicular proteins and block the release of acetylcholine from the presynaptic membrane (Osborne *et al.*, 2007). Clinical signs of botulism are weakness, tremors, recumbency, laryngeal paresis, and other signs of nervous system dysfunction (Braun *et al.*, 2005). Botulism toxins do not appear to be excreted in milk (Galey *et al.*, 2000).

3. Potential Production and Use

Botulism toxins can be produced and incorporated into ingredients used in feedstuffs. The use of dead rodents or other animals can serve as a substrate for *Clostridium botulinum*, providing anaerobic conditions. Contamination of feedstuffs with the carcasses of dead mice or other small animals could be a method of disseminating botulism toxins. For further details of botulism toxins, readers are referred to in Chapter 30.

IV. PLANT TOXINS

A. Background

Poisonous plants and their seeds could be used to adulterate feedstuffs. Seeds of poisonous plants have been incorporated into animal rations and have resulted in animal intoxication (Burrows and Tyrl, 2001). Poison seeds can be used to intentionally adulterate feedstuffs. For example, grain screenings that contain high levels of toxic plant seeds could be used as a terrorist tactic to manufacture a pelleted feed. Pasture lands can be seeded with poisonous plants to increase the presence of poisonous plants in rangelands. Overgrazed pasture lands could be more susceptible to this type of terrorist attack. The genes required for production of poisonous plant proteins could be used to produce selected plant toxins by biotechnology. These terrorist tactics would also undermine the trust in governments to control biotechnologies.

B. Castor Beans (Ricin)

1. Background

Castor beans are grown commercially for castor oil. Castor oil has sought-after industrial and automotive applications. The annual production of castor bean waste mash exceeds 1 million tons (Doan, 2004). Castor bean cake is also sold as fertilizer. Ricin is easy to extract from castor bean meal.

2. Ricin as a Weapon

Ricin (ricin-D) from castor beans (*Ricinus communis*) is considered a potential bioterrorist weapon and has been considered as a chemical weapon since 1918. The ricin content of castor bean varies with the growing conditions and genetics of the plant (Millard and LeClaire, 2008). A general guide is 1 to 2 mg of ricin/g of ground castor seed. Ricin can be 5% of the total protein in castor beans, and is present in the waste mash after the oil has been extracted. The ricin content of the waste mash generally is 1 to 5% (w/w). Ricin is considered to be relatively heat stable at temperatures used for food processing (Audi *et al.*, 2005; Jackson *et al.*, 2006). Ricin is soluble in water over a wide range of pH values.

3. Toxicity

The seed coat of the castor bean needs to be fractured for the ricin to be released. Ricin in waste mash is biologically available. Most animal species including birds are sensitive to castor bean poisoning (Jensen and Allen, 1981; Burrows and Tyrl, 2001; Mouser *et al.*, 2007). The degree of toxicity depends on the dose (number of seeds) ingested and liberation of ricin by degradation of the seed coat by some form of mastication. The liberated ricin is absorbed from the gastrointestinal tract. The estimated lethal dose of castor bean seeds by species is given in Table 48.1 (Burrows and Tyrl, 2001).

TABLE 48.1. Sensitivity of different species to castor bean intoxication

Species	Lethal castor bean mass/kg of body mass	Comment
Human	225 mg	Seed considered to require mastication
Horse	100 mg	Considered the most sensitive species
Duck	255 mg	1 in 8 ducks died at 16 days after treatment. Grinding of the seeds by the proventriculus can be a variable in toxicity
Chicken	400 mg	Grinding of the seeds by the proventriculus can be variable in toxicity
Goose	400 mg	Grinding of the seeds by the proventriculus can be variable in toxicity
Rabbit	1.2 g	Mastication considered required
Pig	1.2 g	Mastication likely required
Goat	5 g	Age of ruminant can be a factor as the rumen may inactivate ricin
Sheep	14 g	Age of ruminant can be a factor as the rumen may inactivate ricin
Cattle	*a*	See text

[a]Cattle have been fed castor bean meal at ingredient levels (Albin *et al.*, 1968)

Ruminants are considered to be more resistant to ricin (Table 48.1) (Albin *et al.*, 1968; Burrows and Tyrl, 2001). The most likely reason is degradation of ricin by microbes in the rumen. This phenomenon also suggests that young ruminants would likely have the same sensitivity to ricin as monogastric animals.

4. Analytical Methods

Analytical methods include immune methods (ELISA) and liquid chromatography/mass spectrometry (LC/MS). Gastric contents can be assayed and ricin can be detected in blood and body fluids by radioimmunoassay and LC/MS (Darby *et al.*, 2001; Mouser *et al.*, 2007).

5. Mechanism of Action

Ricin is a lectin-type globular glycoprotein and consists of A and B chains (Doan, 2004; Li and Pestka, 2008). Ricin is considered a Type 2 ribosome-inactivating protein (RIP) (Millard and LeClaire, 2008). The ricin B chain attaches to the plasma membrane at the galactose receptors, pits are formed and the ricin is internalized by clathrin-dependent and clathrin-independent endocytosis and forms an intracellular vesicle. The cellular uptake of ricin is a slow process and this can explain the lag time between exposure and onset of clinical signs. The vesicles containing ricin coalesce and form endosomes. The internalized ricin can dissociate from the endosomes and be transported to lysosomes for degradation. Ricin can bleb off the endosome and return to the cell surface by vesicular and tubular structures, or be transported to the Golgi apparatus. In the Golgi apparatus, the A chain undergoes retrograde transport to the endoplasmic reticulum (Audi *et al.*, 2005). Cleavage of the A and B chains occurs in the endoplasmic reticulum. From the endoplasmic reticulum, the ricin A chain is translocated to the cytosol. The ricin A chain catalytically inactivates ribosomes. The A chain is an enzymic polypeptide that catalyzes the N-glycosidic cleavage of adenine from position 4324 of the 28S rRNA in the ribosome (Lord *et al.*, 1994; Doan, 2004; Li and Pestka, 2008). The removal of adenine from 28S rRNA in the ribosome destroys its functionality and thereby blocks protein synthesis. One A chain can enzymatically inactivate ~1,500 ribosomes/min.

6. Clinical and Pathological Findings

Clinical signs of ricin intoxication are high morbidity, abdominal pain, emesis, diarrhea, and lethargy. Hematemesis and melena may be observed. Clinicopathology includes elevated hepatic enzymes in serum, hyperphosphatemia, and sometimes hypoglycemia. Electrolyte imbalance can occur due to diarrhea and emesis. Pathological findings in a dog were hepatocellular necrosis, fibrosis of central veins and sinusoids, and lymphocytic infiltrate. Jejunal mucosa can be eroded, infiltrated with leukocytes, and the tunica muscularis can also be affected. Hemorrhage can be observed in the mucosal and muscular layers. Necrosis can be seen in the spleen. Renal pathology is also noted.

7. Other Plant Source Type 2 RIPs

Abrin is a plant source Type 2 RIP. It is found in *Abrus precatorius* (rosary pea, Indian licorice, jequirity bean). The toxicology of abrin is considered to be very similar to ricin. A similar *Abrus* toxin is pulchellin, produced by *A. pulchellus* (Millard and LeClaire, 2008). The rosary pea has been reported to be more toxic than castor beans (Griffiths *et al.*, 1994). Species sensitivity is variable and horses are considered to be the most sensitive. The mature goat is considered to be a more resistant species and 2 g of seed/kg body weight is reported as a lethal dose. The lethal dose for cattle is reported at 600 mg of seed/kg body weight. It is likely that abrin is denatured in the rumen (Burrows and Tyrl, 2001).

There is limited commercial production of the jequirity bean. The rosary pea is grown as an ornamental plant and has escaped into the wild in the warmer climate in the USA.

Abrus sp. is not grown as an oilseed or for other large-scale commercial use.

For further details on ricin, readers are referred to in Chapter 25.

V. RAPIDLY ACTING AND EASILY AVAILABLE SUBSTANCES

A. Cyanide

Cyanide is considered a terrorist weapon (Wismer, 2007; Ballantyne and Salem, 2008). Cyanide has a history of use in controlling problem wildlife and unwanted farrow animals (Westergaard, 1982; Wiemeyer *et al.*, 1986). Cyanide in a solid form could be used to adulterate feedstuffs. Two common forms of cyanide salts are sodium cyanide and potassium cyanide. Cyanide could be incorporated into animal feedstuffs, especially concentrate and mineral mixes.

1. Mechanism of Action

The toxicology of cyanide (CN^-) is complex (Wismer, 2007; Ballantyne and Salem, 2008). The lethal effect of cyanide is blockage of cytochrome C oxidase, which results in loss of electron transfer and where molecular oxygen is unused. In addition to cytochrome C, other cytochromes are adversely affected by forming complexes with the cytochrome iron. The oxidized cytochrome–CN complexes are stable, but the complex is relatively unstable in a reductive environment. The instability of the cytochrome-CN^- complex in a reductive environment is the focus of the majority of treatment regimens.

2. Plant Sources – Ruminants

Certain plants contain cyanogenic glycosides (Nicholson, 2007). Cyanogenic plants and trees are common in some geographical regions, and could be placed in pastures. Plant parts containing cyanogenic glycosides, precursors that form hydrogen cyanide (HCN) in the rumen, could be placed in ruminant rations.

3. Treatment of Cyanide Poisoning

Treatment of cyanide poisoning has been discussed in detail by Wismer (2007) and Nicholson (2007), and in Chapter 19.

B. Insecticides

Large numbers of cattle and other domestic livestock have been poisoned with insecticides that were inadvertently incorporated into feedstuffs. There have been instances where the same transport vessels have been used to transport/store insecticides, seeding crops, and silage, resulting in cross-contamination. Insecticides could be used by terrorists to adulterate animal feedstuffs. The toxicology and treatment of insecticides have been discussed in detail by Plumlee (2004) and Gupta (2006, 2007).

VI. PERSISTENT ORGANIC COMPOUNDS

A. Background

Persistent organic compounds (POCs), also known as persistent organic pollutants (POPs), could be candidates for a terrorist attack on livestock. The majority of the POCs are bioconcentrated in body lipids. Most of these compounds require high doses to cause acute illness in livestock, poultry, and fish. At low doses the resultant illness is generally is less evident. The lack of overt clinical signs increases the risk of edible animals passing inspection and their products being consumed by humans (Fries, 1985; Hayward *et al.*, 1999; Dorea, 2006). The issues of the relay of POCs from feedstuffs to food-producing animals and from animal-source foods to humans are known (Kay, 1977; Fries, 1985; Huwe and Smith, 2005; Dorea, 2006). Environmental sources of POCs are also important in the agro-ecosystem (Stevens and Gerbec, 1988). The widespread flame retardant (polybrominated biphenyls) contaminated feedstuffs found in an incident in Michigan [ball clay contaminated with polychlorinated dibenzodioxins (PCDDs) and dibenzofurans (PCDFs) in animal mineral mixes] and other incidents clearly show that these events can occur over a substantial period of time before low-level contamination is recognized. Recognition of these incidents requires substantial resources consisting of highly trained personnel, sophisticated laboratory procedures and laboratory safety requirements.

B. Economics of a Terrorist Attack Using POCs

The history of incidents clearly shows that feedstuffs contaminated with POCs have caused remarkable economic loss. If the feedstuffs have a wide distribution, the economic impacts can be great. For example, with the ball clay incident, it was estimated that 1.7 million eggs/day and 35% of the catfish were contaminated with PCDDs/DFs (Hayward *et al.*, 1999). Animal-source foodstuffs contaminated with PCDDs/DFs were consumed by humans (Fiedler *et al.*, 1997; Rappe *et al.*, 1998). The polybrominated biphenyl (PBB) retardant incident in Michigan contaminated feedstuffs and large numbers of livestock and poultry were destroyed (Fries, 1985). More than 400 upright silos were coated with a polychlorinated biphenyl (PCB) containing resin (Willett *et al.*, 1987). Subsequently, milk was contaminated with PCBs.

C. Clinical Findings

Clinical signs of a mixture of PCBs and PCDDs/DFs in poultry feed have been reported (Bernard *et al.*, 2002).

A sudden drop in egg production occurred followed by a reduction in hatchability. Ascites and edema were observed along with ataxia. Pathology included degenerative changes in skeletal and cardiac muscle. The source of the contamination was PCB oil that had accidentally been emptied into a tank of recycled fat. The toxicology of PCBs and PBBs in cattle is controversial (Jackson and Halbert, 1974; Cook *et al.*, 1978; Willett *et al.*, 1987). My review of dairy records in herds fed silage from silos sealed with a PCB-containing sealant and accounts from clients who were feeding dairy cattle from PCB-contaminated silos showed that there were signs of intoxication. The observations were an increase in the rebreeding interval due to anestrus and reduced conception. Some cattle had signs of abomasitis. Calf mortality was increased due to failure to thrive syndrome. The occurrence of infections in calves was also increased, suggestive of immune suppression. The calves had decreased weight gains. Milk production was also decreased after feeding from the bottom of upright silos.

D. Human Exposure

Human populations have been exposed to POPs by consuming contaminated animal-source foodstuffs. The incidents have shown that feedstuffs can be contaminated by toxic substances that resembled mineral supplements, and mineral and fat supplements are vehicles for POPs. Contamination of feedstuffs also caused dissemination of POPs in the agro-ecosystem. Past experiences have shown that delays can occur before the contamination is recognized.

VII. HEAVY METALS

A. Background

There are several incidents of heavy metal intoxication and contamination of livestock feedstuffs. The source of the heavy metals was from smelters being deposited on forage or heavy metals as contaminants being incorporated into feedstuffs (Curley *et al.*, 1971; Rice *et al.*, 1987; Coppock *et al.*, 1988; Galey *et al.*, 1990; Swarup *et al.*, 2005). The risk for translocation of lead to milk appears to increase with blood lead levels above 0.20 μg/ml. Human poisoning has occurred when meat from pigs fed methyl mercury was consumed.

B. Deposition in Tissues

Heavy metals are deposited in liver, kidney, and bone. Organ meats (liver and kidney) can contain heavy metals and methyl mercury is deposited in skeletal muscle.

C. Use as Terrorist Weapons

Heavy metals can be used as terrorist weapons by incorporation into animal feeds. There are reports of zinc sulfate imported as a fertilizer supplement being contaminated with cadmium. This raises concerns because zinc sulfate is incorporated into animal feeds.

D. Toxicology

Readers are referred to other books for discussions on the toxicology of heavy metals (Plumlee, 2004; Gupta, 2007).

VIII. CONTAMINATED TRANSPORT VESSELS

Historically, occurrences of feedstuffs becoming contaminated in transport vessels were primarily because of the failure to clean the vessels after use. Increasing energy costs most likely will discourage dedicated use of transportation vessels for feedstuffs. Contamination of transport vessels could be a terrorist activity. Likely chemicals would be pesticides, POPs, heavy metals, and cyanide salts. Contamination of transport vessels used for ingredients such as salt and mineral would ensure wide dissemination.

IX. CONCLUDING REMARKS AND FUTURE DIRECTION

The agro-ecosystem is vulnerable to terrorist activity. Protection of the agro-ecosystem from chemical contamination is a challenge. Farm gate biosecurity protocols are becoming more common, but many of these programs do not include protection from chemical threats. Identification of contaminated feedstuffs and foodstuffs generally occurs after animals and humans have been contaminated. The use of acutely toxic chemicals by terrorists would most likely result in the deaths of a large number of livestock and poultry. The contamination of edible animal-source foodstuffs would be less likely because the animals are dead, sick, recumbent, or debilitated, and would not pass inspection. Few intoxicating substances produce pathognomonic signs, clinicopathologic profiles, and lesions. For toxic substances that cause chronic illness and increase the risk of cancer and endocrine disruption, considerable time is generally required for the pattern of disease occurrences and the etiology associated with feedstuffs and animal-source foodstuffs to be identified. Additional time is required to track and remove the suspect feedstuffs or foodstuffs from storage and commerce. The load demand on laboratories and personnel can be substantial when widespread contamination of feedstuffs/foodstuffs has occurred and the toxic substance is known. The workload can be overwhelming when laboratory personnel are required to identify unknown chemical(s) and develop analytical methods.

Examples exist where interactions of chemicals used as adulterants are required to produce toxicity (Brown *et al.*, 2007; Puschner *et al.*, 2007). Clever use of chemical

mixtures for a terrorist attack on food can delay identification of the etiology. This would help the terrorists to achieve their objective of maximizing psychological, sociological, economic, and political impacts, preventing governments and corporations from ensuring the public that food is safe.

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CHAPTER 49

Threats to Wildlife by Chemical Warfare Agents

R.W. COPPOCK

I. INTRODUCTION

Wildlife as a target of terrorist activity is a relatively new concept. Wildlife has value as a source of food, materials for garments, and other necessities and luxuries. There is also a desire to view wildlife in its natural habitat. Ecotourism is a growing industry especially if a country has unique or rare wildlife species or spectacular habitats. Wildlife is also important for sport and trophy hunting. The role of government is to protect wildlife from overkilling, and to protect the environment necessary for wildlife to feed and breed – this role is enforced by using a variety of laws and regulations. Additional laws and enforcement measures can be used to protect wildlife from human disturbances. Terrorist activities against wildlife can be undertaken to disrupt or destroy income and to remove a food source from aboriginal peoples that depend on wildlife for subsistence and culture preservation. Terrorist activities by their nature are generally carried out to destabilize society.

II. BACKGROUND

Wildlife can be an indirect target for terrorist attack and generally has a similar spectrum of sensitivity to terrorist agents as humans and domestic animals. Terrorist attacks against commercial operations could occur in more remote areas and such attacks could have an immense impact on wildlife including fish and other aquatic organisms. Impoundments of toxic water could be a target for terrorists for the primary purpose of contaminating potable water sources. Not linked to terrorism, there are recent examples of toxic water being released, with subsequent public outcry and economic impact, in several countries. Release of toxic water into the headwaters of large river systems was reported to have devastating impact on wildlife (Cunningham, 2005). One can only imagine the public and political reaction that would have occurred if such an incident was proven to be an act of terrorism.

Fish and other aquatic organisms can be impacted by run-off water from terrorist-linked fire fighting. This water could contain toxic substances and these substances could have negative impacts on wildlife. Run-off waters from burnt-over lands draining into waterways also are high in toxic substances.

III. WILDLIFE PROBLEMS OCCURRING AFTER A TERRORIST ATTACK

Wildlife problems can occur after a terrorist attack. For example, gulls (*Larus* spp.), crows (*Corvus* spp.), rats (*Rattus* spp.), and mice (*Mus* spp.) can become a nuisance in areas used for crime scene investigations (Chipman *et al.*, 2002). Such a problem occurred after the September 11, 2001 attacks on the World Trade Center. Control measures are required to enable investigations to be conducted without the danger of attacks on humans and the destruction of evidence. The need for wildlife control in an emergency could be unforeseen in emergency planning.

IV. ILLICIT AND RESTRICTED SUBSTANCES

A. Background

There are concerns regarding illicit pesticides being used for clandestine activities (Whitlow *et al.*, 2005). Because of their extreme toxicity, over time the use of certain substances was limited and in some cases they were even deregistered. These compounds are especially dangerous to wildlife because residual levels in carcasses can also kill animals that feed on them.

1. Tetramethylenedisulfotetramine

Tetramethylenedisulfotetramine (TETS, tetramine, CAS No. 80-12-6, 2,6-dithia-1,3,5,7-tetraazatricyclo[3.3.1.13,7]-decane,2,2,6,6-tetraoxide) is emerging as an illicit neurotoxin (Barrueto *et al.*, 2003; Whitlow *et al.*, 2005). Tetramine is an odorless white crystalline power that is radially soluble in water, mixes with feedstuffs, and is generally considered to be tasteless. The mechanism of action for TETS is that it selectively and irreversibly binds

with the chloride channel on the γ-aminobutyric acid receptor and disrupts regulation of chloride in the neuron. Inhibitory activity in the central nervous system (CNS) is decreased and clinical manifestations are seizures with onset occurring shortly after TETS ingestion. Tetramethylenedisulfotetramine is considered to be more potent than sodium monofluoroacetate (SMFA, Compound 1080). The oral LD_{50} of TETS for most species is 0.1 to 0.3 mg/kg of body weight, and the total lethal dose for a human is considered to be ∼10 mg. Rabbits dosed with 0.4 mg of tetramine/kg body weight and killed 1 h later had detectable levels (0.07 to 0.238 μg/g) of tetramine in the liver, kidney, heart, and lung (Xiang *et al.*, 2001). Tetramine is excreted in urine and urine can be used for forensic investigations (Zeng *et al.*, 2006). Tetramethylenedisulfotetramine is stable in tissues and relay or secondary poisoning can occur. Scavenging animals and birds can be poisoned with TETS because of the stability of TETS in tissues and body fluids.

The pathology of TETS in humans has been reported (Zhou *et al.*, 1998). Pathology observations in poisoned humans were edema of the brain, hemorrhages in the brain stem, and myocardial degeneration in the papillary muscles. Human tissues and urine can be assayed for TETS (Xiang *et al.*, 2001; Zeng *et al.*, 2006). A reasonable assumption is that similar pathology would occur in other species.

2. Sodium Monofluoroacetate (Compound 1080)

Sodium monofluoroacetate had common use as a poison until the early 1970s (Proudfoot *et al.*, 2006). In addition to being synthetic, SMFA and closely related compounds are found in *Dichapetulum* spp. of plants. The use of SMFA as a poison to eliminate unwanted animals and birds is restricted in many countries. Countries with limited indigenous mammal populations use SMFA to control imported animal species that disrupt and endanger indigenous wildlife (Eason, 2002). The use of SMFA can be limited to kill problem predators. For example, SMFA in some jurisdictions has been limited to sheep collars containing SMFA. In these applications, the SMFA is contained within bladders that are punctured during a predator attack (Burns and Connolly, 1995). The toxicology of fluoroacetamide is similar to SMFA (Osweiler *et al.*, 1976).

Sodium monofluoroacetate essentially is not detected by the sense organs. It is a white powder that has the appearance of flour or sugar, and is stable in storage over a long period of time. SMFA is heat stable to 200°C. It is soluble in water and insoluble in ethanol and lipids. In water, it can be degraded by microorganisms.

Compound 1080 is absorbed from the gastrointestinal tract, respiratory tract, mucous membranes, and wounds (Holstege *et al.*, 2007). Different routes of exposure do not have a remarkable effect on toxicity. The mechanism of action for SMFA is blockage of the Krebs cycle. Metabolic activation by the formation of fluorocitrate is required; a process known as lethal synthesis. Fluoroacetate is converted to fluoroacetyl-CoA and then converted by the enzyme citrate synthase to fluorocitrate. Aconitase catalyzes the conversion of citrate to isocitrate and aconitase is inhibited by fluorocitrate. Inactivation of aconitase blocks the Krebs cycle. Fluorocitrate also blocks the transport of citrate through the mitochondrial membrane. The heart and CNS are simultaneous targets for SMFA. Animals poisoned by SMFA are a hazard to scavengers, opportunists, and carnivores.

Clinical signs of SMFA toxicity (see Chapter 19) are due to the effects on the CNS and heart (Osweiler *et al.*, 1976; Proudfoot *et al.*, 2006). Herbivores can show more cardiac signs. The sequence of cardiac events is arrhythmias, tachycardia, inefficient pumping (weak pulse), and death when the heart fibrillates. Clinical signs observed are ataxia, collapse, and a short interval of agonal struggling (Robinson, 1979). Field observations are usually the discovery of a dead animal with the appearance of sudden collapse and limited evidence of agonal struggling. Carnivores generally have neurological signs of hyperesthesia, aimless wandering, frenzied running, vocalization, incoordination, emesis, opisthotonus, coma, and death. Death occurs from 1 to 24 h after ingestion of SMFA. Birds fall from the sky and die shortly after hitting the ground, or show nervous system signs and an inability to fly. Relay poisoning can be observed wherein animals poisoned by SMFA are consumed by other vertebrates.

Pathological findings in SMFA poisoning are those of acute heart failure and hypoxia. Hypocalcemia can be observed in SMFA intoxication (Proudfoot *et al.*, 2006). Tissues can be assayed for SMFA by gas chromatography methods (Okuno *et al.*, 1984).

V. CYANIDE

A. Background

Cyanide is poisonous to essentially all animal species. Cyanide can be placed in baits including water bait. Sodium and potassium cyanide and other forms of cyanide can be used. Breaching the impoundment can release tons of cyanide into streams, rivers, and lakes. Impoundments of toxic water containing cyanide could be a target of terrorism.

Cyanide is used in the extraction of gold and silver ores. The cyanide used in the process is recycled, but some cyanide escapes into the tailing pond. Bats and other animals drinking from these tailing ponds have been poisoned. There is a historical record of a dam holding a tailing pond being breached by a weather event, and subsequently releasing tons of cyanide into river systems causing an ecological disaster (Cunningham, 2005).

B. Toxicology

Cyanide has a history of being used to control unwanted wildlife and is toxic in the marine environment (Wiemeyer

TABLE 49.1. Estimated LD_{50} of cyanide

Identification	LD_{50} (mg/kg body wt)
Black vulture	4.8
American kestrel	4
Eastern screech owl	8.6
European starling	17
Chicken	21
Japanese quail	10
Coyote	4.1

Sterner (1979); Wiemeyer *et al.* (1986)

et al., 1986). Clinical signs of cyanide intoxication in birds have been described (Wiemeyer *et al.*, 1986). The progression of clinical signs of cyanide poisoning in vultures is ataxia, eye blinking, head bowing, wing droop, increased ataxia, seizures with tail fanning, opisthotonos gasping, and death. The progression of clinical signs in kestrels, owls, and quail is more violent. Time from ingestion to death is dose dependent. The lethal dose 50 (LD_{50}) for some species is given in Table 49.1.

A cyanide disaster can have long-term consequences on a freshwater system (Lakatos *et al.*, 2003). Cyanide can be in run-off water from fire fighting and in run-off from burnt-over lands (Barber *et al.*, 2003). Freshwater fish are highly sensitive to free cyanide. Fish mortality can occur at <20 µg/l (Eisler and Wiemeyer, 2004). The lethal level of cyanide in rainbow trout is changed by water temperature and exercise (McGeachy and Leduc, 1988). Young fish can also be sensitive to the effects of cyanide on the thyroid gland (Brown *et al.*, 2004). Cyanide can reduce the number of viable eggs produced by sexually maturing rainbow trout (Lesniak and Ruby, 1982). The toxicology of cyanide has recently been reviewed (Wismer, 2007; Coppock, 2009).

VI. RICIN (CASTOR BEAN)

A. Background

Castor beans (*Ricinus communis*) and ricin have been identified as potential terror agents of terrorism (Coppock, 2009). Castor beans do not give unique clinical signs of intoxication and in waterfowl can be confused with other acute causes of death (Jensen and Allen, 1981). There are inconclusive reports of Canada geese being killed due to castor bean. In Texas, castor bean intoxication could have been the cause of deaths of 10,000 ducks in 1967, and 2,000 ducks in 1969 to 1970 (Jensen and Allen, 1981). The death of 1,673 ducks occurred again in Texas in 1971. A castor bean was found in the examination of one duck. Castor beans were known to have been grown in the area. Although inconclusive, this report does incriminate castor bean ingestion as the cause of death in ducks and geese.

B. Toxicology

Ducks, taken from the area where 2,000 ducks had died in 1969–1970, were examined by diagnostic procedures (Jensen and Allen, 1981). The toxins of *Clostridium botulinum* and pathogenic bacteria were not identified. Catarrhal enteritis was identified with hemorrhage into the intestine. Seed parts suggestive of *Ricinus communis* were found in scrapings of the wall of the proventriculus. Fatty degeneration of hepatocytes was observed.

Ducks were force-fed whole castor beans (Jensen and Allen, 1981). Progressive signs of acute intoxication were passage of blood-streaked mucus, leg paresis, loss of mobility with wings, sitting, prone recumbency, and death. Necropsy findings were whole and fragments of castor beans, and congestion of the liver. Histopathology changes were severe fatty degeneration of hepatocytes, granulocytic infiltration into portal areas, pulmonary congestion and peribronchial hemorrhage; necrosis and hemorrhage were observed in the spleen. These findings suggest that some waterfowl consume castor beans which can result in castor bean intoxication.

The observation that the duck is the most likely to ingest castor beans provides evidence that castor beans could be used by terrorists against wild ducks.

VII. PESTICIDES

A. Background

Birds and other wildlife are nontarget species for insecticides (Schafer *et al.*, 1983). Observations from the registered and illegal uses of insecticides show that insecticides could be used in acts of terrorism against a targeted wildlife population.

Vertebrates can be nontarget species when insecticides are applied according to the registered label (Marian, 1983; Hunt *et al.*, 1995; Allen *et al.*, 1996; Poppenga, 2007). Vertebrate deaths have been reported from illegal and off-label use of insecticides. Models can estimate the probability of bird deaths resulting from field application of pesticides (Mineau, 2002). Birds, mammals, fish, and other aquatic organisms can be nontarget species from pesticides that enter water systems from surface water.

1. Pesticides

Waterways and flood areas can be contaminated with pesticides, especially insecticides, and result in bird deaths (Hunt *et al.*, 1995; Elliott *et al.*, 1996). Granules of carbofuran in flooded fields have been incriminated as the cause of duck deaths and the cause of death in raptors scavenging dead or debilitated ducks. Illegal use of insecticides resulting in the death of wildlife has also been reported (Allen *et al.*, 1996). The toxicology of insecticides has recently been reviewed (Gupta, 2006, 2007; Poppenga, 2007).

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

Wildlife generally has not been considered in the emergency management of terrorist events using chemical agents. The need to manage problem wildlife at the remote investigation site for wreckage from the World Trade Center brought attention to this aspect of emergency management planning. Regardless of the cause, wildlife can be intoxicated when chemicals and physical agents are released into the environment. Disruption of ecotourism can be an objective of terrorists. Emergency planning needs to include terrorist attacks on wildlife especially in areas where wildlife is concentrated during breeding and migration, and where wildlife congregates during the nonbreeding season. Shooting of the mountain gorillas hopefully is not an indication that these events will become more common (Jenkins, 2008).

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CHAPTER 50

Toxicokinetics of Chemical Warfare Agents: Nerve Agents and Vesicants

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I. INTRODUCTION

Despite the long lasting history of chemical warfare agents (CWAs) first used in large quantities during World War I at the beginning of the 20th century, vesicants and nerve agents especially still represent a commonly admitted threat for military and civilian communities. Therefore, effective medical countermeasures and state-of-the-art medical care are mandatory prerequisites to take the threat of poison attacks seriously. Rising interest in effective treatment of sulfur mustard and especially nerve agent poisoning leads national and governmental authorities to continue programs of medical defense against toxicants. The development and continuous improvement of therapy relies on intensified knowledge of provoked pathophysiological effects, toxicology, and toxicokinetics. The latter is important when understanding the reactivity of poisons in organisms at the molecular level. Awareness of predominant metabolism and efficient elimination processes is essential for the design of a therapeutic regimen of poisoning. For example, the development of protective scavengers is a current challenge in nerve agent defense research.

The toxicokinetic profile of a chemical warfare agent in mammalian organisms depends on numerous factors, including the nature of the poison, route of exposure, and species differences. Sophisticated study design, modern technical and analytical monitoring tools as well as reliable data from literature are indispensable quality criteria which should be met when performing toxicokinetic studies. The present chapter is focused on this topic. For guidance, we will comment on the structure of this chapter to provide the reader with an impression of the content.

We will first introduce the reader to typical invasion processes of exogenous poison. The anatomy of exposed areas of the body is discussed emphasizing interactions with nerve agents and vesicants invading on similar routes following common absorption principles. Afterwards both classes of chemical warfare agents are discussed individually in two subsequent main sections. Organophosphorus (OP) nerve agents are distinguished in terms of their physicochemical properties and toxicity as well as invasion and distribution processes *in vivo* which are of relevance for toxicokinetic characteristics. Subsequently, special focus is given to the basics of elimination and metabolism describing the phenomena of detoxification and concentration decrease at the molecular level. Enzymatic hydrolysis as well as binding to proteins and enzymes is discussed in comprehensive detail. A few comments on excretion will finish this ''journey through the poisoned body''. The last sections are dedicated to concentration–time profiles exemplarily selected for typical routes of nerve agent exposure followed by short introductions to predictive mathematical toxicokinetic models as well as to most modern bioanalytical methods allowing poison quantification and metabolite identification.

Vesicants including sulfur mustard and lewisite are the subject of the second main part of this contribution. Coherences of invasion and distribution are presented and the major processes of metabolism and elimination caused by binding to proteins and more prominently to DNA are discussed. The part closes with comments on current bioanalytical approaches.

This chapter provides readers with a comprehensive overview of the toxicokinetics of OP nerve agents and vesicants.

II. OVERVIEW OF INVASION PROCESSES OF CHEMICAL WARFARE AGENTS

Toxicokinetics is a subfield of toxicology which studies how, how fast, and to what extent toxicants are absorbed by, distributed in, metabolized by, and eliminated from the bodies of living organisms. These processes are dependent on multifaceted conditions, such as the species and gender of intoxicated organism, the physicochemical properties of the toxicant (e.g. hydrophobicity, charge, and molecular weight), its dose and concentration as well as chemical reactivity and stability. Furthermore, the route of exposure is a crucial parameter impacting the effectiveness of incorporation and time for distribution within the organism.

Elaboration of toxicokinetic data of chemical warfare agents is essential for designing effective antidotes, improving first aid, and optimizing therapeutic regimen and medical care. It has to be considered that data obtained from *in vitro* or *in vivo* animal studies need careful extrapolation to humans which, at least, requires sophisticated mathematical models to consider basic interspecies differences (Langenberg *et al.*, 1997; Levy *et al.*, 2007; Sweeney *et al.*, 2006; Worek *et al.*, 2007).

For controlled toxicological studies the poison is most often administered subcutaneously (s.c.), intravenously (i.v.), or intramuscularly (i.m.) whereas more realistic scenarios of chemical warfare agent uptake are percutaneous (p.c.) through unprotected skin, by inhalation of aerosols and vapor (inhal.), or by ingestion (p.o.) of contaminated food and drinks.

Such exposure events that lead to poison uptake and its distribution in the organism are part of the invasion process whereas all steps causing poison decrease, e.g. elimination by degradation, metabolism, or excretion, are part of the evasion process. For a better understanding of the pathophysiology and toxicokinetics of chemical warfare agents, an overview of the routes of poison incorporation follows in the next section with a special emphasis on OP nerve agents and vesicants.

A. Percutaneous Uptake by Contact with Skin

The total skin surface of an adult human is approximately 1.8 m^2 which is quite small when compared to the adsorptive surface of the lungs (100 m^2) and the gastrointestinal tract (GIT, 200 m^2) (Marquardt *et al.*, 1999). The primary function of the skin is to protect the organism against exogenous compounds present in the external environment. Nevertheless, percutaneous uptake is the predominant route of poisoning by nonvolatile and rather lipophilic agents, e.g. VX, which exhibits low vapor pressures thus making respiratory incorporation quite unlikely except the inhalation of aerosols (Czerwinski *et al.*, 2006). In the case of vesicant agents, such as sulfur mustard and lewisite, the skin is both a target organ, susceptible to severe local effects, and a pathway for absorption of the agent, leading to its distribution and subsequent systemic effects. The protective skin architecture is provided by a sophisticated and effective barrier built of two main components, the outer epidermis and the underlying inner dermis.

1. Epidermis

The epidermis is composed of various consecutive complex layers including stratum corneum (horny layer), stratum ludicum (clear layer), stratum granulosum (granular layer), and stratum germinativum (germinative layer), which itself is subdivided into stratum spinosum (spinous or prickle layer) and stratum basale (basal layer) (Marquardt *et al.*, 1999). For a general overview of percutaneous poison uptake we will now restrict the introduction to the horny and germinative layer which are in this sense the most significant epidermal strata.

The stratum corneum is the upper stratum of the epidermis which consists of several avascular, stratified cellular layers of dead keratinocytes characterized by a quite low water content (5–10%) thus making the surface hydrophobic and reducing its permeability for polar compounds. This layer, which exhibits the character of a multilayer lipid membrane, is the major barrier hindering hydrophilic substances from invading the organism. Penetration through the horny layer is most often a passive diffusion-controlled process following Fick's law in a good approximation. Because of their lipophilic nature liquid nerve agents as well as vesicants can readily penetrate the horny layer and absorption is much more effective than for their corresponding vapors (Blank *et al.*, 1957). To estimate the capability of nerve agents for skin penetration the octanol:water partition coefficient (listed as dimensionless log P, Table 50.1) can be used which correlates to the lipophilicity of the agent (Czerwinski *et al.*, 1998, 2006). This parameter can also be used to predict the distribution of OP agents in other tissues and blood (Langenberg *et al.*, 1997; Sweeney *et al.*, 2006).

TABLE 50.1. Physicochemical properties of VX and G-type OP nerve agents

Agent	CASNo.	NATO code	MW (g/mol)	Boiling point (°C)	Vapor pressure (mbar)	Water solubility (g/l)	Hydrolysis rate, $\tau_{1/2}$ (h)	log P(–)
Cyclosarin	329-99-7	GF	180.2	239	0.059 (25°C)[a]	3.7 (20°C)	n.a.	1.04[b]
Sarin	107-44-8	GB	140.1	158	2.8 (20°C)	Miscible	39 (pH 7.0)	0.30[b]
Soman	96-64-0	GD	182.2	190	0.53 (25°C)	21 (20°C)	45 (pH 6.6)	1.82[b]
Tabun	77-81-6	GA	162.1	237–240	0.049 (20°C)	98 (25°C)	8.5 (pH 7.0)	0.38[b]
VX	50782-69-9	VX	267.4	298	$9*10^{-4}$ (20°C)	30 (20°C)	1000 (pH 7.0)	2.09/0.68[b]

log P – octanol:water partition coefficient; MW – molecular weight; n.a. – not available; $\tau_{1/2}$ – period of half-change for hydrolysis. Data are taken from Munro *et al.* (1999) unless otherwise noted,

[a] Committee on Gulf War and Health (2004)

[b] Czerwinski *et al.* (2006)

Skin permeation velocity of toxic substances increases with (1) decreasing molecular weight, (2) increasing lipophilicity, (3) increasing area of contaminated skin, and (4) decreasing thickness of contaminated horny layer. Thickness of the stratum corneum varies depending on the area of the body and the species. Heavily strained areas, e.g. sole of foot, palm of the hand and insides of the fingers, are protected by a 400–600 µm horny layer in humans whereas arms, legs, and body are covered by a barrier of 8–15 µm thickness. Since the first incidents of chemical warfare in World War I, it was observed that skin areas with a very thin horny layer, in particular the axillae and the scrotum, were most susceptible to the effects of sulfur mustard. Effects in these regions, even though they were rarely exposed to the liquid agent, were severe.

Considering thickness and structural composition of the skin (e.g. diameter and density of hair follicles as well as number of cell layers) is essential for choosing appropriate *in vitro* models for skin penetration. Pig skin exhibiting a stratum corneum thickness in the ear of 8.6–28 µm is similar to human abdominal skin (5.5–40 µm) thus representing a good model to study the agent's permeation as recently shown for VX (Vallet *et al.*, 2008). The best model for p.c. *in vivo* exposure studies is thought to be the pig after inner ear-skin application of the agent (Chilcott *et al.*, 2003). In contrast to persistent VX, the major fraction (98%) of a small amount of liquid sarin, which was applied p.c. to six human subjects, evaporated quite rapidly without passing the skin as caused by its high volatility (Marrs *et al.*, 2007). In the presence of organic carrier solutes skin penetration may be enhanced as shown for VX dissolved in isopropanol thus doubling permeation velocity for animal and human skin *in vitro* (Dalton *et al.*, 2006). At the least, experimental conditions should also consider that moisture, heat, and abrasions force transfer and uptake of permeable chemical warfare agents (Blank *et al.*, 1957).

The germinative layer located under the stratum corneum consists of living keratinocytes responsible for the regeneration and proliferation of the skin. It exhibits the highest metabolic activity of all strata towards endogenous and exogenous substances initiating binding to carboxylesterases (CaE or CarbE). Nevertheless, sorption to cutaneous tissue may form a depot as demonstrated for sarin (Fredriksson, 1958; Satoh and Hosokawa, 2006), which explains the delayed local fasciculation of muscles observed after s.c. administration of nerve agents (Czerwinski *et al.*, 2006).

2. Dermis

The dermis (corium) consists of connective vascularized tissue composed of collagen, elastic and reticular fibres anchoring sweat and sebaceous glands, and hair follicles. Capillaries pervading the dermis and hypodermis (subcutis, located beneath the dermis) allow systemic distribution of toxic compounds once they have passed the epidermis. Therefore, percutaneously incorporated poison may be directly transported by the circulation to any compartment of the organism or it may be temporarily retained within the skin layers. In addition, it might penetrate with first-order kinetics into subcutaneous tissues and muscles thus creating a poison depot for delayed release (Chilcott *et al.*, 2005; Wolthuis *et al.*, 1981). If nerve agents are kept in fat tissue metabolic degradation appears rather unlikely thus maintaining an active release system (Sweeney *et al.*, 2006). Correspondingly, Van der Schans and colleagues (2003) reported that it took more than 3 h to reach a maximum concentration in blood of the rather lipophilic VX after percutaneous administration of $1 \times LD_{50}$ to hairless guinea pigs (see Percutaneous uptake, below).

B. Respiratory Uptake by Inhalation

1. Airways and Absorption

Gaseous poison or aerosols of toxic substances are incorporated following inhalation and contact with the respiratory tract. This appears to be the predominant route of poison intake for volatile G-type nerve agents, e.g. tabun, sarin, and soman exhibiting relatively high vapor pressures (Table 50.1). The respiratory tract is composed of three main compartments distinguishable by the areas and organs involved in the breathing process: (1) the extrathoracic or nasopharyngeal compartment comprising mouth, nose, and throat (pharynx), (2) the tracheobronchial compartment containing the voice box (larynx), windpipe (trachea), and right and left bronchi to convey air to the (3) alveolar or pulmonary compartment of the lung, which includes the bronchioles connecting the bronchi with the lobes of the lung and the alveoli. The sacs and cavities of the alveoli allow diffusion-controlled oxygen and carbon dioxide exchange in the blood of pulmonary capillaries (blood–air barrier). This diffusion process follows Fick's law whereupon the diffusion layer exhibits a thickness of 0.4–2.5 µm composed of surfactant and alveolocapillary membranes. The lung of a healthy adult person possesses 300–400 million alveoli spread over a surface of approximately 100 m^2 (Marquardt *et al.*, 1999).

Depending on the physicochemical nature of the toxic gas or aerosol, absorption will take place at different areas of the respiratory tract. Apart from the chemical properties the size of particles and aerosols will also affect the targeted area. Materials with diameters less than 2 µm will reach the alveoli whereas larger ones (about 20 µm in diameter) are retained in the upper respiratory areas of throat and bronchi thus protecting gas exchange in the lung.

a. Absorption in the Upper Respiratory Tract

In contrast to highly lipophilic compounds, hydrophilic toxicants characterized by higher water solubility, e.g. hydrogen chloride and fluorine, are primarily adsorbed by the wet mucosa membranes of the throat and trachea in the upper respiratory tract. This is also the primary area for G-agent first-order absorption according to higher water

solubility (Marrs *et al.*, 2007; Sweeney *et al.*, 2006). Early studies have shown that 80–90% of inhaled nerve agent sarin was readily absorbed by humans when exposed to concentrations ranging from 7 to 43 $mg \cdot min/m^3$ (Oberst *et al.*, 1959, 1968). Slightly lower rates (approximately 70%) were obtained for sarin applied to guinea pigs, dogs, and monkeys (Benschop and de Jong, 2001). More aggressive and reactive agents, e.g. vesicants or pulmonary agents, may harm by chemical burn provoking time delayed inflammation and scarring in these areas. Langenberg and colleagues (1998b) found significant effects of sulfur mustard in the upper respiratory tract of guinea pigs caused by a large fraction of the inhaled agent.

The nasal system of guinea pigs is more complex than that of other mammalian species, including humans. In those species the fraction of agent absorbed or deposited in the upper respiratory system may be smaller. Consequently, the middle and lower respiratory systems are likely to be exposed to a larger portion of the agent.

b. Absorption in the Middle Respiratory Tract

Toxic agents with medium water solubility, e.g. chlorine and sulfur dioxide, reach further into the middle respiratory tract being absorbed in the bronchi and its junctions. As a result, elevated excretion of mucus, coughing, and bronchoconstriction will lead to heavy dyspnea. The respiratory tube system from the nose to the bronchioles is lined with ciliated epithelium allowing transportation of the mucous layer produced by cells of the bronchial system from the lung into the oral cavity. This mechanism, which may be supported by coughing, allows the binding and removal of dust particles thus protecting the alveoli from deterioration. However, once the mucus has reached the oral cavity spontaneous swallowing might provoke poison transport into the digestive tract of the stomach and gut (gastrointestinal tract, GIT).

It is the middle respiratory tract that apparently is the most significant target in the case of respiratory exposure to sulfur mustard. Local effects, such as the formation of pseudomembranes, may both be life-threatening in the acute phase of illness after poisoning and cause long-term disability. The systemic absorption from the middle respiratory tract has never been investigated separately. However, findings from Langenberg's guinea pig model (Langenberg *et al.*, 1998b) suggest that the largest fraction of inhaled sulfur mustard is deposited in that particular area.

c. Absorption in the Alveoli

Several toxic compounds particularly gases of low water solubility or increased lipophilicity, e.g. carbon monoxide, hydrocyanic acid, nitrogen oxides, phosgene and further inorganic and organic irritants, reach the alveoli and are absorbed by the blood. Aggressive and reactive compounds inhaled may damage the epithelial cells of the alveoli causing the liberation of edema fluid which fatally prolongs the diffusion layer and minimizes the permeability for oxygen and carbon dioxide, finally leading to unconsciousness or death by asphyxia (toxic lung edema). Only very few hints exist for OP-induced lung edema following a yet unraveled mechanism of action (Lainee *et al.*, 1991; Delaunois *et al.*, 1992; Niven and Roop, 2004). Similarly, in the case of sulfur mustard exposure the alveoli are only of minor importance in terms of target organ for both local effects and systemic absorption (Langenberg *et al.*, 1998b).

2. Nose-Only Exposure Model for Controlled Respiratory Uptake in Animal Studies *in Vivo*

To elucidate the velocity of nerve agent absorption via the respiratory tract, which may take seconds to hours, Langenberg and colleagues (1998a) designed an apparatus that challenged guinea pigs by nose-only exposure with a constant stream of nerve agent vapor in air held for several minutes. As guinea pigs were not ventilated artificially sublethal doses of soman and sarin (0.4–0.8 LCt_{50}) were applied thus enabling the affected respiratory frequency and minute volume to be monitored. A typical concentration–time profile detected for C(–)P(+)-soman after administration of C(±)P(±)-soman is characterized by a discontinuous curvature reflecting concentration increase during absorption and concentration decrease by elimination (see Respiratory uptake (nose-only model), below). For more detailed technical data on the nose-only apparatus the reader is referred to Langenberg *et al.* (1998a, b) and Benschop and de Jong (2001). The same group also used this apparatus in subsequent experiments to investigate the respiratory uptake of vesicant agents, results of which will be discussed in Respiratory uptake, below.

C. Gastrointestinal Uptake by Ingestion

When drinking poisoned liquids or ingesting contaminated food, toxicants will be incorporated and directly transferred from the mouth, through the gullet (esophagus) into the stomach and bowels (intestine) representing the GIT where transfer into the circulation occurs. The physiological function of the digestive tract includes intake, breakdown, transport, and digestion of food and creation of waste (excrement). Food ingredients as well as toxicants and their digested (metabolized) forms are either absorbed through the walls of the intestine for entering the circulation or eliminated by feces. The surface of the small intestine of an adult human covers more than 200 m^2 and is made up of a huge number of 4–5 million, tiny, finger-like projections (villi and microvilli) covering the surface of the mucous membrane. In contrast the resorbing areas of large intestine (0.5–1 m^2), stomach (0.1–0.2 m^2), rectum (0.04–0.07 m^2), and oral cavity (0.02 m^2) are explicitly smaller due to the lack of villi and are thus of minor importance for poison uptake. Uptake by diffusion through the lipid layers (hydrophobic molecules) and pores (small hydrophilic molecules) of the intestinal mucosa following Fick's law are the most common processes. Nevertheless, facilitated

diffusion and active transport based on affinity binding of the toxicant to carrier molecules might also occur (Marquardt *et al.*, 1999). The processes of pinocytosis and phagocytosis are unlikely for small chemical warfare agents. Following intestinal uptake into the circulation, toxicants are directly transported to the liver, where further metabolism by cytochrome P450 enzymes may happen. This is of essential relevance for toxification of OP pesticides, including parathion, chlorpyrifos, diazinon, and dimethoate, which are transferred into their more toxic oxon derivatives (Butler and Murray, 1997; Furlong, 2007). Liver and intestine are supposed to play very important roles in the elimination of free soman as deduced from rabbit studies at high soman dosing (Li *et al.*, 2002).

In contrast, for sarin it has been shown that the kidney is more important for detoxification than the liver (Little *et al.*, 1986). However, only very limited research efforts have been undertaken to characterize this route of poison uptake for nerve agents (Sidell and Groff, 1974; Sim *et al.*, 1971). Quite high lethal oral doses were reported for tabun (rabbit: 16,300 μg/kg; rat: 3,700 μg/kg; dog: 200 μg/kg) and sarin (rat: 550 μg/kg) indicating extensive hydrolysis in the GIT (Marrs *et al.*, 2007). Very few investigations of toxicokinetics of vesicants after ingestion have been conducted. Nevertheless, this route of exposure is supposed to result in a significant systemic absorption of the agent. The rationale behind this apparent ''blind spot'' of toxicokinetic investigation might be the rarity of gastrointestinal exposure under conditions of military operations. Moreover, should a GIT exposure occur, the local rather than the systemic effects would be life-threatening. See Invasion, below, for an in-depth discussion.

In contrast, ample work has been done with OP pesticides meeting the concerns of more than 500,000 deaths worldwide per year caused by accidental and suicidal ingestion (Eddleston *et al.*, 2005, 2008a, b).

D. Uptake by Intravenous Injection

Intravenous (i.v.) uptake of chemical warfare agents is highly unlikely for realistic exposure scenarios except perhaps for the contamination of open and bleeding wounds. Nevertheless, numerous scientific studies investigating the toxicity and therapeutic treatment of CWA made use of this route of administration. The rationale behind this design is to constitute a defined amount of poison in the blood which is immediately systemically distributed by the circulation to the target compartments under conditions of 100% bioavailability. Therefore, i.v. studies are of undoubted relevance to elaborate systemic toxicity and to characterize the impact on the whole organism. It was shown that sarin was distributed in the central nervous system within 20–30 s after i.v. administration to mice (Waser and Streichenberg, 1988). Therefore, poison acts without delayed uptake caused by diffusion or permeation through skin, tissue, and organs which would increase the extent of metabolic degradation and hydrolysis. The latter processes reduce the amount of toxic agents and hamper direct correlation to poison concentrations in the blood. As demonstrated by Van der Schans and colleagues (2003) only 2.5% bioavailabilty was observed in guinea pigs resulting from a 7 h permeation period after percutaneous adminstration of VX.

As outlined in the sections above, the amount of incorporated poison and its fraction interacting with target molecules to induce more or less specific toxicological effects may vary drastically even under controlled conditions of administration. However, monitoring of quantitative concentration–time profiles of original poison and its metabolites is needed to elucidate the kinetic behavior and to unravel pathophysiological mechanisms. Due to the broad range of such essential variations causing a number of uncertainties, many *in vivo* and *in vitro* studies have been performed during the last 30 years of sophisticated research on protection against OP compounds (OPC) and blister agents. The following sections will summarize the most important consolidated findings underlining the current status of toxicokinetics of the most prominent CWAs: nerve agents and vesicants.

III. NERVE AGENTS

A. OP Compounds as Nerve Agents

For important background information addressing the history, synthesis, basic chemical data, decontamination, toxicity, military use, and political relevance of nerve agents we refer readers to a number of textbooks, reports, and monographs which go beyond the scope of this toxicokinetic contribution (Augerson, 2000; Committee on Gulf War and Health, 2004; Franke, 1977; Koelle, 1963; Langford, 2004; Marrs *et al.*, 2007; Munro *et al.*, 1999; Richardt and Blum, 2008). Nevertheless, in the following section selected chemical and physicochemical properties are considered which are of importance for understanding the toxicokinetic behavior of OP compounds.

Well-known representatives of nerve agents are cyclosarin (*o*-cyclohexyl methylphosphonofluoridate, GF), sarin (isopropyl methylphosphonofluoridate, GB), soman (1,2,2-trimethylpropyl methylphosphonofluoridate, GD), tabun (ethyl dimethylphosphoramidocyanidate, GA), VX (*S*-2-diisopropylaminoethyl *o*-ethyl methylphosphonothioate), and Russian VX [*o*-isobutyl *S*-(*N*,*N*-diethylaminoethyl)methyl phosphonothioate, RVX, RV] (Table 50.1; Figure 50.1). Apart from these substances a large number of additional OP toxicants with high structural similarity are known on the experimental and laboratory level especially when including pesticides for civilian use. However, the above-mentioned compounds are the most prominent nerve agents in the literature. This fact is obviously due to political instructions for national security and defense programs considering historical development, large-scale production, and intended

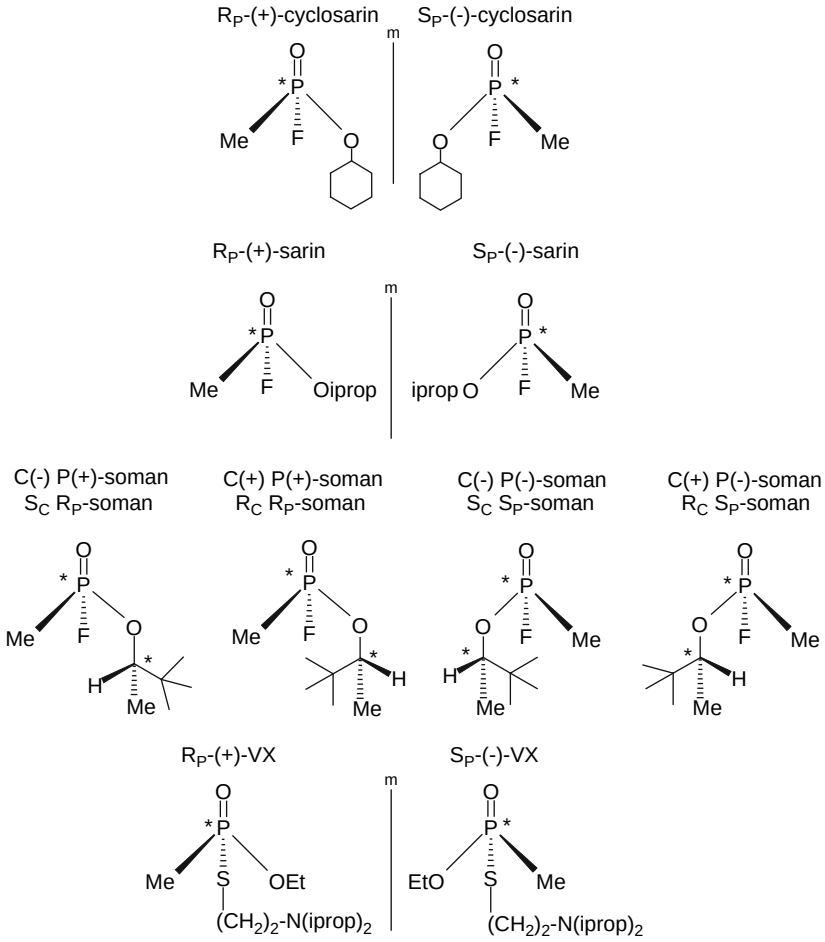

FIGURE 50.1. Structures of stereoisomeric OP nerve agents. Chirality was assigned according to the rules of Cahn, Ingold and Prelog (Cahn *et al.*, 1966; Prelog and Helmchen, 1982) considering oxygen of the P=O bond with minor priority compared to alkoxy substituents (Quin, 2000). Empirically found opticity is, according to Benschop and de Jong (1988), correlated to compounds obtained from stereoselective synthesis (Li *et al.*, 2001). Chirality emerged as a crucial parameter for toxicokinetic properties. S_P-isomers (P(−)-forms) of illustrated G-agents (cyclosarin, GF; sarin, GB; soman, GD) are characterized by higher toxicity combined with higher stability against hydrolyzing mammalian wild-type enzymes as their corresponding R_P-isomers, P(+)-forms (Table 50.3).

military use focusing on the agents most likely relevant for chemical threat. Soman, for example, was produced in large amounts especially by the former Soviet Union thus menacing the Western world during the Cold War period. Therefore, toxicokinetic studies on nerve agents are mainly restricted to sarin, soman, and VX. In addition, following United Nations inspections in Iraq in the 1990s, which revealed the weaponizing of cyclosarin, some studies on the toxicokinetic properties of this agent were also conducted.

1. Physicochemical Properties

If present in high purity all nerve agents are colorless and odorless liquids characterized by different vapor pressures thus causing either quite rapid evaporation with increasing inhalational risk (e.g. G-type agents) or relative high resistance abating p.c. absorption (e.g. V-type agents and cyclosarin) (Table 50.1). Therefore, visual or sensory recognition by smell or taste is nearly impossible.

a. Water Solubility

Water solubility is a crucial parameter affecting the toxicological potency of a compound. Solubility of non-hydrolyzed nerve agents is fundamentally determined by the hydrophobicity and extent of organic substituents. On the one hand, hydrophilic compounds exhibiting high water solubility are less effectively absorbed by skin in the absence of organic carrier solutes but they are easily distributed once they have reached aqueous biological fluids. Therefore, skin penetration of less lipophilic sarin (miscible in water) is not favored whereas nerve agents of significant lipophilicity with low water solubility (VX: 30 g/l; GF: 3.7 g/l, Table 50.1) penetrate the skin and other hydrophobic biological membranes and mucosa quite unhampered (Winkenwerder, 2002). Nerve agent transfer into blood and its systemic distribution are limited thus potentially provoking accumulation in fatty tissue. Nevertheless, noncovalent binding to carrier proteins of the circulation, e.g. γ-globulin and albumin, may support the systemic transport of hardly water-soluble compounds in blood as expected for VX (Vallet *et al.*, 2008) and as commonly known for endogenous fatty acids or exogenous drugs (John and Schlegel, 1999; Weiss *et al.*, 2008; Li *et al.*, 2007). Table 50.1 denotes the corresponding measures of solubility in water.

b. Octanol:Water Partition Coefficient

The lipophilicity of nerve agents and their expected partition behavior are characterized by the octanol:water

partition coefficient (log P). This parameter is often used as an estimate for the tendency of the toxicant to bioaccumulate in the organism. Furthermore, it is helpful to predict penetration of skin and distribution between tissue and blood (Czerwinski *et al.*, 1998, 2006; Poulin and Krishnan, 1995). The log P for the more polar sarin was determined to be 0.30 whereas this measure for lipophilic VX is 2.09 documenting a 50 times increased lipid solubility. However, this ratio might be slightly different when considering inconsistency of data from the literature (Table 50.1). Table 50.1 summarizes log P values of selected nerve agents.

c. Hydrolysis

In aqueous media, nerve agents undergo nonenzymatic hydrolysis which is accelerated by quite acidic and basic pH values (Franke, 1977). Hydrolysis substitutes primarily the reactive leaving group of the OP compound by a hydroxy group thus making the molecule more soluble (Figure 50.2). After cleavage of diisopropyl ethyl mercaptoamine (DESH) from the phosphorus atom of VX (Figure 50.1) the remaining ethyl methylphosphonic acid exhibits a water solubility of 180 g/l: six times higher than VX itself (Munro *et al.*, 1999). Depending on the pH, the alkoxy-group of VX might also be cleaved from the phosphorus atom. Hydrolysis of the reactive electronegative leaving group, which is essential for the primary toxicity (inhibition of acetylcholinesterase), deactivates the molecule thus reducing its toxicity dramatically. The major hydrolysis product of sarin is isopropylmethyl phosphonic acid (IMPA) which is about 10,000 times less toxic than its precursor when administered orally to rats (Munro *et al.*, 1999). IMPA is also the most prominent metabolite of sarin produced *in vivo* (Little *et al.*, 1986). Dramatically increased rates of hydrolysis under acidic conditions are presumably the most important reason for extraordinarily high LD_{50} values found in laboratory animals after oral administration of nerve agents (Marrs *et al.*, 2007). Table 50.1 summarizes hydrolysis rates expressed as periods of half-change in aqueous solutions of nerve agents near neutral pH.

d. Chirality

Typical production batches of nerve agents formerly intended for military use are mixtures of enantiomers obtained from nonchiral synthesis (Figure 50.1). Sarin, cyclosarin, tabun, and VX consist of mixtures of two enantiomers each of which differs in the chirality at the central phosphorus atom thus enabling rotation of linearly polarized light clockwise [P(+)-enantiomers] or anticlockwise [P(−)-enantiomers] (Figure 50.1). In contrast, chirality of soman appears more complex based on two chiral centers, which reside at the phosphorus atom, P(+) and P(−), and additionally in the pinacolyl moiety, C(+) and C(−). Hence, soman occurs in four stereoisomeric conformations as two pairs of diastereomers: P(+)C(+), P(+)C(−), P(−)C(+), and P(−)C(−) (Figure 50.1). To denominate stereoisomers of nerve agents the experimentally found rotational direction of light is typically provided whereas assignments according to the R and S nomenclature are rare. Therefore, Figure 50.1 summarizes both absolute configurations and related optical activities of these most common nerve agents. Chirality was assigned according to the established rules of Cahn, Ingold, and Prelog (Cahn *et al.*, 1966; Prelog and Helmchen, 1982) considering oxygen of the P=O bond with minor priority to alkoxy substituents (Quin, 2000). Empirically found opticity is, according to Benschop and de Jong (1988), correlated to compounds obtained from stereoselective synthesis (Li *et al.*, 2001).

It is well known that chirality of OP nerve agents causes significantly differing toxicological properties determining poison elimination by hydrolysis and kinetics of enzyme inhibition (Benschop and de Jong, 1988). Therefore, special attention should be paid to the corresponding stereoisomers when elaborating toxicokinetics. A number of studies took care of these conformational differences by administration of pure stereoisomers (Benschop and de Jong, 2001) or by enantioselective detection and quantification of poison molecules (Li *et al.*, 2003a, b; Reiter *et al.*, 2007; Spruit *et al.*, 2001; Van der Schans *et al.*, 2003; Yeung *et al.*, 2007). Different techniques were established to produce pure or at least enriched enantiomers of nerve agents, e.g. (1) synthesis by use of chiral adducts subjected to reactions of defined strereochemical outcome, (2) fractional crystallization, chromatographic separation, and isolation of mixed enantiomers, (3) stereoselective binding to serine-esterases, e.g. α-chymotrypsin, and (4) stereoselective enzymatic hydrolysis by phosphorylphosphatases (chemoenzymatic preparation) (Benschop and de Jong, 2001; Li *et al.*, 2001).

Stereoselective enzymatic degradation of nerve agents is also a current issue in developing both novel noncorrosive decontamination systems and new therapeutics making use of recombinant mutated enzymes optimized for fast and exhaustive hydrolysis of most toxic isomers (Blum and Richardt, 2008; Furlong, 2007; Ghanem and Raushel, 2005; Li *et al.*, 2001; Tsugawa *et al.*, 2000).

2. Toxicity

OP compounds, especially nerve agents, represent a class of highly reactive compounds undergoing nucleophilic substitution of their leaving group [e.g. F, CN, S-$(CH_2)_2$-N(^{i}prop)$_2$] (Figure 50.1) by nearly irreversible coupling to nucleophiles, e.g. strongly polarized hydroxyl groups in amino acid side chains or OH functions in aqueous media (Figure 50.2). Toxic effects are mainly due to derivatized enzymes which were subjected to this reaction.

Although most toxicological studies were performed as animal studies a few data for humans exist obtained from (1) military volunteers exposed to nonlethal doses of sarin (NRC, 1982, 1985), (2) accidentally intoxicated industrial

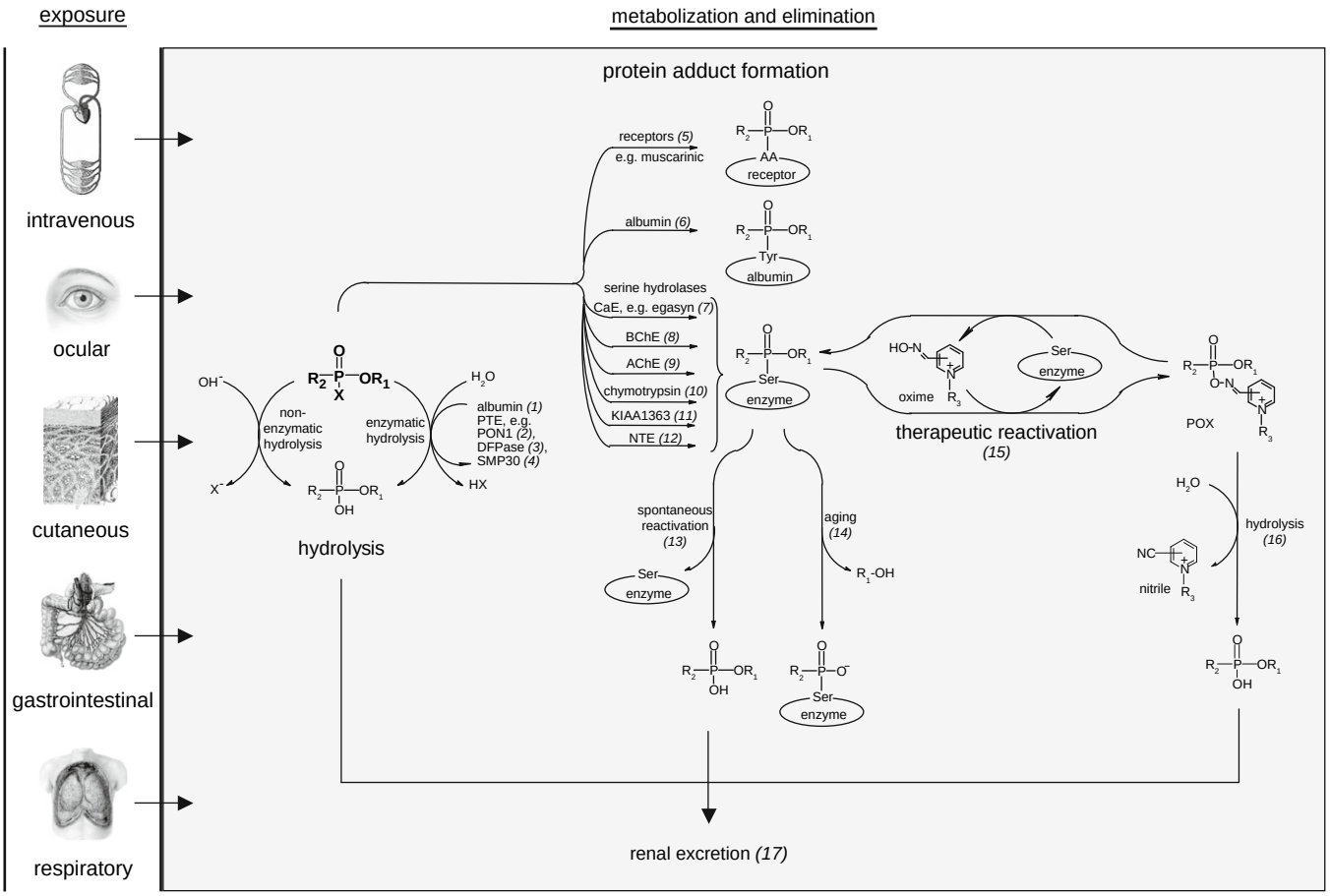

FIGURE 50.2. Elemental steps of toxicokinetics of OP nerve agents in mammalian organisms. OP nerve agents are incorporated following different routes of exposure. Whereas G-agents are predominantly uptaken by the respiratory tract via inhalation the V-agents follow mainly percutaneous invasion. Once the poison has reached the circulation it is distributed systemically causing poisoning of the central and peripheral nervous system by inhibition of acetylcholinesterase. Several processes of metabolism (e.g. enzymatic and nonenzymatic hydrolysis) and elimination (e.g. formation of adducts by binding to proteins and multiple serine esterases followed by marginal spontaneous reactivation and more prominent aging) reduce the amount of circulating poison. Therapeutic causal intervention by oximes reactivates cholinesterases under liberation of a toxic phosphoryloxime intermediate (POX) which itself undergoes immediate hydrolysis. Hydrolyzed nerve agents emanating from these chemical conversions are excreted by kidney more prominantly than by the liver. AA, amino acid; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CarbE, carboxylesterase; DFPase, diisopropyl fluorophosphatase; KIAA1363, acetyl monoalkylglycerol ether hydrolase; NTE, neuropathy target esterase; PON1, paraoxonase 1; POX, phophoryloxime; PTE, phosphotriesterases; $R_{1/2}$, organic substituents, e.g. methyl, cyclohexyl, isopropyl; Ser, serine; SMP30, senescence marker protein 30; Tyr, tyrosine; X, nucleophilic leaving group, e.g. F, CN, $(CH_2)_2$-N(^{i}prop)$_2$. (1) Li *et al.* (2007); Manoharan and Boopathy (2006); Vilanova and Sogorb (1999); (2) Amitai *et al.* (2006); Billecke *et al.* (2000); Furlong (2007); (3) Blum *et al.* (2006); Nordgren *et al.* (1984); (4) Kondo *et al.* (2004); (5) Silveira *et al.* (1990); (6) Black *et al.* (1999); Li *et al.* (2007, 2008); (7) Fujikawa *et al.* (2005); Maxwell and Brecht (2001); Satoh *et al.* (2002); (8) Bartling *et al.* (2007); Kolarich *et al.* (2008); (9) Aurbek *et al.* (2006); Benschop and de Jong (2001); (10) Casida and Quistad (2005); (11) Nomura *et al.* (2008); (12) Costa (2006); Gordon *et al.* (1983); (13) Aurbek *et al.* (2006); Worek *et al.* (2005); (14) Bartling *et al.* (2007); Worek *et al.* (1998, 2005); (15) Aurbek *et al.* (2006); Bartling *et al.* (2007); Kiderlen *et al.* (2005); Sidell and Groff (1974); (16) Kiderlen *et al.* (2005); (17) Minami *et al.* (1997); Shih *et al.* (1994); Waser and Streichenberg (1988).

workers (Duffy *et al.*, 1979), and (3) poisoned civilians affected by the terrorist attacks in Tokyo in 1995, Matsumoto in 1994, and Osaka (Morita *et al.*, 1995; Okumura *et al.*, 1996; Tsuchihashi *et al.*, 1998). For detailed data on these events readers are referred to the reports of the Committee on Health Effects Associated with Exposures During the Gulf War (2000).

a. Inhibition of Acetylcholinesterase

Phosphylation of the OH moiety of the serine residue, being part of the catalytic triad in the esteratic center of acetylcholinesterase (AChE), represents the pathophysiologically most important reaction resulting in enzyme deactivation. Inhibition of AChE was proven to be the predominant major reaction *in vivo* that causes death

within minutes in mammals, insects, and other species depending on acetylcholine (ACh) mediated signal transduction. Maxwell *et al.* (2006) found compelling arguments that inhibition of AChE by nerve agents is the primary mechanism of OP toxicity. They correlated the median lethal doses (LD_{50}) of highly toxic nerve agents determined from rats after s.c. administration with the corresponding bimolecular rate constants of AChE inhibition determined *in vitro* using a probit model for interpreting the mathematical relationship.

AChE is present in the nervous system, where it is most important for toxic effects, and on the surface of red blood cells (RBC) where its biological function is still unknown. Inactivation of AChE hinders the degradation of the neurotransmitter ACh in the synaptic cleft which is of major importance for the regulation of pre- and especially postsynaptic effects. Rising ACh concentrations cause permanent overstimulation of muscarinic (subtypes m1–m5) and nicotinic receptors of effector cells leading to cholinergic crisis and ultimately to death. Clinical symptoms of poison-induced AChE inhibition include (1) muscarinic effects (e.g. miosis, bradycardia, increased secretion of urine, saliva, tears and sweat, bronchoconstriction, and increased gastrointestinal motility) as well as (2) nicotinic effects (e.g. muscular weakness, twitching and tremors, elevated blood pressure, and tachycardia), and (3) central effects (e.g. headache, impaired memory and alertness, anxiety, insomnia, and most importantly respiratory depression and paralysis). Death is caused by respiratory failure as elicited by flaccid paralysis of respiratory muscles, and bronchoconstriction together with increased bronchial secretion and central respiratory depression (Costa, 2006). Liquids or vapors from these agents can cause death within minutes after exposure. Table 50.2 summarizes LD_{50} values for different species and routes of administration for the most common nerve agents. The relative lethality found in animal studies is as follows: VX > soman > cyclosarin > tabun (Sidell and Borak, 1992).

Local irritations do not occur except fasciculation of underlying muscles after percutaneous uptake or miosis caused by excessive stimulation of muscarinic receptors on papillary sphincter muscles resulting from ocular exposure (Dabisch *et al.*, 2008; Sidell and Borak, 1992). However, clinical symptoms related to massive restraints in the motoric and respiratory abilities require the reduction of AChE activity by more than 70% as deduced by Thiermann and colleagues (2005) from murine diaphragm experiments. Accordingly, acute cholinergic syndromes in humans were observed not until the red blood cell AChE activity was inhibited by 75–80% (Sidell and Borak, 1992). Clinical symptoms may depend on the gender of species as recently documented for the extent of miosis after vapor exposure to soman, cyclosarin, and VX (Dabisch *et al.*, 2008). Male rats were approximately three times less sensitive than female rats whereas minipigs show the reversed effect. This phenomenon is referred to different activities of ocular AChE and butyrylcholinesterase (BChE) (Dabisch *et al.*, 2008).

b. Additional Targets with Potential Clinical Relevance

As binding to proteins others than AChE reduces the amount of free OP poison these alternative targets are to be considered when discussing toxicokinetic behavior. Albumin (Black *et al.*, 1997a, b; Williams *et al.*, 2007; Li *et al.*, 2007, 2008), receptor/channel complexes (Pope, 1999), the muscarinic and nicotinic ACh receptors (Bakry *et al.*, 1988; Silveira *et al.*, 1990), and other secondary serine-hydrolase targets were shown to be chemically modified by OPCs thus changing their functionality into potential pathophysiological situations by affecting noncholinergic mechanisms (Casida and Quistad, 2005; Duysen *et al.*, 2001). In general, chemical modification of those proteins and enzymes requires quite high OPC concentrations which are far beyond the lethal dose of nerve agents thus being of minor relevance for acute poisoning scenarios (Pope, 1999). In contrast, elaboration of the toxicity of the much less toxic OP pesticides is more and more focused on these additional targets investigating, for example, genetic susceptibility, developmental toxicity and neurotoxicity, delayed neurotoxicity, and organophosphate-induced delayed polyneuropathy (OPIDP, a distal sensorimotor axonopathy) (Balali-Mood and Balali-Mood, 2008; Costa, 2006). OPIDP is associated with the OP-inhibited enzyme neuropathy target esterase (NTE) that undergoes an essential aging process of phosphylated NTE (elimination of an organic substituent from the central phosphorus atom, Figure 50.2) (Costa, 2006). However, Gordon and colleagues (1983) demonstrated that despite NTE inhibitory potency of the nerve agents tabun, soman, and VX no OPIDP was induced. Nerve agent concentrations inhibiting half of the NTE activity were about three orders of magnitude higher (micromolar range) than for AChE (nanomolar range).

B. Elemental Steps of Nerve Agent Toxicokinetics

Although toxicological characterization in terms of mean acute lethal doses for different species is available for all nerve agents, detailed and extensive toxicokinetic data are rare in the literature for most of them with the exception of soman and sarin (Benschop and de Jong, 2001). For VX and in particular GF only a very limited extent of data is obtainable (Reiter *et al.*, 2007; Van der Schans *et al.*, 2003).

1. Invasion

Supplementary to the more common discussion on invasion processes given in the introductory overview (Section II) we now present some recent findings on skin penetration models. Nerve agents are readily absorbed through skin, eyes, lung, and the gastrointestinal tract. Depending on the individual vapor pressures of nerve agents different routes

TABLE 50.2. Acute lethality of organophosphorus nerve agents

	LD_{50} (µg/kg)			
	Sarin	**Soman**	**Tabun**	**VX**
Intravenous				
Human	14[b]		14 (LD_{Lo})[c]	1.5 (TD_{Lo})[e]
Rat	45–63[c]	44.5[f]	70[c]	7–10[c]
Mouse	83[d]	35[f]	150[e]	20[d]
Guinea pig		27.5[d]		
Rabbit	15[e]		63[e]	
Percutaneous				
Human	24–28*10^3[c]	18,000 (LD_{Lo})[e]	14–21*10^3[c]	86 (LD_{Lo})[e]
Rat	2,500[c]		18,000[f]	
Mouse	1,080[f]	7,800[e]	1,000[f]	
Guinea pig	8,750[g]	9,930[g]	25,840[g]	34[g]
Rabbit	925[f]		2,500[e]	
Subcutaneous				
Human				30 (LD_{Lo})[e]
Rat	103–108[b]	70–165[c]	162[c]	12[e]
Mouse	170[b]	156[d]	250[e]	22[f]
Guinea pig	30[e]	24[f]	120[e]	8.4[f]
Rabbit	30[e]	20[f]	375[e]	14–66[f]
Respiratory[a]				
Human	50–100[b]	70 (LD_{Lo})[e]	150 (LD_{Lo})[e]	5–15[h]
Rat	80–300[b]		30.4[f]	
Mouse	240–380[b]	33.3[e]	0.5[e]	
Guinea pig	100–200[b]		197[e]	
Rabbit	75–144[b]		84[e]	

LD_{Lo} – lethal dose, low: the minimum amount of a chemical which has shown to be lethal to a specified species; TD_{Lo} – lowest toxic dose
Data do not consider chiral distinctions
[a]Given as LCt_{50} [mg·min/m^3]
[b]Winkenwerder (2002)
[c]Subcommittee on Chronic Reference Doses for Selected Chemical Warfare Agents, National Research Council (1999)
[d]Benschop and de Jong (2001)
[e]Maynard and Beswick (1992)
[f]ToxNet, Toxicology Data Network
[g]Czerwinski *et al.* (2006)
[h]Augerson (2000)

of poison uptake are preferred. G-agents, e.g. sarin, soman, and tabun, are quite volatile thus limiting percutaneous uptake due to significant evaporation from the skin (approximately 98% for sarin). In contrast VX exhibits high persistency because of its vapor pressure being 3,000 times lower than that of sarin (Marrs *et al.*, 2007) (Table 50.1). Therefore, percutaneous uptake is most prominent for V-agents characterized by an absorption rate of at least 600 µg/cm^2 per hour as shown for VX after inner ear-skin droplet application of 2 LD_{50} (Figure 50.2) (Chilcott *et al.*, 2005). Nevertheless, VX penetration through skin is characterized *in vitro* by a significant lag time of at least 1 h and a moderate penetration rate of about 1–2%/h (Vallet *et al.*, 2008). These data are in accordance with early studies on human subjects demonstrating that 3 h after percutaneous exposure of VX only 0.4–0.6% was incorporated (Vallet *et al.*, 2008). *In vitro* studies using guinea pig and human skin as well as dermatomed, abdominal skin from domestic pig revealed significant differences for VX permeability. Highest permeability was observed for the skin of the guinea pig whereas no significant differences in the penetration kinetics were found for human and pig skin taken from the animal's flank (Dalton *et al.*, 2006). Therefore, pig skin may serve as an appropriate *in vitro* model for human skin (Vallet *et al.*, 2008) as already established in pharmaceutical research making use of the Franz-type diffusion cell (Franz, 1975; Simonsen and Fullerton, 2006). To predict *in vivo* human VX absorption via skin the human full-thickness abdominal skin was demonstrated to be appropriate also (Vallet *et al.*, 2008).

2. Distribution

Once nerve agents have penetrated into the blood, systemic distribution including crossing of the blood–brain barrier causes toxicity within the central and peripheral nervous system. In mouse and rat studies it has been shown that within 1 min after sublethal single dose i.v. administration of sarin the nerve agent was present in many other compartments, e.g. diaphragm, heart, lung, and brain, and in much higher concentrations in plasma, liver, and kidney (Little *et al.*, 1986; Waser and Streichenberg, 1988). Similar results were observed for VX (Chilcott *et al.*, 2005) and soman, which was also found in cerebrospinal fluid with 100% bioavailability after i.v. bolus injection to pigs (Göransson-Nyberg *et al.*, 1998; Augerson, 2000). For mathematical description Langenberg and colleagues (1997) calculated related tissue/blood partition coefficients for the distribution of soman in guinea pigs revealing approximately a measure of 2 for liver and 1.1 for kidney whereas lung and brain were calculated to be 0.5. Nevertheless sarin present in the brain did not severely inhibit AChE activity in cortex (60%), striatum (40%), and hippocampus (56%) within 24 h (Whalley and Shih, 1989). In contrast soman has caused more severe inhibition in these three areas (83–99%) lowering the synaptosomal sodium-dependent, high affinity choline uptake (SDHACU) within the first 4 h in the hippocampus, whereas from 2–24 h after exposure SDHACU increased in the striatum (Whalley and Shih, 1989). The differences are thought to be due to, e.g. different aging rates and different AChE-inhibiting potencies. However, brainstem and midbrain were influenced neither by sarin nor by soman. It is assumed that active sites of the brain are affected primarily according to increased metabolic action, vasodilation, and increased blood flow in these regions (Scremin and Jenden, 1996). In contrast, 50% inhibition of AChE activity in brain was associated with death or serious signs of toxicity in mice after subcutaneous exposure (Duysen *et al.*, 2001).

The presence of sarin, soman, and VX in the brain demonstrates the necessity for antidotes, especially AChE reactivators, to be capable of passing the blood–brain barrier, representing a current scientific challenge (Lorke *et al.*, 2008; Okuno *et al.*, 2008).

Concentrations of sarin found in the different tissues were decreased by 85% within 15 min after exposure (Committee on Gulf War and Health, 2004). Apart from the active sarin its inactivated metabolite IMPA was found in these tissues in a predominant ratio indicating rapid *in vivo* metabolism as discussed in the next section. Soman was mainly accumulated in the lung after s.c. challenge of rats (Shih *et al.*, 1994) and disappeared from blood and liver largely 2 min after i.p. administration of $0.75 \times LD_{50}$ to mouse (Nordgren *et al.*, 1984). In contrast, VX is more persistent *in vivo* than sarin or soman causing delayed systemic distribution after s.c or p.c. administration. This fact provoked maximum concentrations of VX in blood several hours after p.c. exposure of guinea pigs (Van der Schans *et al.*, 2003).

As outlined in the toxicity section above the toxicological mechanism of action of nerve agents is based on the chemical reactivity of the nucleophilic leaving group. Therefore, metabolism in terms of degradation by hydrolysis and binding to proteins determines bioavailability and elimination processes thus regulating toxicity.

3. Metabolism and Elimination

Enzymes from plasma and tissue are mainly responsible for hydrolysis of OP compounds producing derivatives of phosphoric and phosphonic acids characterized by high water solubility and nearly no toxicity. *In vivo* studies in mice have shown that within 1 min after injection 50% of sarin was rapidly metabolized generating both free hydrolyzed IMPA and bound IMPA attached to esterases by phosphylation as predominant in plasma (Little *et al.*, 1986). In contrast, V-type agents are more stable against enzymatic hydrolysis but may undergo additional metabolism pathways including oxidation of nitrogen and/or sulfur (Van der Schans *et al.*, 2003). Due to the slow reaction velocity of nonenzymatic hydrolysis this process is of minor importance for the elimination kinetics of nerve agents under physiological conditions near neutral pH. As listed in Table 50.1 periods of half-change for nerve agent hydrolysis range from approximately 9 h for tabun to 6 weeks for VX. In contrast, the velocity of enzymatic hydrolysis in blood is much faster thus defining the rate-determining step for poison elimination.

a. Enzymatic Hydrolysis

Enzymes which hydrolyze OP compounds cleave the reactive leaving group of OP compounds, e.g. F or CN, from the central phosphorus atom thus initiating nucleophilic substitution by a hydroxyl group. Hydrolyzing enzyme activity is present in plasma and to a much higher specific extent in kidney and liver thus enabling removal of toxicants from the circulation (Sweeney *et al.*, 2006). Early studies using an isolated hydrolyzing rat liver enzyme demonstrated degradation efficacy for nerve agents in the following order: sarin > soman > tabun (Little *et al.*, 1989). Metabolites deactivated by hydrolysis are nearly nontoxic and easily eliminated from the organism via renal excretion (Munro *et al.*, 1999). Despite this capability to hydrolyze OP compounds the original physiological functions and substrates of the different enzymes vary significantly not allowing assignment to a specific uniform class of enzymes (Figure 50.2). The following section presents the most important enzyme systems relevant for nerve agent metabolism.

i. Phosphotriesterases (PTE)

Based on historical development, different, sometimes inconsistent, nomenclatures were used to assign enzymes that lead to the degradation of OPs. Meanwhile, the

International Union of Biochemistry has introduced systematic rules and numbering classifying the relevant enzymes in the following way: hydrolases (group 3), which cleave ester links (group 3.1) which may represent carboxylester hydrolases (group 3.1.1) or phosphoric triester hydrolases, PTE (group 3.1.8). The latter group contains aryldialkylphosphatase (EC 3.1.8.1) and diisopropyl fluorophosphatase (EC 3.1.8.2) (Vilanova and Sogorb, 1999). As these enzymes catalyze substrate cleavage without self-inhibition they are representatives of A-esterases.

- *Paraoxonase 1 (PON1)*

Paraoxonase (PON1, EC 3.1.8.1, formerly EC 3.1.1.2) is a calcium-dependent liver-expressed P450 phosphotriesterase belonging to the class of A-esterases with broad substrate specificity towards various lactones and esters, which is present in liver and in plasma associated with high-density lipoprotein particles (Vilanova and Sogorb, 1999). P450 isozymes and variants are well known for their dual role in organophophorus metabolism. On the one hand, they bioactivate less toxic phosphorothioates to their highly toxic oxon derivatives via monooxygenase activity. On the other hand, they hydrolyze and detoxify OP insecticides, such as paraoxon, chlorpyrifos, and diazinon by dearylation, as well as nerve agents, e.g. sarin and soman, by defluorination (Davies *et al.*, 1996; Furlong, 2007; Kiderlen *et al.*, 2005) (Figure 50.2).

The original physiological function of PON1 is involved in inactivation of toxic products produced by lipid oxidation (Draganov and La Du, 2004). In addition, PON1 hydrolyzes OP compounds, such as sarin, cyclosarin, tabun, and soman, thus accomplishing enzymatic protection against nerve agents in the circulation (Amitai *et al.*, 2006; Billecke *et al.*, 2000) (Figure 50.2). Levels and genetic variability of PON1 influence sensitivity to these specific substrates caused by Glu/Arg point mutation at position 192 of the human wild-type enzyme (Billecke *et al.*, 2000; Dragonov and La Du, 2004). The Glu^{192} mutant is about three times more active than the Arg^{192} variant (Draganov and La Du, 2004). Despite these differentiations rather low catalytic activity of recombinant human PON1 expressed in HEK cells occurred as a moderate stereoselective process characterized by preferred cleavage of the less toxic P(+)C(+)-enantiomer of soman (k_{cat} 1,030 min^{-1}) which happens twice as fast as for the other three stereoisomers (Yeung *et al.*, 2007, 2008) (Table 50.3). In accordance, Nordgren *et al.* (1984) used an enzyme isolated from swine kidney they called phosphoryl phosphatase to incubate purified enantiomers of soman *in vitro*. Whereas the less toxic P(+)-isomers (R_PS_C- and R_PR_C-soman) were hydrolyzed quite rapidly ($\tau_{1/2} = 2$ min under experimental conditions) both highly toxic P(−)-forms (S_PS_C- and S_PR_C-soman) showed much higher stability ($\tau_{1/2}$ 60–120 min). It appears likely that the predominant enzyme isolated was PON1. In contrast, no stereoselective effects in the catalytic PON1 mediated hydrolysis of cyclosarin and soman using recombinant mammalian material from *E. coli* were detected by Amitai *et al.* (2006). Nevertheless, no (+)-cyclosarin was detected in hemolyzed blood samples taken from swine after i.v. administration of racemic (±)-cyclosarin whereas the (−)-cyclosarin enantiomer was present for at least 20 min after exposure (Reiter *et al.*, 2007). However, the authors suppose that this phenomenon was due to rapid enzymatic and nonenzymatic hydrolysis in blood *in vivo* but do not discuss the potential role of PON1 explicitly. Yeung *et al.* (2007) conclude that variations in the catalytic efficiency of hPON1 towards soman enantiomers are due to differing Michaelis-Menten constants (K_M) characterizing the stability of the enzyme–substrate complex. Consequently, site-directed mutagenesis of recombinant enzymes could cause reduction of K_M for the more toxic enantiomers improving its hydrolyzing capacity. A 10–100-fold increase in catalytic activity of wild-type hPON1 is expected to allow effective protection against incorporated nerve agents (Amitai *et al.*, 2006; Rochu *et al.*, 2007; Masson and Rochu, contribution to this handbook). Accordingly, engineering efficient recombinant human PON1 is a current challenge in medical defense research intending to yield an effective and biocompatible therapeutic applicable for a wide range of nerve agents exhibiting sufficient activity towards all relevant isomers (Amitai *et al.*, 2006). Figure 50.2 displays its role in metabolism and elimination.

- *Senescence Marker Protein-30 (SMP30)*

The human senescence marker protein-30 (SMP30 or regucalcin, primary Swiss-Prot accession No. Q15493) is expressed by hepatocytes and plays a role in the regulation of plasma membrane Ca^{2+}-pumping activity with the potential to rescue cells from high calcium level-induced apoptosis (Kondo *et al.*, 2004). The expression of this liver enzyme decreases with aging. SMP30 was originally identified in rat liver and exhibits a 65% amino acid similarity to PON1 in rat species but does not show catalytic PON1 activity towards the hydrolysis of paraoxon (Billecke *et al.*, 1999). In contrast, mouse and rat SMP30 hydrolyze diisopropyl phosphorofluoridate (DFP), an OP compound related to nerve agents (Kondo *et al.*, 2004). Therefore, it may be speculated that this enzyme is also involved in nerve agent metabolism in the liver. This assumption is supported by the findings of Little *et al.* (1989) who observed hydrolysis of sarin, soman, and tabun by an enzyme derived from rat liver homogenate.

- *Diisopropyl Fluorophosphatase (DFPase)*

Some enzymes were isolated but not unambiguously identified from swine kidney which exhibit diisopropyl fluorophosphate (DFP)-cleaving activity and were thus denominated diisopropyl fluorophosphatase (DFPase) in the older literature (Nordgren *et al.*, 1984). This DFPase belongs to the class of A-esterases acting on DFP, tabun, and organofluorophosphates, e.g. cyclosarin, sarin, and soman. Nowadays, most recent studies define DFPase (EC 3.1.8.2, formerly assigned as EC 3.8.2.1) as a calcium-dependent phosphotriesterase identified in squid *Loligo vulgaris* exhibiting

TABLE 50.3. Catalytic constants for hydrolysis of nerve agents and rate constants of esterase inhibition

	Inhibition rate constant, k_i ($M^{-1}min^{-1}$)								k_{cat} (min^{-1})
	Bovine AChE	Electric eel AchE	Human AChE	Minipig AChE	Pig AChE	Equine BChE	Human BChE	Rat plasma CarbE	hr wt PON1
Soman									
C(±)P(±)-soman	$5*10^{7}$[d]	$1.5*10^{8}$[d]	$9.2*10^{7}$[b,1]			$1.29*10^{7}$[e]	$2.8*10^{8}$[b]	$0.51*10^{7}$[e]	7500[i,3]
C(+)P(+)-soman	$<1*10^{4}$[d]	$<5*10^{3}$[d]	$2*10^{3}$[h,2]			$1.7*10^{6}$[h]	$6*10^{6}$[h]		1030 ± 94[f]
C(−)P(+)-soman	$<1*10^{4}$[d]	$<5*10^{3}$[d]	$2*10^{3}$[h,2]			$1.2*10^{5}$[h]			593 ± 54[f]
C(+)P(−)-soman	$1.75*10^{8}$[d]	$2.8*10^{8}$[d]	$8*10^{7}$[h,2]			$1*10^{7}$[h]	$5*10^{6}$[h]	$3*10^{7}$[j]	553 ± 163[f]
C(−)P(−)-soman	$2.7*10^{7}$[d]	$1.8*10^{8}$[d]	$1.5*10^{8}$[h,2]			$6*10^{7}$[h]	$4*10^{7}$[h]	$1*10^{6}$[j]	501 ± 45[f]
Sarin									
(±)-sarin	$1.51*10^{7}$[e]		$3.2*10^{7}$[b,1]			$0.56*10^{7}$[e]	$3.2*10^{7}$[b]	$0.30*10^{7}$[e]	
(+)-sarin	$<3*10^{3}$[g]								
(−)-sarin	$1.4*10^{7}$[g]								
Cyclosarin									
(±)-cyclosarin			$4.21*10^{8}$[a,1]	$4.84*10^{8}$[a]	$4.84*10^{8}$[a]		$7.2*10^{8}$[b]		25,400[i,3]
VX									
(±)-VX	$3.23*10^{7}$[e]		$9.91*10^{7}$[a,1]	$5.61*10^{7}$[a]	$4.43*10^{7}$[a]	$6.3*10^{7}$[e]		$1.51*10^{3}$[e]	
(+)-VX	$2.0*10^{6}$[g]								
(−)-VX	$4.0*10^{8}$[g]								
VR									
(±)-VR			$4.60*10^{8}$[a,1]	$1.95*10^{8}$[a]	$1.88*10^{8}$[a]				
CVX									
(±)-CVX			$3.06*10^{8}$[c,1]		$1.46*10^{8}$[c]				

Rate constants for inhibition of most prominent serine esterases by nerve agents differ significantly depending on the nature of enzyme, its originating species and stereoisomers of the agent. It appears obvious that for the depicted G-agents and VX the P(−)-isomers are much more effective inhibitors than their corresponding P(+)-forms. In contrast hydrolysis of the latter isomers happens faster than of the toxic P(−)-variants. Hydrolytic stability is determined by PON1 activity characterized by its catalytic constant k_{cat} (last column). Both properties (inhibition and hydrolysis) cause higher toxicity for P(−)-agents *in vivo* selectively (Table 50.4)

AChE – acetylcholinesterase; BChE – butyrylcholinesterase; CarbE – carboxylesterase; CVX – Chinese VX; hr wt – human recombinant wild-type; k_{cat} – catalytic constant for hydrolysis; PON1 – paraoxonase 1; VR – Russian VX

[1]Human red blood cell AChE
[2]Recombinant human AChE
[3]Recombinant rabbit PON1 from *E. coli*
[a]Worek *et al.* (2008)
[b]Bartling *et al.* (2007)
[c]Aurbek *et al.* (2006)
[d]Benschop *et al.* (1984)
[e]Maxwell and Brecht (2001)
[f]Yeung *et al.* (2007)
[g]Benschop and de Jong (2001)
[h]Ordentlich *et al.* (1999)
[i]Amitai *et al.* (2006)
[j]Sweeney *et al.* (2006)

unique structural properties (Blum *et al.*, 2006). This squid-type DFPase has not yet been identified in mammalian organisms.

ii. Non-Mammalian Enzymes

Several other enzymes from bacteria (e.g. phosphotriesterases or OP hydrolases, OPH, from *Pseudomonas diminuta* or *Flavobacterium* sp. and OP acid anhydrolase, OPAA, from *Alteromonas* sp.) or squid (diisopropyl fluorophosphatase, DFPase) are also known to detoxify nerve agents thus being of interesting relevance for novel noncorrosive decontamination approaches (Blum and Richardt, 2008) but do not play a role in the toxicokinetics of OP compounds in mammalian species so far (Amitai *et al.*, 2006; Rochu *et al.*, 2007; Masson and Rochu, contribution to this handbook). However, potential application as detoxifying antidotes remains a future challenge.

b. Formation of Protein Adducts

Besides direct enzymatic hydrolysis of nerve agent substrates numerous additional proteins are present in the

organism that allow covalent binding to OP compounds thus contributing to detoxification of the poison load (Figure 50.2). Serine esterases are especially predominant targets of nerve agents mostly undergoing irreversible adduct formation. Nevertheless, more than 75% of the serine hydrolases (B-esterases) present in plasma and tissues are essentially unknown with respect to their interaction with OPCs (Casida and Quistad, 2005).

i. Carboxylesterase

Ubiquitous glycosylated carboxylesterases (CarbE, EC 3.1.1.1), formerly named ali-esterases, are B-esterases belonging to the multigene enzyme superfamily of α/β hydrolases (Hosokawa and Satoh, 2006; Satoh and Hosokawa, 2006). In principle this class of isozymes plays a major role in pharmacokinetics by hydrolytic biotransformation of exogenous ester-drugs and ester-prodrugs. However, their physiological function still remains unclear (Satoh and Hosokawa, 2006).

Carboxylesterases are very important serine-esterases in plasma of nonhuman species that bind nerve agents with broad specificity thus representing the major determinant for *in vivo* detoxification especially in mice and rats (Maxwell and Brecht, 1991) (Figure 50.2). Apart from catalytic CarbE activity in plasma it is also found in several tissues and organs including brain, lung, kidney, and liver thus realizing poison decrease not only in the circulation (Satoh *et al.*, 2002). Microsomal liver carboxylesterase 1, which is also referred to as egasyn, is loosely associated with a β-glucuronidase (BG) complex (Fujikawa *et al.*, 2005). Organophosphate-inhibited egasyn causes cleavage of this complex liberating elevated quantitites of BG into the circulation. Thus BG plasma activity may serve as a sensitive biomarker for OP poisoning (Inayat-Hussain *et al.*, 2007).

Interaction of CarbE with nerve agents follows a kinetic of first order characterized by inhibition of CarbE at the active site serine residue described by a bimolecular rate constant, k_i (Maxwell and Brecht, 2001). For noncharged nerve agents (e.g. sarin and soman) the k_i of rat serum CarbE was found to be $>10^6\,M^{-1}min^{-1}$ whereas cationic substrates (e.g. VX) are converted with poor reactivity ($k_i < 10^4\,M^{-1}min^{-1}$). This specificity is explained by the electrostatic characteristics of the large active site containing only a few cation-Π bonding and anionic residues (Maxwell and Brecht, 2001; Satoh and Hosokawa, 2006).

Covalent binding of OP compounds to CarbE is considered as an irreversible reaction of 1:1 stoichiometry resulting in adducts that do not age (Maxwell and Brecht, 2001). In contrast, spontaneous pH-dependent reactivation liberates the enzyme thus being accessible for additional detoxification although only to a very limited extent. At physiological pH *in vitro* spontaneous reactivation (specified by the rate constant k_r) is a poison-dependent process showing significantly different velocities depending on the size of the inhibitor (steric demand). Whereas reactivation of VX- and sarin-inhibited rat serum CarbE was faster (k_r $4.2*10^{-3}$ min^{-1} and $3.8*10^{-3}$ min^{-1}) reactivation for soman and VR was about ten times slower (k_r $0.44*10^{-3}$ min^{-1} and $0.52*10^{-3}$ min^{-1}) (Maxwell and Brecht 2001). In addition to these differences CarbE exhibits stereoselective properties. Nordgren and colleagues (1984) have demonstrated that CaE from swine liver bound to the less toxic P(+)C(+)-enantiomer of soman (R_CR_P) most efficiently whereas conversion of highly toxic P(−)C(+)-soman (R_CS_P) happens much more slowly (Table 50.3). This fact indicates that degradation of incorporated nerve agents is primarily targeted on less toxic enantiomers thus minimizing the protective effect of CaE. In contrast to G-agents no prominent enantiomeric selectivity was evident for sequestration of VX after p.c. administration (Van der Schans *et al.*, 2003).

Consequently, striking species-dependent differences in the LD_{50} values of OP compounds are mainly due to the variable concentrations of endogenous CarbE acting as a bioscavenger in blood (Table 50.2). Whereas mice and rats exhibit high CarbE activities, activity in rabbits and guinea pigs appears moderate. In contrast, dogs and primates as well as humans possess only little or no CarbE (Benschop and de Jong, 2001). These relations are obvious from s.c. soman LD_{50} values obtained from mice and guinea pigs after inhibition of CarbE (10.2 and 12.2 μg/kg) which were very similar to those obtained for dogs and primates without inhibition (9.1 μg/kg and 13.0 μg/kg) (Maxwell and Brecht, 1991). In contrast, the corresponding soman s.c. LD_{50} values for untreated CarbE are much higher for mice (113 μg/kg) and guinea pigs (28.2 μg/kg) (Maxwell and Brecht, 1991). For these reasons guinea pigs are most often used today for *in vivo* toxicity and toxicokinetic studies intending to examine a small laboratory animal model predictive for humans. Missing CarbE activity in humans hinders efficient detoxification *in vivo* thus keeping the toxic effect of nerve agents (Due *et al.*, 1993). Consequently, the value of CarbE as a bioscavenger for effective clearance of OP poison from the circulation has led to therapeutic concepts which were applied successfully to rodents and nonhuman primates (Maxwell and Brecht, 2001). Feasibility for human organisms has still to be demonstrated.

ii. Acetyl Monoalkylglycerol Ether Hydrolase (AcMAGE)

KIAA1363 (primary Swiss-Prot accession No. Q6PIU2) is a human serine hydrolase derived from acetyl monoalkylglycerol ether hydrolase (AcMAGE, EC 3.1.1.) present in brain, lung, heart, and kidney which is involved in tumor cell invasiveness and lipid metabolism but can also detoxify OP compounds by hydrolysis of the reactive leaving group following transient binding to the active site serine residue (Nomura *et al.*, 2008) (Figure 50.2). This capability has been demonstrated for the pesticide chlorpyrifos and its more toxic oxon derivative. Therefore, activity towards nerve agent hydrolysis is expected but has not been shown so far.

iii. Acetylcholinesterase (AChE)

Acetylcholinesterase (AChE, EC 3.1.1.7) from vertebrates is deduced from a single gene (Massoulie, 2002) expressing identical enzyme primary structures on the surface of red blood cells (RBC), in synapses and different organs. Inhibition of this serine-esterase causes the most prominent pathophysiological effects determining severity of poisoning. Nevertheless, covalent binding to AChE can also be considered as a step in poison elimination (Figure 50.2) which reduces the amount of incorporated poison even though only to a small extent.

AChE activity in whole blood differs significantly among mammalian species thus being of relevance for toxicokinetic consideration. Humans exhibit a quite high AChE activity in blood (651 mU/µmol hemoglobin, Hb) in contrast to smaller values for minipigs (297 mU/µmol Hb) and pigs (190 mU/µmol Hb) (Worek *et al.*, 2008). Furthermore, the bimolecular rate constants (k_i) for nerve agent-induced inhibition of AChE vary among different species. Table 50.3 gives an overview of corresponding inhibition kinetics. The k_i for inhibition of human AChE by VX, for example, is about twice as high as for pig AChE (Aurbek *et al.*, 2006). These differences have to be considered when assessing interspecies toxicokinetic data.

Nerve agents show enantioselective inhibition kinetics when reacting with AChE. Chirality at the central phosphorus atom plays the predominant role affecting higher rates of inhibition for the P(−)-isomers (S_P) than for the P(+)-forms (R_P) (Nordgren *et al.*, 1984). Benschop and de Jong (2001) made great efforts on this stereoselectivity topic and determined the corresponding inhibition rate constants (k_i) for electric eel AChE *in vitro* (Table 50.3). Both P(−)-diastereomers of soman (SpS_C- and S_PR_C-soman) exhibit very high k_i-values ranging from 1.8–2.8*10^8 $M^{-1}min^{-1}$ thus documenting their high toxicity. In contrast, the less toxic P(+)-diastereomers (RpR_C- and R_PS_C-soman) are characterized by k_i-values being 100,000 times smaller (<5*10^3 $M^{-1}min^{-1}$) (Benschop and de Jong, 2001). Similar results were obtained for the sarin enantiomers which differ by a factor of 5,000 revealing a k_i of 1.4*10^7 $M^{-1}min^{-1}$ for the S_P-(−)-form (Table 50.3). In contrast, VX-enantiomers exhibit the smallest differences as obvious from the k_i of the more toxic S_P-(−)-VX (4*10^8 $M^{-1}min^{-1}$) being 200 times higher than for the R_P-(+)-enantiomer (Table 50.3) (Benschop and de Jong, 2001). These k_i data correspond to the order of experimentally determined LD_{50} values in mouse although not considering the impact of hydrolyzing enzymes *in vivo* which also exhibit stereoselective kinetics for hydrolysis (Table 50.4) (Benschop and de Jong, 2001).

AChE on the surface of RBC shows a turnover rate of about 1% per day thus enabling complete exchange within approximately 100 days after exposure (Sidell and Borak, 1992). In contrast, regeneration of synaptic AChE is assumed to be faster reaching 7–10% turnover rates, an estimation that results from animal experiments (Brank *et al.*, 1998; Eddleston, 2008b; Grubić *et al.*, 1981). Both are of importance for the therapeutic monitoring of poisoning as well as for the verification of exposure following bioanalytical methods.

TABLE 50.4. Acute lethality in mice caused by stereoisomers of OP nerve agents

Nerve agent stereoisomers	LD_{50} (µg/kg) mouse
Soman	
C(±)P(±)-soman	156 (s.c.)[a]
C(+)P(+)-soman	<5,000 (s.c.)[b]
C(−)P(+)-soman	<5,000 (s.c.)[b]
C(+)P(−)-soman	99 (s.c.)[a]
C(−)P(−)-soman	38 (s.c.)[a]
Sarin	
(±)-sarin	83 (i.v.)[b]
(+)-sarin	
(−)-sarin	41 (i.v.)[b]
VX	
(±)-VX	20.1 (i.v.)[b]
(+)-VX	165 (i.v.)[b]
(−)-VX	12.6 (i.v.)[b]

Higher rate constants of P(−)-isomers for inhibition of AChE in combination with their minor susceptibility to enzymatic hydrolysis by mammalian phosphotriesterases cause eminently different lethal doses *in vivo*

[a]Benschop *et al.* (1984)

[b]Benschop and de Jong (2001)

- *Spontaneous Reactivation of AChE*

Although inhibition of AChE is mainly discussed as an irreversible reaction, detailed kinetic investigations consider the process of spontaneous reactivation rereleasing an active enzyme and a hydrolyzed detoxified agent (Figure 50.2). Accordingly, rate constants for spontaneous reactivation (k_S) of human AChE adducts are very small as measured for some sarin analogs (0.01–0.052 h^{-1}) whereas the k_S for sarin, cyclosarin, and VX was too small to provoke an experimentally detectable effect (Bartling *et al.*, 2007). Sidell and Groff (1974) demonstrated that spontaneous reactivation of RBC AChE inhibited by VX happens much faster than for sarin which is in accordance with recent human k_s data on V-agents: VX 0.021 h^{-1}; VR 0.039 h^{-1}; and Chinese VX (CVX) 0.171 h^{-1} (Aurbek *et al.*, 2006). However, the toxicokinetic relevance of spontaneous reactivation is obvious from the liberation of free esterase accessible for reinhibition with 1:1 stoichiometry.

- *Aging of AChE*

The cleavage of an organic substituent from the phosphorus atom which is bound to the side chain of an amino acid residue from a protein is described by the aging process

(Figure 50.2). Promoted by specific ionic interactions of neighboring amino acid residues in AChE (and BChE) the remaining P-OH function will undergo deprotonation thus leaving a negatively charged phosphorus moiety (Figure 50.2). Whereas esterase adducts of sarin and soman age by cleaving alkoxy moieties from the central phosphorus atom, tabun loses its dimethylamine moiety (P-N scission) instead of its ethoxy group (Elhanany *et al.*, 2001).

These aged enzyme adducts are not accessible for reactivation by common antidotal oximes thus limiting the success of therapeutic treatment of poisoning with OP compounds. The soman–AChE adduct is known for very rapid aging ($\tau_{1/2} \approx 2$ min) causing severe problems in clinical treatment (Worek *et al.*, 2005). Aging half-lives of phosphylated AChE as well as phosphylated BChE depend on the nature of the inhibiting OP compound. Whereas GF-inhibited AChE exhibits a half-life of 8.7 h the corresponding BChE derivative ages much more rapidly (2.2 h) (Worek *et al.*, 1998). The order of $\tau_{1/2}$ for aging of human AChE–nerve agent adducts was found to be: soman (2 min) < sarin (3 h) < cyclosarin (7 h) < tabun (19 h) < VX (36.5 h) (Worek *et al.*, 2005). Therefore, V-agent-inhibited RBC-AChE ages quite slowly in humans thus allowing therapeutic intervention with oximes for a longer period lasting several days (Sidell and Groff, 1974; Thiermann *et al.*, 2007).

iv. Butyrylcholinesterase (BChE)

Butyrylcholinesterase (BChE, EC 3.1.1.8), formerly named pseudocholinesterase, is synthezised in the liver and present in blood (5 μg/ml), the synapse of neuromuscular junctions, and glia cells and axons of white matter in the brain in numerous allelic variants (Massoulie, 2002). Although BChE has long been considered a nonfunctional vestigial analog of AChE, recent findings point out a possibly more prominent role especially in mouse where the total amount of BChE in the body is ten times as high as AChE (Duysen *et al.*, 2001). Correspondingly, it was observed that the activity of BChE in human whole blood was significantly higher than of AChE (Worek *et al.*, 2008). This led to speculation that BChE may play a backup role for insufficient AChE activity in neurotransmission as deduced from the physiology of AChE knockout mice (Duysen *et al.*, 2001) and may serve as a safeguard against diffusion of ACh into the bloodstream (Massoulie, 2002). However, mandatory experimental evidence is still missing.

Highly glycosylated BChE is a prominent target of OP compounds thus acting as a protective biological stoichiometric scavenger averting damage to neuronal AChE (Kolarich *et al.*, 2008). However, common nerve agents may exhibit significantly differing inhibition rate constants (k_i) for AChE and BChE being approximately in the range from 10^7 to $10^9\,M^{-1}min^{-1}$ (Bartling *et al.*, 2007). Table 50.3 allows comparison of inhibitory potency of nerve agents against AChE and BChE. *In vivo* studies in humans suggest that VX preferentially inhibits RBC AChE much more effectively than BChE resulting in 70% and 20% inhibition, respectively (Sidell and Groff, 1974). In addition to agent-dependent k_i values there is a striking stereoselective dependency in BChE inhibition. The more toxic soman P(−)-enantiomers (S_P) inhibit with preference when compared to their corresponding P(+)-forms (R_P) (Table 50.3) (Nordgren *et al.*, 1984). However, differences are not as pronounced as for AChE but point out effective detoxification properties of endogenous BChE.

Interestingly, no clinical features result from inhibited BChE *in vivo* (Eddleston *et al.*, 2008b). The status of plasma BChE activity is, despite all concerns, a commonly recommended measure to monitor the progress of chemical injury (Eddleston *et al.*, 2008a).

In contrast to all studies on VX toxicokinetics and toxicodynamics published so far, Dorandeu and colleagues (2008) reported an unexpected and not yet clarified phenomenon which emerged after i.v. administration of VX to isoflurane-anesthetized and ventilated swine without oxime therapy. Time-resolved measurement of esterase activities during the experimental period of poison application revealed very fast rebound of BChE activity (from 70% inhibition to 30% within 1 h) while retaining nearly complete inhibition of whole blood cholinesterase. Design and control experiments allowed the following explanations to be excluded: (1) spontaneous reactivation which should happen much more slowly, (2) induced hypoalbuminemia liberating albumin as scavenger competing with BChE, and (3) stimulated release of hydrolyzing enzymes like paraoxonase (PON1). More probably, this observation is explained by an increased biosynthesis of BChE in the liver and/or an elevated release of BChE from other organs (e.g. heart, lung, or pancreas). Future studies will possibly shed more light on this curious and interesting phenomenon.

As total replacement of BChE by synthesis in the liver happens within a couple of weeks, this rather long period allows experimental verification of OP poisoning even when blood samples from poisoned humans are collected with significant delay to the time of exposure (Sidell and Borak, 1992). Therefore, detection of BChE adducts by means of modern mass spectrometric methods is the state of the art technique to prove exposure to OP nerve agents (Carol-Visser *et al.*, 2008; Noort *et al.*, 2006; John *et al.*, 2008).

Nevertheless, the relevant enzyme adducts may undergo the previously described consecutive reactions: spontaneous reactivation and aging. Aging of BChE nerve agent adducts occurs with highly different agent-dependent periods of half-change ($\tau_{1/2}$) as determined *in vitro* by Worek *et al.* (2005). Whereas the soman adduct exhibits typically by far the shortest $\tau_{1/2}$ of less than 1 min, cyclosarin appears much more stable ($\tau_{1/2}$ 2.2 h), followed by tabun ($\tau_{1/2}$ 7 h) and sarin ($\tau_{1/2}$ 12 h). The adduct of VX was the most stable BChE derivative characterized by a $\tau_{1/2}$ of 77 h. Spontaneous reactivation by simple hydrolysis of the serine—phosphorus bond was observed for cyclosarin with a period of half-change of 20 h and much longer times for VX (63 h) and sarin (63 h)

(Worek *et al.*, 2005). These data demonstrate that inhibition of wild-type BChE is nearly irreversible thus lowering the amount of incorporated toxic OP compounds significantly in a stoichiometric manner. Therefore, protection against nerve agent doses of up to $5.5 \times LD_{50}$ (soman and VX) was achieved with exogenous BChE from different species applied prophylactically i.m. to guinea pig, rhesus monkey, and cynomolgus (Lenz *et al.*, 2007). It is supposed that a dose of 200 mg BChE/70 kg will be sufficiently protective in humans against $2 \times LD_{50}$ of soman (Saxena *et al.*, 2008). Based on this valuable protective capacity current efforts are under investigation to use BChE from recombinant (milk of transgenic animals) or natural sources for therapeutic, mainly prophylactic, treatment of nerve agent poisoning (Chilukuri *et al.*, 2005; Huang *et al.*, 2007; Lenz *et al.*, 2007; Lockridge *et al.*, 2005). Plasma half-life of recombinant preparations is significantly prolonged by fusion to albumin (Huang *et al.*, 2008) or pegylation (attachment of polyethyleneglycol) (Chilukuri *et al.*, 2005). In addition, enhanced rapid spontaneous reactivation will deblock the enzyme thus being accessible for subsequent binding to another OP molecule. This strategy is followed by site-directed mutagenesis of BChE leading to a 10^5-fold increase of dephosphorylating activity (Casida and Quistad, 2005). However, feasibility for the human organism has to be shown.

v. Albumin

Although albumin is present in blood in high concentrations (0.6 mM; 41 g/l; 50–60% of total plasma protein) it does not represent an effective scavenger for nerve agent detoxification *in vivo*, hampered by its slow reaction velocity (Li *et al.*, 2008). Nevertheless, sarin and soman bind to albumin at active site tyrosine residue 411 as shown in neat buffered solution and crude human plasma thus inhibiting the enzymatic acylamidase and esterase activity of albumin (Black *et al.*, 1999; Li *et al.*, 2007, 2008) (Figure 50.2). Albumin also exhibits slight hydrolyzing catalytic activity against OP compounds (Vilanova and Sogorb, 1999). Concerning the enantioselectivity in phosphonylation of albumin by soman contradictory results are presented in the recent literature. Whereas Li *et al.* (2008) did not find any enantiomeric preference, Yeung *et al.* (2008) demonstrated that human serum albumin preferentially binds to the less toxic C(±)P(+)-enantiomer. However, the albumin adduct is quite stable in terms of spontaneous reactivation exhibiting extended periods of half-change (6.5 days at 25°C, pH 8.0 and 20 days at 22°C, pH 7.4) and, in addition, does not show any aging phenomena (Li *et al.*, 2007, 2008). Derivatized albumin may thus serve as a biomarker for nerve agent exposure detectable by modern mass spectrometric techniques (Black *et al.*, 1999; Li *et al.*, 2007, 2008; Peeples *et al.*, 2005; Williams *et al.*, 2007).

vi. Muscarinic Receptors

It was shown that tabun, sarin, soman, and VX bind to the muscarinic receptor subtype m_2, leading to the assumption that the ACh binding site is deactivated thus causing potential additional vulnerability (Figure 50.2) (Silveira *et al.*, 1990). Despite the high affinity of nerve agents, pathophysiological effects seem to be of minor relevance when compared to AChE inhibition. However, binding to the receptors will lower the concentration of toxic and reactive OPCs.

4. Excretion

Sarin and its corresponding nontoxic hydrolysis products (IMPA, and additional methyl phosphonic acids) are predominantly eliminated via the kidneys which are thus more important for detoxification than the liver (Little *et al.*, 1986; Waser and Streichenberg, 1988). Urinary excretion happens quite rapidly as demonstrated for single dose s.c. application of sarin, cyclosarin, and soman to rats (Shih *et al.*, 1994). The terminal elimination half-life was found to be 3.7 ± 0.1 h for sarin and 9.9 ± 0.8 h for cyclosarin. In contrast soman showed a biphasic elimination with terminal half-lives of about 18.5 h and 3.6 h (Shih *et al.*, 1994). Maximum peak levels of sarin metabolites in urine were detected 10–18 h after exposure (Minami *et al.*, 1997) and after 2 days hydrolyzed sarin metabolites had been excreted nearly quantitatively (Shih *et al.*, 1994). In contrast, even at 5 days post-exposure soman metabolite recovery was only 62% (Shih *et al.*, 1994). Excretion of soman from blood, liver, and kidney compartments following chemical and enzymatic hydrolysis is considered a first-order elimination process (Sweeney *et al.*, 2006).

5. Concentration–Time Profiles of Nerve Agents in Blood After Various Routes of Administration

Extensive toxicokinetic studies in animal models using rats, marmosets, and guinea pigs were performed for sarin and most often for soman thereby considering individual concentration–time profiles of the different enantiomers (Benschop and de Jong, 2001). The resulting concentration curvatures reflect the combination of the toxicokinetic factors of distribution and elimination by hydrolysis and protein binding as described above in detail. The following section will briefly summarize the most prominent findings considering stereoselectivity and related blood concentration–time profiles. In contrast to lots of OP pesticides, detailed toxicokinetic data on oral uptake of nerve agents are not available. Therefore, we restrict this section to i.v., s.c., p.c., and respiratory exposure. For more details readers are referred to a concise and extensive overview given by Benschop and de Jong (2001).

a. Intravenous Uptake

Direct poison injection into the circulation allows immediate distribution in the organism but also enables undelayed elimination provoked by plasma components. To illustrate these impacts we introduce some fundamental results obtained for some G-agents as depicted in Figure 50.3A (Benschop and de Jong, 2001).

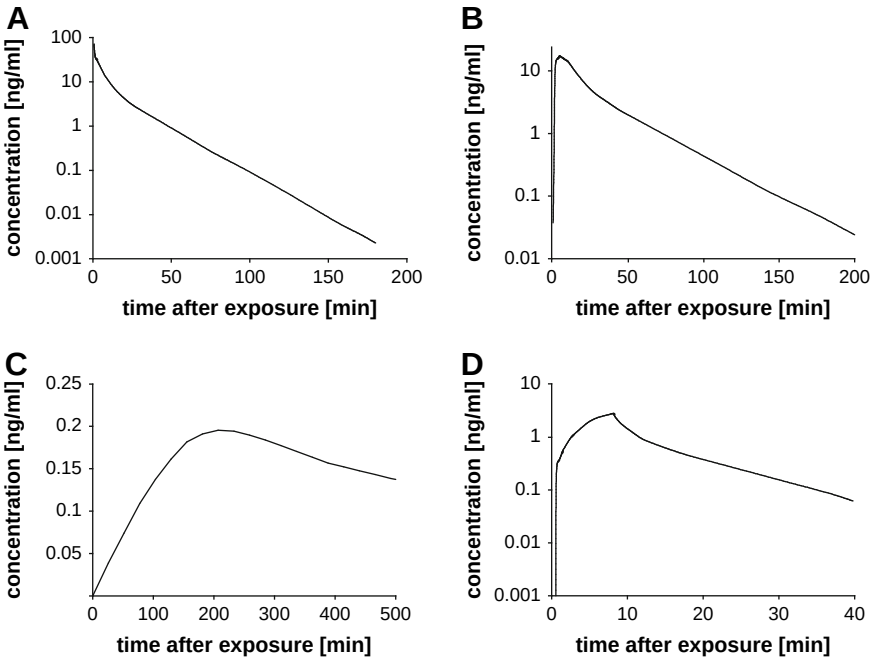

FIGURE 50.3. Concentration–time profiles of highly toxic C(−)P(−)-soman and (±)-VX in guinea pig blood after administration of nerve agents via different routes of exposure. Guinea pigs were challenged with C(±)P(±)-soman (A, B, D) or (±)-VX (C). A: intravenous, 6×LD_{50} (165 µg/kg); B: subcutaneous bolus, 6×LD_{50} (148 µg/kg); C: percutaneous bolus, 1×LD_{50} (125 µg/kg); D: nose-only for soman vapor in air at 0.8×LCt_{50} (48 mg/m^3 for 8 min). Nerve agents were applied to anesthetized, atropinized, and mechanically ventilated animals. Data fits are according to basic studies presented by Benschop and de Jong (2001) (A, B, D) and by Van der Schans *et al.* (2003) (C).

As soon as 0.3 min after i.v. administration of C(±)P(±)-soman to rats, guinea pigs, and marmosets (3–6 LD_{50}) the less toxic C(+)P(+)-enantiomer had been degraded to nondetectable concentration, whereas its diastereomer C(−)P(+)-soman was detectable for a few minutes longer. This rapid decrease of P(+)-enantiomers is caused by fast enantioselective catalytic hydrolysis (Table 50.3). In contrast, the highly toxic P(−)-diastereomers were detected for up to 1 h or longer showing a steep initial concentration decrease caused by systemic diastribution, protein binding, and hydrolysis followed by a more moderate concentration decline during the later elimination phase (Figure 50.3A). CarbE is expected to be the most important scavenger in laboratory animals being essential for nerve agent elimination and causing high species-dependent variations in LD_{50} values (rat > guinea pig > marmoset) which correlate to individual CarbE concentrations (Table 50.2). Resulting concentration curvatures were fitted best by a three-exponential equation (Benschop and de Jong, 2001). Table 50.3A gives a representative example of the concentration–time profile of C(−)P(−)-soman in guinea pig. Corresponding experiments performed with (±)-sarin applied to guinea pigs demonstrated that initial distribution of (−)-sarin happened an order of magnitude faster than P(−)-soman whereas elimination was one order of magnitude slower (Benschop and de Jong, 2001). Reasons for these differences are not clarified yet but are expected to be due to different kinetics of hydrolysis and protein binding causing higher persistence of (−)-sarin.

b. Subcutaneous Uptake

Subcutaneous exposure is often used as a substitute for respiratory exposure due to experimental difficulties in performing controlled inhalational poisoning (Benschop and de Jong, 2001). Exemplarily, we will note a typical study illustrating the s.c. behavior of soman (Figure 50.3B). A C(±)P(±)-soman bolus injection in the scruff of a guinea pig neck resulted in a discontinuous C(−)P(−)-soman concentration–time profile following a mono-exponential equation for the absorption phase and a bi-exponential fit for the distribution phase (Figure 50.3B) (Benschop and de Jong, 2001). The steep initial concentration increase in blood indicated the rapid penetration through capillary vessel walls. Maximum concentration was reached not until 7 min after soman injection yielding in an absorption half-life of about 3.5 min. Despite some certain diastereomer-specific differences [decreased bioavailability of C(+)P(−)-soman] both P(−)-forms exhibited comparable kinetic behavior. Bioavailability was found to be 70–80% when comparing the corresponding area under the curve (AUC) to that of i.v. injection. Very similar to the i.v. characteristics mentioned above, the toxic, more stable, P(−)-isomers were detectable in blood for more than 3 h after exposure whereas the less toxic P(+)-forms of soman could not be determined at any time point. This is attributed to the high hydrolyzing catalytic activity of phosphotriesterases in blood, skin, and other affected tissue (Table 50.3).

c. Percutaneous Uptake

(±)-VX dissolved in isopropanol (1 LD_{50}) was applied p.c. to hairless guinea pigs (Figure 50.3C) (Van der Schans *et al.*, 2003). Typically for p.c. exposure, concentration in blood increased quite slowly reaching its maximum between 3 and 4 h after challenge followed by slight decrease within the next 4 h. However, despite that longer period of monitoring the illustrated concentration–time profile only reflects the distribution and early elimination phase. This slow release is due to the formation of a depot under and in the skin as well as to

reduced hydrolytic degradation of VX *in vivo*. No prominent effects of stereoselective toxicokinetics were observed, therefore depicted curvature reflects the racemic mixture of analytes. Bioavailability of VX at 7 h was found to be quite small when compared to i.v. data, not exceeding 3%. However, the long lasting elimination phase demonstrated high VX persistence in the organism thus resulting in threatening concentrations with acute toxicological relevance for a longer period. Such information is important for therapeutic treatment, pointing out the necessity of long time oxime infusion.

d. Respiratory Uptake (Nose-Only Model)

As pointed out above (see Respiratory uptake by inhalation) respiratory uptake is the most likely route of exposure for G-agents causing 70–80% absorption in the upper respiratory tract. The sophisticated exposure model of the nose-only design applied to guinea pigs challenged the laboratory animals free from potential distortions derived from simultaneous ocular or p.c. uptake as occurring in vapor chambers (Langenberg *et al.*, 1998a).

The toxicokinetics caused by administration of C(±)P(±)-soman to guinea pigs documented a discontinuous process of mono-exponential function for the absorption phase followed by a bi-exponential fit for distribution and elimination (Figure 50.3D). C(−)P(−)-soman was detected in blood as early as 30 s after challenge and a short-term maximum was reached immediately after terminating the 8 min exposure period (Figure 50.3D) (Benschop and de Jong, 2001). Nevertheless, some depot formation might also occur when applying higher doses thus causing maximum levels after completion of exposure. The concentration curvature of C(+)P(−)-soman showed a similar profile but with consistently smaller concentrations which is again due to a higher degree of enzymatic hydrolysis (Table 50.3). In contrast, the less toxic C(−)P(+)-soman was detected in very small concentrations during the exposure phase exclusively whereas C(+)P(+)-soman was not detected at all. Curvatures for S_P-(−)-sarin appeared to be very similar in terms of time for first appearance in blood and for reaching maximum concentration.

These selected representative examples indicate that concentration–time profiles are variable despite common underlying basic chemical reactions of hydrolysis and adduct formation. Despite improving medical treatment of nerve agent poisoning the concurrence of numerous physiological and pathophysiological parameters should be understood. Therefore, establishment of a descriptive and predictive model is of importance for the medical defense of OP compounds.

C. Mathematical Simulation for Prediction of Nerve Agent Toxicokinetics

As is obvious from the huge number of parameters affecting the toxicokinetic behavior, e.g. route of administration, nature of OP compounds, hydrolyzing and bioscavenging enzymes and proteins as well as distinct compartments for distribution and species specificities, mathematical modeling of this complex situation is a big challenge. Nevertheless, based on numerous experimental data on soman toxicokinetics Sweeney *et al.* (2006) introduced a model described by mathematical algorithms that allows the prediction of concentration–time profiles evoked by i.v., s.c., or inhalational soman uptake ($\geq$1 LD_{50}) in common laboratory animals (rat, guinea pig, and marmoset). This physiologically based pharmacokinetic model (PB/PK) benefits from the combination of relevant pharmacokinetic basics and soman-specific experiences realizing both an excellent degree of confidence for theory and laboratory data as well as extrapolation to other species. This concept is a further development of an initial model from the same working group introduced by Langenberg *et al.* (1997). A more detailed description of these models would go beyond the scope of this contribution. Therefore, readers are referred to cited literature.

D. Bioanalytical Techniques Relevant to Toxicokinetics

Elaboration of nerve agent toxicokinetics requires sophisticated analytical tools to detect and, if possible, to quantify the free toxicants as well as adducts with proteins and enzymes. Analysis of OP nerve agents has been performed by capillary electrophoresis (CE), biosensors, matrix-assisted laser desorption/ionization (MALDI) MS, desorption electrospray ionization MS (DESI MS), ion mobility time-of-flight MS (IM-TOF MS), nuclear magnetic resonance spectroscopy (NMR), LC-UV, gas chromatography (GC), and many more techniques (Hooijschuur *et al.*, 2002; John *et al.*, 2008).

1. Determination of Nerve Agents

In contrast to those rather unusual methods, GC coupled to diverse detection systems, e.g. flame ionization detector (FID), nitrogen-phosphorus detector (NPD), flame photometric detector or mass spectrometer, as well as liquid chromatographic (LC) methods, represent the most common techniques for OP determination especially for biological samples. These methods offer high resolution, sufficient limits of detection, good reproducibility, and robust hardware devices. For more detailed information readers are referred to recent review articles (Hooijschuur *et al.*, 2002; John *et al.*, 2008).

However, analysis becomes much more complex when stereoisomers are quantified separately (Figure 50.1). Enantiomers cause identical detector responses in NPD, FID, or MS. Therefore, chiral separation systems are required to overcome these detector limitations. Despite enormous progress in separation media and detector systems within the last two decades the number of reports on chiral analysis of nerve agents valuable for toxicokinetic studies is

still quite small. Chiral separations make use of special chromatographic columns modified with chiral ligands.

Isomers of soman were separated by GC on a Chirasil-L-Val column but lacked baseline separation (Benschop *et al.*, 1981, 1985; Li *et al.*, 2003). In contrast, (+)-sarin and (−)-sarin could be completely separated by the same column (Benschop and de Jong, 2001). A recent study presented a modified method using a Chiraldex γ-cyclodextrin trifluoroacetyl GC-column coupled to an electron impact (EI)-MS which enabled sufficient baseline separation of all four stereoisomers of soman (Smith and Schlager, 1996; Yeung *et al.*, 2008).

Apart from the Chirasil-L-Val method, sarin enantiomers were also separated by a 2D-GC technique on chiral Cyclodex B material prior to NPD monitoring (Spruit *et al.*, 2000, 2001). An additional GC-based approach allowed baseline separation of cyclosarin enantiomers on a GAMMA DEX column monitored by EI-MS (Reiter *et al.*, 2007). VX enantiomers were chromatographed on a Chiracel OD column by LC coupled to an electrochemical detector yielding a lower limit of quantification of about 10 ng/ml blood (Van der Schans *et al.*, 2003). To our knowledge no chiral HPLC-MS separation of OPCs has been described so far although mobile phases are compatible with MS detection.

2. Detection of Enzyme and Protein Adducts of Nerve Agents

In contrast to the measurement of free agent the qualitative detection of protein adducts is a quite novel approach that came about by the overwhelming technical progress in bioanalytical mass spectrometry. Electrospray ionization (ESI) and MALDI as soft ionization methods for mass spectrometric detection are highly valuable for the detection of biomacromolecules like DNA, peptides, and proteins (John *et al.*, 2004, 2005; Schulz-Knappe *et al.*, 2001). Therefore, these technologies are also favorable for the analysis of proteins interacting with nerve agents. Consequently, a number of approaches have been published identifying and characterizing these adducts. Typically, protein and enzyme adducts are first isolated from complex biological matrices (e.g. blood) using affinity chromatographic methods and subsequently they are subjected to enzymatic cleavage by adding selected proteases. Resulting internal peptide cleavage products containing the derivatized (phosphylated) residues are chromatographically separated and analyzed by modern mass spectrometry. Using sophisticated MALDI-MS techniques this general procedure allowed the identification of the albumin adducts of soman (Li *et al.*, 2008) and sarin (Li *et al.*, 2007) as well as the adducts of AChE and tabun, respectively, their aged products (Elhanany *et al.*, 2001). LC-ESI MS was applied to analyze albumin adducts of cyclosarin, sarin, tabun, and soman (Williams *et al.*, 2007) and some OP pesticides (Peeples *et al.*, 2005), BChE adducts of sarin, soman, and VX (Noort *et al.*, 2006; Tsuge and Seto, 2006), and CarbE adducts of OP pesticides (Peeples *et al.*, 2005). For a more detailed description readers are referred to John *et al.* (2008).

This short summary underlines the great importance of modern analytical techniques to unravel pathophysiological situations at the molecular level and to support toxicokinetic studies by enlightening most relevant protein-binding elimination processes.

IV. VESICANTS

A. Sulfur Mustard

1. Overview of Sulfur Mustard

Sulfur mustard is a blistering or vesicating agent that primarily incurs damage at the organs that come into immediate contact with either its liquid or vaporous form. However, severe dermal and respiratory exposure to the agent may also result in the absorption of sulfur mustard that subsequently causes additional systemic damage (Kehe and Szinicz, 2005).

Unfortunately, sulfur mustard has been used in acts of chemical warfare throughout the 20th century, from World War I to the attacks of the former Iraqi regime against Iran and even Kurdish civilians. The simplicity of the agent and its synthesis, combined with devastating medical, psychological, and socioeconomic effects, along with the fact that to the present day, no causative therapy has been established, may convince future aggressors, state and in particular nonstate parties, to use this agent in their attacks. This, in turn, necessitates medical research efforts, including toxicodynamic and toxicokinetic studies, to develop countermeasures against the effects of sulfur mustard. Table 50.5 displays the basic physicochemical properties for sulfur mustard which constitute the fundamental reason for many of its toxicokinetic properties. Data for lewisite, another vesicant agent described in Section IVB, are also shown.

2. Toxicity of Sulfur Mustard

Figure 50.4 depicts the basic chemical mechanism by which sulfur mustard incurs the primary damage to biological molecules which results in subsequent damage to cells, tissues, and organs.

Sulfur mustard forms an intermediate sulfonium ion that further transforms into a carbenium ion, a strong electrophile capable of reacting with nucleophile targets, primarily in DNA and RNA, such as the N7 in guanine (61%) (Ludlum *et al.*, 1994), the N3 in adenine (16%), and the O6 in guanine (0.1%). Even though the latter is considerably less frequent, it is the reason for significant concern as the repair enzyme (O6-alkylguanine-DNA-alkyltransferase) is not capable of reversing this reaction. Therefore, DNA mutation due to mispairing may occur during DNA replication, which is proposed to be the origin of subsequent cancer formation (Ludlum *et al.*, 1986). Because of its bifunctional character, sulfur mustard also forms interstrand

TABLE 50.5. Physicochemical properties of most common vesicant agents

Agent	CAS-No.	NATO code	MW (g/mol)	Melting point (°C)	Boiling point (°C)	Vapor pressure (mbar)	Water solubility (mg/l)	Hydrolysis rate, $\tau_{1/2}$ (h)	log P (–)
Sulfur mustard	505-60-2	HD	159.1	14	217	0.147 (25°C)	0.684[a]	14.7 (20°C)	1.37
Lewisite	541-25-3	L	207.3	−18	190 (decomposing)	0.773 (25°C)	500	Rapid, n.a.	n.a.

log P – octanol:water partition coefficient; MW – molecular weight; n.a. – not available; $\tau_{1/2}$ – period of half-change for hydrolysis. Data are taken from Munro *et al.* (1999) unless otherwise noted

[a]Seidell (1941)

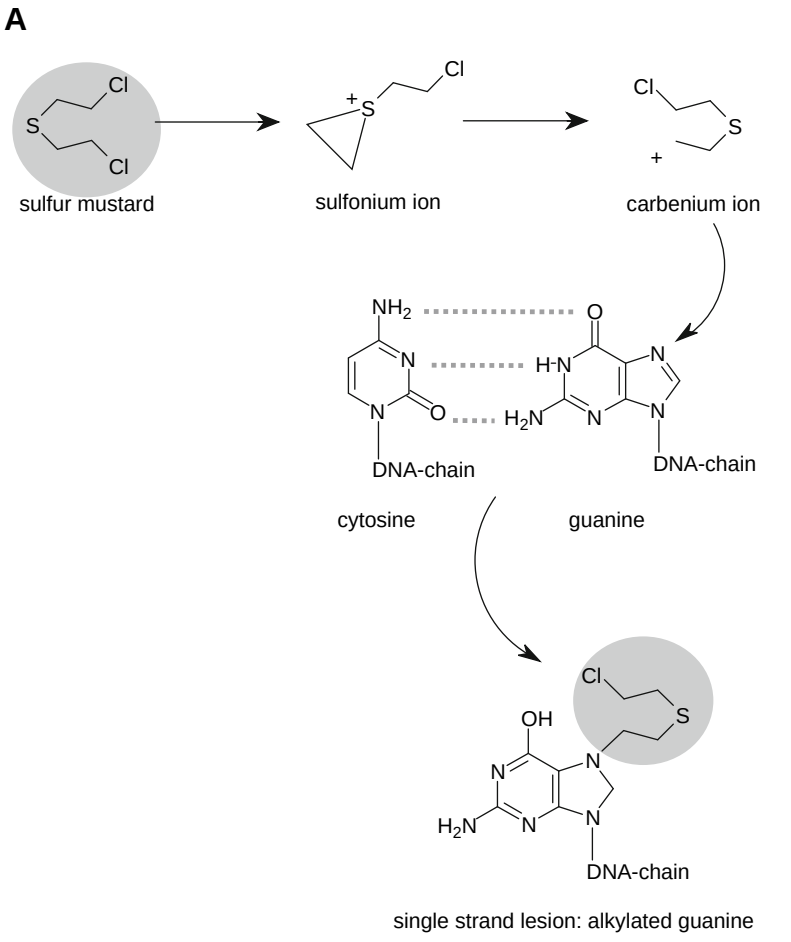

FIGURE 50.4. Sulfur mustard, its structure, mechanism of action, and targets of adduct formation. A: Mechanism of reaction of sulfur mustard and nucleotide guanine. B: Sites of alkylation by sulfur mustard. Arrows mark identified targets in nucleotides and amino acid histidine.

DNA crosslinks, in particular from one guanine to another (17%). It is estimated that a 100 μM concentration of sulfur mustard accounts for 0.28 crosslinks per 10,000 DNA bases (Shahin *et al.*, 2001).

Membrane-bound proteins and enzymes may undergo alkylation in the presence of sulfur mustard (Kehe and Szinicz, 2005). Figure 50.4 depicts targets of alkylation in both DNA bases and amino acids. However, as sulfur mustard-induced DNA damage has the most significant impact on the cell's short- and long-term survivability, it is DNA alkylation that produces the most significant toxic effects, whereas effects from protein alkylation are usually visible only in the presence of high concentrations of sulfur mustard (Lodhi *et al.*, 2001; Peters, 1947). As systemic concentrations will always be several orders of magnitude below local concentrations at the site of immediate contact, the systemic toxic effects of sulfur mustard are also most likely to result from DNA alkylation. DNA alkylation produces the most evident effects in proliferating cells and thus tissues with rapidly proliferating cells such as bone marrow suffer the most obvious damage. Toxic effects are similar to side effects from alkylating antineoplastic drugs (Dacre and Goldman, 1996). (Actually, the first antineoplastic drug had been developed from a structural analog of sulfur mustard, i.e. nitrogen mustard; Goodman *et al.*, 1984.) Those effects include nausea, vomiting, fever, fatigue, apathy, and loss of appetite. Bone marrow toxicity results in an initial leukocytosis, which is soon followed by leukopenia, thrombopenia, eosinopenia, and subsequently anemia (Dacre and Goldman, 1996).

Specific toxic effects of sulfur mustard have also been reported in the central nervous system and may range from agitation to seizures (Balali-Mood and Navaeian, 1986).

3. Invasion

Significant amounts of sulfur mustard may be absorbed from skin that has been in direct contact with sulfur mustard. Vapors of sulfur mustard may also be absorbed from the respiratory system.

In theory, there are two additional pathways for systemic uptake of sulfur mustard: the surface of the eyes and the gastrointestinal system. They have, however, never been investigated for the following reasons: gastrointestinal exposure to sulfur mustard is a rare phenomenon and may only occur in cases of sabotage (deliberate food poisoning) or a considerable lack of basic chemical defense measures, i.e. food consumption in a contaminated area. If occurring, the local effects of exposing the gastrointestinal system to sulfur mustard are severe and life-threatening and include strong abdominal pain, bloody diarrhea and vomiting, and rupture of stomach or duodenum. Subsequent peritonitis is often fatal (Dacre and Goldman, 1996). In comparison to the life-threatening local effects of gastrointestinal exposure, any related systemic effect would be of secondary importance.

Contrary to the rarity of gastrointestinal exposure, ocular exposure is unfortunately frequent among the victims of sulfur mustard attacks and local symptoms of varying severity are likely to occur whenever the individual was not protected by a respirator (mask) at the time of attack (Solberg *et al.*, 1997). As the eye is a very sensitive organ, ocular symptoms are often among the first signs of sulfur mustard exposure. Due to the close proximity of a large number of capillary vessels and as the eye constitutes a relatively weak barrier, xenobiotic substances are often rapidly absorbed (Lama, 2005). However, as the surface of the eye is small in comparison to the skin and the respiratory tract, the total amount of sulfur mustard that may be absorbed from the eye's surface is low. Self-protective effects of the eye such as blepharospasm and pronounced lacrimation may further reduce the amount of the agent that may be absorbed through this pathway. Again, the local effects of exposure appear very severe and are a primary reason for concern in victims of sulfur mustard effects. Whereas blepharospasm and lid edema cause transitory loss of vision – already resulting in immense distress for victims – heavy exposure may result in permanently opaque cornea and blindness. In general early effects in eyes are accessible by therapy with relatively good outcome after several weeks of treatment. In comparison, any systemic effects from transocular absorption alone would be of minor concern. It has to be emphasized that isolated ocular exposure on the battlefield or due to terrorist attacks utilizing sulfur mustard is exceedingly unlikely. Ocular exposure would inevitably be accompanied by cutaneous and respiratory exposure. The specific rate of absorption (i.e. the amount absorbed through a given surface over a specified time) may be lower, in particular in the case of initially intact skin. However, the larger total surface of the skin (and possibly the respiratory tract) would result in an amount that constitutes the major fraction of sulfur mustard absorbed which would subsequently determine the occurrence and severity of systemic effects. Therefore, research regarding the absorption of sulfur mustard has focused on two major pathways which are described in further detail in the subsequent sections.

a. Percutaneous Absorption

Penetration rates of liquid sulfur mustard were determined *in vitro* (71–294 μg/cm^2/h) on human skin with a Franz-type glass diffusion cell and correspond quite well to *in vivo* data derived from human skin (60–240 μg/cm^2/h) (Chilcott *et al.*, 2000). Significant effort has been dedicated to confirm the validity of animal models by comparing data derived from these models with findings from *in vitro* human skin experiments. Chilcott *et al.* (2001) investigated absorption in *in vitro* models of pig ear and heat-separated human skin. Absorption was determined at 411 ± 175 μg/cm^2/h for pig ear skin and 157 ± 66 μg/cm^2/h for human skin, respectively. These data were considered in agreement with earlier *in vivo* findings, even though the authors cautioned that data

on the effectiveness of decontaminants were in complete disparity when comparing human to pig ear skin. Logan *et al.* (1999) determined the sulfur mustard exposure through the skin of the hairless guinea pig at 120 µg/cm^2/h.

Occlusion of the skin, i.e. covering the skin with a material that is impenetrable to air and moisture, can result in a dramatic increase in p.c. absorption. For example, in a study by Chilcott *et al.* (2002) using various *in vitro* models of human skin, absorption rates in unoccluded controls (4.41 ± 1.90 µg/cm^2/h) increased to 538 ± 193 µg/cm^2/h under occluded conditions.

b. Respiratory Absorption

Langenberg *et al.* (1997) conducted inhalation exposure experiments in hairless guinea pigs. LCt_{50} was determined at 800 mg·min/m^3. Following application of one LCt_{50} over 5 min, no unchanged sulfur mustard in blood was found (limit of detection 5 pg/ml). Low concentrations (0.7 adducts per 10^7 nucleotides) of N7-guanine adducts of sulfur mustard were found in the lung. Concentration of N7-guanine adducts was also determined from various tissues along the respiratory tract. Actually, concentrations found were much larger, peaking at approximately 90 adducts per 10^7 nucleotides in the larynx and trachea. Concentration in the carina was lower (approximately 50 per 10^7 nucleotides), but still substantially higher than the above-mentioned value in the lungs. The authors concluded that most of the sulfur mustard inhaled reacts in the upper airways, rather than being absorbed. In animals with complex nasal systems (such as guinea pigs), only very minor fractions would reach the lung. However, they also pointed out that in humans (along with other species with a less complex nasal system), the proportion of sulfur mustard reaching the lung may be larger.

Following inhalation of 3×LCt_{50} (300 mg/m^3 over a period of 8 min), sulfur mustard was found in blood. Its peak concentration during the inhalation period was found to be approximately 5 ng/ml. This concentration soon declined. However, a mean value of 2 ng/ml was found even after 4 h. Nevertheless, a mathematical model explaining the data during the distribution and elimination phase could not be developed. Findings were further complicated by the fact that in two of 12 animals, no sulfur mustard was found in any of the samples. Moreover, no sulfur mustard was detected in any of the animals at 15 and 20 min (7 and 12 min post-exposure, respectively) before reappearing in most of the animals at the above-mentioned concentration of 2 ng/ml. The latter finding may possibly be explained by the hypothesis that the early peak in sulfur mustard concentration is due to direct inhalation (afterwards, concentration declines to levels below the limit of detection), whereas the second, long-term increase in sulfur mustard concentration may be attributed to sulfur mustard absorbed from depots in the upper airways. Figure 50.5 depicts the concentration over time, following the respiratory exposure to 3×LCt$_{50}$ of sulfur mustard. For comparison, concentration over time after i.v. application is also depicted. In summary, the authors concluded that toxicity from sulfur mustard inhalation was due to its local, rather than systemic, effects.

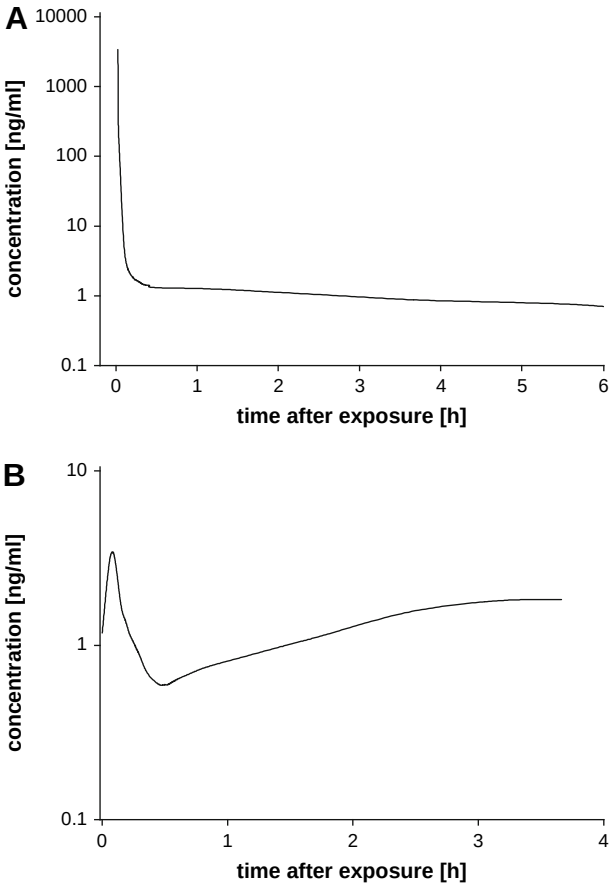

FIGURE 50.5. Concentration over time, following i.v. and respiratory exposure to sulfur mustard in the guinea pig model. A: Decline of sulfur mustard exposure after intravenous injection. B: Concentration over time after respiratory exposure: initial increase in the inhalation phase, followed by a decline and a secondary increase, concentration of approximately 2 ng/ml is sustained for 4 h.

4. Distribution

Sulfur mustard is a strongly lipophilic substance that may accumulate in fatty tissues and has actually been detected at autopsy in a patient who had died 7 days post-exposure. Detailed data, as published by Drasch and colleagues (1987), are displayed in Table 50.6.

Obviously, these findings confirm the theoretical assumption that the lipophilic properties of sulfur mustard result in a distribution, primarily in lipophilic tissues. High concentrations found in the brain may also explain why the central nervous system is one of the organs exhibiting systemic effects of sulfur mustard poisoning, even though it is not a site of rapidly proliferating cells.

TABLE 50.6. Content of sulfur mustard in the tissues of a deceased victim

Tissue/organ	Concentration (mg/kg)
Fat	15.1
Skin with subcutaneous fatty tissue	11.8
Brain	10.7
Skin	8.4
Kidney	5.6
Liver	2.4
Cerebrospinal fluid	1.9
Muscle	3.9
Spleen	1.5
Blood	1.1
Lung	0.8
Urine	Not detected
Blister fluid	Not detected

Data are according to Drasch and colleagues (1987)

It should be noted that some authors have questioned the findings by Drasch and colleagues, considering the absolute concentrations excessively high and therefore unlikely. However, as high-dose sulfur mustard poisoning is fortunately a rare event, it is nearly impossible to verify or falsify these data. One might argue that the amounts of sulfur mustard described may not even permit short-term survival. However, if most of the agent had entered the organism via p.c. absorption, there is a possibility that large amounts of the agent actually had been absorbed and formed a depot without resulting in an instant fatality, but ultimately leading to the death of the patient 7 days later. Whether or not the absolute values are accurate, they at least give an impression of the distribution of sulfur mustard within the organism that correlates with the agent's lipophilic properties.

More recent *in vitro* experiments, using human skin, have confirmed the presence of unhydrolyzed sulfur mustard in the lipophilic stratum corneum and the upper epidermis. Twenty-four hours post-exposure, the distribution ratio between the epidermis and the dermis has been determined at 62 to 38%. Chilcott and colleagues (2000) also suggested that efforts to remove or neutralize the agent from these deposits might have a clinical benefit for the patient.

In summary, these findings suggest that despite the presumably brief half-life calculated (see Elimination, below), sulfur mustard may be present in the organism for a significantly longer period of time necessitating protective measures for the medical personnel providing therapy and an awareness for the possibility of secondary blister formation, even 30 days post-exposure (Balali-Mood and Hefazi, 2005) as well as efforts to remove these deposits. In particular, the use of laser debridement (Graham *et al.*, 2008) and mechanical dermabrasio (Rice, 2008) has been recommended to facilitate the healing process after dermal exposure to sulfur mustard. The effects may at least partly be attributed to the removal of epidermal depots of sulfur mustard.

5. Metabolism

While the previously discussed findings demonstrate the stability of sulfur mustard in lipophilic tissues, the agent is rapidly hydrolyzed whenever situated in an aqueous compartment (Vycudilik, 1987). Thiodiglycol is the primary hydrolysis product, in which the chlorine atoms have been replaced by hydroxyl groups. Thiodiglycol may undergo oxidation to thiodiglycol sulfoxide, which is being conjugated with glutathione to form 1,1′-sulfonylbis [2-*S*-(*N*-acetylcysteinyl)ethane]. Following the β-lyase pathway, the 1,1-sulphonylbis[2-(methylsulfinyl)ethane] and 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane can be formed (Black and Read, 1995). Figure 50.6 summarizes the pathways of sulfur mustard metabolism. No sulfo- or glucuronyl-conjugates were detected in urine after i.v. administration of sulfur mustard (Maisonneuve *et al.*, 1993).

Within cells, sulfur mustard forms adducts with DNA, primarily those described in Toxicity of sulfur mustard, above. Adducts can also be formed with nucleophilic sites of amino acids and proteins. Contrary to DNA adducts, there is no specific mechanism to reverse protein adduct formation. For this reason, there is a strong forensic interest in the detection of protein adducts of sulfur mustard as these may provide evidence of sulfur mustard exposure for prolonged periods after the incident.

A number of adducts to amino acid residues have been identified by Noort and colleagues (1996) and Black *et al.* (1997a, b). Six different histidine residues, three glutamic acid residues, and both of the N-terminal valines were found. Alkylated cysteine, aspartic acid, lysine, and tryptophan were also detected. While the N1 and N3 histidine adducts were found to be most abundant, it was the alkylated N-terminal valine adducts that were most useful for subsequent quantification. See Detection of DNA and protein adducts of vesicants, below, for analytical details.

Noort and colleagues (2008) investigated the persistence of sulfur mustard adducts to albumin and hemoglobin in rats. The albumin adduct (*S*-2-hydroxyethylthioethyl)-Cys-Pro-Tyr was detectable up to 7 days after the exposure, while the adduct to the N-terminal valine in hemoglobin was still detected after 28 days. The decrease of adduct concentration corresponded with albumin half-life and the lifetime of the rat erythrocyte, respectively.

Following two simultaneous cases of accidental human exposure to sulfur mustard, Smith *et al.* (2008) investigated the concentration of the cysteine-34 adduct to albumin and adducts to glutamic and aspartic acids of plasma proteins. In the case of a more severely exposed patient who required hospitalization, both adducts were detected over a 42 day period, though decreasing by approximately 75% towards

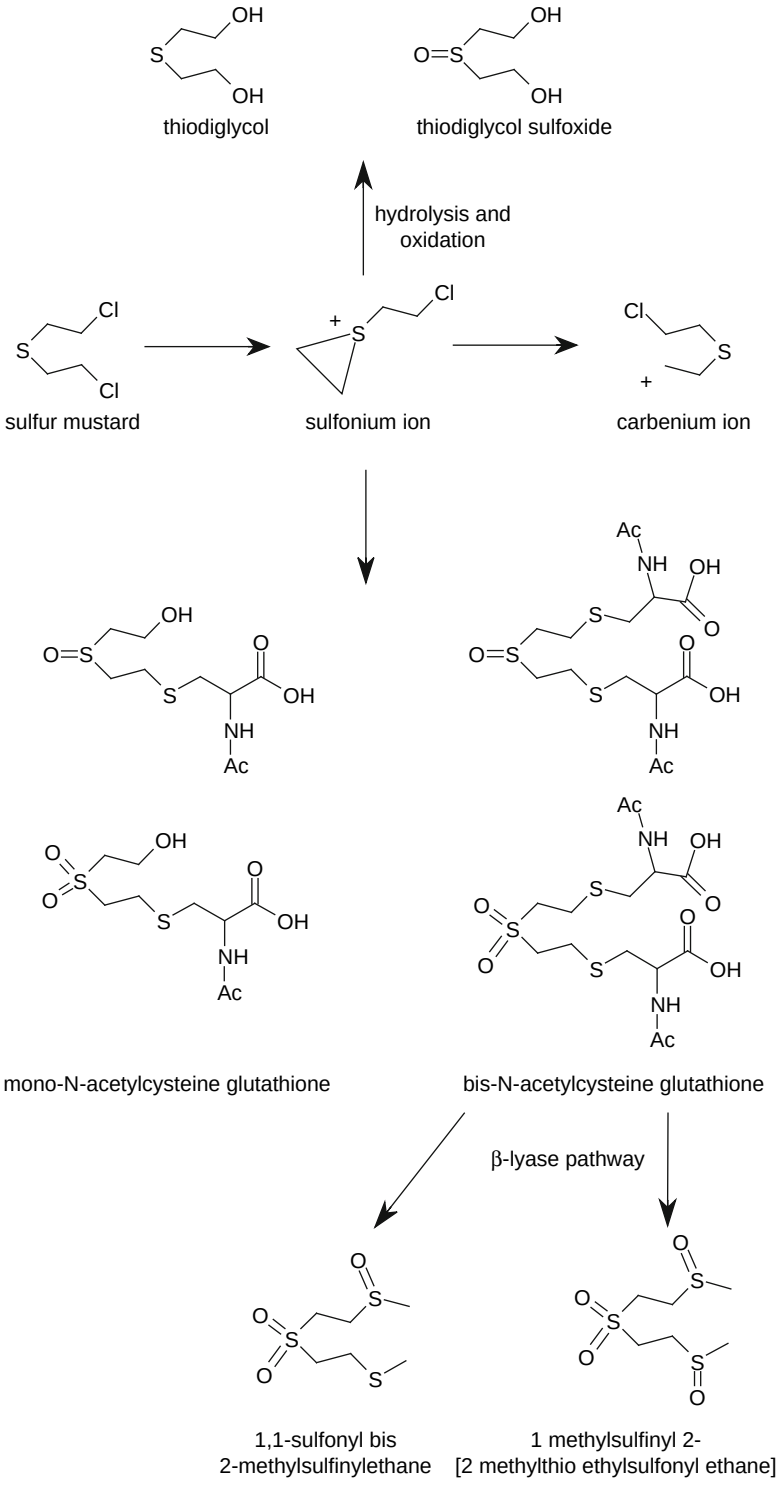

FIGURE 50.6. Metabolism of sulfur mustard.

the end of that period. In a second patient who had developed a single, small blister the albumin adduct was found during a 6 day period post-exposure.

6. Elimination

Following i.v. application of ^{14}C-labeled sulfur mustard in rats, 80% of the radioactivity administered was excreted via the renal pathway. Fecal excretion amounted to less than 3% (Maisonneuve *et al.*, 1993). Metabolites excreted in the urine after accidental human exposure included thiodiglycol, thiodiglycol-sulfoxide and the bis-mercapturate of mustard sulfone (Barr *et al.*, 2008). Comparing the concentrations of thiodiglycol and its sulfoxide, the latter was found in concentrations twice as high. When p.c. exposing weanling

pigs to sulfur mustard, Graham *et al.* (2000) found peak levels of thiodiglycol either in the samples drawn after 6 to 8 h or in other cases after 24 to 48 h. Findings were considered to be in agreement with earlier data from rodent species and cases of accidental human exposure.

It is worth noting that background levels of thioglycol and thiodiglycol sulfoxide have been found in the urine of healthy individuals never exposed to sulfur mustard. For this reason their validity as unambiguous markers for sulfur mustard exposure has been questioned. Black and Read (1995) suggested the determination of β-lyase pathway metabolites which in the urine of exposed patients were found at concentrations similar to those of thiodiglycol sulfoxide, but were not found in unexposed individuals.

B. Lewisite

1. Overview of Lewisite

Lewisite (2-chlorvinyldichlorarsin) is another vesicant agent. Unlike sulfur mustard, there has never been a documented use in armed conflict. It was first synthesized and described by the Belgian priest and chemist Julius Arthur Nieuwland (Nieuwland, 1904). During World War I, the US military chemist Winford Lee Lewis suggested and initiated its development into a chemical weapon, which due to the 1918 armistice in Europe was not used on the battlefield (Vilensky and Redman, 2003).

Lewisite remains a reason for concern because its physical properties, in particular a melting point of −18°C (Table 50.5), might facilitate its use in cold climates. Thus, a belligerent willing to use chemical weapons might feel convinced that lewisite would provide a ''unique'' capability to wage chemical warfare even in winter and/or in mountainous regions. Moreover, large stockpiles of the agent had been abandoned by the Imperial Japanese Army during their retreat from China in the latter stages of World War II, creating a chemical hazard that persists even decades after the war (Hanaoka *et al.*, 2006). Table 50.5 summarizes the physicochemical properties of lewisite.

2. Toxicity of Lewisite

The dominant element in the lewisite structure is arsenic, which is able to react with the sulfhydryl groups of various enzymes, disabling the enzyme in the process (Goldman and Dacre, 1989). Figure 50.7 depicts the structure of lewisite and its toxic mechanism.

Lipoic acid is particularly susceptible to this reaction and one of the most evident consequences is the inhibition of the enzyme pyruvate dehydrogenase (PDH), rapidly disabling the cell's metabolism of glucose and fatty acids. The resulting energy deficiency may lead to swift necrotic cell death. In comparison to sulfur mustard, the latency period (from exposure to first signs and symptoms) is significantly shorter and lewisite injuries have been described as extremely painful from an early stage. Moreover, it is a much more lethal agent and has a large systemic toxicity; 0.5 ml of the agent may produce systemic effects, whereas 2 ml (approximately 3.6 g) can be fatal (Marrs *et al.*, 1996).

3. Invasion

a. Percutaneous Absorption

Precise data, i.e. a diffusion coefficient expressing the p.c. absorption of lewisite (amount absorbed per area and time), are not available. However, Inns and Rice (1993) conducted p.c. toxicity studies in rabbits and determined the LD_{50} at 5.3 mg/kg (3.5–8.5 mg/kg, 95% confidence interval). The exposed area was 2 cm^2 and exposure lasted for 6 h.

Inns *et al.* (1990) had also determined the LD_{50} of i.v. lewisite administration at 1.8 mg/kg (1.6–2.1 mg/kg 95% confidence interval). Thus, it can be concluded that by exposing 2 cm^2 of rabbit skin to a dose of 5.3 mg/kg for 6 h, a dose producing the equivalent effect of 1.8 mg/kg is absorbed. No further calculations that might exaggerate the reliability of available data shall be conducted here.

b. Respiratory Absorption

The LCt_{50} of lewisite in humans has been estimated at 1,500 mg·min/m^3 (ATSDR, 2007), although no experimental

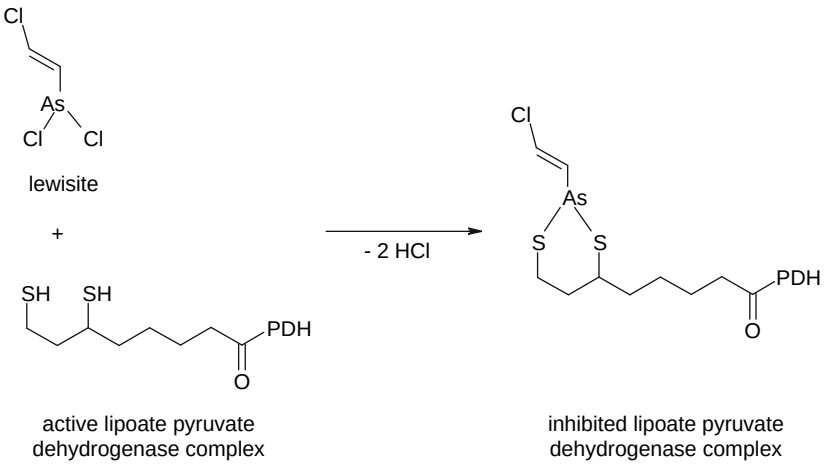

FIGURE 50.7. Lewisite, its structure and mechanism of action. Lewisite forms covalent bonds with lipoic acid, inactivating the enzyme pyruvate dehydrogenase (PDH).

data were cited. Considering its pronounced local effects, however, lethality can at least partly be attributed to the local effects of lewisite on the respiratory tract.

4. Distribution

High distribution volumes per kilogram indicate extensive distribution in tissues, due to the lipophilicity of the substance. In a rabbit model more than seven-fold values, compared to blood concentrations, were found in some tissues, e.g. liver, lung, and kidneys. That ratio was maintained over the sampling period, i.e. for at least 96 h (Snider *et al.*, 1990).

5. Metabolism

Once incorporated, unbound lewisite is quickly hydrolyzed. Its predominant metabolite is 2-chlorovinylarsonous acid, CVAA (Figure 50.8). Analytical methods to confirm lewisite exposure have, at least in the past, focused on the detection and quantification of CVAA. However, Noort *et al.* (2002) also pointed out that due to the high affinity of arsenic towards sulfhydryl groups, adducts of lewisite/CVAA and cysteine residues of proteins are formed. In an *in vitro* study, incubating ^{14}C-labeled lewisite with human blood samples, 90% of lewisite was found in erythrocytes, whereas 25 to 50% of arsenic was bound to globin. From these protein adducts, CVAA can be released to form an adduct with the antidote British Anti-Lewisite (BAL) (Fidder *et al.*, 2000). The authors were also able to identify a specific protein adduct of lewisite formed with the cysteine residues 93 and 112 of β-globin. See Detection of DNA and protein adducts of vesicants, below, for analytical details. Figure 50.8 summarizes the metabolism and reversal of adduct formation by BAL.

6. Elimination

Snider *et al.* (1990) determined the elimination of lewisite from rabbits after p.c. injection. Half-life was determined, ranging from 55 to 75 h. A clearance of 120 ml/h/kg was found. However, these findings only describe the overall elimination of arsenic from the organism, following a lewisite exposure.

In vivo, unbound CVAA is quickly excreted via the renal pathway and cannot be detected in urine samples taken later than 12 h post-exposure. However, the biological half-life of protein adducts is much longer: in blood samples taken 10 days post-exposure and treated with BAL, Fidder *et al.* (2000) were still able to release 10% of the CVAA-BAL concentration found on day 1. Thus, protein adducts of CVAA have an important role in the verification of potential lewisite exposure.

C. Bioanalytical Techniques for Quantification of Vesicants

1. Determination of Vesicants and Direct Metabolites

When Drasch and colleagues (1987) determined the concentration of sulfur mustard in tissues of a deceased victim, they had to employ a combination of dichloromethane extraction, a thin layer chromatography cleanup on silica plates, followed by derivatization with gold chloride,

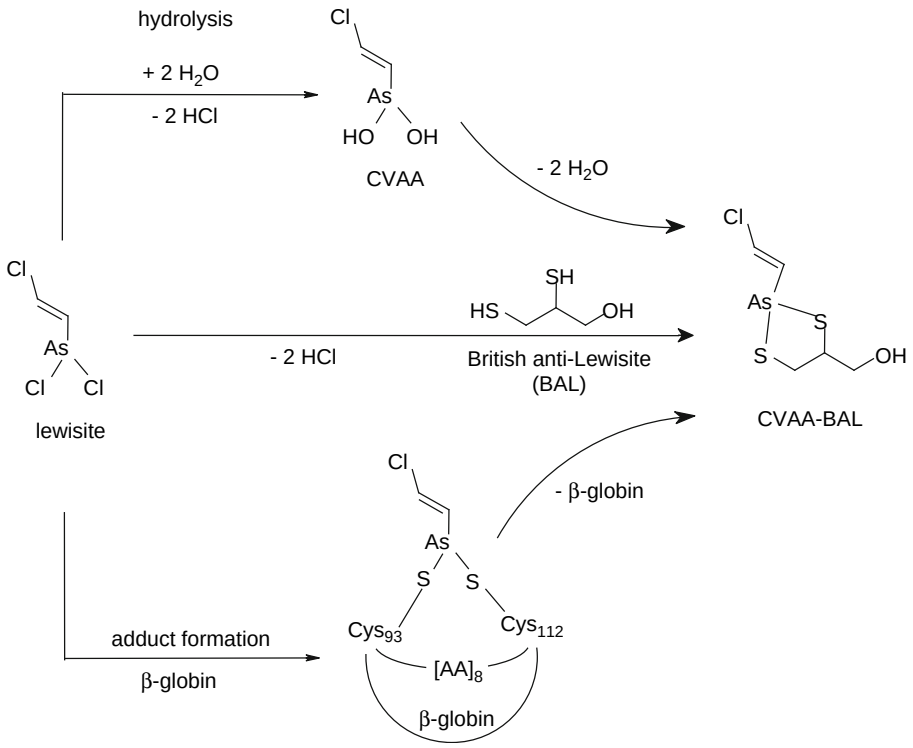

FIGURE 50.8. Lewisite metabolism, adduct formation, and its reversal.

and quantification by electrothermal atom absorption spectroscopy.

Vycudilik (1985) had already used gas chromatography–mass spectrometry (GC–MS) to confirm the presence of sulfur mustard in urine samples. Sodium chloride was added to the sample to facilitate extraction and slow down the hydrolysis in the aqueous sample, and the analyte was then extracted with diethylether. The solvent was evaporated and the residue dissolved in methylene chloride. After purification – by shaking the sample solution for 1 h with silicagel – the solvent was again evaporated. The residue was again dissolved in methylene chloride and used for chromatography.

To detect thiodiglycol, thiodiglycol sulfoxide, and their acid-labile esters, Black and Read (1991) used $TiCl_3$ reduction, converting these metabolites into the single analyte thiodiglycol. Thiodiglycol was then converted to its bis(-pentafluorobenzoyl) derivative and quantified by GC–MS using negative ion chemical ionization. Thiodiglycol sulfoxide could also be extracted directly, using solid phase extraction, followed by a Florisil cleanup. Derivatization and quantification were conducted as described above. $TiCl_3$ was also used by Daly and O'Hehir (2007) to reduce the β-lyase pathway metabolite 1,1-sulphonylbis[2-(methylsulfinyl)ethane] to 1,1′-sulfonylbis[2-(methylthio)ethane] (SBMTE). This was followed by automated solid-phase extraction and liquid chromatography–positive ion-electrospray ionization–tandem mass spectrometry. Methods to detect lewisite exposure have been focused on its main metabolite 2-chlorovinylarsonous acid (CVAA). Initial methods were developed for environmental samples (Bossle *et al.*, 1989). Methods for CVAA quantification in serum were described by Fowler *et al.* (1991) as well as Jakubowski *et al.* (1993). CVAA was derivatized with 1,2-ethanedithiol and quantified using GC–MS. Logan *et al.* (1996) employed a similar method to detect CVAA in the urine of guinea pigs exposed to lewisite.

2. Detection of DNA and Protein Adducts of Vesicants

Adducts of sulfur mustard can be hydrolyzed to release thiodiglycol. Lawrence *et al.* (2008) used this procedure, followed by extraction, derivatization, and gas chromatography–negative ion chemical ionization–mass spectrometry. However, a number of methods have been developed to directly detect and quantify the adducts of sulfur mustard. The most prevalent DNA adduct, N7-(2-hydroxyethylthioethyl)-guanine, was directly detected and quantified by Fidder *et al.* (1994, 1996a), using electrospray LC–tandem MS with multiple reaction monitoring.

Initial efforts by Noort *et al.* (1996, 1997) to detect protein adducts of sulfur mustard focused on the 4-(2-hydroxyethylthioethyl)-L-aspartate, 5-(2-hydroxyethylthioethyl)L-glutamate, the cysteine and the N-terminal valine adduct and two histidine adducts, N1- and N3-(2-hydroxyethylthioethyl)-L-histidine, respectively. Acidic hydrolysis and pronase digestion were used to release these adducts from globin. Pronase is a mixture of proteinases isolated from the extracellular fluid of *Streptomyces griseus*. Adducts were derivatized with 9-fluorenylmethyl chloroformate, followed by identification and quantification, using GC–MS.

The N-terminal valine adducts, even though they constitute only 1–2% of alkylated amino acids, were useful for subsequent quantification efforts. N-alkylated N-terminal valine could be selectively cleaved, using pentafluorophenyl isothiocyanate as reagent in a modified Edman procedure. The product of this reaction, pentafluorophenyl thiohydantoin, was derivatized with heptafluorobutyric anhydride and quantified by negative ion GC–MS–MS. This method was sensitive enough to confirm an *in vivo* exposure of guinea pigs, 48 h after intravenous administration of 0.5 mg/kg, i.e. 6% of the LD_{50} (Fidder *et al*, 1996b).

Noort *et al.* (1999) again used pronase to digest human serum albumin that had been incubated with sulfur mustard. The cleavage product, *S*-[2-[(hydroxyethyl)thio]ethyl]-Cys-Pro-Phe, was analyzed by micro LC-tandem mass spectroscopy.

Incubation of lewisite–protein adducts with BAL is capable of transferring its metabolite 2-chlorovinylarsonous acid (CVAA) into a BAL-CVAA derivative. This derivative can be quantified using GC–MS. The method is able to detect a 1 nM lewisite exposure of human blood *in vitro* (Fidder *et al.*, 2000).

A specific β-globin adduct to Cys93 and Cys112 was identified by the use of electrospray tandem mass spectrometry as well as by chemical transformation with the cysteine-selective reagent vinylpyridine and derivatization by S-carbamylation (Fidder *et al.*, 2000). No method for quantification of this adduct was described. As of mid-2008, no specific adducts other than the above mentioned have been described in the literature.

Despite 90 years of research on the effects of vesicant agents and on medical countermeasures only very limited data on toxicokinetics are available. However, technological advances in analytical chemistry have, in particular in the last two decades, contributed to a better understanding of the toxicokinetic properties of both sulfur mustard and lewisite. Knowledge gaps do exist, but research efforts continue. The Chemical Weapons Convention constitutes enormous progress in the efforts to completely rule out future incidents of chemical warfare, and the risk of chemical weapons being used by state parties is probably lower than in any decade since the 1910s. On the other hand, there is a worldwide asymmetric threat and the risk of terrorist attacks using chemical weapons cannot be ruled out. Defense experts, including medical chemical defense researchers, will be concerned with these issues for the foreseeable future. It is to be hoped that the more comprehensive knowledge on both toxicodynamics and toxicokinetics will contribute to the development of more effective, possibly causative therapies of vesicant poisoning. At the same time, the capability to unanimously prove vesicant exposure, even long after the incident, does certainly increase the possibility that any use

of these chemical warfare agents will ultimately be detected and sanctioned. Thus, state-of-the-art analytical methods may have an important role in deterring and preventing acts of chemical warfare.

V. CONCLUDING REMARKS AND FUTURE DIRECTION

Measurement of toxicokinetic profiles is primarily motivated to improve in-depth understanding of the poison's fate in the organism and thus of pathophysiological consequences. Both are important to optimize antidote treatment and therapy regimens for poisoned humans intending to boost poison elimination and reverse toxic effects or at least minimize harm by causal and symptomatic approaches. As outlined in the sections above, the fate of poison is regulated by multifactorial processes characterized by degradation and enzymatic metabolism as well as elimination by protein or DNA binding. With respect to state-of-the-art techniques and study design only a few animal models are being investigated comprehensively for nerve agents whereas nearly no data on sulfur mustard and lewisite are available in the literature. To make matters worse, almost no human toxicity or toxicokinetic data for chemical warfare agents are available due to ethical reasons, thus hindering specific and tailored medical approaches. Therefore, data are to be obtained from animal studies requiring extrapolation of relevant characteristics to human conditions. As obvious from the tabular compilations on acute lethal doses of nerve agents in different laboratory animals and different routes of exposure (Table 50.2), especially under consideration of stereoisomeric differences (Table 50.4) and underlying kinetic characteristics in terms of rate constants for serine esterase inhibition and catalytic constants for phosphotriesterase mediated hydrolysis (Table 50.3), such data are still incomplete. In addition, inter- and intraspecies variations are enormous, thus limiting comparability and transferability of experimental results. Furthermore, there are only a few data considering specific differences between lethal and sublethal exposure scenarios. Data on tissue distribution in animals are rare and correlation to human organisms is not clarified conclusively to explain, for example, the larger persistence of nerve agents in the human body. This lack of information indicates future demands of medical defense research. Accordingly, there is also a rising need for combined toxicokinetic and pharmacokinetic data obtained from poisoned organisms under therapeutic treatment. This will help to find the most valuable reactivators of OP-inhibited cholinesterases which differ dramatically in efficacy in a poison- and species-dependent manner. Taken together, results of future studies should (1) support the development of mathematical models to describe and predict poison and antidote behavior *in vivo* in humans, (2) unravel novel targets of poison on the molecular or compartmental level, which are useful as biomarkers or to identify additional pathophysiological situations, and (3) establish novel bioanalytical methods and techniques which allow very low quantification limits and sufficient selectivity for stereoisomers in various tissues and compartments. Whereas a number of studies have been performed using the commonly known nerve agents there remains a much higher demand for elaborative work with respect to vesicants.

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CHAPTER 51

Physiologically Based Pharmacokinetic Modeling of Chemical Warfare Agents

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I. INTRODUCTION

Pharmacokinetics (PK) is the quantitative determination of drug or chemical movement (time-course concentration) throughout the body. One of the basic principles of pharmacology and toxicology is that the effect of a drug or chemical is directly related to its concentration at some target site or receptor in the different tissues of the body. This premise is the critical reason and continued justification for the development of improved PK descriptions of chemical warfare agents. Mathematical models are applied to this type of data to simplify or reduce the description of the basic chemical–biological processes of absorption, distribution, metabolism, and excretion (ADME). These model types are driven by the basic premise that significant pharmacological or toxicological understandings are gained by knowing the internal drug/chemical concentration at the target site. Most often termed ''classical'' PK models, they reduce the entire body mass to either one or two mathematical compartments which represent the volume of blood, plasma, and/or readily accessible extracellular spaces, and a deep compartment storage representing other tissues. The advantage of this analytical approach is the determination of a simple global description of a chemical's behavior in the body and potentially the concentration at major target receptor sites. The major disadvantage is that these empirical models are poor at interspecies extrapolation, since the parameters do not have a physiological interpretation, and it is difficult to predict how they change when the underlying physiology changes.

Physiologically based pharmacokinetic (PBPK) models are a special type of PK model that attempts to provide more definition to the model analysis by incorporating physiological factors into the model design, like tissue volumes, blood flow rates, and species-specific enzyme characteristics that can more accurately differentiate the dose–response relationship for a chemical or drug in one species from that of another species. The power of this approach is to be able to perform laboratory studies, both *in vitro* and *in vivo*, in common experimental species to develop a complete PBPK model and then extrapolate these results to predictions in humans based on *in silico* experimentation.

Physiologically based pharmacokinetic (PBPK) models have become useful analytical tools to interpret pharmacokinetic data and to interpret data from complex chemical exposure scenarios. These models are mathematical constructs that allow the coordination of species-specific physiology, chemical-specific information, and the experimental protocol for the chemical or chemicals of concern. The power of PBPK models lies in aiding the ability of scientists and decision makers to simulate the time-course concentration of chemicals in experimental animals and humans, to better determine estimates of actual chemical doses delivered to the target tissue, and thereby provide a better prediction of response. Due to the physiologically based nature of these models, simulations of experimental data can be performed by one exposure route, to validate the PBPK model, and then this model can be used to simulate and predict the kinetics and pharmacodynamics in the human by one or multiple exposure routes. This provides decision makers with a fairly rapid method of comparing results from *in vitro* and *in vivo* laboratory studies, to potentially real world exposure scenarios. Perhaps the most useful application of these models lies in a strongly animal data-based PBPK-PD (PD – pharmacodynamic) model being used to make predictions about human responses to chemical warfare agents (CWAs), across a broad range of doses and CWA types, in light of not being able to actually conduct human exposure studies.

CWAs are represented by any one of a number of chemicals exhibiting a very high toxicity by various mechanisms. The present Handbook exhibits CWAs with structures as simple as carbon monoxide (CO) and as complex as botulinum toxin or ricin proteins. While this chapter could address the development of PBPK models of CWAs in general, the focus will primarily be on the organophosphate (OP)-based nerve agents typically represented by sarin (GB – isopropyl methylfluorophosphonate).

II. DEVELOPMENT OF PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELS

Most attempts at describing CWA PK and PD have used classical kinetic models that often fit one set of animal experimental data, at lethal doses, with extrapolation to low-dose or repeated exposure scenarios having limited confidence. This is due to the inherent nonlinearity in high-dose to low-dose extrapolations. Also, the classical approach is less adept at addressing multidose and multiroute exposure scenarios, as occurs with agents like VX, where there is pulmonary absorption of agent, as well as dermal absorption. PBPK models of chemical warfare nerve agents (CWNAs) provide an analytical approach to address many of these limitations.

There are only a few PBPK models that have been developed to describe the pharmacokinetics and pharmacodynamics of CWNAs. Maxwell *et al.* (1988) developed a pharmacodynamic model for soman in the rat describing the inhibition of acetylcholinesterase (AChE) in different tissues, with mass balance equations including parameters for blood flow, tissue volumes, soman metabolism, and tissue/plasma partition coefficients. This effort resulted in an accurate prediction of AChE activity in eight different tissues after intramuscular soman dosing and was able to reproduce dose–response inhibition from 10% up to 100% of the brain AChE activity. Gearhart *et al.* (1990) used diisopropylfluorophosphate (DFP) as a model compound for CWNAs, to develop a PBPK-PD model describing the pharmacokinetics of DFP and the inhibition of AChE and butyrylcholinesterase (BuChE) in all the pertinent tissues of the body. This model construct was able to predict the pharmacokinetics of DFP and inhibition of AChE and BuChE after acute and repeated doses by three different routes. Recently, the PBPK-PD model for DFP was reparameterized to predict GB kinetics, inhibition of AChE, and regenerated GB from bound AChE sites on the red blood cells (RBCs) (Gearhart *et al.*, 2005).

There are three basic critical components to PBPK models: (1) species-specific physiological parameters, (2) chemical-specific parameters, and (3) specific experimental details for the studies to be simulated. Species-specific physiological parameters are the organ weights and blood flow rates for the defined compartments in the PBPK model. These values are most often available in the published literature and when lacking, are derived from the closest like species. Chemical-specific parameters that are unique for each chemical are the tissue solubility (partition coefficient), metabolism of the parent compound, and plasma and tissue binding characteristics. Tissue solubility is most often measured experimentally for the volatile CWNAs by the vial equilibration method (Gargas *et al.*, 1989). Existing models for the physiological distribution of CWNAs are based on a limited number of data sets for each agent and typically rely on single exposures at relatively high doses, often in the supralethal range (Van der Schans *et al.*, 2003). Mathematical models of these data have been developed but extrapolation to low-level exposures has proven problematic (Sweeney and Maxwell, 1999). Further, there are very few experimental data and essentially no modeling efforts for repeated low-level exposures (Benschop and de Jong, 1991). Thus, new experimental data at the appropriate dose ranges appear to be required for further refinement and extension of existing physiologically based pharmacokinetic (PBPK) models.

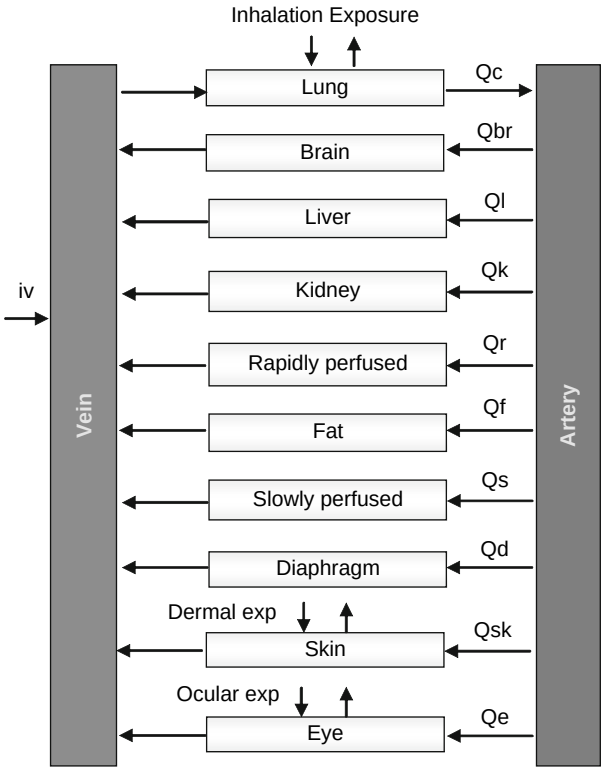

FIGURE 51.1. PBPK-PD model schematic of sarin in Hartley guinea pig. This model structure allows for the simulation of experimental studies with dosing by intravenous or subcutaneous dosing, and inhalation exposure. This model design was after Gearhart *et al.* (1990) and was adapted to simulate the pharmacokinetics and pharmacodynamics of sarin in the guinea pig.

Much of the animal data on the biological effects of low-level exposure to GB and other nerve agents has been collected via the subcutaneous or intramuscular route, particularly in guinea pigs, because of their lack of carboxylesterase. A good deal of data has also been collected by this dosing route or intramuscular dosing in rats, minipigs, and nonhuman primates. The primary exposure route of interest for human exposure is almost exclusively inhalation or dermal contact, so the quantitative implications of these laboratory exposure data for human health risk assessment are not straightforward. The most reliable tool for extrapolating across both species and exposure routes is

PBPK modeling, in which species differences in physiology are taken directly into account, and equivalent systemic and tissue doses for different routes of exposure can be calculated. Such extrapolation procedures, exemplifying the integration of diverse datasets in both animals and humans, are illustrated in Figure 51.2. Here the lower pentagon represents the dermal (or SC dosing) exposure route for different species, while the upper pentagon represents inhalation exposure. The green arrow represents specifically the extrapolation of subcutaneous exposure data in the guinea pig to apply to human inhalation exposure situations. In this example, the relevant biological effects observed in the guinea pig at specific subcutaneous exposures are noted. The guinea pig PBPK model then allows blood and tissue concentration time-courses corresponding to these doses to be calculated. Assuming that similar biological effects occur at similar target-site concentrations across species, the necessary inhalation exposure corresponding to the same blood and/or tissue concentrations in the humans can then be back-calculated using the human PBPK model for the agent. By using PBPK models in this way, human inhalation exposures are thereby quantitatively linked to the rich animal response and health effects databases.

III. NEED FOR IMPROVED MEASURES OF CWNA EXPOSURES – USE OF PBPK ANALYSIS OF DATA

Historically, cholinesterase activity has been used to evaluate exposure of humans and animals to nerve agents. Unfortunately, circulating cholinesterase activity is a relatively insensitive and imprecise marker of nerve agent exposure. By contrast, regeneration of nerve agents from inhibited enzymes using a high fluoride concentration (fluoride regeneration) is well suited to monitoring absorbed doses resulting from low-level exposures to sarin (GB), soman (GD), and other CWNA (Polhuijs *et al.*, 1997; Jakubowski *et al.*, 2001, 2003; Adams *et al.*, 2004). This technique has not yet been applied systematically for kinetic modeling of CWNA exposures in guinea pigs. Recently, a method for regeneration of GB from blood/tissue has been developed and validated in rat, guinea pig, and pig models and provides a sensitive and quantitative means for estimating the kinetics of GB in blood (Jakubowski *et al.*, 2004). This method is based on fluoride ion regeneration of the protein-bound agent. Fluoride ion regenerated sarin (R-GB) was found in blood and tissues of minipigs exposed to sarin vapor levels ranging from miosis to lethality. The R-GB in these samples was analyzed by gas chromatography mass spectrometry (GC-MS) after a C18 solid-phase extraction sample preparation that included fluoride ion addition and pH adjustment. Serial blood samples taken before and during miosis-level GB inhalation exposures resulted in red blood cell R-GB levels that steadily rose during the exposure. This demonstrated the ability of the R-GB assay as a biometric of exposure. The slopes of the lines created by plotting R-GB versus time closely correlated to the experimental exposure level. Therefore, the rates of GB absorption in these animals were proportional to the GB exposure concentration. In contrast to R-GB, monitoring AChE activity was an ineffective indicator of exposure at miosis levels in these experiments.

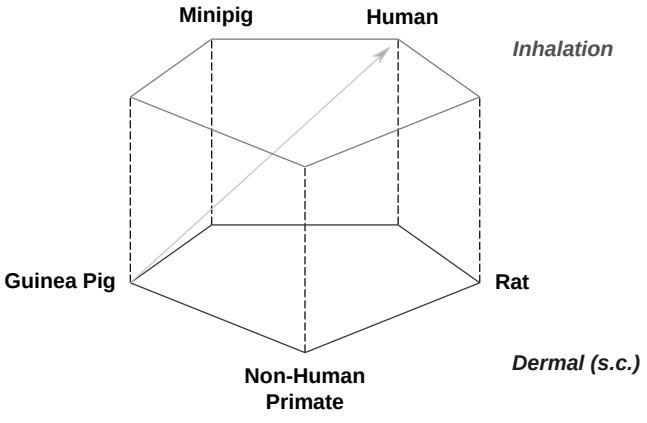

FIGURE 51.2. Schematic illustration of combined interspecies and route-to-route extrapolation. The arrow represents the extrapolation of guinea pig subcutaneous exposure data to human inhalation exposure situations.

A. Relationship Between Regenerated Sarin and AChE Activity and its Use as a Dose Surrogate

Regenerated sarin S_R from red blood cells is experimentally related to AChE activity A in Figure 51.3. It is apparent that the relationship can be adequately described in terms of a Michaelis–Menten equation as follows:

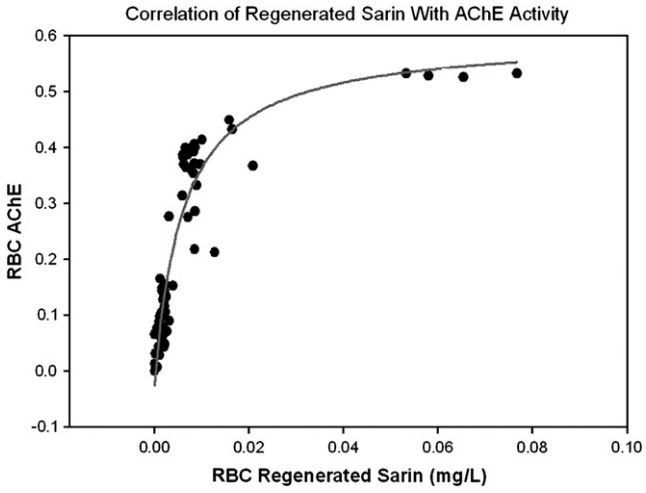

FIGURE 51.3. Plot of red blood cell acetylcholinesterase activity as a function of regenerated RBC sarin.

$$A = A_{\max}\left[1 - \frac{S_R}{S_R + K_m^{eff}}\right] \qquad (51.1)$$

where $A_{\max} = 0.623$, and $K_m^{eff} = 0.006$. Note that K_m^{eff} is not the true K_m for sarin binding to AChE, which is the concentration of sarin needed to fill half the AChE receptors, since we do not know that the regenerated sarin is the same as the original exposure concentration. (If there are additional sarin-binding sites than AChE, for example, the regenerated sarin would underestimate the exposure concentration, and the true K_m would be larger than K_m^{eff} by a certain factor.) The fact that these data fit so well to an M–M-type equation strongly suggests that there is a simple proportionality between K_m^{eff} and the ''true'' K_m, and that sarin regenerated from RBCs is a suitable surrogate for the original exposure to sarin in blood (Figure 51.3).

Regenerated sarin from RBCs at high doses may exceed the total available binding capacity of the AChE receptors associated with the RBCs. There are therefore additional sarin-binding sites proposed to be associated with RBCs that continue to bind sarin well after the AChE receptors are filled, and therefore may comprise the bulk of the regenerated sarin at high concentrations. We thus have proposed two competing binding processes: one high-affinity, low-capacity site that dominates binding of sarin at low concentrations, and which normally releases sarin very slowly if at all (the AChE receptors); and one relatively low-affinity, high-capacity site that releases sarin rapidly compared with the circulation time of the blood (Figure 51.4). This latter site would dominate at higher concentrations, and in the absence of any evidence for its saturation even at these high concentrations, we approximate it as a first-order process described with simple association and dissociation rate constants k_{on} and k_{off}.

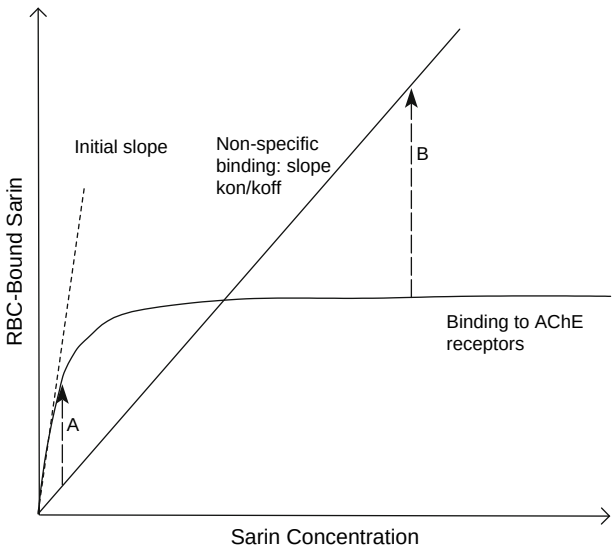

FIGURE 51.4. Hypothetical bound GB vs free sarin concentrations for saturable AChE binding, and linear nonspecific binding. The AChE binding has a higher initial slope, so it will dominate in a competitive situation at low doses (A), but is saturable and so will eventually be swamped by the nonspecific binding at higher doses (B).

B. General PBPK Model Structure

The basic structure of the PBPK model used to describe GB pharmacokinetics and pharmacodynamics (PD) followed that of the PBPK-PD model for DFP (Gearhart *et al.*, 1990). Tissue compartments (Figure 51.1) were added to the previous model structure describing the eye and the skin, where previously these compartments were lumped together in the rapidly perfused or slowly perfused tissues. The eye was added to provide the means of predicting miosis during CW agent exposure, from both the systemic absorption of chemicals, but more importantly, the amount of chemical absorbed directly to the eye structures via the ocular surface via absorption and diffusion. Adding this compartment also provided a mechanism of addressing the pharmacodynamic effects of sarin on the eye, by correlating inhibition of AChE enzyme levels in the iris with levels of miosis. The skin was added primarily to provide an exposure route for those agents that have a significant dermal absorption potential. Decreases in absorption due to upper respiratory tract deposition were accommodated by refining the model as shown in Figure 51.5.

PBPK parameters for individual organ weights were obtained from Breazile and Brown (1976), Altman and Katz (1979) and Peeters *et al.* (1980). Blood flows for most organs were obtained from Peeters *et al.* (1980) or were scaled from other rodent species. The partition coefficients were based on the values used for DFP and soman (Gearhart *et al.*, 1990), as well as the cholinesterase and sarinase values for tissue and blood. The description of the interaction of GB with the mammalian system of the guinea pig followed that as described by Gearhart *et al.* (1990) for the CWA simulant diisopropylfluorophosphate (DFP) in rodents and Gearhart *et al.* (2005) in the Göttingen minipig. The description of sarin binding to AChE is shown in Figure 51.6. Sarin interaction with AChE is described by a bimolecular rate constant, causing loss of AChE activity.

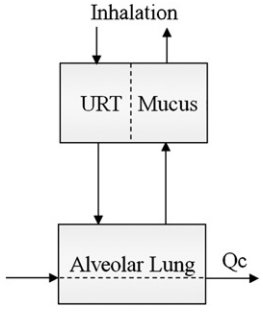

FIGURE 51.5. Detailed schematic for upper respiratory tract (URT) interactions for inhalation exposures.

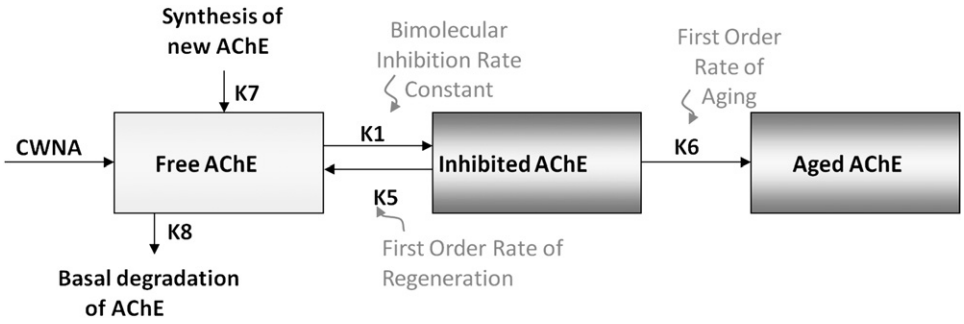

FIGURE 51.6. Schematic for acetycholinesterase inhibition by sarin.

The inhibited AChE sites represent the amount of sarin which can be regenerated from the red blood cells.

IV. PBPK SIMULATION OF CHOLINESTERASE INHIBITION AND REGENERATED GB

The main focus of this effort was to develop and validate methods to relate and integrate CWNA toxicity data across routes of exposure in a common species. The specific goal is to compare uptake and clearance kinetics of similar sublethal doses of GB in the blood of guinea pigs exposed to the agent by acute intravenous (IV), inhalation (IH), or subcutaneous (SC) injection. GB regenerated from blood was used as the dose metric to compare the uptake and clearance kinetics of similar doses of GB administered to guinea pigs by IV, SC, or IH routes of exposure. The resulting database will be used to derive a quantitative ratio (SC/IH) of systemically absorbed agent that will allow predictions of the atmospheric GB concentration relevant to a second species based upon SC injection exposures.

Guinea pigs were exposed to sublethal levels of GB by IV, IH, and SC routes. Serial blood samples were collected in order to determine simple uptake and clearance kinetics using regenerated agent as a dose metric in a single dose (IV, SC, and IH). The resulting database was used to derive the PBPK model. This model will be used to determine a quantitative ratio (SC/IH) of systemically absorbed agent that will allow predictions of the atmospheric GB concentration relevant to the increased guinea pig data sets based upon subcutaneous injection exposures.

Simulations of inhibited cholinesterase and regenerated GB versus the plotted data are exhibited in Figures 51.7 through 51.13. The methods of GB exposures or dosing shown are for either intravenous, subcutaneous, or inhalation. In most cases the experimental data were well represented by the PBPK-PD model simulations. Those instances where the greatest deviation occurred between the model and the data were most likely due to data variability, such that the trend of the data would not follow a reproducible model behavior (Figure 51.11), or cases where there is still some question about the strength of the model parameters being used. Figures 51.7 through 51.10 show the model simulation of some of the key data used to determine model behavior. In each of these cases, the simulations provide a good representation of the overall magnitude and tread of

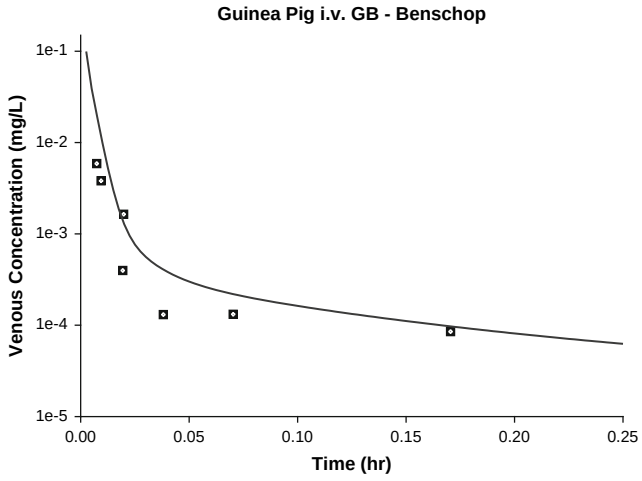

FIGURE 51.7. PBPK-PD simulation of AChE inhibition in the guinea pig after an intravenous GB dose of 0.8 LD_{50} (0.0042 mg/kg body weight) (Benschop and De Jong, 2001 – 19.2 µg/kg).

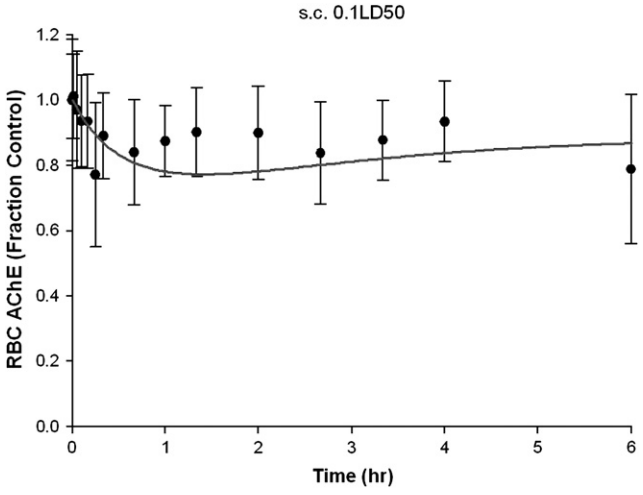

FIGURE 51.8. PBPK-PD simulation of AChE inhibition in the guinea pig after an SC dose of 0.1 LD_{50} (0.0042 mg/kg body weight).

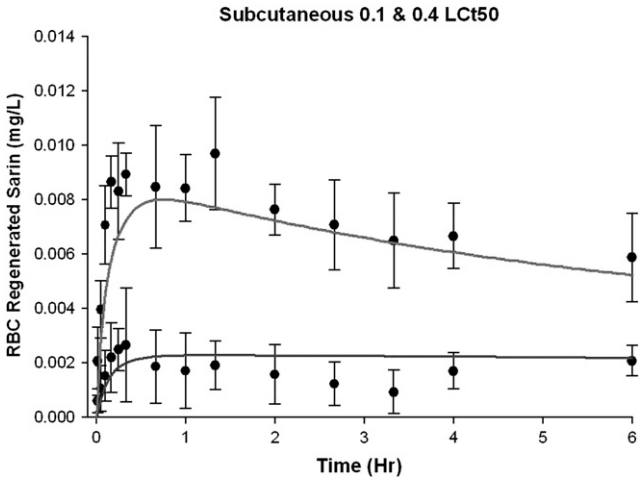

FIGURE 51.9. PBPK-PD simulation of regenerated sarin in the guinea pig after an SC dose of 0.1 LD_{50} (0.0042 mg/kg body weight) and 0.4 LD_{50} (0.0168 mg/kg body weight).

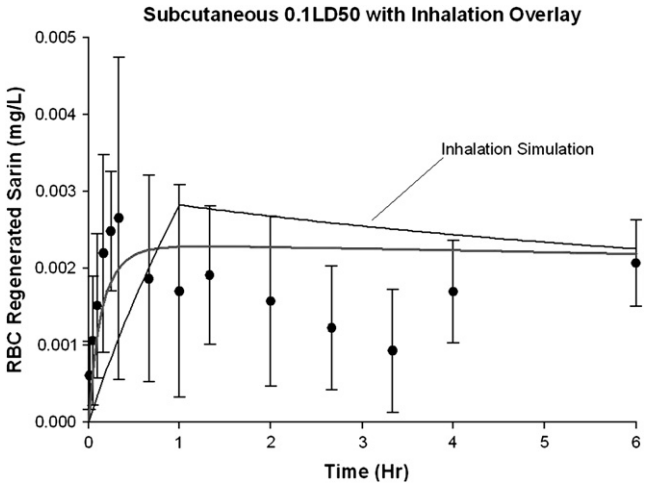

FIGURE 51.11. Overlay of the simulation of regenerated RBC sarin after a 0.1 LD_{50} SC dose of sarin vs IH dosing simulation at the inhalation concentration required to reproduce the SC data.

the data. In some cases, as in Figures 51.7 and 51.9, there are points during the simulation when there is either an over- or underestimation of the data. This type of variation between PBPK-PD simulations and data is a common occurrence whenever there is an attempt to exactly simulate time-course kinetic data for such toxicologically active a compound as GB. If it appears that significant decreases or increases in the actual experimental values are indications of real mechanisms and not just animal to animal variability, then that may only be simulated using more sophisticated methods, as with stochastic processes where actual fluctuations in blood flows or shunting of blood could, for example, cause changes in represented concentrations out of the normal trend of the data. Certainly one major issue not addressed in this chapter is the effects of GB on its own pharmacokinetics which in turn has a significant impact on key PBPK parameters such as blood flow or respiratory ventilation parameters. A well-known response to severe CWNA exposure is a suppression of the cardiac output and respiratory ventilation, both of which could alter measured PK and PD endpoints and if not modulated in the PBPK-PD model, lead to over- or underestimation of data.

V. CONCLUDING REMARKS AND FUTURE DIRECTION

The overall performance of the PBPK-PD model in predicting both the concentration of regenerated GB and the inhibition of RBC AChE was very successful for both the subcutaneous and inhalation routes of exposure, at these doses. Previously published studies showing the significant

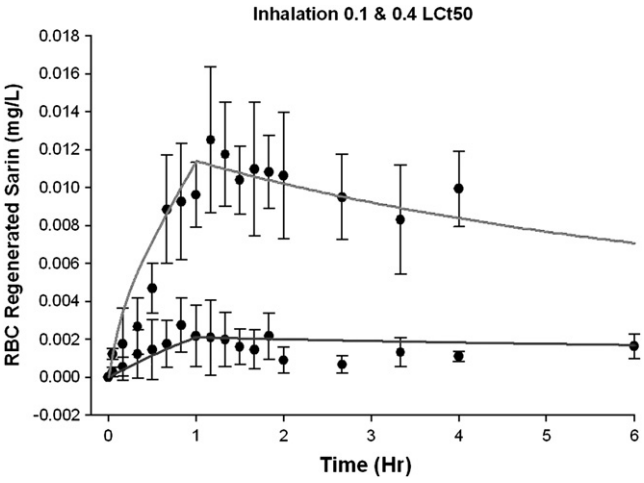

FIGURE 51.10. PBPK-PD simulation of regenerated sarin in the guinea pig after a 1 h inhalation exposure to sarin vapor at 0.1 LC_{50} (0.4 mg/m^3) or 0.4 LC_{50} (1.6 mg/m^3).

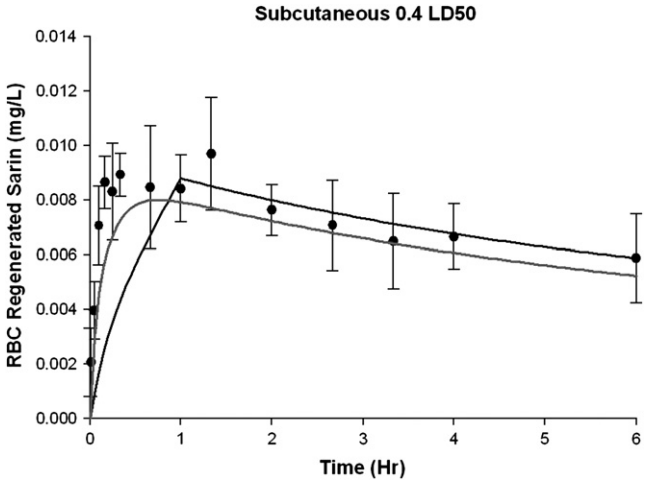

FIGURE 51.12. PBPK-PD simulation of an exposure of guinea pigs to the SC concentration equaling 0.4 of the lethal concentration affecting 50% of the exposure population (LCt_{50}).

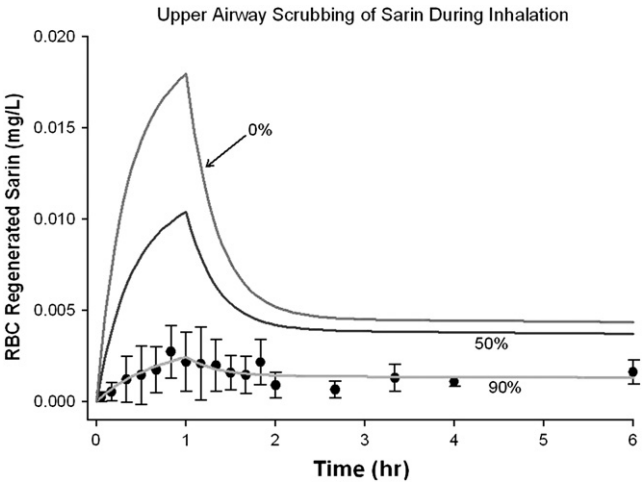

FIGURE 51.13. Upper airway scrubbing simulations. As was previously seen with the simulation of minipig inhalation exposures, a significant percentage of the inhaled dose was scrubbed by the upper respiratory tract, with the best fit to the data requiring the loss of 90% of the inhaled dose.

deposition of GB in the upper respiratory tract were confirmed in this analysis of the inhalation of GB in the guinea pig at 0.1 and 0.4 times the LC_{50} for this species. The PBPK-PD model was utilized to equate the delivered dose of sarin by subcutaneous exposure, with what would be obtained after inhalation. In this analysis, it was determined that at lower GB inhalation concentrations, there is a significant amount of deposition of the agent on the upper respiratory tract (Figure 51.13), material which is both hydrolyzed and resultantly inactivated, or is bound in by such a mechanism that it presently is hypothesized to have limited activity. This is one aspect of this analysis which requires further study. To determine the actual inhalation concentration that would correlate with a particular subcutaneous dose, the PBPK-PD model was exercised repeatedly, and the simulation output was overlaid on the subcutaneous simulations. This process is shown in Figures 51.5–51.11 and 51.12 for the 0.1 and 0.4 SC LD_{50}s. This calculation showed that to reproduce the SC 0.1 LD_{50} of 0.0042 mg/kg (Figures 51.11) required an inhalation concentration of 0.08 ppm for a 1 h inhalation exposure. This value is very close to the experimentally determined value for the inhalation 0.1 LC_{50} of 0.05 ppm. The SC 0.4 LD_{50} of 0.0168 mg/kg required an inhalation concentration of 0.28 ppm for a 1 h inhalation exposure (Figure 51.12). Further simulations will need to be exercised to confirm these apparent relationships.

A previously developed PBPK-PD model for the CWNA surrogate DFP was parameterized to simulate the concentration and effects of low-level chemical warfare agents (CWAs) in the guinea pig after exposure by inhalation and subcutaneous injection. The model code was written to account for absorption of CWAs from multiple sites (respiratory tract – lower and upper, dermal, ocular) after vapor or subcutaneous exposure. Literature references to guinea pig physiology were used for the majority of organ volumes and blood flows, while some parameter values were scaled from other species. Unique features of this PBPK-PD model structure were physiological compartments for the eyes, as a source of external CWA absorption and internally as a site of cholinesterase binding, and skin as a dermal absorption pathway. One initial pharmacodynamic endpoint developed in this model was CWA inhibition of cholinesterases (AChE, BuChE). Covalent binding of sarin to cholinesterases and other proteins was also estimated by a novel parameter based on fluoride ion regeneration of the agent. The PBPK-PD model was used to simulate AChE inhibition after inhalation and subcutaneous injection of CWAs and to predict potential pharmacodynamic effects at different tissue target sites. This preliminary model will provide a quantitative tool to predict the physiological consequences of low-level, nonlethal exposure after CWNA exposure.

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CHAPTER 52

Metabolism of Warfare Nerve Agents

MILAN JOKANOVIĆ

I. INTRODUCTION

The first organophosphorus (OP) nerve agents, tabun (GA) and sarin (GB), were developed in the 1930s by Gerhard Schrader. These, and the even more toxic soman (GD), developed in 1944, are members of the so-called G-agents. Together with VX, developed after World War II in the United Kingdom, these compounds have emerged as the major nerve agents known to have been produced and weaponized. The nerve agents are alkylphosphonic acid esters. Tabun contains a cyanide group. Sarin and soman, which contain a fluorine substituent group, are methylphosphonofluoridate esters. These nerve agents contain a C—P bond that is almost unique in that it is not found in organophosphate pesticides. This C—P bond is very resistant to hydrolysis. VX contains a sulfur and is an alkylphosphonothiolate.

The very high toxicity of these agents can be attributed to the excessive cholinergic stimulation caused by inhibition of acetylcholinesterase (AChE) at neuromuscular junctions and in the central nervous system. Nerve agents react rapidly with a serine hydroxyl group in the active site of AChE with the formation of a phosphate or phosphonate ester. The phosphorylated enzyme regenerates very slowly rendering the enzyme inaccessible for its physiological substrate acetylcholine. The chirality around the phosphorus atom largely influences the toxicity of these agents as documented in the case of soman whose P(−)-isomers are much more toxic than the P(+)-isomers (Van der Schans *et al.*, 2007).

In the case of G-agents the intact agent is present in the organism for only several hours. The dominant metabolic pathway of G-agents is hydrolysis, a process mainly mediated by so-called A-esterases, and the metabolic products formed are corresponding *O*-alkyl methylphosphonic acids in the case of sarin and soman (Figure 52.1). VX is a less suitable substrate for A-esterases. In addition to hydrolysis, binding reactions of nerve agents to esterases such as AChE, serum cholinesterase (ChE), carboxylesterase (CarbE), and other proteins occur. Both OP pesticides and nerve agents lose their acyl moieties when they react with AChE, ChE, and CarbE. After binding to AChE and ChE the phosphoryl residues of soman, sarin, tabun, and VX undergo an intramolecular rearrangement with subsequent loss of one phosphoryl group. This process is called aging and the reaction is further discussed in Chapter 65.

In addition, due to the reversibility of the binding reaction of sarin and soman to CarbE, it appears that CarbEs are involved in metabolic detoxification of these agents to their corresponding nontoxic metabolites isopropyl methylphosphonic acid (IMPA) and pinacolyl methylphosphonic acid (PMPA) (Jokanović *et al.*, 1996).

One of the important proofs which support the significance of detoxification reactions of nerve agents in the body was presented by Fonnum and Sterri (1981) who reported that only 5% of LD_{50} of soman in rats or about 5 μg/kg reacts with AChE causing acute toxic effects, while the remaining 95% undergoes various metabolic reactions.

In this chapter the mechanisms involved in metabolism of warfare nerve agents are discussed. Mechanisms of biotransformation of OP pesticides are beyond the scope of this chapter and interested readers are referred to other publications (Jokanović, 2001; Chambers *et al.*, 2001; Tang *et al.*, 2006).

II. CHEMICAL ASPECTS OF METABOLISM OF NERVE AGENTS

The G-agents are anticholinesterase OP nerve agents which at sufficient concentrations can be toxic or fatal by any route of exposure. Differences in volatility and water solubility result in varying degrees of persistence and variations in the likelihood of exposure by certain routes. Of the G-agents, tabun gives rise to the greatest number of degradation products. The main metabolic product of tabun is EDMPA (Figure 52.1). Toxicity data are available only for a limited subset of the tabun degradation products. Hydrolysis gives rise to dimethylamine, among other substances. Dimethylamine is moderately toxic in terms of acute lethality but causes irritation of the human respiratory tract (Munro *et al.*, 1999).

Sarin is metabolized to IMPA, which slowly undergoes further hydrolysis to the very stable MPA. IMPA also forms in the course of spontaneous reactivation of sarin-inhibited CarbEs and ChEs. IMPA has low oral toxicity in rats and mice, but it produces mild skin irritation in rabbits.

In the study of Little *et al.* (1986) a single sublethal dose (80 μg/kg) of radiolabeled sarin was administered intravenously to mice and the tissue distribution was studied for 24 h. Within 1 min, sarin was distributed to the brain, lungs,

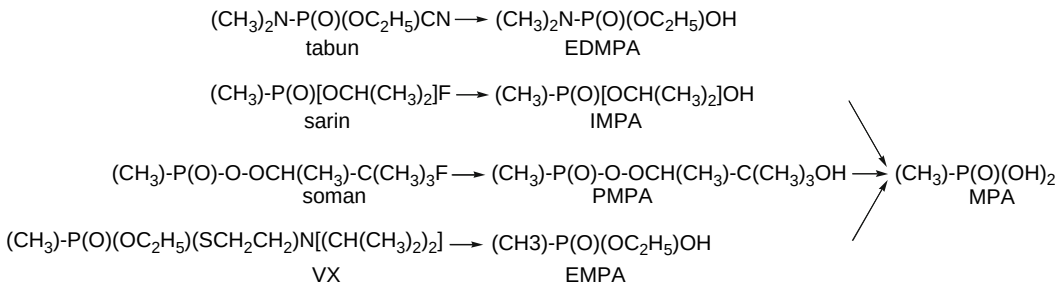

FIGURE 52.1. Metabolic detoxification of warfare nerve agents tabun, sarin, soman, and VX in mammals *in vivo*. Chemical names of metabolites are: EDMPA – ethyl dimethylaminophosphoric acid, IMPA – isopropyl methylphosphonic acid, PMPA – pinacolyl methylphosphonic acid, EMPA – ethyl methylphosphonic acid, and MPA – methylphosphonic acid.

heart, and diaphragm, but the highest concentrations were found in kidneys, liver, and plasma. Thereafter, the concentrations in all tissues rapidly declined and within 15 min only trace quantities of [^{3}H]sarin were found in brain. Within the first minute, about half of the labeled sarin was associated with the major sarin metabolite IMPA. The kidneys contained the highest concentrations of sarin and its metabolites, whereas much lower concentrations of metabolites were detected in the liver suggesting a minor role for the liver in detoxification of sarin.

Shih and colleagues (1994) injected rats subcutaneously with a single dose of sarin (75 μg/kg) and measured excretion of the hydrolyzed metabolites, the alkylmethylphosphonic acids, including IMPA and other methylphosphonic acids. Urinary elimination was found to be quite rapid and the terminal elimination half-life of sarin metabolites in urine was 3.7 h. Most of the administered dose of sarin was retrieved from the urine in metabolite form after 2 days.

Distribution, metabolism, and elimination of sarin in humans appear to resemble findings in animals. Minami and colleagues (1997, 1998) detected the sarin metabolite IMPA in urine of humans after the terrorist attack in Tokyo in 1995. They found peak levels of IMPA or methylphosphonic acid in urine 10–18 h after exposure. The levels of IMPA in urine correlated with the clinical symptoms. They also found evidence of distribution of sarin to the human brain in four of the 12 people who died after exposure. IMPA and MPA were detected in patients from the Matsumoto sarin exposure (Nakajima *et al.*, 1998).

Hydrolysis products of soman include pinacolyl methylphosphonic acid (PMPA) and MPA. No biologic data were found for PMPA. It has been shown that the toxic C(±)P(−)-isomers of soman react rapidly with covalent binding sites. The less toxic C(±)P(+)-isomers are hydrolyzed several orders of magnitude faster than the C(±)P(−)-isomers. The low toxicity of the C(±)P(+)-isomers is primarily due to a low intrinsic reactivity toward AChE and rapid hydrolysis (Van der Schans *et al.*, 2007). The levels of C(±)P(−)-isomers remain toxicologically relevant for periods of 50–100 min in rats, guinea pigs, and marmosets at doses of 2–3 LD_{50} (Benschop and de Jong, 1991).

The organophosphate nerve agent VX is a potent anticholinesterase agent that can act by dermal, oral, or inhalation routes of exposure. The anticholinesterase mechanism of action of VX is due to the oxo (=O) group, but is also influenced by the presence of alkyl substituents. There are some characteristics of VX which make the agent different from G-agents. VX is present in blood as protonated amine, it is hydolyzed at a much slower rate than G-agents, it reacts more slowly with CarbEs and A-esterases, and it can be metabolized by other routes such as oxidation reactions at nitrogen and/or sulfur. Initial metabolic and degradation products may retain some anticholinesterase activity, but hydrolysis of one or more alkyl ester bonds of organophosphonic acids results in the generally nontoxic alkyl methylphosphonic acids. MPA is resistant to further hydrolysis. Limited data for MPA suggest low oral toxicity in the rat and mouse, and irritant effects on human eye and skin. Munro *et al.* (1999) discussed the metabolic degradation products of VX in mammals and found that there are about 25 such products and each had shown different levels of toxicity. Among those only *S*-(diisopropylaminoethyl) methyl phosphonothionate (EA 2192) has anticholinesterase activity and somewhat lower toxicity compared to VX.

Benschop *et al.* (2000) and Van der Schans *et al.* (2003) studied the toxicokinetics of VX stereoisomers in hairless guinea pigs and marmosets. Following an intravenous dose of 28 μg/kg (marmosets) or 56 μg/kg (guinea pigs), VX was found in the blood at toxicologically relevant levels even after 6 h. Detoxification proceeded at a slower rate in marmosets than in guinea pigs. The VX metabolite EMPA was found in the blood of the exposed animals; however, the metabolite contributed only 5% to the recovery of the phosphonyl moieties related to the VX dose. Metabolites of VX were also evaluated in *in vitro* studies by treating liver homogenates and plasma from hairless guinea pigs, marmosets, and humans with the radiolabeled compounds ^{35}S-VX. The potential toxic metabolite VX-N-oxide was not found. Desethyl-VX was found after incubation of VX in plasma of all three species; however, because of its slow rate of formation, Benschop *et al.* (2000) concluded that it would be unlikely that VX would be present at toxicologically

relevant levels after administration of VX *in vivo*. *In vitro* studies with ^{35}S-VX revealed that a significant part of the thiol-containing leaving group (*S*-2-/*N*,*N*-diisopropylamino/ethane thiol) was bound to proteins such as albumin.

Tsuchihashi *et al.* (1998) have developed a method for identification of VX metabolites in serum collected from a victim of the Osaka VX incident. In the serum sample, both EMPA and 2-(diisopropylamino-ethyl)methyl sulfide were detected. The techniques using GC-MS and GC-MS-MS were applicable to biological samples such as serum. These results provided the first documented identification of the specific metabolites of VX in victims' serum and clarified a part of the metabolic pathway of VX in the human body. In addition, methods have been developed for measuring the VX-inhibited AChE hydrolytic product EMPA (Noort *et al.*, 1998; 2002).

III. ESTERASES INVOLVED IN METABOLISM OF WARFARE NERVE AGENTS AND OTHER OPs

Numerous esterases can react with OPs but in a different way. The first classification was given by the late Professor Aldridge (1953). In the first group there were esterases that hydrolyze OPs as substrates, and among them particularly their uncharged esters, which are not inhibited by these compounds. This group of enzymes was named *A-esterases* although in the literature there are many other names for the same group of enzymes given according to the substrate hydrolyzed (paraoxonase, somanase, DFPase, etc.) or their chemical structure (phosphotriesterases, phosphorylphosphatases, anhydrases of organophosphorus compounds). In the second group of enzymes interacting with OPs were *B-esterases* which are inhibited with OPs in the progressive reaction which is time and temperature dependent. This group of enzymes comprises AChE, ChE, CarbE, trypsin, chymotrypsin, and other enzymes. In the third group were *C-esterases* that do not interact with OP. It is paradoxical, but basically true, that OPs can be substrates for both A- and B-esterases because their concentration in blood and tissues is decreased in the presence of these enzymes.

The mechanism of interaction of A- and B-esterases with OP is similar. B-esterases initially form Michaelis complex with an OP inhibitor producing phosphorylated or inhibited enzyme that either reactivates very slowly or does not reactivate at all (see Figure 69.1 in Chapter 65). However, after formation of Michaelis complex with OP A-esterases perform hydrolysis of OP and their catalytic activity and turnover rate are very high. It was already shown that CarbE, as a typical B-esterase, can hydrolyze carboxylic esters that serve as functional groups in OP such as malathion thus performing detoxification of the compound (WHO, 1986; Fukuto, 1990).

A. A-Esterases

In a classification from 1992 (International Union of Biochemistry, 1992) hydrolases of OP were described as a special entity as ''phosphoric triester hydrolases'' which comprise three groups of enzymes: phosphoric monoester hydrolases (EC 3.1.3), phosphoric diester hydrolases (EC 3.1.4), and phosphoric triester hydrolases (phosphotriesterases) (EC 3.1.8). Phosphoric triester hydrolases are further divided in two similar subgroups: aryldialkylphosphatases (EC 3.1.8.1) and diisopropylfluorophosphatases (EC 3.1.8.2).

Aryldialkylphosphatases take part in hydrolysis of aryldialkylphosphates producing dialkylphosphate and aryl alcohol. These enzymes react with substrates such as paraoxon (Figure 52.2a), but also with phosphonates and phosphinates. Other names for this group of enzymes are hydrolases of organophosphate compounds (OPCs), A-esterases, paraoxonases, PON1, aryltriphosphatase, and aryltriphosphate dialkylphosphohydrolase. They are inhibited with compounds that form chelates like EDTA since they require the presence of divalent ions, mainly Ca^{2+} (Walker, 1993). Some fractions of the enzyme purified from human serum were able to hydrolyze both paraoxon and phenylacetate and it was thought for a long time that the same enzyme is responsible for both. However, enzymes hydrolyzing aryl esters are classified as arylesterases (EC 3.1.1.2).

Diisopropylfluorophosphatases take part in hydrolysis of diisopropylfluorophosphate (DFP) and similar compounds producing diisopropylfluorophosphoric acid and fluoride ion (Figure 52.2b). These enzymes react with phosphorus anhydride bonds such as those between phosphorus and acyl radical (F^-, Cl^-, CN^-) in highly toxic OPCs such as soman, sarin, tabun, and DFP, and they were accordingly named somanase, sarinase, tabunase, DFPase, diisopropylfluorophosphate fluorohydrolase and anhydrases of organophosphorus acids. They are also inhibited by chelating agents, and their activity requires the presence of divalent ions such as Ca^{2+}, Mg^{2+}, and Mn^{2+} (Walker, 1993). They exist in several forms, even in different tissues of the same species, which react differently with substrates. However, in the literature published during the last 15 years the term PON1 apparently covers both phosphoric triester hydrolases. In a further text, in order to avoid eventual confusion, the term A-esterase will be generally used for enzymes

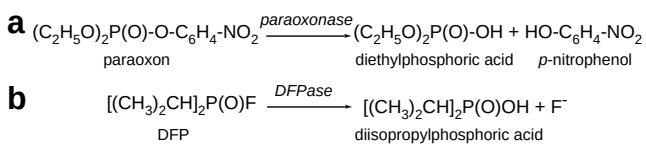

FIGURE 52.2. (a) Hydrolysis of paraoxon with aryldialkylphosphatase (paraoxonase, EC 3.1.8.1) and (b) hydrolysis of diisopropylfluorophosphate (DFP) with diisopropylfluorophosphatase (DFPase) (EC 3.1.8.2).

hydrolyzing OPs along with other terms (such as PON1, paraoxonase, etc.) as they appear in original references.

The molecular weight of human A-esterases is between 43 and 45 kDa. Human A-esterase is a protein of 355 amino acids having two polymorph sites in which arginine or glutamine are located at position 192 and methionine or leucine at position 55 (La Du *et al.*, 1993; Li *et al.*, 1995). The former accounts for three genotypes (QQ, RR, and QR) relating to the catalytic properties of two forms of an enzyme (types R and Q allozymes), which hydrolyze certain organophosphates at different rates. Its three-dimensional structure is also known (Benning *et al.*, 1994; Vilanova and Sogorb, 1999). At the active site of A-esterases there are two metal cations connected via a common ligand, and most of the other protein groups are bound to this binuclear site through imidazolium side chains from histidine groups (Benning *et al.*, 1994). Activity of these enzymes largely depends on Ca^{2+} which represents a necessary factor for maintaining the function of active site and it is also possible that Ca^{2+} directly participates in catalytic reactions or maintains the conformation of amino acids at the active site. In addition, in the case of paraoxon, Ca^{2+} facilitates the release of diethylphosphate from the active site probably by polarizing bond P=O which makes phosphorus atoms much more sensitive to nucleophilic attack of hydroxyl ions (Vitarius and Sultatos, 1995). Human A-esterases can hydrolyze many OPs, among them paraoxon, soman, sarin, tabun, chlorpyriphos and chlorpyriphos oxon, DFP, dichlorvos, diazoxon, and pyrimiphos methyl oxon.

The R allozyme (Arg192) hydrolyzes the organophosphate paraoxon at a high rate; however, it has a low activity toward diazoxon, sarin, and soman. Lower activity means that more sarin would be bioavailable to exert its anticholinesterase effects. The Q allozyme, on the other hand, has high activity toward diazoxon, sarin, and soman (and low activity toward paraoxon). Thus, individuals with the Q allozyme (QQ or QR) are expected to have greater hydrolysis of sarin than individuals homozygous for the R allele (RR) (Costa *et al.*, 1999, 2005). In Caucasian populations, the frequency of the R allele is about 0.3, but the frequency is 0.66 in the Japanese population (Yamasaki *et al.*, 1997; Costa *et al.*, 2006). This would make individuals in the Japanese population more sensitive to the toxicity of sarin, a fact that may have contributed to their morbidity and mortality after the terrorist attacks in 1995. The concentration of PON1 in human plasma (about 60 mg/l) varies between individuals by as much as 13-fold. PON1 activity level is determined by a combination of complex genetic interactions and environmental/dietary factors, giving rise to a 40-fold variation in PON1 for a single individual (Rochu *et al.*, 2007). The relationship between paraoxonase polymorphisms and toxicity of OPCs was further discussed in an excellent article by Costa *et al.* (2006) and other papers from his team.

Paraoxonase (PON1) is a member of a family of proteins that also includes PON2 and PON3, the genes of which are clustered on the long arm of human chromosome 7 (q21.22). PON1 is synthesized primarily in the liver and a portion is secreted into the plasma, where it is associated with high-density lipoprotein (HDL) particles (Costa *et al.*, 2005). The primary physiological role of PON1 appears to be to protect low-density lipoproteins (LDL) from oxidative modifications (Mackness *et al.*, 1993; Aviram *et al.*, 1998; Vilanova and Sogorb, 1999; Rochu *et al.*, 2007). In addition to its role in lipid metabolism and cardiovascular disease and atherosclerosis, PON1 has been shown to play a role in the metabolism of drugs and xenobiotics containing aromatic carboxyl esters (Costa *et al.*, 2003). It is also suggested that PON1 hydrolyses various lactones including naturally occurring lactone metabolites.

The differences in the activity of A-esterases due to polymorphisms may have important effects on the toxicity of OP in humans who are occupationally or accidentally exposed. In this respect it was proposed that humans expressing lower activity of A-esterases could be more susceptible to toxic effects of OP and there were ideas that such individuals should not be exposed (Mackness, 1989). Hernandez *et al.* (2004) have confirmed this hypothesis suggesting the association of paraoxonase phenotypes with susceptibility of humans to anticholinesterase pesticides toxicity. However, additional studies are needed to fully understand the effects of A-esterase polymorphism on the capacity of detoxification and toxicity of OPs.

1. Toxicological Relevance of A-Esterases

For many years it had been suggested that activity of A-esterases in serum can serve as a protective factor in poisoning with OP and among them OP insecticides whose active metabolites are AChE inhibitors and, at the same time, substrates for A-esterases. An important finding in this respect was significantly higher toxicity of pyrimiphos methyl and diazinon in birds than in mammals (Brealey *et al.*, 1980; Walker and Mackness, 1987) (oral LD_{50} of pyrimiphos methyl in hens is 30 mg/kg, and in rats about 1,500 mg/kg). The difference in toxicity of these OPs was explained by protective effect of A-esterases in mammals that express relatively high activity of the enzymes, in contrast to birds, which have no or very low A-esterase activity and are more susceptible to OPs. A-esterase activity in rabbits is about seven-fold higher than in rats, and because of this the rats are about four-fold more susceptible to paraoxon. Direct confirmation of this hypothesis was obtained after administration of purified A-esterases from rabbit serum to rats which provided a significant protection in poisoning with paraoxon or chlorpyrifos oxon (Main, 1956; Costa *et al.*, 1990). Furthermore, it was shown that purified A-esterases given to mice significantly decrease inhibition of AChE in brain and diaphragm after administration of chlorpyrifos or its active metabolite chlorpyriphos oxon (Li *et al.*, 1993, 1995). The increased levels of A-esterases in the serum of mice were maintained for 2 days providing protective effects not only when the enzyme was given before chlorpyriphos but also when it was given 3 h after the insecticide (Li *et al.*, 1995). A-esterases

purified from *Pseudomonas diminuta* and given to mice decreased inhibition of AChE in brain and ChE in serum in poisoning with paraoxon and DFP, and this effect was less significant in poisoning with soman and sarin (Tuovinen *et al.*, 1994). These authors have also observed accelerated reactivation of serum ChE in mice that was inhibited with paraoxon and proposed possible cleavage of the bond between ChE and paraoxon (which provides the same effect as spontaneous reactivation of ChE) and destruction of paraoxon itself. Purified A-esterases have shown protective effect when given to mice before poisoning with tabun (Ashani *et al.*, 1991a). The availability of serum paraoxonase knockout mice has provided an *in vivo* system which enables more direct examination of the role of paraoxonase in detoxification of OPs (Furlong *et al.*, 2000b). These animals demonstrated highly increased sensitivity to chlorpyrifos oxon and diazoxon and moderately increased sensitivity to the respective parent compounds chlorpyrifos and diazinon.

A recent study investigated PON1 genotype and serum enzyme activity in a group of 25 ill Gulf War veterans and 20 controls (Haley *et al.*, 1999). Ill veterans were more likely than controls to possess the R allele (QR heterozygotes or R homozygotes) and to exhibit lower enzyme activity. This study raises the possibility that the R genotype (low sarin-hydrolyzing activity) may represent a risk factor for illness in Gulf War veterans. However, because of the very small size of the study, such findings necessitate further confirmation in a larger population (Furlong, 2000a). La Du *et al.* (2001) have also found reduced sarinase and somanase levels in plasma obtained from Gulf War veterans. In a similar study, in a group of 152 UK Gulf War veterans, the PON1 activity was lower in veterans than in a control group but the differences were independent of PON1 genotype (Mackness *et al.*, 2000). However, in both studies there were no indications about the extent of exposure to nerve agents and possible effects of such exposures.

Standard therapy of OP poisoning consists of the administration of a combination of atropine, oxime, and diazepam with other supportive measures when necessary. However, the possibility of addition of purified enzymes such as AChE, ChE, CarbE, and A-esterases to this therapeutic scheme has been considered and preliminary experiments in animals have shown much better protective effect after addition of exogenous enzymes. In this respect, protective effects of AChE, ChE, and CarbE are based on formation of covalent conjugates or phosphorylated enzymes in the stoichiometric ratio 1:1. Capacity for binding of these enzymes is limited by the number of active sites on the enzyme to which OP molecules can be bound. This means that more enzymes have to be administered in order to achieve better detoxification of OPs which may not always be possible due to adverse effects. This can also be influenced by differences in the extent of spontaneous reactivation of these enzymes inhibited by OP.

It is known that AChE spontaneously reactivates very slowly except when inhibited with dimethyl phosphates, which occurs relatively rapidly at the rate of 25% in 1.3 h (Gallo and Lawryk, 1991) or with a half-time of about 2 h for AChE of rat brain inhibited with dichlorvos *in vivo* (Reiner and Pleština, 1979). CarbE from rat plasma can be spontaneously reactivated at a much faster rate after inhibition with OPs having different structure (Jokanović *et al.*, 1996). However, A-esterases have a very important advantage in eventual therapeutic application in relation to other enzymes in that they can be given in low amounts since their catalytic activity is, in general, proportional to the substrate concentration. Relatively long persistence of exogenous A-esterases in circulation (more than 30 h) (Li *et al.*, 1995) and its considerable activity towards many OP compounds having different chemical structures certainly recommend them for further studies directed to possible addition of these enzymes to the standard therapy of OP poisoning. Possible applications of phosphotriesterases in the prophylaxis and treatment of OP poisoning were recently discussed by Sogorb *et al.* (2004).

Another possible application, probably in the near future, is that A-esterases may find a role in the destruction of large amounts of nerve agents and OP insecticides, and the degradation products formed are generally nontoxic. Eventual mutations on A-esterases can contribute to increased specificity towards substrates of special importance such as warfare nerve agents soman, sarin, and tabun.

B. B-Esterases

B-esterases are the group of enzymes that can be inhibited by OP compounds in the progressive reaction that is time and temperature dependent. This group of enzymes comprises CarbE (EC 3.1.1.1), AChE (EC 3.1.1.7), ChE (EC 3.1.1.8), chymotrypsin, trypsin, and some other enzymes. A common feature of these enzymes is that they have a serine hydroxyl group at the active site that enables them to react with OP in a similar fashion (Figure 69.1 in Chapter 65 of this book). AChE, ChE, and CarbE are members of the α/β hydrolase family and have a high degree of overall homology in their amino acid sequences, but they differ in several critical regions that produce differences in their biochemical properties. The most significant biochemical differences in these esterases are related to the extent of aging of the OP-inhibited esterase, the size of the active site, and the ability of the OP-inhibited enzyme to undergo spontaneous or oxime-induced reactivation (Doctor *et al.*, 2001).

1. Serum Cholinesterase (ChE; E.C. 3.1.1.8)

While the biological role of ChE is presently unclear, it is known that soman, sarin, tabun, and VX bind to ChE, without any apparent toxic effects, decreasing the amount of free agents available for inhibition of AChE in the central nervous system and erythrocytes. Pretreatment with human

plasma cholinesterase has protected laboratory mice (Ashani *et al.*, 1991b) and monkeys (Raveh *et al.*, 1997) from lethal and other acute toxic effects of VX exposure. Thus, variability in plasma cholinesterase activity is a parameter of concern for characterization of population susceptibility to nerve agent exposure. Selective inhibition of ChE had no effect on the acute soman toxicity to mice (Clement, 1984). Since VX reacts with ChE, and very slowly with CarbE due to its positively charged quaternary ammonium group, it appears that ChE may have a significant role in detoxification of VX. Wide variations in ChE activity seen in individuals not exposed to OPC, caused by genetic, physiological, and pathological conditions, as well as interactions with many drugs, may strongly influence the susceptibility of those individuals to OPs (Jokanović *et al.*, 1996). It is possible that individuals with lower ChE activity may be more susceptible to the effects of OPs including nerve agents.

2. Carboxylesterases (CarbEs; EC 3.1.1.1)

The mammalian CarbEs comprise a multigene family whose gene products are located in the endoplasmic reticulum (Hosokawa and Satoh, 2006). CarbEs are the enzymes that hydrolyze esters and thioesters or amide groups of carboxylic acids. They were also mentioned in the literature as aliesterases and esterase D. CarbEs have a very important role in metabolism of lipids, endogenous fatty acid esters, steroids, and a large number of ester-containing drugs and prodrugs such as salicylates, clofibrate, procaine, lorazepam, cilazapril and other angiotensin-converting enzyme inhibitors, narcotics (cocaine, heroin), and capsaicin. CarbEs also participate in detoxification of pesticides (carbofuran, pyrethroids, OPs), acrylates, mycotoxins (T2 toxin), and esters of nicotinic acid (Cashman *et al.*, 1996). Certain isoenzymes of hepatic microsomal CarbE are involved in the metabolic activation of some carcinogens and are associated with hepatocarcinogenesis (Hosokawa and Satoh, 2006). Similar enzymes to CarbEs are arylesterases (EC 3.1.1.2) that hydrolyze aromatic esters of carboxylic acids. However, this classification is not perfect since CarbEs hydrolyze some aromatic esters (i.e. phenyl valerate, phenyl butyrate) and arylesterases hydrolyze certain aliphatic esters. These two enzymes can be clearly differentiated according to their interaction with OPs since CarbEs are inhibited with OPs while arylesterases can hydrolyze some OPs that contain an aromatic group such as paraoxon and chlorpyriphos oxon, and because of this they were often confused with A-esterases.

The CarbE profile in humans is not well known. While CarbEs were considered to be absent from the blood plasma of humans (Li *et al.*, 2005), they are, indeed, present in human erythrocytes and monocytes as well as in human liver, kidney, lung, skin, and nasal tissue (Cashman *et al.*, 1996). Additional literature documents the presence of CarbEs in many human tissues and fluids, including brain, milk, mammary gland, pancreas, small intestine, colon, stomach, placenta, and plasma and serum (Chanda *et al.*, 2002; Kaliste-Korhonen *et al.*, 1996). The lung CarbEs are associated with alveolar macrophages (Munger *et al.*, 1991). Further, CarbEs are present in human tissues, and organs where exposure to nerve agent vapors would likely first occur (nasal tissues and the lung) would be distributed (erythrocytes, monocytes, plasma) and would generate effects (brain, stomach, colon, etc.). CarbE is also present in human serum. Chanda *et al.* (2002) indicate that full characterization of the OP-protective capabilities of CarbEs requires assessment not only of the amount but also of the affinity exhibited by CarbEs for the inhibitor as well as the total CarbE activity unlikely to be inhibited. The detoxification potential of CarbEs is apparently complex and is an area requiring further experimental characterization.

CarbEs are proteins of molecular weight between 47 and 65 kDa which can be found in microsomal fraction of many mammalian tissues (Satoh and Hosokawa, 1998). CarbEs are synthesized in liver and secreted into plasma (via the Golgi apparatus) where they are present in soluble form. Their physicochemical and immunological properties and the sequence of amino acids are very similar, while their specificity towards various substrates is different (Hosokawa *et al.*, 1995; Satoh and Hosokawa, 1998). CarbE belongs to the group of esterases having serine at its active site that hydrolyzes esters of carboxylic acids in a biphasic reaction. In the first phase carboxylic ester acylates the hydroxyl group of serine at the active site, and in the second phase serine is deacylated in the presence of water (Augustinsson, 1958). The active site of CarbE comprises a peptide isoleucine–phenylalanine–glycine–histidine–serine–methionine–glycine–glycine, with serine and histidine directly participating in biochemical reactions. Physiological substrate for CarbE is probably *O*-acetyl sialic acid (Satoh and Hosokawa, 1998). CarbE can be differentiated from other serine esterases, AChE (EC 3.1.1.7) and ChE (EC 3.1.1.8), in that AChE and ChE react with positively charged esters such as acetylcholine and butyrylcholine and can be inhibited with carbamates, while CarbEs do not react with positively charged esters and inhibition with carbamates occurs only at high concentrations. Inhibition of CarbE, except inhibition of neuropathy target esterase associated with organophosphate-induced delayed polyneuropathy, does not cause any known toxic effects. Classification and nomenclature of CarbEs were proposed by Satoh and Hosokawa (1998) and readers are referred to an excellent chapter in Hosokawa and Satoh (2006) for more details.

a. The Relationship Between CarbE Activity and Toxicity of Nerve Agents and Other OPCs

The first finding that made a connection between lower activity of CarbE and increased toxicity of OPs was given by Frawley *et al.* (1957) showing that EPN increases toxicity of malathion by inhibiting CarbEs that hydrolyze malathion. Murphy *et al.* (1959) have further shown that

TOCP, which is a specific inhibitor of CarbE and weak anticholinesterase agent, increases acute toxicity of malathion in rats from 1,100 mg/kg to 10 mg/kg by inhibiting CarbE. Mechanism of action of TOCP was explained by Eto *et al.* (1962) who found that TOCP itself is not a CarbE inhibitor and that under *in vivo* conditions it is converted to its active metabolite CBDP (2-/*o*-cresyl/-4H-1:3:2-benzodioxaphosphorin oxide) which is a potent and irreversible inhibitor of CarbE. Later experiments indicated that TOCP and CBDP strongly potentiate toxicity of other OPCs which do not contain a carbethoxy bond such as paraoxon (Lauwerys and Murphy, 1969), soman, sarin, and tabun (Bošković, 1978; Clement, 1984; Jokanović, 1989), but not of VX agent (Bošković, 1978) probably because VX in physiological conditions is positively charged and a weak inhibitor of CarbE (Maxwell, 1992). Bošković (1978) found that pretreatment of mice with CBDP increased the s.c. toxicity of soman by 19.1-fold, and its i.p. toxicity by 17.8-fold. For other nerve agents he observed an increase of s.c. toxicity of sarin, tabun, and VX by 11-, 5- and 0.24-fold, respectively. Clement (1984) observed that potentiation of soman toxicity in mice after previous administration of TOCP or CBDP was directly related to plasma CarbE and not to activity of CarbE in liver and other tissues. This effect of TOCP and CBDP was explained by phosphorylation of active sites at CarbE that occupies the binding sites for other OPs, increasing its concentration in circulation and therefore its acute toxicity. Binding of soman to CarbE in rodents occurs specifically with the most toxic stereoisomer of the agent (Cashman *et al.*, 1996).

The detoxification potential of endogenous CarbE to protect against the lethal effects of nerve agent exposure was tested by Maxwell (1992) who observed that a wide range in potentiation of toxicity of different OPs *in vivo* cannot be correlated with reactivity of these compounds towards CarbE showing that paraoxon and soman toxicity in rats with inhibited CarbE was potentiated by two- and six-fold, respectively, in spite of their similar inhibitory power for CarbE. It was concluded that detoxification of OP via CarbE is very important for highly toxic OPs such as soman, sarin, tabun, and paraoxon with an LD_{50} of <2 μmol/kg, while it is less important for less toxic OPs such as DFP ($LD_{50} = 9.75$ μmol/kg) and dichlorvos ($LD_{50} = 98.4$ μmol/kg). Having in mind that relatively higher concentrations of OP insecticides have to be achieved in circulation and tissues in order to exert toxicity, dominant factors in the detoxification of less toxic OPs are A-esterases since their catalytic activity is proportional to substrate concentration and their K_m value is in the millimolar range.

Contrary to these findings of decreased CarbE activity increasing the toxicity of many OPs, there are also data showing that increased CarbE activity can decrease toxicity of OPs. Activity of CarbE can be increased by about 80% after repeated administration of phenobarbital to rats and mice by a mechanism of enzyme induction which caused a decrease in soman and tabun toxicity by two-fold, while toxicity of sarin was not affected probably because plasma CarbEs inhibited with sarin spontaneously reactivate very rapidly *in vitro* and *in vivo* with the half-times of 18 and 120 min, respectively (Bošković *et al.*, 1984; Clement, 1984; Jokanović, 1989; Jokanović *et al.*, 1996).

Various OPs inhibit both CarbE and AChE at similar concentrations ranging from 1 to 1,000 nmol/l. CBDP, dichlorvos, DFP, and paraoxon show higher affinity towards CarbE *in vitro* and as a result their acute toxicity is lower in contrast to highly toxic OPs soman and sarin that have 4–6 times higher affinity for AChE. This relationship was confirmed *in vivo* after administration of 0.9 LD_{50} of these compounds (Maxwell, 1992). Rat plasma CarbE appears to be more sensitive to soman and sarin than CarbE in rat liver and brain, and can be completely inhibited at sublethal doses. Significant inhibition of CarbE in liver can be obtained only at multiple lethal doses (Bošković *et al.*, 1984). Even when two-thirds of rat liver was removed by partial hepatectomy 5 LD_{50} of soman was not sufficient for significant inhibition of rat liver CarbE (Jokanović, 1990). Somani *et al.* (1992) found that interspecies variation in response to some nerve agents may be accounted for largely by CarbE binding.

Involvement of CarbE in development of tolerance to paraoxon and DFP was investigated by Dettbarn *et al.* (1999) who found that rat plasma CarbE provides significant protection against paraoxon toxicity because its rapid reactivation can reduce the toxicity of repeated paraoxon applications and contribute to tolerance development. This same mechanism did not apply to DFP toxicity, as inhibition of CarbE of plasma, liver, and lung neither potentiated its toxicity nor prevented tolerance development. These findings confirmed previous observations that CarbE detoxification is of greater importance for highly toxic OPs such as nerve agents and paraoxon than for less toxic ones such as DFP.

In the study of the mechanism of interaction of CarbE with some OPs *in vitro* it was found that this reaction is not irreversible, but reversible due to rapid spontaneous reactivation of inhibited CarbE (Jokanović *et al.*, 1996; Jokanović, 2001). The highest rate of spontaneous reactivation was obtained for plasma CarbE inhibited with sarin and the half-time of reactivation was 18 min. These results were also confirmed in experiments *in vivo* in which rats were treated with 0.5 LD_{50} of soman, sarin, and dichlorvos (Jokanović *et al.*, 1996). Calculated half-times of reactivation for plasma CarbE of the rats treated with 0.5 LD_{50} dichlorvos, sarin, and soman were 1.2, 2.0, and 2.7 h, respectively. Similar results were reported by Gupta *et al.* (1987a) who found 50% of spontaneous reactivation of plasma CarbE 24 h after poisoning of rats with 100 μg/kg soman. Gupta *et al.* (1987b) also reported 94% of reactivation of plasma CarbE in rats treated with 200 μg/kg tabun, but 7 days after poisoning. Spontaneous reactivation of CarbE hydrolyzing phenyl valerate inhibited with paraoxon *in vitro* was observed by Barril *et al.* (1999).

b. The Role of CarbE in Detoxification of OPCs

CarbEs participate in detoxification in three different ways. The first is hydrolysis of ester bonds in OPs that contain them such as malathion (WHO, 1986; Fukuto, 1990). The second is binding of OP to CarbE and other proteins which decreases the concentration of free OPs in circulation that can react with AChE in vital tissues (Clement, 1984; Jokanović, 1989). The third role is related to all OPs that can phosphorylate CarbE by binding to the serine hydroxyl group at its active site (Jokanović *et al.*, 1996; Jokanović, 2001). During spontaneous reactivation this phosphoryl residue is separated from the enzyme, accepting the hydroxyl group from water as its new acyl radical. This newly formed OP (i.e. organophosphoric acid) is a much less potent, if at all, esterase inhibitor which represents nontoxic metabolites of the parent OP. In the case of nerve agents the corresponding metabolites formed EDMPA (for tabun), IMPA (for sarin), and PMPA (for soman) are shown in Figure 52.1. CarbE activity recovered in this reaction can be inhibited again by other OP molecules. The active role of CarbE in this process is in its involvement in metabolic transformation of OP to its nontoxic and biologically inactive metabolites. Because of rapid spontaneous reactivation of CarbE one active site at the enzyme can metabolize several molecules of OP and this reaction does not occur according to stoichiometric ratio 1:1 depending only on the stability of the bond between phosphorus from the OP and oxygen from the serine hydroxyl group. Tissues in which this ''turnover'' is fast, such as plasma, have higher capacity for detoxification of OPs than expected only on the basis of catalytic activity of CarbE. This reaction can be very important under conditions of repeated (subchronic or chronic) exposure to low doses of nerve agents and other OPs that could be detoxified through the reaction with CarbE without any apparent toxic effect.

The role of CarbE as a bioscavenger involved in the detoxification of nerve agents and other OPs was investigated. The ideal OP bioscavenger would have a fast rate of reactivity for a broad range of OP compounds, a slow rate of aging, and the ability to reactivate to increase its stoichiometry as a bioscavenger. Evaluation of CarbE on these criteria suggests that it is an important candidate as an OP bioscavenger (Doctor *et al.*, 2001). One of the most important advantages of CarbE is that OP-inhibited CarbE does not undergo the rapid aging that prevents oxime-induced reactivation of OP-inhibited cholinesterases. This means that OP-inhibited CarbE can be reactivated yielding an active enzyme, involved in further metabolism of OP molecules, and an inactive OP metabolite.

Another advantage of CarbE is the much greater size of its active site compared to AChE (10× difference) and ChE (6× difference) (Saxena *et al.*, 1999). The large active site volume of CarbE minimizes steric hindrance effects at the active site and maximizes the potential for reactivation. In a study investigating the structural specificity of AChE, ChE, and CarbE, Maxwell *et al.* (1998) found that AChE could accommodate OP inhibitors containing only one bulky group (e.g. isopropyl, pinacolyl, phenyl), ChE could accommodate OP inhibitors containing two of the smaller bulky groups (like isopropyl), while the active site of CarbE was sufficiently large to accommodate up to two of the largest bulky groups (e.g. phenyl groups). Therefore, CarbE had the ability to detoxify the broad spectrum of OP inhibitors. The only exception to this observation is due to fewer aromatic residues in the active site of CarbE in comparison to ChE and reduced affinity of CarbE for positively charged OP inhibitors (such as VX). This effect apparently has a small importance for nerve agents (except VX) and pesticides since only a few of them are positively charged (Doctor *et al.*, 2001).

IV. OTHER FACTORS INVOLVED IN METABOLISM AND DETOXIFICATION OF NERVE AGENTS

A. Protein Binding

Proteins are amphoteric structures containing anionic and cationic reactive sites. Proteins can also participate in other interactions with xenobiotics through the formation of hydrogen bonds, polarity, and electrostatic and van der Waals' forces. Many xenobiotics can bind to proteins from blood such as albumin and B-esterases. The high concentration of albumin in plasma (30–60 g/l) may balance the poor reactivity of this protein with OPs. However, the OP scavenging property of albumin *in vivo* has not yet been evaluated. Easy binding to proteins occurs with substances that are ionized at physiological pH and those soluble in lipids such as OP. After binding of OP to proteins such as CarbE, ChE, AChE, and other macromolecules, these agents are metabolized since their acyl radical is released and phosphoryl residue remains bound to proteins. This unspecific binding of OP to blood proteins decreases inhibitor concentration in circulation and tissues thus preserving AChE activity at target sites. Binding of OP to proteins can be limited by steric hindrance and protein conformation factors that do not allow OP molecules to access all binding sites at the protein. The involvement of secondary non-cholinergic targets (neuropathy target esterase, fatty acid amide hydrolase, arylformamidase, acylpeptide hydrolase, and other macromolecules) in OP toxicity was reviewed by Casida and Quistad (2004).

The recent study of Williams *et al.* (2007) found that sarin, soman, cyclosarin, and tabun phosphylate a tyrosine residue on albumin in human blood. The tyrosine adducts with soman and tabun were detected in guinea pigs receiving therapy 7 days following subcutaneous administration of five times the LD_{50} dose of the respective nerve agent. VX also forms a tyrosine adduct in human blood *in vitro* but only at high concentrations.

Li *et al.* (2008) have shown that soman covalently binds to albumin at tyrosine 411. The adduct is stable ($t_{1/2} = 20$ days). However, though the concentration of albumin in plasma is very high, its reactivity with soman is too slow to play a major role in detoxification of the agent. The authors concluded that soman–albumin adducts could be useful for the diagnosis of soman exposure. Tarhoni *et al.* (2008) also found that OP pesticides covalently bind to albumin and that the adduct is stable for more than 7 days.

B. Tissue Depots for Nerve Agents and Other OPs

Deposition of OP in fatty tissue is based on high lipid solubility of these compounds that are stored in its original form or as toxic metabolites. Fatty tissue may have high capacity for deposition of large amounts of OP particularly for phosphorothioates that are more lipophilic than corresponding phosphates. It is not known how significant deposition of nerve agents can be in relation to their toxicity. However, for other OPs it is known that their deposition in fatty tissue decreases concentration of free OP in blood preventing inhibition of AChE. From these depots OP can be mobilized under some physiological and pathological conditions in the form capable of inhibiting AChE in target tissues. Mobilization of these compounds from fatty depots may occur in stress situations for the body such as illness, repeated treatment with some drugs, increased physical activity (important for military personnel), changed dietary regimen, and increased lipid metabolism. This can be very important in patients poisoned with these compounds in which, after completion of the treatment for acute poisoning, release of OP from the depots may occur causing the recurrence of symptoms of OP poisoning. In this respect, Ecobichon *et al.* (1977) described a case of poisoning of a female patient dermally exposed to fenitrothion and its active metabolite fenitrooxon that were partly deposited in fatty tissue. Eight months after completion of the treatment for OP poisoning she tried to lose weight and symptoms of OP poisoning with inhibition of AChE and ChE reappeared because of the release of fenitrothion from fatty tissue where it was deposited for such a long time. Also, Davies *et al.* (1975) have described five patients poisoned with dichlorofenthion in which cholinergic symptoms of poisoning lasted up to 48 days, with the presence of this insecticide in fatty tissue and blood for more than 50 days.

There are some data in the literature indicating possible deposition of nerve agents in other tissues. Van Helden and Wolthuis (1983) and Van Dongen *et al.* (1986) suggested possible deposition of soman and its nontoxic analog called soman simulator (the difference between the two compounds is the methyl group in soman simulator instead of fluoride in soman) in muscles and particularly in the diaphragm. However, they did not further investigate what comprises this depot and its physiological role. In addition, Kadar *et al.* (1985) have observed significant accumulation of ^{3}H-soman in mouse lungs and skin indicating that this agent could be bound to alveolar epithelial cells in the lungs and keratinocytes of skin epidermis. Considerable accumulation of the label occurred in the urine, gall bladder, and intestinal lumen, suggesting that these were the main pathways of excretion.

V. CONCLUDING REMARKS AND FUTURE DIRECTION

After more than three decades of research on metabolism of nerve agents soman, sarin, tabun, and VX, it can be concluded that several enzymes have a significant role in this process. Enzymes capable of hydrolyzing these agents (A-esterase, PON1) were very efficient in breaking down the bond between phosphorus and acyl radical and their activity was proportional to substrate concentration. Esterases such as cholinesterases and carboxylesterases act by binding OP molecules to the hydroxyl group placed in their active site, decreasing free concentration of the agents in blood, thus preventing inhibition of acetylcholinesterase at target sites and subsequently their toxic effects. In addition, binding of OP to carboxylesterases is reversible indicating an active role of the enzyme in metabolic detoxification of nerve agents and other OPs. However, it is necessary to further investigate the role of these enzymes and other macromolecules in the detoxification of OP compounds. Important issues for further research in this field should be related to the assessment of the detoxification potential of carboxylesterases, the significance of A-esterase polymorphism on the capacity of detoxification and their possible application in prophylaxis and treatment of OP poisoning in humans.

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CHAPTER 53

On-Site Detection of Chemical Warfare Agents

YASUO SETO

I. INTRODUCTION

Chemical warfare agents (CWAs) were used in World Wars I and II, during the Cold War, and still continued to be produced and stockpiled (Somani, 1992). In the 1980s, Iraq used sarin (GB) and mustard gas (HD) in the Iran–Iraq conflict (Black *et al.*, 1994). In 1992, a treaty prohibiting the development, production, stockpiling and use of chemical weapons and mandating their destruction was ratified, and in 1997 it came into force (Organization of the Prohibition of Chemical Weapons, 2005). However, the Japanese doomsday cult group, AUM Shinrikyo, used GB in Matsumoto 1994 and in the Tokyo subway in 1995. As a result, many defenseless people were poisoned or killed (Seto *et al.*, 2000). The Tokyo subway sarin gas attack and the US postal anthrax letter attack in 2001 (Inglesby *et al.*, 2002) presented a threat of chemical and biological terrorism all over the world. To realize safe and secure society, the authorities should establish a more strengthened crisis management system at national level for civil defense (Seto, 2006a). In addition, various CWAs have been discovered from former Japanese military force facilities during land excavations (Ohashi, 2004). Injuries were reported due to the direct contact with CWAs leaked from containers in Samukawa (Hanaoka, 2004), and some complaints were received concerning damage to health (neurological disorders) as a result of drinking water contaminated probably with degradation products of arsenic vomiting agents in Kamisu (Ishii *et al.*, 2004).

In crisis management of chemical warfare terrorism cases and chemical weapon disposal, monitoring of CWAs in public places, security checks at territorial borders, airports, large event venues, executive facilities, and demilitarization spots of chemical weapons are performed for protection against terrorism and workers' health. With regard to consequence management, on-site detection is performed by first responders for personal protection; on-site samples are then transported to laboratories for analysis from the perspective of both investigation and emergency lifesaving. In incident management, laboratory analysis is performed to provide evidence for the court in order to prevent future crimes (Figure 53.1). Among these detection schemes, rapid on-site detection is most important for minimization of the disaster, because it takes a long time to carry specimens sampled on-site to a laboratory for analysis (Smith, 2002; Harris, 2002; Fittch *et al.*, 2003). Various kinds of measuring technologies have been used for on-site detection and laboratory identification of CWAs.

II. PROPERTIES OF CHEMICAL WARFARE AGENTS

CWAs are low-molecular weight synthetic compounds, which are fast acting and sometimes lethal, even at low levels (Somani, 1992; Stewart *et al.*, 1992; Marrs *et al.*, 1996), and in physical properties can be classified into gaseous ''blood agents'', gaseous ''choking agents'', volatile ''nerve gases'', volatile ''blister agents'', nonvolatile ''vomit agents'', and nonvolatile ''lacrimators (tear gases)''(Figure 53.2). The physicochemical and toxicological properties of CWAs are widely varied in molecular weight, melting point, boiling point (vapor pressure), vapor density, stability in air and water, lethal concentration, incapacitating concentration, smell, water solubility, effect on skin, and antidote possibility. In particular, except for slow-acting agents, which manifest toxicity after several hours such as phosgene (CG) and mustard gas (HD), CWAs are fast acting. Deadly poisonous nerve gases and toxic blister agents are registered as scheduled compounds in the Chemical Weapon Convention. Although CWAs show toxicity by skin contact and intake, in chemical warfare terrorism cases and chemical weapon disposal, inhalation toxicity against CWA vapor is mainly considered, and analytical technologies are aiming to detect CWAs in vapor phases. To measure the toxicity of a substance, a lethal concentration value (LCt_{50}, $mg \cdot min/m^3$) is used. This value indicates the vapor concentration leading to 50% death in humans with 1 min inhalation.

III. CONCEPT OF ON-SITE DETECTION

In chemical warfare terrorism countermeasures, we must consider the dispersion of various kinds of CWAs including

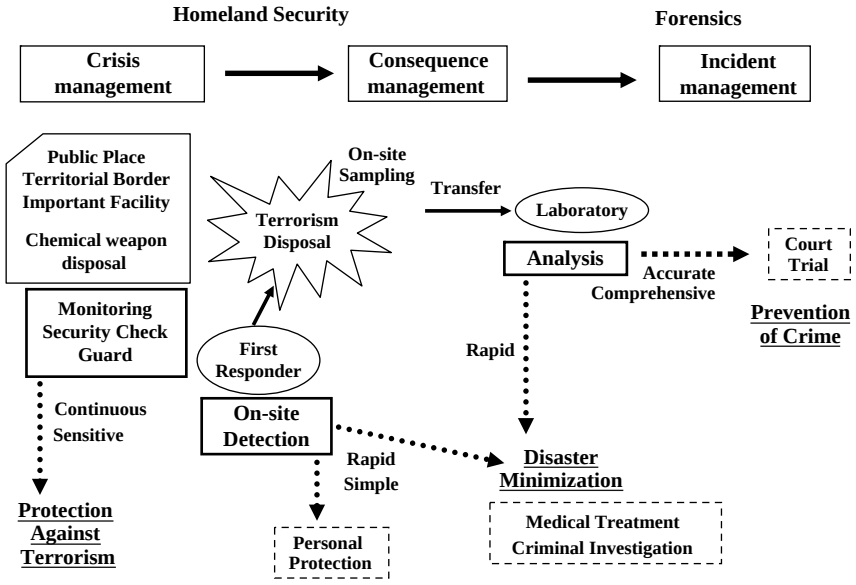

FIGURE 53.1. Detection and identification in chemical terrorism countermeasure and chemical weapon disposal.

toxic industrial chemicals (TIC) except for overt cases such as the previous notice of the criminal acts. If clear symptoms of the CWA-exposed casualties are manifest, it is possible to narrow the kinds of CWAs detected. On the other hand, in chemical weapon disposal, the kinds of the possible dispersed CWAs are apparent in advance. In a situation of exposure to CWA vapor and subsequent intoxication, monitoring technology is very important for exposure minimization and effective medical treatment. However, there is no clear idea of the required performance of the on-site detection technology, and it depends only on previous experience. Namely, the on-site monitoring of CWAs is largely an unknown area compared to the other monitoring situations, and so not only the researchers but also the first responders have little appreciation of how to detect CWAs on-site. The required on-site detection criteria are kinds of CWAs, limit of detection (LOD), response time, time required to start the detector, recovery time after the previous alarm, accuracy (interference, false positivity), state of alarm, operational performance (technology required, training required), maintenance, cost of introduction and maintenance, etc. In addition, machinery operational conditions, durability and portability should be considered.

When considering toxicity manifestation time and vapor dispersion, the detection sensitivity for vapor concentration is one hundredth of LCt_{50} within 1 min. In the case of GB, this required detection sensitivity is 0.15 mg/m^3, and at this level there is no odor and humans show no signs of toxicity. In the chemical weapon disposal situation, because the workers stay in one place for a long time, the time weighted average (TWA) values are the monitoring target for allowed operational conditions. These TWA values are approximately 1/100,000 of LCt_{50}. The desired alarm time is to be less than several minutes. There is a trade-off relationship between LOD, alarm time, detection accuracy and operation, and in detection equipment the mechanism raising the LOD leads to prolonged alarm time and an increased frequency of false positivity.

Before on-site detection technology had become as well developed as it is today, field damage and injury of the victims had been detected using the senses and by transferring on-site samples and victims' samples to specific laboratories and emergency medical hospitals, where CWAs were identified through laboratory analysis and diagnostics. To minimize damage from terrorist activity, it is vitally important to obtain information about the kinds and concentrations of dispersed CWAs using currently available technology. These laboratory analytical technologies have also been brought to the field. Figure 53.3 shows the concept of the determination of CWAs. ''Remote detection (stand-off detection)'' is the method employed to detect CWAs remote from the dispersed site using spectroscopic technology, which is divided into the following two types. ''Passive'' detection senses the (mostly the infrared) light emitted from the target. ''Active'' detection senses the secondary light emitted from the target into which the primary light was irradiated from the equipment remote from the site – usually laser is used as the irradiated light, and the reflected light, fluorescent light and absorbed light (ultraviolet, visible, infrared) are used as the emitted light. The vapor concentration of CWAs used for terrorism is low because of the high toxicity, and as terrorism is predicted to occur at civilian locations where various kinds of interfering compounds exist, it is difficult to fulfill the on-site detection requirement. ''Suction detection'' is the method to detect CWAs by directly measuring CWAs through the suction of vapor in the field, and is divided into the following types. ''Continuous monitoring'' continuously draws on-site air samples with fixed or movable equipment. ''Point detection'' is the method used to detect CWA vapor by first responders moving

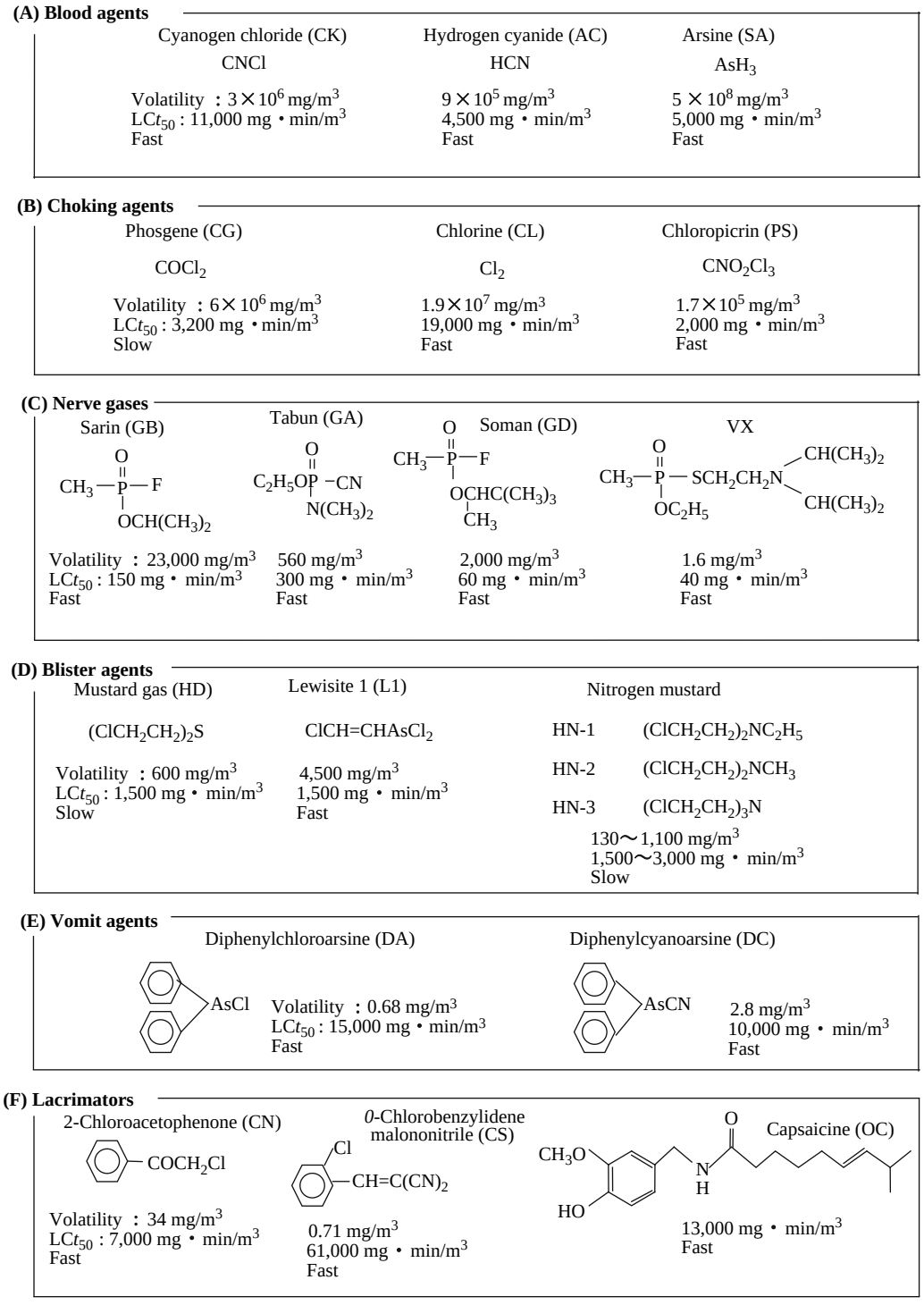

FIGURE 53.2. Chemical structures, volatility (at 25°C) and inhalation toxicity data of chemical warfare agents.

(A) Remote detection
active: LASER (reflection, absorption, excitation fluorescence)
passive: infrared, visible light
(B) Suction detection
continuous monitoring: real-time detection, collection detection, collection off-site analysis
point detection: real-time detection, collection detection, off-site analysis
(C) On-site sampling detection: real-time detection, sampling off-site analysis, sampling on-site analysis

FIGURE 53.3. Concept of on-site detection for chemical warfare agents.

in the field. ''Real-time'' detection detects CWAs by giving an alarm when continuously drawing the air, and ''collection detection'' detects CWAs by giving an alarm after the air sample is collected and detected further with cycling measurement. ''Collection off-site analysis'' detects CWAs in the laboratory from collected samples transferred from the field. As for the solid or liquid samples and the dispersed surface, CWAs are detected by directly contacting the detector, or the sampled or stripped specimens are measured by inserting into the detectors. ''On-site analysis'' uses a large trailer containing chemical laboratory equipped with analytical machines.

It is necessary to predict dangerous terrorist activity and chemical weapon disposal situations by detecting fast-acting CWAs quickly with low concentration levels (Sidell *et al.*, 2003). Any casualties are attended to with proper emergency treatment (securing aeration, antidote administration, etc.). First responders put on protective masks and clothing, and perform the zoning of the dangerous sites. For on-site detection, field deployability, rapid alarm capability, and ease of automated operation are most important – the low frequency of false positivity is a secondary requirement. However, frequent false positive alarms disrupt the responders in the performance of their duties in dangerous situations. Various types of on-site CWA detection devices have been used by military organizations all over the world (Fittch *et al.*, 2003), and some have been introduced for civil defense and counterterrorism such as mobile police teams, fire defense teams, and the coastguard.

CWAs are measurable as the chemicals and the detection technologies adopt different mechanisms depending on the target CWAs (Paddle, 1996). The discrimination level varies from screening to identification methods. At present, detection paper, gas detection tube, ion mobility spectrometer (IMS), flame photometric detector (FPD), photoionization spectrometer (PID), surface acoustic wave detector (SAW), Fourier transform/infrared spectrometer (FT/IR), gas chromatograph (GC), mass spectrometer (MS), and GC–MS are utilized as field equipment. Furthermore, chemical sensors, biosensors, and micrototal analysis systems (μTAS) have been extensively developed. Except for highly discriminating technologies such as chromatographic or spectrometric measurement, many detectors show only evidence of restricted chemical species. The representative species are ''nerve gases (nerve agents)'', ''blister agents'', ''blood agents'' including gaseous choking agents, and ''toxic industrial chemicals (TIC)''. The ''vomit agent'' and ''lacrimators'' are not considered, because of the mechanical impossibility of detecting such nonvolatile agents and their low toxicity. Nerve gas is the chief preferential agent, followed by blister agent and blood agent. So far, CWA detection technology has been developed and utilized for military missions, and so it is not guaranteed that such on-site equipment would work properly for real CWAs in civil defense siuations. Considering the present situation of terrorism countermeasures and on-site needs, on-site detection technology should be developed scientifically. Several research groups are engaged in the evaluation of commercially available detection equipment, and are developing new detection technology. Our laboratory has evaluated some commercially available detection equipment using authentic CWAs. In the following section, the evaluation results obtained in the author's laboratory are stated (Seto, 2006a, b; Seto *et al.*, 2005, 2007).

IV. THE PRESENT SITUATION OF THE DETECTION TECHNOLOGY

As for remote detection technology, passive methods of spectrophotometrically measuring specific infrared light emitted from the CWAs, and the built-in machines, are in part utilized by the military. The machine can detect CWA vapor instantaneously and continuously, but due to background interference practical utilization in the civilian market is far in the future. Active methods of measuring reflected light of the irradiated laser provide high sensitivity compared to the passive method, but the machine is too heavy (over 1 ton). Rapid is a typical machine manufactured by Bruker Daltonics (Germany). By analyzing the obtained spectra, the discriminating capability is significantly raised, and other than ultraviolet, visible and infrared lights, a wide range of other light such as mini-wave and tera-hertz wave light are now available, in addition to laser excitation and Raman scattering.

A. Classical Manual Method

As for suction detection, detection paper and gas detection tube are still used as the classical manual detection tools, which can detect CWAs by the appearance of color changes manifested with the reaction between the reagents and CWAs. ''Detection paper'' shows instant color change for liquid forms of CWAs with a sensitivity of several $\mu g/cm^2$ (Toyobo, Japan). Two kinds of pigments and a pH indicator are impregnated in the cellulose paper, and the paper turns to brown or orange when a droplet of GB, soman (GD), or tabun (GA) (G agent, G stands for Germany) is applied, red when a droplet of HD, lewisite 1 (L1) (H agent) is applied, and black or deep green when VX (V agent) is applied. This coloring reaction is based on the organic solvent solubility, and so almost all organic solvents except for water show false positivity. Dimethylmethylphosphonate, acetone, toluene, and ethyl acetate show G agent false positivity, 2-mercaptoethanol, carbon tetrachloride, and aniline show H agent false positivity, and diethylamine shows V agent false positivity. Due to too many false positivities, detection paper seems to be impractical for civilian defense missions (Seto *et al.*, 2005).

The ''gas detection tube'' shows a color change for vapor CWAs with a sensitivity of mg/m^3 concentration level. The

specific reagents are impregnated into the silica gel support in the glass tube. Both sides of the tube are opened by a cutter, the appropriate volume of the air sample is drawn, and the extent or length of coloring is ascertained using the naked eye. For the detection of blood and choking agents, commercial industrial-use gas detection tubes (Komyo Kika; Gastec, Japan) are available. However, for special agents such as nerve gases, the Dräger Safety gas detection tube (Germany) is adequate (Takayama *et al.*, 2007).

For nerve gases, the ''phosphoric acid ester'' tube shows a red color, which is based on the complicated sequential procedures of butyrylcholinesterase inhibition, substrate butyrylcholine hydrolysis, and pH indication. For HD, the ''thioether'' tube shows a yellow color, which is based on the reaction with silver chloride and chloramines. For L1, the ''arsine and organic arsenicals'' tube shows black; a sequential procedure involving two types of chemical reaction allows for separate detection of both inorganic and organic arsenicals, and based on the reduction by zinc and hydrochloride and a complex formation with gold and mercury, produces a gold colored colloid. For AC, the ''hydrogen cyanide'' tube shows a pink color, which is based on oxidation by mercury chloride and subsequent pH indication. For CK, the ''cyanogen chloride'' tube shows a pink color, which is based on the reaction with pyridine and barbituric acid. The limit of detection (the minimum concentration giving three positive results within three trials), the response time and interference of the stimulants and solvents are shown in Tables 53.1 and 53.2. The operation is a complicated procedure of breaking the inner liquid tube and subsequent incubation in a number of detection tubes. However, the procedure is rather tedious, and several minutes are required from the start of the operation until tube coloration was observed. The gas detection tube is recommended as the supplemental means for ascertainment of CWA species after IMS screening.

TABLE 53.1. Detection performance of Drager gas detection tubes

Agent	Gas detection tube	Limit of detection	Response time
Sarin	Phosphoric acid ester	0.002 mg/m^3	5–6 min
Soman	Phosphoric acid ester	0.02 mg/m^3	5–6 min
Tabun	Phosphoric acid ester	0.5 mg/m^3	5–6 min
VX	Phosphoric acid ester	2 mg/m^3	5–6 min
Mustard gas	Thioether	2 mg/m^3	2 min
Lewisite 1	(In)organic arsine	4 mg/m^3	2 min
Hydrogen cyanide	Hydrogen cyanide	0.3 mg/m^3	1 min
Cyanogen chloride	Cyanogen chloride	0.8 mg/m^3	3 min

TABLE 53.2. False positivity of Drager gas detection tubes

Gas detection tube	Compound
Phosphoric acid	Dichlorvos, methomyl
Thioether	2-Chloroethylethylsulfide, 1,4-tioxane

B. Photometric Method

This type of detection is based on the photometric or electronic response manifested by the physicochemical reaction of CWAs. A flame photometric detector (FPD) detects CWAs by measuring the specific phosphorus or sulfur emission line produced through combustion with hydrogen gas. AP2C, a handy portable automated FPD, was manufactured by Proengin (France). This detector responds rapidly to both vapor and liquid forms of phosphorus containing CWAs in the ''GV'' mode and of sulfur containing CWAs in the ''H'' mode. Approximately 0.1 mg/m^3 and 1 mg/m^3 concentration levels of nerve gases and HD are detected in GV and H modes, respectively (Seto *et al.*, 2005, 2007). The simulants containing phosphorus or sulfur show false positivity, and CWAs which do contain neither phosphors nor sulfur show negative detection. The flame ionization detector (FID) detects nonspecifically combustible CWAs by measuring ionic production manifested by the combustion with hydrogen, and the limit of detection (LOD) is sub-mg/m^3 level of vapor concentration. The photoionization spectrometer (PID) detects all kinds of CWAs nonspecifically, which is based on the measurement of the produced ion current of charged gas ions by irradiation with ultraviolet light. RAE Systems (USA) manufactures ppb RAE, and the LOD is several tens of mg/m^3 level (Seto *et al.*, 2007).

Chemical sensor technology adopting acoustic waves (Harris, 2002) is also available for CWA on-site detection. The CWAs are adsorbed reversibly on the arrayed cells of the specific liquid phase polymer, and the acoustic wave numbers change according to the mass increase due to the CWA adsorption. CWAs can be detected and discriminated by analyzing the respective wave number changes (Harris, 2003; Grate, 2000). By increasing the number of the polymer cells, discriminating power is increased. Portable detector adopted arrayed surface acoustic wave (SAW) devices are commercially available. ChemSentry, manufactured by BAE Systems (USA), adopts ten different polymer cells, and detects CWAs as ''NERVE'' for nerve agents, ''BL'' for blister agents, and ''BLOOD'' for blood and choking agents. As shown in Tables 53.3 and 53.4 (Matsushita *et al.*, 2005), LOD is rather high, the response

TABLE 53.3. Detection performance of arrayed surface acoustic wave detector – ChemSentry

Agent	Alarm	Limit of detection	Response time	Recovery time
Sarin	NERVE	30 mg/m^3	12 s	235 s
Soman	NERVE	50 mg/m^3	12 s	234 s
Tabun	NERVE	100 mg/m^3	13 s	230 s
Mustard gas	BL	38 mg/m^3	8 s	236 s
Lewisite 1	NERVE	280 mg/m^3	109 s	272 s
	BL	57,000 mg/m^3	13 s	394 s
Hydrogen cyanide	BLOOD	28 mg/m^3	100 s	19 s
Cyanogen chloride	BLOOD	940 mg/m^3	103 s	27 s

BL – blister

and recovery time is long, and the false positive frequency is high. This chemo-adsorptive sensor technology provides the possibility of raised discrimination by adopting arrayed cells, but due to the strong adsorption of CWAs disturbs quick response of alarm and recovery.

C. Ion Mobility Spectrometric Method

The ion mobility spectrometer (IMS) is most frequently used for military and civil defense missions to detect not only CWAs but also explosives and illicit drugs (Cottingham 2003; Iceman and Stone, 2004). The drawn air sample is ionized by β emitter or corona discharge under atmospheric pressure, and the ionized water cluster molecules combine with CWA molecules in the reaction region. The produced cluster ions (positive or negative) are periodically traversed through the drift region, and detected on the collector. Ion mobility depends on their charge and molecular weight with a several millisecond cycle (Figure 53.4). CWAs are recognized according to the ion mobility of the peak or peaks, and semi-quantified according to the ion peak height (St Louis and Hill, 1990). In positive ion mode, a protonated water cluster combines with the targets, and in negative mode a water and carbon dioxide cluster attached with oxygen combines with the targets. As the dopant, ammonia or organic solvents are introduced into the drift region, which raises sensitivity and discriminating power. Easily ionizable nerve gases show high sensitivity, and weakly ionizable blister agents do not show high sensitivity. Low molecular weight, blood and choking agents do not produce characteristic cluster ions, and so the detection sensitivity is low. Because of atmospheric pressure ionization, the devices are manufactured in compact form, and the response time is fast. However, the resolution of the produced target ions is low compared to the mass spectrometer, leading to frequent false positivity by many kinds of organic solvents. To raise the sensitivity and accuracy, the ionization mechanism has been improved, by using electrospray, laser desorption, matrix-assisted laser desorption, and the development of new hardware (Cottingham, 2003; Kolakowski and Mester, 2007).

TABLE 53.4. Response of ChemSentry against organic solvents

Alarm	Compound
NERVE	Methanol, ethanol, *n*-propanol, 2-butanol, *t*-butyl alcohol, ethyl acetate, 1,4-dioxane, acetonitrile, acetaldehyde, *N*,*N*-dimethylformamide, pyridine
BL	Dichloromethane, 1,2-dichloroethane, chlorobenzene
BLOOD	Ammonia, acetaldehyde
CONFIDENTIAL	*t*-Butyl alcohol, acetone, diethylether, diethylamine, *N*,*N*-dimethylformamide, pyridine

An aspiration-type device is based on discrimination by the pattern recognition of the several IMS cells of different ion mobility and polarity. Environics OY (Finland) manufactures two types of IMS detectors. The M90 (type D1) is an aspiration-type 80 μCi ^{241}Am-bearing IMS, adopting six IMS cells and one semiconductor cell, and provides vapor detection of GB, GD, and GA (LOD: sub-mg/m^3) as a ''Nerve'' mode alarm with a false-positive alarm against vapors of dimethylmethylphosphonate and trimethylphosphate. HD and L1 vapor (LOD: several mg/m^3) caused a ''Blister'' mode alarm, but 2-mercaptoethanol and 2-chloroethylethylsulfide vapors also caused a false-positive alarm. AC or CK gas (1,000 mg/m^3) showed a false ''blood'' negative alarm (Seto *et al.*, 2004). The same Finnish aspiration-type IMS, ChemPro100 (version 6.2.5), adopts 16 IMS cells and one semiconductor cell, providing more reliable recognition. Tables 53.5 and 53.6 (Maruko *et al.*, 2006) show LOD, response and recovery time for CWAs, and interference. Blood agents were not detected easily, which is the reason for the false negativity and may be the influence of *n*-hexane used as CWA solvent in the detection experiment.

LCD-3.2E, a corona discharge-type IMS, which was developed by Smiths Detection (United Kingdom), is a handy detector showing two kinds of alarm: ''G'' for nerve gases and ''H'' for the other CWAs. Tables 53.7 and 53.8 (Sekioka *et al.*, 2007) show LOD, response and recovery time for CWAs, and interference. This machine adopts an ammonia dopant, raising sensitivity and discriminating power. Maintenance requires the sieve pack to be changed frequently for proper operation. SABRE 4000, manufactured by Smiths Detection, is a high-resolution IMS, providing agent name alarm. Air Sense (Germany) manufactures GDA2, which is a multisensor system consisting of IMS, semiconductor, metal oxide, and PID, enabling identification and quantification for not only

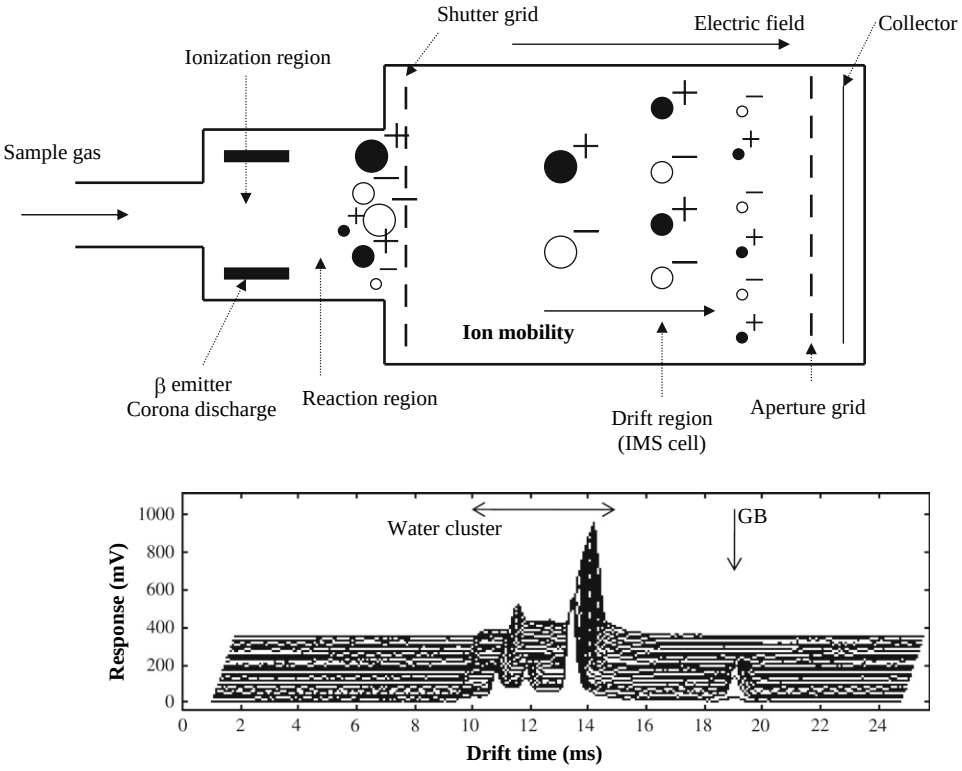

FIGURE 53.4. Ion mobility spectrometry. Detection mechanism and an exemplified spectrum. SABRE4000 (Smiths Detections Ltd, ionization ^{63}N detected GB).

CWAs but also TIC. These two machines need frequent maintenance.

D. Fourier Transform/Infrared Spectrometric Method

Fourier transform/infrared spectrometry (FT/IR) detects and identifies CWAs by measuring the infrared spectrum of the air sample noninvasively and instantly (Mukhopadhyay 2004). Considering the interference of water and carbon dioxide, characteristic absorbance peaks in the low-wave number region are used as the specific marker. Portable FT-IR equipment is commercially available. IGA-1700 (Otsuka Electronics, Japan) and DX-4000 (Temet, Finland) showed HD (1,200 cm^{-1}) and GB (1,000 cm^{-1}) detection with an LOD of 10 mg/m^3 after subtracting background water and carbon dioxide absorption. VIR-9500 (Japan Spectroscopy Co., Japan) showed L1 detection with an LOD (815 cm^{-1}) of 2 mg/m^3 using an 8 m light path gas cell.

E. Gas Chromatography

Gas chromatography (GC) detects CWAs by measuring the peak response appearing on the GC column. CWAs are discriminated with the retention time and the detectability by the specific detection devices (Henry, 1997). Combined with the automated air collection/thermal desorption system, GC provides very sensitive detection (lower than $\mu g/m^3$) for

TABLE 53.5. Detection performance of aspiration-type IMS detector – ChemPro100

Agent	Alarm	Limit of detection	Response time	Recovery time
Sarin	Nerve	0.5 mg/m^3	13 s	17 s
Soman	Nerve	0.3 mg/m^3	15 s	17 s
Tabun	Nerve	0.5 mg/m^3	17 s	18 s
	Blister	10 mg/m^3	18 s	13 s
Lewisite 1	Blister	40 mg/m^3	22 s	23 s
AC	Negative	7,000 mg/m^3		
CK	Unknown	1,000 mg/m^3	38 s	23 s
PS	Unknown	332 mg/m^3	26 s	3 s

TABLE 53.6. Response of ChemPro100 against organic solvents

Alarm	Compound
Nerve	Dimethylmethylphosphonate, trimethylphosphate, triethylphosphate, dimethylformamide
Blister	2-Chloroethylethylsulfide, 1,4-thioxane, 1,4-dithiane, 2-mercaptoethanol, ethanol, benzene, toluene, xylene, chloroform
Blood	None
Unknown	Methanol, acetone, diethylether, acetonitrile, acetic acid, HCl, ammonia, formaldehyde, diethylamine

TABLE 53.7. Detection performance of corona discharge-type IMS detector LCD-3.2E

Agent	Alarm	Limit of detection	Response time	Recovery time
Sarin	G	0.2 mg/m^3	4 s	4 s
Soman	G	0.15 mg/m^3	5 s	4 s
Tabun	G	0.3 mg/m^3	4 s	3 s
Mustard gas	H	12 mg/m^3	2 s	3 s
Lewisite 1	H	6 mg/m^3	4 s	2 s
Hydrogen cyanide	H	15 mg/m^3	4 s	3 s
Cyanogen chloride	H	500 mg/m^3	5 s	4 s
Chlorpycrin	H	13 mg/m^3	6 s	4 s

nerve gases and blister agents every 10 min cycle. The OI Corporation (USA) manufactures Minicams.

F. Mass Spectrometry

By reducing the size of a laboratory-type mass spectrometer (MS), and making it portable and resistant to mechanical shock, the machine can be used to detect CWAs in the field (Wise and Guerin, 1997). MS provides high-resolution power, but to maintain the vacuum, the machine needs to be large, and its operation is complicated. Electron ionization is usually used because of the benefit of the abundant mass spectra data library. On-site MS adopts a hydrophobic membrane inlet system to eliminate oxygen and nitrogen from the ionization region. The adsorbed CWAs are then introduced to the ionization region. The small molecules and strong adsorptive compounds cannot be detected by this ionization-type MS. Field-portable MS, which has been developed for environmental TIC measurement (Horiba, Japan) and adopts time-of-flight mass analyzer and pattern recognition data analysis, detects high vapor levels of GB and HD.

TABLE 53.8. Response of LCD-3.2E against organic solvents

Alarm	Compound
G	Trimethylphosphate, triethylphosphate, *n*-propanol, diethylamine, triethylamine Tobacco smoke
H	2-Chloroethylethylsulfide, 1,4-thioxane, diethylether, acetic acid

The technology of GC–MS can be downsized for field usage and is now commercially available. Inficon (USA) manufactures a field-portable GC–MS, Hapsite. Vapor is withdrawn for 30 s into the Tenax preconcentration system, thermally desorbed into the apolar capillary column with elevated temperature control, and the separated components are finally analyzed by the electron ionization quadrupole mass spectrometer. GB, GD, GA, and HD are detected and identified within 12 min with postulated LOD values of 0.2, 0.5, 8, and 0.3 μg/m^3 (Sekiguchi *et al.*, 2006). Gaseous and weakly volatile (boiling temperature >250°C) CWAs cannot be detected.

G. Other Sensor Technologies

As for sensor technologies, biosensors utilizing the CWA target enzymes have been developed over the years (Guerrieri *et al.*, 2002; Zayats *et al.*, 2003; Walker and Asher,

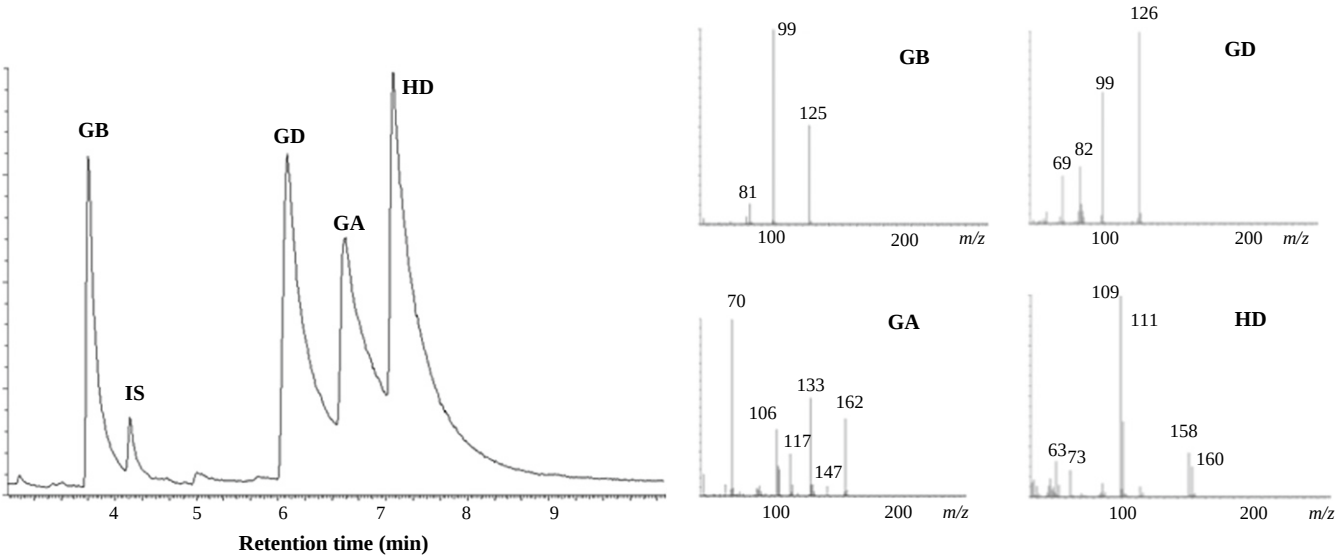

FIGURE 53.5. GC–MS data of portable apparatus, Hapsite. Left: total ion chromatogram; right: electron ionization mass spectra. GB, GD: 1 mg/m^3; GA: 3 mg/m^3; HD: 0.5 mg/m^3. IS: internal standard.

2005). To detect nerve gases, acetylcholinesterase is fixed on the sensor chip, and by the reaction with nerve gases, substrate hydrolysis velocity is lowered due to enzyme inhibition, and the resulting electric response such as hydrogen peroxide formation by choline oxidase is monitored (Palleschi *et al.*, 1992). A fluorescent sensor device utilizing organophosphorus hydrolase (Russell *et al.*, 1999) and a chemical sensor utilizing nerve gas-reactive fluorescent reagent (Zhang and Swager, 2003) have been developed. These types of sensors are still not practical to use. To detect AC, tyrosinase is fixed on glassy carbon, and after concentration of AC on the colloidal mineral the inhibited activity is monitored by electrochemically measuring polyphenol hydrolysis (Shan *et al.*, 2004). Nerve gases and blister agents easily hydrolyze to form the characteristic compounds, and the hydrolyzed CWAs can be analyzed in the wet system utilizing μTAS capillary electrophoresis (Wang, 2004).

V. COMPARISON OF THE EXISTING ON-SITE DETECTION TECHNOLOGIES

First responders require the following criteria: detection sensitivity, detection accuracy, response time, recovery time and operation; Table 53.9 compares these items. The numerical data are derived from our experiment. The evaluation is categorized into the three signs: ''OK'' means acceptable, ''Δ'' means improvement required, and ''X'' means not acceptable. There are no devices that meet all CBWA detection requirements. Low sensitivity, false alarms, and the strong adsorption of CWAs on certain devices are particularly serious problems. The gas detection tube system, permitting the detection of a wide range of CWA vapors, suffers from tedious operation and a slow response. The IMS-based detectors, which permit rapid and sensitive detection of nerve and blistering agents, show low sensitivity for gaseous agents, and a false positive for some compounds. The FPD-based detector, which permits rapid and sensitive detection of nerve gases and HD, shows false-negative detection for nonphosphorus and sulfur CWAs. The PID-based detector shows nonspecific and low sensitive detection. The SAW-based detector does not permit sensitive detection of CWAs. The FT-IR, which permits noninvasive and constant detection, shows inadequate sensitivity. The GC-based detector with concentration system, which permits sensitive detection of nerve gases and blister agents, suffers from tedious operation and a slow response, as does the GC–MS-based detector, which permits sensitive identification of only volatile nerve gases and HD. Nonvolatile vomit agents and lacrimators cannot be detected in any of the above devices.

VI. DEVELOPMENT OF NEW ON-SITE DETECTION TECHNOLOGIES

Figure 53.6 shows a performance map of the physical properties of on-site CBWA detection equipment. CBWAs are represented in terms of volatility and molecular weight, and the target agent territories and drawbacks of the detection equipment are shown. The detection of gaseous agents and nonvolatile CWAs needs to be improved. To enable more sensitive and continuous monitoring of both

TABLE 53.9. Comparison of detection performance of various chemical warfare agent detectors

	Gaseous agent	Nerve gas	Blister agent	Vomit agent Lacrimator	False alarm	Response time	Recovery time	Operation
Detection tube	OK 1 mg/m^3	OK 0.5 mg/m^3	OK 4 mg/m^3	X	Δ	X 1–5 min	–	X
IMS	Δ 10–500 mg/m^3	OK 0.3 mg/m^3	Δ 5–10 mg/m^3	X	Δ	OK 3–20 s	Δ s–min	OK portable
FPD	X ND	OK 0.1 mg/m^3	Δ (non-As) 1 mg/m^3	X	X	OK 2–5 s	OK s	OK portable
PID	Δ 100 mg/m^3	X 100 mg/m^3	Δ 100 mg/m^3	Δ	X	Δ 5–10 s	Δ s	OK portable
SAW	Δ 50 mg/m^3	X 50 mg/m^3	Δ 100 mg/m^3	X	X	Δ 15–13 s	X 4–5 min	OK portable
FT/IR	Δ 50 mg/m^3	X 50 mg/m^3	Δ 50 mg/m^3	Δ	Δ	Δ min	Δ –	Δ movable
GC Tenax	X ND	OK 0.001 mg/m^3	OK 0.001 mg/m^3	X	OK	X 5–10 min	Δ min	Δ fixed
GC–MS Tenax	X ND	Δ (not VX) 0.1 mg/m^3	Δ (HD) 0.1 mg/m^3	X	OK	X 10–15 min	Δ min	Δ movable

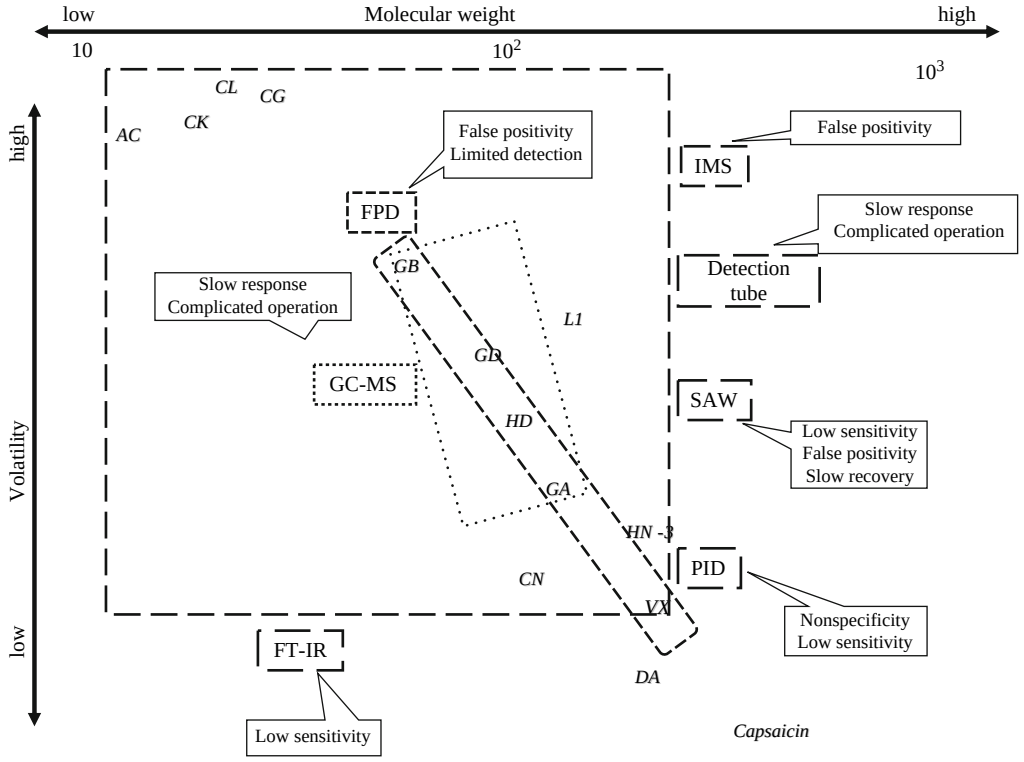

FIGURE 53.6. Performance of on-site chemical warfare agent detection equipment.

volatile and nonvolatile CWAs, our laboratory, coordinated with Hitachi Ltd, has developed on-site detection methods utilizing counterflow introduction atmospheric pressure chemical ionization MS (CFI-APCI-MS, Figure 53.7). CFI-APCI-MS technology provides soft ionization of the suctioned CWAs by corona discharge (APCI), and the introduction of the produced primary target ions into the mass analyzer; the secondary interfering ions are excluded from the ionization region (CFI). CFI-APCI technology is superior in noise ion reduction, realizing ultrasensitive detection (Takada, 2006). Explosive residues and illicit drugs are monitored with this type of machine. Adopting an

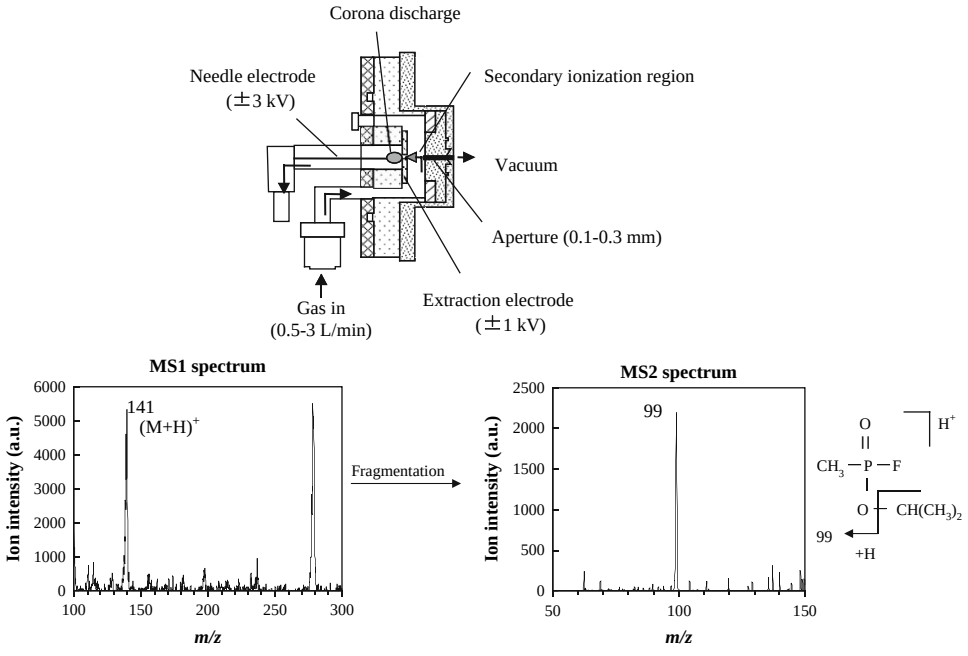

FIGURE 53.7. Counterflow introduction/atmospheric pressure chemical ionization mass spectrometer. Upper: structure of ionization chamber; lower: exemplified mass spectra of GB.

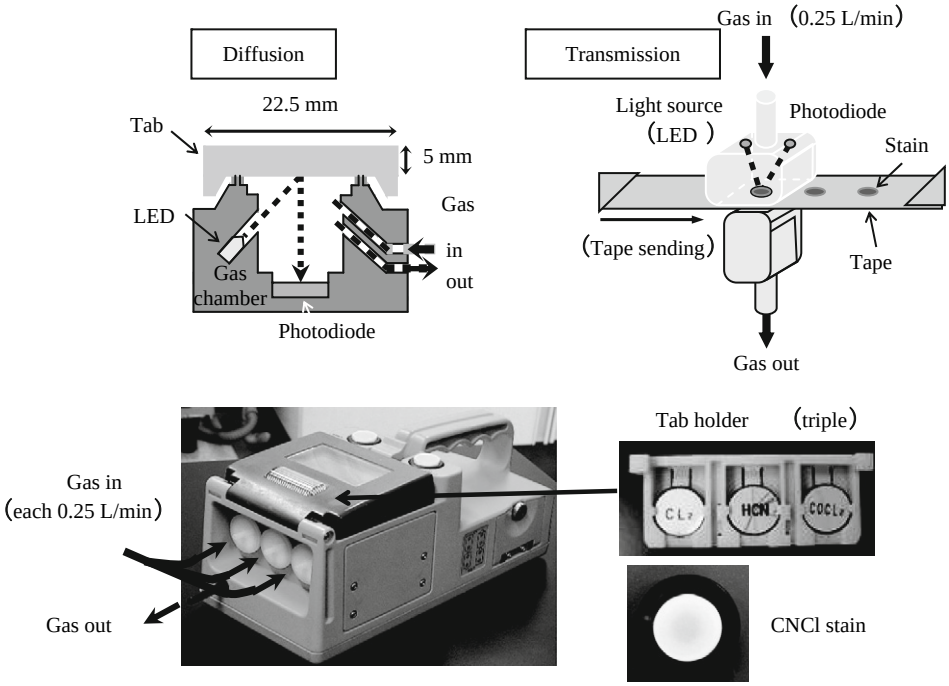

FIGURE 53.8. Monitoring tape method. Schematic detection mechanism of diffusion (upper left) and transmission (upper right) types of apparatus and arrayed diffusion-type detector (lower).

ion trap mass analyzer (MS^n function), all kinds of nerve gases, blistering agents, vomit agents and lacrimators are detected within several seconds with LODs in the sub-$\mu g/m^3$ region (DS-1000, Figure 53.7).

The monitoring tape method (Nakano, 2001) detects hazardous gases by spectrophotometrically measuring the color change on the tape or tab impregnated with specific reagents after the reaction with the suctioned air sample (Figure 53.8). The technology was developed by Riken Keiki Ltd (Japan), and machines are commercially available. Diffusion-type apparatus is used as the portable machine, and by selecting the tab the machine can monitor the desired gases. Transmission-type apparatus is used as the fixed monitor machine, which provides more sensitive detection. Riken Keiki and the author have developed the three tab arrayed detector (FP-100). Three kinds of gaseous CWAs can be detected simultaneously and specifically with LODs lower than 1 mg/m^3 within 1 min. Blood agents (AC, CK, and arsine), choking agents (CL, GC), and L1 can be detected.

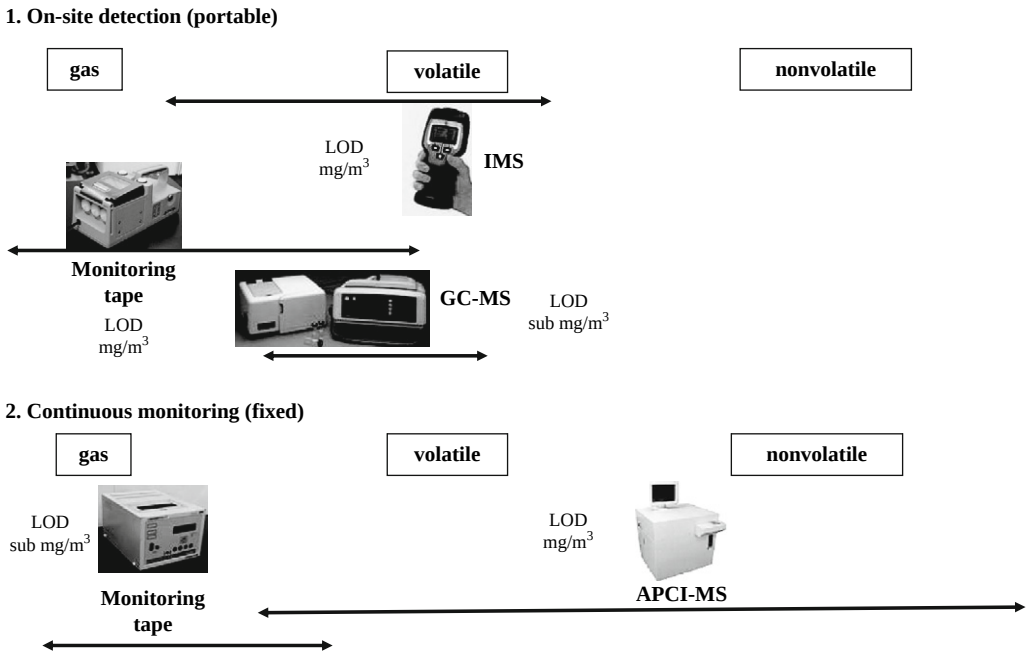

FIGURE 53.9. Recommended combination of on-site detection equipment.

VII. CONCLUDING REMARKS AND FUTURE DIRECTION

When considering on-site CWA detection requirements and the status of the present detection technologies, the recommended on-site detection system is shown in Figure 53.9. For portable-type detectors used by first responders, a combination of IMS and arrayed monitoring tape method machine is desirable. This combination covers gaseous to volatile CWAs. GC–MS helps to identify the detected CWAs. Nonvolatile CWAs are still unable to be detected by portable machines. For fixed-type detectors, highly sensitive detection equipment is necessary, and a combination of CFI-APCI-MS and a lined set of transmission-type monitoring tape method machines is almost perfect, covering all the CWAs with the required sensitivity. It is anticipated that new sensing technology will be developed in the future and will overcome the problems of large size, false alarms, low sensitivity and narrow detection coverage.

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CHAPTER 54

Laboratory Analysis of Chemical Warfare Agents and Metabolites in Biomedical Samples

M. J. VAN DER SCHANS

I. INTRODUCTION

Chemical warfare agents (CWAs) are the most toxic compounds ever produced. In order to develop medical countermeasures against the effects of these agents, analytical procedures to analyze these agents in biological matrices are essential for a better understanding of the toxicological process. The need for the analysis of biomedical samples can have several purposes. First, the agents themselves may be detected in their intact form in the case of toxicokinetic studies. Toxicokinetic studies provide a quantitative basis for the development of new strategies for prophylaxis and therapy against intoxication with chemical warfare agents. Second, verification of exposure to CWAs is another goal that requires analytical methodology for biomedical samples. Verification of exposure is needed for several reasons:

1. In the case of chemical warfare military personnel who are exposed need a fast diagnosis to ascertain the level of exposure and the identity of the agent, in order to give victims adequate treatment.
2. Low-level exposures to nerve agents might be associated with unexplainable phenomena like the Gulf War Syndrome (CIA, 1997).
3. Despite the OPCW treaty, large stockpiles of intact agents still exist, waiting for destruction. Personnel in such destruction facilities might have an elevated risk of exposure; careful biomonitoring could minimize the risks associated with these activities.
4. In the case of a terrorist event, unambiguous verification is needed to verify the exposure.

Methodologies for the verification of exposure to chemical warfare agents have been published recently in special issues of the *Journal of Analytical Toxicology* (Barr, 2004, 2008; Black, 2008). Methodology of the clinical methods has also been reviewed by Noort *et al.* (2002), Black and Noort (2007), and Capacio *et al.* (2008). This chapter is not meant as a duplicate for these references. It will briefly discuss the analytical techniques that are needed for the analysis of biomedical samples. Most of the analytical methods still rely on chromatographic techniques like gas and liquid chromatography. The progress in instrument development on the detection site has been tremendous in the last few decades. Analyses based on mass spectrometry are now more or less routine. Single and triple quadrupole mass spectrometers have become affordable and automated standard configurations with mass spectrometers can now fulfill the analytical need for most type of analyses. However, analyses of chemical warfare agents might need some additional requirements. For example, it might be necessary to measure an intact agent at extremely low concentrations, because only these levels are relevant in view of the high toxicity of the agents. In that case, it might be considered to utilize large volume sample introduction. This puts higher demands on the analytical configurations because the introduction of larger sample volumes also increases the matrix effect, which puts extra demands on the selectivity of the analysis. In the special case of nerve agents, it might be desirable to distinguish the stereoisomers of these compounds from each other, which requires a rather complicated analytical configuration. In this chapter the methods for the bioanalysis of CWAs or their biomarkers will be briefly described and in case the instrumentation for a particular analysis is more sophisticated than a standard configuration, it will be discussed in more detail.

II. NERVE AGENTS

Nerve agents are organophosphorus (OP) compounds that inhibit acetylcholinesterase (AChE) extremely rapidly, which results in an accumulation of acetylcholine (ACh), leads to muscle fasciculations and paralysis, and finally results in death (Dacre, 1984). There are several strategies available to verify an exposure to nerve agents. It is not

feasible to measure the intact reagent, since the half-life of these agents is only a few hours, which means that they disappear within a day after exposure (Benschop and de Jong, 2001; Van der Schans *et al.*, 2008a). Metabolites, often alkylmethylphosphonic acids, are better biomarkers because they circulate for a longer period of time and are gradually excreted in urine (Shih *et al.*, 1994; Fredriksson *et al.* 1995; Nagao *et al.*, 1997; Noort *et al.*, 1998). Several methodologies have been published to analyze these metabolites, which were found in plasma and urine (see Table 54.1 for an overview). Most methods for determination of these compounds are based on liquid chromatography or gas chromatography which requires derivatization (Black and Muir, 2003). Selective MS–MS techniques facilitated by triple quad instruments or ion trap instruments enabled the detection of hydrolysis products down to the pg/ml range (Barr *et al.*, 2004; Riches *et al.*, 2005). This concentration is so low that hydrolysis products can be detected in urine up to 1 week after exposure (Riches *et al.*, 2005).

Nerve agents bind to proteins such as AChE and butyrylcholinesterase (BuChE). These proteins are not excreted or metabolized rapidly (typical half-life is 12 days for BuChE; Hall *et al.*, 1984), which means that adducts to proteins can serve as retrospective biomarkers for exposure to nerve agents (Fidder *et al.*, 2002; Van der Schans *et al.*, 2004a). The enzymatic measurement of AChE activity, known as the Ellman assay, is the easiest method to determine a nerve agent exposure (Ellman *et al.*, 1961; Halbrook *et al.*, 1992). The method is based on the enzymatic cleavage of the substrate acetylthiocholine into thiocholine, which converts 5,5′-dithiobis(2-nitrobenzoic acid) into nitrobenzoate, a yellowish product that can easily be measured with a low-cost colorimeter. Major drawbacks of the method are, first, that the identity of the nerve agents cannot be elucidated from this measurement. Second, the intra- and interpersonal variation of the ChE activity implies that a decrease of ChE activity must be relatively large to be significant (Brock, 1991). Third, the *de novo* synthesis of the enzyme restores the

TABLE 54.1. Major biomarkers for nerve agent exposure

Agent	Matrix	Biomarker	Comment	Analytical technique	Reference
Tabun	Urine	Me_2N-P(O)(OEt)OH	No stable biomarker	GC–MS–MS	Driskell *et al.* (2002)
Tabun	Blood	Adduct to BuChE	Fluoride reactivation	GC–NPD, FPD, MS	Van der Schans *et al.* (2004a)
Tabun	Blood	Adduct to BuChE	Peptic digest of BuChE	LC–MS–MS	Fidder *et al.* (2002)
Sarin	Urine	IMPA	Detected in human exposures	GC–NPD, FPD, MS; LC–MS–MS	Barr (2004); Riches *et al.* (2005); Noort *et al.* (1998)
Sarin	Blood	Adduct to BuChE	Fluoride reactivation	GC–NPD, FPD, MS;	Polhuijs *et al.* (1997); Degenhardt *et al.* (2004)
Sarin	Blood	Adduct to BuChE	Peptic digest of BuChE	LC–MS–MS	Fidder *et al.* (2002)
Soman	Urine	PMPA	Detected in urine of rhesus monkeys	GC–NPD, FPD, MS; LC–MS	Riches *et al.* (2005)
Soman	Blood	Adduct to BuChE	Not found in human exposures because of rapid aging	GC–NPD, FPD, MS	Van der Schans *et al.* (2004a)
Soman	Blood	Adduct to BuChE	Peptic digest of BuChE	LC–MS–MS	Fidder *et al.* (2002)
Cyclohexylsarin	Urine	ChMPA		GC–NPD, FPD, MS; LC–MS	Evans *et al.* (2008)
Cyclohexylsarin	Blood	Adduct to BuChE	Fluoride reactivation	GC–NPD, FPD, MS	Van der Schans *et al.* (2004a)
Cyclohexylsarin	Blood	Adduct to BuChE	Peptic digest of BuChE	LC–MS–MS	Fidder *et al.* (2002)
VX	Urine	EMPA		GC–NPD, FPD, MS; LC–MS–MS	Barr (2004)
VX	Blood	Adduct to BuChE	Fluoride reactivation	GC–NPD, FPD, MS	Polhuijs *et al.* (1997); Degenhardt *et al.* (2004)
VX	Blood	Diisopropylamino-ethylmethylsulfide	Detected in serum after exposure to VX	GC–NPD	Bonierbale *et al.* (1996)
VX	Blood	Adduct to BuChE	Peptic digest of BuChE	LC–MS–MS	Fidder *et al.* (2002)

enzyme activity within several days within the range of the control values. Low-level exposures to nerve agents or exposures that took place several weeks before the biosample could be taken cannot be detected using this method. Instead of looking for a decrease in AChE activity, it is more efficient to detect the ChE fraction that is inhibited by the nerve agent. In that case, it is also better to look at BuChE, which has several advantages over AChE. First, BuChE is a protein present in plasma, which is an easier sample matrix to process than whole blood. Second, the concentration of BuChE is approximately 80 nM, which is approximately ten times higher than the concentration of AChE in blood (Myers, 1952; De Jong and Wolring, 1984). This automatically means that the concentration of that biomarker is higher and therefore easier to detect. The most straightforward method to detect the adduct of nerve agent is the fluoride reactivation method (Polhuijs *et al.*, 1997). During incubation of a plasma sample with fluoride ions, the nerve agent adduct is released from the protein and can be extracted in a GC compatible solvent and subsequently analyzed with GC. The lowest detectable degree of inhibition that can be determined with this method depends on the type of GC detector. Typical reasonably priced detectors like the nitrogen phosphorus detector (NPD), flame photometric detector (FPD), and mass selective detector show absolute detection limits of about 1 pg, which means that a concentration of 1 ng/ml can be detected using an injection volume of 1 µl. A concentration of 1 ng/ml of nerve agent is equivalent to 5–7 nM, which corresponds with approximately 10% BuChE inhibition. In order to be able to determine lower degrees of inhibition, higher sample volumes have to be injected which puts higher demands on the selectivity of the separation and detection method.

A better chromatographic selectivity can be obtained by two-dimensional chromatography based on the heart cutting method, which will be discussed later (see Analysis of nerve agents, below). Another option is to improve the selectivity of the detector. Chemical ionization with ammonia as reaction gas is a relatively soft ionization mode, which ensures a more selective detection of only compounds with sufficient proton affinity (Degenhardt *et al.*, 2004; Jakubowski *et al.*, 2004; Holland *et al.*, 2008; Solano *et al.*, 2008). Another more expensive option is to use an HR–MS instrument, which can provide sensitivity and selectivity for the sensitive detection of low degree inhibition (Degenhardt *et al.* 2004; Solano *et al.*, 2008). When the large volume technique is utilized low degrees of inhibition down to 0.1% can be confirmed.

A major drawback of the fluoride reactivation method is that not all nerve agent adducts are amenable to fluoride reactivation, with the aged adduct of soman the best known example. This problem can be solved by looking at the BuChE enzyme itself. Fidder *et al.* (2002) published a method based on the LC–MS analysis of a nerve agent phosphylated nonapeptide derived after pepsin digestion of inhibited butyrylcholinesterase. The authors presented a procedure to extract BuChE from plasma using home fabricated procainamide gels. The phosphylated nonapeptide is best analyzed with an LC–MS–MS instrument using the single reaction monitoring (SRM) mode. The mass of the parent ion depends on the mass of the nerve agent that is conjugated to the peptide. During the fragmentation process, the phosphyl moiety of the nerve agent is removed first and the characteristic daughter ions are 778, 673, and 602, which are all fragments of the native peptide. This method and the fluoride reactivation method were both successfully applied on plasma samples that were taken from victims who had been exposed to sarin in the Tokyo subway in 1995 (Polhuijs *et al.*, 1997; Fidder *et al.*, 2002). Due to the low concentration of the biomarkers, the analyses are target directed because a full scan would be at the cost of sensitivity, which means that low-level exposures cannot be detected. However, this is a serious problem in the case where the identity of the nerve agent is not known beforehand. Recently, some progress has been made in solving this problem. The number of different OPs exceeds several thousand, but the number of different masses is only 170. Since the OPCW Schedule 1 nerve agents consist only of saturated alkyl groups with mass increments of 14 units the number of mass possibilities can be further reduced to 36 masses, which means that only 36 MRM transitions have to be recorded (Van der Schans *et al.*, 2008a). Another approach was recently published by Noort *et al.* (2006). The phosphylated serine residue of nerve agent inhibited BuChE can be converted under alkaline conditions into a dehydroalanine residue that can subsequently react with a generic tag, yielding a mutual product, whatever the identity of the nerve agent. The resulting product can be detected in the most sensitive single reaction monitoring mode of the mass spectrometer.

A. Analysis of Nerve Agents

1. G-Agents

The requirements for the analytical equipment of the analysis of nerve agents become more sophisticated when the fate of the nerve agents themselves is analyzed. The toxicokinetic studies create insight into the distribution and elimination of the agents and indicate until which time toxicologically relevant concentrations are still present in the circulation (Benschop and De Jong, 2001; Van der Schans *et al.*, 2008b). In view of the high toxicity of nerve agents, these toxicologically relevant concentrations are in the pg/ml range. Using gas chromatography, these levels can only be measured when large volume samples can be introduced on the column. With higher sample volumes, the matrix effect is increased as well and it places higher requirements on the selectivity of the analysis. Another challenging factor is that nerve agents are chiral compounds with an asymmetrical phosphorus atom (Benschop, 1975; Benschop and De Jong, 1988). The difference in toxicity of the two isomers is several orders of magnitude, the P(−)-isomers being the most toxic (Benschop *et al.*, 1984;

Benschop and De Jong, 1988). In the case of toxicokinetic studies, it is important to differentiate between the two isomers because it is essential to know which isomer is still present in the circulation. Chiral gas chromatography can fulfill this requirement for G-agents like sarin and soman. Soman has two asymmetric atoms, phosphorus and carbon in the pinacolyl group. Therefore, the compound consists of four different stereoisomers.

An optical active stationary phase like β-cyclodextrin can be used to separate the isomers of sarin (Spruit *et al.*, 2001). Chirasil-Val columns can be used to separate the four isomers of soman (Benschop *et al.*, 1981, 1985). The Chirasil-Val stationary phases were synthesized at the TNO Prins Maurits Laboratory. Smith and Schlager (1996) reported the use of commercially available gamma-cyclodextrin-based columns that are also capable of separating the four isomers of soman. In the case of toxicokinetic studies the chiral columns were installed in a two-dimensional GC configuration according to the heart cutting method (see Figure 54.2 for an outline). First, the introduction of large volume samples requires additional selectivity from the chromatographic system to alleviate the matrix effect. Second, the fragile optical active phase needs to be protected from the ''dirty'' extracts of the biosamples and the condensating solvent in the column. Samples are introduced by thermodesorption tubes filled with TENAX material. The components are cryo-focused in a cold trap and after a certain desorption time, flash injected into the first column. According to the heart cutting technique one small section of the effluent of the first column is collected in a cold trap and then reinjected on the second analytical column with the chiral selective phase. The isomers of soman could be detected in the blood of guinea pigs at levels down to 10 pg/ml blood (Benschop and De Jong, 1991). Figure 54.1 shows a chromatogram of the analysis of the four isomers of soman together with one isomer of deuterated soman [d_{13}-C(−)P(+)-soman] which was added as an internal standard.

2. VX

Efforts to separate the isomers of VX with gas chromatography have been unsuccessful so far. However, separation was achieved using normal phase liquid chromatography with a Chiracel OD-H column. The separation of the enantiomers of VX was first described by Kientz *et al.* (1994). They used a thermo-ionic detector for the selective detection of phosphorus compounds. Unfortunately, this technique was not robust enough. The same separation was also described by Van der Schans *et al.* (2003) using electrochemical detection. The mobile phase (hexane:ethanol 95:5) was mixed post-column with 0.1 M lithium perchlorate solution to ensure conductivity necessary for electrochemical detection. The system could be used to study the stereoselective degradation of VX in *in vitro* samples such as liver homogenates and plasma. Although the electrochemical detection was rather sensitive (absolute detection limit 25 pg), the method was not selective enough to measure low levels of VX in extracts of biological matrices at a relevant level (<10 ng/ml). Relevant levels of VX in blood could be detected down to 1 ng/ml using a two-dimensional off-line LC–GC separation. The enantiomers of VX were separated on the Chiracel OD-H column and collected in fractions of 100 µl. The mobile phase (hexane:ethanol 95:5) is GC compatible and can be directly injected onto the GC–NPD. Since VX can be sensitively detected using GC–NPD, the amount of VX in each fraction could be quantified and the analyses of ten consecutive fractions from the LC were sufficient to quantify VX and determine the ratio of the enantiomers (Bonierbale *et al.*, 1996; Van der Schans *et al.*, 2003). Smith (2004) published the same separation using a single quadrupole mass spectrometer using atmospheric pressure chemical ionization

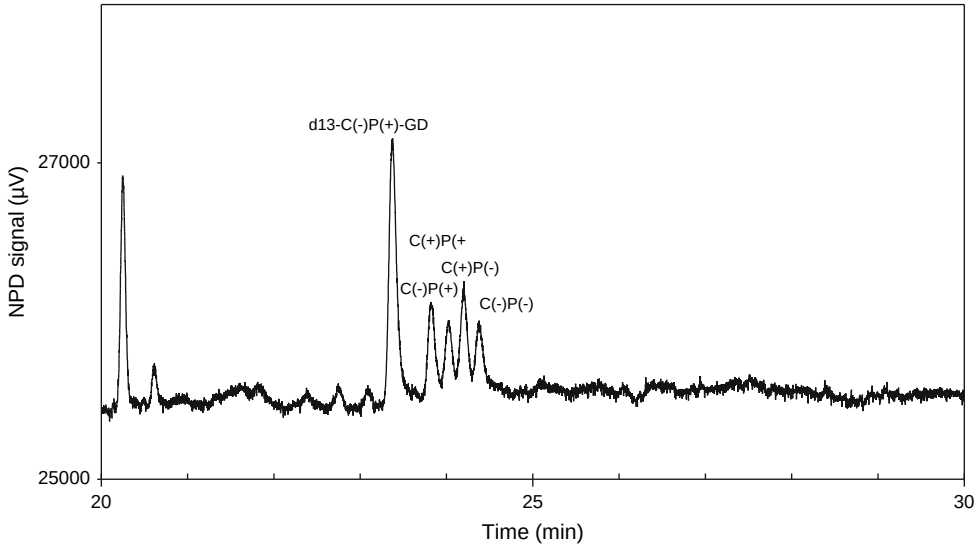

FIGURE 54.1. Large volume (200–400 µl) thermal desorption from TENAX (300 mg, TA 60–80 mesh) followed by 2D-GC and MS detection in EI mode. (1) Precolumn. (2) Analytical column. Small part of the chromatogram of the first column is reinjected on the second analytical column. From: Trap and Van der Schans (2007), Figures 3 and 4.

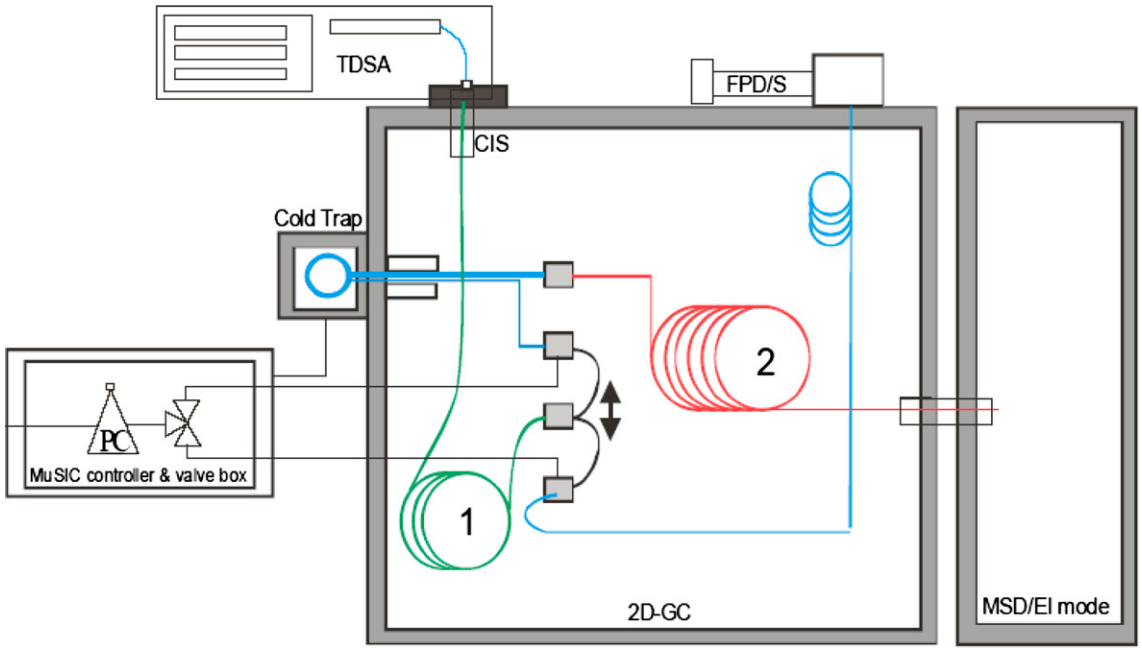

FIGURE 54.2. Typical separation of the stereoisomers of soman and internal standard d_{13}-C(−)P(+)-soman using the 2D-GC configuration. From: Trap and Van der Schans (2007), Figures 3 and 4.

(APCI). In that paper, he focused on the separation and detection of VX to study the stereoselective degradation of VX in plasma. The detection of low levels of VX was not mentioned. Recently, the chiral analysis of VX was performed using the same chromatographic system and connected to a triple quadrupole mass spectrometer with an APCI ionization source. Using this configuration VX isomers could be detected at levels down to 50 pg/ml blood.

TABLE 54.2. Biomarkers for sulfur mustard exposure

Matrix	Biomarker	Comment	Analytical technique	Reference
Urine	Thiodiglycol	Compound also present in nonexposed subjects (<2 ng/ml)	GC–MS	Black and Read (1988); Riches *et al.* (2007)
Urine	Thiodiglycol sulfoxide	Compound also present in nonexposed subjects (<10 ng/ml)	GC–MS	Black and Read (1995); Riches *et al.* (2007)
Urine	1,1′-Sulfonylbis[2-*S*-(*N*-acetylcysteinyl)ethane]	Minor excretion product in humans	GC–MS	Black *et al.* (1991)
Blood	Hemoglobin adduct, N-terminal valine	Detected in human samples	GC–MS	Fidder *et al.* (1996a or b); Noort *et al.* (2004)
Blood	Hemoglobin adduct, histidine N-HETE adduct	Detected in human samples	LC–MS	Black *et al.* (1997)
Blood	Albumin adduct, cysteine adduct	Digestion with pronase	LC–MS/MS	Noort *et al.* (1999, 2004)
Blood	Albumin adduct	Detected as thiodigylcol after alkaline hydrolysis	GC–MS	Capacio *et al.* (2004); Lawrence *et al.* (2008)
Blood/skin	DNA adduct		Immunoslotblot assay, LC–MS	Fidder *et al.* (1996); Benschop *et al.* (1997); Van der Schans *et al.* (2004b)

III. SULFUR MUSTARD AND LEWISITE

There is a variety of biomarkers that can verify an exposure to sulfur mustard. Table 54.2 shows an overview of these biomarkers. Analogous to the nerve agents, biomarkers can be distinguished in metabolites that are excreted in urine and adducts to proteins. A disadvantage of the major metabolite, thiodiglycol, is that this compound is also present in subjects that are not exposed to sulfur mustard (Black and Read, 1995; Boyer *et al.*, 2004). In that respect, the protein adducts are more reliable biomarkers for sulfur mustard exposure because these biomarkers direct unambiguously to an exposure to sulfur mustard. The sulfur mustard adduct to N-terminal valine of globin can be analyzed after a sample preparation procedure known as the modified Edman degradation (Fidder *et al.*, 1996a; Noort *et al.*, 2004a, b). Sulfur mustard binds also to cystein in albumin; this adduct can be analyzed as a tripeptide Cys-Pro-Phe with LC–MS–MS after a digestion with pronase (Noort *et al.*, 1999, 2004a, b). Analogous to the fluoride reactivation method, the sulfur mustard adduct can also be released from the protein by alkaline hydrolysis and measured as thiodiglycol. A precipitation step during the sample preparation removes endogenous thiodiglycol before it is released from the protein (Capacio *et al.*, 2004; Lawrence *et al.*, 2008).

Unlike the nerve agents, the analyses can be target directed and the mass spectrometer can be operated in the most sensitive SIM or SRM mode. Table 54.2 shows that most methods rely on analytical techniques such as gas and liquid chromatography combined with mass spectrometry. Most of the analyses can be performed with standard analytical configurations, without the utilization of large volume sample introduction. The LC–MS technique is already equipped to handle sample volumes up to 100 µl and the quality of the LC separation and tandem MS detection is normally sufficient. The concentrations of the biomarkers are normally so high that they can be detected with normal configurations. Smith *et al.* (2008) showed that after an accidental exposure to sulfur mustard, the albumin adduct could be measured up to several weeks after exposure. Typical detection limits using GC–MS configurations were in the pg range, which means that concentrations down to 1 ng/ml can be detected. This level is also sufficient to detect the levels several weeks after exposure. Noort *et al.* (2008) demonstrated that the sulfur mustard adduct on N-terminal valine in marmosets could be measured up to 4 weeks after exposure using GC–NICI–MS. However, in the case of toxicokinetic studies of sulfur mustard, typical concentrations at 10–100 pg/ml in blood were found and could not be measured with a normal GC–MS configuration. The utilization of large volume sample introduction by thermodesorption and two-dimensional chromatography (analogous to the configuration for chiral nerve agent analysis) enabled the detection of sulfur mustard levels down to 10 pg/ml (Oostdijk *et al.*, 2007).

TABLE 54.3. Biomarkers for lewisite exposure

Matrix	Biomarker	Analytical technique	Reference
Urine	Chlorovinylarsonous acid (CVAA)	GC–MS	Wooten *et al.* (2002)
Blood	CVAA bound to hemoglobin	GC–MS	Fidder *et al.* (2000)

In addition to the methods based on instrumental analysis, sulfur mustard exposure can be detected using immunoassays. Antibodies that can recognize DNA adducts have been raised. In an immunoslotblot assay exposures to sulfur mustard could be verified in DNA that is obtained from blood or skin (Benschop *et al.* 1997; Van der Schans *et al.*, 2004b).

The number of methods to verify an exposure to lewisite is limited. Chlorovinylarsonous acid (CVAA) can be found as the main metabolite in urine, while this compound can also be found as an adduct to hemoglobin (see Table 54.3). The analyte can be analyzed with GC–MS using normal configurations.

IV. CONCLUDING REMARKS AND FUTURE DIRECTION

The development of sophisticated analytical instruments, mainly based on mass spectrometry, enabled several analyses for bioanalysis of chemical warfare agents. Toxicokinetic studies at relevant levels (down to 10 pg/ml blood) can now be performed. Exposures to CWAs can be verified up to several weeks after exposure, because of the persistence of the biomarkers but also thanks to the sensitive and selective instrumentation. It is anticipated that future equipment will be even more sensitive, enabling exposures that occurred weeks after the event to be tracked down.

A better sensitivity is also desirable, in order to meet the criteria that are in place for identification of a compound meeting the forensic standards. Normally, identification by two techniques is needed. If these techniques are not available, special requirements to the MS spectra are demanded. A link with the doping control analysis can be made, which accepts that three ions in an SIM or SRM are needed, provided that the retention time is within a certain time window (Black, 2008). In the case of analyses with tandem MS detection, two transitions are also sufficient for forensic identification. When instrumentation becomes more sensitive it is possible that these requirements can be met. In that view, it will be interesting to watch the developments in miniaturization, such as lab-on-a-chip. Another interesting development is comprehensive GC–GC. Comprehensive GC–GC offers a great selectivity and resolution ideal for

complex samples such as biomedical samples. The combination with time of flight mass spectrometry offers the possibility to analyze in a full scan mode, not target directed, with the availability of a full mass spectrum that will meet the forensic standards. The utility has been demonstrated for the detection of CWAs in complicated matrices such as fuel, but it is expected that this technique will also be soon available for biomedical samples (Reichenbach *et al.*, 2003). Finally, it may be expected that the sample preparation will be more or less automated. Recently, a promising result was published by Carol-Visser (2008), describing the on-line digestion and analysis of sulfur mustard adducts on albumin and the sarin adduct to BuChE.

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CHAPTER 55

Biosensors for the Detection of OP Nerve Agents

JUN-ICHI ANZAI

I. INTRODUCTION

Organophosphorus (OP) pesticides and nerve agents have been detected based on various principles including spectroscopy, chromatography, microgravimetry, and electrical and electrochemical techniques. Among the devices used for detecting OP agents, electrochemical devices such as modified electrodes are relatively inexpensive, small in size, and easy to operate. One of the characteristic features of electrochemical devices is that the function and performance of electrodes can be arbitrarily regulated by modifying the surface with functional molecules, proteins, DNA, etc. Thus, one can develop electrochemical devices that can be used for detecting chemicals in sample solutions and in gas phase. The modified electrodes whose surface is modified with proteins and other biological molecules are often called biosensors. For this reason, modified electrodes or biosensors have been widely used for detecting a variety of analytes including OP agents. So far, the majority of biosensors have been developed not for environmental and toxicological purposes but for biomedical or clinical use because of the large market. A typical example of a biomedical biosensor is a glucose biosensor used for monitoring glucose levels in the blood of diabetics. The present chapter describes recent progress in the development of electrochemical biosensors and related devices for detecting OP nerve agents and pesticides.

II. BIOSENSORS

It is well established that electrochemical techniques are useful for determining ions and molecules in solution and gas phases. Oxidizable and reducible chemical species dissolved in solution can be detected by measuring oxidation or reduction current that is produced upon electrolysis of the species on the surface of electrodes. Usually, the output current of the system depends on the concentration of redox species in the solution. Thus, one can quantitatively determine the concentration of the analyte. Another merit of electrochemical techniques is easy identification of analyte by recording redox potential of the analyte because the redox species can be characterized by its own redox potential. In other words, one has to apply an appropriate electric potential to the electrode to electrochemically oxidize or reduce specific ions or molecules for obtaining output signal from the electrochemical reaction. For example, hydrogen peroxide (H_2O_2) is known to be oxidized according to reaction (1) (Scheme 55.1) on the surface of metal or carbon electrode poised at 0.4–0.6 V, which often depends on the type of electrode material and on the solution conditions, while no oxidation current can be observed at the electrode potential lower than this value because H_2O_2 cannot be oxidized at the lower electrode potential. Thus, one can identify the redox species based on the redox potential applied to the electrode and quantitatively determine the concentration from the magnitude of the output current.

However, this is true only for the sample solutions which contain a single component, H_2O_2 in this case. The electrochemical response is more complicated if sample solutions contain two or more redox species because both species would be oxidized or reduced and thus contribute concurrently to the output current. This is often the case for the electrochemical determination of drugs and metabolites or other biological components in blood, because many kinds of redox-active species are intrinsically contaminated in blood. Ascorbic acid (vitamin C) and uric acid (UA) are redox species found in blood and these compounds often disturb electrochemical measurements of blood because the redox potential of vitamin C and UA overlaps with those of many kinds of drugs and biological components (Anzai *et al.*, 1998; Hoshi *et al.*, 2001). Another drawback of electrochemical measurements is that target analytes are often electrochemically inert and thus no electric signal can be obtained with an electrode. In other words, the redox potential of many target molecules is too high to be oxidized or reduced in the reasonable potential range. Operation of electrochemical systems in high electrode potential is impractical because oxidation of H_2O and reduction of dissolved O_2 often disturb the measurements. For these reasons, analytes and samples to which electrochemical

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^- \quad (1)$$

SCHEME 55.1. Electro-oxidation of H_2O_2

protocol can be successfully applied by using unmodified electrodes are rather limited.

Electrochemical biosensors are devices that are fabricated by combining proteins or other biological molecules and electrodes (Xu *et al.*, 2006; Ramanavicius *et al.*, 2006; Yun *et al.*, 2007). Usually, functional proteins are immobilized on the surface of metal or carbon electrodes to effect selective determination of analytes in sample solution. Figure 55.1 schematically shows the concept and structure of electrochemical biosensors, where the surface of the electrode is modified with biomaterial that can selectively bind target analyte in the sample solution contaminated with many interfering substances. It is also possible to substitute electrodes with other transducers such as thermistors and optical devices to prepare calorimetric and optical sensors. Among the biosensors developed so far, enzyme-based biosensors have been most extensively studied due to their high sensitivity and wide applicability in biomedical and environmental analyses. Enzyme-catalyzed reactions occur on the surface of electrode and the reaction products are oxidized or reduced electrochemically, resulting in generation of electric current as output signal. Thus, chemical signals (type of analyte and its concentration) can selectively be converted into electric signals through the enzyme/electrode interface. For example, the principle of operation of glucose biosensors is based on the glucose oxidase (GOx)-catalyzed oxidation reaction of glucose [reaction (2)] and electrochemical oxidation of reaction product of the enzymatic reaction (H_2O_2) [reaction (3)] (Scheme 55.2). It is clear that 2 moles of electron can be produced from 1 mole of glucose, and the concentration of glucose in the sample solution is related to electric current recorded. It should be noted here that oxidation or reduction potential of glucose is too high to be oxidized or reduced directly on unmodified electrodes. Consequently, electrochemical determination of glucose is not possible without using GOx-modified electrodes (or GOx biosensors). It is clear from reactions (2) and (3) that the role of enzyme in the function of biosensors is to catalytically produce redox-active species which can be electrolyzed by the electrode.

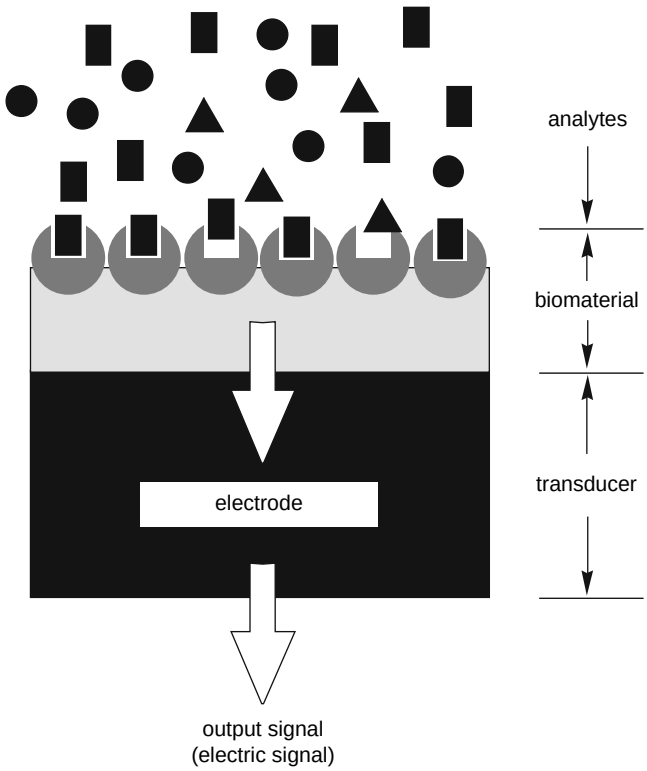


FIGURE 55.1. A schematic illustration of the structure of biosensors. (Reproduced from Anzai, 2006, with permission).

One of the merits of an enzyme biosensor is its versatility. The selectivity of biosensors directly relates to the type of enzyme used for constructing biosensors. Up to now, many kinds of enzyme sensors have been reported, other than glucose biosensors, using a variety of enzymes. A miniaturization of the sensor body is another possible merit of enzyme sensors. In fact, miniature sensors of micron size have been prepared for detecting target analyte in a single cell. The miniaturization of sensor body would make it possible to construct a sensor array on a single tip. However, one should keep in mind that the output current decreases with decreasing the size of electrode, resulting in a suppressed signal-to-noise ratio. For routine purposes, therefore, biosensors of pencil size are usually used for easy handling. A small disk of metal or carbon materials (3–5 mm diameter) is often mounted in an insulating plastic rod (~10 mm diameter and 50–100 mm length) to prepare disk electrodes.

Figure 55.2 illustrates an experimental set-up for biosensor measurements. Electrochemical measurements using biosensors require three electrodes, which are called the working electrode (enzyme-modified electrode), reference electrode, and counter electrode. In some cases, the three electrodes can be assembled into a single body electrode. It is also possible to eliminate the reference electrode and use the counter electrode as a pseudo-reference electrode for specific reasons, such as miniaturization. The experimental set-up shown in Figure 55.2 is a batch system, in which the electrodes are immersed in the sample solution to obtain output signal. For constructing flow systems, the electrodes are set at a suitable place in the fluidic device, which is often used for measuring a large number of samples.

In Section III, recent studies on the determination of OP nerve agents and pesticides based on electrochemical biosensors will be discussed. The electrochemical biosensors

<Enzyme reaction>

$$\text{Glucose} + O_2 \longrightarrow \text{Gluconolactone} + H_2O_2 \quad (2)$$

<Electrochemical reaction>

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^- \quad (3)$$

SCHEME 55.2. Enzyme and electrochemical reactions in glucose biosensor.

used for detecting OP compounds can be divided into three types depending on the type of enzymes used for constructing biosensors: (1) choline esterase (ChE)–choline oxidase (ChOx) bienzyme-modified biosensors, (2) ChE-modified biosensors, and (3) organophosphorus hydrolase (OPH)-modified biosensors. Also, OP biosensors based on transducers other than electrochemical devices are discussed in this chapter.

III. ELECTROCHEMICAL BIOSENSORS FOR DETECTING ORGANOPHOSPHORUS COMPOUNDS

A. ChE–ChOx Bienzyme-Modified OP Biosensors

The primary objective of ChE–ChOx bienzyme biosensors was to determine neurotransmitter acetylcholine in biological samples (Chen *et al.*, 1998). The redox potential of acetylcholine (ACh) is too high to be determined directly using electrochemical reaction. For this purpose, acetylcholine esterase (AChE) was employed as ChE. AChE and ChOx were immobilized on the surface of the electrode to attain electrochemical analysis according to reactions (4) and (5) (Scheme 55.3).

The enzymatic reactions of AChE–ChOx bienzyme biosensor consist of AChE-catalyzed hydrolysis of ACh into choline and acetic acid [reaction (4)] and oxidation reaction

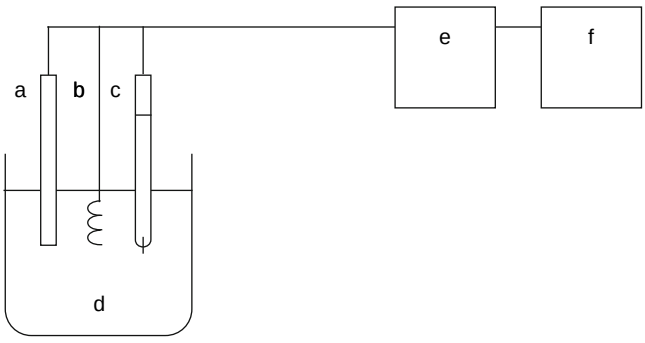
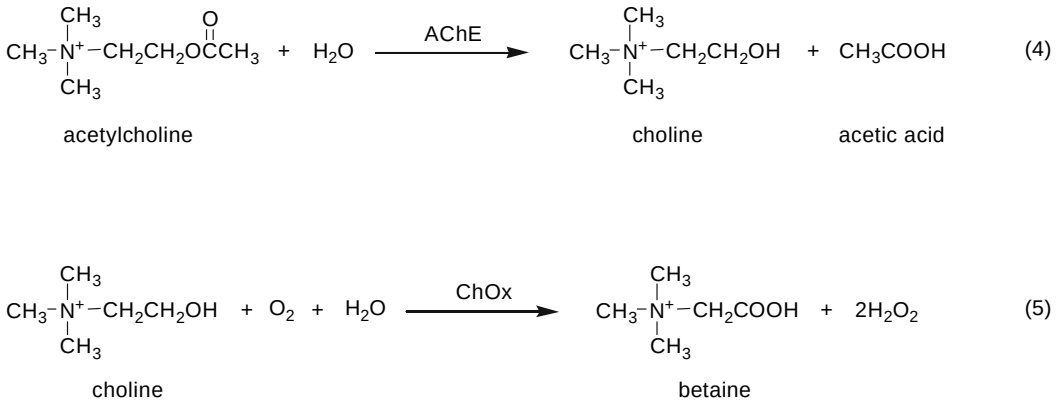


FIGURE 55.2. An experimental set-up for operation of electrochemical biosensors. (a) Biosensor, (b) counter electrode, (c) reference electrode, (d) sample solution, (e) potentiostat, and (f) recorder. (Reproduced from Anzai, 2006, with permission).

of choline catalyzed by ChOx [reaction (5)]. The latter reaction produces H_2O_2, which can be electrochemically oxidized on the electrode to generate output signal depending on the concentration of acetylcholine. It is also possible to use an oxygen electrode in place of a metal or carbon electrode because ChOx consumes dissolved O_2 during the oxidation of choline. Therefore, the O_2 consumption can be detected using a commercially available O_2 electrode. The reason why AChE–ChOx bienzyme biosensors can be used for detecting OP compounds is that the catalytic activity of AChE is inhibited by OP compounds. OP compounds are known to irreversibly bind to AChE to disturb its catalytic activity. It is thus clear that reaction (4) is suppressed in part depending on the concentration of OP and the rate of choline production becomes lower in the presence of OP compounds, resulting in reduced response in the output current of the sensor. In fact, the change in output current of the sensor upon addition of an OP-containing sample is detected in the presence of a known concentration of ACh, as illustrated in Figure 55.3. The ΔI value depends on the concentration of OP compounds in the sample solution. Butylcholine esterase (BChE) is sometimes used in place of AChE, in which butyrylcholine is used as substrate.

According to the above protocol, we have determined trichlorfon (Figure 55.4) in water samples using AChE–ChOx biosensors, which were constructed by a layer-by-layer deposition of the enzymes on the surface of a platinum disk electrode (Shi *et al.*, 2005). Response of the sensor to trichlorfon was evaluated by a successive addition of trichlorfon into the solution of 2 mM ACh. The output current of the sensor decreased with increasing the concentration of trichlorfon over the concentration range of $1 \times 10^{-8} - 2 \times 10^{-5}$ g/ml. The lower detection limit of the sensor was found to be ca. 1×10^{-9} g/ml (1 ppb). A key point for constructing high-performance OP sensors in bienzyme systems may be to suitably control the ratio of the catalytic activity of AChE and ChOx in the sensor. In the present case, the catalytic activity of ChOx should be higher than that of AChE – the overall rate of the reactions is determined by the rate of AChE-catalyzed reaction because OP compounds disturb this step. Therefore, usually, excess amounts of ChOx are mixed with AChE and the ChOx–AChE mixture is immobilized on the surface of the electrode to make the AChE-catalyzed reaction a rate-limiting step. In this situation, the overall reaction rate can be determined by the rate of the ChE-catalyzed reaction because the following



SCHEME 55.3. Enzyme reactions in AChE–ChOx bienzyme sensors.

electrochemical oxidation of H_2O_2 is sufficiently fast. On the other hand, in our sensors, ChE and ChOx were immobilized separately layer by layer for optimization of the amounts of enzymes.

ChE–ChOx bienzyme-modified biosensors have been widely used mainly for detecting OP pesticides, which are summarized in Table 55.1. AChE and BChE are often used as ChE enzyme. This type of ChE–ChOx sensor can be used conveniently for detecting OP compounds, and detection time for qualitative analysis is reasonably fast. For quantitative determination of OP compounds, however, operation of the sensor is somewhat time consuming because multiple steps (a measurement of response to acetylcholine and inhibition) are required.

B. AChE-Modified OP Biosensors

As described in the previous section, use of a bienzyme system sometimes induces complexity in the design of biosensors originating from different catalytic activity of the enzymes. In the ChE–ChOx systems, ChOx is used to oxidize choline that is generated through ChE-catalyzed reaction because the oxidation potential of choline is too high to be electrochemically directly oxidized. It is reasonable to assume that, if the reaction products can be oxidized on the electrode directly, one can remove the second enzyme ChOx from the sensor design, and a ChE-modified electrode may be used for detecting OP compounds. This concept was tested using an AChE-modified electrode as sensor in the presence of acetylthiocholine in place of acetylcholine as the substrate of AChE (Scheme 55.4). It was found that acetylthiocholine is decomposed into thiocholine and acetic acid [reaction (6)], and the resulting thiocholine can be electrochemically oxidized to its dimer (dithiobischoline) on the surface of electrode [reaction (7)]. Therefore, the output current of the sensor in the solution of a constant concentration of acetylthiocholine would be dependent on OP compounds in the solution. Thus, one can determine the OP concentration from the decrease in the output current, as shown in Figure 55.3.

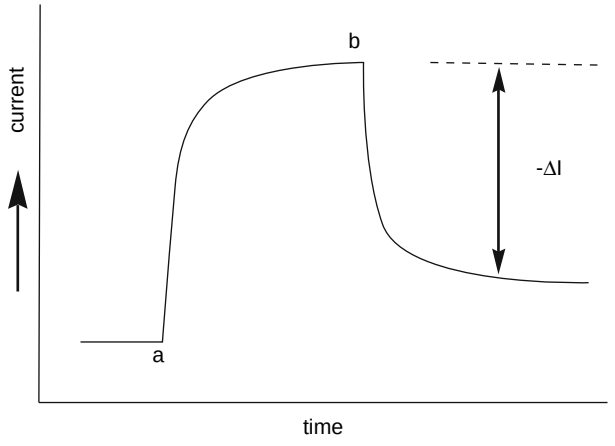


FIGURE 55.3. A typical response of inhibition-mode AChE–ChOx bienzyme OP sensors. (a) Addition of acetylcholine and (b) addition of OP sample. $-\Delta I$ corresponds to the output signal. (Reproduced from Anzai, 2006, with permission).

Table 55.2 collects some properties of AChE-modified biosensors based on acetylthiocholine. As shown in reaction (7), thiocholine can be directly oxidized to dithiocholine. However, oxidation potential of thiocholine is relatively high, ca. +0.7 V, and thus a high background current and a possible interference from oxidizable contaminants cannot be excluded. To circumvent this, electron transfer mediators (Med) are often used to oxidize thiocholine at milder potential as depicted in Figure 55.5, where thiocholine is oxidized by an oxidized form of the mediator, Med (ox), into dithiobischoline and the resulting reduced form of the mediator, Med (red), is oxidized at the electrode to generate output current. In this scheme, 1 mole of thiocholine generates 1 mole of electron. For example, Carlo and co-workers modified the surface of a screen printed carbon electrode with a thin layer of perfluorinated ion-exchanger films containing 7,7,8,8-tetracyanoquinodimethane (TCNQ) (Figure 55.6) as Med. It was found that the reduced form of TCNQ is oxidized at +0.4 V vs pseudo reference electrode (Ag wire) (Carlo *et al.*, 2004). The TCNQ-based biosensors were applied to

TABLE 55.1. AChE–ChOx bienzyme biosensors for detecting OP compounds

Sensor	Organophosphorus compound (lower detection limit)	Reference
Dendrimer-coated	Dichlorvos (5 pM)	Snedarkova *et al.* (2003)
Gold electrode	Carbofuran (50 pM)	
Screen-printed	Methyl parathion (50 nM)	Lin and Wang (2004)
Carbon electrode		
Gold electrode	Trichlorfon (4 pM)	Shi *et al.* (2005)

a recovery test using egg, bovine meat, milk, and honey as food matrices. No false negative or false positive samples were detected in the assay. These results indicate that TCNQ-based biosensors may be useful as a prescreening device in the analysis of food safety.

Another recent topic in detection of OP compounds based on ChE-modified biosensors is to use carbon nanotubes (CNTs) as an electrode modifier. CNTs are known to exhibit excellent electrochemical properties originating from high catalytic activity of edge-plane-like graphite sites at CNT ends and a large effective surface area. Lin *et al.* (2004) have reported that stable CNT-modified glassy carbon (GC) electrodes can be prepared by coating the surface of GC electrode with a suspension of CNT and can be used for sensitive determination of thiocholine (Liu *et al.*, 2005). Voltammetric studies have demonstrated that thiocholine is oxidized at +0.15–0.20 V on CNT-modified GC electrodes as compared to its higher oxidation potential on unmodified GC (+0.7 V), carbon paste (+0.6 V), and gold electrodes (+0.9 V). A lower detection limit of 5×10^{-6} M thiocholine was obtained under the optimal batch conditions, while the detection limit was further improved down to 3×10^{-7} M by the electrochemical detection in flow injection analysis.

The same authors have further improved the CNT-modified biosensors for detection of OP compounds based on a layer-by-layer immobilization of CNTs, polymer, and AChE on the surface of GC electrodes (Liu and Lin, 2006). AChE was sandwiched with poly(diallyldimethylammonium chloride) layers on the surface of CNTs. The OP biosensor thus developed was used to detect paraoxon (Figure 55.7) as low as 4×10^{-13} M with a 6 min response time in flow injection analysis. A high stability of the sensors was also a merit of this biosensor; no deterioration in the response was observed after 1 week of continuous use of the sensor. Only a 15% decrease in the activity of the enzyme was observed after 3 weeks, though the authors

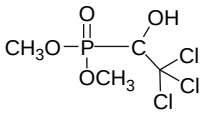
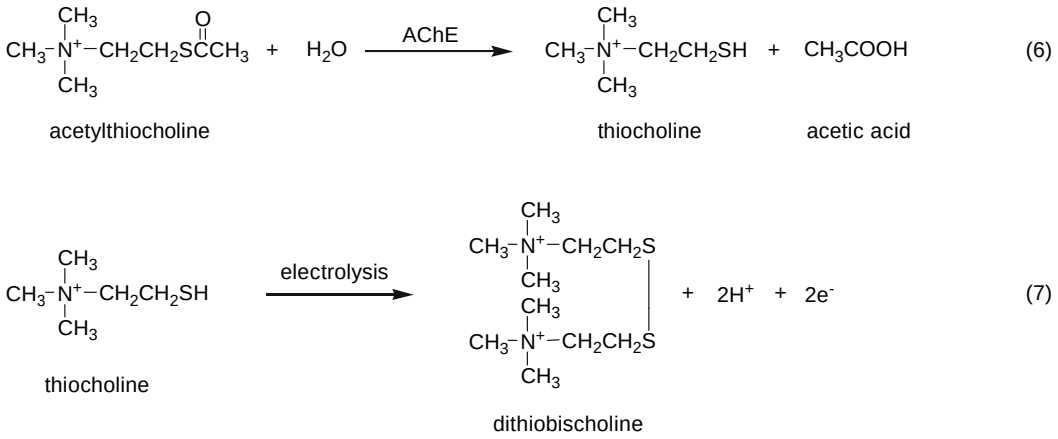


FIGURE 55.4. The chemical structure of trichlorfon.

recommended recalibration after 2 weeks' operation. The high stability of the biosensors was ascribed to the assembling of enzymes in the sandwich-like layer structure, providing a favorable microenvironment to maintain the catalytic activity of the enzyme.

The effects of organic solvents on the performance of ChE-modified sensors were studied because OP compounds are sometimes extracted from samples using organic solvents. For this purpose, 4-aminophenyl acetate (Figure 55.8) was used as the substrate of the AChE enzyme in place of acethylthiocholine because of its high solubility in organic solvents and the redox-active nature of 4-aminophenol produced through enzymatic reaction (Scheme 55.5). Marty and co-workers prepared an AChE-modified carbon electrode by coating AChE-containing polymer film on the electrode (Andreescu *et al.*, 2002). 4-Aminophenol was found to be oxidized on the electrode at +0.1 V, while no redox response was observed in the 0–0.4 V range for 4-aminophenyl acetate. An AChE enzyme modified on the electrode retained ca. 80% catalytic activity even in the aqueous solution containing 1–5% acetonitrile or ethanol, though more than 20% organic solvent induced nearly complete deactivation of the enzyme, suggesting a possible use of the sensor in the presence of a small amount of water-miscible organic solvent in aqueous media. In fact, the AChE-modified sensor could be successfully used for determining 10^{-9}–10^{-8} M level of OP compounds in the aqueous solutions containing 1–5% acetonitrile and ethanol.

Model OP compounds are usually used for studying detection of nerve agents with biosensors because of the difficulty working with nerve agents. Recently, ChE-modified disposable electrodes have been used for detecting sarin and VX (Figure 55.9) in solution and in air stream. Arduini and co-workers have tested ChE inhibition in sarin and VX standard solution and found the lower detection limit of 12 and 14 ppb, respectively (Arduini *et al.*, 2007). For detecting sarin gas in air, the surface of the sensor which had been covered with a small amount of 0.05 M phosphate buffer was exposed to sarin stream for 30 s and then ChE inhibition of the sensor was evaluated in solution. The sensor was found to be effective to detect sarin gas at a concentration of 0.1 mg/m^3. Thus, low concentrations of sarin gas can be detected in a few minutes using ChE-modified electrochemical biosensors.



SCHEME 55.4. Enzyme and electrochemical reactions in acethylthiocholine-based sensors.

C. OPH-Modified OP Biosensors

The detection of OP compounds by biosensors modified with ChE–ChOx or ChE enzymes relies on an inhibition of catalytic activity of ChE enzymes by OPs. Therefore, it is a drawback of the sensors that the magnitude of the output signal correlates inversely with the concentration of OP compounds in the sample. The protocol in measurements is somewhat complicated because the substrate of ChE has to be added in the sample solution before measurements, resulting in multiple steps needed for OP detection. In addition, inhibition of ChE by OP compounds is usually irreversible and thus reactivation of ChE activity is required for repeated use of the sensors, although this problem can be circumvented by using disposable sensor tips.

In contrast to the inhibition mode of detection in the above biosensors, a direct detection of OP compounds has been proposed by means of organophosphorus hydrolase (OPH) enzymes, which hydrolyze OP compounds as shown in Scheme 55.6. OPH enzymes have a rather wide spectrum of substrate selectivity and hydrolyze many kinds of OP compounds including OP pesticides, VX, and sarin. It is obvious from the reactions shown in Scheme 55.6 that the reaction products such as 4-nitrophenol and 2-(diisopropylamino)ethanethiol can be electrochemically oxidized on the electrode to generate electric current as an output signal. Thus, OPH-modified electrodes directly detect OP compounds in a single step, as compared to two steps required for the biosensors constructed using ChE–ChOx and ChE enzymes.

Mulchandani and co-workers (1999) prepared V-type nerve agent biosensors by immobilized OPH on the surface of a CNT-modified carbon electrode (Joshi *et al.*, 2006). They used a mutant OPH that exhibits 20 times higher catalytic activity toward demeton-S (a frequently used mimic of V-type nerve agents) than wild-type OPH. The OP biosensors thus prepared exhibited an electrochemical response to demeton-S at $+0.4$ V, the higher and lower detection limits being 8.5×10^{-5} and 1×10^{-6} M, respectively, with a sensitivity of 8×10^{-6} A/mM. A high response of the biosensors to the hydrolysis product of R-VX [2-(diethylamino)ethanethiol, Figure 55.10] and mimic of the hydrolysis product of VX [2-(dimethylamino)-ethanethiol, Figure 10] was also demonstrated, suggesting

TABLE 55.2. AChE biosensors using acetylthiocholine

Sensor	Organophosphorus compound (lower detection limit)	Reference
Screen-printed	Paraoxon (19 nM)	Andreescu *et al.* (2002)
Carbon electrode	Chlorpyrifos ethyl oxon (1 nM)	
Screen-printed	Carbaryl (55 nM)	Carlo *et al.* (2004)
Carbon electrode	Methy parathion (100 nM)	
Carbon nanotube electrode	Paraoxon (0.4 pM)	Liu and Lin (2006)
Screen-printed	Sarin gas (0.1 mg/m^3)	Aruduini *et al.* (2007)
Carbon electrode[a]	Sarin in solution (12 ppb)	
(Prussian blue)	VX in solution (14 ppb)	

[a]BChE was used in place of AChE

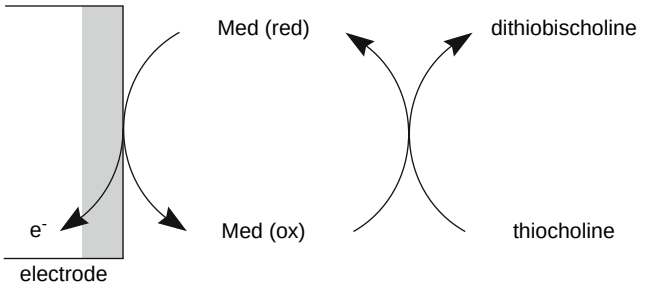


FIGURE 55.5. An electrochemical oxidation of thiocholine through electron transfer mediator. Med (red) and Med (ox) represent electrode transfer mediator in reduced and oxidized forms. (Reproduced from Anzai, 2006, with permission).

possible use for detecting VX and R-VX. A merit of this sensor is its high selectivity to demeton-S over many possible interfering compounds belonging to OP and carbamate families, excluding ''false positives'' in the field use of the biosensors. This biosensor was successfully applied to analyze lake water spiked with demeton-S. The same group developed a flow injection electrochemical detection system using an OPH-modified gold electrode. The response of the system depended linearly on the concentration of OP compounds over the range of 1×10^{-6}–1×10^{-5} M with a lower detection limit of 1×10^{-7} M. The authors suggested a possible use of the flow injection system for rapid screening of OP compounds (Wang *et al.*, 1999).

Wang and co-workers prepared OPH-modified carbon electrodes as a submersible biosensor for remote monitoring of OP compounds (Wang *et al.*, 1999). The biosensor mounted in a plastic tube was connected to a 50 foot long shielding cable via environmentally sealed rubber connectors. It was found that, despite the 50 foot long cable, the noise level of the sensor in the response to paraoxon and methyl parathion is very low. The authors suggested a possible use of the sensor for remote sensing of OP warfare agents in the field. The same group recently reported an interesting electrochemical/optical route for detection of OP nerve agents based on OPH-mediated biometallization (Arribas *et al.*, 2005). They found that the formation of cupric-ferrocyanide (CuFeCN) particles is accelerated by nitrophenol produced through OPH hydrolysis of OP agents. Therefore, the rate of formation of CuFeCN nanoparticles depended on the concentration of OP agents. The detection of CuFeCN particles can be carried out by either electrochemistry or optical measurement. CuFeCN is known to be redox active and CuFeCN

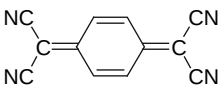


FIGURE 55.6. The chemical structure of TCNQ.

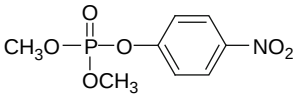


FIGURE 55.7. The chemical structure of paraoxone.

deposited on the surface of a carbon electrode exhibits an oxidation peak at ca. +0.7 V in its voltammogram, peak current being dependent on the concentration. Optical detection of OPs is also possible based on the color changes of the CuFeCN solutions. A light yellow color was observed in the absence of OP, while the solution became darker (orange–red) upon addition of OP due to the formation of a CuFeCN colloid. It is a merit of this system that no seed is required for CuFeCN biometallization in contrast to seed-induced growth of gold nanoparticles (see above).

Potentiometric OP biosensors were also prepared by coupling OPH and a glass pH electrode. The sensors were constructed by immobilizing OPH on the surface of a pH-sensitive layer of a commercially available pH electrode by crosslinking with bovine serum albumin (BSA) to form a thin film. This biosensor detects pH changes (or changes in electrode potential) originating from hydrolysis reaction of OP compounds catalyzed by OPH in the OPH–BSA film (Mulchandani *et al.*, 1999). The best sensitivity of the sensor was obtained in the operation in pH 8.5, 1 mM buffer, in which the sensor could detect as low as 2×10^{-6} M of OPs including paraoxon, ethyl parathion, and methyl parathion. In a similar mechanism of signal transduction, one can use a pH-sensitive field effect transistor (FET) for developing enzyme-modified FETs. Use of a FET provides superiority in miniaturization of the sensor body because a FET is usually produced through mass production on semiconductor materials (Simonian *et al.*, 2004).

An electrochemical OP sensor by the nonenzymatic route was reported based on chemical modification of the surface of a gold electrode with ferrocene derivative (Fc). For this purpose, the gold electrode was modified with dithioFc derivative to form an aminoFc-monolayer-modified electrode (Khan *et al.*, 2007). The principle of operation of the aminoFc-modified electrode for OP sensing is that chloro- or cyano-substitued OP compounds covalently bind to aminoFc moieties, by which the redox potential of the surface-confined Fc can be altered. In fact, ca. 110 and 60 mV shifts in the redox potential were observed, suggesting a possible use of the sensors for detecting OPs from the potential shifts.

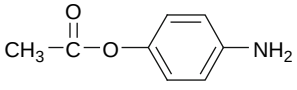
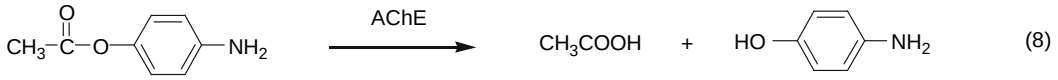


FIGURE 55.8. The chemical structure of 4-aminophenyl acetate.



SCHEME 55.5. Hydrolysis reaction of 4-aminophenyl acetate by AChE.

IV. MISCELLANEOUS BIOSENSORS FOR OP DETECTION

Different types of transducers other than electrodes have also been used for constructing OP biosensors. Recent progress in the study of metal and semiconductor nanoparticles (NPs) stimulated researchers to apply these materials for detecting OP compounds. Willner and co-workers found that thiocholine-stimulated growth of gold NPs is inhibited by OP nerve agents depending on the concentration, enabling the NPs to be used for detecting OP compounds (Figure 55.11; Pavlov *et al.*, 2005). A visible absorption of gold NP solution at ca. 550 nm originating from the plasmon absorption band of NPs increased as a result of NP growth in the presence of AChE and acethylthiocholine because enzymatically produced thiocholine promotes growth of NPs. In the presence of 1×10^{-8}–1×10^{-6} M of OP compounds in the solution, however, the growth rate was significantly suppressed. NP seeds were confined on the surface of a glass plate for constructing an optical sensor system and the absorbance of the glass plate was monitored in the presence of OP agents. The NP-confined glass plate exhibited a maximum absorption at ca. 570 nm and the intensity and wavelength clearly depended on the concentration of OP agent in the sample solution. The optical sensor can detect 10^{-6} M level of OP compounds. Another merit of the NP-confined glass plate is the fact that the glass plate yields a blue-colored surface without OP agents while the OP agents generate a weak pale pink color, enabling OP compounds to be detected with the naked eye.

Polymer microbeads were also used for constructing an OP detection system using fluorescence response of the microbeads. Carboxylate-functionalized polymer microbeads that had been covered with a poly(vinylpyridine) (PVP) layer were modified with fluorescamine (FLA). When the microbeads were exposed to the vapor of diethyl chlorophosphate as a model OP, fluorescence of the microbeads increased rapidly as a result of formation of a phosphoramide of FLA (Figure 55.12; Bencic-Nagale *et al.*, 2006). A PVP layer on the surface of the microbeads served as an acceptor of HCl generated from phosphoramide formation. A potential use of the microbeads for constructing a sensor array was suggested for screening OP vapor.

Microgravimetry has often been used for studying mass changes on the surface using a quartz-crystal microbalance (QCM). A probe of QCM is a thin vibrating quartz resonator sandwiched between metal electrodes. The QCM detects adsorption of substance on the surface of the quartz resonator as changes in resonance frequency of the resonator. Thus, one can detect a nanogram level of mass changes on the surface. It is a great merit of QCM that it can be operated in air for detecting adsorption of gases as well as in solution for detecting dissolved analytes. For this reason, QCM is quite suitable for detecting nerve agents in air. Jiang and coworkers prepared a polymer film-coated quartz resonator for detecting dimethyl methylphosphonate (DMMP) vapor as a stimulant of nerve agents. The poly(vinylidene fluoride) (PVDF) film-coated quartz resonator was found to be sensitive to DMMP vapor in ppm level with a rapid and reversible response (Ying *et al.*, 2007). A zeolite film was also utilized as a coating of QCM for detecting DMMP (Xie *et al.*, 2005).

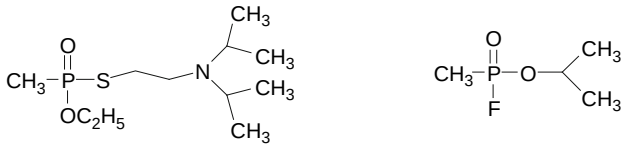
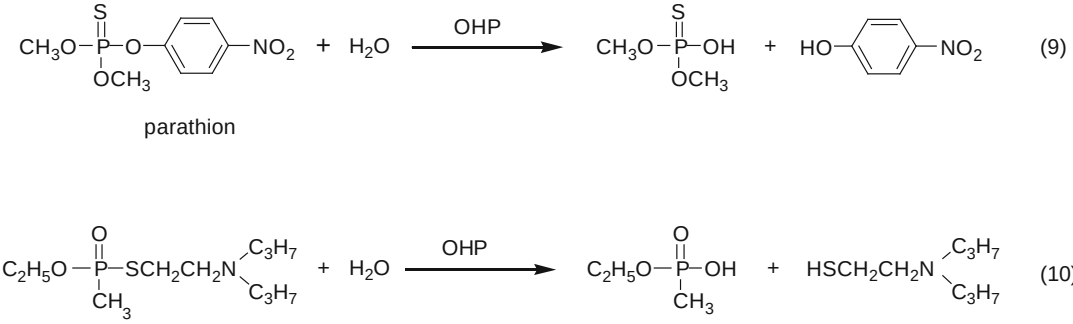


FIGURE 55.9. The chemical structures of VX and sarin.



SCHEME 55.6. OPH-catalyzed hydrolysis of parathion and VX.

FIGURE 55.10. The chemical structures of 2-(diethylamino)ethanethiol and 2-(dimethylamino)ethanethiol.

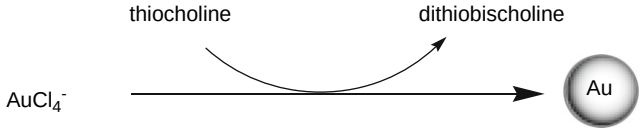


FIGURE 55.11. A thiocholine-induced growth of Au nanoparticles.

The progress in the development of microelectromechanical systems accelerated the use of microdevices for constructing biosensors. Microcantilevers (MCLs) have been chemically modified to use as sensitive probes for detecting OP compounds. It is known that adsorption of molecules on the surface of MCL induces bending of MCL due to changes in surface tension, depending on the amounts of mass adsorbed, in both gas and solution phases. Usually, one side of the MCLs is modified with functional materials to effectively induce bending of MCLs upon binding of analytes. Recently, the surface of V-shaped silicon MCLs, whose dimensions were 180 µm in length, 25 µm in leg width, and 1 µm in thickness, was modified with thin films containing OPH enzyme for preparing OP biosensors (Karnati *et al.*, 2007). The MCL-based sensors exhibited bending response to paraoxon with the dynamic range of 10^{-7}–10^{-3} M. The conformational changes of OPH enzyme associated with the catalytic reaction are suggested to be a main factor inducing the MCL bending. It was also possible to construct MCL-based OP sensors using organic polymers and metal oxides as surface coating in place of OPH enzyme. In these cases, adsorption OP compounds on the surface of MCLs induced bending, depending on the concentration of OPs, resulting in detection of DMMP as a model OP in air (Voiculescu *et al.*, 2005; Zhao *et al.*, 2006).

V. CONCLUDING REMARKS AND FUTURE DIRECTION

This chapter described recent developments of biosensors for detecting OP nerve agents. Different detection modes using different enzymes are applicable for constructing OP biosensors using electrochemical principles. Among them, use of OPH enzyme is superior to other protocols in view of the fact that OPH-based biosensors directly detect OP compounds in a single step as compared to the inhibition-mode biosensors based on ChE–ChOx and ChE enzymes which require a two-step operation. A merit of electrochemical biosensors in OP detection is that a portable device can easily be prepared for in-field analysis. The easy operation of biosensors is another merit as compared to chromatographic or spectroscopic measurements. Therefore, biosensors have high potential for fast on-site screening of OP nerve agents.

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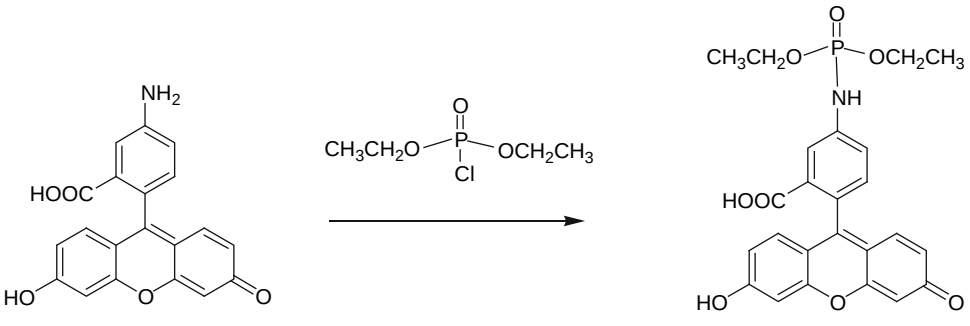

FIGURE 55.12. An enhanced fluorescence upon binding of OP compound to FLA.

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CHAPTER 56

Biomarkers of Exposure to Organophosphorus Poisons: A New Motif for Covalent Binding to Tyrosine in Proteins that have No Active Site Serine

OKSANA LOCKRIDGE, LAWRENCE M. SCHOPFER, AND PATRICK MASSON

I. INTRODUCTION

In 1954, Schaffer *et al.* (1954) reported the astonishing finding that the irreversible inhibition of eel acetylcholinesterase by the organophosphorus agent, diisopropylfluorophosphate, was the result of covalent binding to serine. In 1959, horse butyrylcholinesterase that had been inactivated by treatment with diisopropylfluorophosphate was found to have diisopropylphosphate-labeled serine in the sequence FGESAGAAS (Jansz *et al.*, 1959). Despite this evidence, serine was not immediately accepted as the esteratic site of cholinesterases, because the pKa of serine was so high that significant reaction with organophosphorus (OP) agents was not expected (Bergmann, 1955). The crystal structures of acetylcholinesterase and butyrylcholinesterase confirmed that organophosphorus agents make a covalent bond with serine. These structures also provided an explanation for the special reactivity of the active site serine (Nachon *et al.*, 2005; Sussman *et al.*, 1991). A nearby histidine and glutamic acid form a catalytic triad with serine that lowers the pKa of the serine, consistent with the expectation that the hydroxy-anion of serine is necessary to make a nucleophilic attack on the phosphorus. The oxyanion hole activates the OP for attack by the serine.

The covalent bond between OP and the active site serine of intact cholinesterase is stable, but not irreversible. Hydrolysis can occur with a half-life of between 10 and 35,000 min, depending on the enzyme, OP, temperature, pH, and buffer composition. The adduct becomes irreversibly bound to the enzyme after one of the alkyl groups on the OP is lost in a step called ''aging'' (Benschop and Keijer, 1966; Michel *et al.*, 1967). The dealkylated OP makes a stable salt bridge with the protonated histidine of the catalytic triad, so that histidine is no longer available for the dephosphorylation step that would otherwise have restored the enzyme to an uninhibited state. Hundreds of scientists have contributed to this understanding of the mechanism of OP inhibition of acetylcholinesterase and butyrylcholinesterase activity. Their studies are the foundation for use of acetylcholinesterase and butyrylcholinesterase as biomarkers of OP exposure.

OP-inhibited acetylcholinesterase and butyrylcholinesterase are the established biomarkers of OP exposure. The special features that make them good biomarkers are: (1) they react rapidly with OP at low OP concentrations; (2) symptoms of acute toxicity always correlate with inhibition of acetylcholinesterase and butyrylcholinesterase; (3) acetylcholinesterase is present in human red blood cells, while butyrylcholinesterase is present in human plasma, making it possible to test for OP exposure by measuring enzyme activity in a blood sample; (4) enzyme activity assays for acetylcholinesterase and butyrylcholinesterase are simple and inexpensive; (5) the OP adduct (nonaged and aged) for common pesticides and nerve agents is relatively stable making it possible to detect exposure days after the actual event; and (6) the mechanism of irreversible inhibition of acetylcholinesterase and butyrylcholinesterase activity by OP is understood.

II. USE OF ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE BIOMARKERS IN THE CLINIC

Most hospitals and forensic laboratories are capable of performing cholinesterase activity assays. Two examples of the usefulness of acetylcholinesterase and butyrylcholinesterase activity assays are given below.

A. Tokyo Subway Attack with Sarin

Acetylcholinesterase and butyrylcholinesterase were useful biomarkers for identifying the poison that intoxicated 5,000

people in the Tokyo subway. In March 1995 members of the Aum Shinrikyo sect dispersed the nerve agent sarin in trains on the Tokyo subway. Japanese health workers identified the poison as a cholinesterase inhibitor within 2 h of seeing the first patient (Nozaki *et al.*, 1995). A cholinesterase inhibitor was suspected because victims had physiological signs of cholinergic intoxication including pinpoint pupils. Laboratory assays showed that red cell acetylcholinesterase and plasma butyrylcholinesterase activities were inhibited, thus confirming that the poison was a cholinesterase inhibitor. There are many cholinesterase inhibitors, including OP pesticides and carbamates. In the case of some OPs, AChE inhibiting compounds are precursor molecules that need bioactivation before they are toxic to humans. The rapid onset of toxic symptoms meant that the agent used in the subway was already activated or a direct AChE inhibitor was involved that needed no activation. The fact that people were becoming intoxicated by breathing the air meant the poison was volatile. These characteristics suggested that the poison was a nerve agent, and very likely sarin. The Forensic Science Laboratory used gas chromatography–mass spectrometry to identify sarin in crime scene samples (Seto, 2001). Identification of the poison as sarin was crucial to the police in their search for the perpetrators. Years later, new mass spectrometry methods were developed and used to retroactively identify sarin bound to red blood cell acetylcholinesterase (Nagao *et al.*, 1997) and to plasma butyrylcholinesterase from the Tokyo subway victims (Fidder *et al.*, 2002; Polhuijs *et al.*, 1997).

B. Suicide Attempts

Exposures to OP pesticides are declining in the USA following the banning of chlorpyrifos and diazinon for residential use in 2000 (Sudakin and Power, 2007). In 2004 the number of OP exposure cases reported to the American Association of Poison Control Centers was 7,181. The majority of these incidents resulted in either no symptoms or minor symptoms. The number of OP fatalities in the USA was four in 2006 (Bronstein *et al.*, 2007). Two adult males and two adult females intentionally ingested OP pesticides for the purpose of committing suicide. In contrast, ingestion of OP pesticides in attempted suicide is a frequent occurrence in rural communities of Sri Lanka, India, and China, where an estimated 200,000 persons die annually from OP pesticide poisoning (Eddleston *et al.*, 2008). Diagnosis is made on the basis of cholinergic signs of toxicity, smell of pesticides or solvents, and reduced butyrylcholinesterase and acetylcholinesterase activity in blood. Patients who survive the suicide attempt are monitored in hospital for several days; their plasma butyrylcholinesterase is assayed daily because recovery of butyrylcholinesterase activity is a marker of OP elimination from the body.

III. METHODS TO DETECT OP ADDUCTS ON ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE

A. Cholinesterase Activity Assay

Exposure to a toxic dose of OP results in inhibition of acetylcholinesterase and butyrylcholinesterase activities. The most common method to measure OP exposure is to assay acetylcholinesterase and butyrylcholinesterase activities in blood using a spectrophotometric method (Ellman *et al.*, 1961; Wilson *et al.*, 2005; Worek *et al.*, 1999). The drawbacks of activity assays are that they do not identify the OP. They show that the poison is a cholinesterase inhibitor but do not distinguish between nerve agents, OP pesticides, carbamate pesticides, and tightly bound, noncovalent inhibitors like tacrine and other anti-Alzheimer drugs. In addition, low-dose exposure, which inhibits less than 20% of the cholinesterase, cannot be determined by measuring acetylcholinesterase and butyrylcholinesterase activity because individual variability in activity levels is higher than the percent inhibition.

B. Fluoride Reactivation Followed by Gas Chromatography–Mass Spectrometry

A new method for identifying exposure to nerve agents was introduced by Polhuijs *et al.* in 1997 (Polhuijs *et al.*, 1997). The method is based on the finding that incubation of sarin-inhibited butyrylcholinesterase with 2 M sodium fluoride at pH 4 results in release of sarin. Sarin is then extracted and analyzed by gas chromatography–mass spectrometry. Polhuijs *et al.* applied their new method to positively identify sarin in serum samples from Japanese victims of the Tokyo subway attack. This method has the advantage that it positively identifies nerve agents and other OPs (Van der Schans *et al.*, 2004). The method has been validated by Adams *et al.* (2004) who found that sodium fluoride released sarin and soman from human butyrylcholinesterase as well as from covalent attachment to human albumin.

C. Identification of OP–Butyrylcholinesterase Adducts by Electrospray–Ionization Tandem Mass Spectrometry

In clinical diagnosis of OP exposure, the tissue most readily available for study is blood. OP adducts of butyrylcholinesterase are better candidates for study than OP adducts on acetylcholinesterase for the following reasons. Human blood contains 5 mg of butyrylcholinesterase and 0.5 mg of acetylcholinesterase per liter. The butyrylcholinesterase is in plasma, whereas the acetylcholinesterase is bound to the membranes of red and white cells. Most OPs, with the exception of chemical warfare nerve agents, react more rapidly with butyrylcholinesterase than with acetylcholinesterase.

Fidder *et al.* introduced an electrospray–ionization tandem mass spectrometry method for diagnosing OP exposure by measuring the mass of the OP-labeled active site peptide of human butyrylcholinesterase (Fidder *et al.*, 2002). His starting material was 0.5 ml of human plasma from a victim of the Tokyo subway attack. The mass of the active site peptide was higher by 120 atomic mass units, compared to the mass of the unlabeled active site peptide. This added mass was exactly the added mass expected from sarin. The peptide's MS–MS fragmentation spectrum yielded the sequence of the peptide, and verified that the OP label was on serine 198, the active site serine. Examples of the MS–MS spectra from tryptic peptides of pure, OP-labeled human butyrylcholinesterase are shown in Figure 56.1.

Fidder *et al.* (2002) showed that this method could also detect human butyrylcholinesterase adducts of soman, dimethyl paraoxon, diethyl paraoxon, and pyridostigmine, when pure butyrylcholinesterase was treated with these poisons. Studies from other laboratories confirmed that OP–butyrylcholinesterase adducts from pure butyrylcholinesterase could be identified by this type of mass spectrometry (Li *et al.*, 2008b; Sun and Lynn, 2007; Tsuge and Seto, 2006). A technical difficulty with the method, in clinical settings, is the need to enrich plasma samples for butyrylcholinesterase to make it possible to detect the labeled peptide in the mass spectrometer. At least two purification steps must be employed, either affinity chromatography and HPLC or affinity chromatography and gel electrophoresis. While identification of OP–butyrylcholinesterase adducts is easy when the starting material is pure butyrylcholinesterase, it becomes a challenge when the starting material is plasma.

D. Antibody Detection

Sensors that use an antibody to detect OP–butyrylcholinesterase or OP–acetylcholinesterase adducts are being developed but are not yet commercially available.

IV. WHY ARE NEW BIOMARKERS NEEDED?

A. Not all OPs Inhibit Acetylcholinesterase and Butyrylcholinesterase

Tri-*ortho*-cresyl phosphate, the contaminant in a homemade liquor called ''Ginger Jake'', which is responsible for delayed neuropathy and paralysis of the legs, is bioactivated to a form that inhibits neuropathy target esterase but not acetylcholinesterase (Casida and Quistad, 2004; Glynn, 2006). Large structures with a 12–20 carbon alkyl chain on the phosphorus atom inhibit fatty acid amide hydrolase but not acetylcholinesterase (Casida and Quistad, 2004). These examples clearly show that OPs which do not affect cholinesterases can react with other proteins, sometimes generating toxic symptoms. These examples also suggest that additional, unknown OP targets may exist which are sensitive to OP that do not affect cholinesterases. In order to detect exposure to such OPs, biomarkers other than cholinesterases will be needed.

B. OP Doses Too Low to Inhibit Acetylcholinesterase Cause Toxicity

Workers in India ($n = 59$) engaged in the manufacture of quinalphos had normal red cell acetylcholinesterase activity, but complained of generalized weakness and fatigue. They had significantly low scores for memory, learning ability, and vigilance compared to controls. Plantar reflexes were abnormal in 50% of the workers. The average age of the workers was 30 ± 6 years and the average duration of exposure to quinalphos was 5.7 years. There was no history of acute poisoning. The plant was situated in large tin sheds without adequate ventilation (Srivastava *et al.*, 2000). The most logical explanation for their symptoms was low-dose exposure to OP.

Family tobacco farmers in Brazil ($n = 37$) used chlorpyrifos and acephate for 3 months a year, for 5.4 h a day. The average duration of exposure was 18 years. Their plasma cholinesterase activity was within the normal range and was not different between on- and off-exposure periods. Clinically significant extrapyramidal symptoms were present in 12 subjects during the pesticide application season, though this number was reduced to nine after 3 months without exposure. Generalized anxiety disorder was diagnosed in 13 subjects, and major depression in eight subjects. After 3 months without OP exposure, the number of subjects with psychiatric disorders declined to about half (Salvi *et al.*, 2003). The most logical explanation for their symptoms was low-dose exposure to OP.

More examples of chronic low dose, subclinical exposure to OPs in humans leading to chronic neurotoxicity are cited in reviews (Abou-Donia, 2003; Kamel and Hoppin, 2004).

Animal studies have been directed at understanding the mechanism of low-dose OP toxicity. The studies agree that doses too low to inhibit acetylcholinesterase activity nevertheless have adverse effects on the animals, including disruption of adenylyl cyclase signaling (Song *et al.*, 1997), hyperphosphorylation of calcium/cAMP response element binding protein (Schuh *et al.*, 2002), airway hyperactivity (Lein and Fryer, 2005), changes in expression levels of fibroblast growth factor in the brain (Slotkin *et al.*, 2007), changes in serotonin receptors (Slotkin *et al.*, 2008), and inhibition of acylpeptide hydrolase (Richards *et al.*, 2000).

Rats chronically treated with low doses of chlorpyrifos have long-term cognitive deficits in the absence of clinical signs of exposure (Jett *et al.*, 2001; Terry *et al.*, 2007). The mechanism for impairment of cognitive function may involve disruption of the microtubule transport of vesicles, organelles, and other cellular components which are

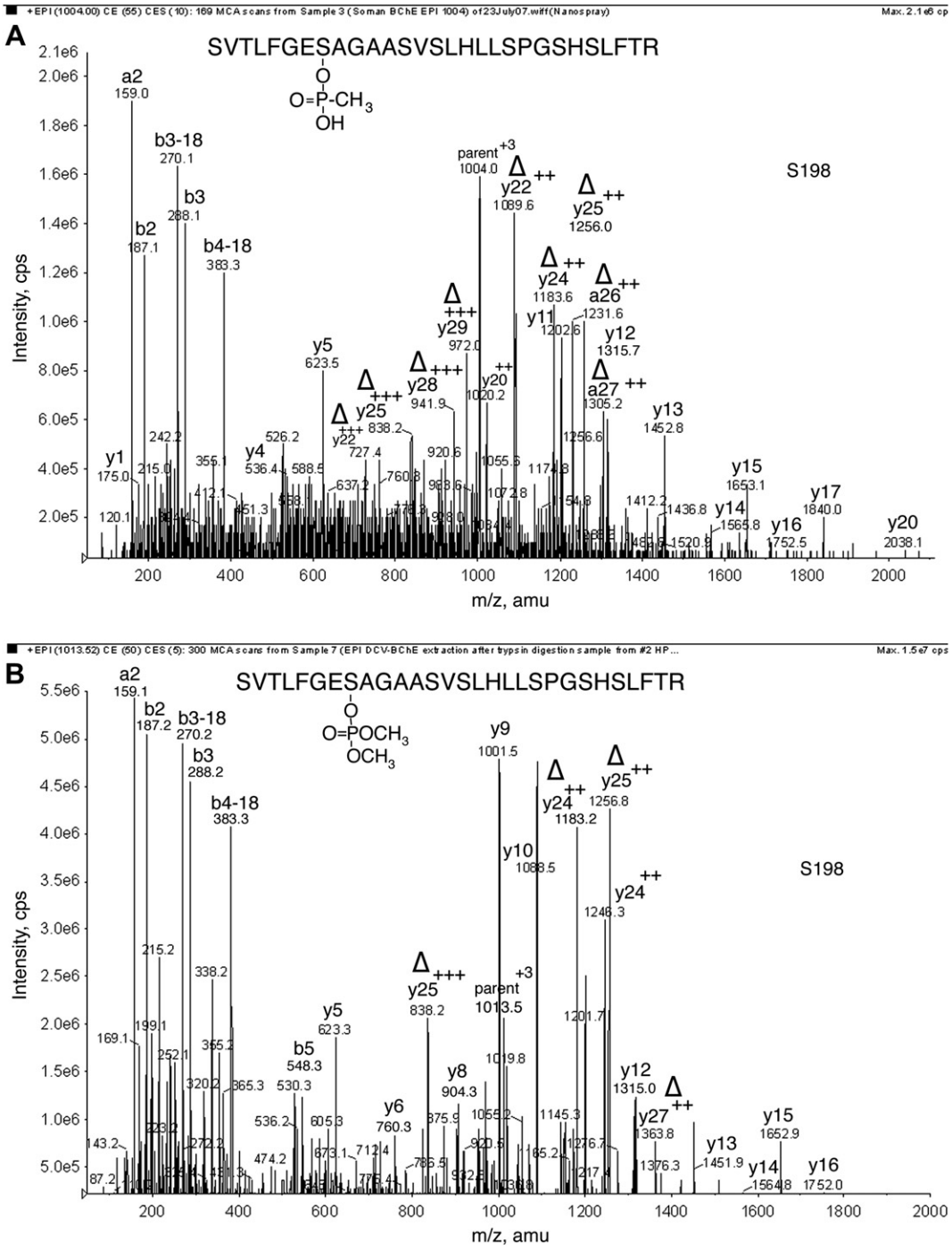

FIGURE 56.1. MS–MS spectra of peptide SVTLFGESAGAASVSLHLLSPGSHSLFTR from human butyrylcholinesterase labeled with aged soman (A), dichlorvos (B), or chlorpyrifos oxon (C). Spectra were acquired on the QTRAP-4000 mass spectrometer using low-energy, collision-induced dissociation. Ions marked with △ have lost the OP as well as a molecule of water, converting the active site serine to dehydroalanine. This type of fragmentation is typical when phosphoserine or organophosphoserine peptides are subjected to collision-induced dissociation in the mass spectrometer. (A) The parent ion for the aged soman-labeled peptide is the triply charged 1004.0 amu ion, where the added mass from aged soman is 78 amu. The scan has nine dehydroalanine masses and no masses other than the parent ion that retain methylphosphonate. (B) The parent ion for the dichlorvos-labeled peptide is the triply charged 1013.5 amu ion, where the added mass from dichlorvos is 108 amu. The scan has four dehydroalanine masses and one mass (y24++) where Ser198 retains dimethoxyphosphate. (C) The parent ion for the chlorpyrifos oxon-labeled peptide is the triply charged 1022.6 amu ion, where the added mass from chlorpyrifos oxon is 136 amu. The scan has four dehydroalanine masses and three masses (y22++, y24++, y25++) where Ser198 retains diethoxyphosphate. To obtain these data, pure human butyrylcholinesterase, accession # gi:158429457, was labeled with OP, digested with trypsin, and peptides were HPLC purified before mass spectrometry. (O. Lockridge, unpublished data)

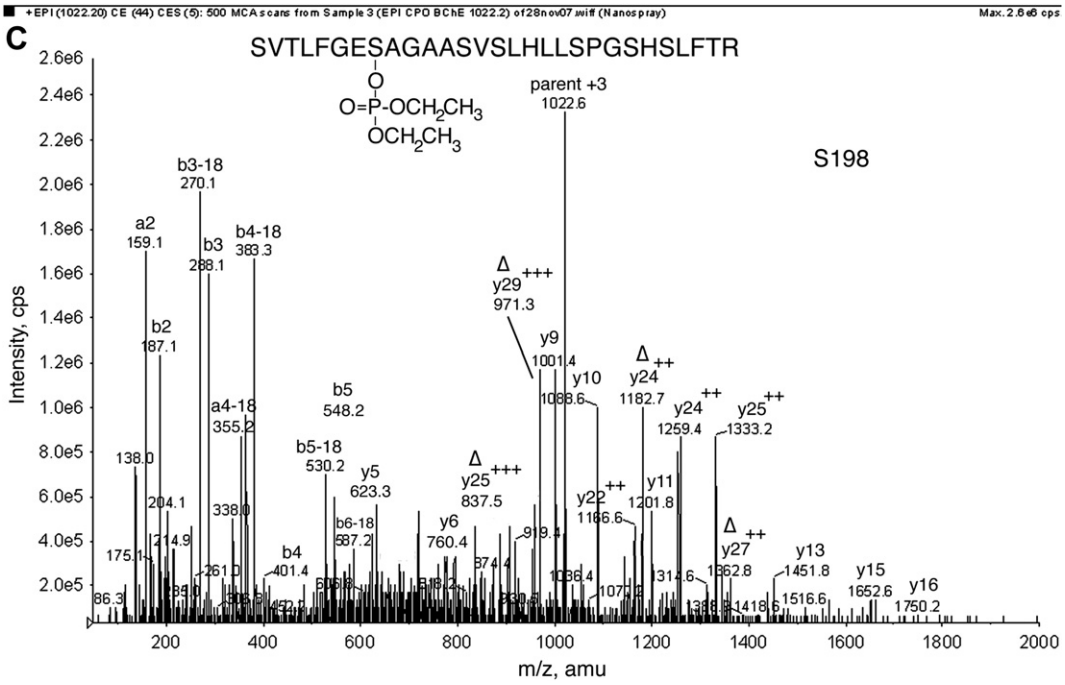

FIGURE 56.1. (*Continued*)

synthesized in the neuronal cell body and moved down long axons to presynaptic sites (Gearhart *et al.*, 2007).

A possible, noncholinesterase-based explanation for the neurotoxic symptoms observed in humans from the examples cited above is that workers were also exposed to a combination of chemicals including heavy metals, solvents, herbicides, and fumigants. The symptoms may actually have been caused by these chemicals and not by the OP (Kamel and Hoppin, 2004). However, the animal data cannot be explained this way because the animals were treated only with OP. In conclusion, OP targets that are not acetylcholinesterase or butyrylcholinesterase are involved in chronic neurotoxicity. These unknown targets bind OP at doses too low to inhibit acetylcholinesterase.

C. Only Some People have Symptoms

Not every person who has been chronically exposed to OP exhibits symptoms. For example, of 612 sheep dippers exposed to diazinon twice a year for 3 days at a time for 40 years, only 19% reported symptoms. In a control group of ceramics workers, who had not been exposed to OPs, 5% reported similar symptoms (Pilkington *et al.*, 2001).

The susceptibility of a minority of the population may be explained by genetic variation in genes affecting OP metabolism. The best studied example is paraoxonase, an enzyme that inactivates OPs (Furlong, 2007; La Du *et al.*, 2001). Paraoxonase polymorphism in humans is hypothesized to explain why some people are resistant to OP toxicity while others are susceptible. Another enzyme that may be involved in resistance to OP toxicity is butyrylcholinesterase. Butyrylcholinesterase scavenges OPs, eliminating the poison before it reaches sites where it could cause harm (Doctor *et al.*, 1991). Humans have a wide range of butyrylcholinesterase activities, with some people having no butyrylcholinesterase whatsoever, due to genetic variation (Manoharan *et al.*, 2007). It is possible, but unproven, that people with butyrylcholinesterase deficiency are more susceptible to OP toxicity. Polymorphisms in liver cytochrome P450 enzymes may also contribute to individual susceptibility to OP toxicity. Specific cytochrome P450 enzymes bioactivate the parent organophosphorothioates to the highly toxic oxon forms. Other cytochrome P450 enzymes detoxify OPs by dearylation (Hodgson and Rose, 2007). The concentration of each isozyme varies among individuals. Thus the relative rates for activation and detoxification will differ between individuals and the circulating levels of the toxic form of the OP will vary between individuals.

Another possibility for variable susceptibility in the population could be genetic variation in the yet unknown OP targets that are responsible for the symptoms. Some forms of these targets may be more sensitive to OPs than are others.

D. Toxic Symptoms Depend on the Identity of the OP

High doses of OPs cause similar toxic effects independent of the identity of the OPs. However, low-dose effects are not identical for all OPs (Moser, 1995). For example, a low dose of fenthion decreased motor activity in rats by 86% but did not alter the tail-pinch response, whereas a low dose of parathion did not affect motor activity but did decrease the

tail-pinch response. In another example, rats given doses of different OPs that inhibited acetylcholinesterase to similar levels had more severe toxicity when the OP was parathion than when it was chlorpyrifos (Pope, 1999). Toxicological studies such as these have led to the conclusion that sites in addition to cholinesterase are targets of OPs. New biomarkers for these effects of OPs are needed.

In summary, acetylcholinesterase and butyrylcholinesterase are not the only proteins modified by OP exposure in humans. Neurotoxicity from low doses of OPs may be explained by OP modification of heretofore unidentified proteins. Toxic symptoms from low-dose exposure to a particular OP are not identical to toxic symptoms from another OP, suggesting that the set of proteins modified by a particular OP does not overlap completely with the set of proteins modified by a different OP. Identification of new biomarkers of OP exposure could lead to new assays for OP exposure, and could lead to an understanding of the causes of low-dose toxicity.

V. NEW BIOMARKERS IN ANIMALS

A. Beta-Glucuronidase in Rat Plasma

A carboxylesterase in rat liver microsomes called egasyn is tightly complexed to beta-glucuronidase. When egasyn binds OP, it releases beta-glucuronidase into blood. A single oral dose of chlorpyrifos (10 mg/kg) increased the level of beta-glucuronidase activity in rat blood 100-fold within 2 h (Fujikawa *et al.*, 2005). The beta-glucuronidase activity decreased to control levels by 24 h. Thus, increased levels of beta-glucuronidase in plasma may serve as a biomarker for OP exposure. However, increase in plasma beta-glucuronidase activity has not been validated in other animal species or in humans.

B. Acylpeptide Hydrolase in Rat Brain

Acylpeptide hydrolase is a member of the serine hydrolase family. It deacetylates the acetylated N-terminus of polypeptides. Rat brain acylpeptide hydrolase was inhibited 93% at a dose of dichlorvos (4 mg/kg, i.p.) which inhibited acetylcholinesterase only 47%. The *in vitro* sensitivity of acylpeptide hydrolase to chlorpyrifosmethyl oxon, dichlorvos, and diisopropyl fluorophosphate (IC_{50}) was 6–10 times greater than that of acetylcholinesterase (Richards *et al.*, 2000). Acylpeptide hydrolase is also found in human erythrocytes where it could potentially serve as a biomarker for low dose exposure to OP in humans, though human cases of OP exposure have not yet been tested for OP-modified acylpeptide hydrolase.

C. Albumin in Mouse and Guinea Pig Plasma

Mice treated with a nontoxic dose of a biotin-tagged OP called FP-biotin had FP-biotinylated albumin in blood and muscle (Peeples *et al.*, 2005). *In vitro* experiments identified the site in human albumin for covalent attachment of a variety of OP as tyrosine 411 (Li *et al.*, 2007).

Guinea pigs treated with the nerve agents soman, sarin, cyclosarin, or tabun had nerve agent-labeled albumin in their blood (Williams *et al.*, 2007). The OPs were bound to tyrosine. The tabun–tyrosine and soman–tyrosine adducts were detected in blood 7 days post-exposure, indicating that the adducts are stable. The adducts did not undergo aging and were not released from tyrosine by treatment of the guinea pigs with oxime, which is a common treatment for OP exposure that induces release of OP from acetylcholinesterase.

These examples show that OPs can bind covalently to albumin under physiological conditions, and that the resultant adducts are relatively stable. OP–albumin adducts could therefore be useful as biomarkers of OP exposure. In addition, unlike cholinesterases, the soman–albumin conjugate does not age (Li *et al.*, 2008a), making it possible to discriminate between sarin and soman exposure. OP–albumin adducts have not yet been reported in humans exposed to OPs.

D. M2 Muscarinic Receptors in Heart and Lung

^{3}H-Chlorpyrifos oxon binds covalently to rat heart M2 muscarinic receptors (Bomser and Casida, 2001). The site of attachment has not been identified. When guinea pigs were treated with chlorpyrifos, diazinon, or parathion at doses too low to inhibit acetylcholinesterase activity, the M2 muscarinic receptors lost their ability to inhibit acetylcholine release from parasympathetic nerves, causing bronchoconstriction (Lein and Fryer, 2005).

VI. COVALENT BINDING OF OP TO TYROSINE

In a 1963 Pedler lecture, Sanger reported that ^{3}H-DFP makes a covalent bond with tyrosine in the sequence Arg-*Tyr*ThrLys from human and rabbit albumin (Sanger, 1963). Mass spectrometry of human albumin treated with soman, chlorpyrifos oxon, FP-biotin, dichlorvos, and DFP confirmed OP modification on Tyr 411 in peptide LVRY*TKKVPQVSTPTL (Li *et al.*, 2007, 2008a), where the underline shows Sanger's sequence and the * indicates the labeled tyrosine.

At the time we confirmed Sanger's observations we thought that albumin was a special case, though we were aware that papain and bromelain had also been reported to bind ^{3}H-DFP on tyrosine (Chaiken and Smith, 1969; Murachi *et al.*, 1965).

We soon learned differently. In a general search for proteins that bind OP, we treated live mice and mouse tissues with FP-biotin, a modified OP designed for identifying unknown targets of OP reaction. The FP-biotinylated

proteins were isolated on immobilized avidin, washed with 0.1% SDS, and separated on an SDS gel. Coomassie blue stained bands were excised, digested with trypsin and the proteins in the bands were identified by mass spectrometry. To our surprise the majority of proteins that were identified were not serine esterases or proteases, which are the classical targets for OPs, nor did the identified proteins contain the consensus sequence, GXSXG, which is characteristic of an active site serine. Typically, we found high abundance proteins such as albumin and tubulin.

Though biotin–avidin complexation has been used widely to isolate specific targets, it is notorious for generating false-positive identifications. To rule out the possibility that our results were an artifact we set out to find the OP-labeled peptide. We reasoned that convincing proof for OP labeling required identification of the labeled peptide and the labeled amino acid.

During years of mass spectrometry analysis, we had consistently identified OP-labeled tubulin in mouse brain. Therefore, we studied pure bovine tubulin (Cytoskeleton, Inc.) by treating it with soman, sarin, FP-biotin, DFP, chlorpyrifos oxon, and dichlorvos. We isolated the OP-labeled tryptic peptides and analyzed them by fragmentation in the QTRAP 2000 and QTRAP 4000 mass spectrometers. We identified five OP-labeled peptides in tubulin (Grigoryan *et al.*, 2008). In every peptide, the OP was covalently attached to tyrosine (Table 56.1).

Similar mass spectrometry experiments with pure human and mouse transferrin (Li *et al.*, 2008c), and with human kinesin showed that the OP label was consistently on tyrosine (Table 56.1). Studies with human plasma identified OP labeling on tyrosine in apolipoprotein and alpha-2-glycoprotein. Aggressive treatment of human albumin with FP-biotin and chlorpyrifos oxon led to identification of seven OP-labeled tyrosines (Ding *et al.*, 2008). Finally, we found that synthetic peptides made a covalent bond with DFP, chlorpyrifos oxon, and dichlorvos (Table 56.1). Mass spectrometry conclusively proved that the OP was attached to tyrosine.

VII. MOTIF FOR OP BINDING TO TYROSINE

Comparison of the sequences of the OP-labeled peptides in Table 56.1 shows no definite consensus sequence around the tyrosine to which the OP binds. What the peptides do have in common is the presence of a positively charged arginine, lysine, or histidine within 5 amino acids of the labeled tyrosine, most being within 3 amino acids. We suggest that these positively charged residues could interact with the phenolic hydroxyl of tyrosine to lower the pKa. Such ion-pair interactions have been shown to lower the pKa for the negatively charged partner, with a comparable rise of the pKa of the positively charged partner by as much as 4 pKa units (Johnson *et al.*, 1981). Tyrosines with a lower pKa value would be better nucleophiles and thus be better able to attack OPs.

Peptide SYSM has no positively charged residue in its sequence. Labeling efficiency on this peptide was poor at pH 8.3. MPCAEDYLSVVLN also contains no positively charged residue but this tyrosine was near a positively charged residue in the albumin structure.

In conclusion, most proteins that we have examined in detail, using sensitive mass spectrometry techniques, have shown the capacity to become labeled by OP on tyrosine. However, only certain tyrosines in a protein are labeled, i.e. those on the surface and near a positively charged residue that could potentially activate the phenolic hydroxyl group. The finding that even small synthetic peptides can be labeled by OP on tyrosine has led to the hypothesis that OP labeling on tyrosine is a general phenomenon. We propose that OP labeling on tyrosine is a new motif for OP binding to proteins.

VIII. CHARACTERISTICS OF OP BINDING TO TYROSINE

A. On Rate

Little is known about the rate of OP binding to tyrosine because the recognition of this OP binding motif is new with the writing of this chapter. Soman binding to Tyr 411 in human albumin has been measured and has been found to be slow with a bimolecular rate constant of $15 \pm 3\ M^{-1}\ min^{-1}$ (Li *et al.*, 2008a). We expect that other proteins will be identified whose rate of OP binding to tyrosine will be fast.

B. Off Rate

The OP adduct on tyrosine 411 of human albumin is stable. The half-life for decay of the soman–Tyr 411 adduct is 20 days at pH 7.4, 22°C (Li *et al.*, 2008a). The chlorpyrifos oxon–Tyr 411 adduct is even more stable (Ding *et al.*, submitted). After 7 months at 22°C in pH 7.4 buffer, 80% of the Tyr 411 was still labeled with diethoxyphosphate, which is the adduct formed by chlorpyrifos oxon. However, at pH 8.3 and 22°C, 50% had lost the OP label in 3.6 months. OP–albumin adducts stored at −80°C are stable indefinitely at pH 8.3, 7.4, and 1.5. One advantage of a stable OP–tyrosine adduct is that it will survive in an animal long enough to be useful for generation of antibodies. Another advantage of a stable OP–tyrosine adduct is that detection of OP can be made on samples long after exposure.

C. No Aging

OPs bound to tyrosine do not age. Aging of OP adducts on acetylcholinesterase and butyrylcholinesterase is defined as the loss of an alkoxy group from the phosphorus atom. Every OP–tyrosine adduct in Table 56.1 has been examined

TABLE 56.1. OP binding motif on tyrosine

	OP labeled	Reference
Human albumin gi:3212456		
ETFLKK[Y]LYEIAR	Y138	Ding *et al.*
IARRHP[Y]FYAPEL	Y148	Ding *et al.*
RRHPYF[Y]APELLF	Y150	Ding *et al.*
LFFAKR[Y]KAAFTE	Y161	Ding *et al.*
FEQLGE[Y]KFQNAL	Y401	Ding *et al.*
NALLVR[Y]TKKVPQ	Y411	Li *et al.* (2007)
MPCAED[Y]LSVVLN	Y452	Ding *et al.*
Bovine albumin gi:162648		
NALIVR[Y]TRKVPQ	Y410	Schopfer *et al.* (2005)
Human transferrin gi:136191		
RKPVDE[Y]KDCHLA	Y238	Li *et al.*, in preparation
RKPVEE[Y]ANCHLA	Y574	Li *et al.*, in preparation
Mouse transferrin gi:21363012		
RKPVDQ[Y]EDCYLA	Y238	Li *et al.*, in preparation
LYLGHN[Y]VTAIRN	Y319	Li *et al.*, in preparation
GIFPKG[Y]YAVAVV	Y429	Li *et al.*, in preparation
QGCAPG[Y]EKNSTL	Y491	Li *et al.*, in preparation
KEEYNG[Y]TGAFRC	Y518	Li *et al.*, in preparation
Bovine alpha tubulin gi: 15988311		
EVRTGT[Y]RQLFHP	Y83	Grigoryan *et al.* (2008)
EDAANN[Y]ARGHYT	Y103	Grigoryan *et al.* (2008)
HFPLAT[Y]APVISA	Y272	Grigoryan *et al.* (2008)
RAFVHW[Y]VGEGME	Y408	Grigoryan *et al.* (2008)
Bovine beta-3 tubulin gi:93140719		
EASSHK[Y]VPRAIL	Y59	Grigoryan *et al.* (2008)
SKVREE[Y]PDRIMN	Y159	Grigoryan *et al.* (2008)
ARGSQQ[Y]RALTVP	Y281	Grigoryan *et al.* (2008)
Bovine beta tubulin gi:119923822		
DPRHGR[Y]LTVAAV	Y310	Grigoryan *et al.* (2008)
Human kinesin KIF3C gi: 27769239		
YLVRAS[Y]LEIYQE	Y137	
Human apolipoprotein gi: 178775		
RTHLAP[Y]SDELRQ	Y172	
Human alpha-2 glycoprotein 1 gi:119597029		
GAFWKY[Y]YDGKDY	Y42	
WEAEPV[Y]VQRAKA	Y78	
VQRAKA[Y]LEEECP	Y85	
Bromelain from pineapple	Tyr	Murachi *et al.* (1965)
papain gi:157833554		
NQGALL[Y]SIANQP	Y123	Chaiken and Smith (1969)
Synthetic peptides		
R[Y]TR	Y2	Li *et al.*, in preparation
S[Y]SM	Y2	Li *et al.*, in preparation
S[Y]SMEHFRWGKPVGKKR	Y2	Li *et al.*, in preparation
RPVKV[Y]PNGAEDESAEAFPLEF	Y6	Li *et al.*, in preparation
KSTEQK[Y]LTL	Y7	Li *et al.*, in preparation

For peptides derived from proteins, six residues on either side of the OP-labeled tyrosine are shown. Synthetic peptides are shown in their complete length. The labeled tyrosine is enclosed in a box.

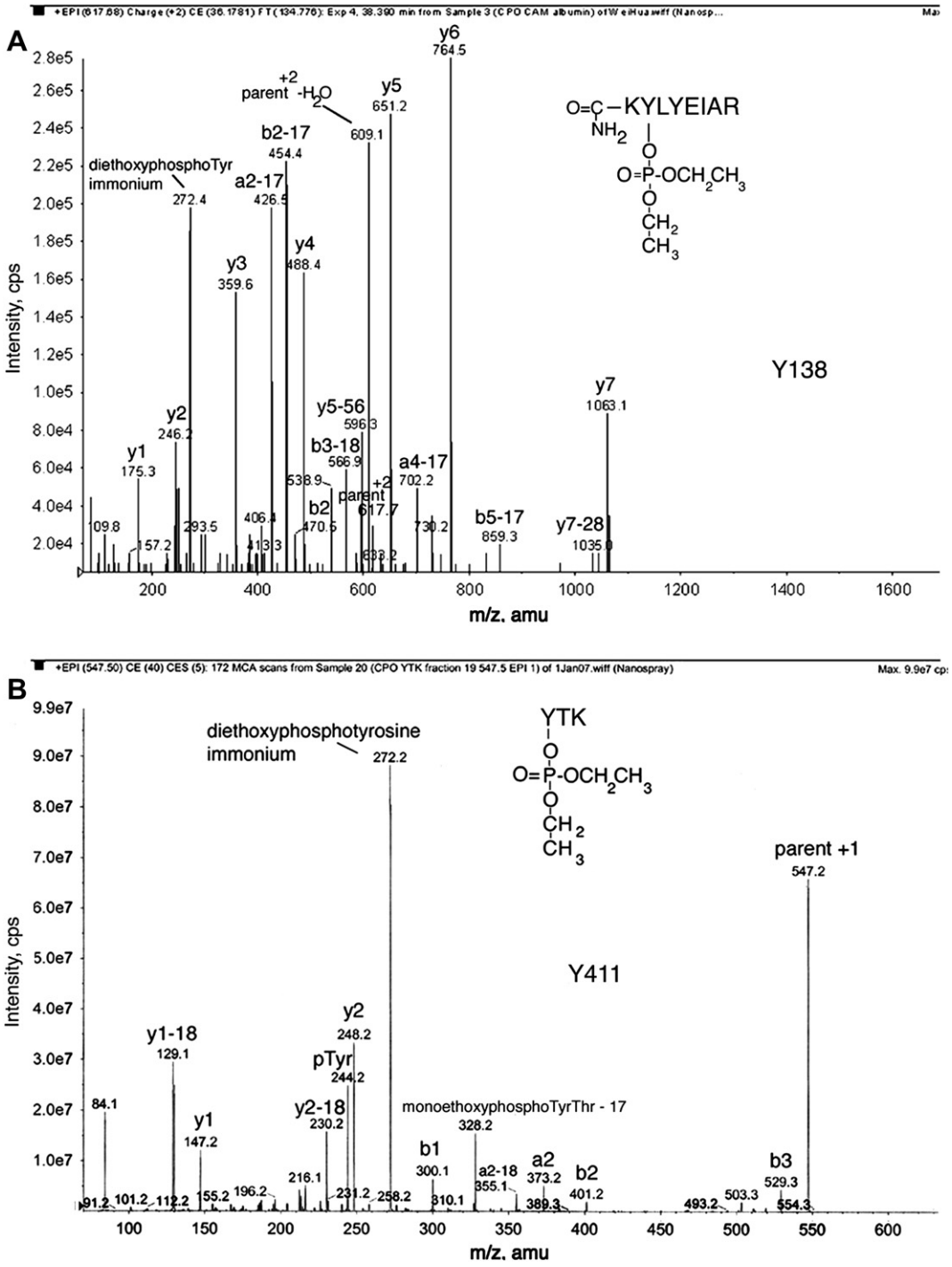

FIGURE 56.2. MS–MS spectra of human albumin peptides labeled on tyrosine with chlorpyrifos oxon. (A) The doubly charged parent ion (617.7 amu) for KYLYEIAR includes a mass of 136 amu from chlorpyrifos oxon and a mass of 43 amu from carbamate. Carbamylation was a by-product of denaturation in 8 M urea. The presence of diethoxyphosphate on Tyr 138 is supported by the masses y7, a2 minus amine (17 amu), b2 minus amine (17 amu), b3 minus water (18 amu), a4 minus amine (17 amu), and b5 minus amine (17 amu), all of which include the 136 amu added mass for diethoxyphosphate. The ion at 272.4 amu is consistent with the mass of the diethoxyphosphotyrosine immonium ion; its presence supports chlorpyrifos oxon labeling of tyrosine. (B) The singly charged parent ion (547.2 amu) for YTK includes an added mass of 136 amu from chlorpyrifos oxon. The presence of diethoxyphosphate on Tyr 411 is supported by the masses of the b1 ion, the y2 ion, and the parent ion, all of which include the 136 amu mass of diethoxyphosphate. Furthermore, the characteristic ions at 272.2 amu for the diethoxyphosphotyrosine immonium ion, at 328.2 amu for the monoethoxyphosphoTyrThr ion minus amine (17 amu), and at 244.2 amu for phosphotyrosine support labeling on tyrosine. Loss of one or both alkyl groups from the diethoxyphospho adduct during collision-induced dissociation in the mass spectrometer is a common observation. These data are original from our laboratory and have not previously been published.

for aging. No masses representing aged OP–tyrosine adducts have been found.

Aging can confound the identification of the original OP. For example, aged sarin and soman adducts on acetylcholinesterase are indistinguishable because they yield the same methylphosphonate derivative. The absence of aging for OP–tyrosine adducts results in a species that is suitable for discriminating between sarin and soman adducts because the unaged adducts have different masses. Absence of aging for OP–tyrosine adducts has also been reported by Williams *et al.* (2007).

IX. METHODS FOR DETECTING OP BINDING TO TYROSINE

A. Mass Spectrometry

If the identity of the OP-labeled protein is unknown, a tagged OP, for example a biotinylated OP, can be used to identify the protein (Schopfer *et al.*, 2005). After the identity of the OP-labeled protein is known, identification of the OP-labeled peptide depends on separating it from contaminating peptides. We have found that the OP-labeled peptide is frequently not found by mass spectrometry unless it has been extensively purified. In some cases it is possible to identify the labeled peptide simply by LC–MS–MS, where an enzymatic digest of the isolated protein is subjected to liquid chromatography on a C18 nanocolumn and the effluent from the column is electrosprayed directly into the mass spectrometer. For other cases, extensive HPLC purification of the enzymatic digest is necessary to obtain a purified fraction of peptides that can be introduced into the mass spectrometer.

Preliminary identification of the labeled peptides in HPLC fractions is made by mass, using MALDI mass spectrometry (MALDI TOF-TOF 4800 from Applied Biosystems). The MS-Digest algorithm on the Protein Prospector website at the University of California San Francisco is a useful tool for predicting the masses of peptides from a proteolytic digest with and without the added mass from a particular OP. Another indispensable tool is the Fragment Ion Calculator from the http://db.systemsbiology.net:8080/proteomicsToolkit/ website, which can be used to calculate the masses of ions generated during MS–MS fragmentation of a peptide. Our laboratory acquires MS–MS spectra with the QTRAP 2000 and QTRAP 4000 mass spectrometers (Applied Biosystems). Examples of MS–MS spectra for human albumin peptides labeled on tyrosine with chlorpyrifos oxon are shown in Figure 56.2.

B. Antibody

An elegant method for identifying OP-labeled tyrosine would be with an OP–tyrosine-specific antibody. A similar antibody toward phosphotyrosine is commercially available. Antibodies to OP–tyrosine adducts are not yet available, though their development is in progress.

X. CONCLUDING REMARKS AND FUTURE DIRECTION

A new motif for OP binding to tyrosine has been identified. Almost all proteins appear to be capable of binding OP covalently on tyrosine. Whether or not OP will bind to tyrosine *in vivo* will depend on the concentration of the protein, the concentration of the OP, and the ionization status of the tyrosine hydroxyl group. The latter factor appears to be dependent on the presence of nearby positively charged residues.

The importance of the first and third factors is amply demonstrated in the case of albumin. The concentration of albumin is so high (600 μM in human plasma) that albumin binds OP even though its rate of reaction is slow. The reaction of albumin's most sensitive tyrosine is aided by the presence of three positively charged residues within a five residue stretch surrounding that tyrosine.

Antibodies to OP–tyrosine will be made. These antibodies will be used to diagnose OP exposure in a biosensor assay with saliva, sweat, or urine. New biomarkers of OP exposure will be identified using mass spectrometry and the new OP–tyrosine antibodies. The identification of new biomarkers for low-dose OP exposure is expected to lead to an understanding of how neurotoxicity is caused by OP doses that are too low to inhibit acetylcholinesterase. For example, it is possible that disruption of microtubule polymerization by OP-adduct formation may explain cognitive impairment from OP exposure.

Identification of new biomarkers of OP exposure may also lead to an understanding of why some people are intoxicated by low doses of OP that have no effect on the majority of the population. The new motif for OP binding to tyrosine may lead to new antidotes for OP poisoning; for example, peptides containing several tyrosines and several arginines may be effective OP scavengers.

Acknowledgments

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CHAPTER 57

Biomarkers and Biosensors of Delayed Neuropathic Agents

RUDY J. RICHARDSON, R. MARK WORDEN, AND GALINA F. MAKHAEVA

I. INTRODUCTION

Delayed neuropathic (DN) agents represent a new functional class of organophosphorus (OP) threats that have heretofore not been used in warfare or terrorist acts. Nevertheless, it is vital to develop effective defenses against them for three reasons. First, these materials can readily be produced by rather straightforward modifications of the structures of conventional nerve agents. Second, warning signs and symptoms of toxicity may be lacking until 1–4 weeks after exposure, when the permanent sensory deficits and paralysis associated with OP compound-induced delayed neurotoxicity (OPIDN) develop. Third, there are no established means of preventing or treating OPIDN. This chapter begins with a review of current understanding about the chemistry and mechanism of action of DN agents; it then describes how existing knowledge can be applied to generate biomarkers and biosensors for discriminating between conventional and DN agents; and it ends by indicating future work needed to address the threat of DN agents.

II. OP COMPOUNDS

A. Conventional Nerve Agents versus DN Agents

Conventional nerve agents, such as sarin and soman, are OP compounds of pentavalent phosphorus that are well known as threats in the context of warfare and terrorism (Sidell and Borak, 1992). These substances produce acute cholinergic toxicity and death via inhibition of acetylcholinesterase (AChE) in the central and peripheral nervous systems (Marrs *et al.*, 1996).

In contrast to conventional nerve agents, DN agents produce permanent neurological dysfunction in the form of OPIDN rather than cholinergic toxicity and death (Richardson, 2005). Like conventional nerve agents, DN agents are OP compounds of pentavalent phosphorus that are readily synthesized, but they are designed to inactivate neuropathy target esterase (neurotoxic esterase, NTE) in preference to AChE (Wu and Casida, 1995). Because of this selectivity, DN agents may elicit little or no warning signs of acute cholinergic toxicity, so that victims of DN agents might not know they have been exposed until OPIDN develops 1–4 weeks later.

Cholinergic toxicity from OP compounds can be elicited solely by inhibition of AChE (Thompson and Richardson, 2004). The AChE–OP conjugate can undergo loss of an OP ligand (''aging'') to yield a negatively charged phosphoryl adduct on the active site serine, but while this reaction has practical implications for therapy, it does not change the qualitative nature of the toxicity (Figure 57.1). On the other hand, if an OP compound is to be a DN agent, it must be capable of inhibiting NTE; moreover, it appears that the resulting NTE–OP conjugate must also be capable of undergoing aging (Figure 57.2) (Richardson, 1992, 2005).

Thus, DN agents are distinct from conventional nerve agents in several respects. Furthermore, especially in view of their ease of synthesis, absence of initial signs or symptoms of exposure, and lack of prophylactic or therapeutic measures, it is conceivable that rogue nations or terrorist groups might consider DN agents attractive as weapons of permanent incapacitation against military or civilian populations. Therefore, part of an effective chemical defense strategy is to develop methods for detecting DN agents via sensitive and selective biomarkers and biosensors (Makhaeva *et al.*, 2003; Malygin *et al.*, 2003).

B. OP Compounds of Pentavalent versus Trivalent Phosphorus

In this chapter, DN agents are defined as OP compounds of pentavalent phosphorus that produce OPIDN. Examples shown in Figure 57.3 are ethyl *n*-octylphosphonofluoridate (EOPF) and 2-(2-methylphenoxy)-4*H*-l,3,2-benzodioxaphosphorin-2-oxide [2-(2-methylphenoxy)-BDPO], the active metabolite of the archetypal DN agent, tri-*o*-cresyl phosphate (TOCP) (Wu and Casida, 1995).

However, there are also OP compounds of trivalent phosphorus that produce neurological injury. Examples

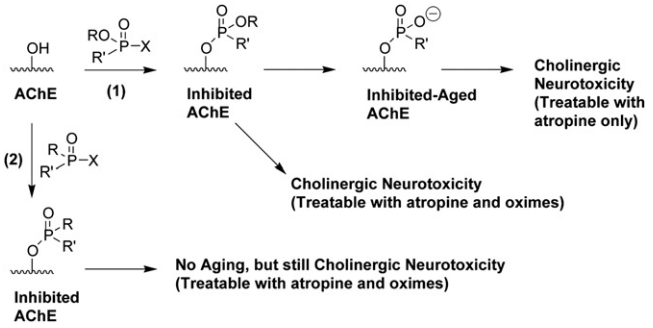

FIGURE 57.1. Reaction of acetylcholinesterase (AChE) with an organophosphonate in pathway (1) yields a phosphonylated (inhibited) enzyme, which can undergo net loss of an R-group to yield an inhibited-aged enzyme. Inhibition alone produces cholinergic toxicity that is treatable with both atropine to counteract excess acetylcholine at muscarinic acetylcholine receptors and oximes to reactivate AChE. Aging does not change the type of toxicity, but it renders the enzyme intractable to reactivation, so that treatment with oximes becomes ineffective. Reaction of AChE with an organophosphinate in pathway (2) yields a phosphinylated (inhibited) enzyme and produces cholinergic toxicity, but because of the stability of the C–P bonds linking the R- and R′-groups to phosphorus the phosphinyl moiety on AChE does not undergo aging, so that both atropine and oximes can be used in therapy. R, R′ = substituted or unsubstituted alkyl or aryl groups, which can be different or equivalent. X = primary leaving group, e.g. fluoride. Reproduced with permission from Richardson (2005).

shown in Figure 57.4 are triphenylphosphite and triphenylphosphine. Comparatively little is known about the neuropathic trivalent OP compounds, but the clinical course and spatial–temporal pattern of lesions that they produce vary from OPIDN, and the mechanisms of action of these compounds appear to differ from that of the DN agents. For example, triphenylphosphite produces a syndrome of delayed axonopathy and other neurodegenerative changes that could represent a variant of OPIDN, possibly via inhibition/aging of neuropathy target esterase (NTE) (Abou-Donia, 1992; Fioroni *et al.*, 1995; Padilla *et al.*, 1987; Tanaka *et al.*, 1990). However, although triphenylphosphite inhibits NTE *in vitro* and *in vivo*, the nature of the adduct formed with NTE has not been elucidated, and the aging reaction of triphenylphosphite-inhibited NTE has apparently not been studied. Triphenylphosphine is another trivalent OP compound that produces delayed axonopathy, but its pathogenesis and mechanism appear to differ from those of triphenylphosphite or neuropathic OP compounds of pentavalent phosphorus. Among other differences, triphenylphosphine does not inhibit NTE *in vitro* or *in vivo* (Davis *et al.*, 1999; Padilla *et al.*, 1987).

With the foregoing in mind, the remainder of this chapter will deal exclusively with OP compounds of pentavalent phosphorus.

III. OPIDN

OPIDN, also known as OP compound-induced delayed polyneuropathy (OPIDP), can be produced within 1–4 weeks of a single exposure to a DN agent; with compounds that are highly selective for NTE, the first week of this period can be clinically quiescent (Davis and Richardson, 1980; Lotti, 1992; Lotti and Moretto, 2005; Richardson, 2005). Pathogenesis involves progressive distal degeneration of sensory and motor axons in peripheral nerves and spinal cord tracts.

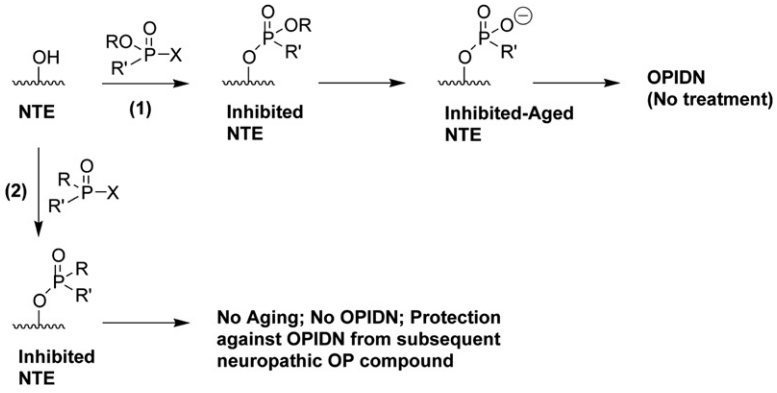

FIGURE 57.2. Reaction of neuropathy target esterase (NTE) with an organophosphonate in pathway (1) yields a phosphonylated (inhibited) enzyme, which can undergo net loss of an R-group to yield an inhibited-aged enzyme. Inhibition alone does not produce organophosphorus (OP) compound-induced delayed neurotoxicity (OPIDN); both inhibition and aging are required, and there is no treatment for the neuropathy. Reaction of NTE with an organophosphinate in pathway (2) yields a phosphinylated (inhibited) enzyme, but because of the stability of the C–P bonds linking the R- and R′-groups to phosphorus the phosphinyl moiety on NTE does not undergo aging, so that OPIDN does not occur. Inhibition of NTE with a nonaging inhibitor is not biologically inert – it protects against subsequent exposures to neuropathic (ageable) NTE inhibitors. R, R′ = substituted or unsubstituted alkyl or aryl groups, which can be different or equivalent. X = primary leaving group, e.g. fluoride. Reproduced with permission from Richardson (2005).

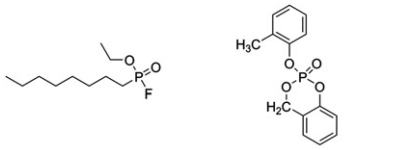

FIGURE 57.3. Examples of delayed neuropathic (DN) agents containing pentavalent phosphorus: ethyl *n*-octylphosphonofluoridate (EOPF) and 2-(2-methylphenoxy)-4*H*-l,3,2-benzodioxaphosphorin-2-oxide [2-(2-methylphenoxy)-BDPO], the active metabolite of the archetypal DN agent, tri-*o*-cresyl phosphate (TOCP) (Wu and Casida, 1995).

Accordingly, symptoms begin with paresthesias in the distal extremities followed by sensory loss, ataxia, and flaccid paralysis. Once the disease is initiated, it advances inexorably. In keeping with its unknown mechanism of neurodegeneration, there is no cure or treatment other than general supportive therapy. Over a period of months to years, some regeneration of peripheral nerve axons may occur to reinnervate muscle. Nevertheless, the concomitant persistence of injury to descending inhibitory pathways in the spinal cord prevents complete functional recovery and permits only a shift from flaccid to spastic paralysis (Richardson, 2005).

Because of the insidious onset and permanent debilitating effects of OPIDN, it is essential to be able to predict the potential of a given OP compound to produce this disease as opposed to causing acute cholinergic toxicity. Furthermore, it is important to develop specific and stable biomarkers and biosensors of exposure to DN agents and devise countermeasures against them (Makhaeva *et al.*, 2003; Malygin *et al.*, 2003). To accomplish these goals, it will be necessary to acquire a level of mechanistic understanding of interactions between DN compounds and target macromolecules akin to what has been achieved for conventional (anti-AChE) nerve agents. Although there is much left to be learned about the pathogenesis and mechanism of OPIDN, sufficient knowledge exists about the apparent initiating events, i.e. inhibition and aging of NTE, to provide a foundation for the development of biomarkers and biosensors of DN agents.

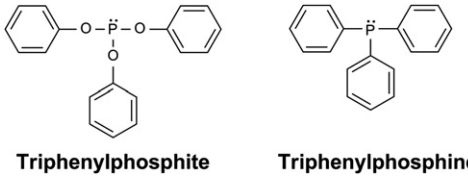

FIGURE 57.4. Examples of neuropathic organophosphorus compounds containing trivalent phosphorus: triphenylphosphite and triphenylphosphine (Eto, 1974).

IV. NTE

A. Definition of NTE and its Potential Normal or Pathogenic Roles

NTE (UniProtKB/Swiss-Prot Q81Y17; PLPL6_HUMAN) has recently been categorized as a lysophospholipase (EC 3.1.1.5), owing to its ability to hydrolyze phosphatidylcholine to glycerophosphocholine (UNIPROT, 2008). It is also known as patatin-like phospholipase domain-containing protein-6, whose gene name is PNPLA6, because the catalytic domain of NTE encompasses a region with sequence homology to patatin, a phospholipase found in potatoes and other plants. Thus, it appears that NTE can function biochemically as a phospholipase, but its precise physiological role remains to be firmly established.

The UniProt entry for human NTE lists three variants attributed to alternative splicing (UNIPROT, 2008). Although UniProt has adopted isoform-1 as the canonical sequence, this chapter defines human NTE as isoform-2, which is the sequence initially reported by Lush *et al.* (1998), consisting of 1,327 amino acids and having a molecular weight without posttranslational modifications of 146 kDa. The protein has a transmembrane domain near the N-terminus (residues 9–31) and three putative cyclic nucleotide binding domains (residues 163–262, 480–573, and 597–689), in addition to the patatin domain (residues 933–1,099), which contains the active site (Wijeyesakere *et al.*, 2007).

The three-dimensional structure of NTE has not been experimentally determined, but a homology model of the patatin domain indicates that the active site serine (Ser^{966}) is located on a ''nucleophilic elbow'' characteristic of serine hydrolases (Wijeyesakere *et al.*, 2007). Moreover, the model indicates that the catalytic site of NTE consists of a novel Ser-Asp catalytic dyad, as in patatin and mammalian cytosolic phospholipase A2 ($cPLA_2$), rather than the classical catalytic triad (Ser-Asp/Glu-His), as found in many serine hydrolases including AChE. Recently, mutations have been identified in NTE that are associated with motor neuron disease (Rainier *et al.*, 2008). The mutations occur within the catalytic domain of NTE, but it is not yet known if they affect the catalytic function of the enzyme or alter some other property of the protein in order to produce disease.

NTE was first identified as the presumptive target of neuropathic OP compounds in the initiation of OPIDN (Johnson, 1970). Its activity in cells and tissues is operationally defined as the enzymatic hydrolysis of the nonphysiological substrate, phenyl valerate, which is resistant to inhibition by diethyl 4-nitrophenyl phosphate (paraoxon) and sensitive to inhibition by *N,N′*-diisopropylphosphorodiamidic fluoride (mipafox) under specified conditions of preincubation with inhibitors and subsequent incubation with substrate (Johnson, 1977; Kayyali *et al.*, 1991; Makhaeva *et al.*, 2007).

Because the full-length protein is difficult to isolate or produce, human recombinant NTE esterase domain (NEST) has been used as an NTE surrogate for studies *in vitro* that require pure protein rather than a crude preparation containing NTE (Kropp *et al.*, 2004). NEST comprises residues 727–1,216 of NTE; it has been shown to be the shortest segment of NTE that retains esterase activity, and the catalytic properties of NEST, including its response to OP inhibitors, closely resemble those of full-length NTE (Atkins and Glynn, 2000; Atkins *et al.*, 2002; Kropp *et al.*, 2004; Van Tienhoven *et al.*, 2002). As noted below, NEST has been incorporated into a biosensor for detection of DN agents (Kohli *et al.*, 2007a).

B. Role of NTE in OPIDN

Early studies indicated that the initial molecular events in OPIDN are the concerted inhibition and aging of a threshold level (>70%) of NTE in the central and peripheral nervous systems (Glynn, 1999; Johnson, 1974, 1982; Lotti, 1992). As with other serine esterases, inhibition of NTE is thought to occur by nucleophilic attack of the active site serine (Ser^{966}) at the phosphorus of the OP compound, with displacement of a primary leaving group. Aging of the phosphylated enzyme presumably involves loss of a second ligand, leaving a negatively charged phosphyl moiety covalently attached to the active site. Note that ''phosphyl'' is used as a generic term for the various types of OP compounds that might be used as inhibitors of serine esterases, e.g. phosphates, phosphonates, phosphinates, and phosphoroamidates.

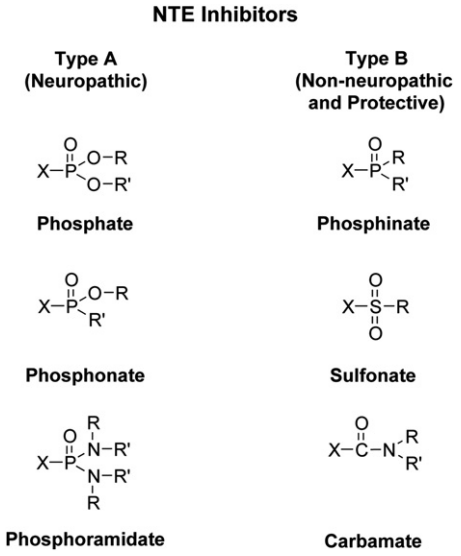

FIGURE 57.5. Subclasses of neuropathy target esterase (NTE) inhibitors. Type A inhibitors include phosphates, phosphonates, and phosphoramidates; these are neuropathic and capable of aging. Type B inhibitors include phosphinates, sulfonates, and carbamates; these are nonneuropathic and not capable of aging. However, inhibition of NTE with a type B inhibitor will protect against organophosphorus compound-induced delayed neurotoxicity (OPIDN) from subsequently administered type A inhibitors. Reproduced with permission from Richardson (2005).

The apparent necessity for aging of NTE as a trigger for OPIDN came about from analyses of structure–activity relationships. These studies revealed that NTE inhibitors fall into two functional categories, type A and type B (Davis and Richardson, 1980; Davis *et al.*, 1985; Johnson, 1975) (Figure 57.5). Type A inhibitors produce OPIDN and include certain phosphates, phosphonates, and phosphoramidates. When type A compounds inactivate NTE, it rapidly becomes intractable to reactivation by powerful nucleophiles, e.g. oximes or fluoride. This inability to be reactivated has been interpreted as evidence of aging. Because loss of reactivatability takes place within minutes of inhibition by type A inhibitors and OPIDN takes 1–4 weeks to develop, aging of NTE is not the rate-limiting step in this disease (Johnson, 1982). Type B inhibitors do not produce OPIDN and include certain phosphinates, carbamates, and sulfonates. NTE conjugates with type B compounds are considered not to undergo aging; those formed from carbamates and phosphinates can be reactivated, and carbamylated, phosphinylated, or sulfonylated NTE cannot undergo further reactions to produce a stable charged species. Despite the fact that inhibition of NTE by type B compounds does not result in OPIDN, this reaction is far from being biologically inert. Pretreatment of animals with type B inhibitors protects them against OPIDN from subsequently administered type A inhibitors, presumably by blocking the formation of aged NTE (Davis *et al.*, 1985; Johnson, 1990; Richardson, 1984) (Figure 57.2). Protection against type A inhibitors by type B inhibitors is effective as long as inhibition of NTE by type B compounds is >30%, thus preventing inhibition and aging of the >70% NTE required for induction of OPIDN.

It would appear that the ostensible requirement for aging of NTE in the development of OPIDN is not merely to ensure irreversible inhibition of the enzyme. Prolonged suprathreshold inhibition (>70%) of neural NTE *in vivo* by repeated dosing of nonaging inhibitors does not cause neuropathy (Johnson, 1970, 1974, 1982). Moreover, repeated low-level dosing with aging inhibitors to produce a prolonged steady-state inhibition that is below threshold (<70%) does not result in OPIDN until a higher single dose is superimposed that raises the inhibition level above threshold. In the latter scenario, there is still a delay of 10–14 days from the high point of inhibition (and presumed concomitant aging) until signs of neuropathy appear (Johnson and Lotti, 1980; Lotti and Johnson, 1980). These results suggest that simple loss of catalytic activity of NTE is not the damaging event in OPIDN. Thus, the notion has arisen that the ''biochemical lesion'' that triggers OPIDN is the formation of a chemically modified (aged) protein that has gained a toxic function in the neuron (Glynn, 2000; Richardson, 1984).

The pathogenic role of aged NTE is not known, but it is tempting to speculate that it may be involved in an axonal self-destruct program that has been proposed to be an important mechanism in neurodegenerative disorders, e.g. motor neuron disease (Raff *et al.*, 2002). Such a program might be triggered by chemically modified NTE acting through a toxic gain of function mechanism. On the other hand, conventional (Moser *et al.*, 2004; Mühlig-Versen *et al.*, 2005; Winrow *et al.*, 2003) and conditional (Akassoglou *et al.*, 2004) NTE knockout experiments, along with demonstrations of a likely role of NTE in membrane lipid metabolism (Quistad *et al.*, 2003; Van Tienhoven *et al.*, 2002; Zaccheo *et al.*, 2004), indicate that lethality or neuropathology can result from a loss of function of NTE (Glynn, 2006). Nevertheless, while the physiological and pathogenic roles of NTE are being deciphered, the fact that an excellent correlation exists between inhibition/aging of NTE and induction of OPIDN is sufficient to use this information for the development of biomarkers and biosensors for DN agents (Makhaeva *et al.*, 2003, 2007).

V. KINETICS OF OP INHIBITOR–SERINE HYDROLASE INTERACTIONS

A. Introduction

The inhibitory and postinhibitory steps in the interaction of an OP compound with a serine hydrolase (E-OH) such as NTE or AChE are illustrated in Figure 57.6. The mathematical relationships describing the kinetics of irreversible inhibition of serine esterases by OP compounds and postinhibitory reactions of the enzyme–inhibitor adduct (conjugate) summarized here were elegantly set forth in the classic work by Aldridge and Reiner (1972), and synopses are available in other sources (Clothier *et al.*, 1981; Main, 1980; Richardson, 1992). The equations featured below provide the foundation for determining the inhibitory potency of OP compounds against serine hydrolases as well as the rates of reactivation or aging of the inhibited hydrolases. Such approaches can be employed to assess the neuropathic potential of compounds and to distinguish conventional nerve agents from DN agents using biomarkers and biosensors.

B. Inhibition

The first step leading to inhibition is a reversible association of the OP compound (depicted in Figure 57.6 as a phosphonate) and enzyme to form a Michaelis-type complex (shown in square brackets); the associated rate constants for the forward (k_{+1}) and back (k_{-1}) reactions are shown on the double arrow. The active site serine hydroxyl of the hydrolase then engages in a nucleophilic attack on the phosphorus atom of the OP inhibitor, displacing the primary leaving group (X^-), resulting in a phosphonylated enzyme with net loss of HX. The primary leaving group, X^-, is typically a conjugate base of a strong acid, HX, such as HF. The phosphonylation rate constant is shown as k_2 (Richardson, 1992).

Considering only the first two steps in the reaction sequence, which is appropriate for most acylating inhibitors, the affinity of the inhibitor for the enzyme is given by the Michaelis-type equilibrium constant,

$$K_a = (k_{-1} + k_2)/k_{+1} \tag{57.1}$$

The overall progress of the reaction from E-OH and inhibitor to phosphonylated enzyme is characterized by the bimolecular rate constant of inhibition, k_i. This important measure of inhibitory potency is determined by measuring the activity remaining as a function of time of preincubation with various concentrations of inhibitor. The substrate is

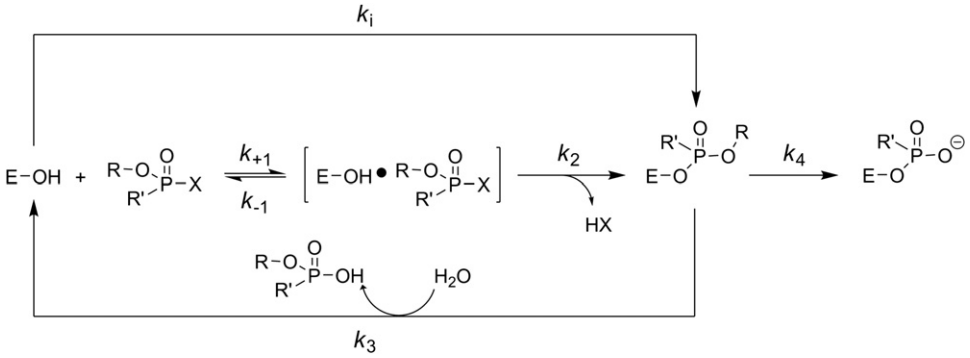

FIGURE 57.6. Reactions between a serine hydrolase and an organophosphonate inhibitor showing associated rate constants. The initial reversible reaction with forward (k_{+1}) and back (k_{-1}) rate constants forms a Michaelis-type complex. The complex can undergo phosphonylation (rate constant k_2) with expulsion of the primary leaving group, X. The overall progress of the reaction from enzyme to phosphonylated (inhibited) product is characterized by the bimolecular rate constant, k_i. The phosphonylated enzyme can undergo spontaneous reactivation (hydrolysis in the presence of excess water) described by the pseudo-first-order rate constant, k_3, or undergo net loss of the R-group (aging) characterized by the first-order rate constant, k_4 (Richardson, 1992).

added after the preincubation interval (Richardson, 1992). When the inhibitor concentration [OP] $<< K_a$,

$$k_i = k_2/K_a \tag{57.2}$$

So-called ideal first-order kinetics of inhibition are obtained under the commonly observed conditions when the concentration of the Michaelis-type complex is small, k_2 is high, k_3 is much smaller than k_2, and [OP] > 10[E-OH], where [E-OH] = concentration of native enzyme. In such cases,

$$\ln(v/v_0) = -k_2[\text{OP}]t/([\text{OP}] + K_a) \tag{57.3}$$

In Eq. (57.3), v is the rate of enzymatic hydrolysis of its substrate at time $= t$, and v_0 is the rate at time zero. Substituting (% activity/100) for (v/v_0) and letting $k' = k_2[\text{OP}]/([\text{OP}] + K_a)$ yields

$$\ln(\%\ \text{activity}/100) = -k't \tag{57.4}$$

Thus, primary plots of ln(% activity/100) versus t will be straight lines with slopes $= -k'$ as shown in Figure 57.7A. In addition, the experimentally determined dependence of % activity remaining on the preincubation time (t) and inhibitor concentration [OP] is

$$\ln(\%\ \text{activity}/100) = -k_i[\text{OP}]t \tag{57.5}$$

Setting Eq. (57.4) and Eq. (57.5) equal to each other gives

$$k' = k_i[\text{OP}] \tag{57.6}$$

Therefore, a secondary plot of $-k'$ versus [OP] will yield a straight line with slope $= k_i$ as shown in Figure 57.7B. The bimolecular rate constant thus obtained is an indication of the inhibitory potency of a given compound against a particular serine hydrolase. As stated above, k_i is a measure of the overall progress of the reaction between the inhibitor and enzyme to yield the organophosphylated product. However, it is important to realize as shown in Eq. (57.2) that k_i is a composite of K_a, which is an indication of the affinity of the inhibitor for the enzyme, and k_2, which is an indication of the rate of phosphylation of the enzyme by the inhibitor. Inspection of Eq. (57.2) and Eq. (57.5) shows that the units of k_i are $[\text{OP}]^{-1}t^{-1}$; for example, the k_i obtained for chlorpyrifos methyl oxon (CPMO) against hen brain microsomal NTE at pH 8.0 and 37°C (Figure 57.7) is $5.82 \times 10^4\ \text{M}^{-1}\,\text{min}^{-1}$ (Kropp and Richardson, 2003; Richardson, 1992).

Under certain conditions, it is possible to determine the K_a and k_2 components of k_i separately. For example, if the secondary plot is not linear, this is an indication of an appreciable concentration of a Michaelis-type complex. In this case, the K_a term must be included; by combining Eq. (57.3) and Eq. (57.4) and rearranging, we have

$$[\text{OP}]/k' = K_a/k_2 + [\text{OP}]/k_2 \tag{57.7}$$

Thus, a Wilkinson plot of $[\text{OP}]/k'$ versus [OP] will yield a straight line with slope $1/k_2$, a y-intercept of K_a/k_2, and an x-intercept of K_a. Moreover, from Eq. (57.2), $k_i = k_2/K_a$; therefore, the reciprocal of the y-intercept gives k_i (Richardson, 1992).

An especially useful relationship is given by substituting a percent activity of interest into Eq. (57.5) to yield the inhibitor concentration at a given time of preincubation with enzyme that would yield the particular percent activity. For example,

$$[\text{OP}]_{50} = I_{50} = \ln 2/(k_i \times t) \approx 0.693/(k_i \times t) \tag{57.8}$$

where $[\text{OP}]_{50} = I_{50} =$ the inhibitor concentration required to produce 50% inhibition of the enzyme at a given time, t, of incubation of enzyme and inhibitor at defined conditions of pH, temperature, and ionic strength before adding substrate. Note that the k_i and I_{50} are reciprocally related and that the I_{50} is time dependent. It is valid to calculate an I_{50} from a k_i value when ideal kinetics are observed. However, it is not valid to calculate a k_i from an experimentally determined fixed-time I_{50}, because the I_{50} alone contains no information

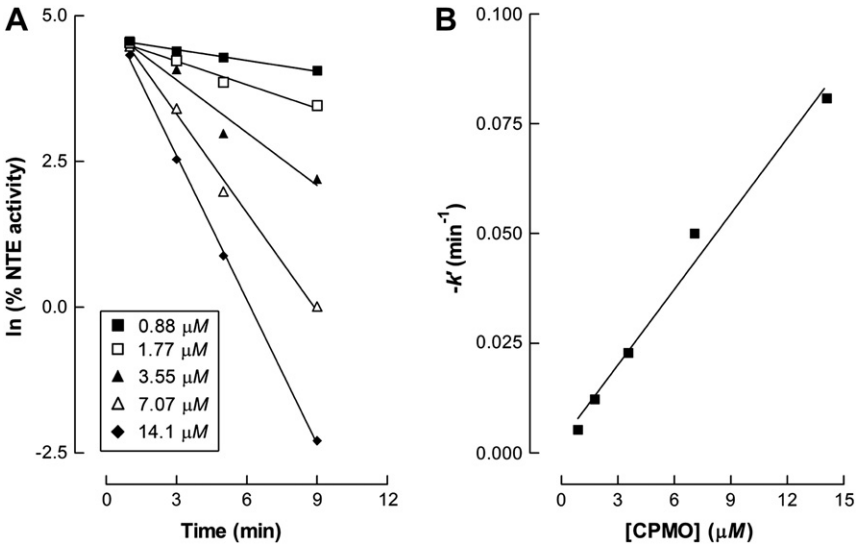

FIGURE 57.7. (A) Primary kinetic plots of ln(% NTE activity) versus time for various indicated concentrations of chlorpyrifos methyl oxon (CPMO). (B) Slopes ($-k'$) of primary kinetic plots versus inhibitor concentration. Data are for hen brain microsomal NTE at pH 8.0 and 37°C. Reproduced with permission from Kropp and Richardson (2003).

about the kinetic behavior of the inhibition reaction. In addition, if inhibitory potency is assessed by measuring fixed-time I_{50} values, it is essential to report the time of preincubation along with the concentration, because the I_{50} decreases as the preincubation time increases (Aldridge and Reiner, 1972; Clothier *et al.*, 1981; Richardson, 1992). For example, using Eq. (57.8), the 20 min I_{50} for chlorpyrifos methyl oxon against hen brain microsomal NTE at pH 8.0 and 37°C may be calculated from the k_i given above to be $0.693/(5.82 \times 10^4\ M^{-1}\ min^{-1})(20\ min) = 5.95 \times 10^{-7}\ M \approx 0.60\ \mu M$ (Kropp and Richardson, 2003).

C. Reactivation

The phosphonylated enzyme can undergo spontaneous reactivation via hydrolysis to yield free enzyme and a phosphonic acid, with an associated pseudo-first-order rate constant, k_3 (the reactivator, water, is present in great excess). The spontaneous rate constant of reactivation, k_3, can be obtained from the relationship

$$\ln(100/\%\ \text{inhibition}) = k_3 t \tag{57.9}$$

for which

$$\%\ \text{inhibition} = [(A - A_t)/(A - A_0)] \times 100 \tag{57.10}$$

where A is the activity of the control, A_t is the activity of the inhibited enzyme at time t, and A_0 is the activity of the inhibited enzyme at $t = 0$. In practice, reactivation is studied by preincubating the enzyme with a concentration of inhibitor that rapidly produces essentially complete inhibition. The inhibitor and enzyme are then rapidly separated or the preparation is diluted sufficiently to prevent further appreciable inhibition of free enzyme, and the return of enzyme activity is measured at timed intervals. The slope (k_3) of the least-squares best-fit line from a plot of ln(100/% inhibition) versus t is determined using linear regression from the initial linear portion of the curve. From Eq. (57.9), it can be seen that the units of k_3 are t^{-1}, e.g. min^{-1}, and that the half-life, $t_{1/2}$, the time required for 50% reactivation, can be determined to be

$$t_{1/2} = \ln 2/k_3 \approx 0.693/k_3 \tag{57.11}$$

The apparent half-life of reactivation can range from minutes to weeks, depending upon the enzyme, inhibitor, pH, and temperature. Reactivation can appear to be slow or nonexistent if aging has occurred, because the aged form of the phosphylated enzyme is stable to hydrolysis and will not reactivate (Jianmongkol *et al.*, 1999; Richardson, 1992).

D. Aging

The inhibited esterase can also undergo aging, e.g. via net loss of the R-group to yield the negatively charged phosphonyl adduct on the active site serine of the enzyme. Aging is characterized by a first-order rate constant, k_4, and the operational definition of this reaction is the time-dependent loss of the ability to reactivate the inhibited enzyme by powerful nucleophiles, such as fluoride ion or oximes. The method of determining aging is similar to that for reactivation. Enzyme is preincubated with a concentration of inhibitor sufficient to produce essentially complete inhibition in a relatively short time. Aliquots are then treated with a reactivator such as fluoride or an oxime and the amount of activity relative to a nonreactivated sample is measured. The first-order rate constant of aging, k_4, can be determined from the relationship

$$\ln[100/(\%\ \text{reactivation})] = k_4 t \tag{57.12}$$

for which

$$\%\ \text{reactivation} = [(AR_1 - AI_1)/(AR_0 - AI_0)] \times 100 \tag{57.13}$$

where AR_1 = activity of reactivated enzyme at t_{aging}, AR_0 = activity of reactivated enzyme at $t = 0$, AI_1 = activity of inhibited enzyme without reactivator at t_{aging}, and AI_0 = activity of inhibited enzyme without reactivator at $t = 0$. The slope (k_4) of the least-squares best-fit line from a plot of ln(100/% inhibition) versus t is determined using linear regression from the initial linear portion of the curve. From Eq. (57.12), it can be seen that the units of k_4 are t^{-1}, e.g. min^{-1}, and that the aging half-life, $t_{1/2}$, the time required for 50% aging, can be determined to be

$$t_{1/2} = \ln 2/k_4 \approx 0.693/k_4 \tag{57.14}$$

The apparent half-life of aging depends upon the enzyme, inhibitor, pH, and temperature. However, NTE inhibited with most neuropathic agents exhibits aging half-lives on the order of several minutes, so that the 1–4 week delay between exposure and appearance of signs of OPIDN is not due to the time required for aging of phosphylated NTE (Jianmongkol *et al.*, 1999; Johnson, 1982; Kropp and Richardson, 2007; Richardson, 1992).

E. Relative Inhibitory Potency (RIP)

The neuropathic potential of an OP compound depends upon its ability to inhibit NTE (and presumably for the NTE–OP conjugate to undergo aging). However, an OP compound will not be able to inhibit NTE to a sufficient degree to produce OPIDN if its cholinergic toxicity is too high to permit survival of a neuropathic dose. Therefore, neuropathic potential of an OP compound is a relative concept that depends upon the ability of the compound to inhibit NTE compared to its anti-AChE activity. Compounds can be screened *in vitro* for relative inhibitory potency (RIP) by measuring k_i or I_{50} values and calculating a ratio:

$$\text{RIP} = k_i(\text{AChE})/k_i(\text{NTE}) \tag{57.15}$$

Because of the reciprocal relationship between the k_i and I_{50} described by Eq. (57.8) above,

$$\text{RIP} = I_{50}(\text{NTE})/I_{50}(\text{AChE}) \qquad (57.16)$$

Thus, when the RIP > 1, the compound is a more potent inhibitor of AChE than NTE, and cholinergic toxicity would be expected to predominate over OPIDN. In fact, when the RIP > 1, the dose required to produce OPIDN tends to be greater than the median lethal dose (LD_{50}) for the compound (Kropp and Richardson, 2003; Richardson, 1992).

For example, although chlorpyrifos methyl oxon (CPMO), the active metabolite of the insecticide chlorpyrifos methyl, would seem to be a potent inhibitor of NTE, with $k_i = 5.82 \times 10^4\ M^{-1}\ min^{-1}$ and 20 min $I_{50} = 0.595\ \mu M$, it is a much more potent inhibitor of AChE, with a $k_i = 1.09 \times 10^7\ M^{-1}\ min^{-1}$ and 20 min $I_{50} = 3.18 \times 10^{-9}\ M$ (3.18 nM), so that the RIP for this compound is 187. This value is $\gg 1$, indicating that OPIDN could not be produced unless the dose were $\gg LD_{50}$ and aggressive therapy for cholinergic toxicity were instituted (Kropp and Richardson, 2003). In contrast, the compound di-*n*-pentyl 2,2-dichlorovinyl phosphate (*n*- pentyl DCV) has an RIP of 0.03 and produces OPIDN at a dose of 2 mg/kg, which is much lower than the LD_{50} dose of 26 mg/kg (Richardson, 1992). OP inhibitors with extremely high selectivity for NTE have been synthesized, e.g. ethyl *n*-octylphosphonofluoridate (EOPF) (Figure 57.3) (Wu and Casida, 1995), which has 20 min I_{50} values for mouse brain NTE and AChE of 0.02 and 54 nM, respectively, yielding an RIP value of 3.7×10^{-4} (i.e. about 2,700 times more potent for NTE than AChE) (Wu and Casida, 1996). Note that EOPF is a chiral compound, and the reported I_{50} and RIP values are for the racemic mixture. Given that inhibitory potency can vary considerably between *R* and *S* forms of OP compounds (Wu and Casida, 1994), it would be of interest to carry out such measurements on resolved isomers.

Because RIP values can encompass several orders of magnitude above and below 1, it is convenient to visualize such data by using log (RIP) as shown in Figure 57.8. It can readily be seen that higher positive numbers correspond to greater cholinergic propensity, as typified by the insecticide active metabolite, CPMO. Likewise, larger negative numbers correspond to greater neuropathic potential, as exemplified by the DN agent, EOPF. It is also interesting to see the steady progression from cholinergic to neuropathic tendency exhibited by the homologous series of dialkyl 2,2-dichlorovinyl phosphates (dichlorvos, DCV) compounds. In this and other series of alkyl OP inhibitors, increasing length of alkyl chains tends to produce decreasing potency against AChE and increasing potency against NTE until maximum selectivity is reached, after which potencies and selectivity decline (Richardson, 1992; Malygin *et al.*, 2003). The highest potency for NTE appears to have been achieved with certain phosphonofluoridates, where the sum of alkyl and alkoxyl chain length is 12–16 atoms, including O and P; in this series, EOPF has the highest potency and selectivity for NTE (Wu and Casida, 1995, 1996).

Determinations of RIP carried out *in vitro* using preparations of AChE and NTE can only assess the neuropathic potential of OP compounds that are direct-acting inhibitors, i.e. compounds that do not require metabolic activation. Such preparations would also not detect effects of detoxification that might occur *in vivo*. To account for the possibility of metabolic activation/deactivation, the system can be augmented by preincubating the compound of interest with xenobiotic metabolic enzymes (or chemical activators, e.g. bromine) before mixing with AChE or NTE preparations (Barber *et al.*, 1999). The concept of the RIP can also be extended to assaying AChE and NTE *ex vivo* and calculating activity ratios after dosing laboratory animals with the compound of interest. Because the adult hen is the species of choice for assessing the neuropathic potential of OP compounds, this species could also be used for *ex vivo* RIP determinations (Richardson, 1992; Richardson *et al.*, 1993). However, despite the fact that rats and mice are not as suitable as hens for studying the clinical and histopathological effects of DN agents, rodent species have AChE and NTE in their nervous systems and can therefore serve as sources of these enzymes for *in vitro* or *ex vivo* determinations of neuropathic potential (Novak and Padilla, 1986; Quistad *et al.*, 2003; Wu and Casida, 1996).

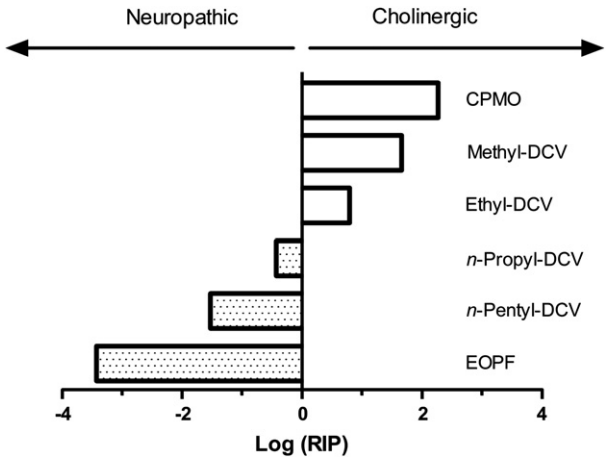

FIGURE 57.8. Spectrum of log values of relative inhibitory potency (RIP) = k_i(AChE)/k_i(NTE), where k_i = bimolecular rate constant of inhibition. As log(RIP) becomes more positive, cholinergic potential increases; as log(RIP) becomes more negative, delayed neuropathic potential increases. CPMO – chlorpyrifos methyl oxon. DCV derivatives refer to symmetrical dialkyl-2,2-dichlorovinyl phosphate (dichlorvos) compounds. EOPF – ethyl *n*-octylphosphonofluoridate. Data from Kropp and Richardson (2003); Richardson (1992); and Wu and Casida (1996).

VI. BIOMARKERS

A. Introduction

A widely quoted definition of ''biomarker'' is that it is ''a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention'' (Atkinson *et al.*, 2001). Although this definition was developed from the standpoint of pharmacology and therapeutics, it can easily be modified to apply to toxicology. In a toxicological setting, a biomarker would consist of an appropriate measure of a chemically induced pathological process, e.g. a response to a toxicant that could be considered injurious or diagnostic of potential injury. Ideally, a toxicological biomarker would represent a biological response that would be pathognomonic of a specific agent or at least a selective indicator of the effects of a class of agents. Thus, a biomarker of a DN agent would involve more than measuring the mere presence of the compound; instead, it would assess a distinctive and preferably early feature of the pathogenesis of OPIDN. Because the earliest known molecular events associated with OPIDN are the inhibition and aging of NTE, the appropriate biomarkers of DN agents would be measurements of these reactions, which could be done via determination of inhibition and aging using enzymology, or through identification of the resulting OP–NTE conjugates using mass spectrometry.

B. Enzymological Measurements of NTE Inhibition and Aging

NTE activity in lymphocytes and platelets was discovered by Dudek and Richardson (1978, 1982), characterized in human populations (Bertoncin *et al.*, 1985; Maroni and Bleecker, 1986), and used in animal (Makhaeva *et al.*, 2003; Richardson and Dudek, 1983; Richardson *et al.*, 1993; Schwab and Richardson, 1986) and human (Lotti *et al.*, 1983, 1986; McConnell *et al.*, 1999) studies to monitor exposures to potentially neuropathic OP compounds. NTE activity is not found in erythrocytes or in plasma or serum. These studies indicate that NTE in lymphocytes, platelets, or whole blood can be assayed and used as a biomarker of exposure to DN agents, particularly if the blood samples are taken within 24 h of acute exposures. Attempts to measure the aging of NTE in lymphocytes or platelets of exposed subjects have apparently not been done. However, given that aging of NTE inhibited by DN agents typically occurs within a half-life of a few minutes (Clothier and Johnson, 1979; Johnson, 1982), aging would be complete by the time that lymphocyte samples could be taken and assayed for activity. Nevertheless, as stated above, given the apparent requirement for aging as well as inhibition of NTE in OPIDN, to help rule out false positives arising from inhibition by Type B (nonaging) inhibitors (Figure 57.5), aging studies ought to be undertaken in future work. As noted below, mass spectrometry can be used to identify the phosphyl adduct on the inhibited enzyme, thus differentiating between inhibited and aged forms.

Recently, a lysophosphatidylcholine (LysoPC) hydrolase activity has been characterized in mouse and human erythrocytes (Vose *et al.*, 2007). This enzyme exhibits a similar inhibitory profile to that of NTE; therefore, it might be used along with lymphocyte or platelet NTE as an additional biomarker of exposure to DN agents. However, high inter-individual variation in LysoPC hydrolase was seen, possibly related to dietary lipids, which would require establishing pre-exposure baseline values for each subject.

In keeping with the concept of the RIP discussed under ''Relative Inhibitory Potency'', above, inhibition of lymphocyte and/or platelet NTE and possibly erythrocyte LysoPC hydrolase should be used in conjunction with inhibition of erythrocyte AChE and plasma butyrylcholinesterase (BChE) to assess the likelihood that an exposure to OP compounds would produce cholinergic and/or delayed neuropathic effects. Erythrocyte AChE inhibition has long been used as a biomarker of exposure to conventional nerve agents or OP insecticides (Lotti, 1995; Wilson and Henderson, 1992). BChE can be sensitive to both conventional and DN agents, and its inhibition could thus serve as a general biomarker for OP agents (Kropp and Richardson, 2007; Van der Schans *et al.*, 2008).

C. Identification of NTE–OP Conjugates using Mass Spectrometry

Mass spectrometry has proved to be an excellent tool for identifying the OP conjugates (adducts) of serine hydrolases such as AChE and BChE (Doorn *et al.*, 2000; Elhanany *et al.*, 2001; Jennings *et al.*, 2003; Kropp and Richardson, 2007; Li *et al.*, 2007; Van der Schans *et al.*, 2008). One approach is to reactivate inhibited esterases with fluoride and to detect the liberated OP fluoridate using gas chromatography–mass spectrometry; however, this technique cannot be used if the esterase conjugate has aged (Degenhardt *et al.*, 2004; Solano *et al.*, 2008). Because NTE inhibited with DN agents undergoes rapid aging (Johnson, 1982), it is important to be able to detect the aged adducts. Therefore, peptide mass mapping would be the current method of choice, whereby the active site peptide containing the phosphyl moiety on the active site serine is identified using mass spectrometry following digestion of the inactivated esterase with trypsin or other proteases (Doorn *et al.*, 2001b). Although some DN agents have extremely high potency and selectivity for NTE, in general there will be some degree of inhibitory overlap with other serine hydrolases (e.g. see Figure 57.8). Accordingly, in addition to examining NTE, it would be useful to acquire mass spectrometry data on OP conjugates of other potential esterase targets, such as AChE and BChE, with particular attention being paid to the aged species.

The classical view of the aging reaction of OP-inhibited serine esterases, based on studies of soman-inhibited AChE, is that aging involves an S_N1 reaction at a ligand carbon and transient formation of a carbocation that dissociates from the enzyme (Michel *et al.*, 1967; Shafferman *et al.*, 1996). The traditional model has been confirmed using mass spectrometry for BChE inhibited by a variety of phosphates and phosphonates and allowed to age in the presence of [^{18}O]-H_2O (Li *et al.*, 2007). Thus, it appears that cholinesterases inhibited by phosphates or phosphonates undergo aging via S_N1 cleavage of an O–C bond rather than S_N2 cleavage of a P–C bond.

However, it is important to realize that mechanisms of inhibition and aging can differ depending upon the esterase, inhibitor, and stereochemistry of the inhibitor. For example, the aging of AChE inhibited by ethyl *N*,*N*-dimethylphosphoramidocyanidate (tabun) was shown to proceed via hydrolytic P–N bond scission with loss of dimethylamine (Elhanany *et al.*, 2001). In addition, the mechanism of inhibition of AChE, BChE, and cholesterol esterase by the resolved stereoisomers of isomalathion has been shown to differ between the (1*R*) and (1*S*) forms, and the mechanism of aging in each case appears to proceed via S_N2 displacement of a secondary leaving group by water, involving cleavage of a P–S bond (Doorn *et al.*, 2000, 2001a, b, 2003). A subsequent study of BChE inhibited with racemic isomalathion confirmed that the major pathway for aging involved scission of the P–S bond, but relatively small peaks in the mass spectrum indicated that O–C and S–C cleavage could also occur as minor pathways (Li *et al.*, 2007).

For NTE inhibited by phosphates or phosphonates, the aging mechanism has been thought to be consistent with an S_N2 reaction at phosphorus (Johnson, 1982), although this hypothesis has not been rigorously tested. With respect to stereochemical influences on aging, limited studies indicate that both enantiomers of certain phosphonates inhibit NTE, but only one enantiomer undergoes aging (Johnson *et al.*, 1985, 1986). As expected, the isomer that inhibits NTE and ages produces OPIDN. In contrast, the isomer that inhibits NTE without aging does not produce OPIDN. Furthermore, the nonaging isomer protects against neuropathy from subsequent administration of a neuropathic OP compound (Johnson and Read, 1987; Johnson *et al.*, 1988). Some racemic phosphonates have been found to produce OPIDN at lower than expected threshold levels of inhibited/aged NTE (i.e. <70%), possibly because of potentiation or promotion of subclinical axonopathy by the nonaging enantiomer, presumably by the action of the nonaging enantiomer on one or more targets other than NTE that could be involved in repair or regeneration of neural lesions (Lotti, 1992).

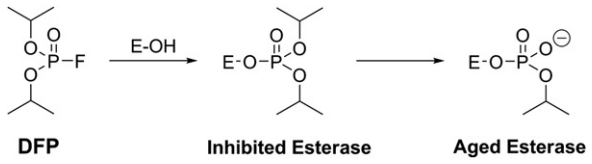

FIGURE 57.9. Inhibition and aging of serine esterases by diisopropylphosphorofluoridate (DFP). The active site serine is organophosphorylated in the inhibition step. Aging results in net loss of an isopropyl group to yield the monoisopropylphosphoryl esterase. This mode of inhibition and aging has been established for acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and neuropathy target esterase catalytic domain (NEST) (Kropp and Richardson, 2007).

Perhaps the most thoroughly investigated compound with respect to inhibition and aging of serine hydrolases is diisopropylphosphorofluoridate (DFP). Using a variety of techniques, this compound has been shown to inhibit AChE (Clothier and Johnson, 1979; Kropp and Richardson, 2006; Millard *et al.*, 1999a, b; Ordentlich *et al.*, 1996, 1998), BChE (Kropp and Richardson, 2007; Li *et al.*, 2007; Masson *et al.*, 1997), NTE (Clothier and Johnson, 1979; Williams, 1983; Williams and Johnson, 1981), and NEST (Kropp *et al.*, 2004) by organophosphorylation of the active site serine followed by aging to yield a monoisopropylphosphoryl adduct, as shown in Figure 57.9. Esterase adducts have been directly demonstrated using mass spectrometry for AChE (Kropp and Richardson, 2006), BChE (Kropp and Richardson, 2007; Li *et al.*, 2007), and NEST (Kropp *et al.*, 2004), and in the case of BChE, the mechanism of aging has been unequivocally demonstrated to involve scission of an O–C bond in an isopropyl group (Li *et al.*, 2007). These results, taken together with those for soman-inhibited AChE (Michel *et al.*, 1967) and BChE inactivated with a variety of phosphonates and phosphates (Li *et al.*, 2007), indicate that it is reasonable to expect that aging of phosphonylated or phosphorylated serine esterases, including NTE and NEST, will proceed via O–C bond scission. This insight enables accurate prediction of the biomarker species to be sought in the mass spectra of serine esterases inhibited with phosphonates or phosphates.

Among the type A inhibitors of NTE (Figure 57.5), the phosphoramidates are perhaps the most enigmatic to be studied to date. For example, mipafox, the phosphoramidate analog of DFP, is capable of producing suprathreshold inhibition (>70%) of neural NTE *in vivo* and causing OPIDN (Davis and Richardson, 1980). Therefore, mipafox-inhibited NTE was expected to undergo aging (Davis *et al.*, 1985). Indeed, early studies showed that NTE inhibited by mipafox was intractable to reactivation, indicating the possibility of aging as a mechanism for yielding a nonreactivatable enzyme (Johnson, 1982). In the case of mipafox-inhibited NTE, however, nonreactivation was thought to be too rapid to measure by standard kinetic approaches (Johnson, 1982). Consequently, aging of mipafox-inhibited NTE had only been inferred and not directly demonstrated. Subsequently, Milatovic and Johnson (1993) showed that NTE inhibited by mipafox could be reactivated by prolonged treatment with KF at acidic pH. This surprising finding led to the conclusion that aging had not

occurred after all. Instead, it was surmised that an unusual property of the bond between the phosphoramidate and the active site serine was responsible for the stability of the complex as well as the perturbation of NTE that leads to OPIDN.

It had been known for some time that esterases inhibited by mipafox are resistant to reactivation, but conventional aging could not be proved or disproved by classical techniques. It is also known that the phosphoramido hydrogen on *N*-alkylphosphoramidates is acidic and that its removal yields a resonance-stabilized anion (Eto, 1974). Such a mechanism would explain the relative stability of phosphoramidated esterases in a manner that does not require loss of an alkylamino group to yield a negative charge on the adduct (Richardson, 1995). Moreover, reprotonation of the phosphoramide anion would generate a neutral species subject to attack by a nucleophile and account for the reactivation of mipafox-inhibited NTE at low pH. Under the acidic conditions usually employed for peptide mass spectrometry, the putative anion would be reprotonated. Thus, it can be hypothesized that the species attached to NTE after its inhibition by mipafox and allowing time for aging is the intact *N*,*N*′-diisopropylphosphorodiamido moiety. Such a result would indicate that the inhibited enzyme has not lost an isopropyl or isopropylamino group due to an aging process analogous to the net loss of an isopropyl group in the aging of DFP-inhibited NTE or NEST.

In contrast to the results for mipafox-inhibited NTE, early limited studies indicated that mipafox-inhibited BChE or AChE undergo irreversible aging, because the enzymes could be reactivated soon after inhibition, but not after 18 h (Milatovic and Johnson, 1993). The chemistry of phosphoramidates suggest that aging could involve hydrolytic loss of an alkylamino group. For example, acid-catalyzed P–N bond fission has been observed for certain *N*-alkyl phosphoramidates (Eto, 1974) and the aging of tabun-inhibited AChE has been shown to proceed via P–N bond scission with loss of dimethylamine (Elhanany *et al.*, 2001).

Considerations of probable mechanisms of aging for mipafox-inhibited serine esterases suggest that mass spectrometry studies would support a deprotonation mechanism for NTE or NEST and hydrolytic P–N bond scission for AChE and BChE. As summarized in Figure 57.10, these expectations were borne out for human recombinant NEST, which was used as a surrogate for NTE (Kropp *et al.*, 2004), and BChE, respectively (Kropp and Richardson, 2007). However, mipafox-inhibited AChE gave the surprising result, confirmed by immunoprecipitation and mass spectrometry, of loss of both isopropylamine groups to yield a simple phosphate adduct on the active site peptide (Kropp and Richardson, 2006). Thus, in the case of phosphoramidates, aging appears to be nonclassical for the three esterases investigated; moreover, the biomarker is distinctly different in each case.

VII. BIOSENSORS

A. Nanostructured Electrochemical Biosensors to Measure Enzyme Activity

Recently developed nanofabrication methods using layer-by-layer (LBL) self-assembly provide exciting new opportunities to design multilayered, functional, biosensor interfaces to measure enzyme activity. Since the LBL technique was first introduced (Decher and Hong, 1991), significant work has been done to fabricate polymer and organic thin films through alternating deposition of positively and negatively charged polyelectrolyte species on solid surfaces (Decher, 1997; Decher *et al.*, 1992; Lvov *et al.*, 1994; Ruths *et al.*, 2000; Schmidt *et al.*, 1999). Polyelectrolyte layers typically have a thickness on the order of a nanometer, allowing ultrathin interfaces to be fabricated that have minimal mass-transfer resistance. LBL self-assembly using polyelectrolytes is thus well suited for biosensor development studies, because, by minimizing

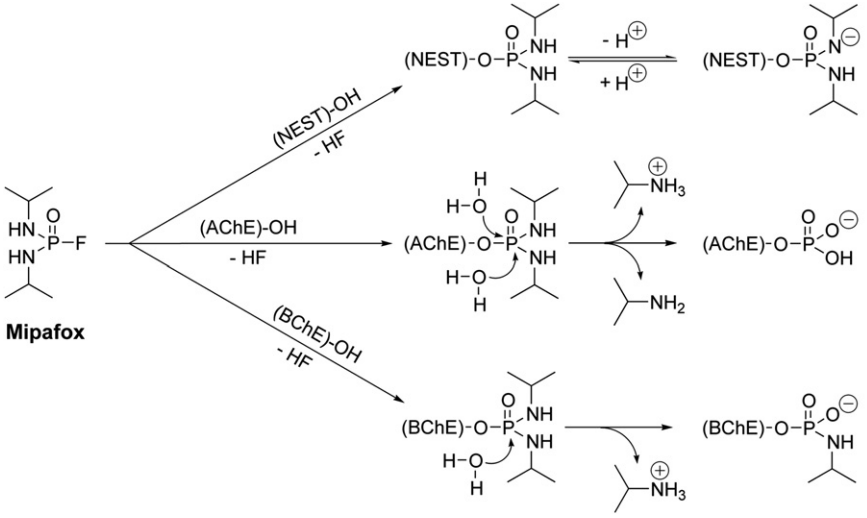

FIGURE 57.10. Inhibition and aging of serine esterases by *N*,*N*′-diisopropylphosphorodiamidic fluoride (mipafox). (A) Neuropathy target esterase catalytic domain (NEST) showing aging by reversible deprotonation of a phosphoramido nitrogen. (B) Acetylcholinesterase (AChE) showing aging by displacement of both isopropylamine groups by water. (C) Butyrylcholinesterase (BChE) showing aging by displacement of a single isopropylamine group by water. Reproduced with permission from Kropp and Richardson (2007).

mass-transfer resistances, the intrinsic enzyme kinetics can be measured. LBL assemblies of polyelectrolytes have been used to develop nanostructured biosensor interfaces that encapsulate enzymes (Hassler *et al.*, 2007; Kohli *et al.*, 2006, 2007a) and functional nanoparticles (Kohli *et al.*, 2005, 2007b). Electrochemical biosensors that measure the activity of a protein have been widely used to determine analyte concentrations, in both research and commercial applications (Prodromidis and Karayannis, 2002). Biosensors can detect protein activity either directly (Hassler and Worden, 2006) or indirectly through reaction coupling (Peteu *et al.*, 1996, 1998). The indirect approach greatly expands the range of enzyme activities that can be characterized using electrochemical biosensors. For example, reaction coupling has been used to measure NTE activity outside of the electrode, e.g. in blood samples (Makhaeva *et al.*, 2003, 2007) or AChE, BChE, and NEST activity incorporated into the electrode (Kohli *et al.*, 2007a).

B. Electrochemical Biosensor Arrays for High-Throughput Analysis

Microsystems technologies, particularly thin film deposition of microelectrodes and formation of microfluidic channels, have been widely applied to biological analysis systems (Bergveld, 1996; Urban, 2000), such as DNA-processing chips (Raisi *et al.*, 2004) and other lab-on-chip implementations (Kovacs, 2003; McFadden, 2002; Ziegler, 2000). Most of these devices rely on optical transduction mechanisms (Rabbany *et al.*, 1994; Vo-Dinh *et al.*, 2003) that often cannot quantitatively measure several important protein activities, such as redox reactions. In contrast, these parameters are readily measured using versatile electrochemical techniques. Traditionally, electrochemical measurements are performed at the ''beaker scale'', but newer technologies allow such systems to be miniaturized for improved performance and high-throughput analysis. Complete three-electrode electrochemical cells, including thin film Ag/AgCl reference electrodes, have been integrated on silicon surfaces (Yun *et al.*, 2004). Thin film reference electrode stability has been improved using Nafion or polyurethane coatings (Nolan *et al.*, 1997) and complex micromachined structures (Suzuki, 2000; Suzuki *et al.*, 1999). A remaining challenge is to adapt these new technologies to the construction of miniaturized electrochemical arrays for high-throughput protein activity characterization. Such biosensor array platforms must be compatible with the self-assembled biosensor interfaces, thus

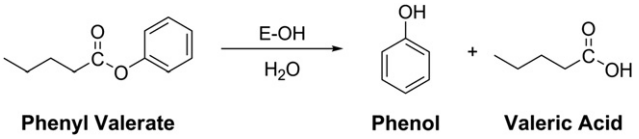

FIGURE 57.11. Reaction of a serine esterase with phenyl valerate to yield phenol and valeric acid (Kayyali *et al.*, 1991).

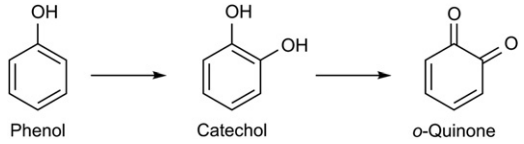

FIGURE 57.12. Reactions catalyzed by the two activities of tyrosinase. Phenol is first oxidized to catechol and then to *o*-quinone. Reproduced with permission from Kohli *et al.* (2007a).

establishing requirements for electrode materials, geometries, surface smoothness, and overall array architecture.

C. Assembly of Electrochemical Biosensor Interfaces for Serine Hydrolases

The natural reaction substrates and products of serine esterases are not electrochemically active. In order to transduce these esterase activities into an electrical signal, a two-enzyme reaction pathway can be assembled on a gold electrode. In the first reaction, the esterase of interest converts a phenyl ester (e.g. phenyl valerate) into phenol (Figure 57.11). In the second reaction, tyrosinase converts phenol first to catechol, and then to *o*-quinone (Figure 57.12), which can be electrochemically reduced back to catechol at an electrode, yielding a measurable current (Eq.57.17) (Kohli *et al.*, 2007a).

$$o\text{-Quinone} + 2H^+ + 2e^- \rightarrow \text{Catechol} \qquad (57.17)$$

Tyrosinase is a copper-containing oxidase (Coche-Guerente *et al.*, 2001; Forzani *et al.*, 2000), which possesses the two different activities illustrated in Figure 57.12. In the first step, referred to as the hydroxylase or cresolase activity, molecular oxygen is used to hydroxylate phenol to form catechol. In the second step, known as the catecholase activity, the enzyme oxidizes catechol to *o*-quinone, which is simultaneously oxidized by oxygen to its original form, with the production of water. The *o*-quinone is electrochemically active and can be reduced back to catechol, as illustrated above in Eq. (57.17).

By co-immobilizing tyrosinase with a serine esterase on a gold electrode, it is possible to establish a multistep reaction pathway that allows the activity of the esterase to be determined indirectly via measurement of *o*-quinone reduction at the electrode. The molecular architecture of a bi-enzyme sensor interface is shown schematically in Figure 57.13.

In practice, the fabrication of these bi-enzyme biosensors is relatively straightforward. Gold electrodes are scrupulously cleaned in ''Piranha solution'' [(concentrated) H_2SO_4: 30% (w/w) H_2O_2, 7:3 (v/v)] and then dipped in a 5 mM solution of thioctic acid in ethanol for 30 min. The electrodes are washed with ethanol, dried under nitrogen, and dipped in positively charged poly-L-lysine (PLL) solution for 45 min. The PLL solution is prepared by adding

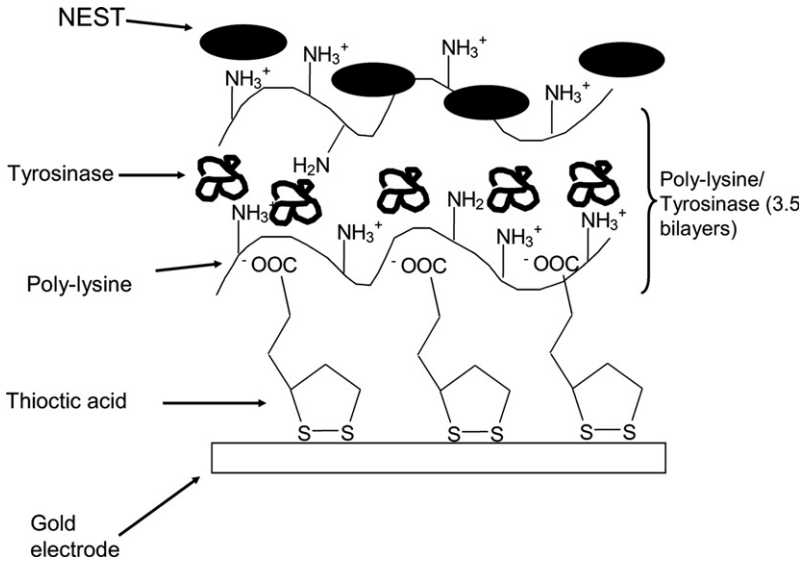

FIGURE 57.13. Molecular architecture of bi-enzyme biosensor electrode containing tyrosinase and neuropathy target esterase catalytic domain (NEST). Reproduced with permission from Kohli *et al.* (2007a).

12 mg of PLL in 50 ml of 20 mM phosphate buffer (pH 8.5). The electrodes are then rinsed with water and dipped in an aqueous solution of negatively charged tyrosinase (0.2 mg/ml) for 1 h. The last two steps are repeated 3.5 times to create 3.5 PLL-Tyr bilayers, with PLL being the topmost layer. The electrodes are washed with water and dipped in a 0.1 mg/ml solution of a serine esterase (e.g. AChE, BChE, or NEST) in 100 mM phosphate buffer, pH 7.0, for 1 h. The electrodes are then washed with water, dried under nitrogen, and dipped in phosphate buffer (0.1 M, pH 7.0) for testing. All steps are done at room temperature (approximately 22°C) (Kohli *et al.*, 2007a).

D. Electrochemical Measurements of Serine Esterase Activity

The electrochemical properties of serine esterase-containing biosensor interfaces are readily characterized. Each biosensor is immersed in a stirred buffer solution and maintained at a potential of −100 mV (vs an Ag/AgCl reference electrode). For example, NEST activity was measured indirectly via the output current of the electrode for a variety of phenyl valerate concentrations. As shown in Figure 57.14, the first step involves the diffusion of the ester (e.g. phenyl valerate) through a stagnant film from bulk solution to the enzyme layer. The NEST enzyme then hydrolyzes phenyl valerate to phenol, which is then oxidized to *o*-quinone by tyrosinase. Reduction of *o*-quinone at the surface of the electrode generates a current and regenerates catechol, which can then be reoxidized by tyrosinase. This internal recycling between catechol and *o*-quinone provides a mechanism for signal amplification, thereby enhancing biosensor sensitivity (Kohli *et al.*, 2007a).

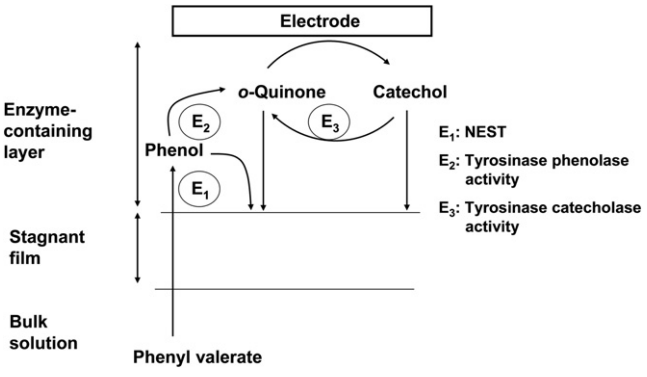

FIGURE 57.14. Schematic representation of the pathway that leads to the generation of current in the bi-enzyme biosensor containing tyrosinase and neuropathy target esterase catalytic domain (NEST). Reproduced with permission from Kohli *et al.* (2007a).

The response of the biosensor to aliquots of ester can be measured at a variety of pH values and applied potentials. For a typical NEST biosensor, the highest signal-to-background ratio is obtained at pH 7.0 and a working electrode potential of −0.1 V relative to an Ag/AgCl reference electrode. At this potential, addition of phenyl valerate to a stirred aqueous solution triggers a rapid increase in the NEST biosensor signal, with a time constant of about 5 s. BChE can be substituted for NEST, with a similar current vs time response using phenyl valerate as substrate. For AChE, phenyl acetate is a better substrate than phenyl valerate. Current increases linearly with phenyl acetate concentration ($R^2 = 0.989$) in the range of 0.5 to 16 µM, reaching saturation at approximately 40 µM. Current vs time response and concentration calibration curves similar to those obtained for NEST and AChE can be obtained for BChE in the bi-enzyme electrode. Sensitivities of the electrodes obtained from the slopes of the initial linear portions of the concentration calibration curves were found to be 180,

25, and 87 $nA\mu M^{-1}\,cm^{-2}$ for AChE, BChE, and NEST, respectively, where AChE used phenyl acetate, and BChE and NEST used phenyl valerate as substrate. Control experiments were also done in which each of the substrates was delivered to a gold electrode containing only PLL-Tyr bilayers. The current sensitivities obtained in these control experiments were negligibly small – always less than $0.5\ nA\mu M^{-1}\,cm^{-2}$, indicating that the signal is mediated by the esterase activity (Kohli *et al.*, 2007a).

The ability of the biosensors to detect chemical agents was confirmed by adding a known serine esterase inhibitor, phenylmethylsulfonyl fluoride (PMSF), to the solution. The electrode response decreased in a concentration-dependent manner (Kohli *et al.*, 2007a). Using a combination of biosensors containing different target enzymes, e.g. AChE and NEST, it would be possible to obtain real-time differential data that could be used to distinguish exposures to conventional versus DN agents.

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

DN agents have not yet been used in warfare or terrorism. However, their possible use must be seriously considered, because it is straightforward to design and synthesize the compounds based on established methods in OP chemistry. Thus, a conventional nerve agent can be reengineered to produce a DN agent. The use of DN agents would be catastrophic, because there are no accepted means of prophylaxis or treatment, and the effects are devastating and permanent. NTE is the presumptive target for initiation of OPIDN. Accordingly, lymphocyte NTE has been used as a biomarker of exposure, and the NTE catalytic domain (NEST) has been incorporated into a biosensor for detection of DN agents. However, the precise physiological function of NTE and its role in the pathogenesis of OPIDN remain to be elucidated. Therefore, further mechanistic understanding is needed in order to improve upon existing biomarkers and biosensors as well as to develop preventive and therapeutic measures.

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CHAPTER 58

Monitoring of Blood Cholinesterase Activity in Workers Exposed to Nerve Agents

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I. INTRODUCTION

Determination of the activity of blood cholinesterases (erythrocyte, RBC acetylcholinesterase, AChE, EC 3.1.1.7 and plasma butyrylcholinesterase, BuChE, EC 3.1.1.8) is currently the most important manner for conformation of the diagnosis of organophosphate (OP) poisoning, for monitoring of the recovery of intoxicated person, or for forensic study purposes (Bajgar, 2004, 2005; Eddleston *et al.*, 2003; Karalliedde *et al.*, 2003; Pope *et al.*, 2005). It is necessary to examine the whole picture of intoxication, i.e. not only biochemical examinations but clinical signs allowing more precise assessment of the prognosis of the intoxication. The evidence supporting AChE as the primary site of both OP nerve agents and pesticides action has been summarized by many authors (Bajgar, 1985, 1991; Lotti, 2000; Marrs, 1993; Mars *et al.*, 1996; Taylor, 1985). Their findings include the following observations: symptoms of OP poisoning are similar to those of the AChE inhibitor physostigmine; the *in vivo* LD_{50} value for a variety of OPs correlates well with the inhibition efficacy to AChE determined *in vitro*; and cholinesterase reactivators (e.g. oximes), anticholinergics (e.g. atropine), and reversible AChE inhibitors (e.g. carbamates) can attenuate OP toxicity. However, there are ample documented data showing that AChE inhibition is not the only important biochemical change during intoxication. These data have described many other changes accompanied by the development of intoxication that might contribute to OP toxicity. They have included changes of other enzymes, neurotransmitters, immune changes, anaphylactoid reaction, behavior, etc. The evidence includes the data indicating that prophylactic/therapeutic drugs might also have multiple sites of action similar to those observed during intoxication (Bajgar, 1991, 1992; Bardin *et al.*, 1987a, b; Cowan *et al.*, 1996, Kassa, 1998). Nevertheless, the first reaction of OP is interaction with cholinesterases in the blood stream (Bajgar, 1985, 1991; Benschop *et al.*, 2001) and then in the target tissues – the peripheral and central nervous system (Bajgar, 1985, 1991; Bardin *et al.*, 1987; Green, 1958, 1983; Marrs, 1993; Mars *et al.*, 1996). The delayed neurotoxic effect is caused by a reason other than cholinesterase inhibition. The neurotoxic esterase has been described as the target site for this symptom; however, only some OPs are neurotoxic in that sense (Abou-Donia *et al.*, 1990; Lotti, 1992; Johnson, 1990, 1992; Johnson and Glynn, 1995; Slotkin *et al.*, 2008).

The mechanism of AChE inhibition for all the OP nerve agents is practically the same, i.e. phosphorylation or phosphonylation of the esteratic site of AChE.

Monitoring the cholinesterase changes during the intoxication is at present the best indication of the severity of OP poisoning as well as a basis for antidotal therapy.

Both the toxicodynamics and toxicokinetics of OP nerve agents can be explained by their biochemical characteristics of interacting with cholinesterases and other hydrolases. Inhibition of cholinesterases in the blood is the first target for OPs according to the principle of ''first come, first served'' (Benschop and de Jong, 2001).

AChE and BuChE differ in their enzymatic properties and physiological function (Massoulié *et al.*, 1993; Darvesh *et al.*, 2003). However, there are other types of cholinesterases such as benzoylcholinesterase, propionylcholinesterase, etc. AChE splits neurotransmitter acetylcholine (ACh) at the cholinergic synapses. A similar reaction is also observed in erythrocytes but its function is not yet known in detail, as is also the case with the function of BuChE activity in plasma, though there is evidence that BuChE plays an important role in cholinergic neurotransmission and could be involved in other nervous system functions, in neurological diseases, and in nonspecific detoxification processes (Darvesh *et al.*, 2003).

II. DETERMINATION OF CHOLINESTERASES

Determination of cholinesterase activity is based on a number of principles. In general, an enzyme is added to the buffered or unbuffered mixture and the enzymatic reaction is initiated by adding the substrate. Different parts of the reaction mixture are determined (continually or noncontinually), i.e. unhydrolyzed substrate or reaction products, either directly or indirectly (Augustinsson, 1971; Holmstedt, 1971; Witter, 1963). The conditions must be

chosen very carefully because of different factors influencing the activity (Reiner and Simeon-Rudolf, 2000, 2006).

According to the procedure and laboratory instrumentation, the most common methods of cholinesterase determination are as follows: electrometric (e.g. Michel, 1949), titration (e.g. Nenner, 1970), manometric (e.g. Witter, 1963), colorimetric detection of the unhydrolyzed substrate (e.g. Hestrin, 1949), measurement by the change of pH using an indicator (Winter, 1960), spectrophotometric (e.g. Siders *et al.*, 1968; Voss and Sachsse, 1970; Worek *et al.*, 1999a), fluorimetric (e.g. Sasaki 1964; Kusu *et al.*, 1990), radiometric (e.g. Israel and Lesbats, 1987), calorimetric (Konickova and Wadso, 1971), polarographic (Fiserova-Bergerova, 1969), enzymatic (Abernethy *et al.*, 1986; Israel and Lesbats, 1987), and others, e.g. near infrared spectroscopy (Domjan *et al.*, 1998). These methods are also suitable for the detection of cholinesterase inhibitors using biosensors (Brimijoin and Rakonczay, 1986; Cremisini *et al.*, 1995; De Jong *et al.*, 1988; Walker *et al.*, 2007) or immunochemical assay for detection of chemical warfare agents (Lenz *et al.*, 1997a, b).

A very sensitive and commonly used method for cholinesterase determination was described by Ellman *et al.* (1961), based on hydrolysis of the thiocholine substrates acetyl- and butyrylthiocholine or others. After enzymatic hydrolysis, the relevant acid and thiocholine are released and thiocholine by its SH-group is detected using 5,5′dithiobis-2 nitrobenzoic acid forming 5-mercapto-2-nitrobenzoic acid determined spectrophotometrically at 412 nm (Figure 58.1).

Sometimes this method is used with specific inhibitors (tetraisopropyl pyrophosphoramide for BuChE inhibition and BW284C51 for AChE inhibition) and there are many modifications described in the literature. This method is in good correlation with other methods. It is sufficiently specific and sensitive and it is used for different purposes in many laboratories around the world. Expression of the activity varies greatly, usually as µmoles of substrate hydrolyzed per minute (time) per ml of material examined (e.g. plasma, serum) or per mg of weight tissue or tissue protein. From these values, the expression of the activity in units can be derived (it is the quantity of enzyme catalyzing µmol of substrate per minute at standard conditions). In the clinical laboratory, the enzyme activity can be also expressed as 1 mol of substrate hydrolyzed per liter or kg (cat/l, kg) per second, which is the hydrolysis of 1 mol of substrate hydrolyzed per second per liter or kg ($mol \cdot s^{-1} \cdot l^{-1}$ or kg^{-1}). There are many publications dealing with the review and modifications of cholinesterase determination. One of the last methodical works improving Ellman's method (Ellman *et al.*, 1961), including a description of the methods, is a paper published by Worek *et al.* (1999a).

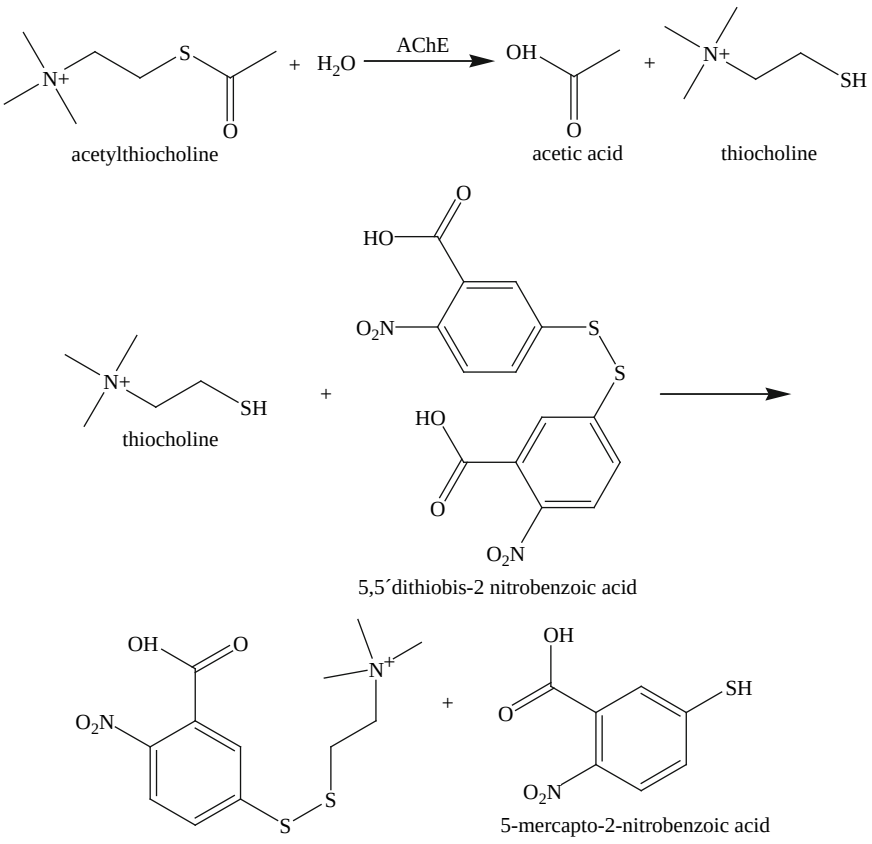

FIGURE 58.1. Acetylthiocholine is hydrolyzed by AChE to acetic acid and thiocholine, which forms yellow 5-mercapto-2-nitrobenzoic acid after reaction with Ellman reagent.

III. FACTORS INFLUENCING ACTIVITY OF CHOLINESTERASES

The influencing of BuChE activity by gamma irradiation, stress, gravidity, certain neurological and psychiatric disorders, hormones, and medical drugs has been demonstrated (Bajgar, 1985, 1998; Brown *et al.*, 1981). The elevation of BuChE activity is not so frequent; an increase in children with nephritic syndrome has been observed; an elevated ratio of BuChE/LDL cholesterol indicates an increase in the risk of cardiovascular diseases (Kutty, 1980; Navratil and Bajgar, 1987). The involvement of BuChE with the fat (cholesterol) metabolism has been suggested by Van Lith *et al.* (1991) and Van Lith and Beynen (1993). The relationship between BuChE activity and experimentally induced diabetes mellitus in rats was also mentioned (Annapurna *et al.*, 1991).

In clinical biochemistry, BuChE determination in the plasma or serum is more frequently used than that of AChE in the red blood cells. Except for intoxication with OP or carbamates (CMs), a BuChE decrease indicates either a reduced enzyme synthesis or a decrease in the number of production of cells in the liver (Masopust, 1983). A special case of diminished BuChE activity is the hereditary affected by the presence of atypical variants of BuChE (Brown *et al.*, 1981; Whittaker, 1980).

There are many other factors influencing BuChE activity and the diagnostic importance of diminished BuChE activity is important for the following states – except hereditary decrease of the activity and poisoning with OP nerve agents and OP/CM pesticides, congenital deficiency, liver damage, acute infection, chronic malnutrition, metastasis (especially liver), myocardial infarction, dermatomyositis, intoxication with carbon disulfide or mercury, and obstructive jaundice (Bajgar, 1991; Bardin *et al.*, 1987a, b; Kutty, 1980; Molphy and Ratthus, 1964; Wyckoff *et al.*, 1968).

Determination of AChE activity is not so widely used in clinical laboratories. A decrease in red blood cell AChE activity in pernicious anemia has been demonstrated; diminished erythrocyte AChE activity is typical for paroxysmal nocturnal hemoglobinemia and ABO incompatibility (Rakonczay, 1988). AChE activity in the erythrocyte membrane can be considered as an indicator of erythrocyte membrane integrity. Increased AChE activity in rectal biopsy is of great diagnostic significance in Hirschsprung's disease, especially in the presence of its atypical molecular form (Bajgar and Hak, 1979; Rakonczay, 1988). There are other publications demonstrating increased AChE activity in the amniotic fluid during pathologic development of the neural tube (Bonham and Attack, 1983). AChE activity in erythrocytes and cerebrospinal fluid is also diminished in some endogenous depressions and other psychiatric disorders; however, the results presented are not quite clear at present (Bajgar, 1985; Bohnen *et al.*, 2007; Martucciello, 2008; Rakonczay, 1988; Skau, 1986).

IV. DIAGNOSIS OF OP POISONING

The determination of cholinesterases in the blood is the basic method for diagnosis and therapy monitoring for OP poisoning though some doubts exist, some preferring the clinical signs of poisoning as a leading tool for OP poisoning diagnosis and monitoring (Bardin *et al.*, 1987a, b). The determination of AChE and BuChE activity in the whole blood is possible. The decrease in these activities is a good marker but the diagnostic validity is limited to the statement that some factors causing a decrease in blood cholinesterases are present. In connection with the anamnestic data (exposure to OP), this is important information. The determination of the red blood cell AChE or plasma BuChE is more informative. There are some discussions dealing with AChE and BuChE activity but which is more important for the diagnosis? In general, AChE activity in the red blood cell can be considered to be more important for diagnosis with nerve agents than plasma BuChE activity. However, there are some discussions dealing with the validity of the BuChE determination. This enzyme was described as a not very good marker of OP poisoning and its determination was proposed for exclusion from the recommended biochemical procedures (Bardin *et al.*, 1987a, b; Molphy and Ratthus, 1964; Wyckoff *et al.*, 1968). The temporal profile of BuChE was studied in a cohort of 25 OP-poisoned patients to examine their relationship to the development of intermediate syndrome. The study suggests that BuChE reflects the clinical course of poisoning and that intermediate syndrome may be associated with a persistent BuChE inhibition (Khan *et al.*, 2001). Israeli authors also described a direct correlation between the degree of BuChE inhibition levels and the severity of intoxication with OP pesticides (Weissmann-Brenner *et al.*, 2002). According to Aygun *et al.* (2002), in the acute phase of OP poisoning, low levels of AChE support the diagnosis of OP poisoning but do not show a significant relationship to the severity of poisoning. The preference of AChE determination has been demonstrated by Worek *et al.* (1999b) – BuChE activity determination for diagnosis and therapeutic monitoring provides no reliable information on AChE status. This is in good agreement with our experimental results (Bajgar, 1998). Plasma BuChE activity is in some cases a good marker for diagnosis of OP pesticide poisoning. It is necessary to exclude a diminishing of BuChE activity caused by other reasons. In all cases, the simple cholinesterase determination provides us with information about the decrease of enzyme activity without specification of the factor causing this phenomenon. A more detailed specification is possible using special methods not available in all clinical laboratories.

For the purposes of occupational medicine, the determination of cholinesterases in the blood of workers intoxicated with OP is obligatory. A decrease in activity below 70% of normal values is an indicator that the worker should

not come into contact with OP. However, the normal values varied within the laboratories depending on the method of determination.

For practical purposes (individual and interindividual variation), determination of individual norm activity is recommended (this approach is better than that of calculating the decrease from an average value) as well as separate determination of cholinesterases, the red blood cell AChE and plasma BuChE. The activity determined in the whole human blood corresponds to about 10% of BuChE and 90% of AChE. This is different to rats where this ratio is 29% of BuChE and 71% of AChE (Bajgar, 1972). Erythrocyte AChE activity seems to be more useful for these purposes than BuChE activity in plasma.

There are other biological materials available for special purposes – amniotic fluid, cerebrospinal fluid, and bioptic materials. From these samples, tissue obtained by rectal biopsy is used most frequently (diagnosis of Hirschsprung's disease). An elevated AChE activity in the rectal tissue/homogenate (detected histochemically/biochemically) is one of the good diagnostic markers indicating a need for surgical treatment of Hirschsprung's disease and a criterion for diagnosis and management of obstipation (Kobayashi *et al.*, 2002). The presence of an unusual AChE band after electrophoretic separation supports the diagnosis (Bajgar and Hak, 1979; Rakonczay, 1988). The same (either AChE elevation or the presence of a new electrophoretic AChE form) in the amniotic fluid can be applied for the diagnosis of malformation of the neural tube development during pregnancy (Bonham and Attack, 1983). AChE activity in cerebrospinal fluid is also changed in some pathological states; however, the diagnostic validity is not so high and can be considered as a complementary examination (Koponen and Riekkinen, 1991).

In OP poisoning, it is necessary to check vital functions (cardiac, ventilation) and other clinical signs and according to the symptoms to apply different biochemical examinations and treatment. Diagnostic criteria are mostly based on anamnesis and the state of ventilation (Bardin and Van Eeden, 1990; Bardin *et al.*, 1987a, b). Serum electrolytes (especially potassium), BUN, and creatinine are indicated to assess the degree of volume depletion by secretory losses. Arterial blood gas, blood pH, glucose, lactate, and pyruvate allow us to assess the degree of hypoxia/hypercapnia/acidosis in the presence of respiratory distress. The neurological examination involves, e.g. recording of muscle action potential of abductor digiti minimi after stimulation of nerve ulnaris. A quantitative correlation is shown between red blood cell AChE activity and paraoxon concentration in plasma. In these cases, muscle function was severely disturbed when the red blood cell AChE activity was inhibited by more than 90% (Thiermann *et al.*, 2002).

Direct determinations of the toxic agent (OP nerve agent or pesticide) in the circulating system are also possible. However, the parent compound will circulate intact for a short period of time and detection will not be possible for more than approximately a few hours after exposure. Metabolites circulate for a longer time and are mostly excreted in urine. A metabolite of sarin (*O*-isopropyl methylphosphonic acid) could be traced in urine and plasma from victims after the Tokyo subway sarin terrorist attack (Noort *et al.*, 1998, 2002). For some OP pesticides (parathion, paraoxon), detection of *p*-nitrophenol in urine is an indicator of exposure (Bajgar, 1985). However, the retrospectivity of these methods is limited. Detection using an immunoassay of nerve agents is now in progress. The antibodies against soman may have the appropriate specificity and affinity for immunodiagnosis of soman exposure (Lenz *et al.*, 1997a, b; Miller and Lenz, 2001).

Methods for the determination of blood cholinesterase inhibition (AChE and BuChE) do not allow for the identification of the OPs and do not provide reliable evidence for exposure at inhibition levels less than 10–20%. Moreover, they are less suitable for retrospective detection of exposure due to *de novo* synthesis of enzymes. Recently, a method was developed which is based on reactivation of phosphylated cholinesterase and carboxylesterase (CarbE) by fluoride ions. Treatment of the inhibited enzyme with fluoride ions can inverse the inhibition reaction yielding a restored enzyme and a phosphofluoridate which is subsequently isolated and quantified by gas chromatography and phosphorus-specific or mass spectrometric detection (De Jong and Van Dijk, 1984; Polhuijs *et al.*, 1997a, b). Human (and monkey) plasma does not contain CarbE but its BuChE concentration is relatively high (70–80 nM; Myers, 1952; De Bisschop *et al.*, 1987), much higher than the concentration of AChE in blood (ca. 3 nM; Heath, 1961). The plasma of laboratory animals, such as rats and guinea pigs, contains considerable concentrations of CarbE in addition to cholinesterases. This method allows partial identification of the OP whereas the lifetime of the phosphylated esterase (and consequently the retrospectivity of the method) is only limited by spontaneous reactivation, *in vivo* sequestration, and aging. The rate of the latter process (aging) depends on the structure of the phosphyl moiety bound to the enzyme and on the type of esterase. Phosphylated CarbEs generally do not age. Based on this method for retrospective detection of exposure to OP, the exposure of victims of the Tokyo incident to an OP, probably sarin, could be established from analysis of their blood samples (Fidder *et al.*, 2002; Polhuis *et al.*, 1997a, b). Fluoride-induced reactivation of OP-inhibited AChE is a reliable and retrospective method to establish OP exposure. It is limited to compounds that regenerate with fluoride ions. A novel and general procedure for diagnosis of exposure to OPs, which surpasses the limitations of the fluoride reactivation method, was described (Van der Schans *et al.*, 2002). It is based on the rapid isolation of BuChE from plasma by affinity chromatography, digestion with pepsin followed by liquid chromatography with the mass spectrometric analysis of phosphylated nonapeptides resulting in the digestion of inhibited BuChE with pepsin. The method can be applied

for the detection of exposures to various OP pesticides and nerve agents including soman. This approach is very valuable and represents a new field for the improvement of diagnosis with OP nerve agents and pesticides. A comprehensive review of the methods for retrospective detection of exposure to toxic scheduled chemicals has been published by Noort *et al.* (2001, 2002).

As was mentioned previously, a decrease in cholinesterase activity is the factor indicating (after the exclusion of other factors) an exposure to OP nerve agents or other cholinesterase inhibitors. This simple determination does not allow us to make certain decisions dealing with the antidotal therapy (especially the repeated administration of reactivators) and has low prognostic validity. Therefore, a new test of the reactivation has been described (Bajgar, 1991). The principle of the reactivation test is double determination of the enzyme, the first without and the second with the presence of a reactivator in the sample. The choice of reactivator is dependent on the availability of the oxime; however, in principle it is necessary to have in these parallel samples the same concentrations of reagents. The concentration of the reactivator (usually trimedoxime, but other oximes such obidoxime, pralidoxime or HI-6 are also possible) must be no higher than the oxime concentration which causes the hydrolysis of the substrate (acetyl- or butyrylthiocholine), i.e. the oxime concentration is lower than 10^{-3} M because these higher oxime concentrations cause artificial hydrolysis of the substrate (Patočka *et al.*, 1973).

OP poisoning is very complex and there exist many biochemical changes to be registered. Though the assessment of sensitivity and specificity was rather subjective, it is clear that there are two or three parameters that are most sensitive: cholinesterase determination (depending on the type of OP, either AChE or BuChE), the possible determination of OP metabolites in the blood and determination of the phosphonyl moiety on the target enzyme (if possible). In the case of developed intoxication when convulsions occurred, tension of the blood gases is also a good marker; however, these changes are not very specific. The same approach can be applied to lactate. This is not surprising because of the existence of convulsions including hyperactivity of the diaphragm (and thus disturbed ventilation, low oxygen saturation, and an increase in acid metabolites). It should also be mentioned that the validity of these parameters is changed during intoxication. The changes in transaminases, LDH and γ-GT, indicating liver damage can be caused by solvents used in commercially available OP insecticides. A low validity in the number of RBC or leukocytes is also indicated. As for CS and TAT stress markers, it is clear that OP intoxication represents a stress situation. In this context, an increase in ALT can also be considered as a stress marker and not indicative of liver damage.

Determination of AChE or BuChE molecular forms is interesting and useful for improvement of the diagnosis of OP poisoning. It was demonstrated that these forms are inhibited in different manners – some of the forms are resistant (a low molecular weight), and some of them are very sensitive (a high molecular weight). When the total AChE activity is determined, the value obtained is a ''mean'' of the activities of these forms. The changes in the cyclic nucleotides are interesting but not valid for blood. They were determined during animal experiments with toxic OPs and are of more interest in connection with the nervous system. Esterases and AP, generally hydrolases, are sensitive but the inhibition potency of different OPs is very variable: for nerve agents these hydrolases are not valid, and, for some OP insecticides like malathion, they are sometimes more sensitive than cholinesterases. In conclusion, diagnosis of OP poisoning represents a serious problem. The development of the new specific methods mentioned (fluoride reactivation, tandem MS analysis of enzymatic digests of BuChE) is of high importance for more precise diagnosis of OP nerve agent poisoning. The extensive review of Noort *et al.* (2002) dealing with biomonitoring of exposure to chemical warfare agents (not only nerve agents) is strongly recommended. From a practical point of view in the clinical laboratory, it is necessary to monitor basic physiological functions, cholinesterases, and other biochemical parameters not only for diagnostic purposes but also preferably for the regulation of treatment.

V. MONITORING OF BLOOD CHOLINESTERASE ACTIVITY IN WORKERS WITH NERVE AGENTS

For the purposes of occupational medicine, the determination of cholinesterases in the blood of workers intoxicated with OP is obligatory. However, the normal values varied within the laboratories depending on the method of determination. Systematic monitoring of workers with nerve agents was performed at our department from 1962 to 1963; however, the results presented contain determinations during 40 years (1964–2004). Because of the large number of results, the results are limited to RBC AChE only although plasma BuChE activity was also determined.

A. Methods for Determination

Modified Hestrin's method (Hestrin, 1949) was used initially. This method is based on colorimetric detection of unhydrolyzed substrate, ACh. Acetylcholine reacts with hydroxylamine quantitatively in alkaline solution after reaction with $FeCl_3$. The activity was determined in RBC hemolysate with distilled water (1:20). The method was modified and activity was expressed as μmoles of hydrolyzed acetylcholine per minute and per ml.

From 1964, a method described by Winter (1960) using an autoanalyzer (Technicon, USA) system was evaluated. The method is based on determination of acetic acid

released from the substrate hydrolyzed (ACh) and detected with phenol red. AChE activity in RBC hemolysate with distilled water (1:10) was determined (pH 7.6, phosphate buffer 37°C). The activity was expressed as µmol of acetic acid per 10 min.

From 1972 to 1975, Ellman's method (Ellman *et al.*, 1961), modified for the autoanalyzer, was used. The activity was expressed as ncat/liter. The same method was used for this determination using kinetic methods and instruments, Vitatron (Eefde, Holland) (1976–1990) and Uvicon 952 (Kontron Instruments, Switzerland), from 1991 to 2004.

B. Correlation Among Methods

To achieve comparable results (unification of the activity, i.e. one scale), all the activities were recalculated to ncat/liter. Recalculation of the activities was performed simply using: U/liter = 16.6667 ncat/liter. However, in the case of different substrates (esters of choline and thiocholine), correction was made according to correlation analysis ($y = 2.0188x + 2.2458$ and $r_{xy} = 0.9998$). Moreover, this correlation was demonstrated previously including not only Ellman's and Winter's methods (Ellman *et al.*, 1961; Winter, 1960) but also the potentiometric method.

C. Subjects

International inspectors: 28 males (two determinations, 2000 and 2002), seven selected (more determinations), age unknown.

Students: 21 medical students, 10 female, 11 male, age 19–20.

Workers in the department: 18 males, from these nine selected for long-term study, and 23 females, from these 13 selected for long-term study. Last determination was performed at age 60 (female) and 65 (male), respectively.

Ages at the beginning of study: 24 to 40 years for males, and 19 to 48 years for females. All determinations were performed during the years 1964 to 2004.

D. Data Analysis and Findings

Because of the large number of examinations, the results are demonstrated only for the RBC AChE. The first step was a simple survey of AChE activities divided between females and males. The next step was analysis of individual results and trends. Some attempts to detect a decrease of AChE activity in the case of potential exposure to nerve agents were made. The results are not fully evaluated and can be considered as preliminary.

The results of Hestrin's method (Hestrin, 1949) are documented as an average activity of the RBC AChE. Normal AChE activity was determined to be 104.9 ± 8.5 ncat/l and the coefficient of variation was 27.1%. The method was relatively difficult and is shown for information only. The results of AChE activities for all persons are shown in Figure 58.2. Selected results of RBC AChE activity determination are shown in Table 58.1.

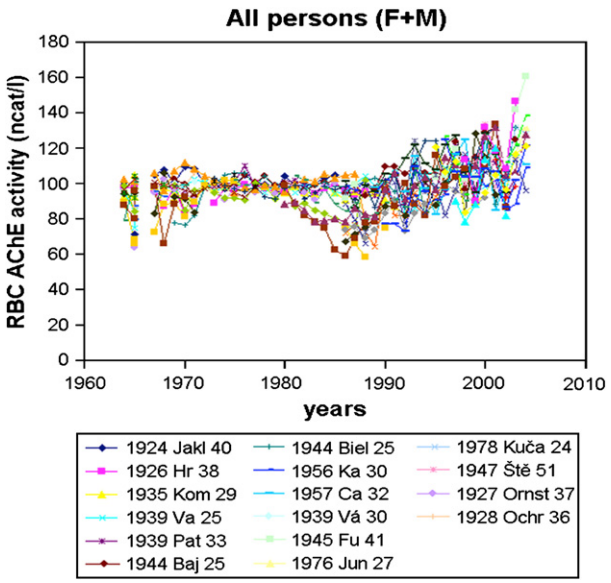

FIGURE 58.2. AChE activities of all monitored persons.

The correlation of activities in hemolysate and whole blood is shown in Figure 58.3.

In general, there were certain tendencies to decrease cholinesterase activity (especially RBC AChE activity) during their work at the department. Some rare examples of suspect intoxications were observed in the cholinesterase monitoring as well as an RBC AChE decrease in workers during their work with high concentrations of nerve agents (e.g. inhalation exposure experiments) (Figure 58.4).

In a practical way, the best appreciation of cholinesterase was performed using individual norms for RBC AChE. It seems to be the most sensitive parameter for monitoring cholinesterase changes in exposed workers, followed by a sensitivity for whole blood hemolysate. Plasma BuChE activity is not so specific and is a sensitive parameter. A decrease of 30% of individual norms seems to be critical for further consideration. In conclusion, cholinesterase determination in blood (especially RBC AChE) is a good parameter for monitoring and laboratory examination of

TABLE 58.1. Selected results of RBC AChE activity determination

Dept Toxicology	All	M	F
(18 + 23)	101.2 ± 7.2	102.2 ± 6.9	100.2 ± 8.4
Selected (9 + 13)	98.0 ± 8.2	99.7 ± 6.3	93.9 ± 7.8
Inspectors (0 + 28)	–	110.7 ± 9.4	–
(2000 + 2002)	–	112.4 ± 14.7	–
Students (11 + 10)	110.2 ± 9.5	115.1 ± 9.4	105.3 ± 9.5
Suspect intoxication (3 + 4)	90.9 ± 4.8*	94.3 ± 6.9*	86.4 ± 2.3

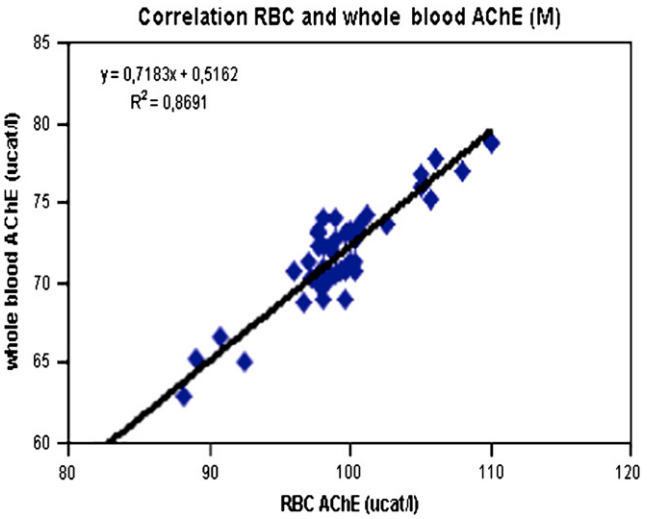

FIGURE 58.3. Correlation between RBC and whole blood AChE.

workers with highly toxic nerve agents, reflecting possible exposure to these agents.

Variation of RBC AChE is relatively high. Therefore individual levels were considered to be better, and the decrease of 30% under individual norms was decided to be a limit for exclusion of workers from active work with nerve agents. A further decrease can be considered a significant diagnostic marker for suspect intoxication. According to the decrease, the classification of steps of intoxication can be assumed: a decrease of 30–50% indicates mild poisoning, 50–70% medium poisoning and 70–90% severe intoxication. This decrease is in good agreement with results for humans and animals (Bajgar, 1992). Increased AChE activity was also observed for males (not significant) and is in agreement with other results (Augun *et al.*, 2002). Similar results were obtained by other authors, for example no

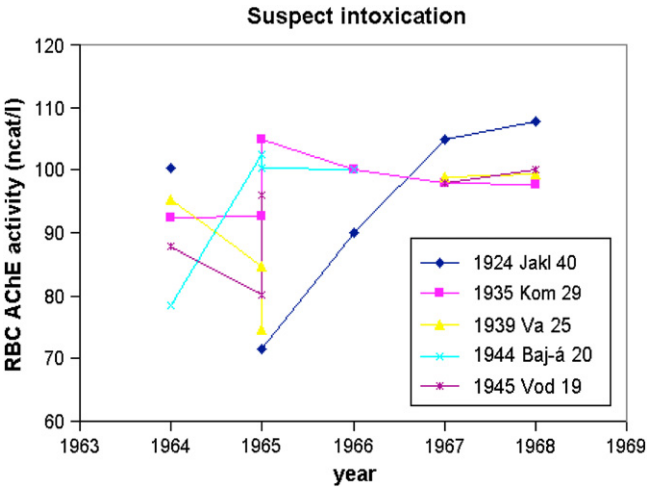

FIGURE 58.4. Suspect OP intoxication.

significant differences in RBC AChE activity between males and females (Rumenjak, 1998), and a tendency to an increase in activity with age (not significant). In general, the values of RBC AChE activity are very good compared with the results of other authors (Augun *et al.*, 2002; Rumenjak, 1998; Zhou *et al.*, 2003).

VI. CONCLUDING REMARKS AND FUTURE DIRECTION

This chapter describes the sensitivity of blood cholinesterase as a diagnostic parameter for monitoring workers exposed to nerve agents. The sensitivity to detect changes in response to nerve agent exposure is ordered as RBC AChE > whole blood cholinesterases > plasma BuChE. Normal cholinesterase activity assayed with different methods is in good correlation. RBC AChE or whole blood cholinesterase can be considered a good marker for the monitoring of nerve agent exposure.

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CHAPTER 59

Strategies to Enhance Medical Countermeasures After the Use of Chemical Warfare Agents on Civilians

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The information in this chapter is provided for educational purposes only and does not necessarily represent endorsement by, or an official position of, the National Institute of Neurological Disorders and Stroke or any other Federal agency.

I. INTRODUCTION

The use of chemical warfare agents (CWAs) has historically been a primary concern of military personnel due to their devastating physical and psychological effects when employed on the battlefield. However, largely due to the 2001 terrorist attacks in the USA, there is now a heightened awareness of biological, radiation/nuclear, and chemical threats to the civilian population. As a result of these new threats, agencies within the Department of Health and Human Services (DHHS) and other federal agencies have sustained a highly focused effort to assess and, if necessary, improve upon current emergency response capabilities in the event of an actual terrorist event. These efforts include both nonmedical countermeasures such as personal protective equipment for first responders who must enter a contaminated site, and medical capabilities such as safe and effective antidotes and diagnostic tools to reduce mortality and morbidity after a chemical attack or accident. In 2006, the US Congress appropriated funds to the National Institutes of Health (NIH) to implement the National Strategic Plan and Research Agenda for Medical Chemical Countermeasures (NIAID, 2007). This program, led by the National Institute of Neurological Disorders and Stroke (NINDS) and National Institute of Allergy and Infectious Diseases (NIAID) with participation from five other NIH Institutes and Centers, is called ''Countermeasures Against Chemical Threats (CounterACT)'' (http://www.ninds.nih.gov/counteract). The goal of the CounterACT program is to develop safer and more effective therapeutics to treat victims exposed during a chemical incident. Complementing this goal, the CounterACT program also supports the research and development of novel diagnostic technologies, which could be utilized after a chemical incident to determine the proper course of medical intervention.

II. SCOPE OF RESEARCH

The CounterACT program supports drug development at all stages from target identification to lead optimization to advanced preclinical and clinical trials. The program focuses on basic and translational research that is relevant to the development of novel therapeutic or diagnostic techniques that will enhance our medical response capabilities during a chemical emergency. New medical countermeasures/technologies that have no practical utility during a mass casualty incident are not considered in this program. A high priority of the program is to identify alternative routes of administration for FDA-approved therapeutics that would be safe, effective, and easy to administer during a mass casualty scenario (e.g. intramuscular injection). Similarly, alternative physical and/or chemical formulation of existing therapeutics to prolong their chemical stability is also a program priority. Enhancement of shelf-life would permit these therapeutics to be stockpiled and strategically positioned for rapid deployment in response to a chemical incident.

The CounterACT program also supports mechanistic studies to identify targets for therapeutic/diagnostic development. There are efforts under way to develop standardized *in vitro* and *in vivo* experimental models to determine the efficacy of promising therapeutics. The program also funds initial and advanced efficacy screening studies performed under good laboratory practices (GLP) using either new and/or established experimental models, to include nonhuman primates. Finally, the CounterACT program also supports clinical studies, including clinical trials, of therapeutics that have advanced past the preclinical phase. In the CounterACT program, special consideration is given to research that is relevant to people who are particularly

vulnerable, including the young, the elderly, and individuals with pre-existing medical conditions.

III. COUNTERACT PROGRAM STRUCTURE

From its inception, the CounterACT program has employed a strategy of building partnerships among laboratories within industry and academic sectors (Figure 59.1), and it has relied on the experience and resources within the Department of Defense (DoD). The DoD has been the primary federal agency responsible for research with CWA, including countermeasure development. The DoD has assembled and supported many of the world's experts in the field of CWA research. The NIH also has a vast pool of experts in scientific areas that are very relevant to some of the central questions being posed in this field. For example, the nerve agents cause seizures and the NIH has funded research on seizures caused by epilepsy and other illnesses for decades. The partnership between DHHS and DoD facilitated by CounterACT has led to excellent collaborations among scientists who would otherwise rarely interact. These collaborations have already produced promising novel approaches in the development of medical chemical countermeasures. The CounterACT program is composed of over 30 individual Research Projects, Contracts, and Research Centers of Excellence located in over 20 states within the USA. Most of these have collaborations with other US and foreign laboratories resulting in over 50 funded primary investigators in over 40 different laboratories. This network of researchers maintains collaborative interaction through sharing new and exciting results at annual meetings sponsored by the NIH. Each year, new collaborations among scientists in varied disciplines such as neuroscientists and pulmonary experts with similar interests are formed.

IV. THE CIVILIAN THREAT SPECTRUM AND SPECIAL CONCERNS

Chemical threats can be defined as highly toxic chemicals that could be used in a terrorist attack, or those that could be released from transportation and storage facilities during an accident or after a natural disaster causing mass casualties. The civilian threat spectrum includes not only traditional CWAs, but also thousands of other highly toxic industrial chemicals (TICs). However, based on availability and the feasibility of mass exposures, the number of chemicals that pose a serious threat is probably much smaller. Some TICs are stored in large industrial facilities and transported by rail and other means across the USA. Accidents and fatalities involving human exposures to these TICs are not that uncommon (Broughton, 2005; Varma and Mulay, 2006; Buckley *et al.*, 2007; Wenck *et al.*, 2007) (Figure 59.2),

FIGURE 59.2. Train wreck and chlorine spill in Graniteville, SC, in 2005. (www.cen.online.org June 23, 2008)

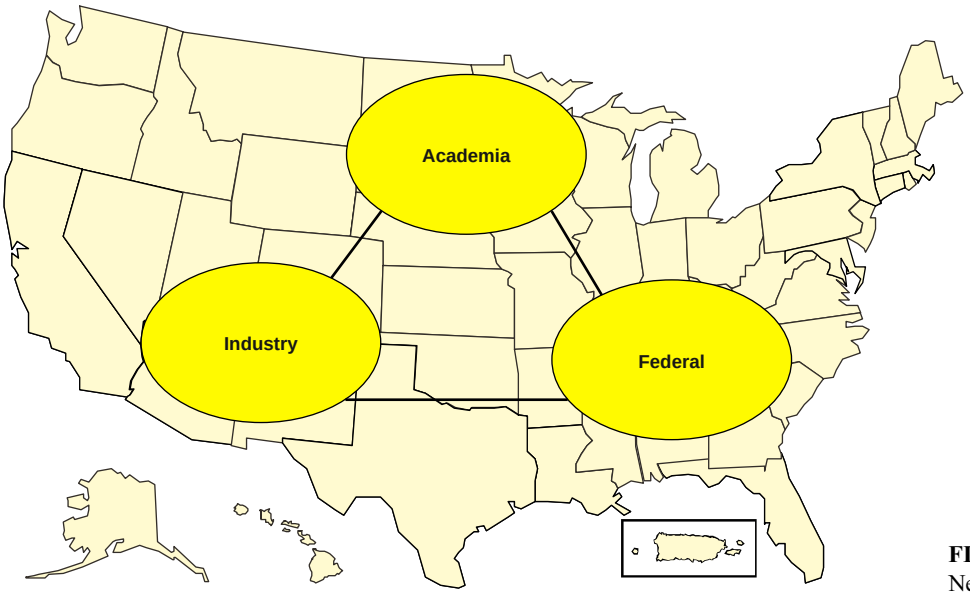

FIGURE 59.1. CounterACT Research Network partnerships.

while CWAs are rarely implicated outside military facilities or off the battlefield. These CWAs can be divided into four general categories: traditional nerve agents such as sarin and VX, vesicating agents such as sulfur mustard, pulmonary agents such as chlorine and phosgene, and metabolic poisons such as cyanide. Detailed descriptions of medical interventions for each of these CWAs can be found elsewhere in this book, and will only be briefly discussed to illustrate the different approaches undertaken by the NIH civilian research community to develop medical countermeasures against threat agents.

Military research on the effects of the organophosphorus (OP) nerve agents and the mustard gases has been ongoing for several decades since their discovery and use in the past world wars. This work along with some efforts in the nonmilitary research community has led to the development of useful therapeutics and diagnostic tools primarily for use to protect those on the battlefield. Of particular note are the developments of more effective personal protective equipment (PPE), prophylactic drugs such as pyridostigmine bromide (PB) that reversibly binds to OP nerve agent (Gordon *et al.*, 2005; Tuovinen *et al.*, 1999), and portable antidote kits like the MARK-1 autoinjector (Rebmann *et al.*, 2008). Differences between the civilian threat and the military threat pose some unique challenges to researchers (Figure 59.3). First, typical military personnel are fit and healthy and between the ages of 18 and 45, whereas the civilian demographic is much broader including pediatric and elderly segments, as well as individuals with preexisting medical conditions such as asthma, heart diseases, or diabetes. These factors will be extremely important when deciding the specific type and dosage of medical countermeasures to be administered during a chemical emergency. Fortunately, some military countermeasures can be adapted to special populations such as children (Baker, 2007; Henretig *et al.*, 2002). However, the development and validation of good animal models to simulate these special segments of the population are still urgently needed.

Another important potential difference between the military and civilian sectors is that military officials may be able to predict with some degree of certainty when CWAs could be used, and thus arm military personnel with pre- and post-exposure countermeasures, such as PB and MARK-1 kits. Since it is impossible to predict when a civilian attack will occur, the NIH has been primarily focused on treating victims after an exposure to CWAs, with the possible exception of some work on prophylactic treatments that could be administered to first responders who must enter a contaminated site.

Military Focus:
- **Warfighter: 18-45 and Healthy**
- **Open air environment**
- **High number of casualties is the goal**
- **Prophylactic measures is focus**

Civilian Focus:
- **Pediatric - Elderly**
- **Pre-existing Medical Conditions**
- **Could happen in closed environment**
- **No need for high casualties**
- **Post-exposure therapies is focus**
- **First responders and DeCon personnel**

FIGURE 59.3. Civilian and military challenges in countermeasure development.

V. PRETREATMENTS

Pretreatments are defined in this chapter as those medical countermeasures to be administered before CWA exposure (see Figure 59.4). Personnel that would be pretreated with medical countermeasures include first responders such as emergency medical technicians (EMTs), other emergency department medical personnel including those that may be exposed within an emergency department, and persons responsible for site decontamination. In theory, prophylactic drugs would be administered in conjunction with proper PPE to first responders before they enter a CWA ''hot zone''. However, depending on possible adverse effects and pharmacokinetics of the proposed drugs, there may be some operational difficulties in the implementation of a prophylaxis program. For example, compliance by otherwise healthy individuals may present an issue if there are potential side effects. Another obstacle to a prophylaxis program is the time needed for the proposed drug(s) to achieve or maintain maximal therapeutic levels when a first responder arrives at the contaminated site. However, if these challenges can be overcome, pretreatments against the toxic effects of chemical agents for emergency first responders would represent a significant advancement in the response capabilities of the nation. In addition to prophylactic drugs, better dermal and ocular medical protectants should also be considered for inclusion as pre-exposure treatments, especially for medical personnel who may have to work in areas where off-gassing may occur by individuals exposed to CWAs.

The only pretreatment currently approved by the FDA for nerve agent is PB, which was first approved for treating myasthenia gravis and is now approved for treating soman

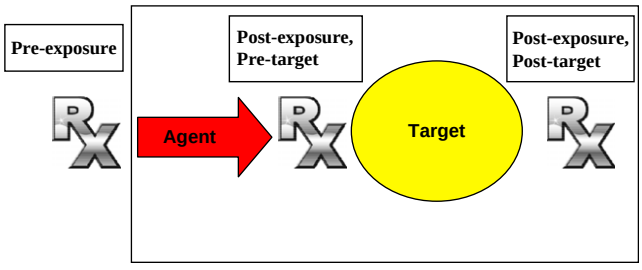

FIGURE 59.4. Different opportunities for medical intervention to reduce mortality and morbidity cause by CWAs.

only. It is to be used by military personnel at high risk of exposure to soman on the battlefield. It is a reversible inhibitor of acetylcholinesterase (AChE) and protects it from permanent inhibition by soman. Because it is itself an inhibitor of the target enzyme in nerve agent toxicity, it is contraindicated after exposure and AChE inhibition has already occurred. The US military has led the effort to explore other potential pretreatments. One effort involves the use of serum-derived or recombinant butyryl-cholinesterase (BuChE) as a stoichiometric scavenger of nerve agent. This could be given as a prophylactic treatment. Previous animal studies have shown that injection of exogenous human BuChE enzyme can protect guinea pigs and Cynomolgus macaques from death against very high doses of soman or VX (Lenz *et al.*, 2006). The challenge with this stoichiometric approach is reaching and maintaining an effective dose when each protein molecule only binds one molecule of nerve agent. The issue of immunogenicity can be a problem with protein and antibody approaches. Alternative approaches with small molecules acting on cholinesterases and related targets are being examined as pre- and post-exposure therapies within the CounterACT program.

VI. POST-EXPOSURE PRE-TARGET PROPHYLACTIC THERAPIES

Most CWAs reach their physiological targets at toxic levels to cause serious illness and even fatality only minutes after exposure. The medical intervention approach in this case is to reverse the toxic action at the target site immediately or treat the physiological response as soon as possible. This latter approach will be further discussed in the section below. Another therapeutic possibility is that some CWAs may exert their toxic effects more slowly and not reach the target site at critical levels until after some period of time. This may be due to the route of exposure such as the relatively slower dermal versus the more rapid inhalation route, or due to slower toxicokinetics and/or toxicodynamics. In this instance, the opportunity for medical intervention may be greater, and a ''post-exposure, pre-target'' approach would be beneficial (Figure 59.4). Under this paradigm, PB (or other reversible cholinesterase inhibitors) may be utilized to protect peripheral AChE before the systemic concentration of irreversibly inhibited OP nerve agent–AChE reaches a critical level. Drugs that act as prophylactic treatments can also act on the agent before it reaches the target. The theoretical post-exposure, pre-target approach is a function of the ratio of bound-to-free agent at the time a therapeutic drug is administered.

Similarly, therapeutics that detoxify the chemical agent from systemic circulation, such as protein scavengers of nerve agent or newer cobalamine-based molecules that bind and scavenge cyanide, could work as well. The Cyanide Antidote Kit has been used for several years to remove cyanide from circulation by forming cyanomethemoglobin (using nitrites) or thiocyanate (using sulfur donors) complexes that are less toxic and more readily excreted. Next generation cyanide antidotes may include drugs that could preserve mitochondrial respiration, sulfur donors with longer half-life, or novel nitric oxide-based approaches.

VII. POST-EXPOSURE POST-TARGET TREATMENT

For many CWAs (e.g. nerve agents, cyanide, etc.), there is little opportunity to medically intervene when the dose is high and/or rapidly delivered to the target site. In these situations, high doses of these CWAs are almost always fatal. If the action at the target site does not cause immediate death, there are several strategies that can be employed to reduce the impending morbidity and fatality. At the molecular level, AChE reactivators such as oximes can be administered during a nerve agent exposure incident to remove nerve agent from the active site of AChE. Administration of oxime therapy will only be successful if irreversible covalent modification of the nerve agent–AChE complex has not occurred (a process called ''aging''). ''Unaged'' AChE enzyme is thus restored to the active state and can continue the hydrolysis of the neurotransmitter acetylcholine (ACh). This intervention approach was developed through an intimate knowledge of the molecular mechanism of nerve agent toxicity.

In cases where less is known about mechanisms at the molecular level, or there are no available drugs that can modify actions at the target site, the intervention approach is to treat the effects of target site activation (or inactivation). For example, at sublethal doses of nerve agents, medical intervention primarily involves combating the effects of nervous system activation and cholinergic crisis. As discussed in other chapters, atropine is used to block overstimulation of cholinergic receptors, and anticonvulsants such as diazepam are used to prevent or treat seizures. Similarly, victims exposed to a sublethal concentration of the fast-acting CWA cyanide are largely treated with ventilation and supportive care to delay mortality and morbidity until transportation to medical facilities. An important area of research in the CounterACT program is to identify new approaches (e.g. better surfactants) to mitigate the pulmonary edemas that typically develop after lung injury caused by agents such as chlorine.

Whether there is sufficient time after an exposure to the CWA for a therapy to be beneficial is dependent on the initial dose. This will depend on the initial level of the agent released and how close a victim is to the point of release, or ground zero. If the dose is low, the risk of an acute life-threatening intoxication will likely not be an issue, but there is evidence that acute sublethal exposures to chemical nerve agent may be associated with long-term neurological sequelae (Hoffman *et al.*, 2007; Yamasue *et al.*, 2007).

Some agents such as sulfur mustard may cause a delayed toxicity after initial exposure or long-term pulmonary effects such as fibrosis years later (Emad and Emad, 2007a, b). Therefore, medical interventions aimed at improving the long-term health outcomes of nonlethal exposures are critical. Since the immune responses observed in the pulmonary system after sulfur mustard or exposure to TICs share similar characteristics, it is likely that therapeutic drugs that prevent, slow, or halt the processes of inflammation and subsequent cellular degeneration will probably have great utility across many different types of chemical agents. The CounterACT program is funding research to determine whether drugs such as anti-inflammatory, anti-oxidant, anti-necrotic/apoptotic compounds, or those that promote cell survival such as growth factors, are effective against CWA threats.

VIII. DIAGNOSTIC TECHNOLOGIES

Proper diagnosis of chemical poisoning can sometimes be an essential part of medical intervention in terms of how to choose the correct treatments and how to monitor effectiveness of a treatment. Because some agents require treatments that may themselves have serious adverse side effects, and some agents may require different kinds of antidotes, technologies that provide medical personnel with information on the chemical or class of chemical agent could significantly improve medical outcomes. Improved rapid diagnostic techniques/technologies for the purpose of differential diagnosis, triage, detection of subclinical exposures, prognosis, and prediction of tissue damage fall into this category. This would include devices for detecting biomarkers (genomic, proteomic, metabonomic) in blood, urine, saliva, lavage fluid, as well as medical diagnosis standards or ''toxidromes'' to help medical personnel identify the best medical interventions. Another example would be when considering the diagnosis of seizure activity and appropriate treatments. The muscular effects of OP nerve agents may mask or mimic signs of seizure activity. A field deployable electroencephalograph (EEG) suitable for use by first responder medical personnel could be used to detect potential nonconvulsive seizures during a chemical event.

IX. BASIC/MECHANISTIC RESEARCH AND TARGET IDENTIFICATION

Support of basic or fundamental research efforts is an important component of the NIH Mission (http://www.nih.gov/about/#mission). Realizing this goal, the NIH CounterACT program supports basic research efforts to identify molecular mechanisms of toxicity exerted by chemical threat agents for the purpose of identifying novel therapeutic targets. For example, the program supports research on understanding the mechanisms of nerve agent-induced seizures with respect to temporal and regional changes, roles of cardiac versus neuronal mitochondria in cyanide toxicity, or the cellular and molecular basis for agent-induced pulmonary edema. Similarly, understanding the toxicokinetics related to the different routes of exposure to chemical agents is also very important for determining therapeutic windows.

X. *IN VITRO* AND ANIMAL MODELS FOR EFFICACY SCREENING

Development and validation of *in vitro* and/or *in vivo* animal models for rapid screening of molecular libraries to identify potential medical countermeasures is another priority of the CounterACT program. These models include seizures in small mammals, models of direct lung injury from an inhaled source, animal models of cyanide intoxication, and medium throughput models of dermal or ocular injuries. It is important that models be amenable to use under GLP methodology so that the data generated are acceptable to the FDA. Since adherence to GLP standards may be expensive, earlier screens to identify potential hits are usually performed under non-GLP conditions.

For efficacy testing, most of these efforts are highly specialized endeavors and are typically performed in-house for each project. However, for some efficacy testing, CounterACT investigators are encouraged to utilize the centralized testing facilities that the NIH has provided. For many years, the NINDS Anticonvulsant Screening Program (ASP) at the University of Utah (http://www.ninds.nih.gov/funding/research/asp/index.htm) has screened compounds for potential therapeutic activity in the treatment of epilepsy. Capitalizing on this considerable experience and the medium-throughput screening infrastructure already in place within the ASP, the CounterACT program created an arm of testing at the ASP. The CounterACT arm specifically tests compounds for potential efficacy in alleviating nerve agent-induced seizures in addition to neuroprotectant screening studies against the neuropathology that results from chemical exposures.

Another key component of the CounterACT program is its collaborative efforts with DoD laboratories. One of the major challenges in developing an NIH program on medical chemical countermeasures is that many of the highest priority chemical threat agents such as the CWAs are highly toxic and can only be used in approved, usually military, research facilities. Consequently, the NIH has partnered with the US Army Medical Research Institute of Chemical Defense (USAMRICD) (http://chemdef.apgea.army.mil/) through the support of four Interagency Agreements and one Research Center of Excellence to conduct chemical countermeasure research against *bona fide* CWAs and other agents.

XI. ADVANCED PRECLINICAL DEVELOPMENT AND CLINICAL STUDIES

Once a lead compound is identified, optimization of that compound into a viable drug is the next crucial step in the development process. Unfortunately, this is the step where many potential therapeutic drugs fail. A very important component of the drug development process is lead optimization using medicinal chemistry to develop the best pharmacokinetic profile for the desired formulation and preferred route of administration. This is an iterative process that is coupled to biological assays of efficacy, safety, and pharmacokinetics. General chemistry and manufacturing support is critical once a lead compound is identified. These studies include current good manufacturing principles (cGMP)-compliant stability studies to support regulatory submission. Manufacturing processes and procedures under cGMP conditions must be developed and reagent-grade and clinical-grade drugs must be synthesized in amounts sufficient for preclinical evaluation and Phase I/II clinical trials. To support these activities, the CounterACT program has utilized several NIH resources, including the CounterACT Preclinical Development Facility at SRI International discussed below, as well as other programs currently in development.

The CounterACT network is supported by several centralized facilities that serve its many investigators. The CounterACT Pre-clinical Development Facility is located at SRI International (http://www.sri.com/) and provides pharmacokinetic, safety, chemistry, and formulation studies under GLP and cGMP conditions. The facility is available to the entire network when a lead compound is ready for investigational new drug (IND)-enabling studies. Alternatively, SRI also provides smaller pilot studies for promising compounds that may require additional characterization before further efficacy testing. This facility and other programs under development also provide regulatory affairs support on an individual basis for the CounterACT investigators. For clinical trials, the CounterACT program leverages itself upon other established resources at the NIH, such as the NINDS Neurological Emergency Treatment Trials Network (NETT) (http://www.nett.umich.edu/nett/welcome). The NETT is currently conducting a Phase II/III clinical trial on a promising anticonvulsant that could potentially be used to treat nerve agent-induced seizures.

XII. CONCLUDING REMARKS AND FUTURE DIRECTION

The NIH CounterACT program employs several strategies for addressing the challenge of developing safer and more effective medical chemical countermeasures. First, the program leverages itself upon the experience and resources of the DoD chemical defense programs which overlap in some areas and differ in others. Second, it engages the NIH research community that otherwise would not be involved in CWA research, but who bring critical expertise in relevant areas of scientific endeavor. The scientific approach is one that looks for opportunities to intervene medically during the course of exposure through acute and long-term responses. Long-term effects are important especially if better acute treatments increase the number of patients surviving an otherwise lethal exposure. This is similar to what could be observed after improved survivability of traumatic injuries, for instance in the military. The CounterACT program is structured with investigator-initiated research projects and facilities to support these efforts. The drug discovery and development process is very expensive and it takes many years to obtain a licensed therapeutic drug ready for market. The future of the CounterACT program will depend on several factors including prior successes of the program and the foresight by leadership to support the program sufficiently through the research and development process in order to obtain the best return on the initial investment.

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CHAPTER 60

Medical Countermeasures and Other Therapeutic Strategies for Sulfur Mustard Toxicity

R. VIJAYARAGHAVAN, ANSHOO GAUTAM, AND MANOJ SHARMA

I. INTRODUCTION

Chemically, sulfur mustard (SM) is bis (2-chloroethyl) sulfide and is well known as mustard gas (Table 60.1). There are various mustard agents. However, SM is one of the most important blistering or vesicating agents. It is regarded as the king of chemical warfare agents and is of historical and current interest. Of all the chemical warfare agents, SM is the one which is used the most, for example during the Iran–Iraq War (Sawyer *et al.*, 2002). The easy availability of precursors, the simple method of synthesis and the extremely stable nature of SM make it a chemical weapon of choice by the military and terrorist groups (Table 60.2) (Borak and Sidell, 1992; Balali-Mood and Hefazi, 2005). It is preferred by the military as it can incapacitate rather than kill, and due to its delayed action it produces mass casualties requiring prolonged and intensive care (Graham *et al.*, 2005). The destructive properties of SM on vital organs, and lack of effective and satisfactory antidotes, are additional benefits of SM as a chemical weapon. SM has been used in more than ten conflicts killing and inflicting severe injuries in millions of military personnel and civilians. In the Iran–Iraq War it caused heavy casualties and thousands of victims still suffer due to late effects (Kehe and Szinicz, 2005; Saladi *et al.*, 2006). SM has been stockpiled by several countries but since the Chemical Weapons Convention (CWC) came into force many are in the process of destroying it. However, the complete destruction of all the declared SM may take some time.

SM forms sulfonium ion in the body and alkylates DNA leading to DNA strand breaks and cell death. Due to the high electrophilic property of the sulfonium ion, SM binds to a variety of cellular macromolecules (Somani and Babu, 1989; Dacre and Goldman, 1996). The most common complications of SM occur in lung, skin, and eye which are the principal target organs due to its direct effect. The systemic toxicity leads to several manifestations (Balali-Mood *et al.*, 2005; Dacre and Goldman, 1996).

The first clinical manifestations of SM poisoning in victims occur in the eyes with a sensation of grittiness, lacrimation, photophobia, blepharospasm, and corneal ulceration (Balali-Mood and Hefazi, 2006). If exposure to vapor is prolonged, rhinorhea, laryngitis, bronchitis, necrosis of mucous membranes of the respiratory tract, and bronchopneumonia will occur. Skin lesions include erythema, blisters, and necrosis (Balali-Mood and Hefazi, 2006; Dacre and Goldman, 1996). SM-exposed victims still suffer from delayed effects like chronic obstructive lung disease, lung fibrosis, chronic conjunctivitis, recurrent corneal ulcers, and abnormal pigmentation of the skin (Kehe and Szinicz, 2005).

Despite rigorous research efforts on the development of an antidote to SM, employing a variety of *in vivo* and *in vitro* systems and using various mustard agents, so far no satisfactory and recommended treatment has evolved (Figure 60.1). In general, antidotes to chemical warfare agents are considered as ''orphan drugs'' as they are not a regular phenomenon and the pharmaceutical industry has very little interest in them (Szinicz *et al.*, 2007). In view of the possible threat from various sources against the military and civilian populations, and the risk during the destruction of the stockpiled mustard agents, development of a suitable antidote is a prime requirement. Physicians should also be aware of the likelihood of mass casualties due to SM exposure and should be knowledgeable about the various medical countermeasures.

The current treatment strategy consists of symptomatic management that prevents infections and promotes healing. There are no standardized or optimized methods of casualty management to reduce the suffering and provide speedy wound healing (Graham *et al.*, 2005). At present, no antidote exists for SM poisoning. The best method of minimizing the injury is by immediate decontamination of the exposed individuals, followed by palliative treatment of symptoms (Geraci, 2008; Munro *et al.*, 1990). Antidotes to SM can be sought in four different directions (Figure 60.2): (1) prevention of SM from entering the system (personal

TABLE 60.1. Synonyms of sulfur mustard

Bis(2-chloroethyl)sulfide
Bis(β-chloroethyl)sulfide
1-Chloro-2-(β-chloroethylthio)ethane
2,2′-Dichlorodiethyl sulfide
Di-2-chloroethyl sulfide
β,β′-Dichloroethyl sulfide
Schwefel-lost
S-Lost
S-Mustard
Sulfur mustard
Sulfur mustard gas
Yellow cross liquid
Yperite

decontamination at the site of contact), (2) prevention of SM from alkylating critical target molecules, mainly DNA, (3) restoration of SM alkylated DNA, and (4) prevention and reversal of the cascade of secondary biochemical reactions following alkylation (Vijayaraghavan *et al.*, 2001a). Of these, most of the research is focused on the decontamination methodologies and prevention or reversal of biochemical complications (Smith and Gross, 2002).

In this chapter the various antidotes and treatment regimes conducted on animal models and the treatment regimens recommended in human casualties are reviewed.

II. SKIN DECONTAMINATION

SM is a lipophillic compound and rapidly penetrates the skin. Hence the decontamination has to be done immediately and should be complete. It is known that decontaminiation done 5 min after SM contact may not be beneficial as a sufficient quantity would have been absorbed by the skin (Vijayaraghavan *et al.*, 2002). A number of proprietary formulations are available that can efficiently decontaminate SM. A useful decontaminant for mustard would be one which is readily available in large quantities, inexpensive, and potentially biodegradable (Table 60.3) (Cerny and Cerny, 1997).

TABLE 60.2. Physicochemical properties of sulfur mustard

Appearance	Yellow, oily liquid
Odor	No odor to garlic, mustard
Molecular weight	159.08
Density	1.27 (specific gravity)
Solubility	Very hydrophobic
Freezing point	14.45°C
Boiling point	215–217°C
Volatility (mg/m^3, 20°C)	610
Persistence	Highly stable

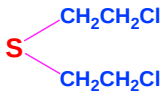
A. Efficacy of Barrier Creams

A number of barrier creams have been developed that prevent the penetration of chemical warfare agents including SM, thereby reducing the morbidity. A barrier cream coded as IB1 (Israel Institute of Biological Research) has been evaluated in pigs and has been found to be safe and effective against SM and VX (Kadar *et al.*, 2003). Barrier creams comprising perfluorinated polymers were effective against SM when evaluated using human skin *in vitro*. Three candidate perfluorinated barrier creams were evaluated in domestic white pigs against SM vapors and found to be very effective. The treated sites looked like the control animals and the untreated animals showed evidence of persistent damage with slow healing (Chilcott *et al.*, 2007). Perfluorinated reactive barrier cream formulations have been developed and evaluated against SM using an *in vitro* diffusion cell system containing human skin. Novel perfluorinated barrier creams were generally more effective under unoccluded conditions. Pretreatment with a proprietary formulation reduced the skin absorption of SM by several fold (Chilcott *et al.*, 2002). The addition of polyoxometalates to perfluoropolyether topical skin protectants enhanced the decontamination efficacy against SM (Johnson and Hill, 1999). Highly reactive nanoparticle-based cream has also been developed that can decontaminate a variety of chemical warfare agents and is safer to use on skin (Koper *et al.*, 1999). Barrier creams known as topical skin protectants have been formulated which can delay the penetration of SM through the skin. One topical skin protectant showed instant decontamination (about 2 s) of SM simulant dibutyl sulfide as studied using the gas chromatographic mass spectrometric technique (Clark *et al.*, 1999). There is a concern that several proprietary formulations may have adverse effects on the effectiveness of the skin decontaminant Fuller's earth (a native form of aluminum silicate) which is generally used as a physical decontaminant (Chilcott *et al.*, 2002). Many countries use approved skin decontaminants for SM such as M291 (US Army), RSDL (Canada), Fuller's earth (UK), and PDK (India). It is also recommended that if the decontaminants are not readily available, talcum powder or flour can be used (Borak and Sidell, 1992). The use of water for decontamination of oily chemical warfare agents is contraindicated, as it may allow the spreading of the agent, facilitating absorption. However, eye contamination can be removed by copious use of water.

B. Efficacy of Fuller's Earth

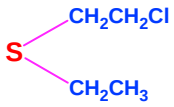
The decontamination efficiency of Fuller's earth, Ambergard, and BDH spillage granules was tested by measuring the skin absorption rates and the percentage of applied dose of SM that penetrated human and pig ear epidermal membranes *in vitro*. Fuller's earth was found to be better than the other two. This was in agreement with an *in vivo*

Sulphur mustard (SM)



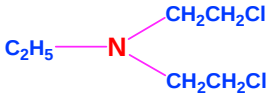
Bis-2 chloro ethyl sulphide

CEES



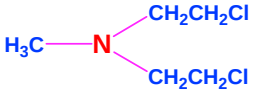
Chloro ethyl ethyl sulphide

Nitrogen Mustard – 1 (HN-1)



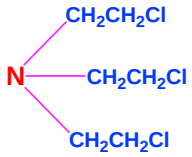
N-ethyl-2,2' –di(chloroethyl)amine

Nitrogen Mustard – 2 (HN-2)



N-methyl-2,2' –di(chloroethyl)amine

Nitrogen Mustard – 3 (HN-3)



2,2',2''–tri(chloroethyl)amine

FIGURE 60.1. Chemical structures of different mustard agents.

test in a rat model and human volunteer study (Chilcott *et al.*, 2001). The Canadian Reactive Skin Decontaminant Lotion has been identified to be better than Fuller's earth when decontaminated 5 min after dermal contact with SM and VX in domestic swine (Taysse *et al.*, 2007). Several proprietary decontaminants are known, such as the Polish formulation ORO and C9 that can react with SM (Popiel *et al.*, 2005).

C. Efficacy of Hypochlorites

The decontamination efficacy of sodium hypochlorite and calcium hypochlorite solutions was found to be similar against low-dose SM in guinea pigs with respect to the wound on the skin. Surprisingly, the skin surrounding nondecontaminated but exposed animals demonstrated less wound showing that the hypochlorite solutions *per se* or the SM–hypochlorite solution interaction might have caused the skin lesions (Gold *et al.*, 1994). The concentration of hypochlorite solution also matters in the efficiency of chemical decontamination. 0.5% is a weak and 4% is a mild decontaminant of SM. If the ratio of hypochlorite to SM was kept at 1,000:1 then 0.5% and 4% effectively decontaminated SM (Wormser *et al.*, 2004). Vaporized hydrogen peroxide is a common gaseous sterilant widely used in industry and it has been found to be an effective chemical

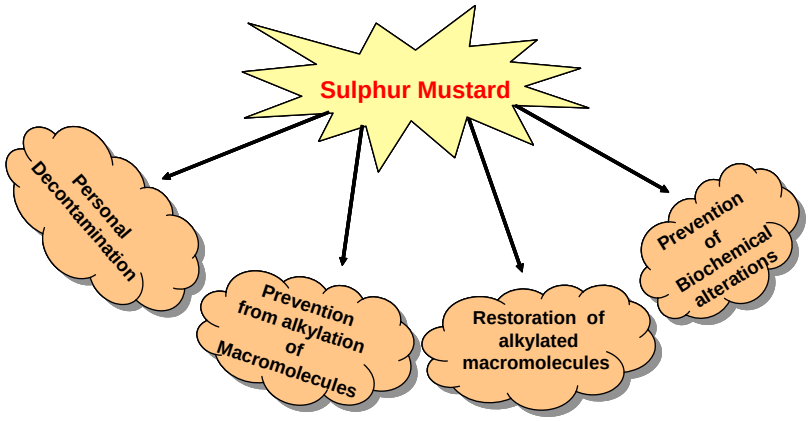


FIGURE 60.2. Possible approaches for antidote development against SM toxicity.

TABLE 60.3. Requirements of a good decontaminant

1. Effective against most of the chemical warfare agents
2. Simple and self-administration without any assistance
3. Safe from any type of toxicity
4. Abundant and easily available
5. Large-scale production possible

warfare agent decontaminant in the presence of ammonia for SM and nerve agents (Wagner *et al.*, 2007).

D. Efficacy of Chloramides

The chloroamide compound 1,3,4,6-tetrachloro-7,8-diphenyl-2,5-diiminoglycoluril (S-330) is a rapid and effective chemical decontaminant of SM, and dermal formulations either in polar media or in perfluoropolyether oil have been developed. A disadvantage of S-330 is that it can tolerate only 5–10% of water. Alcohols and acidic and basic conditions also reduce its efficiency (Shih *et al.*, 1999a, b). A reactive chemical decontaminant, CC2 (*N*,*N*′-dichloro-bis [2,4,6-trichlorophenyl] urea) in various hydrophilic and lipophilic formulations (20%) has been prepared as a personal decontaminant for SM. CC2 reacted with peanut oil and neem oil, and was unstable in povidone iodine and Fuller's earth. Good stability was achieved with petroleum jelly, honey, polyvinyl pyrrolidone, calamine lotion, acacia, and hydroxypropyl cellulose. Though CC2 was stable in lipophilic formulations, it did not protect the animals. The hydrophilic formulations, particularly acacia and hydroxypropyl cellulose, gave very good protection and were highly stable. CC2 is also a very safe compound and compatible with human skin (Kumar *et al.*, 1991; Reddy *et al.*, 1996; Dubey *et al.*, 1999; Vijayaraghavan *et al.*, 2001b, 2002; Lal *et al.*, 2002).

E. Efficacy of Other Decontaminants

The decontamination efficacy of radioprotective agent aminoethylisothiourea and cystamine on rat skin against SM was comparable to standard decontaminants, such as alcoholate, clay, and Fuller's earth (Knezević and Tadić, 1996). A mixture containing bovine hemoglobin, gelatin, and poi (a Hawaiian foodstuff) on the hydrolytic kinetics of the mustard simulants, 2-chloroethyl ethyl sulfide, 2-chloroethyl methyl sulfide and 2-bromoethyl phenyl sulfide, is reported. The kinetic mechanisms and rate constants were dependent upon the mixtures' concentration and viscosity (Cerny and Cerny, 1997).

III. TREATMENT OF SKIN INJURIES

SM is a potent cutaneous vesicant that penetrates rapidly through the skin, causing prolonged injuries and leading to severe incapacitation (Kadar *et al.*, 2000). The metabolically active and proliferating basal cells in the skin are more sensitive to SM (Ray *et al.*, 2000). SM produces blisters with a severe inflammatory reaction in skin of exposed individuals (Casbohm *et al.*, 2004). The development of efficacious countermeasures against SM vesication requires an understanding of the cellular and molecular mechanism of SM-induced tissue injury (Sabourin *et al.*, 2004). Progress towards an effective treatment for SM injuries has been slow due to the lack of a suitable animal model to study the toxicology and pathology.

SM is probably the only chemical showing more toxicity through the percutaneous route compared to subcutaneous route in animal models. For the same dose the percentage body weight loss, hepatic glutathione content, and histopathological lesions were more in the percutaneous route compared to subcutaneous route (Vijayaraghavan *et al.*, 2005). Though 2-chloroethyl ethyl sulfide is used as a simulant for studying the mechanism of toxicity and development of antidotes for SM, the pattern of toxicity of 2-chloroethyl ethyl sulfide is different from SM (Gautam *et al.*, 2006). SM is used in ointment (S-mustard-vaseline), known as Russian ointment, in a concentration of 1:20,000 (0.005%) for the effective treatment of psoriasis (Illig *et al.*, 1979; Janouchek *et al.*, 1987).

A cascade of events follow SM-induced alkylation of macromolecules. SM most likely alkylates purine in deoxyribonucleic acid (DNA). The apurinic endonucleases act at SM–DNA adducted sites to produce backbone breaks in DNA and this activates chromosomal enzyme poly(ADP-ribose)polymerase (PARP). This enzyme utilizes nicotinamide adenine dinucleotide (NAD^+) as a substrate and depletes the NAD^+ content of the cells. The depletion of NAD^+ causes inhibition of glycolysis resulting in the accumulation of intermediates that stimulate the $NADP^+$-dependent hexosemonophosphate shunt. The stimulation of the hexosemonophosphate shunt has been associated with DNA damage and increase in protease synthesis and release. These proteases could be responsible for the development of subepidermal blisters characterized by fluid accumulation in the cavity created by separation of the moribund basal cell layer from the basement membrane (Papirmeister *et al.*, 1985). When a wound was created by scratching human epidermal keratinocytes, the nitric oxide synthase level was increased. SM inhibited the increase in nitric oxide synthase level. Preventing the SM-induced inhibition of inducible nitric oxide synthase may be a prospective strategy to promote wound healing in SM-exposed skin (Ishida *et al.*, 2008).

A. Efficacy of Steroids and Nonsteroidal Anti-Inflammatory Drugs

The mouse ear vesicant model has been used to evaluate pharmacological agents for countering SM dermal injury. The SM-induced skin injury in mouse ear was found to be

very similar to that reported in human skin. The combined topical application of adexone, a steroid, and voltaren, a nonsteroidal anti-inflammatory drug, was found to be effective in reducing the intensity of SM skin injury in the mouse ear vesicant model. But this combination did not protect the cytotoxicity of the epithelial layer (Dachir *et al.*, 2004). The SM-induced inflammation can be reduced only when the treatment is initiated at an early stage (Dachir *et al.*, 2002). The systemic administration of anti-inflammatory drugs, such as hydrocortisone, indomethacin and olvanil, provided a significant reduction of edema induced by SM. They also significantly reduced the subepidermal blisters in the mouse ear vesicant model and olvanil additionally protected the epidermal necrosis (Babin *et al.*, 2000; Casillas *et al.*, 2000).

In the mouse ear model, dimercaprol, octyl homovanillamide, and indomethacin were given 15 min before SM topical application, and ear tissue was harvested for ear weight determination. All three drugs were found to reduce SM damage (Dillman *et al.*, 2006). When SM was applied topically on the inner surface of mouse ear, genes responsible for inflammation, apoptosis, and cell cycle regulation were altered, showing that intervention with these molecular mechanisms could be appropriate targets for developing prophylactic and therapeutic treatments for SM-induced skin injury (Sabourin *et al.*, 2004). The vanilloid olvanil reduced SM-induced edema and also mRNA expression of cytokines and chemokines. This suggests that blocking the inflammatory effects of neuropeptides, such as substance P, may provide protection against SM-induced dermal injury (Casbohm *et al.*, 2004). Exposure to SM on mouse ear edema model showed alteration in interleukin-6 which can be used as an *in vivo* biomarker for evaluating anti-inflammatory drugs (Ricketts *et al.*, 2000). The involvement of tumor necrosis factor-α in SM-induced skin toxicity was confirmed by a reduction in mouse ear edema following administration of anti-tumor necrosis factor-α antibodies. Anti-tumor necrosis factor-α antibodies may be a new approach in the treatment of SM (Wormser *et al.*, 2005).

B. Efficacy of Protease Inhibitors

Anti-inflammatory drugs are effective against SM-induced toxicity, but they are generally evaluated using *in vivo* techniques. Interleukin-8 has been used as a marker for screening SM antidotes in human epidermal keratinocyte cell cultures. A protease inhibitor, ICD 1579 (ethyl *p*-guanidinobenzoate hydrochloride), and an inhibitor of trypsin, *N*-tosyl-L-lysine chloromethyl ketone, were evaluated against SM. ICD and *N*-tosyl-L-lysine chloromethyl ketone dose dependently suppressed SM-induced increase in interleukin-8 (Cowan *et al.*, 2002). Human epidermal keratinocytes exposed to SM were treated with a p38 mitogen activated protein kinase inhibitor, SB203580, and the levels of interleukin-6, interleukin-8, tumor necrosis factor-α and interleukin-1β were significantly decreased, showing that p38 MAP kinase plays a role in SM-induced cytokine production in human epidermal keratinocytes. Inhibition of this may reduce the inflammatory response elicited by SM exposure (Dillman *et al.*, 2004). SM exposure to weanling pigs showed transcriptional activity in the inflammatory response proteins interleukin-6, interleukin-8, interleukin-1β, and matrix metalloproteinase-9 and moderate changes in tumor necrosis factor-α which may contribute to SM dermatotoxicity. Drugs preventing SM-induced inflammation should be one of the medical countermeasures to lessen the toxicity (Sabourin *et al.*, 2002). Pretreatment with caffeic acid phenethyl ester, a known inhibitor of NF-κB signaling, significantly decreased SM and nitrogen mustard (mechlorethamine) induced reporter gene activity in human epidermal keratinocytes (Minsavage and Dillman, 2007).

Epidermal melanocytes have a higher sensitivity to SM compared with other skin cell types, which may be due to the enzymatic production of melanin precursors exerting an additional cytotoxic effect. *In vivo* experiments have shown that melanocytes are more sensitive than keratinocytes for studying the cytotoxic effects of SM. This may be due to the uncoupling of the melanogenic pathway by depletion of cellular glutathione, resulting in the increased production of cytotoxic quinone free-radical species by tyrosinase. Kajoic acid, a depigmenting agent, exerts its effect by acting as a slow-binding, competitive inhibitor of tyrosinase. Kajoic acid can be used as an adjunct for SM skin injuries (Smith and Lindsay, 2001a). Similarly, the control of the cell cycle by protein kinase C modulation and manipulation of melanin synthetic pathways may also have therapeutic benefits (Smith and Lindsay, 2001b). Laminin-5 is a heterotrimer of laminin alpha3, beta3, and gamma2 subunits, and is a component of epithelial cell basement membranes. Studies on human epidermal keratinocytes showed that SM alkylates laminin-5 and degrades it, resulting in basal cell death and separation of the epidermis from the dermis. This information may be useful in developing cutaneous therapies for SM lesions (Jin *et al.*, 2008).

C. Efficacy of PARP Inhibitors

It has been postulated that SM-induced cellular damage may activate PARP, resulting in the depletion of cellular NAD^+ and leading to blister formation. It has been demonstrated that niacinamide, an inhibitor of PARP and a precursor for NAD^+ synthesis, may be useful as a pretreatment compound to reduce SM-induced microvesication in guinea pig skin. When given as a pretreatment and post-treatment it was observed that niacinamide should be repeatedly given to reduce the microblisters induced by SM (Yourick *et al.*, 1992). Pretreatment and post-treatment (4 to 6 h) with a combination of niacinamide, promethazine, and indomethacin reduced the erythema induced by SM in hairless guinea pig skin. But when the combination was applied 24 h

after SM administration no protection was observed (Yourick *et al.*, 1995).

D. Efficacy of Iodine Treatment

Several studies showed that post-exposure treatment with iodine is effective against SM lesions in rodents. SM exposure in guinea pigs showed a marked increase in epithelial nuclear vacuolation, epidermal thickening, and acute inflammation, a few hours after exposure. Topical iodine treatment reduced the severity of these parameters (Brodsky *et al.*, 2006). Povidone–iodine ointment is an efficient protective agent against skin toxicity caused by SM (Wormser *et al.*, 1997). Histopathological examinations in animal models showed that application of povidone–iodine up to 20 min following exposure to the vesicant resulted in marked skin protection. It was also observed that the shorter the interval between exposure and treatment the better the result. Topical iodine preparations have been used as post-exposure treatment against SM-induced skin lesions in fur covered guinea pig skin. Iodine treatment 15 min after SM exposure resulted in statistically significant reductions of dermal inflammation, hemorrhage, and necrosis. It was also observed that SM was not affected chemically by iodine as measured by gas chromatography mass spectrometry analysis (Wormser *et al.*, 2000b). A pig model was used to simulate SM-induced skin lesions and a single dose of iodine formulation was applied. A single application of iodine formulation at 20 min post-SM exhibited no protection as observed by biopsy. This shows that the animal skin may behave differently for SM (Margulis *et al.*, 2007). Apart from being a safe and widely used disinfectant, povidone–iodine ointment is recommended as an efficient protective agent against skin toxicity caused by hazardous chemicals and by heat stimuli (Wormser *et al.*, 2000a).

Topical treatment with iodine or povidone–iodine ointment significantly reduced the skin lesions induced by SM. The combination of anti-inflammatory agents and iodine increased the counterirritating activity. Both human and experimental animal studies demonstrated that the ointment should be applied immediately after SM exposure (Brodsky and Wormser, 2007). Since proteolytic activity is involved in skin damage caused by chemical irritants, the effect of iodine on mechlorethamine-induced skin collagenolytic activity was studied in the guinea pig model. Reduced collagenolytic activity may be one of the mechanisms by which iodine protects the skin against chemical injury (Wormser *et al.*, 2002b). Iodine treatment significantly reduces inflammation and necrosis and increases epidermal hyperplasia. Experiments carried out in animal models show that nitric oxide radicals are involved in SM-induced skin lesions. Reduction of inflammation by iodine ointments may be associated with reduced inducible nitric oxide synthase expression (Nyska *et al.*, 2001). Though some studies showed the effectiveness of topical iodine for 15 to 30 min, in another study the protection was demonstrated for up to 60 min in the mouse ear edema model using an improved topical povidone–iodine preparation termed N66, which contains steroidal and nonsteroidal anti-inflammatory agents. Povidone–iodine preparation combined with anti-inflammatory agent may function as a potent antidote against skin lesions induced by SM at relatively long intervals between exposure and treatment (Wormser *et al.*, 2004).

E. Efficacy of Mechanical Treatments

The SM skin wound is very slow healing. Miniature pig models were used to assess the usefulness of mechanical dermabrasion in hastening the healing of SM vapour-induced skin injuries. Dermabrasion of the SM wound is a valuable procedure in surgical management (Rice *et al.*, 2000). Several treatment modalities for chemical burns, such as surgical excision, laser ablation, and chemical debridement with debridase or trypsin-linked to gauze, were evaluated against SM and nitrogen mustard-induced wounds in the guinea pig model. Debridase was most effective and reduced significantly the burn lesion (Eldad *et al.*, 1998b).

Exposure to SM can produce partial thickness burns which take a long time to cure. To accelerate the rate of healing, laser ablation was given on SM burns on the dorsum of large white pigs, and it was found to be effective (Evison *et al.*, 2006). SM burns were generated on the ventrum of weanling pigs and four treatments were compared: (1) full-thickness CO_2 laser debridement followed by skin grafting, (2) full-thickness sharp surgical tangential excision followed by skin grafting, used in deep thermal burns management, (3) partial-thickness laser ablation with no grafting, and (4) partial-thickness sharp excision with no grafting. It was observed that laser debridement followed by skin grafting was effective in improving the wound healing of deep SM burns (Graham *et al.*, 2002a, b).

When wound healing studies were conducted on SM wounds in weanling pigs, it was observed that re-epithelialization was nearly complete with petrolatum and scarlet red dressings. The use of cultured epithelial allograft material, Xeroform Petrolatum and Scarlet Red Ointment dressings, is an effective and inexpensive treatment for SM injuries (Graham *et al.*, 2006).

An anesthetized domestic swine model was used to investigate whether alteration of skin temperature had any effect either apparently or histopathologically on the development and progression of SM-induced skin lesions. Cooling the SM-exposed skin (15°C) reduced the severity of SM-induced cutaneous lesions (Sawyer *et al.*, 2002). Similarly, the effect of temperature on the development of SM-induced toxicity was investigated in human skin keratinocyte cultures and on hairless guinea pig skin. Moderate cooling (5 to 10°C) of SM exposed sites after 4 to 6 h of treatment significantly reduced the severity of the lesions.

In contrast, *in vitro* cooling only temporarily reduced the toxicity (Mi *et al.*, 2003).

F. Efficacy of Cholinergic Drugs

SM causes blisters because epithelial cells lose their attachments. Since epithelial cell adhesion is under the control of the local cytotransmitter acetylcholine acting through the muscarinic and nicotinic receptor, it is possible that pharmacologic protection from the vesicating action of SM can be achieved by using cholinergic drugs (Grando, 2003). Similar to serine protease inhibitors that can prolong the survival of soman intoxicated animals and also protect against SM toxicity, the PARP inhibitors can reduce both soman-induced neuronal degeneration and SM-induced epidermal necrosis (Cowan *et al.*, 2003).

G. Efficacy of Calmodulin Antagonists

Ointments containing calmodulin antagonists were found to be effective in preventing skin injuries induced by SM. Topically applied pluronic base ointments containing lidocaine or pentamide gave beneficial effects when applied immediately after SM on pig skin (Kadar *et al.*, 2000). Calmodulin antagonists and anesthetics on the skin lesions induced by SM were investigated in hairless mice and it was observed that calmodulin antagonists and anesthetics in water soluble bases may be useful for the treatment of SM-induced skin lesions. The water soluble ointment bases showed some beneficial effects, whereas oily bases made the skin lesions worse. Trifluoperazine (0.5–1%) and thioridazine (2%), potent calmodulin antagonists, in Pluronic F-127 base considerably prevented the development of SM-induced skin lesions. A similar effect was achieved with pentamidine (10%). Anesthetics, such as lidocaine and pentobarbital, showed some protection, although at concentrations above 5% (Kim *et al.*, 1996a). Since *O*-phenanthroline inhibited the ulcerative effect of topically applied meclorethamine in guinea pigs and also protected against meclorethamine-induced toxicity in rat liver slice cultures, it could also be a useful antidote to SM (Wormser and Nyska, 1991).

H. Treatment of Pruritis in Humans

In humans the treatment of skin lesions is mostly symptomatic. Pruritus is a common problem among SM-exposed veterans. A number of treatments like antihistamines, local anesthetics, and corticosteroids are prescribed in order to control pruritus in Iranian patients. When Unna's Boot (a gauze bandage impregnated with glycerine, zinc oxide, and calamine lotion) and betamethasone were compared for the treatment of pruritis, Unna's Boot showed better results (Shohrati *et al.*, 2007a). SM-induced pruritis in chemical warfare-injured veterans was treated with phenol (1%) and menthol (1%), and this combination significantly reduced the pruritis in comparison to placebo (Panahi *et al.*, 2007). A double-blind safety and efficacy study was conducted to compare the safety and efficacy of cetirizine, doxepine, and hydroxyzine as a treatment of chronic pruritus in SM-exposed victims. Though sedation was reported in cetirizine, doxepine, and hydroxyzine groups, hydroxyzine and doxepine gave better results than cetirizine (Shohrati *et al.*, 2007b, c).

IV. TREATMENT OF LUNG LESIONS

Exposure of SM vapors to mice induced sensory irritation during exposure, and there was a concentration-dependent decrease in the respiratory frequency and an increase in tidal volume. The respiratory frequency decreased on subsequent days of exposure depending upon the exposure concentration, and the breathing pattern was characteristic of bronchial obstruction (Vijayaraghavan, 1997; Lakshmana Rao *et al.*, 1999). Administration of aerosolized SM solution through the intratracheal route in guinea pigs induced two main symptoms, asthma-like symptoms reflected by an early bronchoconstriction and late asthmatic responses, and acute respiratory distress syndrome with pulmonary edema and damage to the lung surfactant. Mice exposed to SM vapors showed infiltration of inflammatory cells, swelling and vacuolation of parenchymal cells, and congestion and hemorrhage in the alveoli (Pant and Vijayaraghavan, 1999, 2002).

A. Efficacy of Salbutamol and Curosurf

Intratracheal nebulization of salbutamol solution or bolus administration of curosurf (surfactant) reduced the mortality in guinea pig. Salbutamol appeared to be more effective than curosurf in this respect. Since SM exposure induced damage to the lung surfactant, severe bronchoconstriction, and inflammation of the respiratory tract, a combined treatment consisting of exogenous surfactant, anti-inflammatory drugs, and broncholytics may be a better choice (Van Helden *et al.*, 2004).

B. Efficacy of Corticosteroids

Intratracheal injection of SM in guinea pigs induces airway epithelial damage and the tracheal epithelium appeared disorganized and showed a significant decrease in height and cell density. Corticoids like betamethasone showed significant effects on airway epithelium (Calvet *et al.*, 1996). Intratracheal injection also induces airway muscle hyperresponsiveness to substance P by reducing tracheal epithelial neutral endopeptidase activity. Corticoids like betamethasone inhibit this hyperresponsiveness (Calvet *et al.*, 1994a). Primary alveolar macrophages obtained from guinea pigs were exposed to SM and treated with dexamethasone, and the SM-induced inflammatory effect was reduced. SM intoxication in guinea pigs through intratracheal injection showed severe lesions to the

tracheal epithelium with an increase in respiratory system resistance and microvascular permeability. Pretreatment with capsaicin did not prevent this. Aerosolized substance P and histamine induced airway hyperresponsiveness and pretreatment with phosphoramidon caused a further increase in airway responsiveness to substance P (Calvet *et al.*, 1994b). Accumulation of high levels of TNF-α was observed in guinea pigs exposed to 2-chloroethyl ethyl sulfide, an SM analog, intratracheally. This activates both acid and neutral sphingomyelinases, resulting in the accumulation of ceramides, a second messenger involved in cell apoptosis. Since NF-κB disappears, the cells continue to be damaged owing to accumulation of ceramides and activation of several caspases, leading to apoptosis (Chatterjee *et al.*, 2003).

Chronic bronchitis is the most frequent late respiratory disease among Iranians exposed to SM during the Iran–Iraq War. Oral and intravenous corticosteroid therapy was investigated in improving the lung function in SM-induced chronic bronchitis patients. There was a significant improvement in spirometry indices of oral and intravenous groups (Ghanei *et al.*, 2005). Since short-term corticosteroid therapy has a significant effect on lung function in most of the patients with SM-induced chronic bronchitis, a short-term bolus steroid treatment to triage the patients into responders and nonresponders for subsequent treatment is required (Ghanei *et al.*, 2005). Interferon gamma-1β was administered to patients with bronchiolitis. A 6 month treatment with interferon gamma-1β and a low dose of prednisolone improved the lung function of SM-exposed patients with bronchiolitis (Panahi *et al.*, 2005).

C. Efficacy of Combination of Corticosteroids and β-Agonist

The role of two-combination inhaler therapy was tested in humans exposed to SM. Either fluticasone propionate and salmetrol or beclomethasone and salbutamol were given. Inhaled corticosteroids and long-acting beta 2-agonists are effective in the treatment of patients with chronic bronchiolitis following exposure to SM. However, a medium dose of fluticasone/salmeterol has the same effect on airway reversibility, rather than a very high dose of beclomethasone with the short-acting beta-agonist (Ghanei *et al.*, 2007). Persistent cough is a usual occurrence in SM-exposed victims which is alleviated with an anti-tussive.

D. Efficacy of Doxycycline and Roxithromycin

Respiratory tract lesions induced by SM are characterized by epithelial cell damage associated with inflammatory cell infiltration. SM was injected intratracheally in guinea pigs pretreated with doxycycline. Doxycycline pretreatment resulted in decreased gelatinase activity, decreased inflammation, and protected histological lesions. Doxycycline may be a promising therapeutic agent for SM (Guignabert *et al.*, 2005). Anticytotoxic and anti-inflammatory effects of roxithromycin, a macrolide antibiotic, were tested *in vitro* using normal human small airway epithelial cells and bronchial/tracheal epithelial cells exposed to SM. Roxithromycin has inhibitory effects on the cytotoxicity and inflammation induced by SM. This effect may be due to the down-regulation of proinflammatory cytokines by roxithromycin. Roxithromycin may serve as a potential therapeutics agent for SM toxicity which is independent of its antibacterial activity (Gao *et al.*, 2007).

E. Efficacy of Tetraamines

Hexamethylenetetramine has been shown to protect human lung cells against SM toxicity and has also been shown to be effective *in vivo* against phosgene. The ability of hexamethylenetetramine to protect against the toxicity of SM was investigated in human upper respiratory tract cell lines, BEAS-2B and RPMI 2650. Results demonstrated that it was necessary for hexamethylenetetramine to be available at the time of exposure to SM for effective cytoprotection. No protection was seen when cells were treated with hexamethylenetetramine following exposure to SM, or where hexamethylenetetramine was removed prior to SM exposure. Results suggest that hexamethylenetetramine may be effective as prophylaxis for exposure to SM by inhalation (Andrew and Lindsay, 1998a). The A549 cell line is used as a model to study the toxicity and mechanism of action of SM. The ability of hexamethylenetetramine to protect A549 cells against the toxic effects of SM was studied and it was found that hexamethylenetetramine could protect cells against the effects of SM, though hexamethylenetetramine has to be present at the time of SM challenge (Lindsay and Hambrook, 1997).

F. Efficacy of Methacholine

SM induces asthma in humans. In patients with SM-induced asthma, the airway responsiveness to both methacholine and distilled water depends upon the percentage of bronchoalveolar lavage eosinophils (Emad and Emad, 2007a, b). Hematopoiesis could be affected by SM exposure. The average number of red blood cells and hemoglobin of victims were increased compared with historical data. The mild increase in erythroid cells and hemoglobin concentration may be due to chronic obstructive pulmonary disorder and other respiratory diseases in these patients (Ghanei, 2004).

G. Efficacy of Isopropyl Esters

Human and animal lung cells have been used successfully to model the toxic effects of inhaled SM. The epithelia of the upper respiratory tract are the primary targets of inhaled SM. The potential of monoisopropyl esters and diisopropyl esters of glutathione as cytoprotectants in the human upper respiratory tract cell lines, BEAS-2B and RPMI 2650, was

evaluated against SM. The greatest protection was shown where monoisopropyl esters or diisopropyl esters were present at the time of exposure to SM. The optimum pretreatment times were found to be 1 h for monoisopropyl esters and 2 h for diisopropyl esters. Limited protection of cells treated with monoisopropyl esters or diisopropyl esters immediately following SM exposure was also demonstrated. No protection was observed if monoisopropyl esters or diisopropyl esters were not administered immediately following SM exposure. Results suggest that monoisopropyl esters and diisopropyl esters may be effective treatments for exposure to SM by inhalation (Andrew and Lindsay, 1998b). *N*-acetyl cysteine is also a good antidote for SM lung injury (Mérat *et al.*, 2003).

V. TREATMENT OF EYE LESIONS

The eye is the organ most sensitive to SM vapor. Ocular damage is common in both military and civilian populations exposed to SM in the Iran–Iraq War. Ocular injuries following SM exposure are characterized by an inflammatory response, observed as eyelid swelling, conjunctivitis, corneal edema, cellular infiltration, and photophobia. Patients with significant corneal involvement are at risk for corneal ulceration. Ocular injuries generally heal completely in 1 or 2 weeks. In severe cases, blindness may occur (Safarinejad, 2001). Topically applied steroid treatment and anti-inflammatory drugs are potential therapies, and this can be supplemented with an antibacterial agent if required. The development of SM-induced ocular lesions in rabbits is similar to the lesions described in human casualties (Kadar *et al.*, 2001). Rabbit eyes were exposed to SM vapor and treated with commercial ophthalmic solution of dexamethasone or diclofenac and both treatments gave good protection. Anti-inflammatory agents, though effective, do not have any therapeutic effect on corneal erosions (Amir *et al.*, 2000a, b).

VI. TREATMENT OF SYSTEMIC EFFECTS

A variety of strategies and drugs have been experimented on *in vitro* using a number of cell lines, and *in vivo* using different animal models. None of the agents proved to be effective in all the systems, and hence it could not be recommended as a standard therapy for SM-exposed victims. Moreover, other than SM, its analogs have been used as stimulants without any conclusive evidence of the effectiveness of the therapies (Das *et al.*, 2003; Gautam *et al.*, 2007).

A. Efficacy of Niacinamide, Aminobenzamide, and Cyclohexamide

Human mononuclear leukocytes are a good model for studying SM-induced cytotoxicity. Niacinamide, 3-aminobenzamide, and PARP inhibitors have been shown to be effective in blocking SM-induced cell death when added to the cultures during the first 4 h post-exposure. They offered partial protection when added between 6 and 12 h and were of no benefit when added 12 h post-exposure (Meier, 1996). Upon SM exposure to human lymphocytes the cells lose membrane integrity and viability, the nuclear components get degraded and the cellular function is affected. Treatment of SM-exposed cells with PARP inhibitors prevents or alters the SM-initiated loss of cell viability, membrane integrity, cellular metabolic constituent, and cellular energy, while initiating alterations in nuclear constituents (Meier *et al.*, 2000).

The treatment of human peripheral blood lympocyte cells with selective PARP inhibitors reduced the DNA fragmentation caused by SM. The PARP inhibitors arrested DNA fragmentation at the DNA ladder stage. This effect was observed only when the PARP inhibitors were applied within 8 h of SM exposure (Meier and Millard, 1998). Although endothelial cells and keratinocytes appear to be the primary cellular targets of SM, the role of PARP in SM-induced vesication has not been clearly defined. These observations support the hypothesis that the pathogenic events necessary for SM-induced vesication (i.e. capillary leak and loss of keratinocyte adherence) at higher vesicating doses of SM may depend on NAD^+ loss with PARP activation and subsequent ATP-dependent effects on microfilament architecture (Hinshaw *et al.*, 1999).

SM exposure to normal human epidermal keratinocytes activated the DNA repair enzyme DNA ligase. There is also an active role of PARP in DNA ligase activation and DNA repair in mammalian cells. Concurrent activation of PARP and DNA ligase was also observed when exposed to SM. In human epidermal keratinocytes, when PARP was inhibited by 3-amino benzamide, SM-activated DNA ligase had a half-life that was four-fold higher than that observed in the absence of 3-aminobenzamide. DNA ligase remains activated until DNA damage repair is complete. This suggests that DNA repair requires PARP and PARP is essential for efficient DNA repair. However, PARP participates in DNA repair by altering the chromosomal structure to make the DNA damage sites accessible to the repair enzymes (Bhat *et al.*, 2006). The modulation of a PARP-mediated mechanism may provide a useful approach in preventing SM toxicity (Bhat *et al.*, 2000). Catalytic activation of PARP has been demonstrated to be a major event in response to high levels of DNA damage, and PARP activation may be part of apoptotic signaling (Figure 60.3). In other contexts, overstimulation of PARP triggers necrotic cell death because of rapid consumption of its substrate, NAD^+, and the consequent depletion of ATP. Exposure to SM significantly increased PARP activity in HaCaT cells. The total cell viability was not altered by the administration of 3-aminobenzamide with SM. However, the mode of cell death was influenced by 3-aminobenzamide exhibiting an increase of apoptotic cells and a concomitant decrease in necrotic HaCaT cells (Kehe *et al.*, 2007).

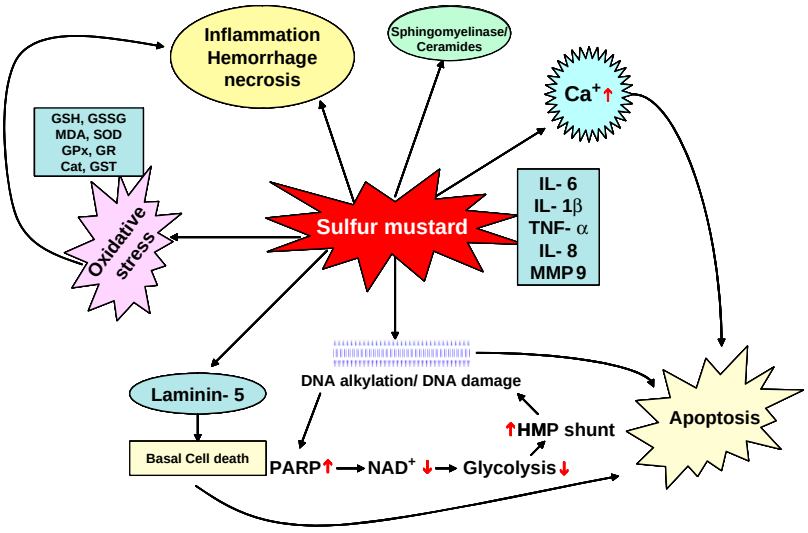


FIGURE 60.3. Possible biochemical alterations after SM toxicity.

When human keratinocytes were exposed to 2-chloroethyl ethyl sulfide, interleukin-1α and related cytokines were depressed, but PGE-2 release was not affected. Histology showed that 2-chloroethyl ethyl sulfide induced the separation of dermal and epidermal regions with severe damage to basal keratinocytes. The adverse changes associated with 2-chloroethyl ethyl sulfide exposure were not ameliorated by niacinamide (PARP inhibitor), CGS9343B (calmodulin antagonist), and leupeptin (cysteine protease inhibitor) (Blaha *et al.*, 2000a, b). Intraperitoneal administration of 2-chloroethyl ethyl sulfide in mice is more potent than its oxidation derivatives 2-chloroethyl ethyl sulfoxide and 2-chloroethyl ethyl sulfone in decreasing organ weight and loss in GAPDH activity, and an increase in GST induction (Kim *et al.*, 1996b). 2-Chloroethyl ethyl sulfide can induce the classical morphological features of apoptosis in the nucleus. Cycloheximide, a protein synthesis inhibitor, does not suppress the 2-chloroethyl ethyl sulfide-induced apoptotic activity, showing that 2-chloroethyl ethyl sulfide-induced apoptosis is a cytotoxic mechanism and is independent of the synthesis of proteins (Hur *et al.*, 1998). Cultured normal human epidermal keratinocytes were used as a model to study and characterize the protease stimulated by the mustards 2-chloroethyl ethyl sulfide, mechlorethamine, and SM. The results suggest that mustard toxicity may involve the stimulation of trypsin/chymotrypsin-like serine protease, dependent on Ca^{2+}. Protease inhibitors may be useful as prospective drugs for SM (Ray *et al.*, 2002).

Inhibitors of mono(ADP-ribose) transferase, lipid peroxidation, and protein synthesis are not effective in reducing SM-induced cell death, while PARP inhibitors are found to be effective in reducing the cytotoxic effects of SM in human lymphocytes. The compound's ability to inhibit PARP has a direct correlation to its ability to reduce SM-induced cell death. PARP plays an important role in SM-induced cell death (Clayson *et al.*, 1993). The effectiveness of various therapeutic agents, namely niacin, niacinamide, and 3-aminobenzamide, was evaluated in human lymphocyte cultures against SM-induced cytotoxicity. Niacinamide and 3-aminobenzamide prevented the cytotoxic effects of SM for up to 2 days (Meier and Johnson, 1992). Lymphocytes exposed to SM show about 30 to 40% of viability, and loss of microvilli, large cytoplasmic vacuoles, extensive blebbing of the perinuclear envelope, loss of cytoplasmic organelles, condensation of nuclear chromatin, and multiple perforations of the plasmalemma. Niacinamide treatment improved the viability to 87% (Petrali *et al.*, 1990). Niacinamide may only be effective as a pretreatment compound to reduce the incidence of SM-induced microvesicle formation (Yourick *et al.*, 1991).

B. Efficacy of BAPTA AM

A Ca^{2+}-dependent mechanism of SM toxicity has been proposed in human epidermal keratinocytes (Figure 60.3). The SM toxicity was inhibited by the Ca^{2+} chelator BAPTA AM [1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester]. The mechanism of protection of BAPTA AM against SM may be by decreasing the metabolic derangements produced by SM (Ray *et al.*, 2000). The role of calcium in SM-induced cell death is well known and modulation of intracellular calcium concentrations may assist in providing protection. SM induces an immediate and irreversible increase in internal free calcium level and it is independent of external calcium concentrations. Treatment of neonatal human skin keratinocytes with thapsigargin, an endoplasmic reticulum calcium ATPase inhibitor, or BAPTA-AM, a calcium chelator, did not change SM toxicity. Furthermore, alteration of external calcium concentrations also failed to improve. Treatment of confluent cultures with ionomycin also failed to give protection against SM showing that in neonatal human skin keratinocytes the changes in intracellular calcium induced

by SM do not play a major role in SM cytotoxicity (Sawyer and Hamilton, 2000).

C. Efficacy of Pyrimethamine and Cimetidine

The suppression of the immune system is one of the major causes of infections, septicemia, and death in patients exposed to SM. The effectiveness of two immunomodulating agents, pyrimethamine and cimetidine, was tested in mice exposed to SM. Pyrimethamine and cimetidine enhanced the antibody titers to sheep red blood cells, augmented delayed-type hypersensitivity responses, and restored splenic follicles compared to control exposure. Spleen weight indices were not augmented by either drug. The immunomodulating drugs may prove effective in countering the immunosuppressive effects of SM (Ebtekar and Hassan, 1993).

D. Efficacy of Doxycycline

Doxycycline inhibits the detachment of SM-exposed HaCaT cells in culture from the growth substrate. However, analysis of the metabolic activity of the adherent cells reveals that doxycycline treatment does not maintain cell viability. It is suggested that doxycycline and other matrix metalloprotease inhibitors may have a role to play in therapeutic intervention against SM, only as a combination therapy (Lindsay *et al.*, 2007). Human keratinocyte cell lines were pretreated with mixtures of methenamine and glutathione prior to SM exposure. Though it is possible to protect the cell cultures from the toxic effects of SM, it will be effective only as a pretreatment (Smith *et al.*, 1997).

E. Efficacy of Glutathione Esters

When A549 cell line was used to assess the toxicity of SM it was found that monoisopropylglutathione ester can protect A549 cells against the toxic effects of SM. Though monoisopropylglutathione ester can protect cells against the effects of SM, it can protect only as a pretreatment. The protection may be due to extracellular inactivation of SM by monoisopropylglutathione ester (Lindsay *et al.*, 1997). Similarly, diisopropylglutathione also protects SM-induced cytotoxicity, but it has to be present at the time of SM challenge for protection. The protection may be due to extracellular inactivation of SM by diisopropylglutathione ester (Lindsay and Hambrook, 1998).

F. Efficacy of Anti-Inflammatory Agents

The development of bifunctional drugs with a nonsteriodal anti-inflammatory agent, diclofenac or ibuprofen, and a cholinesterase inhibitor like pyridostigmine may be beneficial for both nerve and alkylating chemical agents (Amitai *et al.*, 2005, 2006). Anti-inflammatory drugs reduce the dermal, respiratory, and ocular damage caused by exposure to SM. It is also possible to design bifunctional molecules based on a CNS-permeable molecular combination of pyridostigmine, a reversible acetylcholinesterase inhibitor with a hydrophobic spacer to a nonsteroidal anti-inflammatory drug such as ibuprofen or diclofenac. This will act for both nerve agents and blistering agents (Martínez-Moreno *et al.*, 2005).

Calmodulin, poly(ADP-ribose)polymerase and p53 are targets for modulating the effects of sulfur mustard (Rosenthal *et al.*, 2000). It was tested whether calmodulin mediates the mitochondrial apoptotic pathway induced by SM in human keratinocytes. Of the three human CaM genes, the predominant form expressed was CaM1. These results indicate that CaM, calcineurin, and Bad also play a role in SM-induced apoptosis, and may therefore be targets for therapeutic intervention to reduce SM injury (Simbulan-Rosenthal *et al.*, 2006).

G. Efficacy of Nitroarginine Methyl Ester, Nitroarginine, and Thiocitrulline

Pretreatment of chick embryo neuron cultures with the nitric oxide synthase inhibitor L-nitroarginine methyl ester was found to confer significant protection against SM-induced cell death. Protection against the toxicity of SM on primary cultures of chick embryo forebrain neurons was dependent on the continued presence of L-nitroarginine methyl ester in the medium and was persistent up to 48 h after SM exposure. It is reported that this compound is one of the most effective drugs yet identified in protecting against the toxicity of SM and is the only one that exerts its effects therapeutically in *in vitro* models (Sawyer, 1998a). However, these protective effects were not mediated through the inhibition of nitric oxide synthase (Sawyer *et al.*, 1996). Another arginine analog and potent nitric oxide synthase inhibitor, L-thiocitrullline, also gave significant protection against SM cytotoxicity. In contrast to the protection conferred by L-nitroarginine methyl ester, L-thiocitrullline gave protection in immature cultures as well as mature cultures of neurons against SM. In addition, L-thiocitrulline was found to give protection prophylactically compared to L-nitroarginine methyl ester's protection which was post-treatment. This shows that these arginine analogs act differently (Sawyer *et al.*, 1998). L-nitroarginine methyl ester pretreatment and L-thiocitrulline simultaneously with SM potentiated the protective effect (Sawyer, 1998b).

The D- and L-nitroarginine methyl ester, L-phosphoarginine, and L-nitroarginine were found to confer concentration-dependent protection against SM in human neonatal foreskin keratinocyte cultures. Protection was prophylactic in nature, with L-thiocitrullline having almost maximal effect when added to the cultures 1 min prior to SM exposure. No protection was evident when L-thiocitruline was added 30 min post-SM (Sawyer and Risk, 2000). D- and L-nitroarginine methyl ester and eight additional arginine analogs

showed significant, concentration-dependent protection against SM toxicity in primary cultures of chick embryo forebrain neurons. Of these, L-nitroarginine was the most potent in increasing the LC_{50} to more than three-fold (Sawyer, 1998b). Surprisingly, L-nitroarginine methyl ester and L-thiocitrulline administered through a variety of routes did not give any significant protection against topical vapor exposure to SM in hairless guinea pigs. However, both compounds were effective in preventing the toxicity of SM in primary cultures of hairless guinea pig skin keratinocytes, indicating that species differences were not likely to be responsible for the poor efficacy of these compounds *in vivo* (Sawyer and Risk, 2000).

H. Other Treatment Strategies

Ebselen (a selenium containing antioxidant), melatonin, and cyclosporine A markedly prevented mustard-induced anoikis, pointing to these drugs as interesting candidates for the treatment of mustard-induced airway epithelial lesions (Sourdeval *et al.*, 2006). It has been previously suggested that acetylcholinesterase is activated during apoptotic processes and may be involved in apoptosis regulation, and acetylcholinesterase activity may be a potential marker of apoptosis in A549 cells after SM injury. If the mechanism of the involvement of acetylcholinesterase in apoptosis regulation during SM poisoning is known, drugs can be targeted (Steinritz *et al.*, 2007). The therapeutic effects of recombinant human erythropoietin (rh-EPO) and recombinant human granulocyte colony stimulating factor (rhG-CSF) on SM-induced toxicity in dogs was evaluated and they stimulate the growth of the erythrocyte, reticulocyte, and leukocyte. The use of rh-EPO and rhG-CSF can be considered as adjuncts to other therapeutic agents (Cai *et al.*, 2004). Though SM is a blistering agent it can also induce neutropenia in exposed individuals, increasing their susceptibility to infection. Granulocyte colony-stimulating factor (G-CSF) and pegylated G-CSF (peg-G-CSF) have been approved by the US Food and Drug Administration as hematopoietic growth factors to treat neutropenia induced by chemotherapeutic agents. African green monkeys untreated but exposed to SM recovered from neutropenia in 28 days, whereas G-CSF or peg-G-CS-treated animals recovered in 8 to 19 days after exposure. G-CSF or peg-G-CSF may reduce the duration of SM-induced neutropenia (Anderson *et al.*, 2006). Improving the fluid balance and electrolyte concentration also has been proved to be very effective in the treatment of SM poisoning in animals (Callaway *et al.*, 1958; Vojvodić *et al.*, 1985; Sugendran *et al.*, 1998).

The toxicity of SM was found to be dependent on extracellular pH. CHO-K1 cell cultures were exposed to SM and the pH was adjusted to between 5 and 10.0. An eight-fold increase in LD_{50} was observed at pH 9.5 compared to pH 5.0. It is possible that SM causes an extracellular acidification through chemical hydrolysis resulting in cytosolic acidification. This decline in pH initiates the cascade of events that results in SM-induced cell death (Sawyer *et al.*, 2007).

VII. SM-INDUCED OXIDATIVE STRESS AND ITS PROTECTION

The cytotoxic mechanism induced by SM is not well understood. Reactive oxygen and nitrogen species may likely be involved leading to lipid peroxidation, protein oxidation, and DNA damage, and trigger many pathophysiological processes resulting in multiorgan toxicity. To be really effective, treatment against SM must take all molecular mechanisms of cytotoxicity into account. A combination of several individual potent agents, each blocking one of the toxic mechanisms induced by SM, would be beneficial (Korkmaz *et al.*, 2006). Intraperitoneal administration of SM in Wistar rats decreased the body weight dose dependently. SM significantly decreased superoxide dismutase, catalase, glutathione peroxidase, and glutathione *S*-transferase activities in liver and brain, and decreased glutathione reductase activity in liver, which was also associated with a depletion of reduced glutathione and increased malondialdehyde level (Figure 60.3) (Jafari, 2007). The effects of SM toxicity may be local, systemic, or both, depending on environmental conditions, exposed organs, and the extent and duration of exposure (Somani and Babu, 1989). There is evidence showing that oxidative stress is an important factor in SM skin toxicity. Oxidative stress results when the production of reactive oxygen or reactive nitrogen oxide species exceeds the capacity of antioxidant defense mechanisms (Paromov *et al.*, 2007). It is important that the reactive oxygen or nitrogen species should be protected to reduce SM toxicity.

A. Effectiveness of *N*-Acetyl Cysteine

The toxicity of 2-chloroethyl ethyl sulfide appears to be mediated by the generation of reactive oxygen species and consequent depletion of reduced glutathione. Exposure of 2-chloroethyl ethyl sulfide to human Jurkat cells or lymphocytes resulted in a marked decrease in the intracellular concentration of reduced glutathione, and this was potentiated by buthionine sulfoximine, an inhibitor of glutathione synthesis. The effects of 2-chloroethyl ethyl sulfide on the accumulation of reactive oxygen species, decrease in glutathione level, decrease in mitochondrial membrane potential, and increase in caspase-3 activity were all inhibited by pretreatment with *N*-acetyl cysteine or with glutathione ethyl ester. They also prevented 2-chloroethyl ethyl sulfide-induced cell death (Han *et al.*, 2004).

Exposure of SM to macrophage monocyte cell line J774 caused an approximately 15% decrease in the cellular glutathione content, 2 h after exposure. Pretreatment with glutathione elevated cellular reduced glutathione. Glutathione pretreatment also increased cell viability. Similar protection

was also observed after glutathione treatment in the human epidermoid carcinoma cell line. But it is necessary that glutathione should be present extracellularly in order to protect cells from SM toxicity (Amir *et al.*, 1998). Compounds that elevate or reduce intracellular levels of glutathione may produce changes in cytotoxicity induced by SM. Pretreatment of human peripheral blood lymphocytes with buthionine sulfoximine, which reduces intracellular glutathione content, sensitizes the cells to the cytotoxic effects of SM. Pretreatment of the cells with *N*-acetyl cysteine, which elevates intracellular glutathione levels, partially protects the cells from the cytotoxic effects of SM. Augmentation of intracellular levels of glutathione may provide partial protection against cytotoxicity of SM (Gross *et al.*, 1993).

Intratracheal infusion of 2-chloroethyl ethyl sulfide in rats induces lung injury characterized by massive localized hemorrhage, edema in the alveolar compartment, accumulation of collagen, and parenchymal collapse. Significant protection was observed when antioxidants such as catalase, dimethyl sulfoxide, dimethyl thiourea, resveratrol and *N*-acetyl cysteine were administered. Among them *N*-acetyl cysteine, a sulfhydryl donor and antioxidant, showed better protection and the protection could be observed even when given up to 90 min after exposure (McClintock *et al.*, 2002). 2-Chloroethyl ethyl sulfide-induced lung injury can be substantially reduced by the presence of reducing agents or antioxidant enzymes delivered via liposomes (McClintock *et al.*, 2006). The SM administered to anesthetized rats intratracheally significantly increased lactate dehydrogenase, gamma-glutamyltransferase, and glutathione peroxidase. Niacinamide treatment did not improve the condition, while *N*-acetyl cysteine treatment to some extent improved the condition showing that *N*-acetyl cysteine may be useful as a potential treatment compound for SM-induced lung injury (Anderson *et al.*, 2000). SM targets eyes, respiratory system, skin, and possibly other organs. Extensive exposure can affect the bone marrow and damage the immune system. Clinical trials have demonstrated that *N*-acetyl cysteine is an effective chemoprotective agent against many toxic chemicals. Several studies have shown that *N*-acetyl cysteine significantly reduces the toxic effects of SM and its simulants. Radiolabeled *N*-acetyl cysteine was administered orally and its distribution was studied. *N*-acetyl cysteine increased the levels of tissue glutathione and the distribution studies indicated that oral administration of *N*-acetyl cysteine can significantly reduce skin, eye, and lung toxicity associated with SM. Maximum tolerable dose of *N*-acetyl cysteine can be recommended for treatment of SM poisoning (Bobb *et al.*, 2005; Arfsten *et al.*, 2007).

Exposure of SM to endothelial cells caused apoptosis and necrosis. Necrosis was accompanied by a significant depletion of intracellular ATP, while in apoptotic cells ATP remained at a level similar to healthy cells. Pretreatment with *N*-acetyl cysteine all but eliminated the apoptotic features of cell death but did not prevent necrosis in response to SM. *N*-acetyl cysteine pretreatment prevented the loss of cell adherence and cell rounding following exposure to SM. The actin filament organization may be an important element in cellular susceptibility to apoptotic stimuli and *N*-acetyl cysteine pretreatment may prevent it (Dabrowska *et al.*, 1996). Buthionine sulfoximine pretreatment increased the sensitivity of G361, SVK14, HaCaT, and NCTC 2544 human skin cells to SM (Simpson and Lindsay, 2005). In the endothelial cells presence of reduced glutathione improved the cell viability when exposed to SM. Pretreatment with buthionine sulfoximine, an inhibitor of glutathione synthesis, alone did not show appreciable toxic effects. However, pretreatment with buthionine sulfoximine potentiated the toxicity of SM. Pretreatment with *N*-acetyl cysteine provided significant protection. Coadminstration of buthionine sulfoximine and *N*-acetyl cysteine eliminated the protective effect of *N*-acetyl cysteine. This shows that the protective effects of *N*-acetyl cysteine may be mediated by enhanced glutathione synthesis. The increased glutathione may act to scavenge SM and also prevent oxidative activation of NF-κB (Atkins *et al.*, 2000).

Following intratracheal infusion of 2-chloroethyl ethyl sulfide in guinea pigs, high levels of tumor necrosis factor-α, ceramides, and nuclear factor NF-κB accumulated in lung and alveolar macrophages. There was an increase in the ^{125}I bovine serum albumin leakage into lung tissue, indicating severe lung injury. A single administration of *N*-acetyl cysteine orally before 2-chloroethyl ethyl sulfide infusion was ineffective to counteract these effects. However, consumption of the antioxidant in drinking water for 3 or 30 days prior to 2-chloroethyl ethyl sulfide exposure significantly inhibited the induction of tumor necrosis factor-α, activation of neutral and acid sphingomyelinases, production of ceramides, activation of caspases, leakage of ^{125}I bovine serum albumin into lung tissue, and histological alterations in lung. Pretreatment with *N*-acetyl cysteine as a daily supplement may be beneficial in reducing the lung injury against SM vapors (Das *et al.*, 2003). The hairs of guinea pigs exposed to 2-chloroethyl ethyl sulfide intratracheally looked rough and dry and lost their shiny glaze. They came out easily though there was no sign of skin lesions or skin damage. The alopecia was more or less permanent or the recovery was very slow. Treatment with *N*-acetyl cysteine prior to 2-chloroethyl ethyl sulfide exposure could prevent the hair loss completely (Chatterjee *et al.*, 2004). Long-term administration of *N*-acetyl cysteine is effective in free radical-related diseases. The effect of 2 and 4 month administration of *N*-acetyl cysteine was evaluated in patients with bronchiolitis obliterans due to SM exposure. Dyspnea and cough were significantly improved after 4 months of treatment (Shohrati *et al.*, 2008).

A number of candidate molecules were evaluated against SM and meclorethamine toxicity using human bronchial–epithelial cell lines. The molecules tested were *N*-acetyl cysteine and WR-1065 (sulfhydryl containing

molecules), hexamethylenetetramine (nucleophile), niacinamide (energy level stabilizer), dimethylthiourea (antioxidant), L-thiocitrulline and L-nitroarginine methyl ester (L-arginine analogs), and doxycycline (anti-gelatinase). Dimethylthiourea and WR-1065 protected meclorethamine toxicity and doxycycline protected against SM toxicity as individual agents, while *N*-acetyl cysteine and L-thiocitrulline were effective against both SM and meclorethamine toxicity. The combination of *N*-acetyl cysteine and doxycycline is also very interesting as these agents are already in human use. It exhibited good efficacy as a cotreatment against SM (Rappeneau *et al.*, 2000).

B. Effectiveness of Bioflavonoids

SM applied on the skin of mice depleted glutathione in blood and liver. Malondialdehyde levels in the liver showed an increase indicating lipid peroxidation. Intraperitoneal administration of vitamin E or two flavonoids, gossypin and hydroxyethyl rutosides, after dermal application of SM, did not alter the depletion of glutathione but did reduce the malondialdehyde level significantly. Survival time of mice was increased by gossypin and hydroxyethyl rutosides to a greater extent than by vitamin E or sodium thiosulfate (Vijayaraghavan *et al.*, 1991). Quercetin, a naturally occurring bioflavonoid, by intraperitoneal injection in mice, also significantly protected the depletion of glutathione and increased the malondialdehyde level by SM. Quercetin was effective only as a pretreatment or simultaneous treatment with percutaneously applied SM (Gautam *et al.*, 2007). Further, it was reported that percutaneous administration of SM induced oxidative stress and intraperitoneal administration of gossypin (3,3′,4′,5,7,8-hexahydroxyflavone 8-glucoside) significantly protected against it. A very good protection was observed when gossypin was administered 30 min prior to or simultaneous to SM exposure, but not as post-treatment. A significant increase in red blood corpuscle count and hemoglobin, and a decrease in total antioxidant status of plasma, reduced and oxidized glutathione of liver, glutathione peroxidase, glutathione reductase, and superoxide dismutase activities were observed in percutaneously administered SM in mice. The biochemical changes were protected only when gossypin was administered as either pretreatment or simultaneous treatment, not as post-treatment (Gautam and Vijayaraghavan, 2007).

Antioxidants could enhance survival time, protect liver and lung from oxidative damage, and reduce accumulation of purine metabolites in blood following SM intoxication. The protective effects of various antioxidants, trolox, a water soluble analog of vitamin E, quercetin, a naturally occurring bioflavonoid, and reduced glutathione were studied following SM vapor inhalation in mice and by the percutaneous route. A significant decrease in reduced glutathione and an increase in the level of malondialdehyde, indicating oxidative damage to liver and lung tissues following SM inhalation and percutaneous exposure, was observed. Blood and urinary uric acid, an endproduct of purine metabolism, showed an increase following both routes of exposure. The antioxidants, trolox and quercetin, protected the liver and lung tissues from oxidative damage caused by SM exposure through inhalation and percutaneous routes (Kumar *et al.*, 2001, 2002). Quercetin, gossypin, *Hippophae rhamnoides* flavone and vitamin E were evaluated against the systemic toxicity of percutaneously administered SM in mice. The study supported the fact that SM induces oxidative stress and flavonoids are promising cytoprotectants against the toxic effects of SM more so than vitamin E, but they all are effective only as a pretreatment (Vijayaraghavan *et al.*, 2008). Reduced glutathione and oxidized glutathione levels were decreased, and the malondialdehyde level was elevated after percutaneous administration of SM in mouse. Oral administration of *Hippophae rhamnoides* leaf extract and the flavone from fruit significantly protected them (Vijayaraghavan *et al.*, 2006). Oral administration of *Aloe vera* gel gave only a partial protection though it also contains antioxidants (Gautam *et al.*, 2005).

C. Efficacy of Amifostine and DRDE-07 Analogs as Promising Cytoprotectants

Although several approaches have been proposed to counteract the toxic effect of SM, no satisfactory treatment regimen has evolved. The synthetic aminothiol, amifostine, originally developed as a radioprotector and earlier known as WR-2721 [*S*-2-(3-aminopropylamino) ethyl phosphorothioate], has been extensively used as a chemical radioprotector for the normal tissues in cancer radiotherapy and chemotherapy. Since SM is known as a radiomimetic agent various analogs of amifostine were evaluated against SM toxicity in rat liver slices. One analog, DRDE-07 [*S*-2 (2-aminoethylamino) ethyl phenyl sulfide] showed excellent protection (Table 60.4). Both amifostine and DRDE-07 protected only when they were administered as pretreatment in rat liver slices. *In vivo* protection was also evaluated in mice with oral treatment of amifostine and DRDE-07 against percutaneously administered SM. The protection was dose dependent and effective only when the agents were administered either as a pretreatment or simultaneously with SM, and DRDE-07 offered better protection. Both *in vitro* and *in vivo* data indicate the

TABLE 60.4. Chemical structures of cytoprotectants

S. No.	Compound	Chemical structure
1.	Amifostine	NH_2–$(CH_2)_3$–NH–$(CH_2)_2$–S–PO_3–H_2
2.	DRDE-07	NH_2–$(CH_2)_2$–NH–$(CH_2)_2$–S–C_6H_5
3.	DRDE-10	NH_2–$(CH_2)_2$–NH–$(CH_2)_2$–S–C_6H_4–CH_3
4.	DRDE-21	NH_2–$(CH_2)_2$–NH–$(CH_2)_2$–S–C_6H_{11}
5.	DRDE-30	NH_2–$(CH_2)_2$–NH–$(CH_2)_2$–S–C_3H_7
6.	DRDE-35	NH_2–$(CH_2)_2$–NH–$(CH_2)_2$–S–$(CH_2)_3CH_3$

TABLE 60.5. Efficacy of oral administration of various cytoprotectants, prophylactically against percutaneously administered mustard agents

Drug	SM		CEES		HN-1		HN-2		HN-3	
	LD_{50} (mg/kg)	PI[a]	LD_{50} (mg/kg)	PI	LD_{50} (mg/kg)	PI	LD_{50} (mg/kg)	PI	LD_{50} (mg/kg)	PI
No treatment	7.1	–	1,425	–	11.9	–	20.0	–	7.1	–
Amifostine (p.o.)	31.8	4.5	–	–	14.2	1.2	28.3	1.4	11.9	1.7
Sodium thio sulfate (p.o.)	28.3	4.0	–	–	14.2	1.2	23.8	1.2	11.9	1.7
Melatonin (p.o.)	20.0	2.8	–	–	16.8	1.4	28.3	1.4	11.9	1.7
N-acetyl-cysteine (p.o.)	16.8	2.4	–	–	14.2	1.2	20.0	1.0	10.0	1.4
DRDE-07 (p.o.)	80.6	11.4	1,599	1.1	14.2	1.2	28.3	1.4	10.0	1.4
DRDE-10 (p.o.)	56.6	8.0	–	–	16.8	1.4	28.3	1.4	11.2	1.6
DRDE-21 (p.o.)	50.4	7.1	–	–	14.2	1.2	20.0	1.0	10.0	1.4
DRDE-30 (p.o.)	44.9	6.4	–	–	14.2	1.2	28.3	1.4	11.9	1.7
DRDE-35 (p.o.)	50.4	7.1	–	–	14.2	1.2	28.3	1.4	11.9	1.7

[a]PI (protection index) is the ratio of LD_{50} with treatment to LD_{50} without treatment

promising role of DRDE-07 as a prophylactic agent against SM poisoning (Bhattacharya *et al.*, 2001; Vijayaraghvan *et al.*, 2001a; Kumar *et al.*, 2002).

The protection offered by amifostine and DRDE-07 in mice was better than rat and in a search for more effective antidotes against SM, a series of novel *S*-2(omega-amino-alkylamino)ethyl alkyl/aryl thioethers were synthesized and all are water soluble. A number of compounds demonstrated significant protection (Pathak *et al.*, 2004). Few analogs, such as DRDE-10, DRDE-21, DRDE-30, and DRDE-35 including DRDE-07, gave enormous protection in the mouse skin model (Table 60.5). In the rat skin model, DRDE-07, DRDE-10, and DRDE-21 gave about a two-fold protection. Percutaneously administered SM significantly depleted the hepatic glutathione content and increased the percent DNA fragmentation in mice. Some of the compounds, particularly DRDE-07, DRDE-30, and DRDE-35, significantly protected the mice after SM intoxication. The histopathological lesions in liver and spleen induced by percutaneously administered SM were also reduced by pretreatment with these compounds (Kulkarni *et al.*, 2006). These classes of compounds, though giving very good protection against SM but failing to give appreciable protection against 2-chloroethyl ethyl sulfide and nitrogen mustard, showed the mechanism and toxicity of the mustard agents are different. These compounds may prove effective as prototypes for the designing of more successful prophylactic drugs for SM and nitrogen mustard.

D. Efficacy of Other Antioxidants

The superoxide dismutase activity and the malodialdehyde level in blood platelets were higher in SM exposed cells *in vitro* (Buczynski *et al.*, 1999). SM skin burns in guinea pigs were pretreated with oxygen free radical scavengers, like copper–zinc, and manganese superoxide dismutase. Superoxide dismutase compounds dramatically reduced the burn lesions when administered intraperitoneally before SM exposure as assessed by histopathology. When the superoxide dismutase compounds were administered intraperitoneally 1 h after the burn infliction, and repeated daily for 7 days, no protection was obtained (Eldad *et al.*, 1998a).

Glutathione, a tripeptide that exists in high concentrations in cells, reacts with SM and other electrophilic alkylating agents by forming conjugates and detoxifying them. Compounds that can modulate glutathione levels within the cell may reduce the cytotoxicity of SM when used as a pretreatment. L-oxothiazolidine-4-carboxylate, a cysteine precursor, increased the level of glutathione. Pretreatment of human peripheral blood lymphocytes with L-oxothiazolidine-4-carboxylate resulted in a small decrease in cytotoxicity after SM exposure. Post-treatment with L-oxothiazolidine-4-carboxylate was not beneficial (Gross *et al.*, 1997). SM reacts with glutathione to form a glutathione–SM conjugate by the action of glutathione-*S*-transferase. Ethacrynic acid, an inhibitor, and oltipraz, an inducer, were ineffective in modulating this enzyme in cultured normal human epidermal keratinocytes. However, D,L-sulforaphane, a compound obtained from broccoli extract and a potent inducer of this enzyme, increased the level of this enzyme optimally. When SM was challenged with D,L-sulforphane, there was an improvement in survival compared with unpretreated SM controls (Gross *et al.*, 2006).

The production of reactive oxygen species has been proposed to result from electrophilic or oxidative stress with depletion of cellular detoxifying thiol levels including glutathione. The reactive oxygen species initiates a chain reaction with membrane phospholipids to form lipid peroxides, leading to loss of membrane function, membrane fluidity, and finally membrane integrity. Beneficial effects can be achieved by scavengers of reactive oxygen species

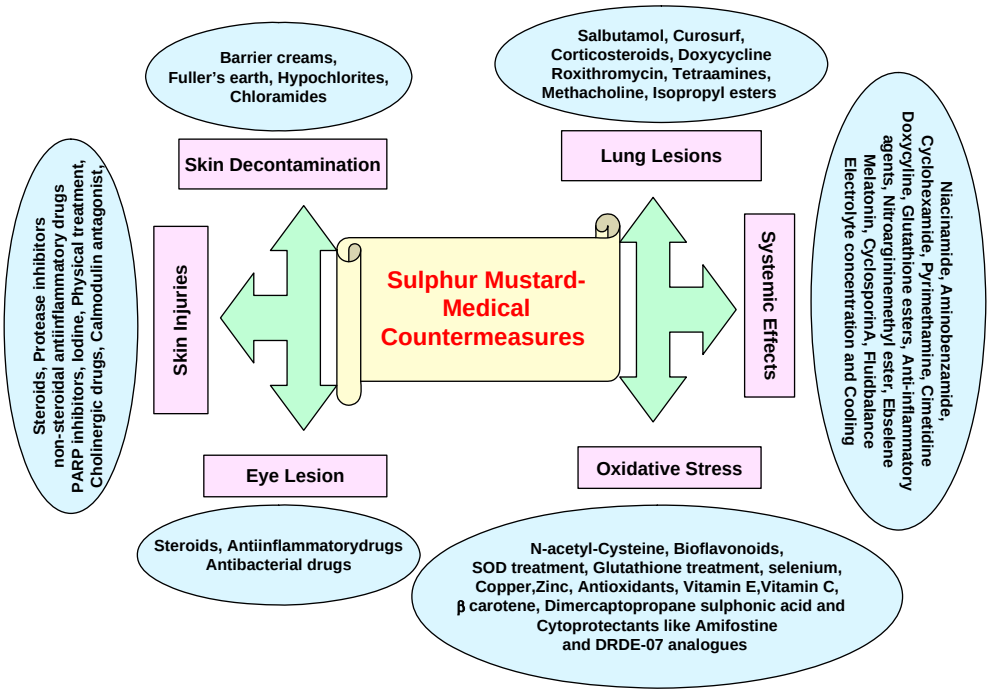


FIGURE 60.4. Schematic presentation of various SM medical countermeasures.

and electrophilic compounds such as glutathione, sulfhydryl compounds, and antioxidants. Substances such as selenium, copper, zinc, and antioxidants including vitamin E, vitamin C, and compounds like beta-carotene may be useful against SM cytotoxicity and lipid peroxidation (Naghi, 2002). Exposure of SM in mice dermally resulted in a significant loss of blood, hepatic, and pulmonary glutathione. These biochemical changes were accompanied by congestion and degeneration in viscera and obliteration of chromatin material in the liver. The biochemical and histopathological changes were less marked in animals pretreated with dimercapto propane sulfonic acid (Pant *et al.*, 2000; Pant and Vijayaraghavan, 2002). SM exposure using the *in vitro* mouse neuroblastoma and rat glioma hybrid cell line model caused a decrease in cellular glutathione level and a time-dependent increase in intracellular-free calcium suggesting a Ca^{2+} mediated toxic mechanism of SM (Ray *et al.*, 1995). Percutaneous administration of SM in mice caused a reduction in glutathione and an increase in malondialdehyde level, and both of them are restored by nifedipine, a calcium channel blocker (Mazumder *et al.*, 1998).

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

A number of animal models and strategies have been used to develop antidotes against the toxicity of SM but none is as successful as decontamination. Decontamination is the best known way to cure or prevent SM-induced toxicity. So far, many decontaminants have been reported but only chloramides and Fuller's earth are to be recommended because of their safety and effectiveness. Various treatment strategies were tried but most of them did not give a satisfactory protection of the *in vivo* system. Povidine iodine, *N*-acetylcysteine and amifostine analogs were found to be effective against SM toxicity *in vivo*. Of these drugs one of the amifostine analogs, DRDE-07, showed maximum protection (Figure 60.4). These types of compounds may prove to be prototypes for the design of more effective antidotes for cytotoxicity of alkylating agents.

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CHAPTER 61

Medical Management of Chemical Toxicity in Pediatrics

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Opinions, interpretations, conclusions, and recommendations are those of the author(s) and are not necessarily endorsed by the US Army.

I. INTRODUCTION

There are essentially millions of chemical compounds known to humanity, but only a limited number are weaponized by conventional militaries. The Organization for the Prohibition of Chemical Weapons (OPCW), the 184-member watchdog agency enforcing the guidelines of the Chemical Weapons Convention (CWC), has identified 55 chemical agents and their precursors that can be used as weapons (OPCW, 2005). Although some of the chemicals are well known (e.g. sarin, soman, VX, mustard), other less obvious choices for chemical terrorism include industrial chemicals such as chlorine and toxic precursors, which are considered ''weapons of opportunity''.

In the hands of terrorists, chemical warfare agents (CWAs) and toxic industrial chemicals (TICs) pose significant threats to civilian populations. A 2002 report to Congress by the Central Intelligence Agency reported that terrorist groups ''have expressed interest in many toxic industrial chemicals – most of which are relatively easy to acquire and handle – and traditional chemical agents, including chlorine and phosgene'' (DCI, 2002). While traditional CWAs like nerve agents are attractive to terrorist groups, these agents require a significant degree of financial resources and capital knowledge to manufacture. Furthermore, the USA and remaining signatory members of the CWC have pledged nonproliferation of CWAs (OPCW, 2005). Unfortunately, millions of tons of TICs continue to be manufactured annually in the USA alone. While they support the wide variety of products generated on a daily basis, including dyes, textiles, medicines, solvents, plastics, paints, and insecticides, they are lethal compounds in the hands of terrorists.

Chemical terrorism is the intentional use of toxic chemicals to inflict mass casualties on an unsuspecting military or civilian population, including children. Such an incident could quickly overwhelm local and regional public health resources and emergency medical services. In addition to utilization of CWAs and TICs, an act of chemical terrorism may involve targeting of industrial factories, tanker cars, or vehicles containing toxic substances with conventional explosives near residential communities or schools. Regardless of the methods used, the release of toxic chemicals by terrorists embodies a real and serious threat to our national security and public health. They can quickly incapacitate those who are exposed and can lead to mortality if not recognized and treated promptly. Moreover, the toxicity of these agents can be enhanced in children due to pediatric vulnerabilities. It is imperative to recognize the different ways children may present with toxicity compared to adults.

II. BACKGROUND

Even though many efforts have been made to protect our nation from threats of terror, it is still paramount for our scientific community to continue building our knowledge base regarding CWAs and better understand the toxicities that can occur when children are exposed. Unfortunately, pediatric treatment recommendations are often extrapolated from adult data, even though it is well recognized that pediatric patients should not be regarded as miniature adults. Children present unique vulnerabilities to these chemicals, and special considerations should be taken.

Due to the possibility of pediatric casualties from chemical agent attacks, several pediatric advocacy groups, such as the American Academy of Pediatrics (AAP), have commented on the urgent need for pediatric chemical casualty research (Blaschke and Lynch, 2003). The Committees on Environmental Health and Infectious Diseases have provided the following consensus statement regarding children and chemical–biological threats (CEH/CID, 2000).

> Because children would be disproportionately affected by a chemical or biological weapons release, pediatricians must assist in planning for a domestic chemical–biological

incident. Government agencies should seek input from pediatricians and pediatric subspecialists to ensure that the situations created by multiple pediatric casualties after a chemical–biological incident are considered.

After September 11, the AAP initiated a number of initiatives to try to address the need to prepare for terrorism against children. For example, the AAP created a Task Force on Terrorism, a comprehensive web resource to disseminate information on terrorism and its impact on children. In addition, the AAP has published several reports and policy statements to provide guidance for health care practitioners preparing for a mass chemical casualty event. These efforts were augmented by the passage of several key Federal legislative acts aimed at improving public health emergencies and their response to chemical terrorism. Finally, the Centers for Disease Control and Prevention created the Strategic National Stockpile site (SNS) as a national repository of antibiotics, chemical antidotes, and antitoxins. The SNS contains a pediatric formulary along with compounding materials that would assist clinicians in creating dosage forms appropriate for pediatric administration of chemical antidotes (CEH/CID, 2006) in the event of chemical terrorism.

Indeed, chemical terrorism on US soil is a very sobering possibility in the future. A significant subset of casualties from a mass chemical exposure event will comprise vulnerable populations such as children and the elderly. In the hopes of better understanding the impact of such an event, it is necessary to learn from historical incidents and case studies where children were exposed to toxic chemicals and treated.

In this chapter, the CWAs and ''weapons of opportunity'' most likely to be used by terrorists to inflict casualties will be examined, following a brief historical account and unique challenges of managing pediatric chemical casualties. The sections for each chemical agent will highlight the pediatric-relevant vulnerabilities and guidelines for medical management. The final two sections will discuss decontamination of children and recommendations to help prepare health care managers and providers in the event of a chemical event. It is hoped that this compilation will provide the necessary guidance and treatment recommendations on how best to treat children involved in a chemical attack.

III. HISTORY OF PEDIATRIC CHEMICAL CASUALTIES

Historically, chemical attacks were limited to the battlefield, and casualties were predominantly military personnel. In turn, the majority of our knowledge concerning management of chemical casualties has come from the experiences of treating our military population. Today, the threat of chemical use is extended to civilian populations as state and nonstate sponsored terrorists target innocent civilians. The risk of chemical and biological terrorism is more tangible since the events of September 11, 2001 and the intentional spread of anthrax through the US Postal Service. Terrorists expanded their scope and threat to inflict mass civilian casualties on a scale never before seen. The threat from attack has moved away from the traditional battlefield to the home front. Innocent civilians, including children, are now prime targets for groups to foment terror and destabilize governments. Even before the attack on the World Trade Center towers, the 20th century witnessed numerous instances where civilian populations were exposed to toxic chemicals or targeted on a grand scale.

During the flurry of chemical barrages across trench lines in World War I (WWI), children from bombed towns in France and Belgium were treated at British, French, and American ''gas'' hospitals as a result of CWA exposure. Numerous reports of civilian casualties from mustard, chlorine, and phosgene are well documented in British archives (Thomas, 1985). After the final tally of civilian casualties from ''gas warfare'' was complete, participants saw how ill-prepared their civilians were against such weapons. School-age children learned the importance of protective measures against chemicals through donning of gas masks and evacuating contaminated areas (Figure 61.1).

Civilians have been unintended and, in some cases, intended targets of CWAs since World War I. While cyanide

FIGURE 61.1. School-age girl with US civilian noncombatant gas mask MI-I-I, child size. Photograph: courtesy of the US Army Research Development and Engineering Command, Historical Research and Response Team, Aberdeen Proving Ground, MD.

was used on Jewish prisoners in World War II, chemical weapons would not be used again during combat on civilian populations until the Iran–Iraq War. In the spring of 1987, Saddam Hussein bombed the Iranian city of Sardasht with mustard munitions, resulting in thousands of civilian casualties (Foroutan, 1996a, b, 1998a, b, c).

Following the attack on Sardasht, Iraq attacked Kurd settlements in early 1988, leading to the infamous attack on Kurdish residents of Halabja in March. Thousands of innocent civilian ethnic Kurds perished during the chemical attack, including 75% women and children. Mustard and nerve agents were dropped on civilians from helicopters and planes, and eyewitnesses reported large smoke clouds causing great morbidity and mortality among children (Hay and Roberts, 1990).

Most recently, there have been incidents in the USA where food has been intentionally contaminated with chemical insecticides. Not surprisingly, children were affected in these incidents. One incident occurred in 1998, where a restaurant's salt supply was contaminated with methomyl, a carbamate insecticide. It was reported that five children became ill after eating the insecticide-contaminated food (Buchholz *et al.*, 2002). In another incident that occurred in 2003, approximately 200 pounds of ground beef were contaminated at a grocery store in Michigan with an insecticide called Black Leaf 40. Approximately 90 people (age range 1–76 years) became ill after ingesting the contaminated beef.

These events confirm the devastating reality that chemical threats pose to our unprotected population today. A military and civilian response to the use of chemical weapons on American soil may not be a matter of if but rather a question of when. These events underscore the need for all pediatric-related health care workers to prepare for a mass casualty incident involving CWAs or TICs.

IV. CHALLENGES TO MANAGING PEDIATRIC CHEMICAL CASUALTIES

A. Overview

Managing pediatric victims of chemical terrorism is an especially difficult challenge. In addition to the obvious physiologic and anatomic differences compared to adults (Table 61.1), there are important psychological and behavioral differences that put children at risk (Rotenberg and Newmark, 2003). Anecdotal reports have claimed that children are likely to be the first to manifest symptoms, to develop more severe manifestations, and to be hospitalized for other related illnesses. In fact, it is anticipated that children will be overrepresented among the initial index cases in a mass civilian exposure to toxic chemicals. Children have many characteristics that make them vulnerable to toxic exposures. The smaller mass of a child automatically reduces the dose of toxic agents needed to cause observable or lethal effects. Studies involving organophosphates (OPs), compounds related to nerve agents, have shown greater vulnerability in immature animals. Some OPs produce the same degree of lethality in juveniles at a fraction of the dose producing lethality in the adult (Rotenberg and Newmark, 2003). Children exhibit an exceptional vulnerability to both the acute and chronic effects of chemicals and are disproportionately susceptible in comparison with adults. The increased toxicity seen in children compared to adults from various routes of exposure can be attributable to a wide variety of factors (shown in Exhibit A). These unique anatomical and physiologic considerations described below cause the rates of absorption, distribution, metabolism, and excretion of toxic chemicals/drugs to differ in children with respect to adults.

B. Respiratory Vulnerability

Inhaled doses in young children may be greater than adults. Some studies have demonstrated a two-fold increase in respiratory tract exposure per unit surface area as compared to adults (Bennett and Zeman, 1998). Deposition of fine particles is higher in young children (ages 7 to 14) relative to adults when the data are normalized by lung surface area (Bennett and Zeman, 1998) and an even greater deposition has been modeled for younger age children (Martonen *et al.*, 2000). The higher respiratory rate and minute volumes per respiratory surface area of a child means that they will inhale a greater dose of a toxic chemical vapor (Rotenberg and Newmark, 2003). Also, children can become intoxicated simply through breathing air that is closer to the ground. Many toxic chemicals display a high vapor density, causing them to distribute closer to the ground (CSMC, 2003). This may lead to greater toxicity for a child compared to an adult. In addition, children have less endurance than adults in the use of their respiratory accessory muscles, putting them at risk for respiratory failure.

Children are especially susceptible from toxic chemicals due to their unique airway anatomy (Figure 61.2). These differences include a greater degree of subglottic narrowing, diminished airway diameter, tendency for nose-breathing, and large tongue size relative to the mouth (Rotenberg and Newmark, 2003). OP nerve agents induce bronchospasm and bronchoconstriction during a cholinergic crisis. In comparing the effect of nerve agent on adult and pediatric airways, Figure 61.2 illustrates that a similar change in airway diameter results in a greater percent increase of airway resistance in children. In addition, copious glandular secretions during a cholinergic crisis may further restrict airflow through an already narrow airway. Therefore, children are at higher risk for toxicity from inhalational chemical exposure.

C. Volume Status Vulnerability

The circulatory system of children can be severely affected by chemical attacks (Rotenberg and Newmark, 2003).

TABLE 61.1. Summary chart of pediatric vulnerabilities and implications for clinical management

Unique vulnerability in children	Implications and impact from chemical toxicity
Body composition	
• Larger BSA/body mass • Lower total lipid/fat content	• Greater dermal absorption • Less partitioning of lipid soluble components
Volume status	
• More prone to dehydration • Chemical agents lead to diarrhea and vomiting	• Children can be more symptomatic and show signs of severe dehydration
Respiratory	
• Increased basal metabolic rate/greater minute volume	• Enhanced toxicity via inhalational route
Blood	
• Limited serum protein binding capacity • Greater cutaneous blood flow	• Potential for greater amount of free toxicant and greater distribution • Greater percutaneous absorption
Skin	
• Thinner epidermis in preterm infants • Greater cutaneous blood flow	• Increased toxicity from percutaneous absorption of chemical agents
Organ size/enzymatic function	
• Larger brain/body mass • Immature renal function/lower renal function • Immature hepatic enzymes	• Greater CNS exposure • Slower elimination of renally cleared toxins, chemicals and metabolites • Decreased metabolic clearance by hepatic phase I and II reactions
Anatomical considerations	
• Short stature: breath closer to ground where aerosolized chemical agents settle • Smaller airway • Greater deposition of fine particles in the upper airway • Higher proportion of rapidly growing tissues	• Mustard significantly affects rapidly growing tissues • Exposure to chemicals can have significant impact on bone marrow, developing CNS • Increased airway narrowing from chemical agent-induced secretions
CNS	
• Higher BBB permeability • Rapidly growing CNS	• Increased risk of CNS damage
Miscellaneous	
• Immature cognitive function • Unable to flee emergency • Immature coping mechanisms	• Inability to discern threat, follow directions, and protect themselves • High risk for developing PTSD

EXHIBIT A

Anatomical and Physiological Considerations Unique to Children

- differences in anatomy
- allometric scaling factors (e.g. increased surface area-to-volume ratio)
- cardiovascular status
- permeability of the pediatric blood–brain barrier (BBB)
- dermatologic factors (e.g. increased cutaneous blood flow) (Fluhr *et al.*, 2000; Simonen *et al.*, 1997)
- increased skin pH (Fluhr *et al.*, 2004; Behrendt and Green, 1958)
- plasma protein binding
- volume of distribution (V_d)
- organ size and maturity
- pharmacokinetic maturity (e.g. metabolic differences) (Fairley and Rasmussen, 1983)

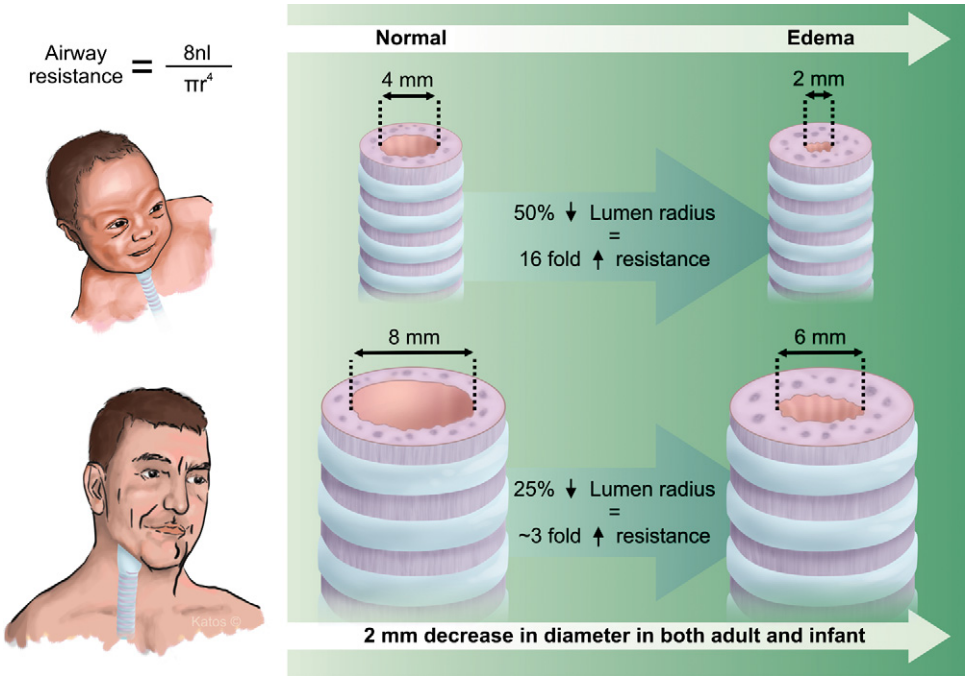

FIGURE 61.2. Comparison of pediatric and adult airways. The most important factor to consider in pulmonary toxicity from cholinesterase inhibition is the airway resistance through the conducting portion of the respiratory system. Airflow from the trachea and mainstem bronchi to the small bronchioles can be characterized as airflow through a series of straight tubes or laminar flow (West, 1995; Guyton and Hall, 2005). Poiseuille's law provides a relationship between flow rate and radius of the tube ($F = P\pi r^4/8nl$), where n is the coefficient of viscosity, P is the pressure difference across the length l of the airway section, r is the radius of the airway, and F is the airway flow rate. Since the resistance to flow R is driving pressure P divided by flow F, using the analogy of Ohm's law, the following relationship for airway resistance R can be derived ($R = 8nl/\pi r^4$). The effect of a 2 mm change in airway diameter on airway resistance is illustrated for both an infant and an adult. *Bottom panel* (adult scenario): a 2 mm reduction in the airway radius as a result of nerve agent-induced bronchoconstriction results in a 25% reduction and a corresponding increase in airway resistance by approximately three-fold. *Top panel* (infant scenario): a similar reduction in a child decreases the airway lumen by 50% and increases airway resistance by 16-fold. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

Children have lower fluid reserves, and small fluid volume losses can cause significant effects. For example, a 5 kg child, experiencing severe dehydration (15% body weight loss), loses 750 ml of fluid. A significant loss of fluid from the gastrointestinal tract as a result of chemical-induced glandular secretions can affect intravascular volume. Also, children are more prone to vomiting and diarrhea than adults. Therefore, children may dehydrate faster during a chemical event (CSMC, 2003).

D. Neurological Vulnerability

The immature central nervous system (CNS) of children can lead to greater toxicity (Rotenberg and Newmark, 2003). Toxic agents can often transverse the immature blood–brain barrier in children. Infants and children are at greater risk of seizures than adults. This is concerning because seizures are common in cases of moderate to severe nerve agent intoxication. Infants are at the highest risk of toxicity due to their susceptibility to imbalances of neurotransmitter systems. Prolonged seizures, or status epilepticus, can cause neuronal injury and deficits of normal brain development in children.

E. Dermatologic Vulnerabilities

Barrier thickness, cutaneous blood flow, surface to volume ratio, temperature, hydration, and skin pH are important factors to consider in the assessment of pediatric dermatological vulnerabilities. The skin of newborns, while appearing vulnerable, has the same histologic features of adult skin with some differences including immaturity of collagen, hair follicles, and sebaceous glands. While newborns and young children are often described as having thinner skin than adults, the stratum corneum, the most superficial layer of the skin, is thinner in premature infants compared to full-term infants, children or adults (Rutter and Hull, 1979; Harpin and Rutter, 1983; Nopper *et al.*, 1996). The skin of a child, however, does not differ significantly compared to adults as evidenced by similar measurements of skin physiological parameters (e.g. transepidermal water loss, skin pH, and stratum corneum capacitance and conductance) (Fluhr *et al.*, 2000). Children 3 months old have the same abdominal skin stratum corneum thickness as 11 year olds and adults (Fairley and Rasmussen, 1983).

Moreover, children have larger surface area-to-volume (mass) ratios, resulting in greater absorption of chemicals.

The skin surface area of infants and toddlers is very large compared to their body weight. An increase in the surface area-to-body weight ratio increases their potential absorption to dermal exposures of chemicals. For instance, a typical infant weighs a fraction (1/20) of an adult 70 kg male, but an infant's surface area is only 1/8 as great. The total skin surface area that is exposed per kg of body weight is therefore 2.5 times higher for infants than adults (Lynch and Thomas, 2004). Burns with extensive skin loss, as seen with certain chemical exposures, can cause significant water loss and toxicity in children (Lynch and Thomas, 2004). Despite the few prospective scientific studies on skin vulnerability in the pediatric population, it can be summarized that the thin, immature skin of preterm infants and the unique dermatological properties of children put them at higher risk for toxicity from percutaneous chemical exposure (CSMC, 2003).

F. Plasma Protein Binding, Volume of Distribution, and Organ Maturity

Children may be at an increased risk of chemical toxicity due to having lower levels of plasma proteins. One factor affecting the amount of free chemical/drug in the circulation is the fraction bound to plasma protein. Neonates have a low protein binding capacity for albumin and alpha-1-glycoprotein (Besunder *et al.*, 1988; Kearns and Reed, 1989; Clewell *et al.*, 2002) and a decreased ability to conjugate and excrete bilirubin, which binds to plasma proteins. This can lead to a smaller pool of available protein binding sites in plasma (Ginsberg *et al.*, 2004). A lower serum protein binding capacity equates to a greater fraction of free chemical available in the circulation and therefore increased toxicity.

The volume of distribution or V_d (liters per kg body weight) of chemicals/drugs is an important factor to consider in pediatrics. Because of the expanded water content in early life (newborns and infants), water soluble chemicals may tend to have a larger volume of distribution. Toxic lipophilic agents, on the other hand, will be decreased in their partitioning to fat because of the lower body lipid content in young children compared to older children and adults (Kearns and Reed, 1989; Clewell *et al.*, 2002; Morselli, 1989). It can be argued that due to lower fat stores, lipophilic agents such as nerve agents will reach higher concentrations in the plasma, leading to an increased risk of chemical toxicity.

Another factor affecting tissue distribution of chemicals in children is organ size per body weight. The brain is disproportionately large in young children. This fact, combined with an immaturity and permeability of the BBB in young children, leads to higher brain concentrations of some chemicals and the potential for enhanced neurotoxicity (Saunders *et al.*, 2000). Liver mass per body weight is greatest in the early postnatal period and other tissues (liver, kidney, lung, and brain) undergo rapid growth during the first 2 years of life (NRC, 1993). These organs are at increased risk for toxicity in children due to their disproportionately larger size per body weight.

Organ maturity in the pediatric population is another factor affecting clearance of toxic agents and therapeutics. In particular, renal clearance is diminished in children compared to adults. Glomerular filtration rate and transporter (secretory) systems in the proximal convoluted tubule are decreased at birth (Kearns and Reed, 1989; Morselli, 1989). In addition, cardiac output, while higher in children, has a lower percentage reaching the kidneys in early life (Ginsberg *et al.*, 2004). This will tend to decrease renal clearance even more, leading to even greater plasma levels of toxic agent. A consequence of immature kidney function and reduced clearance in children can be seen with nerve agents since the parental forms of nerve agents and their metabolites undergo hydrolysis with predominantly renal elimination.

One might consider that renal clearance is faster due to allometric scaling differences in children compared to adults. According to the rules of allometric scaling, smaller organisms have greater respiratory rates, cardiac output, nutrient and oxygen demands, and basal metabolic rates compared with larger organisms. This appears true for children because respiratory rate, cardiac output, and liver mass are greater per body weight than adults. However, faster metabolic rates are not seen in neonates because of immaturity of hepatic enzymes and reduced hepatic clearance, leading to a prolonged toxic agent/drug half-life and longer duration of action.

G. Metabolic Vulnerability

Children are unable to detoxify toxic agents as efficiently as adults because they have less mature metabolic systems (Rotenberg and Newmark, 2003). In particular, phase I oxidative systems, phase II conjugating systems, and miscellaneous other systems (e.g. serum esterases, hydrolases, dehydrogenases) are all immature in children compared to adults. Neonates and children up to 1 year are most affected in their maturing enzymatic function with the greatest effect seen in the first 2 months of life. This leads to slower metabolic clearance of many drugs, toxic chemicals, and activated metabolites, leading to significant toxicity in this age group (Ginsberg *et al.*, 2004). In addition, several authors have reported a reduced activity of acetylcholinesterases, pseudocholinesterases, and arylesterases (paraoxonase) in premature and full-term newborns (Stead, 1955; Lehmann *et al.*, 1957; Augustinsson and Brody, 1962; Ecobichon and Stephens, 1973). These levels do not reach adult levels until 1 year of age (Morselli, 1976). In addition, newborns possess levels of paraoxonase, the enzyme that detoxifies organophosphate pesticides, that are half of those found in the general adult population (Rotenberg and Newmark, 2003). Other studies suggest that newborns have paraoxonase levels four-fold lower and activities three times lower than in their mothers (Holland *et al.*, 2006).

H. Traumatic Injury Vulnerability

Another special challenge to managing pediatric patients is the fact that trauma and injury often accompany chemical attacks (Abraham *et al.*, 2002). Chemical exposures are often dispersed through explosive devices. Traumatic injury patterns differ in children compared to adults. Due to the smaller size of children, multiple trauma occurs more frequently. Compared to adults, children often sustain more head trauma due to their relatively large head size and weaker supportive musculature. Also, their more compliant skeletal system provides less protection to internal organs, leading to greater internal injuries without overlying fractures.

I. Neurobehavioral Vulnerability

Immature cognitive function can put children at risk during a chemical attack (Rotenberg and Newmark, 2003). Children lack the ability to discern threat, to protect themselves, or to follow directions. Infants, toddlers, and young children do not have the motor skills to flee from the site of an incident (CEH/CID, 2000). This can adversely impact their avoidance of a contaminated area and decontamination in the event of exposure. During decontamination, procedures for children who have been separated from their caregivers must be taken into consideration. Without guidance, children may not be able to follow directions for the decontamination process (Wheeler and Poss, 2003).

J. Psychological Vulnerability

Children have fewer coping skills when sustaining or witnessing injury such as parental or sibling death (Henretig *et al.*, 2002b). These events can produce either short- or long-term psychological trauma. It is not unusual for children involved in attacks to suffer from post-traumatic stress disorder (PTSD) related to what they have experienced (CEH/CID, 2000; ARC, 2002). During the management of a chemical event, there are certain behaviors that make the management of children difficult. Children are often influenced by the emotional state of caregivers, requiring providers to remain calm. Also, fear or discomfort may cause children to disobey or act out against providers of care (see Table 61.1) (Blaschke *et al.*, 2003).

Even beyond the behavior of children, there are other barriers to emergency management. The high pressure hoses and cold water that are used to decontaminate victims can expose children to significant additional risk (CSMC, 2003). Use of these items can result in hypothermia and skin damage. Also, emergency care providers often need to wear bulky full protective suits when treating victims. These suits make it difficult to treat very small children who might need intricate procedures such as blood draws. One constant challenge that is consistent with the management of children is the lack of pediatric formulations of specific therapeutics (e.g. autoinjectors containing oxime). Antidotes for chemical agents are often not available in ready-to-administer pediatric dosages, although some progress has been made. In the event of a true chemical event, there is a risk that pediatric centers would be overwhelmed and the ability to expand the number of pediatric hospital beds may be limited (CEH/CID, 2000). Finally, most health care workers are not fully aware of the management or presentation of toxic signs and symptoms from chemical agents. This problem is exacerbated when children typically present differently than adults.

K. Other Vulnerabilities

In addition to the vulnerabilities listed above, there are other factors that can put children at greater risk for toxicity from chemical agents attacks. For instance, the fluid and food intakes of children differ significantly from adults with greater water and milk consumption per weight. Children ingest about 100 ml/kg per day of water compared to the 40–60 ml/kg per day ingested by adults. If water or milk supplies become contaminated, children would feel a greater impact than adults. Also, the diets of children include greater consumption of foods that can be contaminated such as fruits and vegetables (CEH/CID, 2006).

L. Medical Response Vulnerability

Due to the myriad of factors outlined above that make the management of pediatric chemical exposures challenging, it is not surprising that health care practitioners often do not have the knowledge or are not sufficiently trained to handle a mass influx of pediatric casualties. This deficiency was clearly documented in a recent study done by Schobitz *et al.* (2008), where pediatric and emergency medicine residents were tested on the medical management of pediatric victims of biological and chemical terrorism (Schobitz *et al.*, 2008). A test containing essential content was developed and validated by experts. This test was given to volunteer residents and was readministered 5 months after a lecture on the content. The 34 pediatric residents and 15 emergency medicine residents who took the exam scored a median of 65% and 73%, respectively. The authors investigated the benefit of the lecture and found that the 16 residents who attended the lecture and completed the post-test achieved a median score of 70%. For the 20 residents who did not attend the lecture, but completed the post-test, a median score of 66.6% was recorded. The authors concluded that there are significant knowledge deficits among pediatric and emergency medicine residents in their abilities to handle pediatric victims of biological and chemical terrorism. A suggestion was made to incorporate educational curriculum on preparedness into residency curriculums (Schobitz *et al.*, 2008).

EXHIBIT B (Pediatric Case History) – Nerve Agents

Nerve Agent Exposure in Town of Nazhmar, Iran

One victim of the March 22, 1988 attack on the village of Nazhmar was a young child with unreported age and weight. He presented immediately with marked miosis and was comatose. Breathing was irregular and foamy secretions were evident protruding from his mouth and nose. The patient was working very hard to breathe and noted to be using his accessory muscles of respiration. Wheezing was obvious on auscultation, and he showed obvious difficulty on exhalation. Upon suction removal of oral and nasal secretions, the patient was noted to have progressively rigid extremities such that finding venous access became difficult. The secretions were noted to become bloody. Over a 15 min period, a total of 7.5 mg atropine was administered during three treatments. The patient was noted to improve with eye opening, moaning, and two-word phrases. As his muscle tone decreased, his breathing improved, but wheezing was still evident. The child was decontaminated after treatment and subsequently discharged after an hour. At the time of discharge, secretions were not completely dried up, but his pupils were fully dilated and reactive to light.

(*ref:* Foroutan, 1998c)

V. EFFECTS OF SPECIFIC AGENTS

A. Nerve Agents

1. Introduction

Nerve agents pose a real threat to our unprotected civilian population. They can quickly incapacitate those who are exposed and can lead to mortality if not recognized and treated promptly. The toxicity of these agents can be enhanced in children due to pediatric vulnerabilities. Also, it is important to recognize the different ways children may present with toxicity compared to adults.

The major nerve agents are the G-series (tabun, sarin, cyclosarin, soman) and V-series (VX) compounds. These agents are clear, colorless, tasteless, and in most cases, odorless. They have been demonstrated to penetrate normal clothing and skin. Also, these agents are highly toxic as evidenced by the fact that as little as 10 mg of VX on the skin is considered to be an LD_{50} in adults (Rotenberg and Newmark, 2003). In addition, these agents produce toxicity rapidly compared to biological agents. Most G-series nerve agents are highly volatile, and can be dispersed into aerosols that are inhaled by victims. One of the G-series agents, sarin, is volatile and may sink close to the ground (in undisturbed air) where children breathe. Nerve agents may also be disseminated in liquid form. Treatment for dermal exposure begins with rapid topical decontamination.

Although our military experience managing toxicity from nerve agent exposure is limited, exposures to related chemicals such as the OP class occur commonly each year in the USA. In 2006, there were a total of approximately 5,400 OP exposures across the USA (Bronstein *et al.*, 2007). OPs, such as malathion, are commonly used as pesticides. OP toxicity manifests in a similar fashion as toxicity from nerve agents; however, this chemical class is considerably less toxic. One case series of 16 children who experienced poisonings with OPs confirmed that pediatric patients present with toxicity differently than adults (Lifshitz *et al.*, 1999). These children often did not manifest the classic muscarinic effects (such as salivary secretions and diarrhea) seen in adults.

2. Mechanism of Toxicity

Nerve agents cause toxicity by inhibiting esterase enzymes, especially acetylcholinesterase (AChE) (Rotenberg and Newmark, 2003). When nerve agents bind to AChE, they prevent hydrolysis of acetylcholine (ACh). When ACh accumulates in the synaptic space of neurons, this leads to overstimulation of muscarinic and nicotinic receptors. This overstimulation is often termed ‘‘cholinergic crisis’’. Also, it is important to note that the nerve agent–AChE bond undergoes a reaction called ‘‘aging’’ (Dunn and Sidell, 1989). Once this process is complete, the enzyme becomes irreversibly inactivated. This aging process dictates the need for prompt therapy to prevent irreversible toxicity.

3. Clinical Presentation

The signs and symptoms of a cholinergic crisis can be remembered using the mnemonic BAG the PUDDLES (Figure 61.3); these range in severity from lacrimation and urination to seizure activity (Rotenberg and Newmark, 2003). The manifestations of cholinergic crisis seen in a particular individual depend on the dose and route of exposure, as well as the duration of exposure. If death occurs from nerve agents, it is primarily attributed to respiratory failure. Nerve agents affect the respiratory system by causing central apnea, flaccid neuromuscular paralysis, bronchoconstriction, and profound glandular secretions (Hilmas *et al.*, 2006).

Children present a clinical picture that can be very different to that observed in adults. Children in cholinergic crisis may not necessarily manifest with miosis (constriction of pupils) (Rotenberg and Newmark, 2003). In fact, one case series demonstrated absence of miosis in 43% of pediatric victims. Studies involving pediatric exposure to organophosphates have suggested the appearance of isolated CNS effects (such as stupor, coma) in the absence of peripheral muscarinic effects. Pediatric victims of OP intoxication display significant muscular weakness and hypotonia in the absence of glandular secretions in 70–100% of cases involving moderate to severe levels of exposure (Rotenberg and Newmark, 2003). For adults, a presentation of central

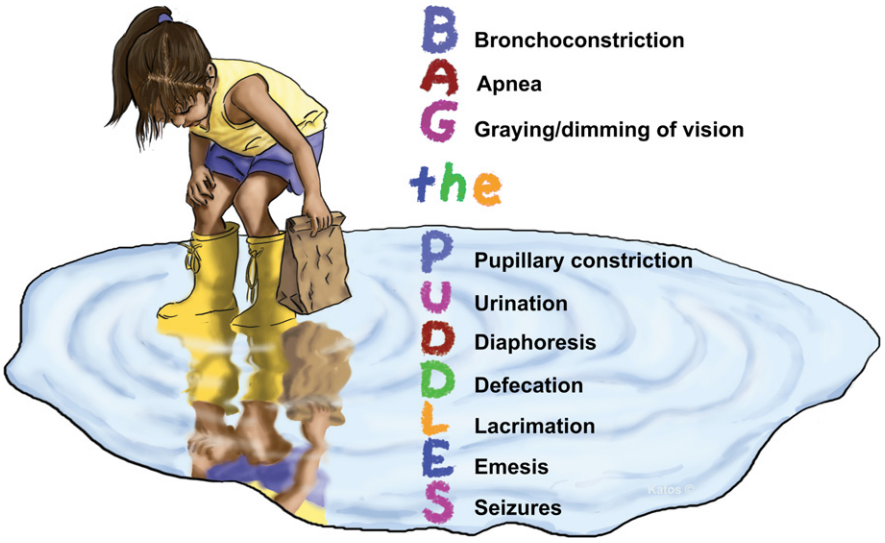

FIGURE 61.3. Helpful mnemonic for cholinergic crisis (BAG the PUDDLES). Illustrations are copyright protected and printed with permission by Alexandre M. Katos (Rotenberg and Newmark, 2003).

intoxication (weakness and hypotonia) from OPs without peripheral muscarinic signs and symptoms would be extremely atypical.

Unfortunately, there are no data on the long-term effects of nerve agent poisoning in children, and the effects must be extrapolated from what has been discovered in the adult population (Rotenberg and Newmark, 2003). Surveillance studies performed on victims of the sarin attacks in Japan revealed a wide range of sequelae, such as continued respiratory problems, vision disturbances, headache, and fatigue. Neuropsychiatric problems were also reported as a delayed effect.

4. Laboratory Findings

Use of cholinesterase levels is limited, especially for confirmation of exposure (Rotenberg and Newmark, 2003). Treatment should not be delayed for these levels to return. Levels should be used after exposure only to confirm diagnosis (after treatment has begun), to monitor recovery, or for forensic investigation.

5. Pediatric Vulnerability

Children have several vulnerabilities, putting them at increased toxicity from this class of chemical agents. A child's smaller mass alone reduces the dose needed to cause symptoms or lethality. For volatile nerve agents, children are especially at risk for respiratory toxicities due to their anatomic differences compared to adults. Their smaller airways can become compromised by the large amount of secretions and the bronchospasm caused by the agents. Also, a greater dose of nerve agent will be inhaled in children due to their higher respiratory rate and minute volumes.

6. Treatment

The overall treatment approach to nerve agent exposure focuses on airway and ventilatory support, aggressive use of antidotes (atropine and pralidoxime), prompt control of seizures, and decontamination as necessary (Henretig, 2002a). Atropine is used for its anti-muscarinic effects, and oxime is used to reactivate AChE. The combination of atropine and pralidoxime chloride (2-PAM Cl) is recommended for the prompt treatment of all serious cases. The timing of atropine and 2-PAM Cl administration is critical. In short, the faster these antidotes are given, the better the outcome. Oxime therapy is rendered ineffective if given after the enzyme aging process has been completed (Dunn and Sidell, 1989). This fact has led to the use of autoinjectors because of their ability to rapidly administer intramuscular doses of these medications. However, there are no currently Food and Drug Administration (FDA)-approved pediatric autoinjectors for 2-PAM Cl. Other administration routes and methods include intravenous (IV) or intraosseous (IO) for atropine and slow IV or continuous infusion for 2-PAM Cl. Data show that peak plasma concentrations of medications administered from autoinjectors are achieved in less than 5 min versus 25 min for intramuscular (IM) administration using a needle and syringe (Rotenberg and Newmark, 2003). The mainstay of adult therapy includes the use of autoinjector technology containing atropine and 2-PAM. Recently, Meridian Medical Technologies™ has developed and received FDA approval for a dual-chambered autoinjector called the ATNAA (Antidote Treatment Nerve Agent Autoinjector) for the military and Duodote™ for civilian emergency medical technicians and first responders (see Figure 61.4). Meridian also produces the older Mark I™ kit (Figure 61.5) which is composed of separate autoinjectors for atropine and 2-PAM.

These products are provided by Meridian Medical Technologies, which has partnered with the US Department of Defense to be the only FDA-approved supplier of nerve agent antidotes. The Mark I™ kit and the single autoinjector devices deliver 600 mg of 2-PAM Cl and 2 mg of atropine (AtroPen®) in seconds. This kit was developed originally

FIGURE 61.4. ATNAA and Duodote™. Photo reproduced with permission from Meridian Medical Technologies™.

FIGURE 61.5. The MARK 1™ kit. Photo reproduced with permission from Meridian Medical Technologies™.

for administration to soldiers, not for children, and with the approval of the Duodote system, the Mark I kit will most likely become antiquated. The autoinjector technology incorporates a spring-loaded needle to disperse medication in an ''all-or-nothing'' fashion. It is impossible to give partial doses of an autoinjector for children. Drug dosing of atropine and 2-PAM Cl in pediatrics is primarily weight based, so a standard dose cannot be used. Pediatric versions of the Mark 1™ kit are available overseas but are not currently available in the USA (PEAP, 2004). In June 2003, the FDA approved pediatric doses of the AtroPen® (atropine autoinjector) to respond to the lack of pediatric specific therapy (Meadows, 2004). Meridian's AtroPen is now available in four dosages, 0.25 mg, 0.5 mg, 1 mg, and 2 mg (Figure 61.6). The 0.25 mg dose should be used for infants weighing less than 7 kg, the 0.5 mg treats patients weighing 7–18 kg, the 1 mg treats patients weighing 18–41 kg, and the 2 mg dose should be used for children/adolescents who weigh more than 41 kg. The needle length for these autoinjectors is 0.8 inches, with a needle gauge of 22. Administration technique of autoinjectors in children is displayed in Figure 61.7. Since the AtroPen® delivers only atropine and not 2-PAM Cl, there continues to be a limitation to the prompt treatment of children. This fact has caused groups such as the pediatric expert advisory panel from the National Center for Disaster Preparedness to recommend the use of the Mark 1™ kit before use of the AtroPen (PEAP, 2004). A table of how to use the Mark

FIGURE 61.6. The Atropen® pediatric autoinjectors. Dose sizes: 0.25 mg – infant, 0.5 mg – child (7–18 kg), 1 mg – child (18–41 kg), 2 mg – adolescent/adults. Photograph reproduced with permission from Meridian Medical Technologies™.

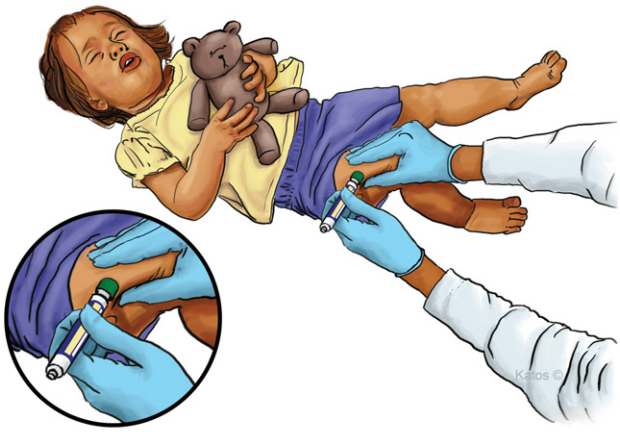

FIGURE 61.7. Technique for using Atropen® pediatric autoinjector.

1 kit for children is displayed in Table 61.2. The use of adult dose-based autoinjectors in children has been addressed. Amitai *et al.* (1992) reviewed 240 instances of accidental pediatric atropine injections using adult dose-based autoinjectors. A low incidence of toxicity was found, with no seizures, arrhythmias, or death. Subsequently, several pediatric guidelines have suggested adult-dose atropine and 2-PAM Cl autoinjectors can be safely used in children larger than 13 kg and inserted to 0.8 inches.

Administration of atropine and 2-PAM Cl must be done cautiously (Rotenberg and Newmark, 2003). Atropine can cause increased heart rate, dry mouth and skin, and near vision can be affected for up to 1 day. Due to the fact that sweating is prevented, elevated temperatures and heat stress may be observed. 2-PAM Cl can cause double or blurred vision and dizziness (Anon, 2002). Doses must be reduced with renal insufficiency. If a medication is given too quickly as an intravenous injection, laryngospasm and rigidity can occur. Higher doses can cause hypertension while lower doses can cause mild electrocardiogram changes (Rotenberg and Newmark, 2003).

Although benzodiazepines are not considered to be an antidote, their use in the treatment of nerve agent exposures is critical (Rotenberg and Newmark, 2003). Status epilepticus can often occur as the nerve agent crosses the blood–brain barrier and causes irritation. Benzodiazepines are the only effective agents that have been proven to treat nerve agent-induced seizures. This group of medications should be used for both prevention and treatment. It is recommended that if more than one organ is impaired, there is impaired consciousness, or muscle twitching, benzodiazepines should be quickly administered. In choosing a specific medication, various agents can be used. Our military department uses the medication diazepam that is administered as an autoinjector (Figure 61.8). In Israel, there is a move towards using midazolam for their population. Some physicians are recommending the use of lorazepam in the pediatric population. Regardless of which medication is used, repeat doses may be needed. For the pediatric population, benzodiazepines should be considered if there is any suspicion of seizure activity. Nonconvulsive status and subtle seizures are common in infants and children, making it difficult for health care providers to recognize nerve agent toxicity.

For each of the medications used to treat nerve agent toxicity, there is weight-based dosing recommendations for pediatric patients. The exact dose to utilize for a specific patient will depend on two critical factors: the severity of the exposure and the weight or age of the patient. Pediatric dosing recommendations for medications used to treat mild to moderate nerve agent exposures are displayed in Table 61.3. Dosing recommendations to treat severe nerve agent exposures are displayed in Table 61.4.

7. Perioperative Care of Children with Nerve Agent Intoxication

As mentioned earlier, it is not uncommon for chemical exposures and trauma to occur at the same time, necessitating the need for surgery. It is important to realize that many drugs used for perioperative management can exacerbate the side effects encountered with nerve agent exposure. Nerve agents can cause drug interactions with medications typically used for resuscitative efforts (Abraham *et al.*, 2001). Anesthetics, such as sodium penthothal and propofol, cause cardiac depression, an effect exacerbated by the excessive muscarinic activity induced by nerve agents. Doses of these drugs may need to be reduced. Use of volatile anesthetics may be preferable because they bronchodilate and reduce the need for nondepolarizing drugs. When nondepolarizing drugs are used, they are often reversed by the use of neostigmine, which affects AChE activity. Halothane should be avoided in infants because the

TABLE 61.2. Dosing of the Mark 1™ kit for children with severe, life-threatening nerve agent toxicity[a] (PEAP, 2004)

Approximate age	Approximate weight	Number of Mark 1™ kit autoinjectors to use	Atropine dosage range (mg/kg)	Pralidoxime dosage range (mg/kg)
3–7 yrs	13–25 kg	1	0.08–0.13	24–46
8–14 yrs	26–50 kg	2	0.08–0.13	24–46
>14 yrs	>51 kg	3	0.11 or less	35 or less

[a]If an adult MARK I kit is the only available source of atropine and pralidoxime, it should not be withheld from even the youngest child (i.e. <3 y.o.)

FIGURE 61.8. The diazepam autoinjector. Photograph reproduced with permission from Meridian Medical Technologies™.

cardiac side effects can be accentuated in the presence of nerve agents. Depression of the cardiovascular system by halothane may cause further bradycardia, hypotension, and reduction in cardiac output. In general, the use of muscle relaxants is not recommended in the setting of nerve agent toxicity. Nerve agents provide a depolarizing block and in the presence of inhibited AChE activity, drugs such as succinylcholine can have longer effects than expected (Rotenberg, 2003b).

Careful use of analgesia is important when caring for victims of nerve agent exposure (Abraham *et al.*, 2001). In general, opioids are considered safe to use because they do not act on the cholinergic system directly. However, some side effects of the drugs, such as histamine release and rare muscle rigidity, can cause difficulty in patient management. Careful dose titration and monitoring for side effects is critical. However, there is one opioid that can have an interaction with nerve agents. Remifentanyl, a potent opioid, contains an ester linkage susceptible to hydrolysis because it is partially metabolized by plasma cholinesterase. This is the same enzyme that is inactivated by nerve agents, resulting in a prolonged duration of action for remifentanyl. Therefore, use of remifentanyl in the post-operative care of nerve agent-exposed victims is not recommended as other analgesics are available (Rotenberg, 2003b). Compared to other chemical warfare agents, patients exposed to nerve agents pose unique challenges for medical and surgical management.

8. Summary

Nerve agent exposures must be handled quickly and efficiently. When children are exposed, it is important to remember that antidote dosing will be determined by the patient's weight and the severity of exposure. Progress has been made to provide pediatric-specific autoinjectors; however, since 2-PAM Cl is not yet available in a pediatric autoinjector form, it is possible to carefully use adult autoinjectors to manage pediatric patients.

B. Carbamates/Organophosphates

1. Introduction

Carbamates and OPs are chemicals that are often used as fungicides, insecticides, or pesticides and possess actions similar to nerve agents. These compounds are considered ''weapons of opportunity'' since their primary use is not by conventional militaries. In the USA, toxicity from these compounds is fairly rare. In 2006, there were approximately 1,200 cases of carbamate exposures and 1,500 organophosphate exposures documented for children 19 years old and younger (Bronstein *et al.*, 2007). Although there were a few fatalities reported in 2006 from these substances, these fatalities occurred only in older individuals.

In general, the toxicity of these compounds resembles that of nerve agents, but is less severe. Pediatric cases of toxicity reported in the literature are often due to accidental poisoning when a young child ingests chemicals placed in unsecured or unlabeled containers. Exposure also can come by consuming foods that have been sprayed with pesticides. Most of the literature on pediatric toxicity from these agents includes retrospective reviews coming out of Israel, where use of these substances as a pesticide for the home and agriculture is common. A retrospective review of 37 cases from the USA has also been published (Zwiener and Ginsburg, 1988).

2. Mechanism of Toxicity

These compounds inhibit the hydrolysis of the neurotransmitter acetylcholine by the enzyme acetylcholinesterase within the mammalian nervous system (Zwiener and Ginsburg, 1988). This inhibition causes acetylcholine levels to rise, thus causing cholinergic hyperstimulation at muscarinic and nicotinic receptors. There are important differences in the way carbamates and OPs bind to acetylcholinesterase as well as their abililty to affect the CNS. Carbamates are reversible inhibitors of cholinesterase enzymes. Carbamates create a reversible bond to the cholinesterase enzyme through carbamylation which can spontaneously hydrolyze, reversing toxicity. Carbamate poisoning produces toxicity similar to that of OPs; however, the toxicity is usually of a shorter duration and less severe in nature (Lifshitz *et al.*, 1994). In contrast, OPs inhibit cholinesterase via an irreversible bond of phosphate radicals

TABLE 61.3. Management of mild/moderate nerve agent exposures (Rotenberg and Newmark, 2003; Anon, 2002)

		Management			
		Antidotes[a]		Benzodiazepines (if neurological signs)	
Nerve agents	Severity of symptoms	Age	Dose	Age	Dose
Tabun, sarin, cyclosarin, soman, VX	**Mild/moderate symptoms** • localized sweating • muscle fasciculations • nausea • vomiting • weakness/floppiness • dyspnea • constricted pupils • blurred vision • rhinorrhea • excessive tears • excessive salivation • chest tightness • stomach cramps • tachycardia or bradycardia	Neonates and infants <6 mo	**Atropine** 0.05 mg/kg IM/IV/IO to max 4 mg or 0.25 mg AtroPen® and **2-PAM** 15 mg/kg IM or IV slowly to max 2 g/h	Neonates	**Diazepam** 0.1–0.3 mg/kg/dose IV to a max dose of 2 mg OR **Lorazepam** 0.05 mg/kg slow IV
		Infants (6 mo–4 yrs)	**Atropine** 0.05 mg/kg IM/IV/IO to max 4 mg or 0.5 mg AtroPen® and **2-PAM** 25 mg/kg IM or IV slowly to max 2 g/h	30 days–5 yrs	**Diazepam** 0.05–0.3 mg/kg IV to a max of 5 mg/dose OR **Lorazepam** 0.1 mg/kg slow IV not to exceed 4 mg
		Children (4–10 yrs)	**Atropine** 0.05 mg/kg IV/IM/IO to max 4 mg or 1 mg AtroPen® and **2-PAM** 25–50 mg/kg IM or IV slowly to max 2 g/h	5 yrs and older	**Diazepam** 0.05–0.3 mg/kg IV to a max of 10 mg/dose OR **Lorazepam** 0.1 mg/kg slow IV not to exceed 4 mg
		Adolescents (>10 yrs) and adults	**Atropine** 0.05 mg/kg IV/IM/IO to max 4 mg or 2 mg AtroPen® & **2-PAM** 25–50 mg/kg IM or IV slowly to max 2 g/h	Adolescents and adults	**Diazepam** 5–10 mg up to 30 mg in 8 h period OR **Lorazepam** 0.07 mg/kg slow IV not to exceed 4 mg

[a]In general, pralidoxime should be administered as soon as possible, no longer than 36 h after the termination of exposure. Pralidoxime can be diluted to 300 mg/ml for ease of IM administration. Maintenance infusion of 2-P at 10–20 mg/kg/h (max 2 g/h) has been described. Repeat atropine as needed every 5–10 min until pulmonary resistance improves, secretions resolve, or dyspnea decreases in a conscious patient. Hypoxia needs to be corrected as soon as possible.

to the active esteratic site of the enzyme (Lifshitz *et al.*, 1999). Thus, the toxicity is more severe.

3. Clinical Presentation

OPs and carbamates have different receptor activities in the mammalian nervous system. OPs have effects on muscarinic and nicotinic receptors and can cause neurological effects in the CNS (Levy-Khademi *et al.*, 2007). Carbamates are thought to cause only parasympathetic muscarinic effects with limited nicotinic and CNS effects (Sofer *et al.*, 1989). However, there are case reports in children that have revealed the presence of CNS effects with carbamate exposures (de Tollenaer *et al.*, 2006). One pediatric case series stated that the signs and symptoms from carbamate poisoning were indistinguishable from OP exposures, with severe CNS depression with stupor and coma occurring in eight cases (Sofer *et al.*, 1989).

Muscarinic hyperstimulation leads to a clinical presentation of miosis, lacrimation, salivation, bradyarrhythmias, urinary incontinence, and intestinal hypermotility (Levy-Khademi *et al.*, 2007). Nicotinic hyperstimulation leads to fasciculations, weakness, and paralysis of skeletal muscles. CNS effects include depression and agitation with coma and seizures occurring in the most severe cases for adults. Generalized tonic–clonic seizures have been seen in several pediatric exposure cases reported in the literature (Zwiener and Ginsburg, 1988).

Additional toxicities that have been reported in the children include diarrhea, pulmonary edema which was associated with OP exposures but not carbamate exposures (Lifshitz *et al.*, 1999), acute pancreatitis, hyperglycemia (Weizman and Sofer, 1992), dyspnea, sweaty cold skin (Sofer *et al.*, 1989), respiratory distress or failure, lethargy, and tachycardia (Zwiener and Ginsburg, 1988).

4. Laboratory Findings

Key findings that have been reported include significant hypoxia, acidosis, and carbon dioxide retention (Sofer *et al.*, 1989). Also hyperglycemia, hypokalemia, and leukocytosis were observed in a case series of organophosphate exposures (Levy-Khademi *et al.*, 2007). A prospective study done on 17 children with typical organophosphate or carbamate poisoning looked at laboratory abnormalities that are associated with acute pancreatitis. Five of the patients (30%) had laboratory values consistent with pancreatitis with elevated immunoreactive trypsin, amylase, and serum

TABLE 61.4. Management of severe nerve agent exposures (Rotenberg and Newmark, 2003; Anon, 2002)

Nerve agents	Severity of symptoms	Management			
		Antidotes[a]		Benzodiazepines (if neurological signs)	
		Age	Dose	Age	Dose
Tabun, sarin, cyclosarin, soman, VX	**Severe symptoms** • convulsions • loss of consciousness • apnea • flaccid paralysis • cardiopulmonary arrest • strange and confused behavior • severe difficulty breathing • involuntary urination and defecation	Neonates and infants <6 mo	**Atropine** 0.1 mg/kg IM/IV/IO or 3 doses of 0.25 mg AtroPen® (administer in rapid succession) and **2-PAM** 25 mg/kg IM or IV slowly OR 1 Mark 1 kit (atropine + 2-PAM) if no other options exist	Neonates	**Diazepam** 0.1–0.3 mg/kg/dose IV to a max dose of 2 mg (PDH) OR **Lorazepam** 0.05 mg/kg slow IV
		Infants (6 mo–4 yrs)	**Atropine** 0.1 mg/kg IV/IM/IO or 3 doses of 0.5 mg AtroPen® (administer in rapid succession) and **2-PAM** 25–50 mg/kg IM or IV slowly or 1 Mark 1 kit (atropine + 2-PAM) if no other options exist	30 days–5 yrs	**Diazepam** 0.05–0.3 mg/kg IV to a max of 5 mg/dose OR **Lorazepam** 0.1 mg/kg slow IV (not to exceed 4 mg)
		Children (4–10 yrs)	**Atropine** 0.1 mg/kg IV/IM/IO or 3 doses of 1 mg AtroPen® (administer in rapid succession) and **2-PAM** 25–50 mg/kg IM or IV slowly OR 1 Mark 1 kit (atropine + 2-PAM) up to age 7, 2 Mark 1 kits for ages >7–10 yrs	5 yrs and older	**Diazepam** 0.05–0.3 mg/kg IV to a max of 10 mg/dose OR **Lorazepam** 0.1 mg/kg slow IV (not to exceed 4 mg)
		Adolescents (>10 yrs) and adults	**Atropine** 6 mg IM or 3 doses of 2 mg AtroPen® (administer in rapid succession) and **2-PAM** 1,800 mg IV/IM/IO OR 2 Mark 1 kits (atropine + 2-PAM) up to age 14, 3 Mark 1 kits for ages >14 yrs	Adolescents and adults	**Diazepam** 5–10 mg up to 30 mg in 8 h period OR **Lorazepam** 0.07 mg/kg slow IV (not to exceed 4 mg)

[a]In general, pralidoxime should be administered as soon as possible, no longer than 36 h after the termination of exposure. Pralidoxime can be diluted to 300 mg/ml for ease of IM administration. Maintenance infusion of 2-PAM at 10–20 mg/kg/h (max 2 g/h) has been described. Repeat atropine as needed every 5–10 min until pulmonary resistance improves, secretions resolve, or dyspnea decreases in a conscious patient. Hypoxia needs to be corrected as soon as possible.

glucose. None of the patients had hypocalcemia, renal dysfunction, or acidosis and all had complete recovery of pancreatic function. The authors concluded that acute pancreatitis, due to anticholinesterase intoxication, is not uncommon in the pediatric population (Weizman and Sofer, 1992). Pancreatitis has been described in adult exposures and the association has been investigated in animal studies (Weizman and Sofer, 1992).

EXHIBIT C (Pediatric Case Histories) – Carbamates/ Organophosphates

Carbamate Exposure in a Child

A 7-year-old female, previously healthy, was exposed to an unknown quantity of the fungicide pesticide maneb (manganese ethylene-bis-dithiocarbamate). She was admitted to the pediatric intensive care unit with status epilepticus. She had experienced abdominal pain with nausea, vomiting, and headache for approximately 3 days prior to admission. On the day of admission, she was pale, unconscious, staring, and hypotonic. Upon examination, it was determined that she also had severe hypothermia (32.5°C) and hypoventilation. In addition, she was found to have a combined metabolic and respiratory acidosis and elevated blood glucose levels. Routine diagnostic investigations such as blood counts, electrolytes, liver/renal function, CSF, and blood cultures, were normal. Urine/blood toxicology screens for barbiturates and benzodiazepines were negative. Liquid chromatographic-mass spectrometry confirmed the presence of maneb in her blood. Upon admission, the patient was intubated and her convulsions were treated with benzodiazepines. Within 24 h, there was a complete recovery of all neurological signs. Repeat physical and neurological exams were normal at 48 h. She was discharged 3 days after admission in good condition.

(*ref:* de Tollenaer *et al.*, 2006)

Case History: Organophosphate Exposure in a Child

A 2-year-old, previously healthy male ingested approximately 10 ml of the organophosphate insecticide demeton-*S*-methyl. Upon discovery of the ingestion, the child was disrobed and washed in the bathtub and then taken to the local hospital. Upon admission, which was approximately 30 min following the ingestion, the child was vomiting and salivating. Atropine was administered. He was then transferred to a larger hospital. During transport, he continued to have excessive salivation and experienced bradycardia. These episodes were treated with additional doses of atropine. Upon admission to the larger hospital, he was salivating, vomiting, and experiencing bronchial hypersecretion. At that time, his pupils were dilated with no reaction to light and he was tachycardic with a pulse rate of 150 bpm. At the hospital, gastic lavage was performed and additional doses of atropine administered. He was intubated prior to the completion of gastric lavage and given diazepam for sedation. Bradycardia (<100 bpm) stimulated the physicians to administer additional atropine. Due to the complexity of the case, the patient was transferred again to a hospital better equipped to handle his case. Upon that admission, the patient's blood pressure was 110/60 mm Hg, pulse 100 bpm, temperature 37.6°C, and his pupils were still dilated and unresponsive. Due to continued bronchial hypersecretion, additional atropine was administered. Obidoxime was administered twice, once at 9.5 and 11.5 h after the ingestion. After the oxime therapy, his condition stabilized and he was able to be extubated approximately 13 h after ingestion. Over the next 4 days, atropine was administered periodically to control mild bronchial hypersecretion. Electrolytes and chest X-rays were normal during the entire course of illness. A low hemoglobin and high alkaline phosphatase were the only abnormal labs. The patient was discharged 8 days after the ingestion in good condition. Plasma cholinesterase levels were initially decreased (<400 U/l), but they rose into the normal range by discharge. Twelve-month follow-up revealed that the child had no signs of neurological sequelae.

(*ref:* Rolfsjord *et al.*, 1998)

Another laboratory value that is often obtained in these exposures is serum pseudocholinesterase. Serum pseudocholinesterase activities are often assessed as normal in children because the reference standards may not be reliable when assessing children. To add to the complexity, the normal range of serum cholinesterase activity is wide (Sofer *et al.*, 1989). Authors have described the limitations of this measurement in determining therapy for children. In fact, it is recommended that a therapeutic and diagnostic trial of atropine should be given whenever there is any possibility of intoxication with these chemicals (Sofer *et al.*, 1989).

Additional laboratory abnormalities that have been reported in children are cardiac disturbances. Prolonged QTc intervals were reported in a few children exposed to organophosphates. However, there was spontaneous resolution with no evidence of ventricular dysrhythmia on electrocardiogram (Levy-Khademi *et al.*, 2007).

5. Pediatric Vulnerabilities

The clinical picture of anticholinesterase intoxication in children is very different to that of adults. Often, clinicians have difficulty in diagnosing the exposure in pediatric patients. In fact, in a retrospective review of OP/carbamate toxicity cases that were admitted to a Children's Medical Center in the USA, the transfer diagnosis was incorrect in 80% of the patients (Zwiener and Ginsburg, 1988). Patients were misdiagnosed with a wide variety of disease states that ranged from head trauma to cranial aneurysm to diabetic ketoacidosis. It was noted that the difficulty in identifying nicotinic and muscarinic signs in children may have contributed to the high misdiagnosis rate. For example, it may be difficult to distinguish normal infant crying from excessive lacrimation (Zwiener and Ginsburg, 1988).

The lack of classic muscarinic effects does not exclude the possibility of cholinesterase inhibitor poisoning in young children with central nervous system depression. In one case series, tearing and diaphoresis were not observed in pediatric patients (Lifshitz *et al.*, 1999). Miosis was absent in a number of pediatric patient cases reported in the literature with 27% of children in one case series lacking miosis on admission (Zwiener and Ginsburg, 1988). The percentage was 20% in another case series of pediatric patients (Sofer *et al.*, 1989).

Adult literature states that the most important signs of organophosphate toxicity are fasciculations and miosis. In one published pediatric case series, fasciculations were quite infrequent, occurring in only 16% of cases (Sofer *et al.*, 1989). Another pediatric case series verified this result, with the frequency of fasciculations being 22% (Zwiener and Ginsburg, 1988).

Another difference is the cardiac manifestations that are seen in adults compared to children. In one pediatric case series, cardiopulmonary manifestations were the least common, with tachyarrhythmias being more common than the bradyarrhythmias that are typically seen in adults (11 patients vs one patient) (Levy-Khademi *et al.*, 2007).

Compared to adults, neurological manifestations of toxicity were the most common in children. In one case series, significant hypotonia and muscle weakness were observed in all children (Lifshitz *et al.*, 1999). In addition, severe CNS depression with stupor and coma occurred in all the cases (Lifshitz *et al.*, 1999). In another case series, coma occurred in 54.8% and seizures in 38.7% of children who were accidentally exposed to organophosphates (Levy-Khademi *et al.*, 2007). It can be theorized that the more permeable blood–brain barrier in young children permits penetration of the toxic agents into the brain, thus causing CNS depression. Another theory is that in young children, cholinesterase inhibitors have a stronger affinity to acetylcholinesterase in the CNS and less affinity to cholinergic synapses in the autonomic ganglia (Lifshitz *et al.*, 1999). Accumulation of toxic compounds or their metabolites in the brain could also result in severe CNS dysfunction. Another thought is that the hypoxemia that has been observed in several pediatric cases could contribute to CNS depression (Sofer *et al.*, 1989).

6. Treatment

In all pediatric cases, supportive care was used to balance electrolyte disturbances and oxygen was administered for hypoxic episodes. Often, patients were intubated and given mechanical ventilation due to the excessive salivation and bronchial hypersecretion. Gastric lavage was utilized in one case of organophosphate ingestion (Rolfsjord *et al.*, 1998). The treatment of organophosphate intoxication mimics that of nerve agent exposures. Atropine and an oxime (such as pralidoxime) are the agents of choice for organophosphate exposures. Atropine therapy alone is recommended for carbamate exposure due to the fact that carbamates reversibly inhibit acetylcholinesterase, so there is little need for an agent such as an oxime, which reactivates the enzyme. In cholinesterase inhibitor poisoning, atropine will alleviate most of the muscarinic signs, few of the CNS symptoms, and almost none of the nicotinic symptoms (Lifshitz *et al.*, 1999).

Although atropine use is standard, clinicians are sometimes faced with the dilemma of administering atropine to a pediatric patient with an elevated heart rate. Due to the fact that children may manifest tachycardia with toxic exposures and the fact that the chronotropic effects of atropine may be minimal in infants and small children compared with healthy young adults, one group of authors suggests that atropine should not be withheld or administered in subtherapeutic doses in tachycardic infants and children with organophosphate or carbamate exposures. Their experience with pediatric patients showed that for patients with tachycardia at the time atropine was administered, their heart rate decreased and none of the patients developed cardiac arrhythmias (Zwiener and Ginsburg, 1988).

Use of oximes is well accepted for organophosphate exposures, but their role in carbamate poisoning is controversial. Animal studies have shown oxime use can increase toxicity when treating carbaryl exposures (Lifshitz *et al.*, 1994). Therefore, there is a general guideline that oximes should be avoided if a carbamate exposure is suspected. One case series reported the routine use of oxime therapy for carbamate exposures in children (Lifshitz *et al.*, 1994). Marked clinical improvement was observed in all patients regardless of whether they were exposed to an organophosphate or a carbamate. In addition to the retrospective review of cases, the authors completed an *in vitro* study of oxime use with carbamate toxicity and discovered that oximes play a minor role in direct reactivation of human carbamylated acetylcholinesterase. Due to this result, the authors concluded that the current guideline to avoid oxime use in a carbamate exposure is valid.

Fortunately, in most cases of organophosphate or carbamate toxicity, pediatric patients had a full recovery if they were diagnosed rapidly and appropriate treatments were administered in a timely fashion.

7. Summary

There is a limited amount of literature available describing the toxicities of organophosphate and carbamate exposures in pediatrics. What literature is available describes major differences between the manners children manifest toxicity compared to adults. It is critical to understand these differences so that patients are not misdiagnosed and appropriate therapy is not delayed. In general, the CNS toxicities are greater in children than in adults with coma, stupor, and seizures being common. It is important to recognize that if therapy is given in a timely manner, complete recovery is often the outcome for children exposed to these toxic agents.

C. Vesicants

1. Introduction

Blister agents or vesicants are chemicals that cause blister or vesicle formation upon dermal contact. Agents such as mustards or lewisite have been used as chemical warfare agents in the past (Yu *et al.*, 2003). Although these agents have less toxicity than nerve agents, they cause prolonged morbidity. There are two types of mustard, termed sulfur mustard (HD) and nitrogen mustards (HNs). HD caused

EXHIBIT D (Pediatric Case Histories) – Vesicants

Mustard Gas Exposure in 14 Children/Teenagers from Halabja, Iraq

Mustard gas was used on the civilian population during the Iraq-Iran War (1980–88). A case series of 14 children and teenagers affected by mustard gas was reported by Momeni *et al*. They found that facial involvement was the most frequent disorder (78%), followed by genital (42%), trunkal and axillar lesions (both 14%). The most prominent laboratory abnormality was eosinophilia (12% of patients). As far as the time course of toxicity, skin lesions appeared 4–18 h after exposure and then erythema developed within 20–30 h. After the erythema, blisters would appear. The authors concluded that the time of onset of toxicity was shorter and more severe in children and teenagers compared with adults.

(*ref*: Momeni and Aminjavaheri, 1994)

Clinical Cases from Mofid Medical Center (Mustard Exposure Following the Halabja Attack on March 17, 1988)

A 3-year-old (yo) male presented to Mofid Medical Center 8 days after the Halabja chemical attack with fever (39.5°C), tachycardia (HR 140), and tachypnea (RR 60). Cutaneous skin lesions were mild, but erythema and edema covered 45 % of his skin surface area. Ocular and respiratory findings were as previously described. Laboratory findings were unremarkable except for a mild anemia. Chest roentograms revealed hilar congestion and consolidation bilaterally. The fever continued despite antibiotic therapy. On day 10 of admission (18 days after exposure), the patient developed leukocytosis with 82% PMNs and worsening respiratory distress. The patient finally died 21 days after exposure.

An 8-yo Iranian male presented at 5:30 pm with fever (40°C), severe agitation, delirium and somnolence 24 h after exposure to chemical agents the previous day in Halabja. BP was 110/70 mm Hg and the patient was notably tachycardic (HR 120), and tachypneic (RR 42). The patient was noted to have serious dermatologic, ocular, and respiratory impairment. Erythema, vesicles, erosions, bullae, ulcerations, and edema was present on 35% of the body. Ocular manifestations included conjunctivitis and palpebral edema. At this point, the patient was working hard to breathe as evidenced from accessory muscles of respiration (sternocleidomastoid). On physical examination of the lungs, wheezing and crepitation were noted throughout all lung field. Laboratory findings were the following: Na^+ 139, K^+ 4.1 mEq/l, BUN 25 mg/dl, calcium 7.3 mg/dl, and WBC 9,900/mm^3 with 90% neutrophils. Arterial blood gases: pH 7.30, pCO_2 31, pO_2 65, and HCO_3 15.1. Chest roentograms showed bilateral infiltrates. The patient died 24 h after admission and 48 h after exposure, despite receiving supportive care.

(*ref:* Azizi and Amid, 1990)

more casualties in WWI than any other chemical weapon. It caused a significant number of casualties, both civilian and military, during the Iran–Iraq War of the 1980s. HD vapor is the route most likely to be used by terror groups (Yu *et al.*, 2003). It affects multiple organ systems including skin, eyes, respiratory and gastrointestinal tracts, and bone marrow (Yu *et al.*, 2003). Nitrogen mustards, on the other hand, have never been used on the battlefield and will not be discussed further.

Lewisite, a vesicant with HD-like properties, causes a similar constellation of signs and symptoms involving the skin, eyes, and airways as well as systemic effects (e.g. increased capillary permeability) after absorption. However, it does not produce immunological suppression like mustard. Another difference is that the management of lewisite toxicity includes an antidote, British Anti-Lewisite (BAL) (Yu *et al.*, 2003).

2. Mechanism of Toxicity

HD rapidly penetrates cells and generates a highly toxic reaction that disrupts cell function and eventually causes cell death (Sidell *et al.*, 1997). It is classified as an alkylating agent, targeting poorly differentiated and rapidly reproducing cells (Yu *et al.*, 2003). Death is a result of massive pulmonary damage complicated by infection.

3. Clinical Presentation

Mustard can cause local effects on skin, airways, and eyes; however, large doses can cause fatal systemic effects (Yu *et al.*, 2003). In a study of clinical findings among children exposed to vesicants, ocular, cutaneous, and respiratory signs were the most prevalent (Azizi and Amid, 1990). Ocular findings consisted of the following: conjunctivitis (94%), palpebral edema (81%), eye closure (63%), keratitis (38%), blepharospasm (25%), corneal ulceration (19%), and chemosis (6%). Cutaneous signs included erythema (94%), hyperpigmentation (75%), ulceration (69%), erosion (63%), blister (56%), edema (50%), vesicles (31%), and hypopigmentation (13%). Respiratory signs included dyspnea (63%), crepitation (50%), and wheezing (25%).

Other pediatric signs of mustard exposure were photophobia, lacrimation, ophthalmalgia, and eye burning (94%). Dry cough (81%), dermal pain, and burning (94%) were also frequent complaints. Less frequent complaints were diplopia, itchy eyes, sore throat, sneezing, nasal secretions, dyspnea, burning sensation of the upper respiratory tract, suffocation, and dysphonia (Azizi and Amid, 1990).

Initial dermal signs of toxicity consist of erythema, occurring 4–8 h after exposure. Pruritus can occur with or prior to erythema (Yu *et al.*, 2003; Azizi and Amid, 1990). Over the next 24 h, large yellowish blisters form in areas of

thin skin such as the groin and underarms. Eye damage can occur, ranging in spectrum from pain and irritation to blindness. Mustard also causes clinical effects that can be delayed for hours (Yu *et al.*, 2003; Sidell *et al.*, 1997; Azizi and Amid, 1990). This causes victims not to recognize toxicity until well after exposure. During this time lag, sulfur works to subclinically damage the skin. This latent period is significant because the shorter the latent period, the more severe the exposure, and the worse the outcome.

The CNS and bone marrow can also be affected, as displayed by symptoms of fatigue, headaches, and depression (Sidell *et al.*, 1997). HD can also lead to pneumonia, the cause of death for many HD casualties in WWI due to lack of antibiotics. A leukopenic pneumonia usually occurs between 6 and 10 days after HD exposure. The manifestation of leukopenia (specifically lymphopenia) results from the myelosuppressive effects of mustard agents.

4. Laboratory findings

While there is no diagnostic confirmatory test for mustard exposure, some laboratory tests can prove useful. As described above, inflammation and infection will show up as fever and leukocytosis. Erythrocyte sedimentation rate (ESR) has been shown to be elevated in patients after mustard exposure (Motakallem, 1988). Some of these patients were in the pediatric age range of 0–18 years. CBC determinations may show abnormalities depending on the severity of the vapor inhalation or exposure (Yu *et al.*, 2003; Azizi and Amid, 1990). The CBC may show a low hematocrit and leukopenia if the exposure was severe. There may be only a transient decrease in WBC with subsequent recovery. In pediatric cases of HD vapor exposure, decreases in hematocrit and/or WBC were likely to occur in the first 2 weeks, with the lowest levels of Hb, Hct, WBC, and neutrophil count observed in the 6th to 10th day samples after exposure (Azizi and Amid, 1990).

These pediatric patients also suffered from clear signs of hypoxemia and renal failure (Azizi and Amid, 1990). Unfortunately, serum creatinine and renal function tests (RFTs) were not found in the charts. Arterial blood gases (ABGs) may provide useful information, but they may show a varied picture. In one pediatric study of mustard casualties, most (43%) cases showed a simple metabolic acidosis. The other groups showed showed the following:

- mixed metabolic acidosis and respiratory alkalosis (29%)
- simple respiratory alkalosis (14%)
- mixed metabolic and respiratory acidosis (7%)
- mixed metabolic alkalosis and respiratory acidosis (7%).

Blood urea nitrogen can be elevated with mustard exposure, but it does not necessarily predict outcome or mortality. Blood urea nitrogen was significantly elevated in severe mustard exposure cases in the Azizi study; three of the four pediatric mustard victims that died showed very high blood urea nitrogen (Azizi and Amid, 1990). While elevations in blood urea nitrogen were found in many of the pediatric casualties from mustard exposure, blood urea nitrogen returned to normal levels soon afterwards in survivors.

5. Pediatric Vulnerability

The effects of sulfur mustard on children are more severe than on adults (Yu *et al.*, 2003). Premature infants have thinner skin, and the dermal–epidermal junction is not fully developed in children (Rutter and Hull, 1979; Harpin and Rutter, 1983; Nopper *et al.*, 1996; Seidenari *et al.*, 2000; Mancini, 2004); therefore, the time between exposure and onset of blisters is shortened, and the number and severity of blisters will be more severe (Yu *et al.*, 2003). In fact, lesions in children exposed to mustard have been shown to be more severe. Initial index cases of mass casualties are typically children. Eye findings tend to be greater in children because of their inability to protect themselves and tendency to rub their eyes (Yu *et al.*, 2003; Azizi and Amid, 1990). Children are also shown to be more susceptible to pulmonary injury for reasons previously discussed. One case report looked at the long-term effects of mustard exposure in a child (Dompeling *et al.*, 2004). Acutely, the child suffered a severe chemical pneumonia; the long-term consequence was chronic bronchiolitis. Finally, signs of gastrointestinal toxicity may be greater in children secondary to fluid losses in combination with lower intravascular volume reserves (Yu *et al.*, 2003).

While the decision to evacuate and hospitalize HD casualties is based on the extent [total body surface area (TBSA) $> 5\%$], severity of the skin lesions, and the recognition of multiple organ involvement (Graham *et al.*, 2005), the threshold to hospitalize children with HD injuries should be lower. One reason is that vapor mustard used by terrorists may cause extensive pulmonary involvement while producing mild skin blisters.

6. Treatment

While decontamination and supportive therapy are the mainstays of treatment, antidotes to counteract HD vapor, aerosol, or liquid exposures do not exist (Yu *et al.*, 2003). Adult decontamination may include bleach solutions; however, this method can cause greater toxicity in children. Soap and water are the preferred agents to use for decontamination in children. Supportive care consists of the management of pulmonary and skin manifestations such as the use of cough suppressants and/or topical silver sulfadiazine for burns (Yu *et al.*, 2003; Sidell *et al.*, 1997; Azizi and Amid, 1990). Pediatric dosage and treatment recommendations for vesicant exposures are displayed in Table 61.5.

There are currently no standardized guidelines of casualty management or drugs available to prevent HD effects on skin and mucous membranes (Sidell *et al.*, 1997; Graham *et al.*,

TABLE 61.5. Management of vesicant exposures (Momeni and Aminjavaheri, 1994; Yu *et al.*, 2003)

Vesicant agents	Symptoms	Antidotes/treatment
Mustard	• Skin erythema and pruritis • Development of large yellow blisters leading to ulcers • Eye damage • Inhalational damage: hoarseness and cough, mucosal necrosis, toneless voice, nausea, vomiting	**Decontamination:** soap, water, no bleach Copious water irrigation for eyes **Pulmonary management:** cough suppressants, throat lozenges **Skin management:** topical agents used for burns (1% silver sulfadiazine), antibiotics for secondary infections (Neosporin®), antihistamines for itching (diphenhydramine 1 mg/kg/dose orally q6–8 h max 300 mg/day, hydroxyzine 0.5 mg/kg/dose orally q6–8 h) **Immune system management:** G-CSF (filgrastim) 5–10 micrograms/kg/day subcutaneous for neutropenia
Lewisite	• Shock • Pulmonary injury • Blisters	**Decontamination:** soap, water, no bleach **Antidote:** BAL-dimercaprol may decrease systemic effects of lewisite **Pulmonary management:** BAL 3–5 mg/kg deep IM q4 h × 4 doses (dose depends on severity of exposure and symptoms) **Skin management:** BAL ointment **Eye management:** BAL ophthalmic ointment

2005). The mainstay of treatment is prompt decontamination, blister aspiration or deroofing (epidermal removal), physical debridement, irrigation, topical antibiotics, and sterile dressing application for cutaneous HD injuries. Current treatment strategies rely on symptomatic management to relieve symptoms, prevent infections, and promote healing. The general recommendations are described in the *Medical Management of Chemical Casualties Handbook* (USAMRICD, 2000a), the Field Management of Chemical Casualties Handbook (USAMRICD, 2000b), and other references (Graham *et al.*, 2005). We will discuss the aspects of treatment that relate to the pediatric population. Most pediatric casualties will have involvement of multiple organ systems (skin, ocular, gastrointestinal, bone marrow, respiratory, etc.) as documented by Iranian physicians treating pediatric casualties of HD vapor during the Iran–Iraq War (Azizi and Amid, 1990).

a. Dermatological Management

Managing mustard skin lesions is especially challenging in the pediatric population. The goal of blister management is to keep the patient comfortable, keep the lesions clean, and prevent infection. Children especially will be extremely anxious at the sight of bullae and erythema in addition to the burning, pruritis, and allodynia associated with HD blisters (Sidell *et al.*, 1997). Anxiolytics may be appropriate to calm them down and prevent them from picking at bullae. Burning and itching associated with erythema can be relieved by calamine or soothing lotion/cream such as 0.25% camphor, menthol corticosteroids, antipruritics (i.e. diphenhydramine), and silver sulfadiazine cream (Sidell *et al.*, 1997; Azizi and Amid, 1990). Pain and discomfort can be relieved with systemic analgesics. Systemic analgesics such as morphine should be given liberally before manipulation of the burned area.

Vapor mustard typically causes a first- or second-degree burn, while liquid mustard produces damage similar to a third-degree burn. In either case, tense bullae are the hallmark of HD injuries. Bullae are typically dome shaped, thin walled, 0.5 to 5.0 cm in diameter, superficial, translucent, yellowish, multiloculated, honeycombed (Moradi *et al.*, 1986), and surrounded by erythema (Sidell *et al.*, 1997). Preventing children from breaking the blisters can be a challenge, especially when constant friction from clothing and blankets is irritating to the skin. These areas should be wrapped in protective dressings. Graham *et al.* (2005) have made an important point about the existence of a reservoir of unbound HD in human skin following a vapor (Logan *et al.*, 2000) or liquid exposure, leading to an off-gassing period. They suggested that this off-gassing period can last for 24 to 36 h, whereby application of an occlusive dressing is not beneficial to prevent vapor build-up (Graham *et al.*, 2005).

It is recommended that small blisters (<1 cm) should be left alone on the child, but the immediately surrounding area should be cleaned, irrigated daily, and covered with topical antibiotic (Sidell *et al.*, 1997). Petroleum gauze bandage dressings should be wrapped around these unbroken blisters and changed every few days. Larger blisters (>1 cm) should be unroofed and irrigated several times a day with saline, sterile water, clean soapy water, or Dakin's solution and covered with topical antibiotic cream or ointment. It should be noted that blister fluid does not contain mustard (Buscher and Conway, 1944) and therefore does not represent a hazard to the health care worker. Options for topical

antibiotic creams in children include silver sulfadiazine, and triple combination antibiotic (bacitracin, neomycin sulfate, and polymyxin B sulfate) (Sidell *et al.*, 1997) but not mafenide acetate, which can cause toxicities in children (Geffner *et al.*, 1981; Ohlgisser *et al.*, 1978). These topical antibiotics should be applied to the area of bullae and surrounding areas of erythema. There is no information comparing use of this combination (triple antibiotic topical ointment) in children with use in other age groups.

While skin healing can take months for completion, pigment changes (hyper- or hypopigmentation) can persist (Sidell *et al.*, 1997; Graham *et al.*, 2005). It is also important to note that not all burn injuries require treatment at a burn center. Patients will require aggressive pain management and close observation for the systemic effects of HD exposure. Skin grafting, although rare, has been successfully used for deep burns (Ruhl *et al.*, 1994).

b. Ophthalmology

The objective for any ophthalmology consultation on pediatric HD injuries involving the eye is prevention of scarring and infection (Sidell *et al.*, 1997). Eyes exposed to HD should be irrigated to remove traces of vesicant. Severe ocular involvement requires topical antibiotics (tobramycin OD) applied several times a day. Topical steroids may be useful in the first 48 h after exposure. Temporary loss of vision may also occur after mustard exposure (Sidell *et al.*, 1997; Azizi and Amid, 1990; Motakallem, 1988). The patient should be reassured that vision loss is not permanent and is due to palpebral edema and not corneal damage.

c. Respiratory System

The conducting and ventilation portions of the respiratory tract are affected with HD vapor, necessitating a pulmonary examination (Dompeling *et al.*, 2004; Sidell *et al.*, 1997; Azizi and Amid, 1990). Bronchodilators are useful to diminish hyperreactive airways and should be used if a prior history of asthma or hyperreactive airways is documented. Further support with humidified oxygen may be required. Ventilatory support may be required for severe cases of HD vapor exposure before laryngeal spasm makes intubation difficult. Bronchoscopy is critical for diagnosis, therapeutic dilation against HD-induced tracheobronchial stenosis, and removal of pseudomembranes that cause airway obstruction.

Since the toxic bronchitis produced by HD is nonbacterial, antibiotic therapy should not be given during the first 3 to 4 days (Sidell *et al.*,1997). Continuous monitoring of sputum for Gram's stain and culture growth is necessary to identify the specific organism responsible for the late developing superinfection. The presence of leukopenia in children, a grave sign of HD exposure, will necessitate aggressive support with combination antibiotic treatment.

d. Gastrointestinal Tract

Atropine or common antiemetics can be given to provide relief from nausea and vomiting, early signs of HD intoxication (Yu *et al.*, 2003). Excellent choices for pediatric-specific antiemetics include medications such as promethazine, metoclopramide and ondansetron (Sidell *et al.*, 1997). Persistent vomiting and diarrhea are a later sign of systemic toxicity and require prompt fluid replacement.

e. Bone Marrow Suppression

As a radiomimetic, HD affects rapidly dividing tissues like bone marrow in addition to the gastrointestinal tract (Sidell *et al.*, 1997; Graham *et al.*, 2005). HD destroys hematopoietic precursor cells; WBCs have the shortest life span and decrease in number first, followed by RBCs and thrombocytes. The bone marrow suppression that is sometimes seen can be treated with filgrastim injections. This medication stimulates the bone marrow to create and release white blood cells.

f. Other Treatment Considerations

Fluid status, electrolytes, and urine output should be monitored in the HD-intoxicated patient. Tetanus prophylaxis should also be administered because fatal tetanus may occur even after a small partial-thickness burn (Marshall *et al.*, 1972).

7. Summary

Pediatric exposures to vesicants can be quite toxic; however, in contrast to nerve agent exposures, HD causes significantly greater morbidity than mortality. While mustard did not cause many deaths in WWI, death from HD exposure is usually due to massive pulmonary damage complicated by infection (bronchopneumonia) and sepsis. Children often show a quicker onset and greater severity of toxicity. Skin and eye toxicity occurs in the form of blisters or irritation that can result in blindness for the most severe cases. Except for lewisite, vesicant exposures must be managed with supportive care and rapid decontamination.

D. Pulmonary Agents

1. Introduction

In January 2002, a Central Intelligence Agency (CIA) report stated that terrorist groups may have less interest in biological materials compared to chemicals such as cyanide, chlorine, and phosgene (DCI, 2002) which are able to contaminate food and water supplies (Sidell *et al.*, 1997; Graham *et al.*, 2005). The targeting of children has the potential to destabilize governments and create widespread terror. Industrial chemicals, such as chlorine and phosgene, have advantages that make them potential candidates to be used by terrorists in the future. Both chlorine and phosgene are fairly easy to manufacture and handle, prompting national concern over their future use. In the USA alone, millions of tons of chlorine and phosgene are produced annually toward the manufacture of various products (Burklow *et al.*, 2003). A detailed discussion of the general mechanisms of chlorine and phosgene toxicity can be found

EXHIBIT E (Pediatric Case History) – Pulmonary Agents

Chlorine Gas Exposure in an Adolescent

A 14-year-old male, previously healthy except for a history of asthma, was exposed to chlorine gas when he mixed household bleach with vinegar. Immediately, he began to cough and have difficulty breathing. His symptoms worsened over the next hour, leading to an admission to the local emergency room. Upon admission, the physical exam revealed that the patient was in respiratory distress with bilateral crackles and diffuse wheezing. He also had conjunctival irritation. Vital signs were pulse 100 bpm, blood pressure of 130/80 mm Hg, respiratory rate of 20/min and a temperature of 97°F. His initial oxygen saturation while breathing room air was 92%. A venous blood gas suggested mild CO_2 retention with a pH of 7.35, PCO_2 of 53 mm Hg, and a PO_2 of 33 mm Hg. Chest radiograph showed bilateral alveolar infiltrates with a normal heart size, which is indicative of acute lung injury. Sinus tachycardia was demonstrated on the electrocardiogram. The patient was treated with oxygen, multiple doses of nebulized albuterol and oral prednisone. Despite these measures, his overall respiratory status continued to decline and a repeat pulse oximetry while on 50% oxygen showed a saturation of 85%. Due to his worsening course, he was intubated and transported to another hospital with a pediatric intensive care unit. Upon intubation, it was noted that the patient had copious secretions. After admission to the PICU, the patient developed acute respiratory distress syndromes (ARDS) and needed ventilatory management for 19 days along with additional doses of albuterol and methylprednisolone. After extubation, he was placed on a prednisone taper and discharged with no evidence of residual pulmonary dysfunction.

(*ref:* Traub *et al.*, 2002)

elsewhere in this textbook and will not be explored further here.

2. Clinical Presentation

Pediatric signs and symptoms of chlorine gas exposure include predominantly ocular, nasal, oropharyngeal, and pulmonary irritation of membranes (Burklow *et al.*, 2003). The hallmark of intoxication by these choking agents involves respiratory complaints. Minor chlorine exposures can lead to burning of the eyes and throat, indicative of mucous membrane irritation. More severely exposed patients may complain of cough, choking, sore throat, shortness of breath, chest tightness, difficulty breathing, and other respiratory-related complaints. Clinical findings may also include lacrimation, rhinorrhea, laryngeal edema, hoarseness, aphonia, stridor, expiratory wheezing, tracheitis, and cyanosis (Güloğlu *et al.*, 2002; Traub *et al.*, 2002). Tachypnea may develop as a direct result of pulmonary irritation, and tachycardia has been demonstrated in some studies. Many pediatric patients with a prior history of reactive airway disease are at increased risk of chlorine-induced bronchospasm (Burklow *et al.*, 2003).

Pulse oximetry may indicate low oxygen saturation (Traub *et al.*, 2002). While arterial blood gases usually indicate hypoxemia, carbon dioxide levels have been shown to be decreased, increased, or normal (Güloğlu *et al.*, 2002; Traub *et al.*, 2002). A hyperchloremic metabolic acidosis may show up on blood chemistries due to systemic absorption of hydrochloric acid.

Pulmonary edema, the most significant morbidity from pulmonary agents, can be seen on chest roentograms (Burklow *et al.*, 2003). Pulmonary edema may develop as early as 2 to 4 h after exposure; radiographic evidence typically appears later. Pulmonary edema may progress to the point of producing Kerley B lines on chest x-rays. These lines are often described as rungs of a ladder running perpendicular to the lateral margin of the lungs, beginning at the costophrenic angle. Chest radiographs will often show opacities of acute lung injury. Pneumomediastinum has also been reported in chlorine gas exposure (Traub *et al.*, 2002).

Pulmonary function tests (PFTs) are not helpful (Traub *et al.*, 2002; Pherwani *et al.*, 1989). A study of school children exposed to a chlorine gas leak reported a predominantly obstructive pattern on PFTs. This could be explained by congestion and edema narrowing the central airways rather than smaller airways.

3. Pediatric Vulnerability

Chlorine is a pungent green–yellow gas, twice as heavy as air (Güloğlu *et al.*, 2002) and settles near the ground (Burklow *et al.*, 2003; Traub *et al.*, 2002). This poses a problem for children, leading to increased exposure for this population in the event of accidental release or an act of terror. Children can be exposed as a result of the following activities: inhaling chlorine vapors at swimming pools (Burklow *et al.*, 2003), mixing of household bleach (sodium hypochlorite) with acidic cleaning agents (Traub *et al.*, 2002), and industrial accidents (Pherwani *et al.*, 1989). Phosgene, a dense gas heavier than air, is a more lethal pulmonary agent than chlorine. While the smell of chlorine is associated with swimming pools, phosgene odor is described as freshly mown hay (Burklow *et al.*, 2003).

Initially, both agents cause intense irritation of the mucosal membranes (Burklow *et al.*, 2003) and coughing (Güloğlu *et al.*, 2002; Traub *et al.*, 2002). This is typically followed by a feeling of suffocation (Burklow *et al.*, 2003). Morbidity from pulmonary agents is the direct result of pulmonary edema, appearing 2–4 h after chlorine exposures. Since children have a smaller fluid reserve (Rotenberg and Newmark, 2003), pulmonary edema can cause rapid dehydration or even shock (Burklow *et al.*, 2003). Due to the higher respiratory rates and minute

TABLE 61.6. Management of pulmonary agent exposures (Burklow *et al.*, 2003)

Pulmonary agents	Symptoms	Treatment
Chlorine	• Lacrimation • Rhinorrhea • Conjunctival irritation • Cough • Sore throat • Hoarseness • Laryngeal edema • Dyspnea • Stridor • ARDS • Pulmonary edema	**Decontamination:** Copious water irrigation of the skin, eyes, and mucosal membranes to prevent continued irritation and injury **Symptomatic care (no antidote):** Warm/moist air, supplemental oxygen, positive pressure O_2 for pulmonary edema **Bronchospasm:** Beta-agonists (albuterol) **Severe bronchospasm:** Corticosteroids (prednisone) (also used for PTS with H/O asthma but use unproven) **Analgesia and cough:** Nebulized lidocaine (4% topical solution) or nebulized sodium bicarbonate (use unproven)
Phosgene	• Transient irritation (eyes, nose, throat, and sinus) • Bronchospasm • Pulmonary edema • Apnea • Hypoxia	**Decontamination:** Wash away all residual liquid with copious water, remove clothing **Symptomatic care:** ABCs, hydrate, positive pressure O_2 for pulmonary edema **Bronchospasm:** Beta-agonists (albuterol), corticosteroids INH/IV, furosemide contraindicated **Hypoxia:** Oxygen

volumes of children (Rotenberg and Newmark, 2003), exposure to pulmonary agents will be greater (Burklow *et al.*, 2003). Concerning the effects on children exposed to pulmonary agents and subsequent treatment, there are many documented clinical case studies in the literature as a result of accidental exposures and industrial accidents (Güloğlu *et al.*, 2002; Traub *et al.*, 2002; Pherwani *et al.*, 1989).

4. Treatment

The first line of treatment for children exposed to pulmonary agents is decontamination. Decontamination can be as simple as removing the victim from the source to fresh air, followed by the removal of contaminated clothing (Burklow *et al.*, 2003). Supportive care includes administration of humidified air, supplemental oxygen, water irrigation, and high flow oxygen delivered via positive pressure for pulmonary edema (Burklow *et al.*, 2003; Traub *et al.*, 2002). Further treatment may include surgical debridement, supportive care with medications such as albuterol for bronchospasm, corticosteroids for inflammation, and antibiotics for any secondary bacterial infections. Antidotes or specific post-exposure treatments do not exist for this class of agents. Supportive treatment recommendations are shown in Table 61.6.

5. Summary

Chlorine and phosgene are two chemicals that can cause severe pulmonary toxicity due to pulmonary edema and direct damage to the lungs. Treatment involves decontamination and supportive care. Special care needs to be provided for exposed children because they are at higher risk for toxicity because of their unique vulnerabilities.

E. Cyanide

1. Introduction

Cyanide is used in plastic processing, electroplating of metals, metal tempering, extraction of gold and silver, fumigants, and photographic development (Rotenberg, 2003a; Baskin and Brewer, 1997). It is also found in vehicle exhaust, tobacco smoke, certain fruit pits and bitter almonds. The major cyanide containing compounds used by the military in WWI were hydrogen cyanide, cyanogen chloride, and cyanogen bromide. Cyanide is also liberated during the combustion or metabolism of nitrogen containing polymers of natural and synthetic origin (Riordan *et al.*, 2002). Cyanides can cause lethality through inhalation of cyanide vapor or ingestion (Prajapati *et al.*, 1992). Cyanide poisoning leads to death in minutes but can be effectively treated with antidotes if diagnosed early. Pediatricians, medical first responders, and firefighters need to recognize victims of cyanide poisoning in order to initiate immediate intervention (Rotenberg, 2003a; Baskin and Brewer, 1997). Cyanide is one of the few chemicals for which an effective antidote exists.

2. Mechanism of Toxicity

The cyanide ion kills aerobic organisms by shutting down oxidative phosphorylation in the mitrochondria and therefore the utilization of oxygen in cells (Baskin and Brewer, 1997; Riordan *et al.*, 2002). Cyanide has a propensity to affect certain organs (e.g. brain, heart, and lungs) more than

EXHIBIT F (Pediatric Case History) – Cyanide

Case History: Cyanide Exposure in a Child

A 2-year-old, previously healthy, 12 kg male ingested an unknown quantity of an acetonitrile-containing sculptured nail remover. The product contains an aliphatic nitrile that releases inorganic cyanide upon human metabolization. The child was brought into the emergency room because of lethargy approximately 10 h after the ingestion. Although the child was acting normally at the time of ingestion, 8 h later, he was found to be moaning, poorly responsive, and having just vomited. In the emergency room, he was pale and lethargic, responding only to deep pain. Abdomen and neck exam was normal and the lung exam revealed bilateral coarse breath sounds with a normal chest roentgenogram. Extremities were mottled and cool with a delayed capillary refill time. Vital signs showed a temperature of 36.9°C, pulse of 140 bpm, respiratory rate of 56/min, and blood pressure of 70/30 mm Hg. Arterial blood gas measurements showed a pH of 6.95, PCO_2 of 11 mm Hg, and PO_2 of 114 mm Hg. His electrolytes revealed a sodium of 137 mmol/l, potassium of 5.1 mmol/l, chloride of 114 mmol/l, bicarbonate of 4 mmol/l, serum creatinine of 70.7 µmol/l, glucose of 15.8 mmol/l, and a blood urea nitrogen of 5 mmol/l. The white blood cell count was 9.5×10^9/l and the hematocrit was 31%. Sinus tachycardia, at a rate of 160 bpm, with normal intervals and axis, was observed on the electrocardiogram. Serial whole-blood cyanide levels were obtained with the initial level being 231 µmol/l (600 µg/dl) 12 h after the exposure. The patient was given oxygen, sodium bicarbonate, and fluid resuscitation. Electrolyte and acid–base disturbances were corrected and no antidotal therapy was administered due to prompt response on supportive therapies. The patient was discharged from the hospital 3 days after admission in good condition.

(*ref:* Caravati and Litovitz, 1988)

others (Rotenberg, 2003a; Baskin and Brewer, 1997). Significant exposure can lead to central respiratory arrest and myocardial depression. Cyanide also acts as a direct neurotoxin (Rotenberg, 2003a), disrupting cell membranes and causing excitatory injury in the central nervous system (CNS) (Baskin and Brewer, 1997; Riordan *et al.*, 2002).

3. Clinical Presentation

Cyanide is an uncommon cause of childhood poisoning. In 2006, there were only 12 reported cases of cyanide exposure in the pediatric population (<19 years) (Bronstein *et al.*, 2007). Since signs of toxicity (see Exhibit F) are so similar to carbon monoxide poisoning, which accounts for the largest group of poisoning deaths among children, clinicians must have a high index of suspicion to make the diagnosis (Riordan *et al.*, 2002; Prajapati *et al.*, 1992). Rotenberg describes a typical toxidrome induced by cyanide (Rotenberg, 2003a). This includes a rapid progression from hyperpnea, anxiety, restlessness, unconsciousness, seizures, apnea, and finally death. Skin, blood, and fundi may be cherry red upon physical examination due to the inability of mitochondria to extract oxygen. In reported cases of accidental cyanide ingestion by children, other signs of toxicity included nausea, vomiting, abdominal pain, headache, lethargy, slurred speech, ataxia, stupor, coma, and respiratory depression. In addition, delayed vomiting occurred due to the slow metabolization of the chemical compound acetonitrile to cyanide, a process that can take 6 to 14 h after the ingestion (Geller *et al.*, 2006).

4. Laboratory Findings

Arterial blood gases can provide clues for cyanide exposure. Classic cases are presented with severe metabolic acidosis, elevated anion gap and high lactate concentrations (Rotenberg, 2003a). Impaired cellular respiration will lead to a high oxygen content in venous blood (Rotenberg, 2003a; Riordan *et al.*, 2002). Thus, a reduced arterial-venous oxygen saturation difference suggests this diagnosis. Blood cyanide levels are confirmatory (Rotenberg, 2003a; Baskin and Brewer, 1997; Riordan *et al.*, 2002) but will only delay the diagnosis, which must be based on the initial clinical presentation. Immediate therapeutic intervention with provision of 100% supplemental oxygen and administration of specific antidotes is paramount. An

EXHIBIT G

Mnemonic for Recognition of Cyanide Toxicity

F-A-T R-E-D C-A-T-S

Flushing of skin
Almonds (bitter almond smell)
Tachycardia

Red (Red/pink skin, bright red retinal vessels)
Excitation of nervous system
Dizziness, **D**eath, recent **D**epression history

Confusion, **C**oma, **C**onvulsions
Acidosis (metabolic or lactic), **A**nion gap
Tachypnea
Soot in nose

almond-like odor on the breath may alert a clinician that a person may have been exposed to cyanide, but up to 40% of the general population is unable to detect this odor.

5. Pediatric Vulnerability

Children are especially vulnerable to cyanide attacks (Rotenberg, 2003a). A larger exposure to cyanide vapor occurs due to the higher respiratory rates and higher surface-to-volume ratios in children. Cyanide liquid causes greater and more rapid absorption when it comes against the immature skin barrier of children. Lower body mass and immature metabolic processes can render children more susceptible than adults to toxicity from cyanide exposure. It has also been noted that children seem more susceptible to ingestion poisoning as demonstrated by various cassava and apricot pit exposures where the severity of toxicity was greater than that seen in adults who were also ingesting these products. In fact, it has been theorized that due to children's higher gastric acidity, leading to greater absorption, they experience more severe toxicity than adults when cassava is ingested (Geller *et al.*, 2006). A case report of potassium cyanide ingestion among ten children reported that the initial symptoms seen included abdominal pain, nausea, restlessness, and giddiness (Prajapati *et al.*, 1992). Cyanosis and drowsiness were also noted, but the signature cherry red skin color was not reported. Post mortem examination from the two children that died showed bright red blood and tissues with congestion. These two children consumed powder packets of potassium cyanide mixed in water, while the other eight children licked the powder, leading to less toxicity. The survivors were managed with aggressive supportive care including gastric lavage, oxygen, and intravenous fluids.

6. Treatment

The mainstay of treatment in cases of cyanide toxicity in the USA consists of supportive treatment and use of a multistage antidote kit (Rotenberg, 2003a; Baskin and Brewer, 1997; Riordan *et al.*, 2002). Table 61.7 details pediatric doses used for the medications in this kit, which contains amyl nitrite, sodium nitrite, and sodium thiosulfate. Antidotes should be provided only for significantly symptomatic patients, such as those with impaired consciousness, seizures, acidosis, hypotension, hyperkalemia, or unstable vital signs (Goldfrank *et al.*, 1998). Even when patients are rendered comatose by the inhalation of hydrogen cyanide gas, antidotes may not be necessary if the exposure is rapidly terminated, the patient has regained consciousness on arrival to the hospital, and there is no acidosis or abnormality of the vital signs (Peden *et al.*, 1986).

7. Supportive Therapy

Irrespective of the antidote treatment available, treatment will always consist of supportive therapy (Rotenberg, 2003a). Supportive therapy alone may reverse the effects of cyanide even in the face of apnea (Rotenberg, 2003a; Baskin and Brewer, 1997; Peden *et al.*, 1986). Supportive therapy includes decontamination, which includes gastric lavage and/or administration of activated charcoal if appropriate, oxygen, hydration, and anticonvulsants. Decontamination measures should take place prior to patient transport to a medical center. First responders and health care professionals should in turn take precautions not to intoxicate themselves through direct mouth-to-mouth resuscitative efforts (Riordan *et al.*, 2002). They must also wear personal protective equipment when transporting the victims to areas with adequate ventilation (Rotenberg, 2003a). Clothes are an obvious source for recontamination of the victim and must be removed. The skin should be subsequently flushed with copious volumes of water. The temperature of the water becomes a major consideration for children who may not tolerate extremes of cold or hot. Depending on the hospital size, antidote kits may or may not be available. Therefore, the time when supportive care is implemented becomes extremely important.

8. Antidotal Therapy

The US standard cyanide antidote kit uses a small inhaled dose of amyl nitrite followed by intravenous sodium nitrite and sodium thiosulfate (Rotenberg, 2003a; Anon, 1998). This antidote converts a portion of the hemoglobin's iron from ferrous iron to ferric iron, converting the hemoglobin into methemoglobin. Cyanide is more strongly drawn to methemoglobin than to the cytochrome oxidase of cells, effectively pulling the cyanide off the cells and onto the methemoglobin (Baskin and Brewer, 1997; Berlin, 1970). Once bound with the cyanide, the methemoglobin becomes cyanmethemoglobin (Anon, 1998). Therapy with nitrites is not innocuous, since methemoglobin cannot transport oxygen in the blood. The doses given to an adult can potentially cause a fatal methemoglobinemia in children or may cause profound hypotension. Treatment of children affected with cyanide intoxication must be individualized and is based upon their body weight and hemoglobin concentration. Once an ampule of amyl nitrite has been broken one at a time into a handkerchief, the contents should be held in front of the patient's mouth for 15 s, followed by 15 s of rest. This should be reapplied using this interrupted schedule until sodium nitrite can be administered. Continuous use of amyl nitrite may prevent adequate oxygenation. Taylor Pharmaceuticals, the manufacturer of the kit, recommends the dose for children of sodium nitrite to be 6 to 8 ml/m^2 (approximately 0.2 ml/kg body weight) but not to exceed an adult dose of 10 ml of a 3% solution (approximately 300 mg). While excessive sodium nitrite can cause methemoglobinemia, it should be noted that in the 70-year history of using the kit, the only reported fatality of such toxicity from using the kit involved a child without serious cyanide poisoning who was given two adult doses of sodium nitrite (Berlin, 1970; Hall and Rumack, 1986). In fact, the scientific literature recommends pediatric dosing based on monitoring hemoglobin levels. The next part of the cyanide

TABLE 61.7. Management of cyanide exposures (Anon, 1998; Berlin, 1970; Hall and Rumack, 1986)

Agent	Severity of symptoms	Antidotes/treatment			
		Multistage antidote kit (for unconscious patients)			
		Age	Amyl nitrite ampules	Sodium nitrite (for Hb = 12)	Sodium thiosulfate (for Hb = 12)
Cyanide	• Tachypnea • Restlessness • Anxiety • Flushing • Tachycardia • Dizziness • Apnea • Respiratory failure • Seizures • Coma	Child <30 kg	1. Crush 1 amp. in gauze close to the mouth and nose of breathing victim 2. Inhale for 15 s, rest for 15 s 3. Replace pearls every 30 s until sodium nitrite can be administered	1. 0.19–0.39 ml/kg not to exceed 10 ml of 3% solution slow IV over less than 5 min or slower if hypotension develops 2. For every 1 g/dl increase or decrease change in Hb, change dose by approximately 0.03 ml/kg accordingly 3. May repeat dose at 1/2 original dose in 30 min if needed	1. 0.95–1.95 ml/kg not to exceed 50 ml of 25% solution IV over 10–20 min 2. For every increase or decrease change in Hb of 1 g/dl, change sodium thiosulfate by 0.15 ml/kg accordingly 3. May repeat dose at 1/2 original dose in 30 min if needed
		Adult	See above	10 ml of 3% solution slow IV over no less than 5 min or slower if hypotension develops	50 ml of 25% solution IV over 10–20 min

Other treatment: evacuation, decontamination, 100% O_2, and correction of acidosis, hypovolemia, and seizures

antidote kit is sodium thiosulfate, which is administered intravenously (Rotenberg, 2003a; Baskin and Brewer, 1997; Anon, 1998; Hall and Rumack, 1986). The sodium thiosulfate and cyanmethemoglobin become thiocyanate, releasing hemoglobin; thiocyanate is excreted by the kidneys. Table 61.8 provides a dosing chart for the safe dosing of sodium nitrite and sodium thiosulfate with continuous monitoring of hemoglobin levels. Before treating pediatric patients with nitrites, it is imperative that prescribers inquire about conditions that may predispose a victim to anemia and, if there are concerns, doses should be decreased. Methemoglobin levels must be monitored sequentially in children and should not exceed 20% (Rotenberg, 2003a). Due to concerns about the excessive methemoglobinemia, along with the complicated administration procedures associated with the cyanide antidote kit, experts have suggested that alternative therapies may be preferable to use in children such as hydroxocobalamin (Geller *et al.*, 2006).

9. Alternative Strategies

Alternative methods of treating cyanide intoxication are used in other countries. For example, the antidote used primarily in France is hydroxocobalamin (a form of vitamin B_{12}), which combines with cyanide to form the harmless vitamin B_{12a} cyanocobalamin (Rotenberg, 2003a; Baskin and Brewer, 1997). In France, this medication is used for children at a dose of 70 mg/kg. A study of 41 French children with fire smoke inhalation showed a prehospital mortality rate of 4% for those given hydroxocobalamin and not found in cardiac arrest (Geller *et al.*, 2006). Authors of the study noted that for those children found in cardiac arrest by paramedics, administration of hydroxocobalmin

TABLE 61.8. Variation of sodium nitrite and sodium thiosulfate dose with hemoglobin concentration (Anon, 1998; Berlin, 1970; Hall and Rumack, 1986)

Hemoglobin (g/dl)	Initial IV dose sodium nitrite 3% (ml/kg) *Do not exceed 10 ml total dose*	Initial IV dose sodium thiosulfate 25% (ml/kg) *Do not exceed 50 ml total dose*
7	0.19	0.95
8	0.22	1.10
9	0.25	1.25
10	0.27	1.35
11	0.3	1.50
12	0.33	1.65
13	0.36	1.80
14	0.39	1.95

did not prevent any mortality. Another case series detailed eight pediatric patients exposed to cassava where four of the most severely affected children were given the cyanide antidote kit, while the four others were given 500 mg of hydroxocobalamin. All children improved regardless of which therapy they were given and were discharged from the hospital with no sequelae. This medication appears to have a good safety profile with adverse effects reported such as transient reddish-brown discoloration of the urine and mucous membranes. Some elevations in blood pressure and rash have also been reported (Geller *et al.*, 2006). The FDA approved hydroxocobalamin for use in the USA in December of 2006 to treat cyanide exposure victims in a product called Cyanokit®, manufactured by EMD Pharmaceuticals, Inc. The package insert for this medication provides adult dosing and a statement that the safety and effectiveness of the Cyanokit has not been established in the pediatric population. However, there is a reference to the 70 mg/kg dose which is used in Europe (Anon, 2006).

10. Summary

Cyanide is found in a wide variety of industrial processes and has been explored by Al Qaeda for use as a weapon of terror (Rotenberg, 2003a). Whether ingested or inhaled, cyanide is very lethal. Cyanide produces toxicity through impairment of mitochondrial enzymes, disrupting the electron transport chain, and preventing their utilization of oxygen. The mainstay of treatment of cyanide toxicity consists of use of a multistage antidote kit. The management of children with cyanide toxicity should include appropriate antidote dose adjustments and proper monitoring to prevent fatal methemoglobinemia. Another antidote, hydroxocobalamin, may gain favor over time as the treatment of choice for pediatric cyanide exposures, due to its preferable safety profile and its ease of administration (Geller *et al.*, 2006).

VI. DECONTAMINATION OF CHILDREN

Decontamination after a chemical terrorist attack needs to be well planned, efficient, and cognizant of the special needs of children. It is well recognized that the unique vulnerabilities of children may lead to a disproportionate number of pediatric victims after a chemical attack. Without proper planning and consideration as to how children will be decontaminated, the potential for preventable pediatric casualties is increased due to time loss and confusion. It is highly recommended for pediatricians to be involved in the development of each hospital's plans for decontamination. Over the last several years, many advances have been made in the management of the critically injured child. In fact, studies have shown that children managed in a pediatric intensive care unit (PICU) have better outcomes than children managed in an adult intensive care unit (Wheeler and Poss, 2003). Not all hospitals have the resources to have their own PICU, but they need to be able to provide the initial resuscitation and stabilization of pediatric victims of a terrorist attack. It is highly recommended that predetermined, written transfer agreements exist between emergency departments in community hospitals and centers that specialize in pediatric care. These agreements will allow the rapid transport of critically injured children to the sites that can ensure the best outcomes.

The first step in the decontamination process is the appropriate triage of patients (Burklow *et al.*, 2003). If this step is done quickly and accurately, patients will be appropriately managed and outcomes will improve. The key to triage is the ability to ration care when resources are limited. Victims are usually classified into tiered categories. Classic categories that have been used on the battlefield include minimal, delayed, immediate, and expectant. Patients in the minimal category have minor injuries that may not require medical care or can be managed with self-care. However, it should be noted that it is difficult for children to manage themselves, in addition to the fact that the category they are placed within can change more rapidly than that seen in adults. The delayed category describes patients who have injuries that will require medical intervention, but the injuries are not immediately life threatening. Logically, the immediate category describes patients who are critically injured and need medical intervention to save life or limb. Finally, the expectant category describes those patients who are so critically injured that they are not expected to survive. The expectant category poses a special challenge to civilian health care workers who are used to expending vast resources and personnel to maximize survival. In a mass casualty event, clinicians need to come to grips with the fact that the most ill may not be treated. Although the classic categories of triage are fairly well known, they are not consistently used among hospitals. Some categories are developed specific to chemical attacks. An example of this are triage categories that separate patients as ''exposed'' or ''not exposed''. At the University of Maryland Medical Center, the biochemical response triage categories differentiate between exposed and not exposed individuals. Furthermore, recognizing that not all exposed individuals will necessarily be symptomatic but may still need to be isolated, the categories differentiate between those who are asymptomatic, exposed and symptomatic, exposed and asymptomatic, and those with unrelated emergent conditions. Regardless of what categories get utilized, triage must focus on the fact that the best outcome is achieved for the greatest number of victims. To achieve this outcome, appropriate identification of the causative agent is critical. This can be a challenge because often full identification is delayed. To protect those involved in triage, full personal protective equipment is highly recommended. Work in full personal equipment can be cumbersome and uncomfortable, but when triage is done correctly, unnecessary decontamination can be avoided.

After triage, the decontamination process should begin (Wheeler and Poss, 2003). All workers who are involved in

this process must be appropriately protected with butyl rubber aprons and gloves, double layers of latex gloves, waterproof aprons, and chemical resistant jumpsuits. Personal protective equipment should also include an appropriately selected air-purifying or atmosphere-supplying respirator, depending upon how well the threat environment has been categorized. It is important to note that this equipment often needs to be changed to prevent health care worker exposure.

The set up and use of the decontamination area must be carefully thought out. Often, the area is split into different zones (Rotenberg *et al.*, 2003). At a minimum, there must be a dirty contaminated zone and a clean decontaminated zone. It is critical to emphasize that traffic must go one-way between zones. This will eliminate the possibility of a cleaned patient becoming cross-contaminated or an exposed patient entering a health care facility before being decontaminated. Security personnel must be utilized to make sure patients do not consciously or unconsciously violate the rules. A secondary triage will be needed as patients enter the clean zone to allow patients to receive antidotes or be referred for further care. Keep in mind that for severely ill patients, antidote administration may precede decontamination.

The selection of the appropriate decontamination agent is important. The gold standard for decontamination is plain water (Rotenberg *et al.*, 2003). Other agents that have been used for decontamination include carbonaceous adsorbent powder, dilute (0.5%) hypochlorite solution, water with soap, and dry decontaminants such as flour or talcum powder. For children, the use or water or water with soap is preferred. In addition to agents used to decontaminate, other cornerstones to management include exposure to fresh air when patients have been exposed to chemicals in the gaseous form, a change of clothing, and showers.

Conducting decontamination in children can be especially difficult. At every step of the process, there are special considerations that need to be addressed (Rotenberg *et al.*, 2003). Starting at triage, clinicians need to understand how chemical toxicities manifest in children; also an understanding of what normal vital signs should be for a child will be critical. Pediatric-specific triage tools often consider different vital signs such as heart rate and respiratory rate parameters and the differing ability of patients to communicate. It is important for the triage to include the examination of the child's mouth and eyes because of frequent hand-to-mouth and hand-to-eye activity. If antidote administration is needed, pediatric references should be readily available, and an understanding of pediatric doses will be needed. When there is a lack of experience with managing children, the otherwise efficient decontamination process can get bogged down. Some hospitals have decided to set up pediatric-specific areas to address the specific needs of children.

Clinicians may need to handle uncooperative or nonverbal children. This becomes especially challenging when an intravenous line needs to be started. Placing a line in a child while in full protective equipment is no small feat. Also, keep in mind that the unfamiliar presence of a clinician in full personal protective equipment can cause fear and distress in a child. Children undergoing decontamination will benefit from a guardian to guide them through and reassure them. For those children who present alone, a guardian will need to be appointed and a system for parental identification will be needed. Hospitals will need to plan for this extra resource. In fact, one Israeli hospital has employed social workers in their disaster preparation to help manage patient/family needs and psychological distress (Rosenbaum, 1993). It is recommended not to separate parents and children during a time of crisis. Plans should be made for the decontamination and treatment of parent–child pairs (Rotenberg *et al.*, 2003).

A range of specially sized supplies is needed to appropriately manage children, which range from pediatric-sized emergency equipment to basic needs such as formula for feeding and diapers for hygiene. Since decontamination often includes disrobing, pediatric-sized clothing would be needed. For children who may need to be observed for hours, toys will be needed.

Also, the agents used to decontaminate children should be carefully selected. Bleach or hypochlorite solutions are not recommended for use with children due to the possibility of skin irritation or damage (Rotenberg *et al.*, 2003). Water is the gold standard for decontamination. When employing water decontamination, the temperature of the water must be addressed. Children, especially newborns and infants, are prone to hypothermia and hemodynamic instability from cold water. Water at a comfortable temperature is recommended along with a good supply of blankets which can be used to quickly warm up pediatric patients after water decontamination. In some situations, indoor sprinkler systems have been used when outdoor conditions were inhospitable.

VII. PREPARATION FOR A CHEMICAL EVENT

Understanding chemical agents used for terrorism and knowing how to manage toxicity is just the first step in preparing for a chemical event. Appropriate training on how to manage pediatric patients in these scenarios is critical. Pediatricians are uniquely trained to participate in the management of pediatric casualties and to advocate for children so that their needs are addressed in emergency planning (Bradley *et al.*, 2003). Many hospitals have held emergency exercises to see how prepared they are for these situations. Beyond this, the assessments should identify deficits and should be used to forge partnerships and relationships and share assets in the community to manage every possible scenario (Blaschke *et al.*, 2003). Health care facilities responsible for treating pediatric victims in

TABLE 61.9. Example pediatric specific hospital emergency drug cache

Drug	Strength	Dosage form	Pediatric dosing	Therapy or prophylaxis	Disease
Albuterol MDI	17 gm	INH	2–4 puffs q4h	Respiratory distress from chemical agents	Chemical exposure
Amoxicillin oral suspension	400 mg/5 ml 100 ml	Oral suspension	15 mg/kg q8h – up to 40 kg, >40 kg 500 mg q8h	Chemoprophylaxis	Anthrax
Atropine	1 mg/ml	Injection	See dosing table	Chemotherapy	Nerve agent exposure
Ciprofloxacin oral suspension	250 mg/5 ml 100 ml	Oral suspension	20–30 mg/kg/day divided q12h for 60 days	Chemoprophylaxis	Anthrax, plaque
Clindamycin	600 mg/NS 50 ml	IVPB	30 mg/kg/day q8h (max 4.8 g/day)	Chemotherapy	Anthrax
Cyanide antidote package	1 kit	Kit	See dosing table	Chemotherapy	Cyanide poisoning
Diazepam IV	5 mg/ml ×2 ml	Injection	See dosing table	Seizures post chemical exposure	SZ post-chemical exposure
Doxycycline oral suspension	25 mg/5 ml 60 ml	Oral suspension	2.5 mg/kg q12h – up to 40 kg, >40 kg 100 mg q12h for 60 days	Chemoprophylaxis	Anthrax, cholera, brucellosis, plague
Oseltamivir suspension	12 mg/ml 25 ml	Suspension	For children ≥1–12 yrs: ≤15 kg: 2 mg/kg/dose (max 30 mg) BID × 5 days, >15–23 kg: 45 mg/dose BID × 5 days, >23–40 kg: 60 mg/dose BID × 5 days, >40 kg 75 mg/dose BID × 5 days	Chemotherapy	Avian influenza
Potassium iodide	65 mg	Tablet	4–18 yrs: 65 mg, 1 mo–3 yrs: 32.5 mg, <1 mo: 16.25 mg	Chemotherapy	Radiation emergency
Pralidoxime	1 gm/20 ml SDV	Powder for injection	See dosing table	Chemotherapy	Nerve agent exposure
Ribavirin solution	40 mg/ml 100 ml	Solution	LD 30 mg/kg followed by 15 mg/kg/day BID × 10 days	Chemotherapy	Viral hemorrhagic fever

a chemical–biological event could be easily strained and overwhelmed. Often large-scale chemical–biological incidents necessitate the use of alternative areas to triage patients such as auditoriums and arenas. These alternative triage areas need to know how to manage pediatric victims (CEH/CID, 2000). Planning for an attack begins with the development of local health resources. Unfortunately, with chemical releases, clinical effects can occur extremely quickly, limiting time to borrow resources from nearby communities. First responders must be educated to recognize pediatric signs and symptoms from each chemical agent, how to wear protective gear in the face of persistent agents, handle pediatric patients, and be able to manage field decontamination. It is critical that adequate supplies of protective gear are available. When planning for decontamination procedures, pediatric vulnerabilities and challenges need to be considered such as the temperature of the water and the ability of children to follow directions.

Since children spend the majority of the day at school, community preparation for a threat must include the local educational system. Development of a rapid evacuation plan and the establishment of in-school shelters are critical. Schools can play a valuable role for the management of pediatric casualties.

Another key element to appropriate preparedness is the development of a pharmaceutical cache of antidotes, antibiotics, and vaccines. This cache will play a key role in the initial management of a chemical attack. Even though the SNS is now in place throughout the USA, it may be several hours before it reaches a hospital and the supply is divided among several sites. The SNS has made efforts to include pediatric-ready medications such as suspensions and solutions. Efforts must be made for local pharmaceutical caches to address pediatric needs. An example of a pediatric pharmaceutical cache is displayed in Table 61.9.

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

Much progress has been made in understanding how pediatric patients need to be managed when they are affected by chemical agents. Several pediatric organizations, such as the AAP, have given guidance on how best to handle these situations. It is a special challenge to gather information regarding pediatric chemical casualties because our experience is so limited. Further research and resources are needed to fully understand all the physical and psychological impacts a terror attack has on children. The intention of this work is to provide a framework from which local and national efforts can grow. In the event of a chemical attack, prior preparation and planning will make the difference to whether lives are saved or further lives are lost. Efforts to improve upon current recommendations for managing pediatric chemical casualties must continue in order to better protect this vulnerable population.

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CHAPTER 62

Physiologically Based Pharmacokinetic/ Pharmacodynamic Modeling of Countermeasures to Nerve Agents

ELAINE MERRILL, CHRIS RUARK, JEFF GEARHART, AND PETER ROBINSON

I. INTRODUCTION

Physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) modeling has proven useful in many areas of toxicology and therapeutics. This quantitative, mechanism-based approach has allowed limited experimental *in vivo* and *in vitro* data to be quantitatively integrated with physiological data so as to facilitate predictions of the behavior of organisms under different exposure conditions. Specifically, it has been used for:

- extrapolation across species (particularly experimental animals to humans)
- extrapolation across dosing scenarios (such as high to low doses)
- extrapolation across routes of exposure.

Models have been developed for a number of chemical warfare (CW) agents (Sweeney *et al.*, 2006; Worek *et al.*, 2005; Aurbek *et al.*, 2006; Van der Merwe *et al.*, 2006; see also Chapter 46 by Gearhart in this book) and surrogates, including diisopropyl fluorophosphate (DFP) (Gearhart et al., 1990, 1994), diazinon (Poet *et al.*, 2004), and chlorpyrifos (Timchalk *et al.*, 2002a, b). In the case of agent countermeasures, PBPK/PD modeling also allows the prediction of therapeutic efficacy under varying exposure scenarios. Specifically, it will allow therapeutic regimens to be optimized to particular exposure scenarios (routes, exposure times, concentrations). In order to do so, models of individual agents and countermeasures must be combined to take into account their interactions, whether they be pharmacokinetic or pharmacodynamic. Such modeling of agent/(multiple) counteragent combinations is an example of mixtures modeling (Krishnan *et al.*, 1994).

There is little yet in the literature approaching a complete model of the kinetic and dynamic interaction of a nerve agent and its current (multi-agent) or potential countermeasures. There have been, however, a number of important steps in that direction, both on the level of model development and in the experimental generation of data useful for the development and validation of such models.

We restrict our discussion here to countermeasures of nerve agents (NA), and will not discuss other chemical agents such as mustards and cyanide, and their antidotes. However, the modeling ideas outlined here can also apply to other agents.

II. BACKGROUND

Organophosphate compounds (OPs), such as the NAs – sarin (GB), soman (GD), tabun (GA), and VX – exert their toxicity by rapidly inhibiting serine esterases, the most critical of which is the ubitiquous nervous system enzyme, acetylcholinesterase (AChE). The inhibition of AChE occurs via phosphorylation of the serine hydroxyl group in the enzyme's active site. Many NAs ''age'' the enzyme, in which an R (alkyl) group attached to the oxygen is removed, leaving a phosphonate or phosphate anion irreversibly bound to AChE (Talbot *et al.*, 1988). The aging rate varies with the NA and is an important therapeutic consideration. AChE is involved in the rapid hydrolysis of the neurotransmitter acetylcholine (ACh). Acetylcholine is involved in the numerous cholinergic pathways in the body, i.e. the central nervous system as well as several aspects of the peripheral nervous system: the somatic nervous system innervating skeletal muscles and both the sympathetic and the parasympathetic divisions of the autonomic nervous system. Inhibition of AChE leads to the accumulation of ACh in the synapses and neuromuscular junctions (NMJs) of these cholinergic pathways (Figure 62.1), resulting in hyperstimulation of muscarinic and nicotinic cholinergic receptors. Clinical symptoms include salivation, lacrimation, urination, and defecation arising from impacts on the autonomic nervous system, tremors arising from the impact on the somatic nervous system, potentially convulsions from impacts on the central nervous system, and death (Myhrer *et al.*, 2007). At moderate to high doses, seizures

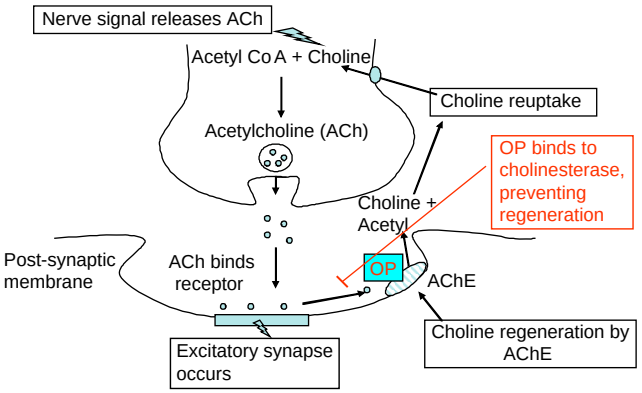

FIGURE 62.1. OPs bind to and inhibit acetylcholinesterase (AChE), the enzyme responsible for degrading the neurotransmitter, acetycholine (ACh), into choline and acetate. When AChE is inhibited by an organophosphate nerve agent, the subsequent reduced hydrolysis of ACh results in an accumulation of ACh within the synaptic cleft and overstimulation of the post-synaptic nerve. Seizures and possibly paralysis and/or death may occur.

rapidly progress to status epilepticus (SE), which can lead to irreversible brain damage (Carpentier *et al.*, 2001; Filliat *et al.*, 1999). Survivors suffer long-lasting cognitive deficits and behavioral changes (McDonough and Shih, 1997) due largely to neuronal degeneration of hippocampal structures. Under acute high-level exposures, the focus of countermeasure treatment is the prevention of seizures, respiratory failure, and bradycardia due to convulsive spasms. For low-level exposures, and for the survivors of high exposures, the concern shifts to minimizing the cognitive impact of the agent which may or may not be related to the degree of seizure activity.

McDonough and Shih (1997) hypothesized that the neurotransmitter systems involved in initiating and maintaining NA-induced seizures follow three main phases. Initially, about 5 min after exposure, hypercholinergic activity triggers the seizure. A transitional phase of cholinergic and glutamatergic hyperactivity follows, as a self-sustaining glutamate–NMDAR-mediated positive feedback loop begins to develop. After approximately 40 min, a predominantly glutamatergic phase sustains seizures, leading to neuronal damage predominantly in the hippocampus, amygdala, piriform cortex, thalamus, and entorhinal cortex across species (Olney *et al.*, 1983; Wade *et al.*, 1987; Fosbraey *et al.*, 1990; Carpentier *et al.*, 1990; Lallement *et al.*, 1992; McDonough and Shih, 1997; Solberg and Belkin, 1997).

High levels of glutamate itself are directly neurotoxic because overstimulation of glutamate ionotropic receptors allows excessive influxes of Na^+ and Ca^{2+}, causing prolonged depolarization of postsynaptic membranes (Bittigau and Ikonomidou, 1997). A cascade of events results from the delayed Ca^{2+} overload, leading to excitotoxic cell necrosis (McDonough and Shih, 1997; Solberg and Belkin, 1997). In addition, as the time lapse before anticonvulsant treatments increases, the Ca^{2+} influx induces transcription factors which mediate significant increases in damaging pro-inflammatory peptides, such as IL-1β, IL-6, and mRNA of TNF-α (Chapman *et al.*, 2006).

The content of this chapter is not inclusive, in the sense of taking one through the complete development and validation of a complex PBPK/PD model, which would describe the kinetics of each component, together with the pharmacodynamic interactions between selected NAs and countermeasures. Rather, this chapter briefly describes some examples of progress made in quantitative modeling and explores how specific countermeasures interfere in this NA-induced cascade of events, and how such quantitative approaches could be used to develop improved treatment regimens.

III. CURRENT COUNTERMEASURES

Conventionally, NA poisoning is treated by a combination of prophylactic and post- exposure therapy, which target the three post-exposure phases of neurotransmitter systems described above. Prophylactic treatments are designed to circumvent aging of the NA–AChE complex and consist of carbamate anticholinesterases, e.g. pyridostigmine, to bind AChE reversibly. Current carbamate pretreatment regimes bind 30–40% of available red blood cell AChE, thereby protecting some of the enzyme from irreversible OP binding (McDonough and Shih, 1997). Carbamates, however, are not without side effects. Partial AChE blockage by pyridostigmine results in a transient overstimulation of the AChR, mimicking mild NA poisoning with nausea, diarrhea, shortness of breath, and dizziness (Abraham *et al.*, 2002). Moreover, repeated prophylactic administration of carbamates is associated with detrimental and debilitating changes in nerve and muscles function (Hudson *et al.*, 1986).

Standard post-exposure treatments include concurrent administration of anticholinergics, such as the muscarinic cholinergic blocker atropine sulfate, and AChE reactivators, such as obidoxime and pyridine-2-aldoxime methochloride (also known as 2-PAM). Oximes cannot reactivate OP-inhibited AChE that has already ‘‘aged’’. Therefore, traditional oxime treatment is considered to be less effective for those agents such as soman, for which aging is rapid (Worek *et al.*, 2005).

If seizures develop, treatments which attempt to attenuate the hyperglutamatergic phase are required. Anticonvulsants, such as benzodiazepine or diazepam, may be used. Diazepam is a lipophilic agonist of the γ-amino butyric acid A ($GABA_A$) receptor, the most important inhibitory receptor of the CNS. The $GABA_A$ receptor is ubiquitously distributed. Therefore, its activation or inactivation affects virtually all brain regions, making dosage critical. Treatments with benzodiazepines depend on

administration within a very narrow time span to be effective because profound neuropathological changes are usually detected within 20 min of the onset of seizures (Lallement *et al.*, 1994; McDonough *et al.*, 1995; Leadbeater *et al.*, 1985; Shih *et al.*, 1999). By themselves, benzodiazepine anticonvulsants provide limited protection, as seizures recur with desensitization of $GABA_A$ receptors (Goodkin *et al.*, 2005). In addition, diazepam has consistently failed to eliminate the neuropathological changes resulting from NA-induced seizures (McDonough and Shih, 1997). Furthermore, in many battlefield and civilian exposure scenarios for NAs, pretreatments are unrealistic, and post-exposure anticonvulsants are likely to be delayed. Epidemiological studies have shown that time to seizure treatment in the USA varies broadly, with only about 41% of all patients receiving their first anti-epileptic drug within 30 min (Pellock *et al.*, 2004).

IV. NOVEL COUNTERMEASURES

Due to the undesirable side effects of the standard treatment regimes, and limitations of $GABA_A$ agonists to adequately control seizures, the need for alternative therapeutics still exists. The focus of OP intoxication treatment is also expanding from immediate survival to include the prevention of long-term cognitive deficits. Other antidotes have been proposed that demonstrate promise in reducing excessive glutamatergic activity and/or prevent destabilization of Ca^{2+} homeostasis, as excitotoxic mechanisms of seizure activity ultimately result in Ca^{2+} overload and activate various enzymes that lead to necrosis of the cell. Attempts have been made by blocking the *N*-methyl-D-aspartate (NMDA) or alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, two subtypes of CNS glutamate receptors (Braitman, 1989; Raveh *et al.*, 2003; Lallement *et al.*, 1994; McDonough and Shih, 1997). NMDA-type glutamate receptors are glutamate-gated cation channels whose permeability to Ca^{2+} underlies aspects of normal synaptic plasticity. Excess Ca^{2+} influx through NMDA receptors (NMDAR) mediates cell death in certain neurodegenerative pathologies. Therefore, neurons must precisely control NMDAR synaptic density, which is negatively regulated by global neuronal activity.

A number of glutamate receptor antagonists have demonstrated neuroprotective and anticonvulsive effects in animals (Urbanska *et al.*, 1998). The high-affinity NMDAR antagonist, ketamine, has shown promise, when administered intramuscularly with atropine sulfate up to 30 min post-poisoning (Dorandeu *et al.*, 2005). Other noncompetitive NMDA antagonists, like dizocilpine (MK-801) and *N*-(1-[2-thienyl]cyclohexyl)3,4-piperidine (TCP), display the ability to terminate seizures when treatment is delayed by 40 min, but seriously depress respiration (Carpentier *et al.*, 1990; McDonough and Shih, 1997; Shih, 1990; Shih *et al.*, 1991).

An unusual NMDAR antagonist that recently demonstrated potent neuroprotection against GD-induced seizure-related brain damage in rats is dexanabinol. Dexanabinol is a nonpsychoactive synthetic cannabinoid, which acts as a highly selective, low-affinity NMDAR antagonist (Eshhar *et al.*, 1993; Feigenbaum *et al.*, 1989). In GD-exposed rats (1.6 LD_{50}, s.c.) dexanabinol, administered interperitoneally (i.p.), at either 5 or 40 min after the onset of seizures, reduced median brain lesion volume 86% and 81%, respectively (as assessed by microtubule-associated protein 2 (MAP2)-negative staining (Filbert *et al.*, 1999). When administered 5 min after seizure onset and repeated every 6 h up to 24 h, 98% reduction was seen. However, it is not known if the same degree of protection would be afforded if measurements were taken later than 24 h post-seizure onset. Dexanabinol did not diminish seizure activity in any of the GD treated rats, suggesting that its neuroprotective effects may be due to its properties as a potent antioxidant and free radical scavenger, as well as its ability to inhibit the production of tumor necrosis factor-alpha (TNF-α) (Shohami *et al.*, 1997), thus also blocking the signal transduction pathway that activates nuclear factor B (NFB).

Impressive data to date also indicate that enzyme-based bioscavengers show tremendous promise for the next generation of pretreatments or antidotes, with potentially no observable side effects. Candidate bioscavenger proteins either bind and sequester toxic OPs (such as serum cholinesterases and carboxylesterases) or catalytically break down the OP into nontoxic products [such as human organophosphorus acid anhydride hydrolases (OPAH) or paraoxonase (HuPON)]. These scavengers as well as carboxylesterase are each capable of providing protection against 2 to 16 LD_{50}s of GD, GB, or VX depending on the scavenger and the test species (rat, mouse, rabbit, guinea pig, or rhesus monkey) with little to no deficits in behavioral testing (Li *et al.*, 2005). Human clinical trials of several bioscavengers are now under way that could lead to FDA licensure.

Human serum paraoxonase (HuPON1) is a Ca^{2+}-dependent enzyme that effectively hydrolyzes many toxic AChE-inhibiting organophosphates (Blum *et al.*, 2008). HuPON1 is present in high-density lipoproteins of human plasma and plays an important role in protecting lipoproteins and cell membranes from oxidative damage. Because it is a human enzyme (Li *et al.*, 2005), it could potentially be used as either a prophylaxis or medical post-treatment for NA exposure, without causing adverse immune reactions (Draganov and LaDu, 2004). Human PON1 has catalytic activity against the pesticide paraoxon and the NAs sarin (GB), soman (GD), and cyclosarin (GF) but shows only minimal activity against the nerve agent VX (Blum *et al.*, 2008). The advantage of catalytic scavengers is the very small amount of the exogenous enzyme needed to hydrolyze and detoxify large amounts of NA.

V. PBPK/PD MODELING

A. Purpose of Mechanism-Based Modeling

For obvious ethical reasons only animal experiments can be used to evaluate new NA antidotes. However, the extrapolation of data from animals to humans is hampered by marked species differences. Currently, quantitative analyses addressing the co-treatment of countermeasures and medicinal treatments, simultaneous with, or immediately preceding or following NA exposures, have been very limited. The current paradigm thus relies on experimentation in various animal models to determine efficacy and then, following the classical model for pharmaceutics, scales the animal results to the human exposure scenario. In the case of NA countermeasures, short of controlled human experimental studies, this leaves a large uncertainty factor as to the protection of military personnel in the face of NA threats, and may severely limit or compromise operational risk management (ORM). One solution to this dilemma is to develop validated mathematical models to predict the biological impact of simultaneous and sequential low-level exposures to specific nerve agents, together with specific countermeasures, countermeasure combinations, and dosing regimens.

Physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) models can be used to explore the interactions between agents, countermeasures, and the organism with a view to integrate multiple animal and human datasets, and to develop tools for countermeasure design and dosing regimen selection to maximize countermeasure administration efficacy. The result of this approach is the development of an interaction model quantifying competition for AChE binding sites between specific agents and countermeasures, as well as other relevant pharmacodynamic and pharmacokinetic modes of interaction; and the development of methods to optimize countermeasure therapies for specific nerve agent exposure scenarios.

The process of applying mathematical constructs to describe experimental results often reveals patterns in the agent's pharmacokinetics or dynamics that might not otherwise be discernible. Failure of a model's simulations to predict experimental measurements sometimes prompts questioning of the data, such as the reliability of the quantitative methods, or sample collection or exposure techniques. More often, it may indicate that greater complexity in the model's structure is required to capture the data's behavior. This is another primary reason for developing models: to create hypotheses (model structures) that are falsifiable, leading to improved models and improved predictions in an iterative process.

There are currently a number of critical toxicological data gaps related to NA exposure, at both acute high levels and repeated low levels. Many of these gaps are due to the impossibility of conducting controlled human experiments involving nerve agent exposure. Filling these data gaps for exposure to nerve agents is essential for predicting performance degradation of personnel, enhancing risk assessment modeling tools, and defining detection thresholds which are physiologically relevant. The purpose of filling such data gaps is to: (1) develop a valid method for predicting dose–response effects for exposures to low NA agent concentrations/doses over long durations, and (2) identify threshold exposure conditions at which toxicologically significant effects occur. PBPK/PD modeling provides a means to fill these gaps. Due to the physiologically based nature of these models, simulations of experimental data can be performed by one exposure route in a particular species to develop and validate the PBPK model, and then that model can be used to simulate and predict the kinetics and pharmacodynamics in another species such as the human exposed via another exposure route. The power of PBPK/PD models thus lies in aiding the ability of scientists and decision makers to make reliable quantitative predictions concerning the potential health effects of real-life human exposures. In addition, this modeling approach can be useful for the development of meaningful therapeutic animal models. After all, therapeutics that have been successfully tested in animals can ultimately be tested in human clinical trials. However, their therapeutic efficacy against nerve agents can only be tested in animals.

VI. DEVELOPMENT OF PBPK/PD MODELS

Critical components to PBPK models include species-specific physiological parameters, and chemical-specific parameters. Species-specific physiological parameters include the organ weights and blood flow rates for the defined compartments in the PBPK model. These values are most often available in the published literature (Altman and Katz, 1979; Peeters *et al.*, 1980; Brown, 1997) and, when lacking, can be derived by appropriate scaling factors from similar species. The basic structure of the PBPK model used to describe nerve agent pharmacokinetics and pharmacodynamics follows that of the PBPK/PD model for DFP (Gearhart *et al.*, 1990) (see Figure 62.2).

Figure 62.3 shows a schematic representation of the pharmacodynamic processes involved in the interactions between nerve agents such as sarin with the organism (via AChE inhibition), together with interactions with pretreatments [pyridostigmine bromide (PB)] and countermeasures (oxime, atropine, and diazepam). This PD submodel is linked to the PBPK via the simulated concentration of sarin, in the selected target tissue (i.e. RBCs, diaphragm, and brain). It provides a framework for describing these biochemical processes quantitatively. And, when linked with the PBPK model, it puts the biochemical processes into a physiological context. This will allow extrapolation of results to multiple species, and to describe relevant exposure

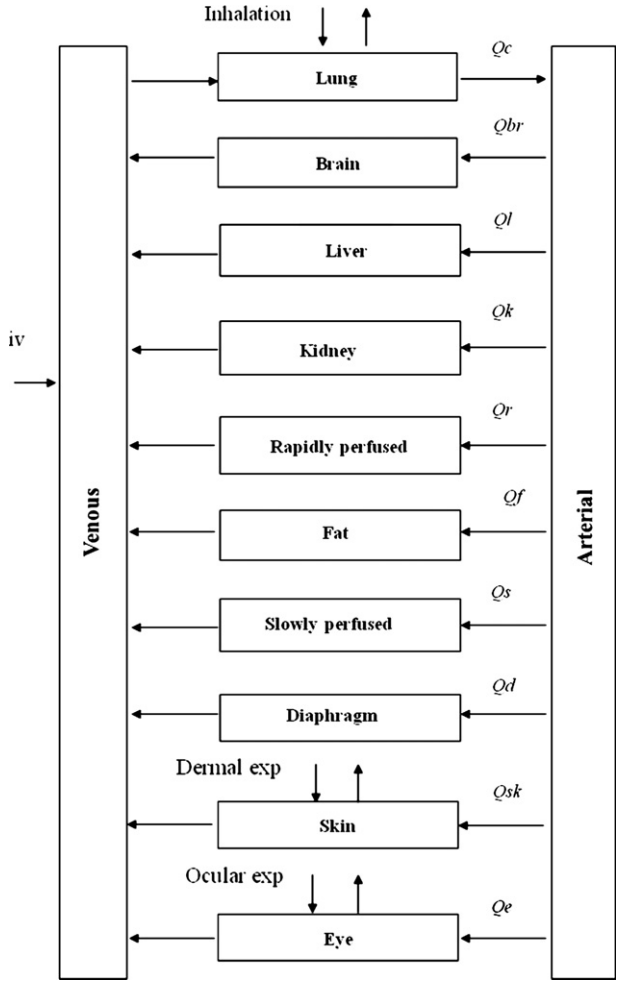

FIGURE 62.2. PBPK model schematic of the distribution of an organophosphate (be it DFP or a NA) in Hartley guinea pig. This model structure allows for the simulation of experimental studies with dosing by intravenous or subcutaneous dosing, and inhalation exposure. This model design was after Gearhart *et al.* (1990) and was also adapted to simulate the pharmacokinetics in the Göttingen minipig.

scenarios so as to address potential health effects and to optimize pretreatment and countermeasure delivery.

VII. EXPERIMENTAL AND QUANTITATIVE STRUCTURE–ACTIVITY RELATIONSHIP (QSAR) METHODOLOGIES TO PREDICT BLOOD AND TISSUE PARTITION COEFFICIENTS

As shown in Figure 62.2, PBPK modeling utilizes mathematical compartments that conceptually represent the physiology of an organism to depict the pharmacokinetics of chemicals exposed to the body. Traditional PBPK models depend on knowing the partitioning of the agent in key tissues of the organism. The distribution of a chemical within a tissue/compartment is most commonly represented by the equation (Krishnan and Andersen, 2001):

$$\frac{dA_t}{dt} = PA(CV_t - \frac{C_t}{P_t}) \tag{62.1}$$

where $A =$ amount of chemical in the tssue, $PA =$ permeation area cross product for the tissue, $CV_t =$ concentration in venous blood emerging from the tissue, $C_t =$ concentration in tissue, and $P_t =$ tissue blood partition coefficient. Note that $A = C_t \times V_t$, where V_t is the volume of the tissue.

PBPK modeling relies heavily on a proper definition of the partition coefficient as this parameter drives chemical uptake into the tissue. It is generally identified for PBPK modeling as the concentration ratio of the free fraction in the tissue to the free fraction in the blood at equilibrium (Schmitt, 2008) as shown in Eq. (62.2).

$$P_t = \frac{C_{Tissue-free}}{C_{Venous-free}} \tag{62.2}$$

The vial equilibration method is the most common *in vitro* method for determining partition coefficients for volatile or semi-volatile materials and has been used most successfully for volatile organic solvents (Gargas *et al.*, 1988). Tissues are harvested from the species of interest, and incubated with the test compound until equilibrium is reached between the tissue and the headspace in the vial. The blood–air or tissue–air partition coefficients are given by the ratio of the concentrations of the chemical in the blood or tissue relative to its concentration in the headspace. Tissue–blood partition coefficients are calculated from the respective tissue–air and blood–air values. A number of operational equations have been derived to calculate these ratios under specific experimental conditions. Time to steady state is critical and should be optimized for the test compound. Metabolism of the compound in exposed tissue samples must be controlled for. Analysis is done by gas chromatography in a verified linear range. Human tissues can be obtained from tissue bank organizations to provide species specificity to models developed with human data. To estimate partitions for compounds of low or nonvolatility, the *in vitro* filtration method of Jepson *et al.* (1994) can be used. Limitations of all such *in vitro* methods include issues of how representative the tissue sample is of the often quite heterogeneous tissue in the animal, and how closely the *in vitro* tissue preparation approximates the *in vivo* state. In addition, for highly lipophilic compounds, issues related to adsorption of the material onto glass and other surfaces need to be addressed.

Identifying the partition coefficient of a chemical for PBPK modeling is difficult due to the fact that most experimental methods fail to address this proper definition. *In vivo* methods most commonly track the kinetics of the chemical (radiolabeled or not). However, if enzymes are not properly inhibited, metabolites can also be tracked instead of the

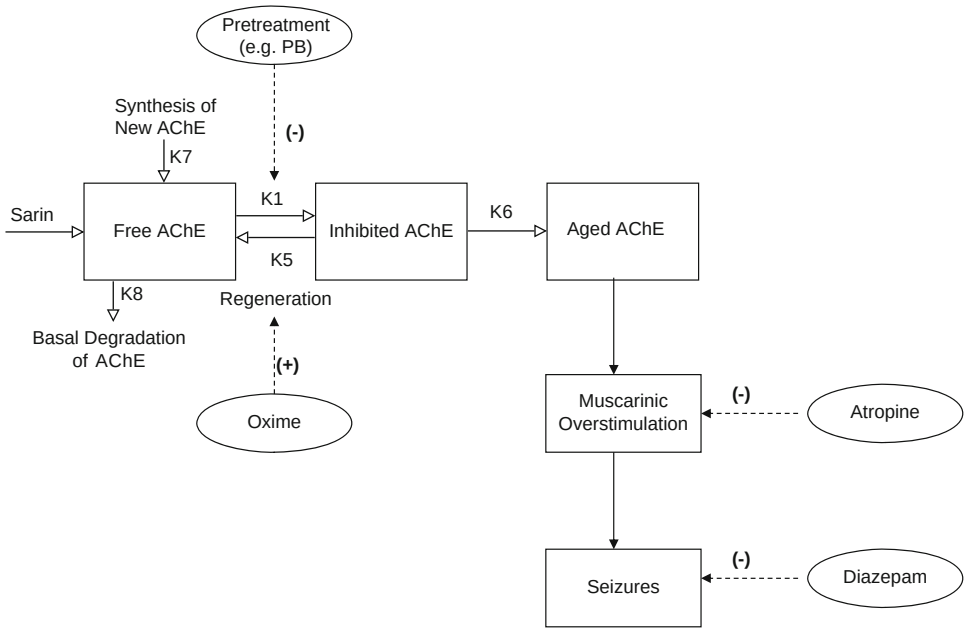

FIGURE 62.3. Schematic for AChE inhibition by nerve agents such as sarin, together with the interaction of pretreatment [pyridostigmine bromide (PB)] and countermeasure treatment (oxime, atropine, and diazepam). Pyridostigmine acts as a reversible cholinesterase inhibitor, oxime acts as an AChE regenerator, atropine acts as a muscarinic receptor antagonist, and diazepam acts as a positive allosteric modulator of GABA.

parent compound leading to contamination of the measured partition coefficient by the more water soluble metabolite. Experiments are most commonly performed via a single dose at different routes of exposure, hardly ever leading to steady state. To reach steady state, a critical point at which measurements should be made, a constant i.v. infusion of a chemical over an extended period of time until the concentration in the blood and tissue becomes constant must be made. Finally, protein binding is most commonly not accounted for, leading to a total concentration measured instead of the available free fraction. Compounds bound to proteins such as albumin, globulin, and lipoproteins reduce a chemical's tendency to partition into or out of tissues. This can be an extremely important issue depending upon the chemical as some are upwards of 90–95% bound in the blood (Poulin and Krishnan, 1996a). These *in vivo* experimental issues are rarely addressed, which leads to utilization of *in vitro* measurements where there is greater control of the system.

Since experimental determinations of partition coefficients are slow and expensive, and are fraught with difficulties, particularly for NAs, an alternative is to predict tissue partitioning from more readily available chemical properties (particularly chemical structure), together with key biological properties of the tissue itself, such as its lipid composition, by means of quantitative structure–activity relationships (QSAR) (Sterner *et al.*, 2008; Ruark *et al.*, 2008). The pharmaceutical industry has utilized QSAR to obtain information on new drug candidates rapidly to help alleviate bottlenecks in the discovery process. A drawback to these models is that they have limited predictive power for scaling across species and to other compounds. However, by utilizing novel mechanistically based methods specifically suited to PBPK modeling, estimations of chemical warfare nerve agent partition coefficient properties can be developed.

Tissue distribution is predicted utilizing a variety of descriptors that range from chemical specific to tissue specific allowing for universal application of the methods across all species, tissues, and chemicals. A compound's structure and its physiochemical properties such as the log of the octanol:water distribution (logP) and the octanol: water distribution mixture of different ionic forms (logD) are commonly utilized in partition coefficient QSAR, or quantitative structure–property relationship (QSPR) models because they describe the relative affinity for a chemical between hydrophilic and hydrophobic phases. Octanol, being amphiphilic, represents the phospholipid bi-layer which provides a barrier for the cell, separating the extracellular from its intracellular components. However, many studies have shown that octanol is not a perfect surrogate for all lipids within organisms and many are beginning to pursue other surrogates such as vegetable oil and specific phospholipid membranes (Schmitt, 2008; Poulin *et al.*, 1999). Other relevant properties include a wide range of descriptors predicted from *ab initio* calculations such as H-bonding acidity and basicity parameters, polarity, polarizability, molar refraction, McGowan volume, energies of the lowest unoccupied molecular orbital (E_{lumo}) and the highest occupied molecular orbit (E_{homo}), the maximum positive and negative atomic charge ($\sum Q$), the solvation of free energy (ΔG), as well as many others (Zhang and Zhang, 2006).

As far as tissue characterization is concerned, the fraction of lipid and water tends to be the dominating factor that influences partitioning into or out of the tissue. Some QSPR studies have included proteins which account for an electrostatic interaction with the amino or carboxyl terminal ends of an ionized chemical at physiological pH as well as some nonspecific binding for neutral compounds. However, a recent QSPR model developed by Schmitt (2008)

identified that nonspecific binding to proteins is approximately 40-fold weaker than to lipids. Therefore, proteins may only become of importance for tissues such as muscle and lung that contain a greater fraction of proteins than that of lipids. There have also been attempts at differentiating between phospholipids and neutral lipids, most notably by Poulin and Krishnan (1995a, b, 1996a, b, c), Poulin and Theil (2000, 2002), and Poulin *et al.* (2001) in the early 1990s. Rodgers and Rowland (2006, 2007) later differentiated between acidic and neutral phospholipids to account for another electrostatic interaction with ionized chemicals within the tissue. There is also a need to distinguish between tissue and interstitial space, as the interstitial space is highly representative of blood plasma. Other tissue components that may be of interest include DNA, RNA, and intracellular organelles. Binding to DNA appears to be highly correlated with β-blockers; however, the fraction of DNA in tissue is too low to make a substantial difference in the current predictions (Rodgers *et al.*, 2005a, b). Some authors have also suggested differences in intracellular organelle pH as another means of accounting for differences in tissue partitioning for ionized chemicals (Daniel and Wojcikowski, 1999; Ishizaki *et al.*, 1996; Siebert *et al.*, 2004; MacIntyre and Cutler, 1988); however, the overall volume of these organelles in relation to the cytoplasm is very low, most likely not resulting in substantial differences.

Optimization of predictions can be made utilizing linear as well as nonlinear relationships by means of statistical methods to correlate chemical and physiological descriptors to experimental datasets. These statistical methods include multilinear partial least square analysis, principal component analysis, and neural networking. Many of these tools are included in QSPR/QSAR packages through companies such as Advanced Chemistry Development, SemiChem, EduSoft, BioByte, TOPKAT, MDL, ChemSilico, Pallas, Pharma Algorithms, and others.

A review of the literature will reveal that several QSPR equations have been developed to predict PCs of drugs for use in PBPK models and many are suitable for a wide variety of chemicals. It appears that the greatest deviations in predicted versus experimental measurements of PCs are largely due to an experimental uncertainty or misinterpretations of the data rather than incorrectness of the models. Strategies still need to be developed for other macromolecular binding (α'-acid glycoprotein) and processes such as blood–brain barrier permeation and active transport but it appears that the methods thus developed are suitable for preliminary PBPK modeling.

VIII. INTERACTION PBPK/PD MODEL FOR NAs AND COUNTERMEASURES

As stated earlier, a number of PBPK/PD models have been developed for individual nerve agents (sarin, VX, soman, cyclosarin) in multiple species. Chapter 51 by Gearhart discusses the development of such models. Standalone PBPK or compartmental models have also been developed that describe the pharmacokinetics of certain countermeasures, such as diazepam (Igari *et al.*, 1983) and oximes (Stemler *et al.*, 1990). However, to date, few models for specific countermeasures (i.e. various oximes, pyridostigmine) have been harmonized and linked to NA PBPK/PD models, so as to be able to quantitatively describe their pharmacokinetic and pharmacodynamic interactions. This is probably due to the fact that most PBPK/PD models developed for NAs and other OPs described the inhibition of ChEs as the critical endpoint. The lack of a mathematical description of the disruption of other complex biochemical pathways, beyond the initial NA-induced ChE inhibition, presents a problem for linking PBPK models of various countermeasure agents to those of NAs. For example, the conventional NA countermeasures, atropine and diazepam, as well as many novel countermeasure agents, do not directly impact ChE kinetics because they act at sites distinct from the esterase's active site. Such agents act at receptor sites such as muscarinic, $GABA_A$, or NMDA receptors (Figure 62.4).

Interactive models, which have been developed and validated, exist for countermeasures which compete with NA inhibition of ChE or the regeneration of the free enzyme from its inhibited state, such as pyridostigmine and oximes, respectively. Numerous data in the literature describe the kinetics of interaction between nerve agents and other OPs and specific countermeasures at active enzyme sites of concern. For example, Davies and Green (1956) have measured the rate of reactivation of inhibited erythrocyte

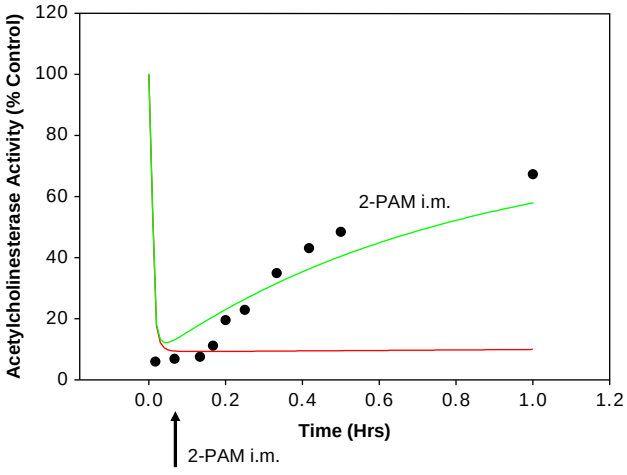

FIGURE 62.4. RBC AChE inhibition in rhesus monkey following administration of sarin [0.75 LD_{50} (15 μg/kg) i.v.] and with 2-PAM (25.8 mg/kg) administered IM at 9 min postsarin i.v. administration. Atropine was administered (0.4 mg/kg) IM 15 min prior to sarin administration. The filled circles indicate experimental data (Woodard *et al.*, 1994); the curves show our PBPK model simulations of AChE activity after sarin, both with (upper curve) and without (lower curve) 2-PAM administration.

TABLE 62.1. Rate of activation of inhibited erythrocyte ChE by various oximes and hydroxamic acids (Davies and Green, 1956)

	Inhibitors		
Compound	**TEPP**	**DFP**	**Sarin**
Diisonitrosoacetone	8.4	0.8	24.3
Monoisonitrosoacetone	6.8	0.7	22.1
*iso*Nitrosoacetophenone	10.7	5.1	4.1′
*iso*Nitrosoacetylacetone	0.7	NA	1.1
Picolinohydroxamic acid	2.9	0.6	0.2
Micotinohydroxamic acid metabolide	0.3	0.05	0.3

Rates of reactivation are given in l/mol/min: temp = 25°C, pH = 7.4
NA–not available

ChE by various oximes (Table 62.1). More recently, Worek *et al.* (2004) determined the kinetic rate constants of inhibition, reactivation, and aging for different NA, pesticides, and oximes with human erythrocyte AChE (see Table 62.2), described by the reactions listed below:

$$[E] + [OP] \xrightarrow{k_1} [EP]$$

$$[EP] + H_2O \xrightarrow{k_2} [EA]$$

$$[EP] + H_2O \xrightarrow{k_3} [E] + [OP]$$

$$[EP] + [OX] \overset{k_D}{\leftrightarrow} [EPOX] \xrightarrow{k_r} [E] + [POX]$$

where [E] is the active enzyme (i.e. AChE), [OP] is the organophosphorus compound, [EP] is the reversibly phosphylated AChE, [EPOX] is the Michaelis–Menten-type phosphyl AChE oxime complex [EPOX], [OX] is the oxime, [POX] is the phosphylated oxime, and [EA] is the dealkylated, ''aged'' AChE. The reaction rates used describe the bimolecular inhibition (k_i), aging (k_a), spontaneous (k_s) and oxime-induced reactivation. The dissociation rate, K_D, is equal to the ratio [EP] × [OX]/[EPOX] and (k_r) represents a first-order rate constant for the displacement of the phosphyl residue from [EPOX], resulting in regenerated enzyme and a phosphorylated oxime.

TABLE 62.2. Rate constants for interactions of AChE, OPs, and oximes (Worek *et al.*, 2004)

		Reactivation constant			
Inhibitor	**Oxime**	k_r (min^{-1})	K_D (μM)	k_{r2} ($mM^{-1}\,min^{-1}$)	**(Oxime) (mM)**
MFPCh	Obidoxime	0.020	1,133	0.018	0.5–4
	2-PAM	0.004	2,949	0.001	1–5
	HI 6	0.076	1,233	0.062	0.3–2
	HLö 7	0.051	606	0.084	0.1–4
MFPβCh	Obidoxime	0.015	1,658	0.009	0.5–4
	2-PAM	0.002	3,131	0.001	0.5–10
	HI 6	0.090	859	0.105	0.5–4
	HLö 7	0.078	458	0.170	0.5–4
MFPhCh	Obidoxime	0.009	3,547	0.003	1–5
	2-PAM	0.003	3,837	0.001	1–5
	HI 6	0.028	1,037	0.027	0.5–4
	HLö 7	0.011	599	0.018	0.3–3

Hence both a high affinity of the oxime to the ''inhibited'' or phosphylated AChE and a high regeneration rate (k_r) are critical for oxime efficacy. Using *in vitro* enzyme kinetic constants and *in vivo* inhibitor and oxime concentrations, Worek *et al.* (2005) were able to extrapolate the percent of AChE inhibition in humans following sarin or cyclosarin (i.v.) and a simultaneous intramuscular injection of various oximes. Similarly, kinetic models have been developed to predict a dose range of human butyrylcholinesterase (HuBChE) required to maintain adequate residual RBC AChE level, following an exposure to sarin, soman, or VX (Ashani and Shlomi, 2004). These and other datasets can readily be integrated into the full PBPK/PD modeling framework, shown previously in Figures 62.2 and 62.3. For example, Figure 62.5 shows a simulation of RBC AChE inhibition and regeneration in the rhesus monkey after exposure to sarin (0.75 LD_{50} i.v.), followed by 2-PAM, administered (i.m.) (25.8 mg/kg) at 9 min post-sarin.

Efforts to develop interactive PBPK models to integrate diverse datasets for both nerve agents and countermeasures continue in order to design an optimized countermeasure delivery program focused on a particular exposure scenario. This involves not just the development of kinetic models for the relevant molecular interactions, but also placing these interactions into an appropriate physiological context wherever possible. It is therefore necessary to take into account the dosimetry of key chemicals at the appropriate site(s) of action. This allows one not only to describe and interpret animal experimental data, but also to extrapolate these kinds of data wherever possible to humans using human physiological parameters and (wherever possible) human *in vitro* data (given the likely absence of human *in vivo* data).

Specific model features relevant to the agent's route of exposure and its biological effect can be incorporated when appropriate, for example quantitative models for ciliary removal of NAs in the upper respiratory tract during inhalation exposure. In addition, it is important to take into account (and incorporate into the model) the limitations of the analytical methods used to quantify circulating and tissue levels of NA, such as the regeneration of NA from its binding sites.

Given that multiple countermeasure agents are currently prescribed for NA treatment, and that future therapeutics will likely also involve multidrug combinations, multiple PBPK models can be used to calculate the co-location of specific compounds at specific sites at specific times, where

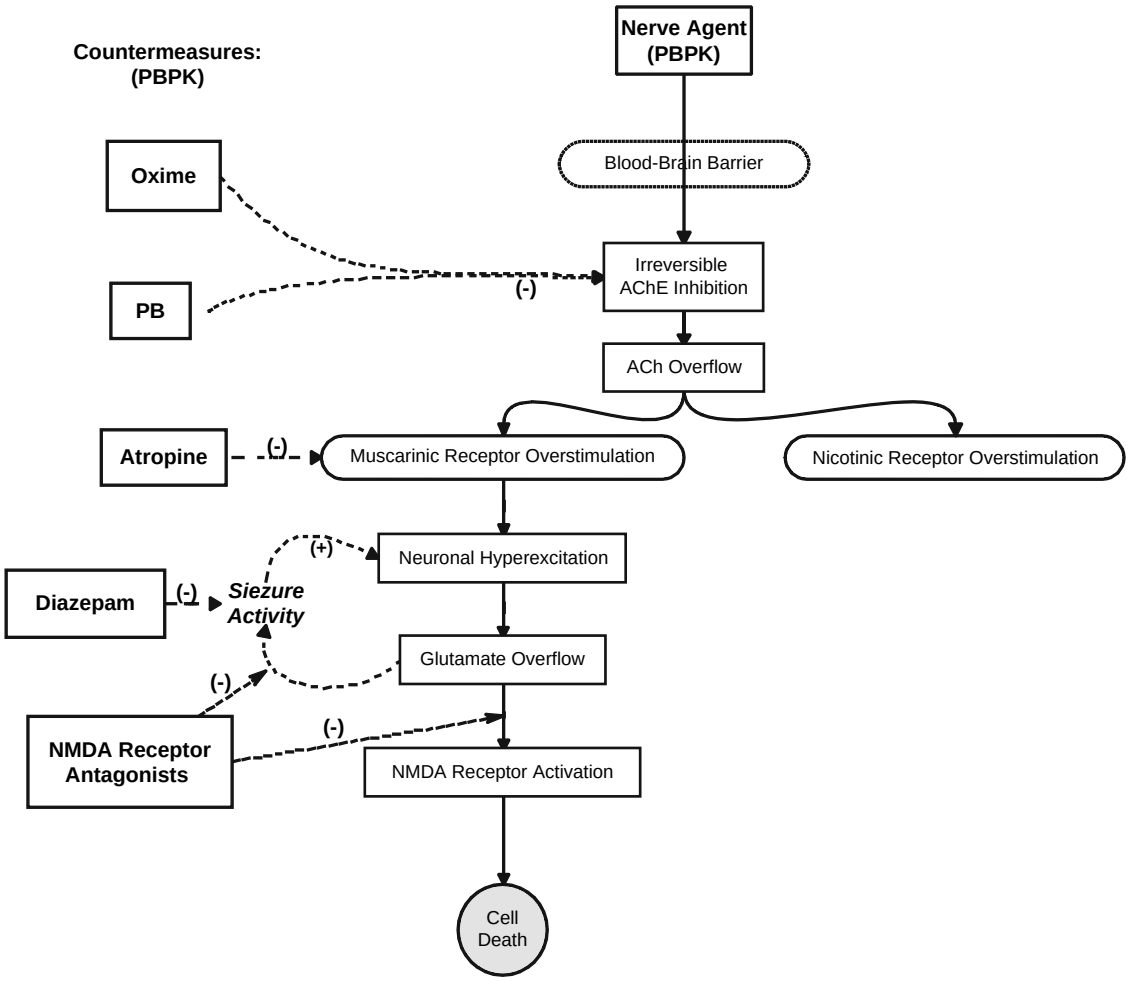

FIGURE 62.5. Schematic showing the progression of biological effects of a nerve agent together with potential points for intervention with countermeasures. It also shows a simplified outline of the scope of a potential set of models, showing the linkage between PBPK models, the nerve agents and countermeasures via their possible interactions.

their pharmacodynamic interactions may then be modeled. For example, it is desirable to model competition for AChE binding sites at erythrocytes and at target sites in the brain; the former since this regenerated NA from red blood cells can be interpreted to calculate a measure NA systemic exposure; and the latter, of course, because of the health impact. In general, the PBPK models can be linked via interaction processes at target sites.

Pharmacokinetic/PD models have been developed and used to predict the pharmacological effect of seizure activity for several anticonvulsants for which drug–receptor kinetics were available from either *in vitro* or *ex vivo* binding studies. Diazepam, an agonist of the γ-aminobutyric acid A ($GABA_A$) receptor, is among the NA antidotes for which the pharmacodynamic impact on electroencephalogram (EEG) activity has been modeled. Danhof and colleagues have developed compartmental kinetic models for various anticonvulsants and modeled their ligand–receptor pharmacodynamics. Electroencephalogram parameters have been used as pharmacodynamic endpoints (Van der Graaf *et al.*, 1999; Bueters *et al.*, 2003; Danhof *et al.*, 2007; Tuk *et al.*, 1999). These receptor binding models (Eq. 62.3) predict changes in receptor binding and pharmacodynamics as a function of changes in the pharmacokinetics of the ligand.

$$E(C) = E_{maxA} \cdot \left(\frac{C_A^N}{K_A + C_A} \right) \tag{62.3}$$

Here $E(C)$ is the observed effect at concentration C, $E_{\max}$ is the maximal effect (based on EEG components), K is the concentration at half-maximal effect, and N is the Hill factor, a constant expressing the sigmoidicity of the concentration–effect relationship. Subscripts A refer to the specific ligand of a ligand-gated ionotropic receptor.

Unfortunately, the impact of anticonvulsants on NA-induced seizures has not been modeled in this way. Given many countermeasures have different ligand–receptor interactions, such an approach would need to be expanded to the other excitatory and inhibitory neurotransmitters and

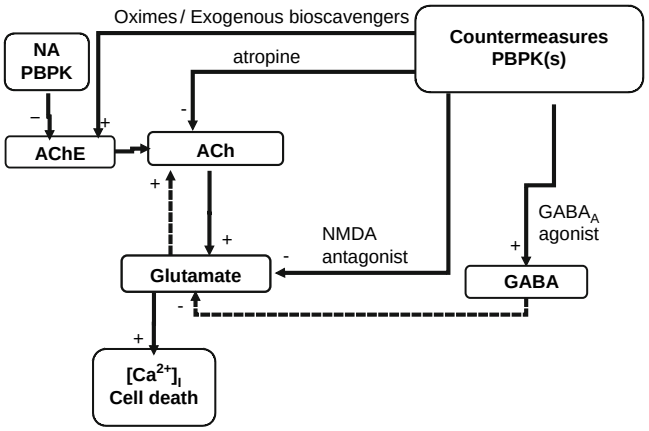

FIGURE 62.6. Simplified PD framework linking NA and various countermeasure PBPK models. Arrows indicate either agonistic (+) or antagonist (−) effect on the cholinergic, glutamatergic, or GABAergic systems. Dashed lines indicate feedback mechanisms. Arrows are synthesis or direct effect.

their respective receptors, which are involved in seizure induction and sustainment. Figure 62.6 suggests such a hypothetical framework that would include modeling of NA-induced increases in ACh and the countermeasures' interactions with associated receptors (esterases, $GABA_A$, mACh, and/or NMDA receptor sites). However, validation of such an approach, using components of gross EEG recordings, may not be plausible, due to the large number of ionotropic channels represented. An endpoint, such as sustained elevated glutamate, may be more appropriate.

While the receptor targets of various NA countermeasures vary distinctly, be they ion channel linked, G-protein linked or enzyme linked, their pharmacodynamic effects often converge upon the final steps leading to the disruption of intracellular Ca^{2+}, beyond which cell death is inevitable. Hence, ultimately a systems biology approach may provide the best predictions of countermeasure efficacy, especially for modeling long-term low-level effects. The Systems Biology Markup Language (SBML) project is an effort to create a machine-readable format for representing computational models in biology. SBML provides an input and output format, so that different software tools can operate on the same representation of a model, removing chances for errors in translation. SBML also provides convenient model databases, such as Biomodels (www.biomodels.net) for sharing these models. Neural Open Markup Language (NeuroML) similarly facilitates building, simulating, testing, and publishing of models, describing channels, neurons, and networks of neurons. Future integration, via a common computational language, of these molecular and cellular level models with PBPK models will permit the development of multiscale models that will link external exposure and tissue dosimetry with NA-induced neurotoxicity. This multiscale approach is necessary for quantitative assessments of the health risks associated with NA exposure.

IX. HEALTH EFFECTS ASSESSMENT AND COUNTERMEASURE OPTIMIZATION

Once validated, these models can be used to predict efficacy of specific countermeasures, countermeasure combinations, and dosing regimens for specific NAs and combinations thereof. In order to do so, appropriate measures of efficacy need to be defined. Perhaps the simplest way to estimate the effectiveness of a particular countermeasure is to determine the degree of a receptor activation at the target site of concern (e.g. brain or brain region) at a certain time or period of time following the administration of the countermeasure(s) compared to the situation in which no countermeasure is administered. Expressed as a simple ratio, countermeasure effectiveness (CE) may be written in terms of the time weighted average concentration (from time $t = T_1$ to $t = T_2$) of receptor occupancy (obtained from the PBPK/PD model):

$$CE = \frac{\int_{T_1}^{T_2} [E]_c dt}{\int_{T_1}^{T_2} [E] dt} \tag{62.4}$$

Here the numerator is calculated by running the model in the presence of the countermeasure, while the numerator is obtained in the absence of countermeasures. The ratio CE can be calculated under a variety of countermeasure dosing regimens to determine an optimum for each specific NA exposure scenario. Clearly, if such an exploration of countermeasure effectiveness were to be conducted experimentally, without the benefit of modeling, it would be prohibitively expensive and time consuming, thus demonstrating the usefulness of the modeling. The information obtained in this way would provide a rational basis for designing countermeasure delivery systems for optimized effectiveness. Ultimately, such an approach would also aid in the design of novel countermeasures, either to be used alone or integrated into an optimized countermeasure delivery ''package''.

A separate though related application of the proposed modeling approach is the development of meaningful therapeutic animal models, based on rational animal to human scale-up. In such an application, dosing regimens for existing countermeasures and countermeasure combinations, and particularly novel countermeasures, can be tested in experimental animals with the knowledge that the results obtained can be extrapolated to humans by taking into account the appropriate species differences incorporated into the models.

X. CONCLUDING REMARKS AND FUTURE DIRECTION

It can be expected that in the near future, PBPK/PD models will be developed that can predict the interaction between mixtures of NAs and countermeasures and their opposing

pharmacodynamic effects. Validation of such complex modeling efforts is data intensive and will rely on compilation of existing *in vitro*, *in vivo,* and *ex vivo* data. Unfortunately, the majority of toxicological studies on NA measure lethality, with little or no additional collection of time-course data of pharmacodynamic endpoints. Similarly, interaction studies between NAs and antidotes often measure only efficacy, in terms of a reduction in cell death. This is understandable due to the difficulty and cost in measuring these NAs in biological matrices and the large number of animals involved in collecting time-course data. Measurements of neurotransmitter changes following OP and countermeasure exposures are very difficult and often there is uncertainty in deciding which biomarker is most predictive of an effect that is not easily quantifiable, such as cognitive deficits. PK/PD analyses may not always be possible if the relationships between the input (the concentrations of the NA and countermeasure) and the output become too indirect. However, it should always be remembered that even without formal concentration–effect analyses, there is always much to be gained if a pharmacological countermeasure test is performed with intensive sampling of PK and PD parameters. Differences in PK/PD parameters give quantitative information about different neurotransmitter systems in the central and peripheral nervous system, which cannot be distinguished by pure statistical group comparisons. PK/PD analyses largely eliminate pharmacokinetic variability, and provide detailed and functional characteristics of pharmacological systems in the brain, or other target organs.

With specific regard to finding novel therapeutics for preventing or ameliorating NA-induced brain damage, PBPK/PD modeling provides a means to extrapolate experimental findings in animals to humans. Differences seen in the efficacy of different therapeutics may sometimes be explained by pharmacokinetics. Perhaps the therapeutic dose reaching a critical tissue, such as the diaphragm, heart or brain stem is too little, too late or not long enough. In addition, many of the animal toxicity studies performed to assess the efficacy of a therapeutic against NA measure a biomarker, such as cell death within 24 h from NA exposure. Biomarkers measured at 24 h may not always be indicative of outcome. Whether or not any protection seen at that point would still have to remain if the biomarker (e.g. cell death) were measured days, weeks or months later then remains unknown. For example, damage seen in thalamus at 24 h might later extend to areas with which it has strong reciprocal connections, such as the cerebral cortex. Lastly, in the quest for antidotes that may rescue one from cognitive deficits down the road, when realistically administered beyond when the glutamatergic phase takes control of seizure activity (i.e. >40 min post-NA exposure), predicting biomarkers that indicate the promotion of physiological balance in the brain, rather than the attenuation of seizure activity, may become the most important endpoints for predicting the efficacy of novel countermeasures.

Given the unsustainable cost and time involved in bringing novel therapeutics to market, quantitative techniques to improve countermeasure development are being used increasingly more. Several opportunities exist in drug development, which could lead us on a more direct path toward finding suitable countermeasures; for example, more complete integration of the available knowledge of therapeutic candidates early in their development, using PBPK/PD model(s) for NAs and countermeasures together. This should be started early, in the preclinical phases of drug development. Extrapolation of animal PBPK/PD models to humans for simulating initial clinical trials should be performed to evaluate the design of proposed clinical trials of a drug development plan and to provide proof of concept. This ability to mathematically explore various exposure–response relationships of NA(s) and countermeasure(s) is useful for designing optimal countermeasure regimens and identifying the limits of its therapeutic window following NA exposure. Uncertainty analysis of the model's parameters may identify biomarkers that may be critical in describing the data. Ways of defining and decreasing the uncertainty in efficacy and safety (risk assessment) in the drug and disease model are needed.

Lastly, the mechanisms (e.g. diffusion-limited transport, receptor binding, protein up-regulation or desensitization) described in a particular NA/countermeasure model should be updated as new information is generated, thereby using the models as knowledge management tools. Thus, modeling the PK/PD of neuroprotectants in the presence of NA may provide direction and guidance early in clinical development when there is opportunity to change direction and plans.

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CHAPTER 63

Prophylactic and Therapeutic Measures in Nerve Agent Poisoning

TROND MYHRER

1. INTRODUCTION

Nerve agents are regarded as the most toxic among all chemical weapons. Nerve agents were originally synthesized during the 1930s in Germany in attempts to achieve more efficient pesticides based on organophosphorus compounds. Tabun was the first to be synthesized, followed by sarin and soman. VX is another type of nerve agent which was originally developed in the UK during research for new insecticides. The organophosphorus nerve agents are highly potent irreversible inhibitors of the enzyme acetylcholinesterase (AChE) that hydrolyzes acetylcholine (ACh). Accumulation of ACh in the synaptic cleft results in overstimulation of muscarinic and nicotinic receptors. This cholinergic overactivity can affect all organ systems. The toxic signs in humans include pinpoint pupils (miosis), bronchoconstriction, hypersalivation, increased lung secretions, sweating, diarrhea, loss of consciousness, seizures, and respiratory arrest. Miosis appears to be a very sensitive index of direct exposure, and can be painful for several days. Chest tightness, rhinorrhea, and increased salivation can occur within seconds/minutes of inhalation of nerve agents. If exposure is substantial, death may occur from respiratory arrest within minutes. Individuals moderately exposed usually recover completely, although EEG abnormalities have been reported in those severely exposed to sarin 6–8 months after the terror attack in Japan (Murata *et al.*, 1997). This chapter describes prophylactic and therapeutic measures to combat organophosphate nerve agents.

II. BACKGROUND

Exposure to nerve agents requires immediate medical treatment. For this purpose, military personnel are issued with autoinjectors, or ''buddy aid'', containing countermeasures for self-administration. Antidotes against nerve agents are based on drugs acting at the muscarinic receptors and $GABA_A$ receptors (McDonough and Shih, 1997). In addition, partial protection against nerve agents can be obtained by the use of reversible (carbamate) AChE inhibitors shielding a critical portion of AChE from irreversible inhibition by nerve agents prior to nerve agent exposure. Furthermore, reactivation of any unaged AChE by an oxime is regarded as important immediate treatment after nerve agent exposure.

A number of armed forces have based their therapy against nerve agent intoxication on an oxime (obidoxime, 2-PAM, HI-6), an anticholinergic (atropine), and a $GABA_A$ agent (diazepam, avizafone) combined with carbamate (pyridostigmine) pretreatment (Aas, 2003). However, such treatment regimens can reduce immediate lethality, but they do not attenuate the occurrence of nerve agent-induced seizure activity and concomitant convulsions, unless atropine is given early and at a high dose (McDonough and Shih, 1997). Such seizures rapidly progress to status epilepticus, a condition that is strongly associated with mortality and brain damage in experimental animals (Shih *et al.*, 2003). Thus, there is an urgent need to search for novel strategies able to prevent or terminate nerve agent-induced seizures. For this reason, the present chapter focuses on animal studies (guinea pigs, rats, monkeys) designed to acquire novel insights into the neuropharmacological mechanisms of nerve agent-induced seizures and neuropathology.

III. MECHANISM OF ACTION

It has been hypothesized that several neurotransmitter systems become involved sequentially in the initiation and maintenance of seizures elicited by nerve agents (McDonough and Shih, 1997). The progression of events can conceptually be divided into three phases. An early cholinergic phase lasting from the time of exposure to about 5 min after onset of seizures is dominated by high cholinergic activity followed by a transitional phase of cholinergic and glutamatergic hyperactivity and finally a predominantly glutamatergic phase after about 40 min (McDonough and Shih, 1997). In the latter study, a substantial body of evidence has been provided in support of the above model.

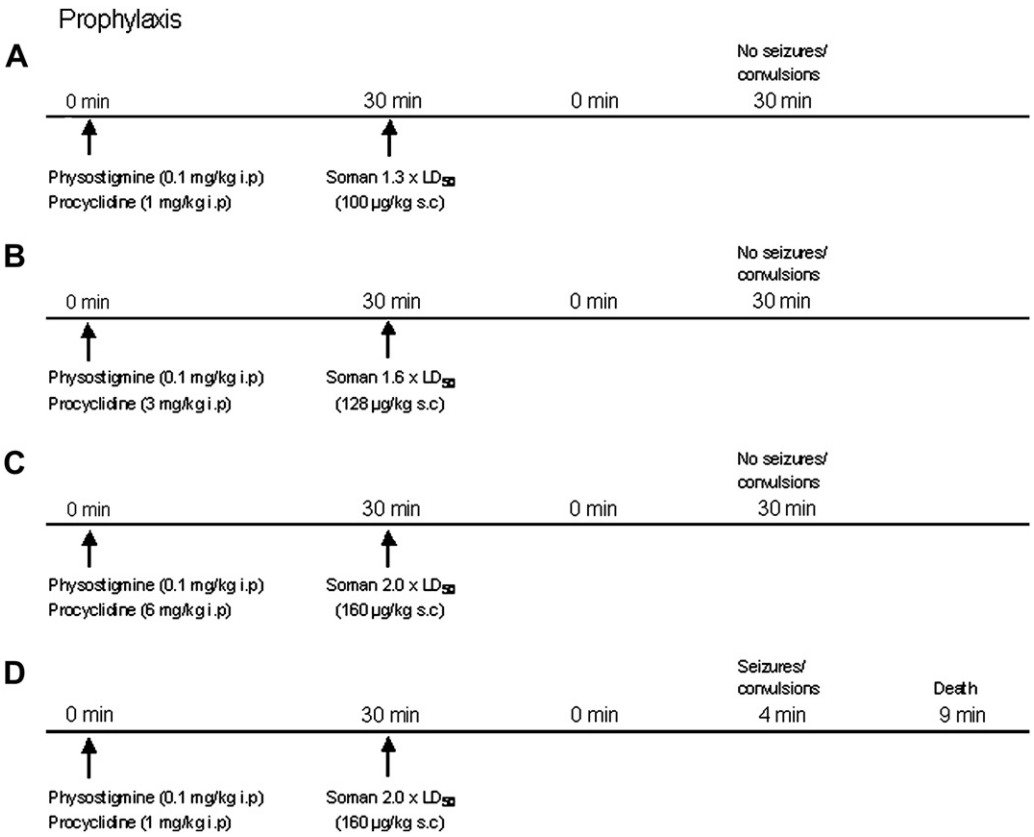

FIGURE 63.1. Experimental design for demonstration of anticonvulsant effects of a fixed dose of physostigmine combined with increasing doses of procyclidine relative to increasing doses of soman (A–C). Insufficient dose of procyclidine compared to soman results in early death (D).

Nerve agent intoxication results in inhibition of AChE and a tremendous buildup of ACh leading to protracted muscarinic receptor activation. This activation seems to happen in a dose-related manner. The higher the soman dose, the shorter the latency to onset of seizures and the greater the likelihood of early death in rats (Myhrer *et al.*, 2006a). The brainstem respiratory drive is vulnerable to increased cholinergic input, because the function of the respiratory center is disrupted. Both muscarinic and nicotinic antagonists can protect the respiratory center against cholinergic overstimulation (Kubin and Fenik, 2004). In compliance with this notion, the nicotinic antagonist mecamylamine in high doses can prolong survival in mice intoxicated by soman (Hassel, 2006). The functional integrity of the diaphragm is not significantly compromised by soman, unless a dose of $14 \times LD_{50}$ is used in cats (Rickett *et al.*, 1986). After a soman dose of $5 \times LD_{50}$ the latency to seizure onset is about 1 min, whereas the latency is about 14 min when the soman dose is $1 \times LD_{50}$ in rats (Myhrer *et al.*, 2003, 2006a). The change in ACh levels throughout the brain has been reported to precede that of any other neurotransmitter (Fosbraey *et al.*, 1990). The increases in glutamate occur in a number of brain regions with different time courses after seizures have started (McDonough and Shih, 1997). According to the three-phase model, efficacious pharmacological countermeasures against nerve agents should preferentially exert cholinergic and glutamatergic antagonism along with GABAergic agonism.

IV. PROPHYLACTIC MEASURES

A. Animal Studies

It takes a higher dose of anticonvulsants to terminate seizures induced by soman than other classical nerve agents such as tabun, sarin, cyclosarin, and VX (Shih and McDonough, 2000). This suggests that a drug capable of stopping soman-generated seizures would be equally effective for the treatment of seizures evoked by other nerve agents. This is the reason why soman is the nerve agent most frequently used in animal models to evaluate potential anticonvulsant drugs.

Pyridostigmine does not penetrate the blood–brain barrier to any extent and therefore provides very limited protection of the brain (Aas, 2003). For this reason, some recent investigations of prophylactic treatment in animals have focused on physostigmine as AChE inhibitor, because this carbamate (in contrast to pyridostigmine) crosses readily the blood–brain barrier (Birtley *et al.*, 1966). Although physostigmine in optimal doses does not protect against a lethal dose of soman, it appears effective when used along with an anticholinergic drug in guinea pigs (Philippens *et al.*, 2000). Physostigmine in combination

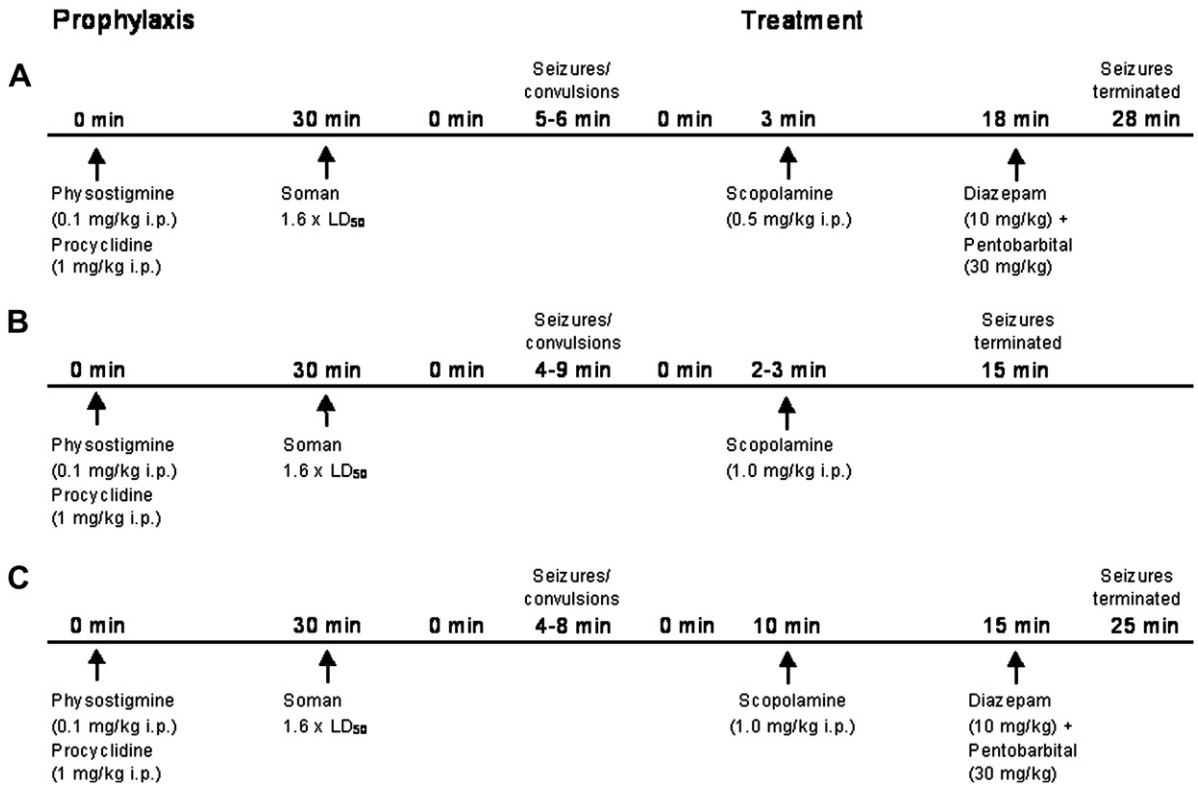

FIGURE 63.2. Experimental design for demonstration of how insufficient prophylactic treatment can be compensated for by adjunct post-exposure treatment. Low dose of scopolamine requires additional treatment (A). High dose of scopolamine is sufficient to terminate seizures (B). High dose of scopolamine given outside the cholinergic window requires additional treatment (C).

with scopolamine has been reported to protect guinea pigs effectively against soman, sarin, and tabun (Philippens *et al.*, 2000; Wetherell *et al.*, 2002). Similar protective effect has also been obtained with monkeys. Pretreatment of rhesus monkeys with physostigmine and scopolamine or physostigmine and trihexyphenidyl 25 min before exposure to $2 \times LD_{50}$ of soman results in survival without convulsions or loss of consciousness (Von Bredow *et al.*, 1991).

The cholinergic and glutamatergic antagonist, procyclidine, has in combination with physostigmine been demonstrated to assure full protective effects against convulsant doses of soman in rats and guinea pigs without any need for post-exposure treatment (Kim *et al.*, 2002). Because physostigmine has a rather short half-life, it will preferably require continuous administration. In a study of rats, osmotic minipumps were implanted subcutaneously affording sustained delivery of physostigmine and procyclidine for 3 days before exposure to a soman dose of $1.3 \times LD_{50}$. This treatment exerted full protective effects against lethality, convulsions, learning and memory impairments, and physical incapacitation (Choi *et al.*, 2004). In a subsequent study in beagle dogs, a matrix-type patch attached to the abdominal side of the animal delivered physostigmine and procyclidine for 2 days before being challenged with various doses of soman ($2–10 \times LD_{50}$). The patch delivery protected against seizures and death up to $4 \times LD_{50}$ of soman. When the patch was combined with HI-6 and atropine injected intramuscularly 1 min after soman exposure, protection was achieved up to a dose of $6 \times LD_{50}$ (Kim *et al.*, 2005).

In a study from our laboratory, the combined effects of physostigmine and procyclidine were tested against various doses of soman. Physostigmine (0.1 mg/kg) in combination with procyclidine at the doses of 1, 3, or 6 mg/kg effectively prevented the development of convulsions and hippocampally monitored seizures when the doses of soman were 1.3, 1.6, or $2 \times LD_{50}$, respectively (Figure 63.1). Results from [^{3}H]MK-801 binding experiments show that procyclidine inhibits the phencyclidine site at the NMDA receptor in a concentration-dependent manner (Myhrer *et al.*, 2004a). Physostigmine (0.1 mg/kg) and procyclidine in a dose of 1 mg/kg do not prevent convulsions/seizures when the soman dose is $1.6 \times LD_{50}$. Subsequent treatment with scopolamine in doses of 0.5 or 1 mg/kg immediately after (3 min) seizure onset shows that only the highest dose produce a reliable termination (Figure 63.2). When scopolamine (1 mg/kg) is given later (10 min) after onset of seizures no effect is obtained. The sustained seizures were subsequently treated with diazepam (10 mg/kg) and pentobarbital (30 mg/kg) and finally terminated 25 min after onset. In rats given inadequate prophylaxis, both modified convulsions and seizures are seen. It is recommended that

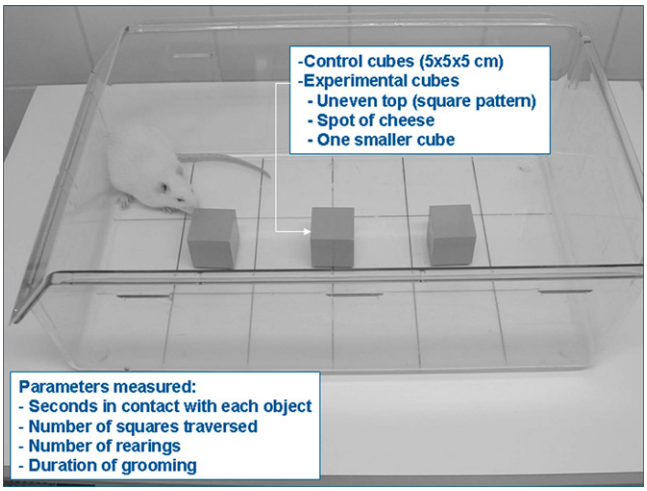

FIGURE 63.3. Novelty test measuring rats' innate preference for novelty, locomotor activity, rearing, and grooming. Testing is carried out in three sessions (one each day). Each session consists of 5 min exploring with neutral cubes present (Phase 1) and Phase 2 (5 min) after 10 min in the home cage during which the central cube has been replaced by a novel one. The novelty is represented by an uneven top (Session I), a spot of cheese on one side (Session II), or a smaller cube (Session III).

moderate doses of prophylactics should be used to avoid adverse effects on cognitive functions, because insufficient prophylaxis can be compensated for by adjunct treatment (Myhrer *et al.*, 2004a).

In order to avoid the problem with a short-lasting prophylactic drug, the AChE inhibitors huperzine, donepezil, and galantamine may offer alternatives with long-lasting efficacy. Both rats and guinea pigs are well protected against soman-induced seizures and neuropathology when pretreated with huperzine, whereas pyridostigmine does not have such effects (Lallement *et al.*, 1997; Tonduli *et al.*, 2001). Donepezil alone does not protect well against seizures evoked by soman, but when donepezil is combined with procyclidine a significant protection is obtained in rats (Haug *et al.*, 2007). Galantamine in combination with atropine administered before a convulsant dose of soman can effectively protect guinea pigs against seizures (Albuquerque *et al.*, 2006).

When pyridostigmine is combined with benactyzine and trihexyphenidyl (exerting both cholinergic and glutamatergic antagonism) in rats, effective protection is obtained against signs of neurotoxicity induced by soman. However, the combination with pyridostigmine and biperiden is reported to be even more efficacious than the former combination against soman poisoning (Kassa *et al.*, 2003).

The results from studies of prophylactic treatment presented so far are in compliance with the notion that seizures are triggered by cholinergic hyperactivity followed by sustained high cholinergic activity for about 5 min after seizure onset. An exception has been seen when MK-801 given before soman can prevent the occurrence of seizures in both guinea pigs and rats (Braitman and Sparenborg, 1989; Shih *et al.*, 1991). In the latter studies, however, MK-801 was combined with other drugs (pyridostigmine, atropine, and pralidoxime or HI-6, respectively). Hence, it is difficult to rule out the possibility that MK-801 interacted with effects of the additional drugs. HI-6 has recently been shown to decrease the release of ACh when hippocampal slices are exposed to soman (Øydvin *et al.*, 2005). Microinfusions of MK-801 or ketamine into the seizure controlling region, area tempestas, do not cause anticonvulsant effects against soman intoxication, whereas microinfusions of atropine or scopolamine do (Myhrer *et al.*, 2008a). Moreover, systemic administration of solely MK-801 or TCP before soman does not prevent the occurrence of seizures (Carpentier *et al.*, 1994; Myhrer *et al.*, 2008a).

It is crucial that prophylactics used to protect military personnel and first responders against lethal doses of nerve agents do not by themselves produce impairment of cognitive capability. This issue appears to be neglected in nerve agent research (Myhrer *et al.*, 2004b). In the latter study, it was examined whether physostigmine, scopolamine, and various doses of procyclidine might reduce rats' innate preference for novelty (Figure 63.3). When these drugs were tested separately, the results show that physostigmine (0.1 mg/kg) and procyclidine (3 mg/kg) do not affect preference for novelty, whereas scopolamine (0.15 mg/kg) and procyclidine in a higher dose (6 mg/kg) result in a preference deficit. The combination of physostigmine and scopolamine or physostigmine and procyclidine (6 mg/kg) causes a marked deficit in preference for novelty. A much milder deficit is observed when physostigmine is combined with lower doses (1 or 3 mg/kg) of procyclidine. The latter combinations also have milder adverse impact on the animals' interest in the test environment and activity measures than the former combinations.

Administration of physostigmine and procyclidine for 3 days by means of minipumps (72 µg/kg/h and 432 µg/kg/h, respectively) in rats does not affect passive avoidance performance (Choi *et al.*, 2004). A similar administration of physostigmine and scopolamine for 2 weeks in marmosets results in normal performance in a two-choice discrimination serial task. However, the concentration of scopolamine used is equivalent to an acute dose of 0.009 mg/kg which is considerably lower than doses which have been shown to affect marmoset cognitive performance (e.g. 0.06 mg/kg) (Muggleton *et al.*, 2003).

In a recent behavioral study of rats, potential cognitive effects of a group of anti-Parkinson drugs (with excellent anticonvulsant properties against nerve agents) were examined in the novelty test (Myhrer *et al.*, 2008b). It was shown that benactyzine (0.3 mg/kg), caramiphen (10 mg/kg), and trihexyphenidyl (0.12 mg/kg) reduce rats' innate preference for novelty, whereas biperiden (0.11 mg/kg) and procyclidine (3 mg/kg) do not. When benactyzine, caramiphen, or trihexyphenidyl is combined with physostigmine, the cognitive impairment disappears. This counteracting effect,

however, produces changes in locomotor and rearing activity not seen by each drug alone. AChE inhibitors and anticholinergics used as prophylactics can offset each other, but exceptions are observed in a previous study (Myhrer *et al.*, 2004b) when a very potent anticholinergic (scopolamine) or a high dose of procyclidine still results in cognitive deficits in spite of coadministration with physostigmine. The half-life of physostigmine is 17 min in plasma and 16 min in the brain of rats (Somani and Khalique, 1986). A crucial issue is how physostigmine is able to antagonize receptor blocking effects of anticholinergics by increasing the level of ACh and thus reduce or prevent cognitive impairment. This apparently subtle balance is probably attained in different ways for other relevant AChE inhibitors used as pretreatment against nerve agents, such as donepezil, huperzine, and galantamine. To equalize cognitive side effects of potent anticonvulsants by use of AChE inhibitors is not recommended, because accurate adjustments for differences in half-life would be required for acute doses. Use of continuous delivery, however, may circumvent the problem with different half-lives of combining drugs.

B. Human Use

Pretreatment with the carbamate pyridostigmine is a well-established method to protect against nerve agent intoxication in the armed forces in a number of nations. A tablet (30 mg) of pyridostigmine bromide is supposed to be taken every 8 h by service personnel. The rationale behind this use is that carbamate occupies a portion of the available AChE (15–40% of the erythrocyte AChE) and renders it inaccessible to nerve agents in the blood, since nerve agents only bind to unprotected enzymes (Leadbeater *et al.*, 1985). The AChE that has been reversibly inhibited by pyridostigmine spontaneously decarbamoylates, and the enzyme is again able to hydrolyze ACh. Because pyridostigmine does not penetrate the blood–brain barrier to any extent under normal circumstances, it only protects the peripheral nervous system. Pyridostigmine has been reported to have negligible physiological and psychological side effects (Leadbeater *et al.*, 1985). However, in a recent meta-analysis, it is stated that carbamate can cause side effects in predisposed individuals (Golomb, 2008).

The carbamate physostigmine, which readily crosses the blood–brain barrier, has been considered a convenient replacement for pyridostigmine. Based on a series of animal studies, the combination of physostigmine and the cholinergic antagonist hyoscine (scopolamine) has been brought to the level of clinical trials in the UK (Scott, 2007). The problem with the relatively narrow therapeutic window and potential side effects of physostigmine has been solved by continuous coadministration with hyoscine in terms of transdermal delivery. The pharmacological antagonism between these two drugs probably alleviates the potentially adverse effects produced by each drug alone. This pretreatment regimen is supposed to be less reliant on supporting therapy than the use of pyridostigmine (Scott, 2007).

The prophylactic combination of pyridostigmine with trihexyphenidyl and benactyzine has been introduced into the Czech Army. The presence of two anticholinergics made it possible to increase the dose of pyridostigmine and thus enhance the prophylactic efficacy (Bajgar, 2004). This combinational regimen (designated PANPAL) has been reported to have no side effects as demonstrated in volunteers: no changes in the psychic state or dysfunctions were observed. Actually, an improvement of tapping test performance was found after administration of PANPAL (Bajgar, 2004).

The use of human plasma-derived butyrylcholinesterase to neutralize the toxic effect of nerve agents *in vivo* has been shown to both increase survival and protect against attenuated cognitive function following nerve agent exposure. No post-exposure autoinjectors are necessary (Lenz *et al.*, 2007). This bioscavenger is currently being produced by using outdated human blood and is ready for clinical trials. Both military personnel and first responders are relevant groups to be protected by biological scavengers.

V. THERAPEUTIC MEASURES

A. Animal Studies

Post-exposure treatment of animals consists of two categories: immediate and subsequent therapies. In the first category, drugs with anticholinergic effects will be of preference, whereas drugs with antiglutamatergic effects will be of preference in the second category according to the three-phase model. Drugs enhancing $GABA_A$ neurotransmission (diazepam and other benzodiazepines) are effective during all phases of nerve agent intoxication (McDonough and Shih, 1997). Immediate therapy also includes an oxime in order to reactivate inhibited AChE.

Reactivation of inhibited acetylcholinesterase is considered to be an important element in post-exposure treatment. Bis-pyridinium oximes can reactivate the phosphorylated enzyme if they are administered prior to the change from the reactivatable to the unreactivatable state, the process referred to as ‘‘aging’’. The aging process is quickest with soman in brain and diaphragm preparation (a few minutes) compared to other nerve agents (Sun *et al.*, 1979). However, administration of oximes after aging has already occurred can enhance survival (McDonough and Shih, 1997). The oximes pralidoxime and obidoxime have been widely used, but HI-6 appears to be a promising substitute in treatment of intoxication with nerve agents. HI-6 has relatively low toxicity and is efficient in the treatment of soman intoxication (Clement, 1982; Hamilton and Lundy, 1989; Kusic *et al.*, 1985; Van Helden *et al.*, 1992; Walday *et al.*, 1993). A disadvantage of HI-6 is its

lack of stability in aqueous solutions that requires storing as powder in a separate chamber in the autoinjector (Thiermann *et al.*, 1996).

Anticholinergic drugs and an oxime are usually applied after prophylactic treatment (pyridostigmine). Anticholinergics have optimal efficacy during the cholinergic phase, i.e. from exposure to about 5 min after onset of seizures. Such drugs are classified as muscarinic receptor antagonists, and they are not able to counteract the nicotinic signs of intoxication like muscle fasciculation, muscle fatigue and weakness (McDonough and Shih, 2007). The rat brain is provided with the muscarinic receptor subtypes M1–M5. However, effective drugs against nerve agents have been shown to be anticholinergics with high specificity toward M1 (McDonough and Shih, 1997). The antinicotinic anticholinergic agent mecamylamine does not have anticonvulsant efficacy in rats poisoned with soman (Shih *et al.*, 1991). The effectiveness of potent centrally active anticholinergics has been shown to be at least as good as diazepam in terminating nerve agent-induced convulsions (McDonough and Shih, 1997). Rats pretreated with HI-6 (to reduce lethality) were given a soman dose of $1.6 \times LD_{50}$ and received anticonvulsants 5 or 40 min after onset of seizures. The results showed that anticholinergics (scopolamine, atropine, benactyzine, trihexyphenidyl) can effectively terminate seizure activity at 5 min, but not at 40 min after onset of seizure (McDonough and Shih, 1993). These anticholinergics were as effective and potent as anticonvulsants as if they had been given as pretreatment.

In our laboratory, we have examined the efficacy of a triple combination of drugs with adequate anticonvulsant effects and a dual combination with inadequate anticonvulsant effects followed by adjunct therapy (Myhrer *et al.*, 2006a). The results show that combined (mixed as a single compound) i.m. injections of HI-6 (42 mg/kg), atropine (14 mg/kg), and avizafone (3 mg/kg) administered 1, 16, and 31 min after exposure to a soman dose of $4 \times LD_{50}$ completely terminate seizures with a moderate mortality rate (25%). When the soman dose is lowered to $3 \times LD_{50}$ the anticonvulsant effect is complete, and no rats die within 24 h. Rats challenged with $5 \times LD_{50}$ of soman all die within 10 min. Without avizafone in the combination, seizures induced by 3 or $4 \times LD_{50}$ of soman cannot be terminated unless an adjunct therapy consisting of procyclidine (6 mg/kg), diazepam (10 mg/kg), and pentobarbital (30 kg/kg) is given, and the mortality rate is comparatively high (78%). Administration of the adjunct therapy alone 6–16 min after $4 \times LD_{50}$ of soman stops the seizure activity, but all the rats die within 24 h (Myhrer *et al.*, 2006a). The latter study shows that administration of countermeasures (mimicking autoinjectors) shortly after exposure to a relatively high dose of nerve agent can prevent death, even without pretreatment of the animals.

The great challenge in treatment of nerve agent poisoned victims seems to be the cases of long delays before treatment can start. Seizures lasting beyond 40 min are increasingly difficult to terminate (Carpentier *et al.*, 2001; Lallement *et al.*, 1999). Even conservative estimates suggest that it will take at least 30 min for emergency personnel to access individuals unprepared for exposure to nerve agent. Even soldiers properly provided with protective mask, gloves, and clothes may need medical help, because bad training, bad discipline, or bad luck can lead to intoxication of nerve agent (Lallement *et al.*, 1999). Hence, there is a pertinent need to search for novel strategies capable of controlling nerve agent-generated seizures well after their onset.

Drugs that enhance $GABA_A$ neurotransmission have been shown to be effective in intoxicated animals during all phases of seizures by counteracting the elevated level of glutamatergic activity (McDonough and Shih, 1997). Seizures induced by kainic acid or lithium/pilocarpine in rats can be terminated 1 h after onset by the administration of diazepam and pentobarbital (Du *et al.*, 1995). This potent combination of two $GABA_A$ agonists may create a promising anticonvulsant therapy, if it is supplied with an anticholinergic agent. Procyclidine was chosen for this purpose, because this drug exerts antagonism on both muscarinic and nicotinic receptors and additionally has antagonistic effect on NMDA receptors (Kim *et al.*, 2002). Procyclidine (6 mg/kg), diazepam (10 mg/kg), or pentobarbital (30 mg/kg) does not stop soman-induced seizures when administered separately, but both convulsions and seizure activity are terminated when these agents are given together. This triple therapy is 100% effective, when administered 30–40 min (5 min between each injection) following onset of convulsions (Figure 63.4) (Myhrer *et al.*, 2003).

In both military and civilian contexts, it would appear somewhat inconvenient for medical personnel to administer three injections of antidotes to achieve termination of seizure activity. It would be more expedient to replace diazepam and pentobarbital by a single $GABA_A$ modulator assuring corresponding anticonvulsant impact. However, systemic administration of $GABA_A$ modulators does not differentiate well between their anticonvulsant properties (Shih *et al.*, 1999). In a recent study based on micro-infusion of GABAergic modulators into seizure controlling areas in the rat brain (area tempestas and substantia nigra), it was found that muscimol, ethanol, and propofol possess the best anticonvulsant potencies against soman seizures among the agents examined (Myhrer *et al.*, 2006b). In a subsequent study, it was shown by systemic administration that procyclidine (10 mg/kg) combined with either muscimol (20 mg/kg), ethanol (3 mg/kg), or propofol (50 mg/kg) can effectively terminate soman-induced seizures when administered 5 min apart 30–35 min after onset. Procyclidine and propofol are considered the most relevant double regimen to replace the previous triple regimen consisting of procyclidin, diazepam, and pentobarbital (Myhrer *et al.*, 2006c).

It has been demonstrated that untreated seizures/convulsions induced by soman result in extensive

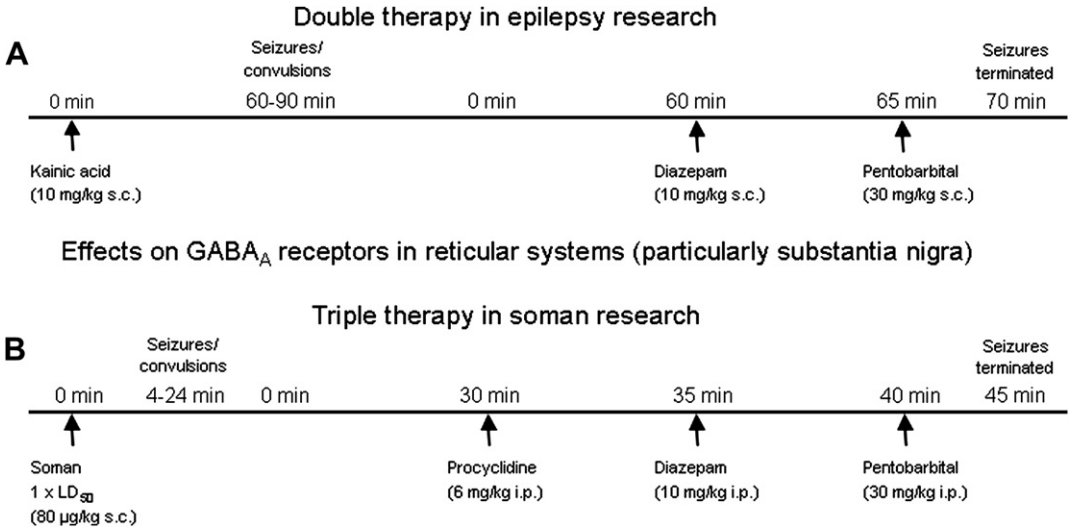

FIGURE 63.4. Experimental design revealing anticonvulsant effects of diazepam and pentobarbital in epilepsy research (A). Anticonvulsant effects achieved by triple regimen when administered 30–40 min after onset of seizures (B).

neuropathology and pronounced cognitive deficits in the Morris water maze (Filliat *et al.*, 1999; Raveh *et al.*, 2002, 2003). In our laboratory, we wanted to investigate whether there is a relationship between the extent of neuronal pathology and degree of dysfunction in the performance of cognitive tasks following soman-induced convulsions that had lasted for 45 min (Myhrer *et al.*, 2005). One behavioral task employed was the novelty test (Figure 63.3) that has proven particularly sensitive in revealing cognitive dysfunctions following selective disruptions of entorhinal projections (Myhrer 1988, 1989). The other behavioral task employed was a simultaneous brightness discrimination test (Figure 63.5) for assessing memory function. A three-choice version of this task has proven sensitive to selective hippocampal or rhinal lesions (Myhrer, 2000). When the convulsions are stopped after 45 min with the triple regimen (procyclidine, diazepam, pentobarbital), the results show that the neuronal pathology (assessed with Fluoro-Jade B staining) varies from none at all to 30% damage in the index areas (hippocampus, amygdala, piriform cortex). Cognitive deficits are seen in the novelty test (11 days post-exposure) (Figure 63.6) and retention of the brightness discrimination task (28 days post-exposure) among the rats with neuropathology (Figure 63.7). Furthermore, significant correlations between neuropathology scores and behavioral measures are found for the animals that convulsed. It is concluded that

FIGURE 63.5. A three-choice simultaneous brightness discrimination test. The rats (deprived of water) learn that they receive water from the well in the positive cylinder (in this case gray, which is used for half of the rats in each group; the other half uses the black cylinder). The criterion (five correct choices in succession) is obtained in 2 days of training. Thirteen days following acquisition the rats are tested for what has been retained of the learning principle (gray or black positive cylinder).

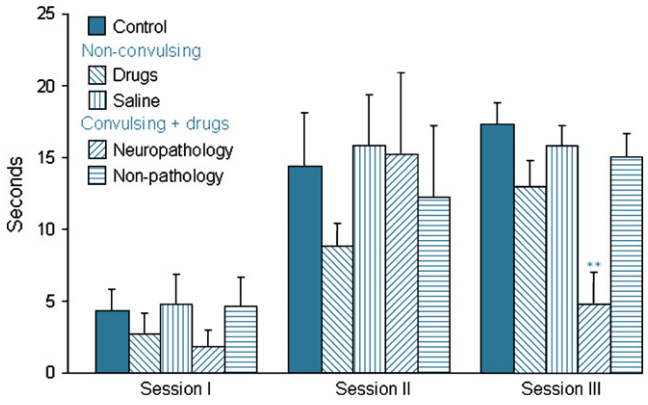

FIGURE 63.6. Mean (SEM) measures of preference for novelty 9–11 days after exposure to soman. Preference for novelty is expressed as the difference in seconds between exploring novel versus neutral objects. Drugs given are procyclidine (6 mg/kg), diazepam (10 mg/kg), and pentobarbital (30 mg/kg). ** Significantly different from all other groups ($p < 0.01$).

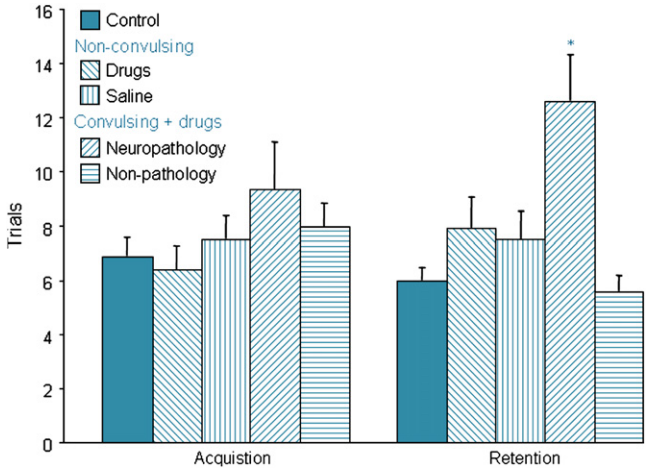

FIGURE 63.7. Mean (SEM) performances (trials to criterion) of acquisition (14–15 days post-soman) in the brightness discrimination task. Drugs are as in Figure 63.6. * Significantly different from all other groups ($p < 0.05$).

early management of soman-induced convulsions is of major importance in preventing neuropathology and accompanying cognitive impairments (Myhrer *et al.*, 2005).

In a number of studies, attempts have been made to terminate soman-triggered seizures/convulsions well after their onset. However, unlike the studies from our laboratory (Myhrer *et al.*, 2003, 2005, 2006c), previous studies have been based on animals also treated before exposure to soman. Marked anticonvulsant effects have been obtained in guinea pigs pretreated with pyridostigmine, given atropine 1 min after a convulsant dose of soman and treated with ketamine 30–120 min post-poisoning (Dorandeu *et al.*, 2005). Treatment with the combination of NBQX and TCP 30 or 50 min after onset of soman-induced seizures has pronounced anticonvulsant impact on rats pretreated with HI-6 and atropine (Lallement *et al.*, 1994b). When pretreated rats (HI-6) are given a single drug of a number of anticholinergics or antiepileptic agents 40 min after onset of soman-evoked seizures the anticonvulsant efficacy is rather modest (McDonough and Shih, 1993; Shih *et al.*, 1999).

Seizures evolving to status epilepticus (seizure activity for 30 min or more) are strongly associated with death or brain damage. The first signs of neuropathology are usually detectable about 20 min after onset of soman-generated seizures in rats (Lallement *et al.*, 1994a; McDonough *et al.*, 1995). The mechanism behind sustained epileptiform activity may be related to increased inactivation of $GABA_A$ receptors along with an up-regulation of NMDA receptors (Chen and Wasterlain, 2006). The net outcome of these changes would require comparatively high doses of anti-glutamatergic drugs to control nerve agent-induced seizures well after onset. This situation appears to make up the biggest challenge within nerve agent research. Animal studies modelling the battlefield/terror situation in which individuals are unprepared to exposure of nerve agent should ideally not include pretreatment. However, in order to assure survival of a reasonable number of animals seizing for 1–3 h, pretreatment appears inevitable.

B. Human Use

Oximes should be used as soon as possible after exposure to nerve agent, because of the short time window of the aging process. The human *in vitro* half-life for soman-inhibited AChE is 1.3 min, for sarin-inhibited AChE 3 h, and for tabun-inhibited AChE 13 h (Vale *et al.*, 2007). When aging has occurred, new AChE has to be synthesized. It is the extremely short aging time of soman that leads to the development of carbamate (pyridostigmine) prophylaxis for nerve agent poisoning. A number of nations use the oxime obidoxime (e.g. Finland, Germany, Norway, the Netherlands), while others use P2S (UK), 2-PAM (USA), or HI-6 (Canada, Sweden) (Aas, 2003). To date, there is no single oxime available that has a high efficiency in reactivating AChE by any known nerve agent. However, HI-6 is considered by most countries to be a very promising oxime following exposure to most nerve agents (Aas, 2003).

Most nations have issued their military personnel with two to three autoinjectors containing atropine and an oxime. Additionally, some nations have provided their soldiers with one autoinjector containing diazepam or avizafone (Aas, 2003). Atropine has to be administered at relatively high dose (2 mg), because of its competitive nature (competes with ACh). The use of autoinjectors speeds up uptake of the drug, due to a spraying effect as the needle plunges into the muscle, resulting in the distribution of drug within a larger muscle area, allowing for more rapid drug uptake than by using conventional needle and syringe (Sidell *et al.*, 1974). However, atropine in too high doses can result in inhibition of sweating and the potential for inducing heat casualties. Even a dose of 2 mg combined with heavy work in a hot environment may induce too high core temperature.

VI. CONCLUDING REMARKS AND FUTURE DIRECTION

This chapter has focused on the development of novel countermeasures against nerve agents in order to improve existing prophylactic and post-exposure treatments. Effective prophylactic treatment can be achieved by using a fixed dose of physostigmine in combination with varying doses of procyclidine. Increased lethal doses of soman can be counteracted by a corresponding increase in the procyclidine dose. However, the higher the doses of procyclidine, the more pronounced the cognitive side effects. Moderate doses of procyclidine should be preferred, because insufficient prophylaxis can be compensated for by adjunct treatment in terms of diazepam and pentobarbital. Since a slight cognitive impairment might be inevitable with effective prophylactics, reduced reliance on pretreatment appears preferable.

Immediate treatment with atropine and an oxime is very efficacious against nerve agent intoxication. However, subsequent treatment well after onset of seizures represents a huge problem in nerve agent research, because of the refractory nature of the epileptiform activity. A triple regimen consisting of procyclidine, diazepam, and pentobarbital has been shown to terminate effectively soman-evoked seizures in rats when administered 30–40 min following onset. A refinement of the triple regimen resulted in a double regimen composed of procyclidine and propofol that can stop seizures 30–35 min after they have been triggered by soman. However, both the triple and double regimens would need monitoring of vital functions, because pentobarbital and propofol can suppress normal function of the respiratory center in the brainstem. Thus, alternative approaches making it possible to design anticonvulsants predominantly affecting the forebrain will be needed. The ultimate aim should be the development of well-tolerated anticonvulsants that can be administered by the soldiers themselves regardless of the point of time after nerve agent intoxication. There are experimental approaches and techniques used in epilepsy research that with great benefit can be adopted in nerve agent studies. A major aim in experimental epilepsy is the identification of seizure controlling brain areas for rational drug designing (Löscher and Ebert, 1996). Microinfusions into area tempestas (AT) or substantia nigra can differentiate more clearly between the anticonvulsant properties of $GABA_A$ modulators against soman-triggered seizures than by systemic administration (Myhrer *et al.*, 2006b). A differentiation between cholinergic and glutamatergic antagonism against soman-induced seizures can be made when anti-Parkinson agents are microinfused into AT (Myhrer *et al.*, 2008a). Thus, it is possible for the microinfusion technique to serve as a tool for screening among drugs with anticonvulsant properties. Furthermore, selective lesions can be used to map potential trigger sites and/or propagation pathways for epileptiform activity induced by nerve agents.

Identification of target areas for nerve agents can allow specification of neurochemical receptors to obtain optimal anticonvulsant effects. Drugs with focal effects may have smaller side effects than drugs acting globally on common denominators for seizure propagation. Simple criteria derived from the three-phase model have been used in the search for effective anticonvulsants against nerve agents. The approach of identifying critical target areas for drug designing used in epilepsy research would probably also become efficient in the search for improved therapies against nerve agents (Myhrer, 2007).

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CHAPTER 64

Pharmacological Prophylaxis Against Nerve Agent Poisoning: Experimental Studies and Practical Implications

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I. INTRODUCTION

Inhibitors of cholinesterases are very important chemicals in the group of organophosphates (OPs). These compounds are used in industry, in veterinary and human medicine, and unfortunately in military (chemical warfare agents/nerve agents) and terrorist activities (Japanese incidents in 1994–1995). The bulk of these compounds is used as pesticides (insecticides, acaricides, etc.). According to the World Health Organization, more than one million serious accidental and two million suicidal poisonings with insecticides occur worldwide every year, and of these approximately 200,000 die, mostly in developing countries (Bajgar *et al.*, 2004, 2007a; Eyer, 2003). The mechanism of action, prophylaxis and treatment of intoxications with OP is a very hot topic at present.

The term prophylaxis is sometimes unclear; generally, it is limited to medical countermeasures applied just before penetration of a toxic agent into the organism without further antidotal therapy. It is also described as a pretreatment but usually antidotal treatment is performed. Thus, when treatment is unnecessary, it can be described as prophylaxis. Although successful prophylaxis can be observed for some OPs, full protection of the organism without post-exposure treatment, especially for soman poisoning, remains open. When treatment is given after the exposure, the term post-treatment is used. It is obvious that when the drug is administered prior to nerve agent exposure with the aim of protecting the organism, exposure to these agents is expected, and therefore post-exposure therapy can be used, i.e. pretreatment could be used as the right term. The term prophylaxis used in this chapter is limited to medical countermeasures applied relatively shortly before penetration of a toxic agent into the organism with the aim of protecting the organism against the toxic agent(s).

Prophylaxis against nerve agents has been described and summarized in various publications (Bajgar *et al.*, 2004, 2007a; Layish *et al.*, 2005; Patocka *et al.*, 2006). The basic mechanism of action of OPs is based on their ability to inhibit the enzyme acetylcholinesterase (AChE, EC 3.1.1.7) at cholinergic peripheral and central synapses (Bajgar *et al.*, 2004; Marrs *et al.*, 1996). In general, prophylaxis can be focused on protection of AChE against the inhibition using reversible cholinesterase inhibitors. The level of OP can be diminished by using enzymes hydrolyzing these agents or enzymes binding the agents (to specific proteins or to antibodies) and thereby reducing the OP level and inhibition of cholinesterases – AChE and butyrylcholinesterase (BuChE, EC 3.1.1.8) (scavenger effect) in the organism; this can be described as detoxification. Another approach to prophylaxis is based on using present antidotes and other drugs (Bajgar *et al.*, 2004, 2007a). Different drugs or their combinations can also be used. A simple scheme describing the fate of the agent in the organism and possible target sites for prophylaxis is shown in Figure 64.1.

II. PROTECTION OF AChE AGAINST INHIBITION

Intact AChE is a basic requirement for normal function of the organism and thus for effective prophylaxis. The enzyme is changed in a way that will make it resistant to OP. This can be achieved by using reversible inhibitors (preferably carbamates), which are able to inhibit AChE reversibly, and after spontaneous recovery of the activity (decarbamylation) normal AChE serves as a source of an active enzyme.

The ability of some carbamates to protect an organism poisoned with OP has been known for many years (Koelle, 1946; Koster, 1946). Physostigmine and neostigmine have been used to protect animals against DFP. The number of OPs studied for protection was enlarged, as well as the number of carbamates studied. The results are very dependent on experimental conditions; nevertheless, the protective effects of physostigmine, aminostigmine, pyridostigmine,

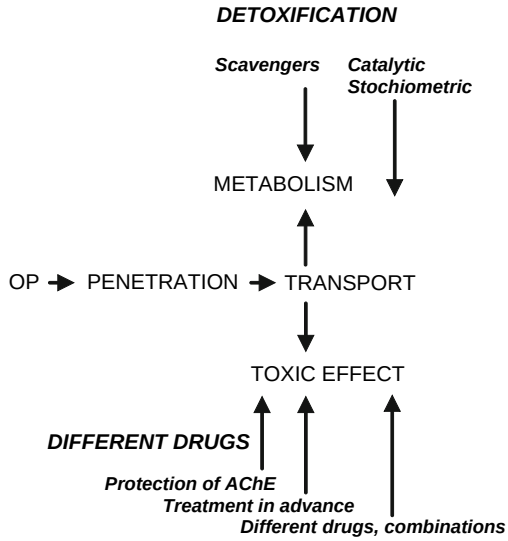

FIGURE 64.1. Four basic reactions of OP in the organism (in capitals) and possible approaches for prophylaxis (in italics)

and others against AChE inhibition caused by different OPs (mostly soman) have been demonstrated (Patocka, 1989; Marrs *et al.*, 1996; Tonkopii, 2003). It has been described that weak inhibitors are able to protect cholinesterases from strong inhibitors (paraoxon) (Petroianu *et al.*, 2005a, 2007). It appeared from the many studies that cholinesterase inhibitor pyridostigmine was the most promising prophylactic drug especially against soman poisoning (Anderson *et al.*, 1992; Bajgar *et al.*, 1994; Gordon *et al.*, 1978, 2005; Kassa and Bajgar, 1996; Kassa and Fusek, 1998, 2000; Kassa *et al.* 2001b, c; Koupilova and Kassa, 1999; Koplowitz *et al.*, 1992; Maxwell *et al.*, 1993; Patocka *et al.*, 1991; Tuovinen, 1998). On the basis of these results, pyridostigmine was introduced into some armies as a prophylactic drug against nerve agents. Its prophylactic effect (like the effects of other carbamates) is increased with its dose; however, with a higher dose, the side effects were more expressed, too. This problem can be solved by adding the pyridostigmine antagonizing drugs – anticholinergics. Many anticholinergics have been tested to protect the organism against intoxication with soman (and other nerve agents) and, on the basis of this research, the prophylactic combination PANPAL composed of pyridostigmine with trihexyphenidyle and benactyzine (Bajgar *et al.*, 1994, 1996; Fusek *et al.*, 2000, 2006; Kassa and Bajgar, 1996; Kassa and Fusek, 1998, 2000; Kassa *et al.*, 2001a) was introduced into the Czech army, and later on into the Slovak army. The presence of anticholinergics allowed us to increase the pyridostigmine dose and, simultaneously, to increase its prophylactic efficacy (Bajgar *et al.*, 2004, 2007a; Fusek *et al.*, 2006; Kassa *et al.*, 1997). Moreover, prophylaxis with PANPAL improves following post-exposure antidotal therapy with anticholinergics and oxime (Bajgar *et al.*, 2004; Kassa, 2006). The prophylactic antidotal combination called PANPAL has no side effects as demonstrated on volunteers: no statistically significant different changes in the actual psychic state as well as no negative changes in the dysfunction time were observed. An improvement of tapping test following PANPAL administration was demonstrated. A decrease in the heart frequency 60 min following PANPAL administration lasting 480 min and returning to normal values within 24 h was demonstrated (Fusek *et al.*, 2006).

Other carbamates also have a good prophylactic efficacy, especially physostigmine and aminostigmine (due to their central effect which is contrary to pyridostigmine) (Kim *et al.*, 2002; Tuovinen and Haninen, 1999; Tonkopii, 2003). Experimental studies with transdermal administration of physostigmine suggest a serious interest in the prophylactic use of this drug (Jenner *et al.*, 1995; Kim *et al.*, 2005; Levy *et al.*, 1992; Walter *et al.*, 1995).

Structurally different inhibitors from the carbamate and OP groups were also studied. From these compounds (preferably binding to the AChE anionic site), tacrine, its 7-methoxyderivative (7-MEOTA) and huperzine A, were considered and experimentally studied with respect to prophylaxis *in vitro* and *in vivo* (Bajgar, 2004). The most interesting results were obtained with huperzine A (Bajgar *et al.*, 2007b; Gordon *et al.*, 2005; Lallement *et al.*, 2002).

III. SCAVENGERS

Keeping AChE intact can be achieved by elimination of the agent before its penetration to the target sites containing the enzyme. In this case, the detoxification principle can be used in two different ways: administration of enzymes splitting the OP or specific enzymes (cholinesterases) which bind the OP. The enzymes hydrolyzing nerve agents are called catalytic scavengers and the enzymes binding nerve agent are described as stoichiometric scavengers. OP is bound to the exogenously administered enzyme or it is decomposed by the enzyme and thus the nerve agent level in the organism is decreased (the action of a ''scavenger''). Enzymes hydrolyzing OPs were thoroughly studied by Li *et al.* (1995) and Raveh *et al.* (1992). Catalytic scavengers are enzymes displaying a turnover with OP/nerve agents as substrates, allowing rapid and efficient protection (Masson *et al.*, 1998). Paraoxonase and similar enzymes are prospective hydrolyzing scavengers (Aharoni *et al.*, 2004; Rochu *et al.*, 2008). On the other hand, many studies have been made with cholinesterases as scavengers. AChE was used as a prophylaxis against organophosphate poisoning and correlation has been found between protection and blood–enzyme level in mice (Raveh *et al.*, 1989; Wolfe *et al.*, 1987). BuChE and AChE were observed to be very effective in protection against OP intoxication (Ashani *et al.*, 1991; Doctor *et al.*, 1991, 1997, 2002; Maxwell *et al.*, 1993, 1998; Moore, 1996, Saxena *et al.*, 1997). The idea to use the plasma as a source of cholinesterase to eliminate nerve agent action after peritoneal dialysis with the human plasma in rats was also described (Bajgar *et al.*, 1982) but without further

development. The administration of enzymes as scavengers seems to be very promising: the enzyme is acting at the very beginning of the toxic action, without interaction with the target tissues and without side effects (Doctor *et al.*, 1997, 2002; Huang *et al.*, 2007; Saxena *et al.*, 2004). All of these features are of great interest and they are yielding practical results – isolation of the enzyme, examination for lack of an autoimmune response, and establishment of pharmacokinetic and pharmacodynamic properties (Saxena *et al.*, 2002). Recombinant human BuChE can be produced from the milk of transgenic goats (Cerasoli *et al.*, 2005). Moreover, BuChE pretreatment also showed protective effects on AChE inhibition in the brain regions following low-level sarin inhalation exposure: the enzyme (BuChE) administered into the blood stream does not influence erythrocyte AChE, plasma BuChE activity is increased, and the brain AChE is not affected. Following intoxication with nerve agent, the agent is bound to plasma BuChE (now existing in excess), and proportionally bound to erythrocyte AChE; both enzymes are inhibited, however, to a lesser extent in comparison with the unpretreated situation. The part of the nerve agent bound to both enzymes represented a loss of the agent and the diminished level of nerve agent is able to penetrate the brain. Therefore, AChE activity in the brain is protected (Bajgar *et al.*, 2007c; Sevelova *et al.*, 2004).

The efficacy of cholinesterases as a bioscavenger of OP can be enhanced by combining enzyme pretreatment with oxime reactivation, since the scavenging capacity extends beyond a stoichiometric ratio of cholinesterases to OP. Human BuChE has previously been shown to protect mice, rats, and monkeys against multiple lethal toxic doses of organophosphorus anticholinesterases (Allon *et al.*, 1998; Maxwell, 1992). An interesting approach can be simultaneous administration of BuChE and reactivators; BuChE acts as a scavenger binding the nerve agents. A reactivator acting as a pseudocatalytic bioscavenger reactivates BuChE simultaneously and reactivated enzyme serves as a new scavenger (Jun *et al.*, 2007).

Given our increasing knowledge of bioengineering and biotechnology, recombinant human BuChE with a good protective effect against nerve agents was obtained from transgenic animals. A connection between these two enzymes (binding and hydrolyzing) is possible with the aim of obtaining a modified enzyme splitting OPs and simultaneously reacting with AChE as a scavenger (Broomfield *et al.*, 1997). Antibodies against OPs are at the research stage and are more focused on the detection of OPs (Lenz *et al.*, 1997; Miller and Lenz, 2001).

IV. PROPHYLAXIS WITH CURRENT ANTIDOTES

The aim of this approach was to achieve a sufficient level of antidotes in the blood circulation before intoxication, so the antidotes can be tested as prophylactics. This can be characterized as ''treatment in advance''. The problem with their use, however, is the timing, duration, and achievement of sufficient levels of these antidotes after administration. The antidotes currently used for the treatment of OP poisoning in this context include anticholinergics, reactivators, anticonvulsants, and others (Bajgar *et al.*, 2004, 2007a). The prophylactic efficacy of antidotes (as demonstrated in treatment studies) is good – administration of the antidotes typically takes place very shortly (minutes) after intoxication. Another study showed good prophylactic efficacy of pyridostigmine or ranitidine with pralidoxime against paraoxon poisoning (Petroianu *et al.*, 2005b, 2006). Extension of the duration of the effects of the antidote by achievement of their sufficient level in the blood by oral administration is not possible (especially reactivators, as they are decomposed in the gastrointestinal tract) and therefore is excluded. Therefore other routes of administration were sought. Transdermal administration of one of the most effective reactivators (HI-6) was shown to be the most realistic approach (Dolezal *et al.*, 1988; Bajgar, 1991). The final result was the new prophylactic transdermal antidote TRANSANT containing the patch impregnated with HI-6. This preparation was clinically tested (including dermal sensitivity) without any harmful effects; field testing was also successful and TRANSANT was introduced into the Czech army and later into the Slovak army (Bajgar *et al.*, 2004; Fusek *et al.*, 2007). If its administration is combined with PANPAL, the two anticholinergics contained in PANPAL are able to prevent and treat symptoms of nerve agent poisoning. Therefore, a combination of TRANSANT and PANPAL administered as a pretreatment provides the best prophylactic efficacy in comparison with pyridostigmine or PANPAL administered alone (Bajgar *et al.*, 2004). The anticonvulsant drugs benzodiazepines (diazepam, midazolam, alprazolam, triazolam, clonazepam) were studied, but isolated prophylactic administration did not offer very good effects (Herink *et al.*, 1990, 1991; Kubova *et al.*, 1990).

V. PROPHYLACTIC USE OF OTHER DRUGS

Prophylactic administration of different drugs (alone or in combination) against intoxication with OPs was studied. Calcium antagonists (nimodipine), neuromuscular blockers (tubocurarine), adamantanes (memantine), and the opiate antagonist meptazinol (Galli and Mazri, 1988; Gupta and Dettbarn, 1992; McLean *et al.*, 1992; Marrs *et al.*, 1996; Karlsson *et al.*, 1998; Stojiljkovic *et al.*, 1998) were also tested, and offered different results with limited practical utility. On the other hand, a positive prophylactic effect has been demonstrated with procyclidine (antimuscarinic, antinicotinic, and the anti-NMDA receptor drug) (Myhrer *et al.*, 2002, 2003), metoclopramide (Hasan *et al.*, 2004), clonidine (Loke *et al.*, 2001), or procyclidine and donepezil (Haug *et al.*, 2007). The prophylactic effect of a group of drugs

TABLE 64.1. Drugs used in the prophylaxis against OP nerve agent poisoning (relatively perspective drugs are in bold)

Principle	Drug group	Drug	Duration	Equipment of the army	Efficacy	Comment
Protection of cholinesterase against inhibition	carbamates	**Pyridostigmine, Aminostigmine, Physostigmine** Syntostigmine, Eptastigmine, Mobam Decarbofuran, Heptylphysostigmine	8 h	PYRIDOSTIGMINE BROMIDE	+++	Dose limited, side effects. Alone is not very effective, following antidotal treatment enhances its effect
	others	**Huperzine A** Tacrine, Methoxytacrine				
	organophosphates					
		TEPP, Paraoxon Ethyl-4-nitrophenylphosphonate				
	aminophenols	Eseroline				
Simulation of treatment	anticholinergics	**Biperidene, Scopolamine, Benactyzine** Atropine, Aprophen, Hyoscine Adiphenine, Caramiphen Pentamethonium, Mecamylamine Trihexyphenidyle				Transdermal administrations of scopolamine, physostigmine and HI-6 were studied
	reactivators	HI-6				
		PAM, Obidoxime, Trimedoxime Methoxime	8 h	TRANSANT (HI-6, transdermal admiministration)	+	Alone is not effective
	others	**Suramine** Benzodiazepines, Tubocurarine Memantine, Procyclidine Nimodipin, Clonidine				
Detoxification	cholinesterases	**Butyrylcholinesterase, Mutants**				
	enzymes hydrolyzing OP	Acetylcholinesterase				Very perspective PROTEXIA®
	monoclonal antibodies against OP	Triesterase Paraoxonase				
Combinations			8 h	PANPAL (pyridostigmine, trihexyphenidyle, benactyzine)	++++	Efficacy is increased with following antidotal treatment
				PANPAL + TRANSANT	+++++	In combination, the best prophylactic efficacy

with anticholinergic and/or antiglutamatergic properties (benactyzine, biperiden, caramiphen, procyclidine, and trihexyphenidyl) with respect to their anticonvulsant properties was studied to prevent damage of the central nervous system induced by seizures. Only procyclidine and caramiphen antagonized soman-induced seizures (Myhrer *et al.*, 2008a). Among the different drugs tested, procyclidine appears to be an effective anticonvulsant with a few cognitive side effects (Myhrer *et al.*, 2008b). Procyclidine with physostigmine (administered transdermally) showed very good prophylactic efficacy against soman in dogs; moreover, this efficacy was increased using antidotal therapy with HI-6 and atropine (Kim *et al.*, 2005). Special importance can be focused on suramine (a protease inhibitor). Administration of this compound prior to soman intoxication (and followed by administration of atropine) showed good prophylactic effect (Cowan *et al.*, 1996). However, all these studies are experimental ones and they have not reached the practical output stage. The combinations of various drugs as prophylactics can be of very different character. They can be used simultaneously (a combination of different drugs) or as pretreatment and posttreatment with different antidotes. Administration of pyridostigmine (or other AChE inhibitors) prior to intoxication and treatment with different drugs is a typical example (Anderson *et al.*, 1992, 1997; Bajgar *et al.*, 1996; Kassa, 1995; Kassa and Bajgar, 1996; Kim *et al.*, 2002; Tuovinen and Haninen, 1999). There are other combinations such as the administration of triesterase (Tuovinen and Haninen, 1999; Tuovinen *et al.*, 1999), procyclidine (Kim *et al.*, 2002; Myhrer *et al.*, 2002, 2003), clonidine (Loke *et al.*, 2001), or sustained release of physostigmine and scopolamine (Meshulam *et al.*, 2001). The results are dependent on experimental conditions but this approach – administration of different drugs – has yielded some good results though up to now they have been on an experimental level. Only three prophylactics have been introduced into different armies – pyridostigmine alone, PANPAL composed of pyridostigmine, benactyzine, and trihexyphenidyle, and TRANSANT (dermal administration of HI-6).

It appears from these results that simple prophylaxis (without post-exposure treatment) against OP is not sufficient. Therefore, pyridostigmine has importance as a prophylactic drug especially when it is combined with post-exposure antidotal treatment. For further development, it is necessary to search for novel prophylactic drugs and new routes of administration. In this context, preparations of cholinesterases are of special importance for the development of more effective prophylactics.

VI. CONCLUDING REMARKS AND FUTURE DIRECTION

There are many drugs being tested for their prophylactic efficacy against nerve agent intoxication. However, only three prophylactics (pyridostigmine alone, PANPAL, and TRANSANT) have been introduced into military medical practice. The perspective approach seems to be searching for more effective drugs or their combinations, including new routes of administration, and the use of purified enzymes, especially cholinesterases or paraoxonases produced by biotechnology.

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CHAPTER 65

Pyridinium Oximes as Cholinesterase Reactivators in the Treatment of OP Poisoning

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I. INTRODUCTION

Organophosphorus compounds (OPs) have been used as pesticides and developed as warfare nerve agents such as tabun, soman, sarin, VX, and others. Exposure to even small amounts of an organophosphorus compound can be fatal; death is usually caused by respiratory failure resulting from paralysis of the diaphragm and intercostal muscles, depression of the brain respiratory center, bronchospasm, and excessive bronchial secretions. The mechanism of OP poisoning involves phosphorylation of the serine hydroxyl group in the active site of acetylcholinesterase (AChE) leading to the inactivation of this essential enzyme which has an important role in neurotransmission. AChE inhibition results in the accumulation of acetylcholine (ACh) at cholinergic receptor sites, producing continuous stimulation of cholinergic nerves throughout the central and peripheral nervous systems. Currently, a combination of an anti-muscarinic agent, e.g. atropine, AChE reactivator such as one of the recommended pyridinium oximes (PAM-2, TMB-4, HI-6, LüH-6), and diazepam are used for the treatment of organophosphate poisoning in humans. This chapter describes the mechanisms of action of OPs and the role of pyridinium oximes used as AChE reactivators in the treatment of OP poisoning.

II. INTERACTION OF CHOLINESTERASES WITH ORGANOPHOSPHATES

There are different types of cholinesterases in the human body, which differ in their location in tissues, substrate affinity, and physiological function. The principal ones are acetylcholinesterase (AchE, EC 3.1.1.7), found in the nervous system and also present in the outer membrane of red blood cells, and plasma cholinesterases (EC 3.1.1.8, ChE), which are a group of enzymes present in plasma, liver, cerebrospinal fluid, and glial cells. Under normal physiological conditions, AChE performs the breakdown of acetylcholine (ACh), which is the chemical mediator responsible for conduction of nerve impulses at the sites of cholinergic transmission. However, its physiological role in blood is not understood. On the other hand, ChE is a circulating plasma glycoprotein synthesized in the liver, which does not serve any known physiological function. Recent evidence indicates that ChE may have roles in cholinergic neurotransmission and involvement in other nervous system functions (cellular proliferation and neurite growth during the development of the nervous system) and in neurodegenerative disorders (Darvesh *et al.*, 2003).

The function of AChE is termination of action of ACh at the junctions of the various cholinergic nerve endings with their effector organs or post-synaptic sites. Organophosphorus compounds and carbamates are the most important AChE inhibitors and are often called anticholinesterases. In the presence of inhibitors, AChE becomes progressively inhibited and is not further capable of hydrolyzing ACh to choline and acetic acid (Jokanović and Maksimović, 1997). ACh accumulates at cholinergic receptor sites and produces effects equivalent to excessive stimulation of cholinergic receptors throughout the central and peripheral nervous systems.

Both substrate and inhibitors react covalently with the esterases in essentially the same manner, because acetylation of the serine residue at AChE catalytic sites is analogous to phosphorylation. In contrast to the acetylated enzyme which rapidly separates acetic acid and restores the catalytic site, the phosphorylated enzyme is stable (Figure 65.1). Inhibited enzyme can be spontaneously reactivated at different rates depending on the inhibitor – for carbamates it occurs very rapidly, while for organophosphates having branched alkyl groups it may occur at a very slow rate. In addition, AChE inhibited with organophosphorus insecticides, having mainly a dimethyl and diethyl phosphate structure, can spontaneously reactivate with half-times for dimethyl phosphates of 1.3 h *in vitro* and 2.1 h *in vivo*. The rate of spontaneous reactivation for diethyl phosphates bound to AChE is much slower (Reiner and Pleština, 1979; Eyer, 2003). As a result, carbamates and dimethyl phosphates are recognized as reversible AChE inhibitors, while other organophosphorus compounds

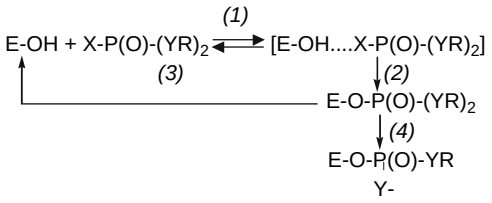

FIGURE 65.1. Interaction of acetylcholinesterase (AChE) and other esterases (E) with organophosphorus esters. Reaction 1 shows interaction of organophosphate molecule with the serine hydroxyl group at the active site of AChE via formation of an intermediate Michaelis–Menten complex leading to phosphorylated enzyme (Reaction 2). Reaction 3 is a spontaneous reactivation of inhibited AChE which occurs very slowly for most OP compounds. Reaction 4, called ''aging'', represents nonenzymatic time-dependent loss of one alkyl group (R) bound to the phosphorus. The aging reaction depends on the chemical structure of the inhibitor and leads to a stable nonreactivatable form of phosphorylated AChE. Y stands for O-, N-, or S-.

having branched alkyl groups are practically irreversible AChE inhibitors.

The variations in the acute toxicity of OPs are the result of their different chemical structures and rates of spontaneous reactivation and aging. The aged form of phosphorylated AChE is resistant to both spontaneous and oxime-induced reactivation. For example, when complexed with VX, AChE in red blood cells spontaneously reactivates at a rate of about 1% per hour for about the first 48 h while the VX–AChE complex ages very little during this period. The soman–AChE complex does not spontaneously reactivate since the half-time for aging is about 2–4 min. The half-time for aging of the sarin–AChE complex is between 5 and 12 h (depending on experimental conditions) and only about 5% of the enzyme undergoes spontaneous reactivation. The half-time for aging of the tabun–AChE complex is about 46 h (Karczmar, 1984; Sidell, 1997; Stojiljković *et al.*, 2001). For OP pesticides containing dimethyl phosphate groups the half-time of spontaneous reactivation of phosphorylated AChE *in vitro* is about 1 h and the half-time of aging is 3.7 h, while these for diethyl phosphates are slower being about 31–57 h and 31 h, respectively (Worek *et al.*, 1999; Mason *et al.*, 2000; Eyer, 2003).

The aging reaction, although appearing with many phosphorylated AChE complexes, has the only major clinical importance and an imperative problem particularly in the treatment of soman poisoning. Aging with soman occurs so fast that no clinically relevant spontaneous reactivation of AChE can occur before aging has taken place. Hence, recovery of function depends on resynthesis of AChE which is a relatively slow process. After soman exposure, it is important to immediately administer atropine and oximes so that some extent of AChE reactivation occurs before all AChE has aged. Even though aging occurs slowly and reactivation relatively rapidly in the case of other nerve agents and OPs, early oxime administration is clinically important particularly in patients poisoned with these agents.

The aging reaction is not the unique property of cholinesterases since it also occurs on other serine esterases such as neuropathy target esterase (NTE) (Johnson, 1982). Although the toxicological consequences of inhibition of AChE and NTE are different, the mechanism of aging on both enzymes is the same as shown in Figure 65.1, and it occurs at a comparable rate for most tested OPs. Earlier studies have shown that atropine can significantly decrease the rate of aging on hen brain NTE inhibited with DFP with a half-time of 7 min (without atropine) and 108 min (with atropine) and this result was confirmed under *in vivo* conditions (Jokanović *et al.*, 1998; Stepanović Petrović *et al.*, 2000). It was also shown that atropine can slow down the aging reaction of soman-inhibited AChE from red blood cells (Schoene and Wulf, 1972; Kuhnen *et al.*, 1985; Van Dongen *et al.*, 1987). Even though no data have been published, it is possible that atropine can show a similar effect on AChE inhibited by other OPs.

III. CLINICAL ASPECTS OF ACUTE OP POISONING

Signs and symptoms of acute poisoning with anticholinesterase agents are predictable from their biochemical mechanism of action and are directly related to the levels of AChE activity (Table 65.1). Three types of effects evident in these poisonings are muscarinic, nicotinic, and central signs and symptoms. The duration of effects is determined mainly by the properties of the compound: its liposolubility, the stability of the OP–AChE complex and whether it is reactivatable after the use of cholinesterase reactivators (such as oximes). It is important to note that only OPs containing the P=O bond (known as direct inhibitors) are potent AChE inhibitors; those having a P=S group (indirect inhibitors) must be metabolically activated to the P=O group (Jokanović, 2001). The signs and symptoms of poisoning with direct inhibitors appear quickly during or after exposure, while those with indirect inhibitors appear slowly and last longer, even up to several days after cessation of exposure.

Clinical diagnosis is relatively simple and is based on medical history, circumstances of exposure, and the presence of clinical symptoms of poisoning. Confirmation of diagnosis can be made by measurement of red blood cell AChE or plasma ChE. Activities of these enzymes are accepted as biomarkers of exposure and/or toxicity of OPs and carbamates. Red blood cell AChE is identical to the enzyme present in the target synapses and its levels are assumed to reflect the effects in target organs. Thus, red blood cell AChE is regarded as a biomarker of toxicity of these compounds. However, it must be kept in mind that the above assumption is only correct when the inhibitor has equal access to blood and synapses. It is difficult to know

TABLE 65.1. Signs and symptoms of poisoning with organophosphorus compounds

Signs and symptoms			Severity of poisoning	Red blood cell AchE (% of control)
Muscarinic	**Nicotinic**	**Central**		
Nausea, vomiting, diarrhea, salivation, lacrimation, bradycardia and arrhythmia, bronchoconstriction, bronchosecretion		Headache, dizziness, drowsiness, anxiety	Mild	>40
As above plus miotic pupils (unreactive to light), involuntary defecation and urination	Twitching of fine muscles, hyperreflexia, fasciculations	As above plus ataxia, psychosis, tremor, dysarthria, slurred speech	Moderate	20–40
	As above plus muscle weakness, reduced tendinous reflexes, paralysis affecting diaphragm and respiratory muscles	As above plus coma, convulsions, respiratory depression	Severe	<20

how closely AChE inhibition in red blood cells reflects this in the nervous system since access to blood is always easier than access to brain. Thus, the inhibition of AChE in red blood cells may be overestimated relative to that in brain (Jokanović and Maksimović, 1997). In addition, AChE in brain is restored by *de novo* synthesis more rapidly than in red blood cells.

The rate of spontaneous reactivation (Figure 65.1, Reaction 3) can be accelerated by pyridinium oximes that have a chemical structure which ''fits'' the structure of the inhibited AChE. The oximes can only be of benefit as long as inhibited AChE is not completely converted to the aged form.

IV. ANTIDOTES IN THE TREATMENT OF OP POISONING

A. Atropine

Atropine acts by blocking the effects of excess concentrations of acetylcholine at muscarinic cholinergic synapses following OP inhibition of AChE. Atropine is the initial drug of choice in acute OP poisoning. Atropine sulfate in combination with an oxime has been used in traditional therapy for intoxication with OP nerve agents as well as with OP insecticides. Atropine can relieve the following symptoms of OP poisoning: sweating, salivation, rhinorrhea, lacrimation, nausea, vomiting and diarrhea, and can help control of bradycardia and circulatory depressions, dilating the bronchi and abolishing bronchorrhea. Atropine does not bind to nicotinic receptors and cannot relieve nicotinic effects of OPCs.

Support for an anticonvulsant property of atropine has been presented by McDonough *et al.* (1987) who found that atropine pretreatment prevents the development of convulsions and brain damage induced by soman and VX. Other authors have stated that atropine can only partly block convulsions after exposure to these agents since other transmitter systems (GABA, glutamate) become involved in cholinergic overstimulation in brain (Zilker, 2005; Antonijević and Stojiljkovic, 2007).

The effects of atropine in OP poisoning are far more complex than muscarinic blockade. In a study in rats, Pazdernik *et al.* (1986) investigated the effect of atropine pretreatment on local cerebral glusose use during seizures induced with soman. Atropine treatment reduced local use of cerebral glucose and brain damage as well. Atropine may be effective in treating acute dystonic reactions and primary position upbeat nystagmus occasionally observed in acute OP poisoning (Heath *et al.*, 1992). Although the clinical efficacy of atropine in OP poisoning is well established, no controlled studies have been published.

The standard dosing of atropine depends on the severity of OP poisoning. The initial dose is usually 2 mg in an adult (0.02 mg/kg in a child) given every 5–10 min until bronchosecretion is cleared and the patient is atropinized (dry skin, sinus tachycardia) which should be maintained during further treatment. The dose may be increased as required. Patients poisoned with OPs appear to be resistant to toxic effects of atropine and may require relatively large doses of atropine administered during prolonged periods. According to IPCS (2002), in severe OP poisoning the total dose of atropine given during 5 weeks of treatment can be as high as 30,000 mg.

B. Diazepam

Benzodiazepines are CNS depressants, anxiolytics, and muscle relaxants. Their main site of action is at the

gamma-aminobutyric acid (GABA) receptor. The $GABA_A$ receptor is a ligand-gated chloride ion channel and part of a superfamily of receptors which also includes the nicotinic acetylcholine receptor and the glycine receptor. GABA is the major inhibitory neurotransmitter in the mammalian central nervous system. Benzodiazepines, including diazepam, alter GABA binding at the $GABA_A$ receptor in an allosteric fashion but these drugs do not directly activate the receptors (Sellström, 1992; Marrs, 2004).

Currently, the most important anticonvulsant is diazepam. The combination of atropine and diazepam is more effective in reducing mortality than atropine or oxime alone. It was also shown that diazepam enhanced the efficacy of low doses of atropine. In the cholinergic nervous system, diazepam appears to decrease the synaptic release of ACh. The main consequence of the action of benzodiazepines in CNS is hyperpolarization of neurons making them significantly less susceptible to cholinergically induced depolarization. The ultimate result is cessation of propagation of convulsions (Sellström, 1992; Marrs, 2004; Antonijević and Stojiljkovic, 2007).

In patients poisoned with OPs, benzodiazepines may have a beneficial effect in reducing anxiety and restlessness, reducing muscle fasciculation, arresting seizures and convulsions, controlling apprehension and agitation, and possibly reducing morbidity and mortality when used in conjunction with atropine and an oxime. Diazepam should be given to patients poisoned with OPs whenever convulsions or pronounced muscle fasciculation are present. In severe poisoning, diazepam administration should be considered even before these complications develop. The recommended dose of diazepam in cases of OPS poisoning is 5–10 mg i.v. in the absence of convulsions and 10–20 mg i.v. in cases with convulsions, which may be repeated as required (Johnson and Vale, 1992; Antonijević and Stojiljkovic, 2007).

C. Oximes

Extensive studies over the past decades have investigated the mechanism of oxime action. There is convincing evidence that the antidotal potency of oximes is primarily attributed to their abilities to reactivate the phosphorylated cholinesterases. Oximes reactivate phosphorylated cholinesterases by displacing the phosphoryl moiety from the enzyme by virtue of their high affinity for the enzyme and their powerful nucleophilicity. Reactivation proceeds as a two-step reaction via formation of an intermediate Michaelis–Menten complex leading to formation of more stable phosphoryl residue bound to the hydroxyl group of serine at the active site of AChE. The rate of reactivation depends on the structure of the phosphoryl moiety bound to the enzyme, the source of the enzyme, the structure and concentration of oxime which is present at the active site, and the rate of post-inhibitory dealkylation known as aging. Phosphorylated oximes are formed during reactivation reaction (Figure 65.2) and some of them appear to be potent inhibitors of AChE (Luo *et al.*, 1999; Ashani *et al.*, 2003; Worek *et al.*, 2007). It was shown that phosphorylated oximes of 2-substituted pyridinium compounds (e.g. 2-PAM, HI-6) are rather unstable while those of 4-pyridinium aldoximes are markedly stable (Ashani *et al.*, 2003; Kiderlen *et al.*, 2005; Worek *et al.*, 2000).

Oximes bind to AChE as reversible inhibitors and form complexes with AChE either at the acylation (catalytic) site, at the allosteric site, or at both sites of the enzyme and protect AChE from phosphorylation. When the reversible inhibitor binds to the catalytic site, the protection is due to direct competition between OP and reversible inhibitor. Binding of a reversible inhibitor to the allosteric site induces indirect protection of the active site. Differences in the mechanisms of enzyme reactivation and protection demonstrate how stereochemical arrangements of oximes can play a role in the potency of their therapeutic efficacy. Direct pharmacological effects, such as direct reaction with OPs (Van Helden *et al.*, 1996), anticholinergic and sympathomimetic effects may also be relevant for the interpretation of antidotal potency of oximes.

In addition to performing AChE reactivation in OPs poisoning, oximes might also show some direct pharmacological effects (Jokanović and Stojiljkovic, 2006). Previous studies supported observations that in some studies the antidotal effects of oximes could be explained only on the basis of AChE reactivation (Schoene *et al.*, 1976; Clement, 1981; Van Helden *et al.*, 1983). Former studies involving adult rhesus macaques (Hamilton and Lundy, 1989) or marmoset monkeys (Van Helden *et al.*, 1992) showed that only limited or no reactivation of soman-inhibited cholinesterase activity occurred when HI-6 was administered in conjunction with atropine and diazepam. These results suggested that some additional mechanism of action of HI-6, other than cholinesterase reactivation, is partly responsible for this enhanced protective activity of HI-6 (Hamilton and Lundy, 1989; Van Helden *et al.*, 1992). HI-6 may have effects on the GABAergic neurotransmission in the CNS (Melchers *et al.*, 1994a).

Furthermore, it was observed that the oxime HI-6 might show direct pharmacological effects in the cholinergic nervous system in skeletal muscles. It has been found that HI-6 reduces the miniature endplate potentials and increases the quantal content by a dose-dependent decrease in the miniature endplate potential amplitude (Melchers *et al.*, 1991). Other possible explanations have been suggested for oximes at other targets in the nervous system, such as

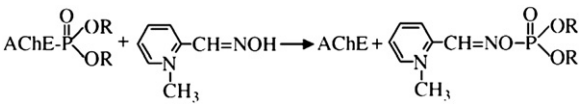

FIGURE 65.2. Reactivation of phosphorylated acetylcholinesterase with pralidoxime and formation of reactivated enzyme and phosphorylated oxime.

alteration of the release of neurotransmitters (Clement, 1979; Kloog *et al.*, 1986), inhibition of acetylcholine release by HI-6 (Van Helden *et al.*, 1998), inhibition of synthesis of neurotransmitters (Clement, 1979), interaction with presynaptic cholinergic nerve terminals (Aas, 1996), and interaction at post-synaptic receptors (Van Helden *et al.*, 1996). It has been shown that both 2-PAM and HI-6 interact with the nicotinic receptor ion complex (Alkondon *et al.*, 1988). The two bispyridinium oximes HI-6 and HLö-7 had significant dose-dependent inhibitory effects on the potassium-evoked release of [^{3}H]acetylcholine from the rat hippocampal slice preparation *in vitro* when the AChE was inhibited with soman. When the AChE was inhibited with soman, two bispyridinium oximes HI-6 and HLö-7 had significant dose-dependent inhibitory effects on the potassium-evoked release of [^{3}H]acetylcholine from the rat hippocampal slice preparation *in vitro*.

Toxic effects of soman and enhanced cholinergic activity might be reduced by suppressing ACh release from cholinergic nerves, thereby reducing the enhanced activation of both muscarinic and nicotinic cholinergic receptors in the brain (Øydvin *et al.*, 2005). It was shown that bispyridinium oximes can slow down the aging reaction of soman-inhibited AChE from red blood cells (Schoene and Wulf, 1972). Oximes may also act as cholinergic blocking drugs at the nicotinic sites (Sidell, 1997). In addition, it has also been proposed that the oximes could participate in hydrolysis of OPs and show direct anticonvulsant effects as well as respiratory stimulation. Patients treated with HI-6 following OP intoxication have shown more rapid improvement than expected on the basis of AChE reactivation. Such effects were attributed to direct pharmacological properties of HI-6 (Kušić *et al.*, 1991).

Mono- and bis-pyridinium oximes are effective against OP-inhibited AChE in the peripheral nervous system, but have a limited penetration across the blood–brain barrier. Limited penetration of HI-6 is due to its pharmacokinetic profile and the two quaternary nitrogen atoms in its structure. However, it appears that oxime penetration through the blood–brain barrier is underestimated since soman can cause seizure-related opening of the blood–brain barrier (Carpentier *et al.*, 1990; Grange-Messent *et al.*, 1999) and thus enable passage of higher oxime concentrations into the brain. Abdel-Rahman *et al.* (2002) have shown that 0.5–1.0 LD_{50} of sarin caused a dose-dependent increase in permeability of the blood–brain barrier in midbrain, brainstem, cerebrum, and cerebellum in rats 24 h after poisoning. Sakurada and coworkers (2003) have determined the amount of 2-PAM passing across the blood–brain barrier at approximately 10% of the given dose which may be effective in reactivation of OP-inhibited AChE in brain. H oximes penetrate the blood–brain barrier at a concentration sufficient to produce biochemical and physiological actions in soman poisoning (Kassa, 2002, 2005). Also, additional data indicate that in OP poisoning, when given with atropine, 2-PAM can pass the blood–brain barrier at higher concentrations.

Possible reasons why oximes may not be effective in OP poisoning were discussed in detail by Johnson *et al.* (2000) and Eyer (2003). Among those reasons the most important are the following:

(a) The oxime dose may be inadequate to produce the optimal concentration required to achieve the desired reactivation and it may not be present at target sites when needed most (i.e. when AChE inhibition reaches its maximum and when OP is present in blood at high concentrations). Proposed minimum-effective plasma levels for oximes of 4 mg/l were also supported by Bokonjić *et al.* (1987), Shiloff and Clement (1987), and Kušić *et al.* (1991). In severe cases of OP pesticide poisoning, higher oxime concentrations may be necessary, especially in the case of pralidoxime. However, Eyer (2003) disagrees with the 4 mg/l concept suggesting necessity of a higher oxime concentration. He proposed that for the most frequently used OPs, pralidoxime plasma concentrations of around 80 µmol/l (13.8 mg/l pralidoxime chloride) or 10 µmol/l of obidoxime (3.6 mg/l obidoxime chloride) should be adequate and maintained for as long as the OP is present in the body. Oxime treatment may be required for up to 10 days.

(b) Besides an inadequate initial dose, subsequent treatment with oxime may not be sufficiently persistent. Oximes are rapidly cleared from the body and although some reactivation may be achieved, another cycle of inhibition and eventually aging of inhibited AChE, due to continuing presence of OPs in blood, may follow. This is particularly possible in the case of massive overdose where residual OP pesticide may persist in the body for several days. In such cases only persistent administration of oximes, by means of continuous infusion or repeated oxime administration, can be expected to result in permanent clinical improvement. Eyer (2003) suggests that the most appropriate dosing regimen consists of a bolus short infusion followed by a maintenance dosage. For pralidoxime chloride a 1 g bolus over 30 min followed by an infusion of 0.5 g/h appears appropriate to maintain the concentration of 13 mg/l. For obidoxime chloride the proposed dosing regimen is a 0.25 g bolus followed by an infusion of 0.75 g/24 h. It is important that the concentrations are well tolerated and effective in keeping the active levels of AChE.

(c) Treatment with oxime may be started too late or terminated too soon. In all cases of poisoning with OPs it would be ideal to start antidotal treatment as soon as possible and to maintain the treatment as long as needed. In cases of poisoning with persistent OP pesticides it is appropriate to start oxime therapy at adequate dose levels up to 10 days after exposure or even later. Eddleston *et al.* (2006) suggest that oximes may be effective if given within about 120 h for diethyl

organophosphorus poisoning and 12 h for dimethyl organophosphorus poisoning. The treatment should be continued until obviously no longer needed and the decission about this can be made on the basis of clinical status of the patient, relatively high AChE activity in red blood cells when compared to control values, and the absence of OPs and/or OP metabolites in urine.

Detailed therapeutic regimens used in the treatment of poisoning with OP compounds are presented in several excellent reviews (WHO, 1986; Lotti, 1991; Johnson and Vale, 1992; Bismuth *et al.*, 1992; Johnson *et al.*, 2000; Eyer, 2003; Marrs and Vale, 2006).

V. PYRIDINIUM OXIMES USED IN THE TREATMENT OF POISONING WITH NERVE AGENTS AND THEIR EFFICACY

Among the many classes of oximes investigated so far, those with clinical application can be divided in two groups – the monopyridinium and bispyridinium oximes. Currently, the only used monopyridinium oxime is pralidoxime (2-PAM), while the most significant bispyridinium oximes comprise trimedoxime (TMB-4), obidoxime (LüH-6, Toxogonin), HI-6 and HLö-7 (Figure 65.3) (Dawson, 1994). There is still no international consensus on the choice of most effective oxime and on dosing regimen.

A. Pralidoxime (PAM-2)

Pralidoxime was synthesized in the USA in 1955 (Wilson and Ginsburg, 1955). Its four salts – chloride (2-PAM Cl), methiodide, methylsulfate, and mesylate (P2S) – were investigated and introduced into practice. 2-PAM is very efficient in reactivating of AChE inhibited with sarin or VX (Johnson and Stewart, 1970; Sidell and Groff, 1974; Harris and Stitcher, 1983; Mesić *et al.*, 1991; Masuda *et al.*, 1995; Nozaki and Aikawa, 1995), but was not successful in reactivation of the tabun- or soman-inhibited enzyme (Inns and Leadbeater, 1983; Koplovitz and Stewart, 1994). Since 2-PAM, as quaternary pyridinium salt, does not readily penetrate across the blood–brain barrier, pro-2-PAM was synthesized as a pro-drug of 2-PAM to gain access to the central nervous system. However, pro-2-PAM was less effective than 2-PAM against experimental poisoning with paraoxon (Bošković *et al.*, 1980). The amount of 2-PAM passing across the blood–brain barrier was quantified as approximately 10% of the given dose, indicating that this level might be effective in reactivation of OP-inhibited AChE in brain (Sakurada *et al.*, 2003). However, in OP poisoning, it appears that 2-PAM, when given with atropine, can pass the blood–brain barrier at higher concentrations.

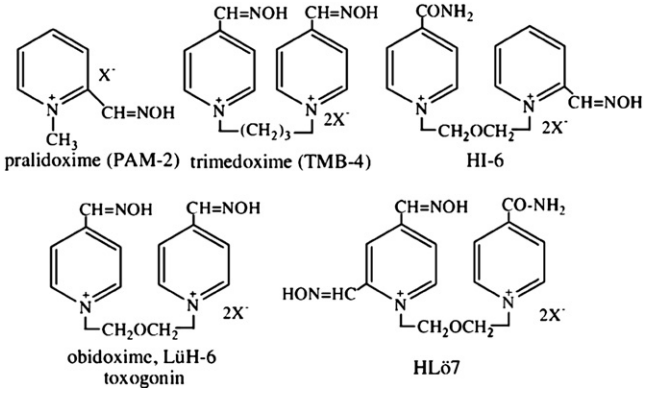

FIGURE 65.3. Chemical structure of pyridinium oximes used in treatment of OP poisoning. X stands for an anion.

Studies with nonhuman primates proved that the combination of atropine and 2-PAM protected against up to five times the LD_{50} of all known nerve agents except soman (Stojiljković *et al.*, 2001).

Pralidoxime administered to human volunteers at a dose of 10 mg/kg by intramuscular route produced a plasma concentration of >4 mg/l within 5–10 min and maintained levels above this threshold for an hour (Sidell and Groff, 1971). Adverse effects of 2-PAM iodide in volunteers include dizziness, blurred vision, occasional diplopia, impaired accommodation, nausea, and headache (Jager and Stagg, 1958; Sidell and Groff, 1971).

Clinical experience with the use of PAM-2 iodide, given with atropine and diazepam, in the treatment of the victims of the Tokyo sarin attack in 1995 was extremely favourable (Stojiljković and Jokanović, 2005). Still, 2-PAM should not be recommended as the drug of choice due to its lack of efficacy against tabun and soman (Kassa, 2005).

B. Trimedoxime (TMB-4)

TMB-4 Cl_2 was synthesized in the USA in 1957 (Poziomek *et al.*, 1958). It is the only one of the major bispyridinium oximes with a propylene bridge between the two pyridinium rings. It was shown that TMB-4 is a more potent reactivator of the DFP-inhibited AChE than 2-PAM (Hobbiger and Sadler, 1958) and better reactivator than LüH-6 in the case of the tabun-inhibited enzyme (Hobbiger and Vojvodić, 1966). TMB-4 was the first oxime to prove its efficiency in the treatment of the animals intoxicated with tabun (Schoene and Oldiges, 1973; Maksimović *et al.*, 1980; Bokonjić *et al.*, 1993). It could also protect the animals poisoned with sarin or VX, but not the ones intoxicated with soman (Maksimović *et al.*, 1980; Inns and Leadbeater, 1983; Binenfeld, 1986). However, TMB-4 was the most toxic oxime among the ''great four'' – it was shown in mice that its LD_{50} is 3, 4 and 8 times less than that for LüH-6, 2-PAM and HI-6, respectively (Clement, 1981).

C. Obidoxime (LüH-6, Toxogonin)

After introduction into medical practice in 1964 obidoxime showed a significant potential as an antidote in poisonings

with OPs (Erdmann and Engelhard, 1964). Given with atropine, obidoxime efficiently protected experimental animals against poisoning with tabun (Inns and Leadbeater, 1983; Maksimović *et al.*, 1989), sarin (Inns and Leadbeater, 1983; Maksimović *et al.*, 1989), and VX (Maksimović *et al.*, 1989). Obidoxime was more effective than TMB-4 as an antidote against intoxication with tabun (Heilbronn and Tolagen, 1965). Similarly to 2-PAM and TMB-4, obidoxime was also inefficient in soman poisoning in mice (Maksimović *et al.*, 1980), guinea pigs (Inns and Leadbeater, 1983), and primates (Hamilton and Lundy, 1989). Obidoxime was more efficient than HI-6 against tabun intoxication. Similarly, obidoxime also was more effective as a medical countermeasure following intoxication with most cholinesterase inhibiting insecticides, while HI-6 is considered to be a better drug against soman inhibited acetylcholinesterase (Aas, 2003). In contrast to TMB-4, obidoxime, when administered with atropine in pyridostigmine-pretreated guinea pigs, could provide some protection against soman as well (Inns and Leadbeater, 1983). Although being less toxic than TMB-4, obidoxime can exert transient hepatotoxic effects (Marrs, 1991), which occur in about 10% of severely poisoned patients (Eyer, 2003).

When administered to human volunteers by the intramuscular route, obidoxime (5 mg/kg) produced a plasma concentration >4 mg/l, from 5 min after injection to 3 h (Sidell and Groff, 1970). Adverse effects of obidoxime in male volunteers were described as pallor, nausea, burning sensation, headache, generalized weakness, sore throat, and paresthesia of the face (Simon and Pickering, 1976; Marrs and Vale, 2006; Eyer, 2003).

D. Asoxime (HI-6)

The first oxime that could reactivate soman-inhibited AChE and provide at least some protection of the animals experimentally poisoned with this nerve agent was synthesized in 1966. It was shown that HI-6 is more potent than LüH-6 and HS-6 in the protection of various rodent species from intoxication with soman (Inns and Leadbeater, 1983; Oldiges and Schoene, 1970; Mesić *et al.*, 1991), as well as sarin and especially VX (Maksimović *et al.*, 1980; Inns and Leadbeater, 1983). HI-6 could not reactivate tabun-inhibited AChE (Clement, 1982; Ćetković, 1984) and it was inefficient, when used as the only oxime, against poisoning with tabun (Maksimović *et al.*, 1980; Inns and Leadbeater, 1983; Mesić *et al.*, 1991). However, other studies found that HI-6 can exert a similar degree of protection against tabun and soman intoxication in primates (Hamilton and Lundy, 1989). It is also suggested that sufficiently high doses of the oxime could even protect rats from the multiple lethal doses of tabun (Lundy *et al.*, 1989). HI-6 given with atropine protected guinea pigs from poisoning against $5 \times LD_{50}$ of soman or cyclosarin (Lundy *et al.*, 2005). The acute toxicity of HI-6 is the lowest among the five oximes (Maksimović *et al.*, 1980; Clement, 1981; Rousseaux and Dua, 1989), with an LD_{50} value after i.m. administration to rats of 781 mg/kg or 2,071 μmol/kg. A clinical study performed on 22 healthy human volunteers did not show any adverse effects when HI-6 was given in doses up to 500 mg by the oral route (Jovanović *et al.*, 1990).

Clinical studies showed that HI-6 dosed at either 250 or 500 mg by the intramuscular route reached plasma concentrations >4 mg/l in 4–6 min. This concentration was maintained for 125 min following the lower dose (250 mg) and 200 min following the higher dose (500 mg) (Kušić *et al.*, 1985, 1991).

HI-6 is considered to be a very promising bispyridinium oxime in medical treatment following exposure to most nerve agents. A disadvantage of HI-6 compared to other available oximes is its lack of stability in aqueous solutions. Both HI-6 and obidoxime are apparently more effective against nerve agents than the monopyridinium oximes P2S or 2-PAM (Aas, 2003).

E. HLö-7

The fourth and the last important ''Hagedorn oxime'' (after LüH-6, HS-6, and HI-6) is HLö-7, which was synthesized in Freiburg, Germany, in 1986 (Löffler, 1986). Only HLö-7 could reactivate AChE inhibited by any of the four major nerve agents *in vitro* and *in vivo* (DeJong *et al.*, 1989; Worek *et al.*, 1994a, b, 1995), as well as AChE inhibited by cyclosarin (Lundy *et al.*, 1992). In addition, HLö-7 more efficiently restored the neuromuscular transmission impaired by *in vitro* superfusion of the neuromuscular preparation with tabun, sarin, soman, or cyclosarin compared to PAM-2, LüH-6, and HI-6 (Alberts, 1990). It was found that HLö-7 induced a significant reactivation of AChE in mice diaphragm inhibited with tabun, sarin, soman, and cyclosarin (Clement *et al.*, 1992). Although both HI-6 and HLö-7 can antagonize sarin-induced hypothermia (proving that, when given with atropine, they can pass the blood–brain barrier and gain access to the CNS) (Clement, 1992; Clement *et al.*, 1992), the toxicity of HLö-7 was 2.5 times higher than that of HI-6 (Clement *et al.*, 1992). The cardiovascular tolerability of HLö-7 was similar, but still not as good as HI-6, at least in anaesthetized guinea pigs (Worek and Szinicz, 1993). HLö-7 appears to be more effective than HI-6 against tabun and VX poisoning and less effective against sarin, soman, and cyclosarin intoxication (Lundy *et al.*, 1992; Eyer *et al.*, 1992). HLö-7-induced protection against tabun poisoning in guinea pigs was significantly better compared to HI-6, while HI-6 was only slightly more efficient than HLö-7 in soman poisoning (Melchers *et al.*, 1994a, b).

The pharmacokinetic profile of HLö-7 was similar to that of HI-6. The mean absorption half-time of HLö-7 was about 14 min after intramuscular administration. Maximum HLö-7 concentration in plasma was reached after 30 min and the half-time of elimination was about 45 min (Eyer *et al.*, 1992).

VI. EFFICACY OF PYRIDINIUM OXIMES IN POISONING WITH OP PESTICIDES

A particular problem in interpreting the beneficial role and efficacy of oximes in clinical practice is a deficiency of published data, especially those evaluated in controlled clinical trials. Studies related to the efficacy of oximes in a clinical setting showed the heterogeneity of therapeutic approaches (i.e. dose regimen, oxime choice, and final outcome of the treatment).

Jokanović and Maksimović (1995) have studied the acute oral toxicity of 25 OP insecticides and one OP fungicide (pyrazophos) in the rat and the efficacy of antidotal treatment involving TMB-4, LüH-6, 2-PAM, and HI-6 (given with atropine and diazepam 1 min after poisining) in animals dosed with $2 \times LD_{50}$ of the OP insecticides. The success of therapy was dependent on the chemical structure of OPs. The oximes were potent antidotes in poisoning with the insecticides having phosphate structure, and provided some extent of antidotal protection in poisoning with phosphonates, phosphorothiolates, phosphorothionates, and phosphorodithioates. However, none of them was an effective antidote against dimethoate and pyridafenthion. The study has shown that TMB-4 was the most effective oxime in the treatment of OP insecticide poisoning.

Kušić *et al.* (1991) have tested the oxime HI-6 in OP pesticide poisoning in 60 patients. HI-6 was administered four times a day as a single i.m. injection of 500 mg with atropine and diazepam treatment. Oxime therapy was started on admission and continued for 2 to 7 days. Most patients were treated with HI-6 and nine patients severely poisoned with quinalphos were treated 2-PAM Cl (1,000 mg four times per day). HI-6 rapidly reactivated human red blood cell AChE inhibited by diethoxy OPs (phorate, pyridaphenthion, quinalphos) as well as that inhibited by dichlorvos (a dimethoxy OP). AChE inhibited with other dimethoxy OPs (dimethoate and phosphamidon) was reported to be resistant to HI-6 treatment, whereas reactivation with malathion was slow (reactivation half-time 10 h). Both HI-6 and 2-PAM successfully reactivated AChE in quinalphos-poisoned patients, with HI-6 acting as a faster AChE reactivator than 2-PAM.

Willems *et al.* (1993) reported that ethyl parathion and methyl parathion could be effectively treated with 2-PAM methylsulfate (plasma concentrations 4 mg/l) and atropine when pesticide concentrations in plasma were relatively low. In severe poisoning with pesticide levels in plasma above 30 µg/l, high 2-PAM concentrations in plasma (14.6 mg/l) did not provide any improvement. In addition, 2-PAM at concentrations of 6.3 mg/l was not effective in AChE reactivation in dimethoate poisoning where AChE was inhibited with its active metabolite omethoate.

Thiermann *et al.* (1997) reported that in parathion poisoning obidoxime (250 mg i.v. administered as a bolus followed by infusion of 750 mg per day) was effective, but AChE reactivation following severe poisoning did not occur until the concentration of paraoxon in plasma was low.

In a clinical study of 63 patients poisoned with organophosphorus insecticides, patients were divided into three groups: one was treated with atropine only, while the other two received atropine and either 2-PAM or obidoxime. Initial and maintenance intravenous doses for 2-PAM were 30 mg/kg and 8 mg/kg/h, respectively, and 8 mg/kg and 2 mg/kg/h, respectively, for obidoxime. The major clinical findings or AChE activities at the time of admission did not show statistically significant differences among the groups. Although the severity of intoxications (based on respiratory complications and duration of hospitalization) was higher in the atropine plus oxime groups, 12% and 50% of patients in the atropine and atropine plus obidoxime groups died, respectively. No mortality was found in the 2-PAM plus atropine group. Incidence of recurrent twitching and convulsions, repeated respiratory arrest, required mechanical respiration, required intensive care unit therapy, and duration of hospitalization were smaller in the atropine plus obidoxime group than in the atropine plus 2-PAM group. Three of the patients who received the obidoxime combination therapy developed hepatitis and two of them died due to hepatic failure, which may indicate overdosage of obidoxime (Balali-Mood and Shariat, 1998).

The only two randomized controlled clinical trials performed so far did not result in a final proof of the efficacy of the oximes in the treatment of poisonings induced by the OP insecticides in humans due to methodological problems (Eddleston *et al.*, 2002). However, experimental and clinical experience suggests that among the pyridinium oximes, obidoxime and trimedoxime, although relatively toxic, could provide reactivation and antidotal protection against most of the OP insecticides. In addition, HI-6 has proved to be effective in the treatment of soman-poisoned animals and safe and effective in patients poisoned with diethoxy OPs.

VII. CONCLUDING REMARKS AND FUTURE DIRECTION

There is no universal, broad-spectrum oxime capable of protecting against all the known OPs. In military toxicology, HI-6 seems to be the best choice for nerve agent poisoning because it was able to protect experimental animals from toxic effects and improve survival of animals poisoned with sublethal doses especially in the cases of soman and cyclosarin. With this exception, available experimental evidence suggests that there are no clinically important differences between pralidoxime, obidoxime, and HI-6 in the treatment of nerve agent poisoning. On the other hand, obidoxime appears to be the best choice for the treatment of poisoning with OP insecticides. The best alternatives for the proposed solutions would be HLö-7 and trimedoxime, respectively. However, it is necessary to rigorously test

these statements in randomized clinical trials conducted according to the latest guidelines. These trials should include objective parameters such as measurement of acetylcholinesterase status, concentration of oximes in blood as well as OPs and their metabolites at different time points in blood and urine. This may be possible in the near future.

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CHAPTER 66

Novel Oximes

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I. INTRODUCTION

Because of the continuous threat of nerve agent misuse, the development of novel antidotes with greater potency is warranted. In this chapter, we would like to turn readers' attention to antidotal therapy with acetylcholinesterase (AChE) reactivators. Although this group of compounds has been known probably for more than half a century, there is no single compound that can counteract every nerve agent's potential to poison. This chapter describes in detail the synthesis of the new AChE reactivators that have been developed within the last few years. During synthesis, special emphasis is given to the structural requirements of these compounds. Finally, several new trends in the development of new reactivators are discussed.

II. ORGANOPHOSPHORUS ACETYLCHOLINESTERASE INHIBITORS

Organophosphorus (OP) compounds are widely used in agriculture as pesticides (DDVP, diazinon, chlorpyrifos, parathion, etc.) and in industry and technology as softening agents and as additives to lubricants. Some OPs are declared as chemical warfare agents or nerve agents. Sarin, cyclosarin, soman, tabun, VX, and Russian VX belong to the well-known members of the OP nerve agent family (Table 66.1; Watson *et al.*, 2006; Bajgar, 2004; Marrs *et al.*, 1996).

The history of nerve agents began prior to World War II in Germany. The first known nerve agent – tabun (*O*-ethyl-*N*,*N*-dimethyl phosphoramidocyanidate) – was synthesized in laboratories of IG Farben by Dr Schrader in 1936 (Germany). Although pesticides were the original aim of Schrader's studies, German authorities identified the deadly potential of organophosphates (OPs), and many other nerve agents such as sarin, cyclosarin, and soman were developed. A few decades later, new nerve agent VX was developed. Other new nerve agents are the intermediate volatile agent (IVA; also named agent GP) and Novichok (Figure 66.1) (Halamek *et al.*, 2007).

During the Cold War, nerve agents were stored and prepared for potential military use but were not used in military conflicts. However, they were misused by Sadam Hussein in Iraq in the Kurdish village of Birjinni (1988) and by the Japanese Aum Shinrikyo sect in Matsumoto (1994) and Tokyo (1995) (Black *et al.*, 1994; Bolz *et al.*, 2002; Tu, 2000).

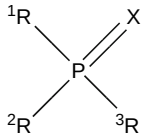
Nerve agents phosphorylate or phosphonylate the serine hydroxyl group at the esteratic part of the active site of the enzyme acetylcholinesterase (AChE; EC 3.1.1.7) (Figure 66.2). AChE plays a key role in cholinergic transmission in the peripheral and central nervous system and, consequently, its inhibition is life-endangering (Taylor, 1996; Marrs, 1993).

Depending on the particular nerve agent, AChE is irreversibly phosphorylated (a time-dependent process). This process is called aging. Aged enzymes cannot be restored by any nucleophilic agent. Aging is defined as dealkylation of the phosphoryl adduct to give a negatively charged adduct that is stabilized by interaction with the catalytic His 440 (Millard *et al.*, 1999). Because of the irreversible inhibition of AChE, the enzyme is not able to fulfill its physiological role in the organism – splitting the neuromediator acetylcholine (ACh) at the synaptic clefts – and, subsequently, ACh accumulates at the cholinergic synaptic junctions (Taylor, 1996; Patocka *et al.*, 2005).

The acute toxicity of nerve agents is usually attributed to the excessive cholinergic stimulation caused by the above-mentioned excess of ACh, followed by subsequent overstimulation of the cholinergic pathways and desensitization of the cholinergic peripheral and central receptor sites (Dawson, 1994).

Symptoms of intoxication are as follows: when an individual is exposed to low amounts of a nerve agent, the initial symptoms include runny nose, contraction of the pupils, miosis, deterioration of visual accommodation, headache, slurred speech, nausea, hallucination, pronounced chest pain, and an increase in the production of saliva (muscarinic central and peripheral symptoms). At higher doses of a nerve agent the aforementioned symptoms are more pronounced. Coughing and breathing problems also occur. The individual then may begin to experience convulsions (nicotinic symptoms) and a subsequent disturbed ventilation, coma, and death. At even higher doses an exposed individual would almost immediately go into convulsions and die from ventilation and cardiovascular failure because of the

TABLE 66.1. Some AChE inhibiting OP nerve agents, pesticides, and prototype compounds



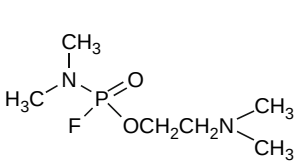
Compound	^{1}R	^{2}R	^{3}R	X	Class
Tabun	O-Et	N-Me_2	CN	O	NA
Sarin	O-*isopropyl*	Me	F	O	NA
Soman	O-(*3,3-dimethylbut-2-yl*)	Me	F	O	NA
Cyclosarin	O-*cyclohexyl*	Me	F	O	NA
VX	S-[*(2-diisopropyl)aminoethyl*]	Me	O-Et	O	NA
Russian VX	S-[*(2-diethyl)aminoethyl*]	Me	O-isobutyl		NA
DFP	O-*isopropyl*	O-*isopropyl*	F	O	Pesticide
DDVP	O-(*2,2-dichloroethenyl*)	O-Me	O-Me	O	Pesticide
Paraoxon	O-(*4-nitrophenyl*)	O-Et	O-Et	O	Pesticide
Parathion	O-(*4-nitrophenyl*)	O-Et	O-Et	S	Pesticide

Et – ethyl; Me – methyl

simultaneous shutdown of the nervous and respiratory systems. The initial stages of symptoms of an individual exposed to a nerve agent may vary depending on the particular nerve agent and amount of agent the individual was exposed to (Dawson, 1994; Rotenberg and Newmark, 2003).

III. ACETYLCHOLINESTERASE (AChE; EC 3.1.1.7)

AChE is a serine hydrolase enzyme that belongs to the esterase family within higher eukaryotes. This family acts on different types of carboxylic esters. AChE's biological role is the termination of transmission impulse at cholinergic synapses within the nervous system by rapid hydrolysis of the neurotransmitter ACh (Schumacher *et al.*, 1986). The monomer of AChE with a molecular weight around 60,000 is an ellipsoidal molecule, whose size is approximately $45 \times 60 \times 65$ angstrom (Å), consisting of a 12 stranded central mixed β-sheet surrounded by 14 α-helices (Sussman *et al.*, 1991). Each monomer contains one catalytic center composed from two compartments: the esteratic subsite containing the catalytic triad and the anionic subsite that accommodates the positive quaternary compartment of ACh. The esteratic subsite contains the catalytic machinery of the enzyme: a catalytic triad of Ser 200, His 440, and Glu 327. The anionic subsite is defined by Trp 84, Phe 330, and Phe 331. Its role is to orient the charged part of the substrate that enters the active center. This role is the main function of the Trp residue (Sussman *et al.*, 1991). The recent rendition of the x-ray structure for AChE places the active catalytic site deep within a gorge-like fold of the protein. The aromatic gorge in the protein is approximately 20 Å deep and penetrates halfway into the enzyme. The active site lies at the base of this gorge only 4 Å above the base, leading some to label this the active gorge. The aromatic gorge is a more appropriate term, because 40% of its content is composed of 14 aromatic residues located in the gorge, which is highly conserved from different species of AChE (Harel *et al.*, 1993). The high aromatic content may explain studies that have proposed hydrophobic and anionic binding sites independently on the active site. Only a few acidic residues are present within the gorge. Markedly, the aromatic residues play an important role in the stabilization of the enzyme–substrate complex. Electrostatic as well as hydrophobic effects are of importance here (Berman and Leonard, 1990). The electrostatic potential map of AChE suggests that this enzyme, like the other enzymes with charged substrates, steers its substrate toward its gorge and into the active site.



Agent IVA

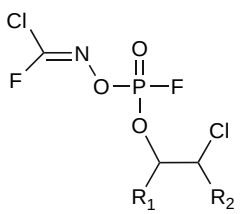


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FIGURE 66.1. Proposed structures of new nerve agents.

The second anionic site of AChE, the so-called peripheral anionic site, is located at the active center gorge entry, and encompasses overlapping binding sites for different

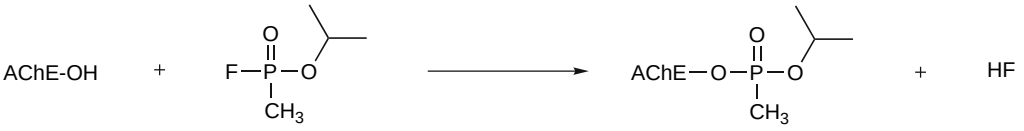


FIGURE 66.2. Inhibition of acetylcholinesterase by sarin.

activators and inhibitors. The peripheral anionic site consists of residues Asp 74 and Trp 286 as a common core. Binding of ligands to these residues may be the key to the allosteric modulation of AChE catalytic activity (Bourne *et al.*, 2003).

IV. ANTIDOTES FOR AChE INHIBITING ORGANOPHOSPHORUS POISONING

Antidotes developed for treatment of nerve agent intoxication could be divided into two parts: prophylaxis as pre-exposure administration of antidotes and post-exposure treatment consisting of anticholinergic drugs, AChE reactivators, and anticonvulsants. Among the most known prophylactic means are carbamates (pyridostigmine and physostigmine), oximes (HI-6; Transant patch – Czech Republic), or scavengers (butyrylcholinesterase, paraoxonase, or phosphotriesterase). The main drugs used for post-exposure treatment are anticholinergics (functional antidotes) that antagonize the effects of accumulated ACh at the cholinergic synapses, and AChE reactivators (called oximes according to the functional oxime group) reactivating AChE inhibited by the organophosphorus inhibitor (causal antidotes). Their effects are synergistic. Central sedative-hypnotics such as benzodiazepines are also used as anticonvulsants (Patocka, 2004).

A. Reactivators of AChE

Pralidoxime (2-PAM; 2-hydroxyiminomethyl-1-methylpyridinium chloride), obidoxime [1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxapropane chloride], methoxime [MMC-4; MMB-4; 1,1-bis(4-hydroxyiminomethylpyridinium)-methane chloride], trimedoxime [TMB-4; 1,3-bis(4-hydroxyiminomethylpyridinium)-propane bromide], and oxime HI-6 [1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane chloride] are considered to be the most important commercially available AChE reactivators (Figure 66.3a). Other AChE reactivators are currently under development in different countries: HLö-7 (1-[2,4-bis(hydroxyiminomethylpyridinium)]-3-(4-carbamoylpyridinium)–2-oxapropane chloride), and K027

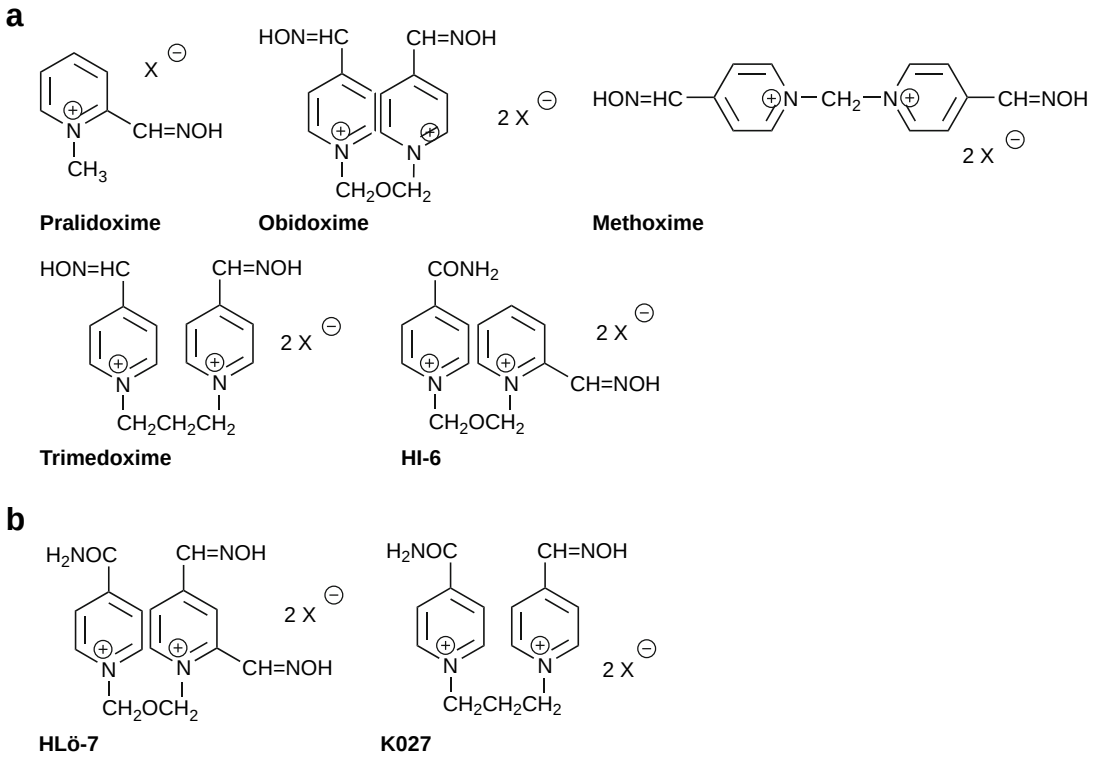


FIGURE 66.3. Selected AChE reactivators.

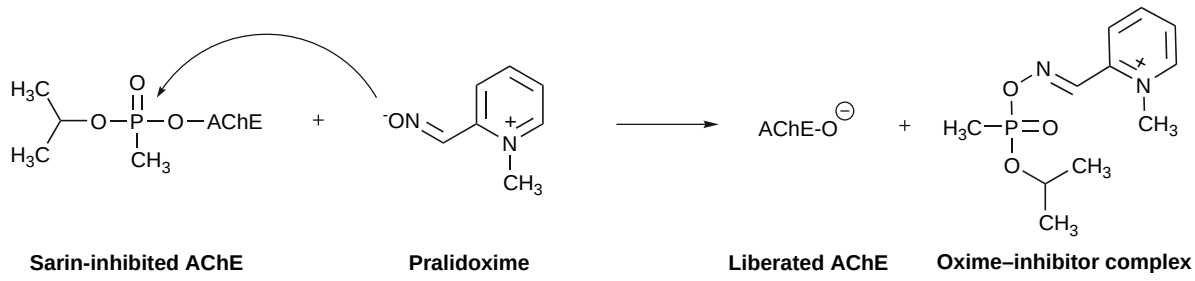


FIGURE 66.4. Reactivation of sarin-inhibited acetylcholinesterase.

[1-(4-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)–propane bromide] (Figure 66.3b).

From a chemical point of view, AChE reactivators are mono- or bis-quaternary pyridinium salts bearing in their molecule a functional oxime group able to split the bond between the organophosphorus inhibitor and the enzyme, to release free functional enzymes to once again be physiologically active in the organism (Figure 66.4).

Unfortunately, none from the above-mentioned AChE reactivators is sufficiently effective against all known nerve agents and pesticides (Table 66.2; Kuca *et al.*, 2007a).

V. SYNTHESIS OF NEW AChE INHIBITORS

At present, many scientific institutions around the world are interested in the synthesis and improvement of new antidotes against chemical warfare agents, especially nerve agents, because of the continuous threat of the nerve agent misuse by terrorists. Regarding the AChE reactivators, finding a suitable oxime sufficiently effective against inhibited AChE, regardless of the type of OP compounds, is a very important task. There are several reviews dealing with the synthesis of new cholinesterase reactivators (Petrova and Bielavsky, 2001; Primozic *et al.*, 2004). Several studies discussing recent developments in the synthesis of novel reactivators are discussed below.

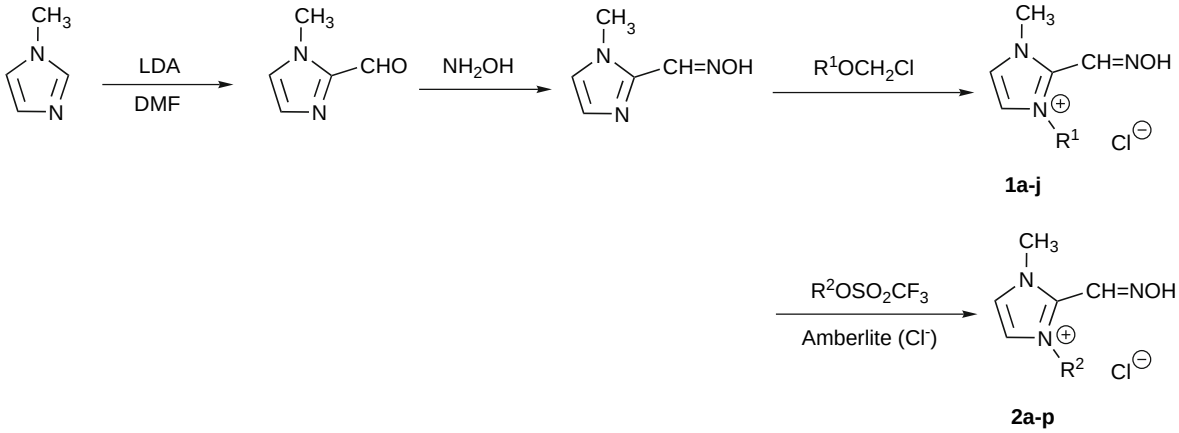
In 1991, Goff *et al.* reported synthesis and activity testing of monoquaternary imidazole derivatives with various substituents such as ether, silicone, nitrile, ester, halogen, nitro, sulfone or amino group (Goff *et al.*, 1991). Published literature reveals that syntheses started from 1-methylimidazole (Figure 66.5).

Synthesized compounds were tested together with atropine sulfate *in vivo* on mice intoxicated by soman and tabun. Afterwards, they were also tested *in vitro* against ethyl(4-nitrophenyl)methylphosphonate-inhibited human erythrocyte AChE using pralidoxime and HI-6 as reference compounds. The experiments showed that derivatives

TABLE 66.2. Reactivation potencies of five commercially available oximes (Kuca *et al.*, 2007a)

	Pralidoxime	Trimedoxime	Obidoxime	Methoxime	HI-6
VX	34/ 0 ++ / –	66/ 10 +++/+	79/ 8 +++/+	59/ 0 +++/–	28/ 13 ++/+
Russian VX	70/ 0 +++/–	30/ 4 ++/–	66/ 17 +++/+	79/ 36 +++/++	42/ 53 +++/+++
Sarin	31/ 0 ++/–	54/ 7 +++/+	26/ 3 ++/–	43/ 0 +++/–	47/ 50 +++/+++
Cyclosarin	4/ 0 –/–	0/ 0 –/–	4/ 2 –/–	37/ 90 ++/++++	70/ 71 +++/+++
Tabun	4/ 0 –/–	28/ 10 ++/+	37/ 28 ++/++	0/ 0 –/–	2/ 4 –/–
Soman	0/ 0 –/–	0/ 0 –/–	4/ 1 –/–	24/ 4 ++/–	5/ 3 +/–
Chlorpyrifos	38/ 4 ++/–	66/ 38 +++/++	63/ 35 +++/++	45/ 10 +++/+	20/ 11 ++/+

Reactivation (%) at oxime concentration 10^{-3} M/10^{-5} M



Product	R^1	Product	R^2
1a	$CH_2OCH_2CH_2OCH_3$	**2a**	CH_2CH_2N
1b	$CH_2OCH_2CH_2CH(OCH_3)CH_3$	**2b**	$CH_2CH_2CH_2CN$
1c	$CH_2OCH_2CH_2Si(CH_3)_3$	**2c**	$CH_2CH_2CH_2CH_2CN$
1d	$CH_2OCH_2CH_2CH_2Si(CH_3)_3$	**2d**	$CH_2CH_2CH_2CH_2CH_2CO_2CH_3$
1e	$CH_2OCH_2CH_2CH_2CH_2Cl$	**2e**	$CH_2CH_2CH_2CH_2OC(O)Ph\text{-}2'\text{-}OH$
1f	$CH_2OCH_2CH_2CH_2Br$	**2f**	CH_2CH_2F
1g	$CH_2OCH_2C(CH_3)_2CH_2Br$	**2g**	$CH_2CH_2OCH_2CH_2F$
1h	$CH_2OCH_2CH_2NO_2$	**2h**	$CH_2CH_2OCH_2CH_2Cl$
1i	$CH_2OCH_2C(CH_3)_2NO_2$	**2i**	CH_2CH_2Br
1j	$CH_2OCH_2CH_2SO_2CH_3$	**2j**	$CH_2CH_2NO_2$
		2k	$CH_2CH_2SO_2CH_3$
		2l	$CH_2CH_2N^+H(CH_3)_2 \cdot Cl^-$
		2m	$CH_2CH_2N^+H\text{-pyrolidinium} \cdot Cl^-$
		2n	$CH_2CH_2N(CH_3)SO_2CH_3$
		2o	$CH_2CH_2N(CH_3)SO_2CF_3$
		2p	$CH_2CH_2N(CH_3)SO_2Ph$

FIGURE 66.5. Synthesis of monoquaternary imidazole derivatives (Goff *et al.*, 1991).

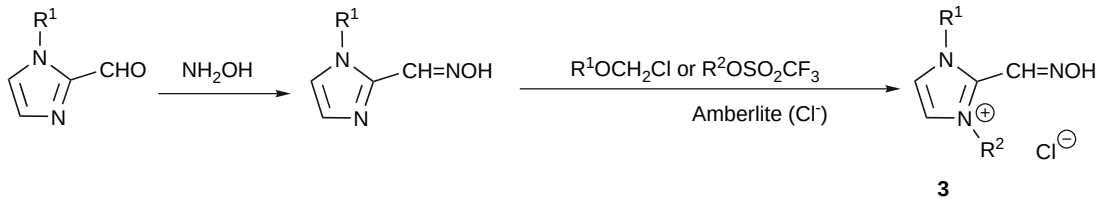


FIGURE 66.6. 2-Hydroxyiminomethylimidazole derivatives (Koolpe *et al.*, 1991).

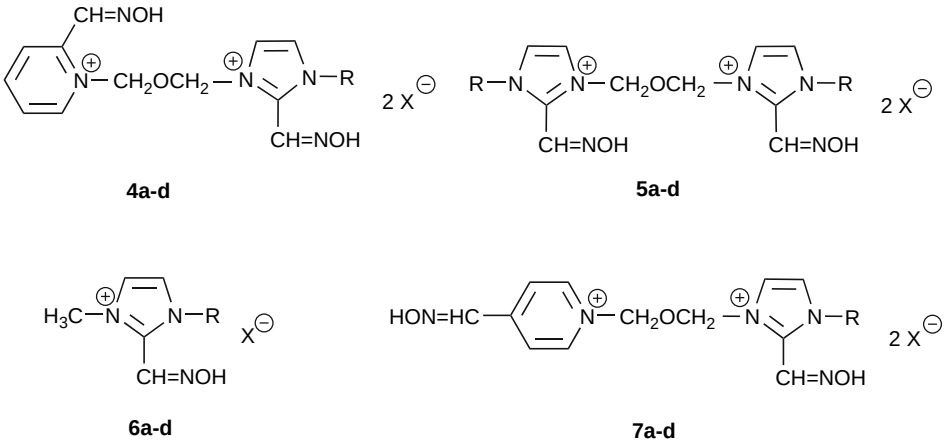


FIGURE 66.7. Mono- and bisquaternary imidazolium or imidazolium–pyridinium compounds (Mesic *et al.*, 1991).

bearing halogen, nitro, sulfone, amine, or aminosulfonyl side-chain functional groups (e.g. **2i–k**, **2n**) greatly improved antidotal protection relative to pralidoxime, whereas those bearing ether, silyl, nitrile, or ester side-chain substituents (e.g. **1a**, **1d**, **2c–d**) were essentially ineffective. Nitro, sulfone, amine, or aminosufonyl functional groups were determined as those which would improve the effectiveness of the quaternary 2-(hydroxyiminomethyl) imidazole. Unfortunately, the ability of tested compounds to reactivate inhibited human AChE was poor and did not correlate with *in vivo* tests on mice.

Koolpe *et al.* (1991) synthesized and studied quaternary salts derived from 2-hydroxyiminomethyl imidazole with various substituents on both nitrogen atoms on the imidazole ring (Figure 66.6), where R^1 is represented by methyl, ethyl, or butyl, and R^2 by a very large spectrum of substituents, e.g. 2-nitropropyl, 2-(ethylsulfonyl)-ethyl, 2-dimethylaminoethyl hydrochloride.

All the synthesized compounds were tested both *in vivo* on mice intoxicated by soman or tabun and *in vitro* on human erythrocyte AChE. In conclusion, nitro, sulfone, amino, and aminosulfonyl side-chain substituents caused high antidotal activity, where the optimum length of side-chain lies between two and four atoms, a cationic quaternary heteroaryl ring is essential for activity, and the presence of an oxime or other nucleophilic moiety is not essential. *In vitro* tests indicated that all these compounds are not particularly effective as AChE reactivators, but they are potent reversible and competitive inhibitors of intact AChE.

Mesic *et al.* (1991) prepared four types of compounds including monoquaternary or bisquaternary imidazolium (imidazolium–pyridinium) salts (Figure 66.7), where R is methyl (**4a**, **5a**, **6a**, **7a**), benzyl (**4b**, **5b**, **6b**, **7b**), phenyl (**4c**, **5c**, **6c**, **7c**) or 4-methoxyphenyl (**4d**, **5d**, **6d**, **7d**), and X is an iodine or a chlorine (only **5a**) anion.

Reactivation activity of the above-mentioned compounds was tested *in vitro* on human erythrocyte AChE and compared to pralidoxime, obidoxime, and HI-6. Some of these (**6b**, **7b**) had better reactivating activity against sarin or tabun than commonly used reference compounds.

Mesic *et al.* (1992) prepared four fluorinated compounds (**8–11**) of mono- and bis-imidazolium type (Figure 66.8).

Reactivation *in vitro* was performed on human erythrocyte AChE inhibited by sarin, VX, tabun, or soman without promising results. Toxicity of all these compounds was tested *in vitro* on human erythrocyte AChE.

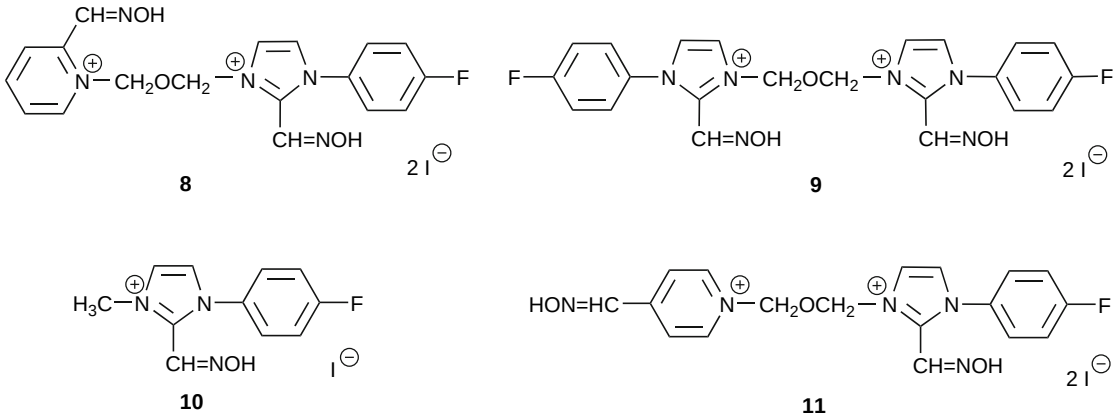


FIGURE 66.8. Fluorinated compounds of mono- and bis-imidazolium type.

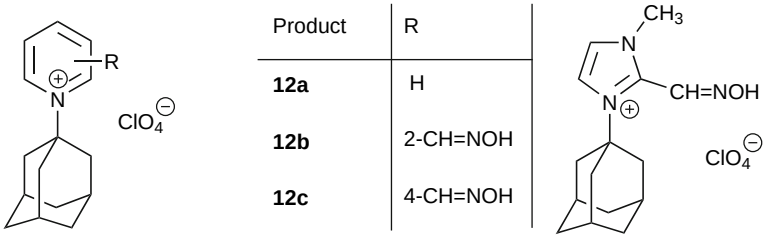


FIGURE 66.9. Monopyridinium and monoimidazolinium adamantane derivatives

Bregovec *et al.* (1992) prepared adamantane derivatives of monopyridinium and monoimidazolinium type (Figure 66.9). In monopyridinium salts, R is hydrogen or hydroxyiminomethyl in position two or four (**12a–c**).

Reactivation *in vitro* was performed on human erythrocyte AChE inhibited by sarin, VX, tabun, or soman compared with HI-6. No newly prepared compound showed reactivation potency stronger than the control substance.

Eyer *et al.* (1992) summarized a study about the methanesulfonate salt of HLö-7 (Figure 66.10). A new synthetic strategy, using bis(methylsulfonoxymethyl)ether linker, was presented.

A broad spectrum of pharmacological data was acquired by *in vivo* experiments on mice, guinea pigs, and dogs. The reactivation experiments were performed *in vitro* on bovine erythrocyte AChE with soman, sarin, or tabun compared to HI-6, and showed that the methanesulfonate salt of HLö-7 is superior to HI-6 in reactivating soman- or sarin-inhibited AChE and exceeds HI-6 in reactivation of tabun-inhibited AChE.

Hsiao *et al.* (1992) patented a preparation of bis-methylene ether-linked bispyridinium compounds (Figure 66.11). Bis(methanesulfonoxymethyl)ether was used to form a linker between pyridine rings, and methanesulfonate anions were replaced by chloride anions.

The patent describes only synthetic pathways and chemical experimental data of prepared compounds (**14**). No biological data were reported. Deljac *et al.* (1992) described compounds of symmetric bisquaternary imidazolium type with a four-carbon linker (Figure 66.12), where R is methyl (**15a**, **16a**), phenyl (**15b**, **16b**), benzyl (**15c**, **16c**), 4-methoxyphenyl (**15d**, **16d**), and 4-fluorophenyl (**15e**, **16e**).

Reactivation *in vitro* was performed on human erythrocyte AChE inhibited by sarin, VX, tabun, or soman. Only one compound, (*E*)-1,4-bis(2-hydroxyiminomethyl-3-methylimidazolinium) but-2-ene dibromide (**16a**), showed promising results in reactivation of AChE inhibited by the above-mentioned inhibitors, except soman (because of aging). In the same publication, two already known AChE reactivators, BDB-108 (**17a**) and BDB-109 (**17b**), and new compounds, BDB-107 (**17c**) and BDB-111 (**17d**), with four carbon linkers and hydroxyiminomethyl in position two or four, are discussed (Figure 66.13) (Deljac *et al.*, 1992).

Reactivation ability and *in vitro* toxicity were performed on human erythrocyte AChE inhibited by sarin, VX, tabun, and soman. All compounds had no reactivation potency against soman-inhibited AChE. Substances with the oxime group in position four on the pyridinium ring (**17a–b**) had high reactivation potency (>70%) against sarin and tabun. Promising potency against VX-inhibited AChE was also achieved. Substances with the oxime group in position two on the pyridinium ring (**17c–d**) had high reactivation potency (>70%) against sarin. However, they were poor reactivators of tabun-inhibited AChE.

Sikder *et al.* (1993) synthesized two new series of 3,3′- and 3,4′-substituted bispyridinium monooximes and two bisoximes bridged by 2-oxapropane (Figure 66.14) and propane (Figure 66.15) connecting chain.

Reactivation potency of the compounds was tested *in vitro* on lyophilized eel AChE inhibited by diisopropylfluorophosphate (DFP). The compounds with a 2-oxapropane linker between both pyridinium rings were weaker reactivators compared to the corresponding propane derivatives.

According to the previous results published in 1992, Mesic *et al.* (1994) synthesized four new *p*-methylphenyl imidazolium derivatives (**20–23**) (Figure 66.16) and compared their biological activity to the corresponding

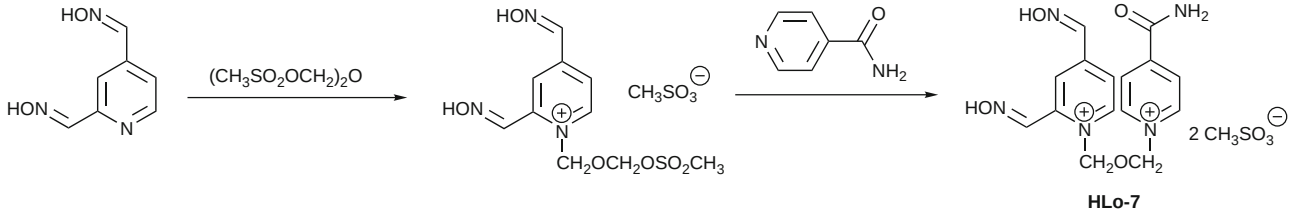
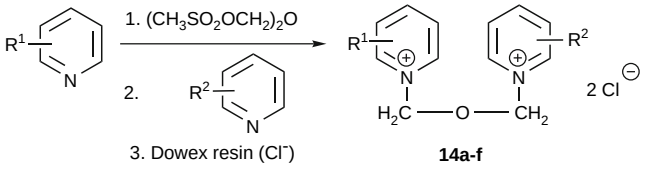


FIGURE 66.10. Preparation of HLö-7 dimethanesulfonate.



Product	R^1	R^2
14a	4-CH=NOH	4-CH=NOH
14b	2-CH=NOH	3-COC_6H_5
14c	3-COC_6H_5	3-COC_6H_5
14d	2-CH=NOH	2-CH=NOH
14e	2-CH=NOH	4-$CONH_2$
14f	4-$CONH_2$	4-$CONH_2$

FIGURE 66.11. Bismethylene ether bispyridinium compounds (patented by Hsiao *et al.*, 1992).

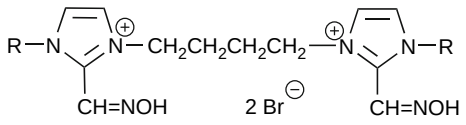
previously prepared *p*-substituted analogs. *In vitro* tests showed that prepared compounds are poor reactivators of soman-inhibited AChE, except the promising results of compound **22**.

Hampl *et al.* (1995) synthesized some already known and new AChE reactivators derived from pyridinecarbaldoxime (Figure 66.17) and pyridazinecarbaldoxime (Figure 66.18) using it for the purpose of cleavage of mimic nerve agent.

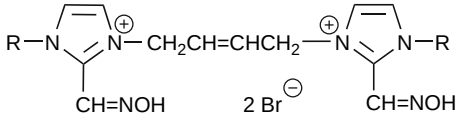
Carvalho and Miller (1995) described derivatives, in which carbon-bonded hydrogen in the 2-hydroxyimino-methyl group of pyridinium monoquaternary salt was replaced by 4-substituted aryl (Figure 66.19). Biological data of the synthesized compounds are not mentioned in the article.

Skrinjaric-Spoljar *et al.* (1999) synthesized some inhibitors and also one reactivator (**27**) of AChE (Figure 66.20), which was found to be weak in reactivation of VX-inhibited human erythrocyte AChE.

Yang *et al.* (2003) used bis(methylsulfonoxy-methyl)ether for synthesis of already known reactivators (obidoxime, HI-6, **14d**) as shown (Figure 66.21). No biological data of synthesized compounds were mentioned in the article.



15a-e



16a-e

FIGURE 66.12. Bisquaternary imidazolium compounds (Deljac *et al.*, 1992).

Pang and Brimijoin (1999) used the method of Poziomek *et al.* (Pang *et al.*, 2003; Poziomek *et al.*, 1958) for synthesis of bispyridinium dicarbaldoximes (**28a–h**, **29a–h**) (Figure 66.22). They found a new efficient method for the synthesis of bis-2-hydroxyiminomethylpyridinium salts using bistriflates as alkylating agents (**30a–h**). This work was previously patented by other authors.

Human erythrocyte AChE inhibited by echothiophate [(2-mercaptoethyl) trimethylammonium-*O*,*O*-diethyl-phosphorothioate iodide] was used for *in vitro* reactivation screening. A compound with three membered carbon linkers (**28b**) was the most effective from the *p*-series and a compound with seven carbon connecting chains (**30f**; Ortho-7) seemed to be the most effective from the *o*-series. These results were explained by the authors using 3D structures.

Kuca *et al.* (2003a) tried to find a reactivator with good efficacy against tabun. The synthesis was made using a subsequent approach under the following scheme (Figure 66.23).

The *in vitro* test on rat brain homogenate showed that oxime K048 has higher efficacy to reactivate tabun-inhibited AChE than pralidoxime or HI-6, which is comparable with obidoxime.

Kuca *et al.* (2003b) prepared a similar potential reactivator of AChE 1-(4-hydroxyiminomethylpyridinium)-3-(carbamoylpyridinium)-propane dibromide (K027) differing from the compound K048 in the length of the connecting chain. Two synthetic pathways were used to reach the proposed structure (Figure 66.24). The activity of new reactivator (K027) was tested *in vitro* on sarin-inhibited AChE with promising results.

Kuca *et al.* (2004a) also synthesized and *in vitro* tested activity of symmetric bispyridinium dicarbaldoximes against cyclosarin-inhibited AChE (Figure 66.25).

Compound 1,4-bis(2-hydroxyiminomethylpyridinium)-butane dibromide (31, K033) gave promising results in *in vitro* reactivation of cyclosarin-inhibited AChE in comparison with commonly used pralidoxime. In the same laboratory, a new series of four monoquaternary compounds using original synthetic strategy was prepared (Figure 66.26) (Kuca *et al.*, 2004b).

The reactivation potency was examined *in vitro* on rat brain AChE. All tested compounds were less effective reactivators of sarin, cyclosarin, VX, or tabun-inhibited AChE compared to pralidoxime.

In India in 2005, Chennamaneni *et al.* (2005) repeated the synthesis of oximes K027 and K048 and moreover they added four more new reactivators (**34**) derived from these compounds (Figure 66.27).

Consequently, they tested their reactivation activity on tetraethylpyrophosphate-inhibited mouse brain cholinesterases. Their activities were compared with pralidoxime. All the compounds (except that with pentylene bridge) were found to be more effective reactivators than pralidoxime. Bispyridinium monooximes with a 3-carbamoyl group were

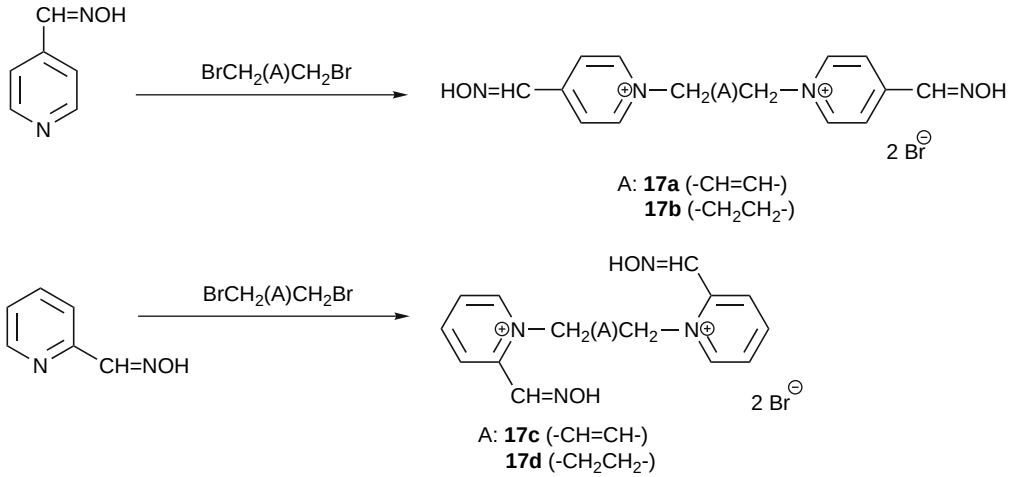


FIGURE 66.13. Bisquaternary pyridinium four carbon-linked compounds (Deljac *et al.*, 1992).

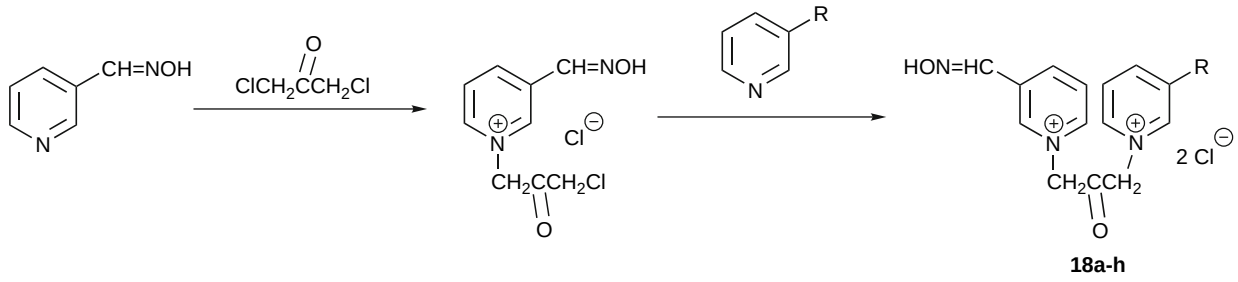
found to be more potent reactivators than the corresponding 4-carbamoyl compounds.

Picha *et al.* (2005) returned to monopyridinium compounds (**35**) with a hydroxyiminomethyl group modified in the side-chain, with the aim of improving the nucleophilicity of this group (Figure 66.28). From the eight oximes prepared and tested, none achieved better reactivation potency if compared with pralidoxime, obidoxime, or HI-6 (Picha *et al.*, 2005; Jun *et al.*, 2008).

In 2006, a new group of 3-oxapentane linker-containing reactivators (**36**) was prepared in Korea (Figure 66.29) (Kim *et al.*, 2005). Among them, several derivatives were found to be promising in case of pesticide and tabun-inhibited cholinesterases reactivation (Kuca *et al.*, 2006; Kim *et al.*, 2006).

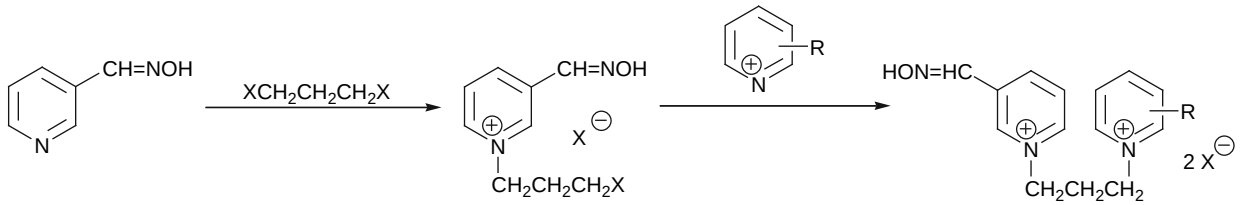
Very promising compounds (K074, K075), which are at present being tested in many laboratories throughout the world, were resynthesized in 2005 in the Czech Republic (Figure 66.30) (Kuca *et al.*, 2005a). As mentioned previously, there has been very promising reactivation activity in the case of tabun and pesticides treatment (Kassa *et al.*, 2007; Lorke *et al.*, 2008a). It will be more thoroughly discussed further in this chapter.

A new xylene linker connecting two pyridinium rings was investigated. Within this investigation 18 new oximes (**37**) were prepared and tested for their ability to reactivate



Product	R
18a	$CONH_2$
18b	$CONHCH_3$
18c	CONH-*i*-Bu
18d	$CONHC_5H_9$
18e	$CONHC_6H_{11}$
18f	$CONHC_6H_5$
18g	COC_6H_{11}
18h	CH=NOH

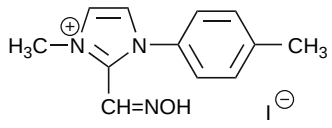
FIGURE 66.14. 3,3′-Bispyridinium compounds bridged by oxapropane linker (Sikder *et al.*, 1993).



19a-o

Product	R	X
19a	3-$CONH_2$	Br
19b	4-$CONH_2$	Br
19c	3-$CONHCH_3$	Br
19d	3-$CONHC_5H_9$	Br
19e	3-$CONHC_6H_{11}$	Br
19f	3-$CONHC_6H_5$	Br
19g	3-$COCH_3$	I
19h	3-$COOCH_3$	I
19i	3-$COOCH_3$	Br
19j	3-$COOC_3H_7$	I
19k	3-$COOC_6H_{11}$	Br
19l	3-COC_6H_5	I
19m	3-CH=NOH	Br
19n	3-CH_3	I
19o	4-CH_3	I

FIGURE 66.15. 3,3′- and 3,4′-bispyridinium compounds bridged by propane linker (Sikder *et al.*, 1993).



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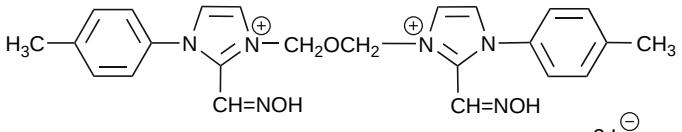

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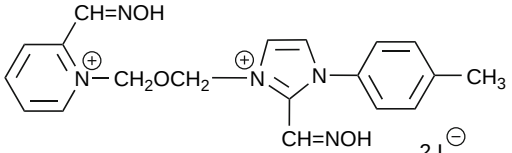

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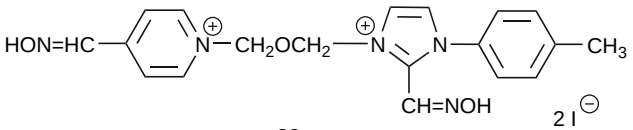

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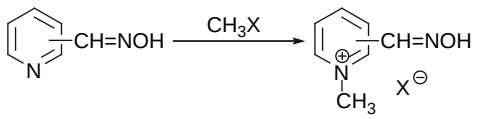
FIGURE 66.16. *p*-Methylphenyl imidazolium derivatives (Mesic *et al.*, 1994).



24a-d

Product	Oxime position	X
24a	2	I
24b	3	I
24c	4	I
23d	4	Br

FIGURE 66.17. Pyridinecarbaldoximes.

tabun and pesticide-inhibited AChE (Figure 66.31) (Musilek *et al.*, 2005, 2007a; Hrabinova *et al.*, 2006).

Chennamaneni *et al.* (2005) prepared four new series of asymmetrical bisquaternary monooximes (**38**) having the aldoxime group in position four at the pyridinium ring and carbamoyl groups in positions three/four at the second pyridinium ring (Figure 66.32). Similar work was published by this group in 2005. The main difference from their results published earlier was the change in the length of the connection chain between two pyridinium rings. The length was six, seven, eight and nine methylene groups. The ability of tested compound to reactivate tetraethylpyrophosphate-inhibited AChE on mouse brain was compared to pralidoxime. It was found that with the increased chain length (from seven to nine methylene groups) the reactivators potentiated the inhibitory effect of OP (Srinivas Rao *et al.*, 2006).

Musilek and colleagues changed regular connection chains between two pyridinium rings (*n*-methylene or 2-oxapropane) to but-2-ene. When considering the sterical rearrangement of the but-2-ene linker, there could be two possible geometrical isomers (*E* and *Z*). Both groups of bisoxime reactivators were prepared (**39**, **40**) (Figure 66.33). They were tested for their possibility to reactivate tabun and pesticide-inhibited AChE. From the obtained results, several candidates were considered as promising for further evaluation of their reactivation potencies (Musilek *et al.*, 2006a, b, c, 2007d).

Monoquaternary pyridines with an oxime group in positions two (pralidoxime), three, and four (**41**) were prepared together with bisquaternary ones (**42**) connected via

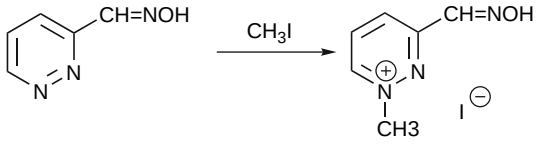


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FIGURE 66.18. 1-Methyl-3-hydroxyiminomethylpyridazinium iodide.

a propane linker (Figure 66.34). Their reactivation activity was tested on tabun and pesticide-inhibited cholinesterases. As predicted, trimedoxime reached the best activity compared with other oximes synthesized throughout the study (Musilek *et al.*, 2006b, c).

Dr Jung's group prepared new oximes with a novel linker having two oxygen atoms in its structure (**43**) (Figure 66.35). Their reactivation activity was tested on rat brain homogenate inhibited by agent VX. As a result, 1,6-bis(4-hydroxyiminomethylpyridinium)-2,5-dioxahexane dichloride was the most potent and appeared to be the most promising compound as a potential reactivator for AChE inhibited by VX agent. Furthermore, the prepared oximes were tested on housefly AChE and bovine red blood cell AChE inhibited by DFP and paraoxon. Also, in the case of pesticide-induced AChE inhibition, the above-mentioned oxime achieved promising results (Oh *et al.*, 2006; Yang *et al.*, 2007). Acharaya *et al.* (2008a) prepared many other compounds of similar structure in 2008. The whole set consisted of 11 compounds differing in the length of the connection chain and position of the oxime groups. They were tested on the electric eel AChE inhibited by sarin. Two promising oximes were found that should be considered for further investigation.

Musilek *et al.* (2006d) prepared bisquaternary reactivators with a cyano group instead the carbamoyl group (**44**), which is present in the structure of HI-6, K027 or K048 (Figure 66.36). Only one derivative seemed to be promising against paraoxon-inhibited AChE. None of the tested substances was able to satisfactorily reactivate tabun-inhibited AChE.

Owing to the fact that HI-6 is at the present time considered as the reactivator of first choice, there are many efforts to improve its application. One approach is the choice of the right counteranion of the reactivator. The anion could influence the solubility and stability of the reactivator in the solution. In 2007, 12 different salts of HI-6 (sulfate, chloride, acetate, bromide, phosphate, mesylate, tartarate, iodide, malonate, salicylate, maleinate, tosylate) were prepared and tested to discover how the anion can influence the self-reactivation process. It was found that there is no difference in the reactivation of cyclosarin-inhibited AChE (Kuca *et al.*, 2007b).

Because of the presence of promising oximes among those with the but-2-ene linker, this pharmacophor was introduced into several other compounds having an oxime group in position four. At the second pyridinium ring, other functional groups were introduced in position four (**45**) (Figure 66.37). Thanks to this structural combination, many new promising oximes were found (Musilek *et al.*, 2007e).

Among them, oxime K203 seemed to be the best one (Figure 66.38). According to our present knowledge, it is considered to be number one in the case of reactivation of tabun-inhibited AChE (Musilek *et al.*, 2007f).

Because of this promising efficacy, many other derivatives having an oxime group on the first pyridinium ring and

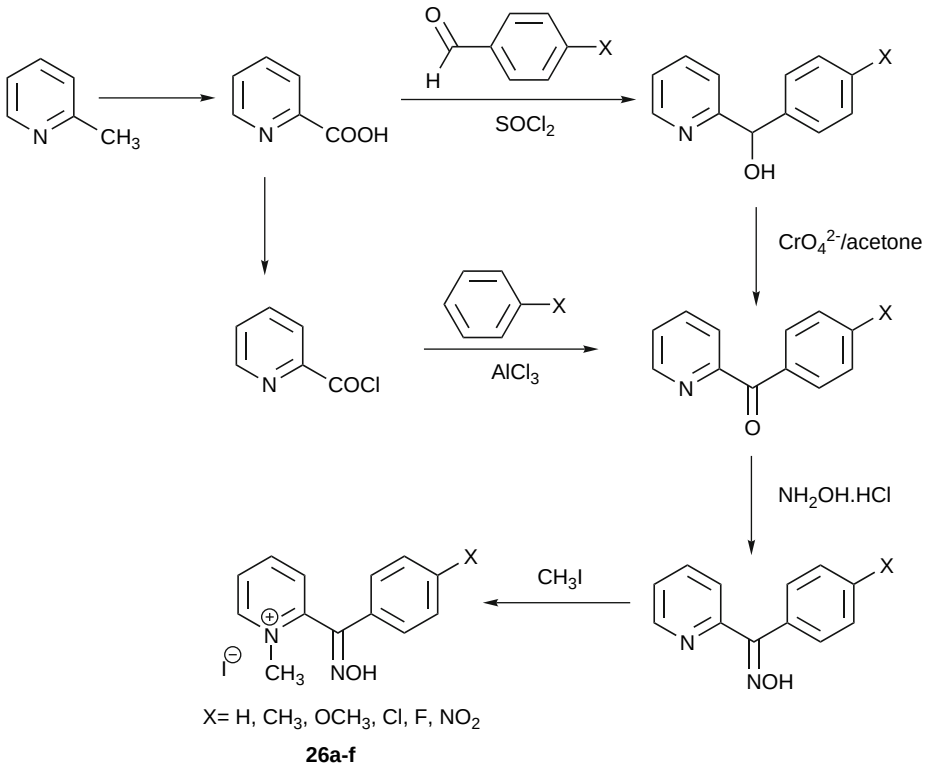


FIGURE 66.19. Preparation of 4-aryl substituted 1-methyl-2-hydroxyiminomethylpyridinium salts.

a carbamoyl group on the other ring were prepared (**46**) (Figure 66.39). Among them, no better reactivator of tabun-inhibited AChE was found. However, many other oximes with promising activity for the treatment of paraoxon-inhibited AChE were found (Musilek *et al.*, 2008).

Another five monoquaternary oximes were prepared and tested in Croatia by Dr Kovarik's group (**47**) (Figure 66.40). All oximes inhibited human AChE reversibly. All prepared oximes reached reactivation of tabun-inhibited AChE within 24 h from 30 to 80% (Odzak *et al.*, 2007).

With the aim of decreasing the size of the reactivator, one oxime group was withdrawn from the trimedoxime structure (**48**) (Figure 66.41). Such modification of the trimedoxime structure decreased its reactivation activity in the case of tabun-inhibited AChE. On the contrary, it increased its reactivation activity in the case of cyclosarin-inhibited AChE (Kuca *et al.*, 2007c).

A Korean group published synthesis and *in vitro* evaluation of several structurally different cholinesterase reactivators (Figures 66.42 and 66.43). Two of them (**28f**, **30f**) were those published already earlier by Pang *et al.* (2003) and Oh *et al.* (2008).

Two other oximes (**49**, **50**) are very interesting when considering blood–brain barrier penetration. They have instead only the quaternary nitrogen, the tertiary nitrogen having no charge. Unfortunately, such structure modification decreased their reactivation potency (Oh *et al.*, 2008).

In 2006 Ohta *et al.* (2006) published a large group of monoquaternary reactivators (**51**) differing in the position of the oxime groups and length and shape of their alkylating chains (Figure 66.44). Subsequently, Okuno *et al.* (2008) examined BBB penetration of selected derivatives using *in vivo* rat brain microdialysis with liquid chromatography–mass spectrometry (LC–MS/MS).

Quite new synthetic results have been achieved in 2008 (Acharaya *et al.*, 2008a, b, c). The findings of Acharya *et al.* (2008b) are focused on the same topic published by other investigators (Musilek *et al.*, 2005, 2007a; Hrabinova *et al.*, 2006; Acharaya, 2008a) – a synthesis of the series of bis-pyridinium oximes connected by xylene linker (**37**). The synthesized compounds were tested for their reactivation of sarin-inhibited electric eel AChE. The other article by Acharya's group describes the synthesis of unique compounds (**52**) having in their structure *para* and

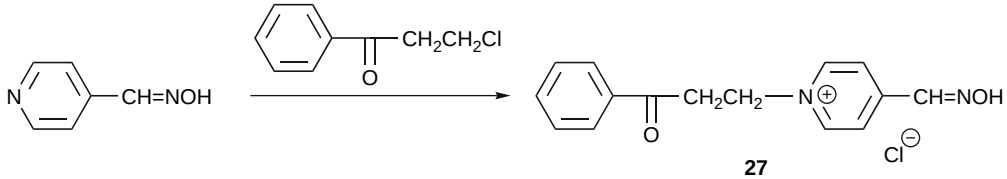
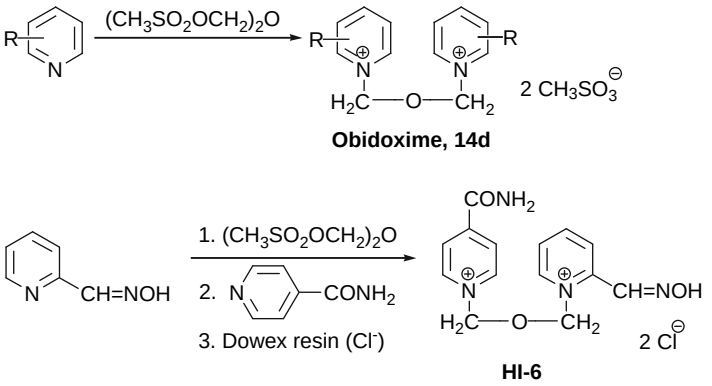


FIGURE 66.20. A novel reactivator tested by Skrinjaric-Spoljar *et al.* (1999).



Product	R
obidoxime	4-CH=NOH
14d	2-CH=NOH

FIGURE 66.21. Synthesis of bis(methylsulfonoxymethyl)ether.

meta-xylene, *cis*-but-2-ene and but-2-ine groups (Acharaya *et al.*, 2008c) (Figure 66.45). All these compounds were tested on electric eel AChE inhibited by DFP. Unfortunately, none of the newly prepared compounds was able to surpass the pralidoxime activity.

VI. *IN VITRO* EVALUATION OF SELECTED AChE REACTIVATORS

There are several techniques to evaluate reactivation activity of structurally different AChE reactivators. Probably the most frequent are Ellmann's method (Ellman *et al.*, 1961; Bajgar, 2005) and the potentiostatic method (Kuca and Kassa, 2003). Each one has its advantages and disadvantages. For example, Ellmann's method does not use real substrate ACh, while the potentiostatic method is very time consuming.

Although the results obtained by both methods are proportionally comparable, to discuss the influence of structural moieties on final reactivator activity only one method should be used. Because of our experiences with the second potentiostatic method, the structure–activity relationship mentioned in this chapter is based on these results.

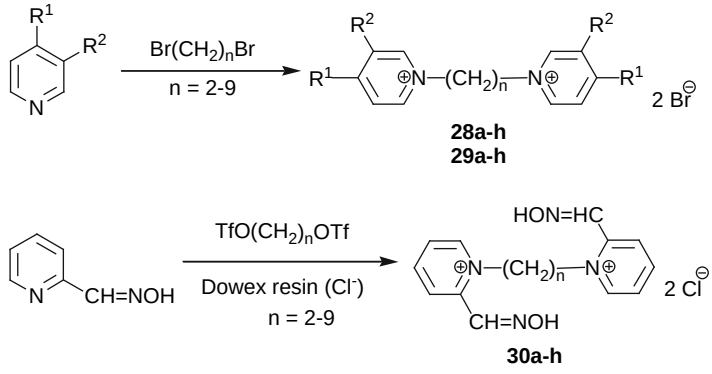
Moreover, species differences in reactivation should be taken into account (Worek *et al.*, 2002; Kuca *et al.*, 2005b). Thus, all results considered in the structure–activity relationship listed below are obtained only with one species (rat).

VII. STRUCTURE–ACTIVITY RELATIONSHIP OF AChE REACTIVATORS

There are five important structural factors which influence the affinity of the AChE reactivators toward inhibited AChE: (1) presence of the quaternary nitrogens in the reactivator's molecule; (2) length and shape of the linker between two pyridinium rings; (3) presence of the oxime group; (4) position of the oxime group at the pyridinium ring; and (5) number of oxime groups in the reactivator structure.

A. Presence of the Quaternary Nitrogen in the Reactivator's Structure

It is generally known that the anionic center of AChE binds the charged quaternary group of the choline moiety of ACh,



Product	R^1	R^2
28a-h	CH=NOH	H
29a-h	H	CH=NOH

FIGURE 66.22. Synthesis of bispyridinium carbaldoximes (Pang and Brimijoin, 1999).

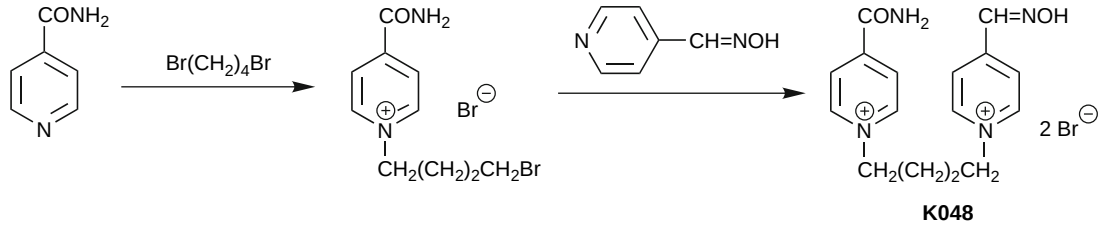


FIGURE 66.23. Synthesis of reactivator K048 for tabun-inhibited AChE.

and also other quaternary ligands including quaternary AChE reactivators (Whiteley and Ngwenya, 1995; Kuca *et al.*, 2004c). Owing to the presence of the quaternary nitrogen in the structure of the AChE reactivator, oximes have increased affinity to both intact and inhibited AChE. In fact, this structural feature allows the entrance of the molecule of the AChE reactivator into the enzyme cavity.

On the other hand, there has been discussion regarding the penetration of the quaternary charged compounds (especially AChE reactivators) through the blood–brain barier (BBB). The partial ability of monoquaternary (Sakurada *et al.*, 2003; Petroianu *et al.*, 2007b) and bisquaternary (Lorke *et al.*, 2007) oximes to penetrate the BBB was published recently. *In vivo* reactivation of nerve agent-inhibited brain AChE was determined many times before and this topic is well described in two recent review articles (Lorke *et al.*, 2008b; Bajgar *et al.*, 2007).

Commonly used AChE reactivators have one (pralidoxime) or two (HI-6) quaternary nitrogens in their molecules. According to our results, affinity of bisquaternary oximes towards intact and inhibited AChE is generally higher in comparison with monoquaternary ones (Kassa and Cabal, 1999; Kuca and Kassa, 2004).

B. Length and Shape of the Connection Chain Between Pyridinium Rings

The length of the connecting chain between both pyridinium rings (for bisquaternary pyridinium reactivators) plays an important role in its potency to reactivate nerve agent-inhibited AChE (Pang *et al.*, 2003; Kuca *et al.*, 2003c). For *n*-methylene linkage chain, there exists dependence between the length of the connection chain and nerve agent used for inhibition. This dependence is shown in Figure 66.46 (Kuca *et al.*, 2003c).

As shown, the ideal length of the reactivator's linker for satisfactory activity of tabun, sarin, or VX-inhibited AChE is three or four methylenes. On the other hand, one methylene group seems to be the most potent reactivator of cyclosarin-inhibited AChE.

Unfortunately, this rule is satisfied just in the case of *n*-methylene linkage chains and oxime groups in position four at the pyridinium ring. Compounds with oxygen, sulfur or other structural fragments differing from the methylene incorporated into the connection chain and with different position of oxime groups do not fulfill the above-mentioned rule (Pang *et al.*, 2003; Musilek *et al.*, 2005, 2006a; Oh *et al.*, 2006; Acharaya *et al.*, 2008c).

These differences could be caused by the presence of the free electrons in the connection chain and subsequent interactions of this part of the reactivator's molecule with the inside of the enzyme cavity.

An important structural factor influencing the reactivation process could also be the ''rigidity'' of the linking chain. Owing to the rigidity of the connection chain, spatial orientation of the pyridinium rings in the enzyme cavity is limited. Compounds with a certain level of rigidity in the connection chain were synthesized with the aim of

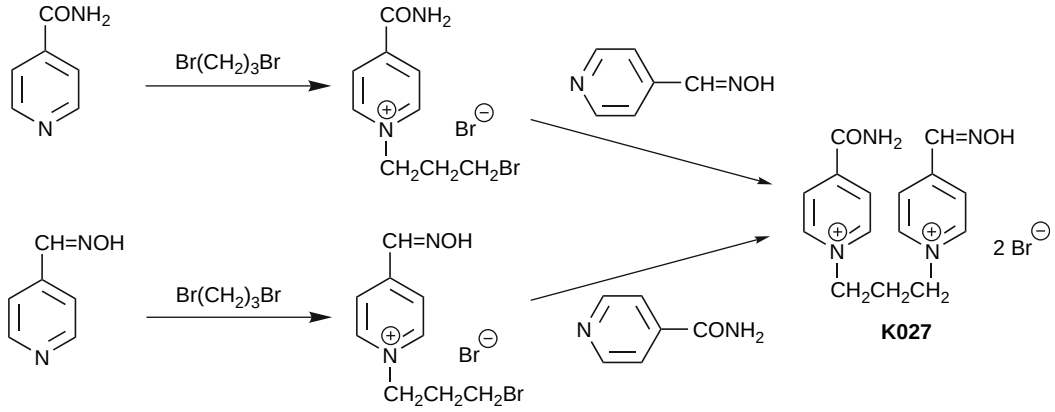


FIGURE 66.24. Synthesis of AChE reactivator K027 (Kuca *et al.*, 2003b).

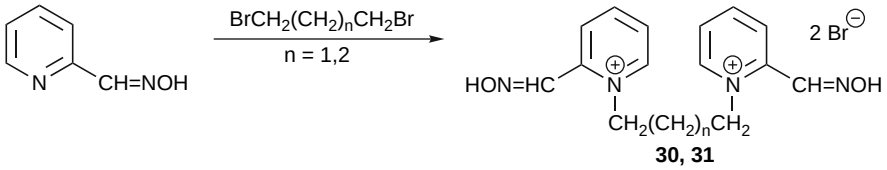


FIGURE 66.25. Synthesis of symmetric bispyridinium dicarbaldoximes (Kuca *et al.*, 2004b).

elucidating the influence of rigidity on reactivation potency. *Z*- and *E*-but-2-ene and *ortho*-, *meta*-, *para*-xylene moieties were inserted into the linkage chain (Musilek *et al.*, 2005, 2006a, 2007c). In Figure 66.47, the angles in the linker caused by incorporation of the rigid moiety are shown.

C. Presence of the Oxime Group in the Oxime Structure

The presence of the oxime group in the structure of the reactivator is another substantial structural factor. Former hydroxyiminoacetone, hydroxamic acids, geminal dioles, and ketoximes were used as potential nucleophilic agents for breaking the bond between inhibitor and enzyme (Picha *et al.*, 2005; Heath, 1961). The aldoxime group currently seems to be used entirely, and therefore it is involved in all newly synthesized AChE reactivators (see Synthesis part of this chapter).

The oxime group (–CH=NOH) in the human body dissociates to the oximate anion (–CH=NO$^-$) which acts as a nucleophile and cleaves the bond between the enzyme and the phosphorus atom of the inhibitor (Patocka *et al.*, 2005; Heath, 1961).

D. Position of the Oxime Group

The other important structural factor is also connected with the oxime group: the position of the oxime group at the quaternary pyridinium ring. As can be seen in Table 66.3, there are significant differences in the reactivation potency of the oximes regarding the oxime group position.

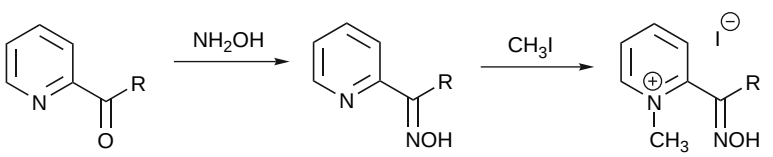
There exists no general rule that all oximes in the given position are able to reactivate all nerve agent-caused inhibitions. However, the general standard is that reactivators containing the oxime group in positions two and four are more effective compared to the oximes in position three. This fact is due to the difference in pKa between oximes in positions two and four versus those in position three.

Although the rule governing the oxime position for all nerve agents is unrealistic, there are some relationships between the position of the oxime group and used nerve agent. This means that the position of the oxime group, as well as the length of connecting chain between quaternary pyridinium rings, are nerve agent dependent. For example, cyclosarin-inhibited AChE is best reactivated by reactivators with an oxime in position two (Kuca and Patocka, 2004). On the other hand, reactivators with an oxime group at the pyridinium ring in position four are currently considered to be the most potent for reactivation of tabun-inhibited AChE (Cabal *et al.*, 2004). Also, pesticide-induced poisonings are best treatable with AChE reactivators with an oxime at position four (Worek *et al.*, 1996; Petroianu and Kalasz, 2007). Very good reactivability of sarin and VX-inhibited AChE has been found with both reactivators (with oxime groups in positions two and four) since they are potent reactivators of inhibited AChE (Kuca *et al.*, 2005c, d).

E. Number of Oxime Groups in the Reactivator's Molecule

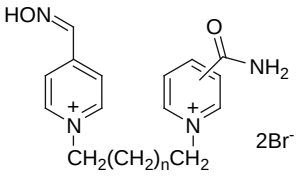
The number of oxime groups in the reactivator's molecule is the fifth structural requirement that needs to be discussed in this section. One molecule of the AChE reactivator can contain one, two, three, or four oxime groups with one, two, or three quaternary nitrogens in one structure. Examples of different structures having one, two, three, or four oxime groups are shown in Figure 66.48.

As can be seen, the number of oxime groups does not increase reactivation potency of the potential AChE reactivator. This fact could be connected with increased size of the AChE reactivator's molecule. Owing to the fact that the main role in the reactivation process has just the first oxime group (with the lowest pKa), the presence of the second oxime group is not so strict.



Pralidoxime R = H
26a R = phenyl
32 R = CH_3
33 R = pyridin-2-yl

FIGURE 66.26. Monoquaternary compounds prepared by Kuca *et al.* (2004b).

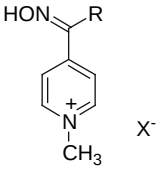


34

n = 1,2,3

$CONH_2$ position … 3,4

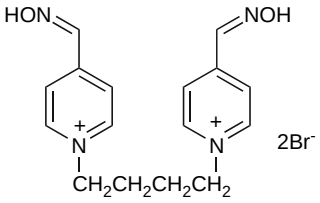
FIGURE 66.27. Structure of new reactivators derived from oxime K027 and K048.



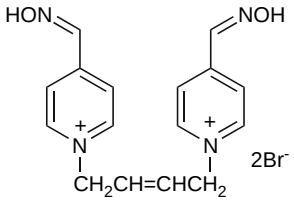
35

R = H, CH_3, Ph, CN, NH_2, $COCH_3$, $CH_2SO_2CH_3$, $CH_2SO_2N(CH_2CH_3)_2$, $CH_2SO_2C_6H_5$

FIGURE 66.28. Structures of compounds prepared by Picha *et al.* (2005).

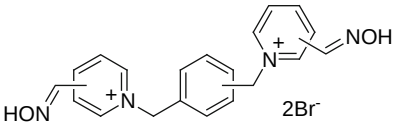


K074



K075

FIGURE 66.30. Structures of oximes K074 and K075.

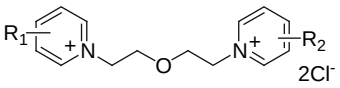


37

Ortho-, *meta*-, *para*-xylene

Oxime positions – all combinations of 2, 3, 4

FIGURE 66.31. Structure of xylene containing linker reactivators.



36

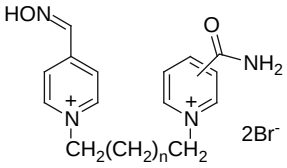
36a … R_1 = 2-CH=NOH; R_2 = 2-CH=NOH

36b … R_1 = 4-CH=NOH; R_2 = 4-CH=NOH

36c … R_1 = 3-CH=NOH; R_2 = 4-$CONH_2$

36d … R_1 = 4-CH=NOH; R_2 = 4-$CONH_2$

FIGURE 66.29. Structures of reactivators synthesized by Kim *et al.* (2006).

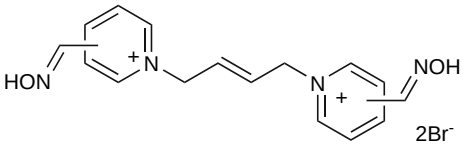


38

n = 4,5,6

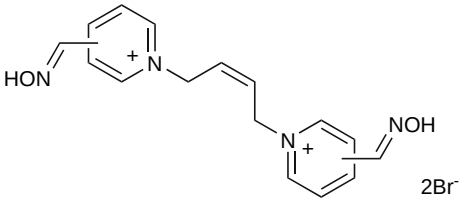
$CONH_2$ position … 3,4

FIGURE 66.32. Structures of reactivators (Chennamaneni *et al.*, 2005).



39

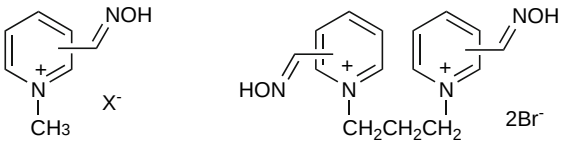
E-isomers



40

Z-isomers

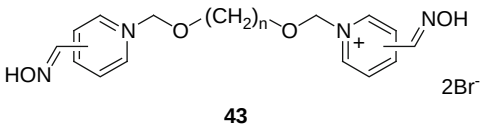
FIGURE 66.33. Newly prepared oximes having but-2-en linker in their structure.



41 **42**

Monoquaternary compounds **Bisquaternary compounds**

FIGURE 66.34. Mono- and bisquaternary pyridines.

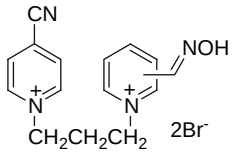


43

n = 2,3,4,5

Oxime position 2,2, 3,3 and 4,4

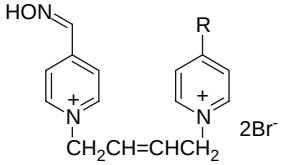
FIGURE 66.35. Structures of oximes prepared in Korea and India.



44

Oxime position 2-, 3-, 4-

FIGURE 66.36. Structures of oximes with cyano group.



45

R = CH_3, t-But, Ph, Benz, CH_2OH, COOH, $COCH_3$, COOEt, CN, SCH_2COOH, $C(NH_2)$=NOH

FIGURE 66.37. Structures of promising oximes with but-2-ene linker.

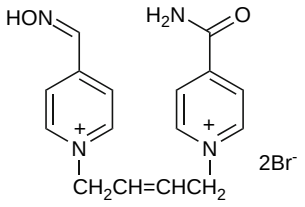
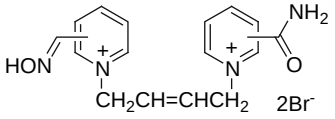


FIGURE 66.38. Structure of oxime K203.

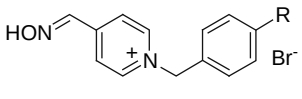


46

Oxime group position … 2,3,4

Carbamoyl group position … 3,4

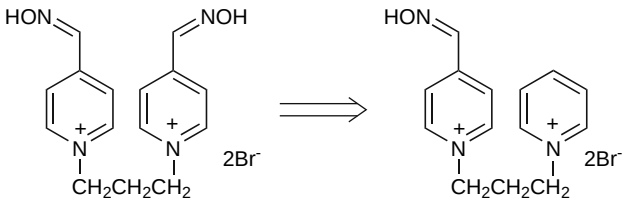
FIGURE 66.39. Other derivatives of oxime K203.



47

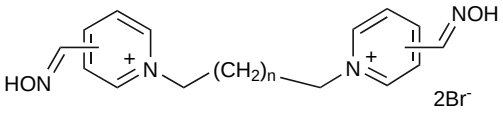
R = H, CH_3, Br, Cl, NO_2

FIGURE 66.40. Monoquaternary reactivators synthesized in Croatia.



Trimedoxime **48**

FIGURE 66.41. Modification of trimedoxime with the aim of decreasing the reactivator's size.



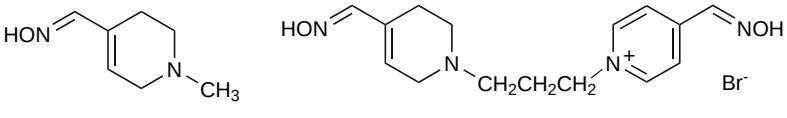
28f, 30f

n = 5

28f Oxime position 4,4

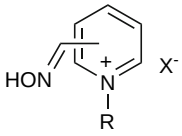
30f Oxime position 2,2

FIGURE 66.42. Structures of the promising Pang's reactivators resynthesized in Korea.



49 **50**

FIGURE 66.43. Structures of the reactivators with tertiary nitrogen.

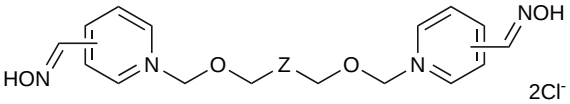


51

Oxime group position 2,3,4

R = ethyl, *n*-butyl, *n*-hexyl, *n*-octyl, *n*-decyl, *n*-octadecyl, isoamyl, benzyl, *p*-methylbenzyl, *p*-tert-butylbenzyl, 2-phenylethyl, 3-phenylpropyl

FIGURE 66.44. Structures of monoquaternary oximes.



52

Z = *para*-xylen, *meta*-xylen, *cis*-but-2-en, but-2-ine

FIGURE 66.45. Structures of Acharaya's reactivators.

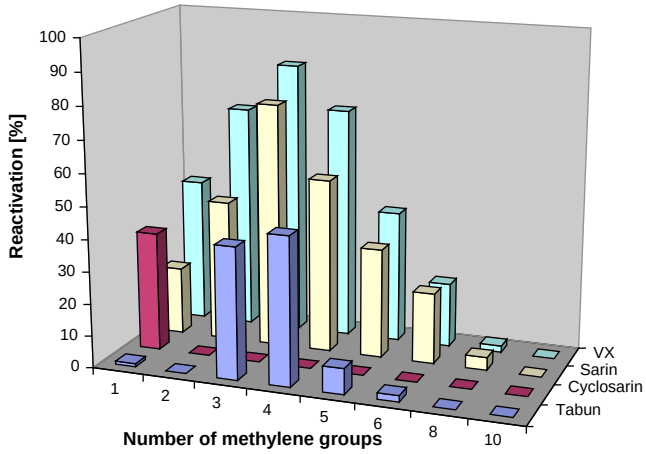


FIGURE 66.46. Dependence of the reactivation activity on the length of the linker. Source of enzyme – rat brain homogenate; inhibitor – chlorpyrifos; time of inhibition – 30 min; time of reactivation – 10 min; pH 7.6; 25°C; oxime concentration 10^{-3} M. (Kuca *et al.*, 2003c).

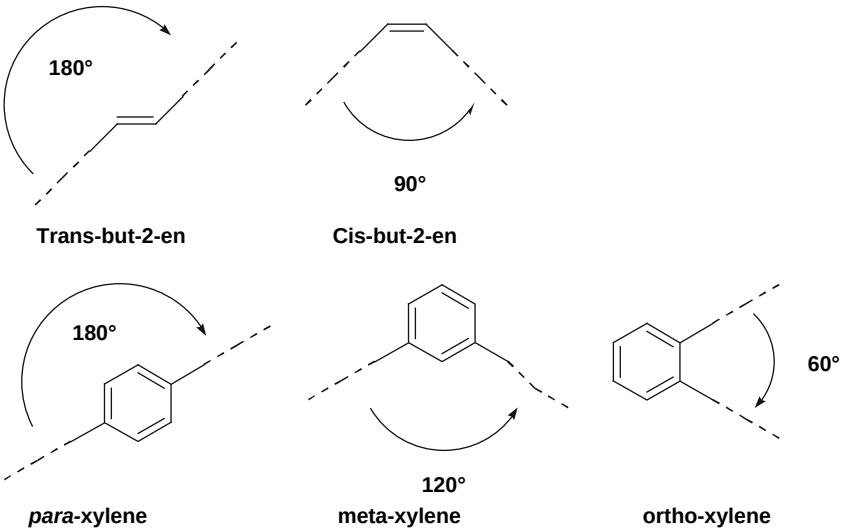
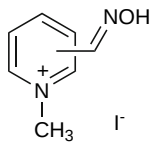
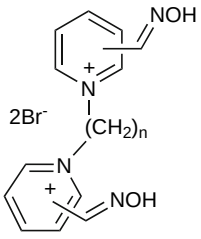


FIGURE 66.47. Rigidity of linkers caused by incorporation of several moieties.

TABLE 66.3. Reactivation potency of selected reactivators influenced by oxime group number and position

Structures	Reactivation potency (%)		Structural factors			
	0.00001 M	0.001 M	Number of oxime groups	Oxime group position	Length of the connection chain	Number of quaternary nitrogens
	9	80	**1**	**2**	–	1
	3	0	**1**	**3**	–	1
	0	7	**1**	**4**	–	1
	6	19	**2**	**2,2′**	3	2
	1	16	2	**3,3′**	3	2
	33	79	**2**	**4,4′**	3	2
	10	24	2	**2,2′**	4	2
	0	6	**2**	**3,3′**	4	2
	30	50	**2**	**4,4′**	4	2

Source of enzyme – rat brain homogenate; inhibitor – chlorpyrifos; time of inhibition – 30 min; time of reactivation – 10 min; pH 7.6; 25°C; oxime concentrations 10^{-5} M and 10^{-3} M (Racakova *et al.*, 2006)

VIII. PROMISING OXIMES

Along with those AChE reactivators which are at the present time commercially available (pralidoxime, trimedoxime, methoxime, obidoxime, and HI-6), there are several oximes which are considered promising.

We can also consider the development of a new reactivator salt as a new compound: HI-6 dimethanesulfonate is probably the number one oxime under development in many countries because of its superior stability and solubility when compared with HI-6 dichloride. It is well known as a broad-spectrum reactivator for nerve agents (except tabun) (Lundy *et al.*, 2006).

In the Czech Republic, great efforts have focused on the development of new reactivators used in the case of tabun poisoning. During the last 5 years, three new generations were prepared. The best reactivators of the first generation (K027, K048) are currently being tested in many countries for their extraordinary potency and low toxicity (Kassa *et al.*, 2006; Calic *et al.*, 2006; Kalasz *et al.*, 2006; Tekes *et al.*, 2006; Gyenge *et al.*, 2007; Benko *et al.*, 2007; da Silva *et al.*, 2008). Subsequently, the second generation was developed. Among the oximes tested, oximes K074 and K075 were recommended as the best ones. However, as they reached the highest reactivation activity, they were too toxic for further thorough investigation (Kassa and Humlicek, 2008; Kuca *et al.*, 2007d). Currently, the best recommended oxime of the third generation for tabun-inibited AChE seems to be oxime K203 (Musilek *et al.*, 2007f; Kassa *et al.*, 2008). Except for the promising results of developed oximes obtained with tabun, these oximes seem to be satisfactory also in case of pesticide poisoning (Petroianu *et al.*, 2006a, b). Among them, oxime K027 was tested in the United Arab Emirates as the best oxime (when compared with obidoxime, trimedoxime, pralidoxime, and HI-6) against pesticides (paraoxon, DFP, methyl paraoxon) both *in vitro* and *in vivo* (Petroianu *et al.*, 2007a, c; Csermely *et al.*, 2007).

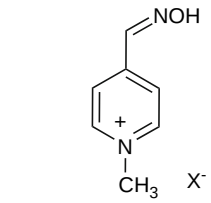
There are still efforts being made to compare oxime HLö-7 with HI-6 to see if its activity is comparable or better. Structures of both compounds are almost similar, differing slightly in one added oxime group at position four on the pyridinium ring. However, the reactivation potency of HLö-7 is not much better to perform all the steps, which are needed for introduction to the market.

In USA, oxime MMB-4 (methoxime) will probably replace the currently available pralidoxime, which is already considered an old fashioned antidote. There are many studies focused on this topic (Singh *et al.*, 2007).

According to recent literature, several laboratories are also focusing on Ortho-7, which has been described by many investigators (Pang *et al.*, 2003; Oh *et al.*, 2008; Ekstrom *et al.*, 2006, 2007).

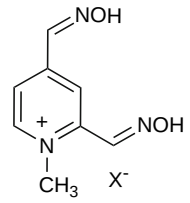
IX. NEW TRENDS IN DEVELOPMENT OF NEW AChE REACTIVATORS

New trends to improve reactivator potency depend on the purpose of their action. There are many directions that should be considered.



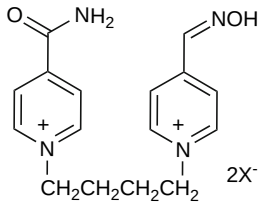
[0;6]

One on one



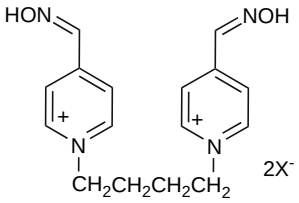
[0;2]

Two on one



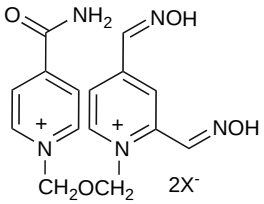
[6;25]

One on two



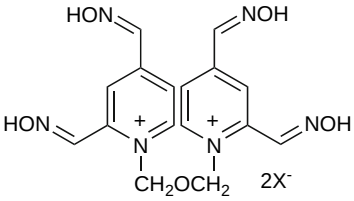
[15;45]

Two on two (a)



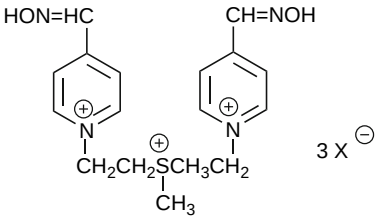
[2;3]

Two on two (b)



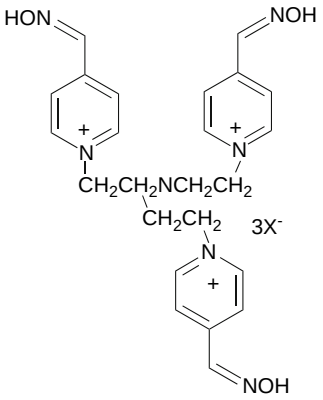
[0;1]

Four on two



[0;9]

Two on three



[0;2]

Three on three

FIGURE 66.48. Structurally different compounds having different oxime groups. Source of enzyme – rat brain homogenate; inhibitor – tabun; time of inhibition – 30 min; time of reactivation – 10 min; pH 7.6; 25°C; oxime concentrations – 10^{-5} M and 10^{-3} M (Cabal *et al.*, 2004).

Probably the most discussed factor is the possibility of a broad-spectrum reactivator. Every new oxime could be tested for its broad-spectrum potency. However, the probability of finding such an oxime is low. Alternately, we can use a combination of two oximes. Such an approach was already applied in the former Yugoslavia (Kovacevic *et al.*, 1989a, b) two decades ago. Candidates for this approach should be oxime HI-6 and various other oximes which are applicable in the case of tabun and pesticide poisoning.

Another factor that needs to be discussed is BBB penetration. Such an increase could be solved by chemical modification of the reactivator's structure, for example modifications like withdrawal of the quaternary nitrogen or connection of quaternary moiety with lipophilic compounds, which can serve as carriers into the brain (amino acids, steroids, Alzheimer's disease drugs, sugars, etc.), or the use of special carriers (e.g. nanoparticles, complex systems). However, do we really need to increase the oxime level in the brain? If there are higher concentrations of oximes in the brain, its toxicity could increase. This needs to be taken into account.

A new nucleophilic group instead of the oxime group could also be considered as a promising approach. However, during the last 50 years, no better nucleophilic group has been found.

New techniques used currently for drug design could be used. Among them, molecular docking studies, prediction of new structures using chemometric tools, click chemistry, or random chemistry approaches could be applied to reach the target.

Finally, oximes could be applied not only for the reactivation of AChE as life-endangering enzymes, but their use as BuChE reactivators could contribute to finding the ''pseudocatalytic scavenger''. Such a scavenger could catch the nerve agent (BuChE as stoichiometric scavenger) and then be renewed by oxime to be used once again for nerve agent binding.

X. CONCLUDING REMARKS AND FUTURE DIRECTION

As mentioned above, the threat of the misuse of nerve agents continues. Also, the requirements on agricultural production increase the use of pesticides and, because of this, deaths of agricultural workers increase. Due to the fact that there is no universal oxime that could be applied in every situation of nerve agent or pesticide intoxication, the development of new oximes still continues. We hope that in the near future, new more promising candidates will be found, especially if novel techniques and data from structure–activity studies are used as a starting point for the synthesis of new reactivators.

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CHAPTER 67

Paraoxonase (PON1) and Detoxication of Nerve Agents

LUCIO G. COSTA AND CLEMENT E. FURLONG

I. INTRODUCTION

More than half a century ago, certain organophosphorus (OP) insecticides were found to be enzymatically hydrolyzed by plasma, and it was determined that this hydrolysis was catalyzed by enzymes designated A-esterases (Mazur, 1946; Aldridge, 1953). Aldridge's proposal that an A-esterase hydrolyzed both phenylacetate and paraoxon (the active metabolite of the OP insecticide parathion) was conclusively proven several decades later, when it was shown that recombinant paraoxonase/arylesterase catalyzed both activities (Gan *et al.*, 1991). Studies in the late 1970s and early 1980s indicated that the plasma hydrolytic activity toward paraoxon was polymorphically distributed in human populations (Playfer, 1976; Eckerson *et al.*, 1983; Mueller *et al.*, 1983), suggesting a genetically based differential susceptibility to OP toxicity. The molecular basis of the paraoxonase (PON1) activity polymorphisms (Humbert *et al.*, 1993; Adkins *et al.*, 1993) and its role in the toxicity of OP compounds (Costa *et al.*, 2002, 2006) have been elucidated. Furthermore, novel important roles of PON1 in the metabolism of oxidized lipids, of certain drugs and, more recently, of quorum sensing, have also emerged, highlighting the multifaceted roles and importance of this enzyme. These latter aspects of PON1 are not discussed in this chapter but the reader can refer to other recent articles and reviews (Durrington *et al.*, 2001; Costa and Furlong, 2002; Mackness *et al.*, 2002; Costa *et al.*, 2003a; Draganov and LaDu, 2004; Ozer *et al.*, 2005).

II. PON1 POLYMORPHISMS: DEFINING PON1 STATUS

PON1 is a member of a family of proteins that also includes PON2 and PON3, the genes of which are clustered in tandem on the long arms of human chromosome 7 (q21.22). PON1 is synthesized primarily in the liver and a portion is secreted into the plasma, where it is associated with high density lipoproteins (HDL) (Sorenson *et al.*, 1999; Deakin *et al.*, 2002). PON1, which received its name from its ability to hydrolyze paraoxon, its first and most studied OP substrate, hydrolyzes the active metabolites of several other OP insecticides (e.g. chlorpyrifos oxon, diazoxon), as well as nerve agents such as sarin, soman, and VX (see below). The crystal structure for a recombinant PON1 indicates that it is a six-bladed β-propeller, with two calcium ions in the central tunnel, one of which is essential for enzyme activity and the other for enzyme stability (Harel *et al.*, 2004).

Activity of paraoxonase in plasma of human populations exhibits a polymorphic distribution, and individuals with high, intermediate, or low paraoxonase activity can be identified (Eckerson *et al.*, 1983; Mueller *et al.*, 1983; reviewed in Geldmacher von Mallinkrodt and Diepgen, 1988). Studies in the early 1990s led to the purification, cloning, and sequencing of human PON1 (Gan *et al.*, 1991; Furlong *et al.*, 1991; Hassett *et al.*, 1991), and the molecular characterization of its polymorphisms (Humbert *et al.*, 1993; Adkins *et al.*, 1993). Two polymorphisms were observed in the PON1 coding sequence: a Gln(Q)/Arg(R) substitution at position 192, and a Leu(L)/Met(M) substitution at position 55 (Humbert *et al.*, 1993; Adkins *et al.*, 1993). PON1 192 and 55 genotypes have been established in several populations utilizing PCR-based methods. The polymorphism at position 192 has been the most studied, with gene frequencies of $PON1_{Q192}$ ranging from 0.75 for Caucasians of northern European origin, to 0.31 for some Asian populations (Brophy *et al.*, 2002).

In addition to these two polymorphisms in the coding region of PON1, 13 polymorphisms have been found in the noncoding region of the PON1 gene (http://pga.gs.washington.edu) and five of these have been characterized to date (Brophy *et al.*, 2001a; Leviev and James, 2000; Suehiro *et al.*, 2000). The most significant of these promoter region polymorphisms is that at position –108, with the –108C allele providing levels of PON1 about twice as high as those seen with the –108T allele (Leviev and James, 2000; Suehiro *et al.*, 2000; Brophy *et al.*, 2001b).

The coding region polymorphisms of PON1 have been investigated for effects on the catalytic efficiencies of hydrolysis of specific substrates. The L/M polymorphism at position 55 does not affect catalytic activity, but has been

associated with plasma PON1 protein levels, with $PON1_{M55}$ being associated with low plasma PON1 (Blatter Garin *et al.*, 1997; Mackness *et al.*, 1998). However, this appears to primarily result from linkage disequilibrium with the low efficiency of the –108T allele of the –108 promoter region polymorphism (Brophy *et al.*, 2002), although some data indicate that $PON1_{M55}$ may be somewhat less stable than $PON1_{L55}$ (Leviev *et al.*, 2001; Roest *et al.*, 2007). The Q/R polymorphism at position 192 significantly affects the catalytic efficiency of PON1. Initial studies indicated that the $PON1_{R192}$ allozyme hydrolyzed paraoxon more readily than $PON1_{Q192}$ (Humbert *et al.*, 1993; Adkins *et al.*, 1993). Further studies indicated that this polymorphism was substrate dependent, as the $PON1_{Q192}$ alloform was found to hydrolyze diazoxon, sarin, and soman more rapidly than $PON1_{R192}$ *in vitro* (Davies *et al.*, 1996). In the case of diazoxon, however, more recent studies have shown that, under physiological conditions, both PON1 alloforms hydrolyze this compound with the same efficiency (Li *et al.*, 2000).

Complete resequencing of PON1 from 47 individuals, as part of the Environmental Genome Project, has led to the identification of more than 160 new single nucleotide polymorphisms, some in the coding regions and others in introns and regulatory regions of the gene (Jarvik *et al.*, 2003). These polymorphisms have for the most part not yet been characterized, but may affect splicing efficiency, message stability, or efficiency of polyadenylation. A few of them, however, have explained discrepancies found when comparing PON1 status (see below) and PCR analysis of codon 192 (Jarvik *et al.*, 2003).

Most studies investigating the association of PON1 polymorphisms with diseases have examined only the nucleotide polymorphisms (Q192R, L55M, C-108T) with PCR-based assays. A functional genomic analysis, however, provides a much more informative approach, as measurement of an individual's PON1 function (plasma activity) takes into account all polymorphisms that might affect activity. This is accomplished through the use of a high-throughput enzyme assay involving two PON1 substrates (usually diazoxon and paraoxon) (Richter *et al.*, 2004). This approach, in addition to providing a functional assessment of the plasma PON_{192} alloforms, provides the plasma level of PON1 for each individual, thus encompassing the two factors that affect PON1 levels or activity (position 192 amino acid and plasma alloform levels). This approach has been referred to as the determination of PON1 ''status'' for an individual (Richter and Furlong, 1999).

The catalytic efficiency with which PON1 degrades toxic OPs determines the degree of protection provided by PON1. In addition, higher concentrations of PON1 provide better protection. Thus, for adequate risk assessment, it is important to know PON1 levels and activity. In a given population, plasma PON1 activity can vary up to 40-fold (Eckerson *et al.*, 1983; Mueller *et al.*, 1983; Davies *et al.*, 1996; Richter and Furlong, 1999), and differences in PON1 protein levels up to 13-fold are also present within a single $PON1_{192}$ genotype in adults (Davies *et al.*, 1996). Studies investigating the role of PON1 in cardiovascular disease have indeed provided evidence that PON1 status (encompassing genotype and activity levels) is a much better predictor of disease than PON1 genotype alone (Jarvik *et al.*, 2000; Mackness *et al.*, 2001).

III. PON1 AND THE TOXICITY OF OP INSECTICIDES

Evidence that PON1 plays a role in modulating the toxicity of OPs *in vivo* has emerged slowly over the last 30 years. Initial indirect evidence was provided by comparison across animal species which differ in the levels of their plasma PON1 activity. Birds that have very low PON1 activity (Machin, 1976; Brealey *et al.*, 1980; Furlong *et al.*, 2000) display a much higher sensitivity, compared to rats, to the acute toxicity of some OPs (e.g. pirimiphos-ethyl, diazinon) (Brealey *et al.*, 1980). Among mammals, rabbits have a five- to 20-fold higher plasma PON1 activity than rats (Aldridge, 1953; Zech and Zurcher, 1974; Costa *et al.*, 1987), and are four-fold less sensitive to the toxicity of paraoxon (Costa *et al.*, 1987). Though several other factors, such as rates of absorption and distribution of OPs, rates of activation and of detoxication by other metabolic pathways, and intrinsic susceptibility of target enzymes, can affect the overall toxicity outcome, these initial observations provided some supporting, albeit indirect, evidence to the hypothesis that low serum PON1 activity would lead to an increased sensitivity to the acute toxic effects of OPs.

A more direct approach was provided by studies in which exogenous PON1 was injected into rats or mice. Background and impetus for such an approach were provided by an earlier study by Main (1956) who showed that intravenous administration of partially purified PON1 from rabbit serum to rats would protect them from the toxicity of paraoxon. Subsequent experiments in rats and mice utilizing pure rabbit PON1 (Furlong *et al.*, 1991) confirmed and expanded this early observation. Administration (via the tail vein) of the enzyme to rats increased serum PON1 activity toward paraoxon by nine-fold, and that toward chlorpyrifos-oxon by 50-fold (Costa *et al.*, 1990). Thirty minutes after PON1 injection, rats were challenged with an acute dose of paraoxon or chlorpyrifos oxon given by the i.v., dermal, i.p. or oral route, at doses causing similar degrees of AChE inhibition in plasma, red blood cells, brain, and diaphragm. Four hours later, at sacrifice, AChE activity measurements indicated a much lower degree of inhibition in animals that had been pretreated with PON1. Protection was more pronounced in the case of chlorpyrifos-oxon exposure, and was more prominent in two target tissues for OP toxicity, brain, and diaphragm (Costa *et al.*, 1990). Additional experiments were carried out in mice, as much less purified enzyme is required for injection, and since this species was

deemed to be ideal for studies involving genetically modified animals with altered PON1 levels (see below). An initial experiment followed the same protocol previously utilized in rats, and provided evidence that i.v. administration of pure rabbit PON1 increased serum chlorpyrifos-oxonase activity by 30- to 40-fold, and protected animals against AChE inhibition by dermally applied chlorpyrifos oxon (Li *et al.*, 1993). As with i.v. injection of rabbit PON1 the increased serum PON1 activity was short lasting ($t_{½}$ = ~6 h), further experiments were aimed at investigating other routes of administration for PON1. Administration of PON1 by the i.v. + i.p. routes increased plasma enzyme activity toward chlorpyrifos oxon by 35-fold and extended the half-life to 30 h. An even longer half-life, albeit with lower peak activity levels, was found when PON1 was given by the i.v. + i.m. route (Li *et al.*, 1993).

Rabbit PON1 also provided protection against the toxicity of the parent compound, chlorpyrifos, when the OP exposure occurred 30 min after i.v. injection of PON1 (Li *et al.*, 1993), or 24 h after an i.v. + i.p. administration of PON1 (Li *et al.*, 1995). Further experiments also showed that PON1, when given 30 min after dermal administration of chlorpyrifos, prevented the reduction of AChE activity in all tissues; when PON1 was injected 3 h after chlorpyrifos, a protective effect was still seen in brain and diaphragm (Li *et al.*, 1995). Altogether, this series of studies indicated that increasing serum levels of PON1 (by injection of purified rabbit enzyme) decreased the acute toxicity of specific OPs. Also of relevance is the finding that PON1 exerted a protective effect when given after OP exposure, indicating the therapeutic potential for use of recombinant PON1 in cases of OP poisoning, possibly in combination with other conventional treatments.

In the past decade, PON1 knockout and transgenic animals have provided important new tools to investigate the role of PON1 in modulating OP toxicity. PON1 knockout ($PON1^{-/-}$) mice were produced by targeted disruption of exon 1 of the PON1 gene, and have normal appearance and body weights (Shih *et al.*, 1998). Plasma from $PON1^{-/-}$ mice has no detectable hydrolytic activity toward paraoxon and diazoxon, and very limited chlorpyrifos-oxonase activity. A similar pattern of activity is also found in liver, indicating that both plasma and liver PON1 are encoded by the same gene (Li *et al.*, 2000). PON1 homozygous mice ($PON1^{+/-}$) have approximately 40% of plasma and liver PON1 activity compared with wild-type mice ($PON1^{+/+}$). As expected, PON1 knockout mice did not differ from wild-type animals in their sensitivity to demeton-*S*-methyl, an OP insecticide with a structure similar to malathion, and which is not a substrate for PON1 (Li *et al.*, 2000). As also predicted, $PON^{-/-}$ mice showed a dramatically increased sensitivity to chlorpyrifos oxon and diazoxon (Shih *et al.*, 1998; Li *et al.*, 2000). $PON1^{+/-}$ mice showed an intermediate sensitivity to diazoxon toxicity (Li *et al.*, 2000). PON1 null mice showed only a slight increase in sensitivity to the toxicity of chlorpyrifos and diazinon (Shih *et al.*, 1998; Li *et al.*, 2000). The most surprising observation was that PON1 null mice did not show an increased sensitivity to paraoxon, the substrate after which the enzyme was named, in spite of having no paraoxonase activity in plasma and liver (Li *et al.*, 2000).

Additional experiments were designed to determine whether administration of exogenous PON1 to $PON1^{-/-}$ mice, to restore serum PON1, would also restore resistance to OP toxicity. For this purpose, either human pure $PON1_{Q192}$ or $PON1_{R192}$ was injected, by the i.v. route, into PON1 knockout mice; the effects of various OPs on brain and diaphragm AChE activity was then determined. $PON1_{R192}$ provided significantly better protection than $PON1_{Q192}$ toward chlorpyrifos oxon, a finding confirmed by a subsequent study by Cowan *et al.* (2001), who administered recombinant adenoviruses containing PON1-LQ or PON1-LR genes to BALB/c mice before challenge with chlorpyrifos oxon. Both alloforms were equally effective in protecting against the toxicity of diazoxon (Li *et al.*, 2000), while neither $PON1_{R192}$ nor $PON1_{Q192}$ afforded protection against paraoxon toxicity (Li *et al.*, 2000). The results of these experiments in PON1 knockout mice prompted a re-examination of the *in vitro* catalytic efficiencies of the two human PON1 alloforms under more physiological salt concentrations (lower NaCl concentration).

Results from kinetic analysis of substrate hydrolysis by purified human alloforms provided an explanation for the *in vivo* finding. In the case of chlorpyrifos oxon, the catalytic efficiency of both PON1 alloforms was very high, and was higher for the $PON1_{R192}$ alloform. Catalytic efficiency was still high in the case of diazoxon, albeit lower than with chlorpyrifos oxon, but no alloform-specific difference was evident. With paraoxon, the $PON1_{R192}$ alloform was much more efficient than the $PON1_{Q192}$ alloform; however, its overall catalytic efficiency was too low to protect against exposure. This confirms the hypothesis (Chambers *et al.*, 1994; Pond *et al.*, 1995) that PON1 is not efficient at hydrolyzing paraoxon at low concentrations, suggesting that PON1 may not degrade paraoxon efficiently *in vivo,* and that other pathways (e.g. cytochromes P450, carboxylesterase) are primarily responsible for detoxifying paraoxon *in vivo*.

Further experiments carried out in PON1 transgenic mice (mice expressing either human PON_{Q192} or human $PON1_{R192}$ on a knockout background, and mice carrying the human $PON1_{R192}$ on top of mPON1) provided additional evidence for such conclusions. A transgenic mouse line that carries the human $PON1_{R192}$ allele over its mouse PON1 was tested for its sensitivity to paraoxon. These mice, whose serum paraoxonase activity was 3.5-fold higher than wild-type mice, showed similar sensitivity to paraoxon as wild-type mice (Li *et al.*, 2000). On the other hand, $hPON1_{R192}$-TG mice (expressing human $PON1_{R192}$ on a knockout background) were significantly less sensitive to the toxicity of chlorpyrifos oxon than $hPON1_{Q192}$-TG mice, despite having the same level of PON1 protein in liver and plasma (Cole *et al.*, 2005).

Altogether, these animal experiments indicate that the ability of PON1 to modulate the acute toxicity of OPs varies among compounds. In the case of chlorpyrifos oxon, both the level of expression and the Q192R genotype are important determinants of susceptibility, highlighting the importance of assessing PON1 status in potentially exposed individuals. With regard to diazoxon, protection or susceptibility is dictated primarily by the level of expression of PON1, independently of the Q192R genotype, stressing the importance of knowing PON1 levels. Perhaps ironically, PON1 status does not appear to play a role in modulating sensitivity to paraoxon toxicity.

IV. PON1 AND THE TOXICITY OF NERVE AGENTS

A number of nerve agents, such as soman, sarin and VX, are OPs, and have been shown to be metabolized *in vitro* by PON1 (Davies *et al.*, 1996; Rochu *et al.*, 2007). Information is scarce about VX, while more information is available regarding the other two compounds. Both sarin and soman are hydrolyzed to a higher degree by $PON1_{Q192}$ than by $PON1_{R192}$ (Davies *et al.*, 1996). Plasma somanase activity and sarinase activity (both in U/l) were found to be 2143 and 335 for QQ homozygotes, and 992 and 38 for RR homozygotes, respectively (Davies *et al.*, 1996). Thus, homozygotes for the $PON1_{Q192}$ allele hydrolyze sarin approximately ten times better than individuals homozygous for the $PON1_{R192}$ allele, while the ratio is about two for soman (Davies *et al.*, 1996). Results of a kinetic study confirmed these findings; catalytic efficiency for sarin was determined as 0.91 and 0.07 (ratio = 13) for $PON1_{Q192}$ and $PON1_{R192}$ homozygotes, respectively, while values for soman were 2.8 and 2.1 (ratio = 1.3) (Rochu *et al.*, 2007). Soman exists as four stereoisomers (C+P+, C+P−, C−P+, C−P−) (Benschop and de Jong, 1988), with both P- isomers displaying the highest *in vivo* toxicity; wild-type recombinant human PON1 was shown to hydrolyze soman stereoselectively, with a six-fold overall difference in catalytic efficiency (P+ > P−) (Yeung *et al.*, 2007).

In vivo studies in animals have shown that intravenous administration of naked DNA bearing the human $PON1_{Q192}$ cDNA to mice could elevate plasma somanase activity by about two-fold (Fu *et al.*, 2005). The pcDNA/PON1-treated mice survived in greater numbers with a pcDNA/PON1 dose dependence and for a longer period after an acute dose of soman (0.2 mg/kg, s.c.) (Fu *et al.*, 2005).

The 1995 terrorist attack in the Tokyo subway system that left 12 people dead and over 5,000 injured (Suzuki *et al.*, 1995; Nagao *et al.*, 1997) provided the opportunity to investigate the role of PON1 in modulating the toxicity of sarin in humans. The prevalence of the $PON1_{R192}$ genotype in the Japanese population is 0.66, compared with 0.25–0.30 in various Caucasian populations (Brophy *et al.*, 2002; Yamasaki *et al.*, 1997). Thus, Japanese individuals may have been more prone to sarin toxicity because of the low sarin-hydrolyzing ability of the $PON1_{R192}$ allozyme. However, among ten of the victims of the Tokyo attack, seven expressed the $PON1_{192Q}$ genotype, with six Q/R heterozygotes and one Q/Q homozygote (Yamada *et al.*, 2001). Thus, the genotype which confers high hydrolyzing activity toward sarin did not appear to provide protection from acute sarin poisoning. Several issues need, however, to be considered. First, only the Q192R genotype of those ten individuals was analyzed, with no information on their PON1 status. In a Caucasian population, the range of sarinase activity among individuals with the QQ or QR genotype ranged from ~0 to 758 U/l (Davies *et al.*, 1996). Second, exposure to sarin in these seven QQ or QR individuals was indeed massive, as it caused death instantly or, with one exception, in less than 48 h (Yamada *et al.*, 2001). Such high-dose exposure would be expected to overcome any potential protection afforded by the $PON1_{Q192}$ genotype. Third, and most importantly, the catalytic efficiency of sarin hydrolysis by PON1, even in QQ homozygotes, is low; the situation is thus similar (albeit reversed) to that of paraoxon, with one $PON1_{192}$ alloform hydrolyzing sarin with better efficiency, but still not efficiently enough to provide protection.

A few studies have also investigated PON1 polymorphisms in US and UK troops that were deployed in the Persian Gulf area in 1990–1991. Individuals who served in the Gulf War theater were potentially exposed to a wide range of biological and chemical agents, including sand, smoke from oil well fires, solvents, petroleum fuels, depleted uranium, anthrax and botulinum toxoid vaccinations, insecticides, pyridostigimine bromide, and nerve agents (IOM, 2000, 2003). A large number of these veterans have complained of a range of unexplained illnesses including chronic fatigue, muscle and joint pain, loss of concentration, forgetfulness, and headache, symptoms that are often referred to as Gulf War Syndrome (IOM, 2000, 2003). PON1 genotypes and plasma enzyme activity were investigated in a group of 25 ill US Gulf War veterans and 20 controls (Haley *et al.*, 2000). $PON1_{R192}$ homozygotes or $PON1_{Q/R192}$ heterozygotes were more likely to have neurologic symptoms than individuals homozygous for PON_{Q192}. Furthermore, low activity of the plasma $PON1_{Q192}$ isoform appeared to better correlate with illness than the PON1 genotype or the activity levels of the $PON1_{R192}$ genotype (Haley *et al.*, 2000). This study would suggest that low PON1 status may represent a risk factor for illness in Gulf War veterans, though such findings necessitate further confirmation in a larger population (Furlong, 2000).

A similar study in a group of 152 UK Gulf War veterans, who self-reported the presence of symptoms associated with the Gulf War Syndrome, yielded somewhat different results (Mackness *et al.*, 2000). Plasma paraoxonase activity and levels of PON1 protein were lower in veterans than in a control group, and these decreases were independent of the PON1 genotype (Mackness *et al.*, 2000). Thus, while in both

studies a reduced plasma paraoxonase activity was found, in one case it was attributed to an overrepresentation of the low activity PON1 isozyme (Haley *et al.*, 2000), whereas in the other it was common to all PON1 genotypes (Mackness *et al.*, 2000). Though the latter study suggests that this group of veterans may have a decreased capacity to hydrolyze some OP insecticides, such as chlorpyrifos oxon, its significance is hampered by the lack of information on the extent of exposure to such compounds among veterans (Costa *et al.*, 2003b).

A third study compared PON1 genotypes and plasma paraoxonase activity in groups of UK veterans from the Persian Gulf War who were symptomatic by self-reporting ($n = 115$), healthy Persian Gulf War veterans ($n = 95$), symptomatic Bosnia peacekeeping veterans ($n = 52$), and symptomatic nondeployed military controls ($n = 85$) (Hotopf *et al.*, 2003). No differences in genotype distribution or PON1 activity were found between healthy and ill Gulf War veterans. However, individuals who were deployed to the Gulf had 25–35% lower median PON1 values than the other two groups, and these differences were not explained by differences in $PON1_{192}$ genotypes between groups. Thus, $PON1_{192}$ genotype and activity were not associated with Gulf War Syndrome, but appeared to be the results of deployment in the Persian Gulf. Possible explanations suggested for such findings were the potential exposure of those who served in the Gulf to yet unknown agents which led to a long-term decrease in PON1 activity, and/or an overrepresentation in those two groups of individuals with the –108T allele, which is associated with lower PON1 levels (Hotopf *et al.*, 2003).

V. PON1 AS A THERAPEUTIC AGENT

Current therapy for OP poisoning relies on the use of atropine, a cholinergic muscarinic antagonist, and on oximes, such as pralidoxime (2-PAM), to reactivate phosphorylated acetylcholinesterase (Lotti, 2000; Eyer *et al.*, 2007). Anticonvulsant drugs such as diazepam may also be utilized to control OP-induced convulsions (Lotti, 2000). More efficacious pyridinium 4-aldoximes, such as obidoxime and trimedoxime, have also been developed; however, their ability to reactivate phosphorylated acetylcholinesterase is not fully exploited because of the formation of phosphoryloximes, which have themselves very high anticholinesterase activity. Interestingly, phosphoryloximes are rapidly hydrolyzed by PON1, with the $PON1_{Q192}$ allozyme being about 50-fold more active than $PON1_{R192}$ (Stenzel *et al.*, 2007), suggesting that PON1 status would also influence the effectiveness and safety of these oximes (Eyer *et al.*, 2007).

While current therapies are effective in preventing lethality, it has been suggested that they may not prevent behavioral deficits, incapacitation, loss of consciousness, or the potential for permanent brain damage caused in particular by OP nerve agents (Lenz *et al.*, 2007). Complementary approaches have thus focused on the use of human proteins that would act as biological scavengers for OP compounds. Such biological scavengers should have no effects on their own, should not present an antigenic challenge to the immune system, should act rapidly and specifically, and should remain in circulation for a prolonged period of time (Lenz *et al.*, 2007). Out of this general concept, two parallel approaches have emerged, one relying on stoichiometric, the other on catalytic, bioscavengers. Among stoichiometric bioscavengers, B-esterases, such as acetylcholinesterase and butyrylcholinesterase, which react with OPs but do not catalyze their hydrolysis, have been utilized in several studies. Administration of these two proteins to rodents has been shown to afford protection toward two- to five-fold LD_{50} doses of sarin, soman, or VX (Wolfe *et al.*, 1987; Doctor and Saxena, 2005; Lenz *et al.*, 2007). Of particular interest in this regard is human butyrylcholinesterase, either isolated from human plasma, or recombinant (Cerasoli *et al.*, 2005).

While stoichiometric scavengers afford good protection toward OP toxicity, high doses are needed to neutralize an equimolar amount of nerve agent (Ashani and Pistinner, 2004). In contrast, a catalytic scavenger would afford similar or even higher protection at relatively low doses (Sweeney and Maxwell, 2003). For example, it has been estimated that 3 μM sarin would be neutralized by 765 mg of human butyrylcholinesterase, but only 120–550 mg of $huPON1_{Q192}$ (depending on time) (Rochu *et al.*, 2007), or a much lower dose of recombinant, engineered huPON1 with significantly increased catalytic efficiency of agent hydrolysis. A number of arguments would support the use of PON1 as a catalytic bioscavenger. First, PON1 is a human protein, suggesting an expected absence of immunological response. Second, experiments in mice have clearly shown that administration of purified human PON1 protects animals against the toxicity of OP insecticides such as chlorpyrifos oxon and diazoxon (Li *et al.*, 2000). Third, PON1 has been shown to hydrolyze soman, sarin, and VX. Nevertheless, as said, the catalytic efficiency of PON1 (even the more active $PON1_{Q192}$) toward OP nerve agents, in particular sarin, is very low. It has been calculated that to provide a valuable medical countermeasure against intoxication by nerve agents, the catalytic efficiency of PON1 will need to be enhanced by one or two orders of magnitude (Rochu *et al.*, 2007). Some direct evolution variants of a recombinant PON1 were found to exhibit a ten-fold faster detoxication of cyclosarin and soman (Amitai *et al.*, 2006). Other huPON1 mutants with enhanced catalytic activity have also been described, though not in relationship to OP nerve agents (Yeung *et al.*, 2004). Until recently, no one had succeeded in expressing active human PON1 in *E. coli*. However, recently, our research team has produced and purified engineered variants of untagged human PON1 from an *E. coli* expression system. The recombinant PON1 is nontoxic when injected into PON1 null mice, persists in

plasma beyond 2 days, and protects against OP toxicity (Stevens *et al.*, 2008).

An additional approach would be that of stimulating the biosynthesis of natural PON1. A number of compounds have been shown to stimulate the expression of PON1 (Costa *et al.*, 2005), among them, low doses of ethanol, some fibrates, statins, or dietary polyphenols such as quercetin (Gouedard *et al.*, 2004a). The red wine ingredient resveratrol has been shown to increase PON1 activity nearly three-fold *in vivo* (Gouedard *et al.*, 2004b) and less than two-fold in liver cells *in vitro* (Curtin *et al.*, 2007). Interestingly, in the latter study, the increased PON1 afforded protection of cells against the toxicity of soman and sarin (Curtin *et al.*, 2007). Modulation of PON1 activity by drugs or dietary agents is of interest; however, more information of the regulation of the PON1 gene is needed to devise approaches that would offer substantially larger increases in PON1. Further, the low catalytic efficiency of nerve agent hydrolysis by native human PON1 is not adequate to provide efficient protection against nerve agent exposure. The $PON1_{R192}$ alloform, however, does provide good protection against both diazoxon and chlorpyrifos-oxon exposure (Li *et al.*, 2000).

VI. CONCLUDING REMARKS AND FUTURE DIRECTION

Evidence from *in vitro* studies has shown that PON1 can hydrolyze a number of OP compounds, including nerve agents. *In vivo* studies in rodents have also indicated that PON1 status can influence the acute toxicity of certain OPs, and that administration of exogenous PON1 can protect animals from OP toxicity. In the case of nerve agents, the catalytic efficiency of PON1 is relatively low, and PON1 status does not greatly influence an individual's susceptibility to their toxicity. However, as noted above, recombinant variants can be engineered with enhanced catalytic activity toward nerve agents. The nonglycosylated, engineered recombinant PON1s with high catalytic efficiency for OP hydrolysis should be excellent candidates for use as catalytic biological scavengers, with both prophylactic and therapeutic applications.

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CHAPTER 68

Role of Carboxylesterases in Therapeutic Intervention of Nerve Gas Poisoning

SIGRUN HANNE STERRI AND FRODE FONNUM

I. INTRODUCTION

Carboxylesterases (CarbEs) constitute an important group of enzymes that plays a major role in the hydrolytic biotransformation of a large number of pro-drugs. In the present context we are, however, more interested in their ability to act as a scavenger towards the highly toxic organophosphorus (OP) compounds. The scavenger function of CarbE in blood plasma is the principal reason for the variation in nerve gas toxicity between the species, in both the presence and absence of carbamate prophylaxis. The scavenger role is primarily a consequence of the different content of CarbE protein in the plasma of different species, and is not necessarily linked to its enzymatic activity. Knowledge of the enzymology of CarbEs is, however, necessary in an attempt to define this scavenger protein, and is also a basis for understanding the role of CarbE in nerve gas poisoning with or without therapeutic intervention. The fact that plasma CarbE in rodents is a natural scavenger towards nerve gases makes it possible for us to handle nerve gas toxicity data in an adequate way. This is of vital importance when comparing and analyzing OP toxicity data for experimental animals such as rodents and species that do not have this specific blood protein such as rhesus monkey and humans. It is apparently a paradox that the plasma CarbE scavenger function in rodents allows us to estimate the protection factor of therapeutic intervention of nerve gas poisoning in humans. This seems to be true for interventions such as carbamate prophylaxis or the use of any artificially supplied scavenger to mimic rodent plasma CarbE. Although experimental protection factors with such interventions in rodents may be relatively low due to their natural scavenger content in plasma, it is worth noting that the estimated factors appear to be high for species including humans that are not supplied with plasma CarbE from nature. Prospective victims of nerve gas poisoning might therefore strongly benefit from further exploitation of any therapeutic intervention which involves the role of CarbE either directly or indirectly.

II. ENZYMOLOGY

A. Classification

Aldridge (1953) separated various esterases into two groups. The first group was A-esterases capable of hydrolyzing OP compounds; the second group was B-esterases, which could bind the OP compounds. CarbE belongs to this B-group of enzymes, which contains the serine molecule in its active site. To this group also belong several other esterases including acetylcholinesterase (AChE), butyrylcholinesterase (BuChE), and chymotrypsin. CarbEs have subsequently been connected to the alpha/beta hydrolase fold – which is common to several hydrolytic enzymes of widely differing phylogenetic origin and catalytic function. The core of each enzyme is similar: an alpha/beta sheet, not barrel, of eight beta-sheets connected by alpha-helices. These enzymes have diverged from a common ancestor and comprise a large family of proteins. They are important as hydrolyzing substrates containing ester or amide groups to free acids and alcohols. They are found in several tissues such as liver, small intestine, kidney, lung, and are also important for the scavenger function in plasma. At present, the database for this family contains over 450 orthologous or homologous members with 44 distinctive functions. Mammalian liver CarbEs belong to a family of proteins encoded by multiple genes. The isoenzymes were initially classified by their substrate specificity and isoelectric point (pI) (Mentlein *et al.*, 1987). However, this classification is now ambiguous because of overlapping substrate specificities, and is imprecise because the CarbEs as glycoproteins exhibit multiple bands with different pI values upon electrophoresis. They are very difficult to classify due to their wide substrate specificity. Satoh and Hosokawa (1998) and Hosokawa and Satoh (2006) have separated all mammalian CarbEs into five main groups based on their molecular properties. The two most important groups are CES1 and CES2. The CES1 group is the largest group and comprises members that have more than 60% homology to human liver CarbE. It contains the liver enzymes of human,

monkey, rat, and mouse. CES1 group enzymes preferentially hydrolyze substrates with a small alcohol group esterified to a large acyl group. Examples of substrates are cocaine, meperidine, methylphenidate, and temocapril (Zhang *et al.*, 1999) all of which contain methanol or ethanol linked to a large acyl group. CES2 includes human, monkey, mouse, rat, and hamster intestinal CarbE. In contrast to CES1 they hydrolyze esters with a large alcohol group and relatively small acyl group. Examples of substrates are heroin, 6-acetylmorphine, and methylprednisolone which contain acetate and succinate as acyl groups. CES3 includes ES male and human hCE3 and is expressed at low level in liver and the gastrointestinal tract. CES4 includes a protein excreted in cat urine with molecular weight 70 kDa (Miyazaki *et al.*, 2003). The CES5 group contains proteins with a much lower molecular weight, namely 46.5 kDa, and with a different structure.

B. Active Site

Through the work of Cohen *et al.* (1955) it was demonstrated that all the esterases in Aldridge group B contain an active serine which binds to the OP group or participates in the ester hydrolysis. Further comparison of the structure of a large number of esterases and lipases confirmed that they all contained a catalytic triad consisting of Ser, His, and Glu (sometimes Asp instead). This catalytic triad was preserved in AChE, BuChE, a series of lipases, and CarbE (Cygler *et al.*, 1993). Two adjacent Gly molecules formed an oxyanion hole. Frey *et al.* (1994) showed for chymotrypsin that the formation of low barrier hydrogen bonds facilitates a nucleophilic attack of the serine–OH group on the acyl carbonyl group of peptides. In the same way these would allow for the reaction between substrate esters or OP esters and the serine–OH of ChEs or CarbEs. CarbEs also contained a catalytic triad consisting of Ser, Glu, and His. When the catalytic triad consisting of Ser203, Glu336, and His450 in CarbE was transferred to The 203, Als 336, and Ala 450, respectively, the mutant had reduced CarbE activity (Satoh and Hosokawa, 1998). The active site gorge of the enzymes has a different size. For torpedo AChE it is 296 D3, for BuChE it is 496 D3, and for CarbE 3,014 D3. This explains why DFP cannot inhibit AChE, only BuChE and CarbE. The steps for interaction of CarbE with substrate or soman (as an example of an OP inhibitor) are the following: the ester bond or the P–F bond is attacked by the serine–OH. The hydrogen bond between the negatively charged oxygen of a tetrahedral complex and the NH groups of glycine stabilizes the charged carbonyl oxygen or phosphate oxygen of the substrate or inhibitor. The ester bond or P–F bond breaks and the leaving group (alcohol or F) diffuses away. A water molecule attacks the serine-*o*-acyl or *o*-phosphoryl group on the enzyme. Then His delivers a hydrogen atom to the serine-*o*-acyl which releases the acyl or phosphoryl group. This reaction is fast for esters but slow in the case of soman.

In the case of AChE there is an intermediate step where serine-*o*-phosphoryl is dealkylated or ''aged''. This is a process where the alkoxy group of the soman residue on the enzyme is hydrolyzed and the soman residue is resistant to the last hydrolysis step. The orientation of the sequence in CarbE allows the alkoxy group in soman residue and other nerve agents to be further away from the His group (7 Å instead of 3.5 in AChE) and therefore it is not dealkylated.

C. Substrate Specificity

Several different carboxylic esters may be hydrolyzed by CarbEs, and among them several may also be hydrolyzed by BuChE and A-esterases. For CarbEs the aromatic butyryl esters are better substrates than the acetyl esters, whereas the opposite holds for A-esterases (Augustinsson, 1961; Aldrige, 1953; Ecobichon, 1970). The hydrolysis of two simple aliphatic and aromatic butyryl esters, namely methyl butyrate and 4-nitrophenyl butyrate, is catalyzed by CarbE with only minimal contribution by ChE and A-esterase in a series of rat tissues, except for duodenum, based on the use of specific inhibitors of the three enzymes (Sterri *et al.*, 1985b). The two substrates are therefore appropriate to use for comparative evaluation of rat tissue CarbEs with respect to both inter- and intra-tissue diversity. Thus, the ratio between activities towards the two substrates in different tissues correlated to the plasma ratio seems to separate CarbEs of rat tissues into two main groups. The CarbEs of cerebrum, kidney, and duodenum display similar relative activity to plasma, whereas liver, lung, heart, and diaphragm constitute the other group with far higher (13–17-fold) relative preference for methyl butyrate than the first group (Sterri *et al.*, 1985b). This may indicate that the two groups are predominantly constituted by CES1 and CES2 CarbEs (see Classification, above) with preference for a substrate with smaller (methanol) and larger (4-nitrophenol) alcohol, respectively, esterified to the butyric acid. Also, the two CarbE isoenzymes of rat plasma seem to differ with respect to their relative specificity for methyl butyrate and 4-nitrophenyl butyrate, as do the three isoenzymes of guinea pig plasma. For each species the ratio between substrate activities of the plasma isoenzymes differs five- to seven-fold in favor of 4-nitrophenyl butyrate, as measured in the top fraction of activity peaks separated by chromatofocusing (Sterri and Fonnum, 1987, 1989).

D. Enzyme Inhibition by OP Compounds

Paraoxon, sarin, and soman inhibit AChE, BuChE, and CarbE almost to the same extent. DFP inhibits BuChE and CarbE to the same extent and 1,000-fold more than AChE. Diphenyl *p*-nitrophenyl phosphinate inhibits CarbEs more than 1,000-fold that of the ChEs. This explains why bis-*p*-nitrophenylphosphate is a specific inhibitor of CarbEs. A problem for CarbE as a scavenger is that VX is a 10,000-fold better inhibitor of the ChEs, and that ecothiophate is

a 10,000-fold better inhibitor of AChE than CarbE. As described under Active site, above, OP-inhibited CarbEs do not age. Instead, they spontaneously reactivate similar to a slow substrate. The half-life for spontaneous reactivation of VX or sarin-inhibited CarbE is 2 h, whereas the half-life for spontaneous reactivation after soman, DFP, or paraoxon inhibition is 20 h (Maxwell and Brecht, 2001). This is in accordance with results from a series of experiments with repetitive injection of $0.5 \times LD_{50}$ of soman in guinea pig at different time intervals. They demonstrated that soman was completely removed within 24 h and at the same time plasma CarbE (tributyrinase) activity was fully recovered (Sterri *et al.*, 1981).

E. Reactivation by Oxime of Nerve Gas-Inhibited CarbE

The original finding by Askew (1956) that diacetylmonoxime (DAM) has an antidotal effect towards sarin poisoning in several species including guinea pig may be due to the reactivation by DAM of sarin-inhibited CarbE in plasma (Myers, 1959; Polak and Cohen, 1970a; Cohen *et al.*, 1971). The authors also reported species differences between rodent plasma CarbEs with respect to reactivation by DAM after sarin poisoning. In addition, they found less effect of DAM reactivation for sarin-inhibited erythrocyte, brain, and lung CarbEs than for plasma CarbE of both rat and mouse (Myers, 1959).

Similar sensitivity to DAM reactivation is reported for various soman-inhibited CarbEs. Following *in vitro* inhibition by soman, the CarbE isoenzymes with low pI in plasma of both rat and guinea pig are partially reactivated (50–60%) by DAM within 5 min and are not further reactivated during the next 30 min (Sterri and Fonnum, 1987; Sterri, 1989). The two isoenzymes in rat plasma cannot be discriminated based on the DAM reactivatability (Sterri, 1989), whereas the one with high pI (6.1) in guinea pig plasma is about half as sensitive to DAM reactivation as the other two (Sterri and Fonnum, 1987). Also, two out of three CarbE isoenzymes in rat small intestine display similar reactivatability as the plasma CarbEs (Sterri, 1989), whereas none of the three CarbE isoenzymes in guinea pig liver can be reactivated by this oxime after soman inhibition (Sterri and Fonnum, 1987). The latter results correspond well with the poor effect by DAM on soman-inhibited commercial CarbEs from porcine liver (Fonnum *et al.*, 1985).

III. ORIGIN OF PLASMA CARBOXYLESTERASE

It follows from their role in nerve gas poisoning that the regulation of plasma CarbEs is of great interest. Possible tissues of their origin may be the liver, according to Yan *et al.* (1995); the intestine as has also been suggested (Sterri, 1989). In contrast to the other rat liver CarbEs, which are microsomal hydrolases A, B, and C with COOH-terminal HXEL consensus sequence for retention in endoplasmic reticulum (Yan *et al.*, 1994), the so-called hydrolase S lacks the COOH-terminal consensus sequence and is therefore presumed to be secreted into the blood (Yan *et al.*, 1995). The authors performed nondenaturing gel electrophoresis of rat serum, followed by staining for esterase activity or immunoblotting with antibody against recombinant hydrolase S. The results were interpreted as evidence that hydrolase S (67 kDa) is synthesized in the liver and undergoes extensive post-translational modification prior to its secretion as a 71 kDa protein in serum. Incomplete processing of hydrolase S was hypothesized to produce the amount of 67 kDa protein in liver microsomes. Results of the same study were taken to support this hypothesis in as much as Western immunoblotting with antibody against recombinant hydrolase S detected a 71 kDa protein in both serum and the medium of cultured hepatocytes. One objection is, however, that since the recombinant hydrolase S antibody also detects hydrolase A and B it may be relatively unspecific and one cannot exclude that other CarbEs may be detected as well. Also, the 71 kDa protein in serum has esterase activity towards 1-naphthylacetate and *para*-nitrophenylacetate, but immunoprecipitation with the hydrolase S antibody led to only a small decrease in the rate of hydrolysis of the two substrates by rat serum. The NH_2-terminal amino acid sequence of the 71 kDa protein is reported to be identical to that deduced from the cDNA encoding hydrolase S beginning with residue 19. Interestingly, based on the results from immunoblots with hydrolase S antibody and mRNA levels detected by Northern blot, the 67 and 71 kDa proteins in liver and serum, respectively, seem to be co-regulated by treatment of rats with various xenobiotics including phenobarbital (approximately 1.7-fold induction) (Yan *et al.*, 1995). Such co-regulation is not supported by the results obtained on plasma and liver CarbE activities with methyl butyrate or 4-nitrophenyl butyrate following phenobarbital treatment of rats by Sterri *et al.* (1985b). In this study the induction was 1.5–1.8-fold in liver and maximally 1.2-fold in plasma. However, phenobarbital induction of CarbE in mice led to similar enhanced activities in both liver and plasma (Clement, 1983).

Some interesting results on soman toxicity, thyroid deficiency, and plasma CarbE were reported by Swisher *et al.* (1986). They observed reduced effect of soman intoxication in hypothyroid rats with respect to both seizure activity and lethality. The resistance to soman was ascribed to increased binding of soman to plasma proteins, since both ChE and CarbE (tributyrinase) of plasma displayed nearly two-fold higher activity in hypothyroid than euthyroid rats. The latter result inspired us to investigate hypothyroid rats with respect to possible parallel coregulation of CarbE and ChE in plasma and liver. Plasma ChE is undoubtedly synthesized in liver (Augustinsson, 1948; Koelle, 1963) and the ChE activity in rat plasma as well as liver is known to differ between males and females (Leeuwin, 1966; Edwards

and Brimijoin, 1983; Andersen *et al.*, 1983; Sterri *et al.*, 1985a) and to respond on thyroidectomy (Leeuwin, 1966). Thus, male and female rats received rat chow supplemented with 0.05% thiouracil for 22, 30, or 38 days, a dosage which led to hypothyroid rats within 3 weeks (Stakkestad and Bremer, 1983). The results on CarbE activities in rat plasma and liver after 38 days were published previously (Sterri and Fonnum, 1989), but the complete set of results including comparison with ChE activities is presented in Figure 68.1. The activities with methyl butyrate increased significantly in liver but did not change in plasma of both male and females after 22 and 38 days of thiouracil feeding, whereas the hydrolysis of 4-nitrophenyl butyrate only varied slightly and inconsistently. The ChE activities of plasma and liver were simultaneously decreased in females and increased in males. The results show that the ChE activities in plasma and liver of both male and female rats are strongly co-regulated, whereas the CarbE activities of the two tissues are definitely not co-regulated (methyl butyrate hydrolysis) or are only marginally influenced by thyroid deficiency (4-nitrophenyl butyrate hydrolysis) (Figure 68.1). This investigation therefore does not indicate that rat plasma CarbE may be synthesized in liver.

Results on the various CarbE isoenzymes with respect to reactivatability by DAM after soman inhibition (see Reactivation by oxime of nerve gas-inhibited CarbE, above) might suggest intestinal origin of plasma CarbE. Two isoenzymes in plasma of both rat and guinea pig are reactivated to a similar degree as the two isoenzymes in the rat small intestine, and are quite different from the three liver isoenzymes of guinea pig which are not sensitive to DAM (Sterri and Fonnum, 1987; Sterri, 1989). Since the results are obtained with the same nerve gas and the same oxime under the same conditions, they may reflect both similarities and differences in enzymatic properties. It is interesting in this context that the DAM-sensitive isoenzymes of rat plasma and small intestine display correspondingly low isoelectric points (4.0–4.8) (Sterri, 1989), whereas higher pI values (5.0–6.4) are reported for six CarbE isoenzymes of rat liver (Mentlein *et al.*, 1987).

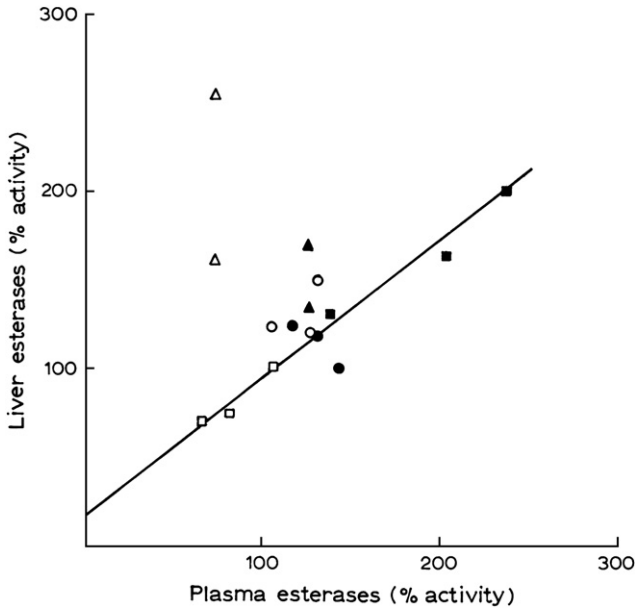

FIGURE 68.1. CarbE and ChE activities in liver versus blood plasma of thiouracil fed rats. The animals received rat chow supplemented with 0.05% thiouracil for 22, 30, or 38 days. Methyl butyrate hydrolysis (▲ △) and 4-nitrophenyl butyrate hydrolysis (● ○) were measured in liver homogenate and plasma in accordance with Sterri *et al.* (1985b), and acetylcholine hydrolysis (■ □) by the method of Sterri and Fonnum (1978). The activities are percent of corresponding control (=100%) in male (closed symbols) and female (open symbols) animals fed standard rat chow.

IV. ROLE OF PLASMA CARBOXYLESTERASE

A. Scavenger Function

The results by Myers (1959) on prophylactic action of DAM against sarin poisoning in rat suggested that most of the sarin required to kill a rat may actually be used up by the inhibition of plasma CarbE, and the sarin may be destroyed in the blood stream before it reaches target AChE in the vital organs of rat. The importance of plasma CarbE for detoxification of sarin in the blood was also suggested by results from the work of Polak and Cohen (1969, 1970a, b) on ^{32}P-sarin injection and its distribution in the rat body. In the case of soman, a completely irreversible AChE inhibitor (Coult *et al.*, 1966), similar conclusions with respect to the function of plasma CarbE were based on results from a series of experiments with acute or repetitive injection of soman in rodents (Sterri *et al.*, 1980, 1981, 1985a; Sterri and Fonnum, 1984; Sterri, 1981; Fonnum and Sterri, 1981). The results showed that the reaction rate between plasma CarbE and soman or sarin may be rapid enough to inactivate a large proportion of the OP-compound before it leaves the blood. Also of special relevance for repetitive exposure, the plasma CarbE seemed to be spontaneously and sufficiently recovered within 24 h, ready to detoxify another dose of soman or sarin.

A scavenger function of plasma CarbE able to prevent the highly toxic OP compounds from reaching AChE in brain and diaphragm requires that an adequate amount of CarbE is present in the plasma. This is fulfilled for rodents, since CarbEs by several authors are reported to be especially abundant in rodent plasma (Myers, 1952; Aldridge, 1953; Goutier, 1956; Augustinsson, 1959; Christen and Cohen, 1969; Polak and Cohen, 1970a; Gupta *et al.*, 1985). In fact, rat and guinea pig plasma may contain as much as 2.8–3.0 μM and 0.5–0.6 μM of CarbE catalytic centers, respectively, as found with ^{32}P-sarin binding to plasma proteins and subsequent polyacrylamide gel electrophoresis or reactivation of CarbE by lowering the pH (Christen and

Cohen, 1969; Christen *et al.*, 1969; Cohen *et al.*, 1971). Interestingly, as this binding capacity has nothing to do with the catalytic enzyme activity, a much higher enzymatic activity was found in guinea pig than in rat with tributyrin as substrate for the plasma CarbE (Christen and Cohen, 1969). However, since CarbE is a B-esterase (Aldridge, 1953; Jansz *et al.*, 1959; Aldridge and Reiner, 1972) it is able to bind and detoxify the highly toxic OP compounds at the active site in equimolar proportions.

The fact that rodent plasma CarbE has a scavenger function against the highly toxic OP compounds is the basis for the CarbE concept, which is an outline of how the toxicity of soman may be influenced by the different content of plasma CarbE in different species in both the absence and presence of therapeutic interventions such as pyridostigmine prophylaxis or any artificially supplied scavenger in the plasma (Sterri and Fonnum, 1989; Sterri, 1989). This is further described below (see Toxicity of nerve gases and Therapeutic interventions).

The various isoenzymes of plasma CarbE might have different importance with respect to their scavenger function. Results show a 2.5-fold higher plasma CarbE activity with methyl butyrate in adult female rats compared to males, whereas the soman toxicity does not differ between them (Sterri *et al.*, 1985a). The same authors also report adult values of soman toxicity in 31 day old rats of both sexes, whereas at that time the methyl butyrate hydolyzing capacity of female plasma is far from fully developed. Thus, the isoenzyme with the highest preference for 4-nitrophenyl butyrate as substrate (Sterri and Fonnum, 1989) may be the most effective soman scavenger of plasma, at least in rats.

B. Toxicity of Nerve Gases

Several investigations have demonstrated that the concentration of CarbE protein in rodent plasma is critical for the tolerance to an acute exposure to highly toxic ChE inhibitors such as soman. For example, a strong correlation exists between the LD_{50} of soman and the plasma CarbE content in developing young rats (Fonnum *et al.*, 1985; Sterri *et al.*, 1985a). In these rats the plasma CarbE activities with 4-nitrophenyl butyrate increased from negligible (5 days old) to adult value (31 days old) and in the same period the acute LD_{50} of soman increased eight- to ten-fold (toxicity decreased) and reached adult value in the 31 day old rats. In addition, young rats (14 days old) with low content of their own plasma CarbE displayed strongly reduced mortality to soman poisoning when a partly purified CarbE protein from rat liver was prophylactically supplied by intravenous injection into their blood before the soman exposure (Fonnum *et al.*, 1985). Also, a linear correlation between soman toxicity and changes in plasma content of CarbE in aging rats is reported by Maxwell *et al.* (1988).

The specific CarbE inhibitors tri-*ortho*-cresyl-phosphate (TOCP) (Mendel and Myers, 1953) and 2-[*o*-cresyl]-4H-1,2,3-benzodioxa-phosphorin-2-oxide (CBDP) (Casida *et al.*, 1961) have been used by several investigators to evaluate the effect on LD_{50} of highly toxic OP compounds. In mice the LD_{50} of soman was reduced to about one-eighteenth by pretreatment with 35–50 mg/kg CBDP (McKay *et al.*, 1971; Boskovic, 1979) whereas pretreatment with 100 mg/kg TOCP reduced LD_{50} of soman to one-third in guinea pig and rat (Sterri, 1981; Fonnum and Sterri 1981; Sterri *et al.*, 1981). In rat the LD_{50} of sarin was reduced to about one-fifth by pretreatment with 40–50 mg/kg TOCP (Myers, 1959; Polak and Cohen, 1969). From studies of the CBDP effect on LD_{50} for several OP compounds by Maxwell (1992) significant protection by plasma CarbE against acute toxicity of the highly toxic soman, sarin, tabun, and paraoxon was reported. Interestingly, for malathion more than a 100-fold increase in acute toxicity by pretreatment with CarbE inhibitor tri-orthotolyl-phosphate is reported by Murphy *et al.* (1976). However, malathion is a less toxic OP compound, which will be hydrolytically detoxified by enzymatic activity of CarbE due to its content of the carboxylic ester group.

Some very interesting results on the prophylaxis of sarin poisoning with DAM in normal and TOCP-pretreated rats are reported by Myers (1959). The author observed that with DAM prophylaxis the LD_{50} of sarin in the atropinized TOCP-pretreated rats was only one-seventeenth of LD_{50} in the atropinized normal rats. This may be regarded as a consequence of sarin binding to plasma CarbE in the normal rats, since any direct reaction between sarin and the prophylactic administered DAM should be similar in the normal and TOCP-pretreated animals.

The scavenger function of plasma CarbE may explain various aspects of soman toxicity and soman inactivation in rodents, but it may also be of value for an adequate understanding of soman toxicity in other species. Rat, guinea pig, and primates including humans are species with respectively high, medium, minor (marmoset), and zero (rhesus monkey and human) concentration of plasma CarbE (Myers, 1952; Aldridge, 1953; Christen *et al.*, 1969; Christen and Cohen, 1969; Cohen *et al.*, 1971). By including these species in an outline describing the influence of plasma CarbE on soman toxicity, Sterri and Fonnum (1989) presented a concept which in principle could explain the differences in LD_{50} of soman between the species. The predicted LD_{50} values of soman for rat, guinea pig, marmoset, and rhesus monkey (or human) agreed well with the experimental values for subcutaneous LD_{50} of soman in rat (Sterri *et al.*, 1980, 1985a), guinea pig (Sterri *et al.*, 1981), marmoset, and rhesus monkey (Dirnhuber *et al.*, 1979). Our general knowledge of experimental LD_{50}s of soman might suggest that rodent plasma CarbE may be a main scavenger for soman by inhalation and intravenous and subcutaneous administration, whereas additional dermal or hepatic factors may influence the LD_{50} of soman by percutaneous or intraperitoneal administration (Sterri, 1989).

The CarbE concept by Sterri and Fonnum (1989) assumes that the concentration of target AChE in the

nervous tissue is small and may therefore be regarded as constant in the different species, whereas the concentration of CarbE in plasma differs. A strongly simplified visual representation of their proportional contribution to LD_{50} of soman in rat is shown in Figure 68.2. The concentrations of target AChE and plasma CarbE in rat are represented by the quadrangles **1** and **2**, respectively, and their proportions are similar to those previously presented in the CarbE concept (Sterri and Fonnum, 1989). At a glance one can see from Figure 68.2 that without therapeutic intervention the LD_{50} of soman is represented by (**1** + **2**) which is strongly influenced by the concentration of plasma CarbE (**2**). Also, it would be reduced in species with lower CarbE concentrations than rat, until it is exclusively represented by the target AChE concentration (**1**) in species such as rhesus monkey and humans which do not have CarbE in the plasma.

C. Therapeutic Intervention

The effect on soman toxicity by pyridostigmine prophylaxis in different species could in principle also be explained by the CarbE concept of Sterri and Fonnum (1989). This means that the protection factors of carbamate prophylaxis as calculated for the different species were similar to the experimental protection factors of pyridostigmine prophylaxis against soman as observed for rat and guinea pig (Gordon *et al.*, 1978) as well as marmoset and rhesus monkey (Dirnhuber *et al.*, 1979). Thus, due to the CarbE concept, we were fairly convinced that the human species would achieve a similar protection by pyridostigmine prophylaxis as rhesus monkey, since both species lack the plasma CarbE. The proportional contribution by pyridostigmine prophylaxis to LD_{50} of soman in rat is visualized in Figure 68.2, as represented by quadrangle **3**. Since this prophylaxis leads to protection of a constant factor, namely target AChE, the contribution to LD_{50} by pyridostigmine is regarded as constant in the species (30 × target AChE concentration) in accordance with the CarbE concept (Sterri and Fonnum, 1989). At a glance one can see from Figure 68.2 that the protection factor of carbamate prophylaxis, which is LD_{50} with prophylaxis (**1** + **2** + **3**) divided by that without prophylaxis (**1** + **2**), is relatively low in rat due to the large concentration of plasma CarbE (**2**), whereas in species such as rhesus monkey and humans the factor would be equal to 30 (Figure 68.2, **1** + **3**) since plasma CarbE is not present.

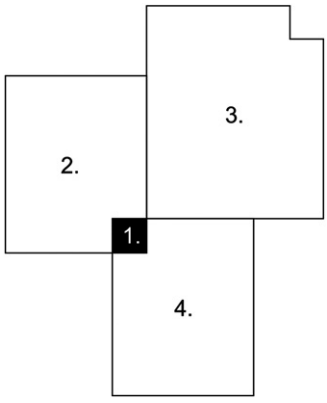

FIGURE 68.2. Visual representation of soman LD_{50} in rat as proportionally influenced by **1.** target AChE, **2.** plasma CarbE, **3.** carbamate prophylaxis, and **4.** artificially supplied scavenger (at rat plasma CarbE concentration). The concentration of target AChE (**1**) or its ''enhancement'' by carbamate prophylaxis (**3**) is constant between the species, whereas the concentration of plasma CarbE (**2**) is varying. The representation is based on the CarbE concept outlined by Sterri and Fonnum (1989) and Sterri (1989). LD_{50} decreases (toxicity increases) when plasma CarbE (**2**) is decreased or absent.

Since plasma CarbE is established as a functional scavenger towards highly toxic OP compounds, this has inspired several experiments on the use of artificially supplied scavengers to improve our protection against the nerve gases. Efficient protection has been observed against OP poisoning by prophylactic injection of either CarbE, AChE, or BuChE into the blood stream of rodent or primate (Fonnum *et al.*, 1985; Wolfe *et al.*, 1987; Raveh *et al.*, 1989, 1997; Broomfield *et al.*, 1991; Maxwell *et al.*, 1991, 1992; Doctor *et al.*, 1993). In the future additional scavengers which could mimic the function of plasma CarbE might hopefully be developed. Based on the CarbE concept (Sterri and Fonnum, 1989) the protection factors of any artificial scavenger added into the plasma of the different species at an adequate concentration could be estimated, and in principle such estimates have been presented by Sterri (1989). The proportional contribution to LD_{50} of soman in rat by an artificially supplied scavenger at a similar concentration as the original plasma CarbE is visualized in Figure 68.2, as represented by quadrangle **4**. At a glance one can see from Figure 68.2 that such a supplement would increase the LD_{50} by a factor of only 2 in rat (**1** + **2** + **4**), but of higher fold in species with a lower concentration of plasma CarbE (reduction in **2**). For rhesus monkey and humans which do not have natural plasma CarbE such a supplement would lead to similar LD_{50}s (**1** + **4**) as for a normal untreated rat (**1** + **2**), which means a protection of about 20-fold in accordance with the CarbE concept (Sterri and Fonnum, 1989; Sterri, 1989). Also, the protection factor in humans would be increased if the concentration of artificially supplied scavenger is enhanced (increase in **4**) (Sterri, 1989).

V. CONCLUDING REMARKS AND FUTURE DIRECTION

Plasma CarbE has proved to have an important role in nerve gas poisoning both with and without therapeutic intervention, and is nature's own example of an effective scavenger of nerve gases being present in the blood. Both CarbE and

other B-esterases have proved to be effective as artificially supplied scavengers, but since they are high molecular weight proteins some practical problems may be difficult to overcome for practical use. It is tempting to speculate, as has been done previously (Sterri, 1989), that a low molecular weight scavenger associated with the large amount of albumin in human plasma would be a better alternative, if possible. Perhaps resources should be utilized so that these possibilities may be worth investigating.

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CHAPTER 69

Protection of Acetylcholinesterase from Organophosphates: Kinetic Insight into Bioscavengers

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I. INTRODUCTION

Organophosphorus (OP) neurotoxicant-induced inhibition of acetylcholinesterase (AChE, EC 3.1.1.7) results in an accumulation of acetylcholine (ACh) in the neural synaptic junctions. The resulting enhancement of nerve impulses leads to a wide variety of hypercholinergic effects that ultimately result in muscular dysfunction and nerve damage (Qian *et al.*, 2006). Traditional treatment of OP poisoning has utilized three categories of drugs: (1) muscarinic receptor blocking agents, such as atropine, (2) cholinesterase reactivators, such as pralidoxime chloride (2-PAM), and (3) emetics, cathartics, and adsorbents to decrease further absorption. While reversal of the muscarinic manifestation is the objective of atropine therapy, the nicotinic cholinergic effects from OP intoxication, such as muscle fasciculations and muscle paralysis, can still lead to death. In some cases, oximes are employed as cholinesterase reactivators in an attempt to release active cholinesterase from the OP–cholinesterase conjugates. The effectiveness of reactivation decreases with time after exposure, and the rate at which this occurs varies with the strength of the nucleophile, its orientation with respect to the phosphate conjugated to the active center serine, and irreversible aging of the OP conjugate (Wong *et al.*, 2000).

Although successful, the current treatments for acute nerve agent poisoning do nothing to avoid the victim suffering a toxic insult that subsequently requires therapeutic management. In contrast, the mechanism of OP toxicity suggests that an approach based on the reduction of the concentration of OP toxicant in the blood before it can reach its site of action (synaptic endplates) should be particularly effective, and incapacitating or even lethal exposures could be mitigated to comparatively mild symptoms. With this in mind, recent efforts have focused on identifying proteins that can remain stable in the intravascular circulation for extended periods of time and act as biological scavengers of OPs. There are several methods by which this can be accomplished: (1) elevating intravascular levels of the cholinesterases, (2) utilizing endogenous OP-hydrolyzing enzymes, (3) providing exogenous OP bioscavengers, or (4) selecting combinations thereof. This chapter describes a kinetically focused, *in vitro* AChE protection assay that aids in the analysis of bioscavengers' efficacy. This analysis discusses the critical role that binding affinity (K_m) and substrate specificity (k_{cat}/K_m) play in protecting AChE against OP-induced AChE inhibition.

II. PROTECTIVE BIOSCAVENGERS

A. Endogenous Enzymes as Protective Bioscavengers

The sensitivities of various organisms to OP compounds are quite variable and appear to be related to the endogenous OP detoxifying capacity of two families of enzymes which are often categorized as the A- and B-esterases. These include the catalytic, high-density lipoprotein-(HDL) associated PON1 (aryldialkylphosphatase EC 3.1.8.1 or aromatic esterase EC 3.1.1.2), and the B-esterases, where the main mechanism of disposition of OP compounds is inhibitory binding and sequestration. This latter category includes naturally occurring proteins that bind and/or react with nerve agents on a one-to-one basis (stoichiometrically), and includes enzymes such as the cholinesterases (ChEs) and carboxylesterases (CarbEs).

Stoichiometric scavengers have the capacity to bind one molecule of nerve agent per molecule of protein scavenger. Although this approach has been proven to be effective (Table 69.1), it is limited in that the extent of protection is directly proportional to the concentration of unreacted, active scavenger in the blood stream at the time of nerve agent exposure (Cowan *et al.*, 2004; Broomfield *et al.*, 1991). Given that the molecular weights of many proteins that are being considered as stoichiometric scavengers are in

TABLE 69.1. Protection of rhesus monkeys from CWA poisoning by BChE (butyrylcholinesterase) (modified from Broomfield *et al.*, 1991)

OP	BChE (nmol)	OP (nmol)	Challenge[a]	Protection[b]
Soman	460	340	$4 \times LD_{50}$	1/1
Soman	460	253	$3 \times LD_{50}$	1/1
Sarin[c]	460	366	$1 \times LD_{50}$	2/2
Soman	503	220–260	$2 \times LD_{50}$	4/4

[a]Median lethal dose is weight dependent
[b]Ratio of survivors to total test animals
[c]Sarin administered in two doses: 183 nmol at 1 and 2 h post-BChE

the 60–80,000 Da range, and the molecular weights of the nerve agents are about 180 Da, the mass ratio of scavenger to nerve agent is approximately 400:1. Thus, a high concentration of scavenger protein in circulation would be required to achieve the equal or greater molar ratio that would be required for protection.

Alternatively, endogenous scavengers with catalytic activity provide the advantage that small amounts of enzyme could be sufficient to detoxify large amounts of nerve agent. This has been demonstrated by studies in which rats or mice injected with purified rabbit PON1 demonstrated an increased resistance to the OP pesticides, paraoxon, chlorpyrifos, and chlorpyrifos oxon (Li *et al.*, 1995, 2000; Costa *et al.*, 1990; Main, 1956). PON1 is a member of the PON family of proteins, which reside in high-density lipoprotein complexes and appear to prevent lipid oxidation in low-density lipoprotein. Although its natural substrate may be homocysteine thiolactone, it is the only member of the PON family that also hydrolyzes OP compounds. This has been further verified by the creation of PON1 null mice, which were found to have increased sensitivity to chlorpyrifos oxon, chlorpyrifos and diazinon, but, surprisingly, not to paraoxon (Shih *et al.*, 1998).

Studies with human populations have determined that the protection afforded by PON1 is complex, as (1) the intravascular expression varies between individuals, (2) the enzyme has significant sequence polymorphisms, and (3) the resulting allozymes have different substrate specificities. Pedigree analysis has established a polymorphism at position 192 as concordant with the serum paraoxonase phenotypes, with the arginine 192 (192R) allozyme hydrolyzing paraoxon more rapidly than the glutamine 192 (192Q) form (Adkins *et al.*, 1993; Furlong *et al.*, 1993; Humbert *et al.*, 1993; Smolen *et al.*, 1991). This is reversed for the hydrolysis of other OP compounds, and especially sarin as the serum of 192Q homozygotes displayed an almost ten-fold greater activity against sarin than the 192R homozygotes. In studies in which purified human PON1-192Q or PON1-192R was injected into the serum of the PON1 null mice, either alloform protected equally well against diazinon exposure, consistent with their equivalent catalytic efficiency for diazinon hydrolysis. However, the PON1-192R alloform provided significantly better protection against chlorpyrifos exposure, as predicted due to its higher catalytic efficiency with that substrate (Li *et al.*, 2000). These observations have been confirmed in transgenic mice expressing one or the other PON1-192 alloform (Cole *et al.*, 2005).

Similar results have been reported for polymorphisms at amino acid 55, with the PON1 paraoxonase activity in blood serum from 55M (methionine) homozygotes reduced compared to either the 55L (leucine) homozygotes or the LM heterozygotes (Mackness *et al.*, 1997). While it is intuitive that the rate of detoxification of a substrate would be dependent on the expression levels of these endogenous enzymes, the effects of the genetic polymorphisms suggest that catalytic efficiency is an equally important consideration.

B. Exogenous Enzymes as Protective Bioscavengers

In addition to PON1, OP-hydrolyzing enzymes have been identified from a variety of prokaryotic sources, such as the organophosphorus hydrolase (OPH, EC 3.1.8.1) from *Pseudomonas diminuta* (Serdar and Gibson, 1985; Serdar *et al.*, 1982) and the OP acid anhydrolase/prolidase (OPAA) from *Alteromonas* sp. (DeFrank and Cheng, 1991; Cheng *et al.*, 1993, 1996). These enzymes have the potential to compete with physiological targets for OP binding and to catalytically hydrolyze a broad range of OPs (Table 69.2). The hydrolytic capability of the OPAA class of enzymes appears to be limited to the phosphonofluoridates, which includes OP nerve agents of the G series (such as soman and sarin), and warfare agent surrogate DFP. In contrast, the OPHs hydrolyze a broader spectrum of substrates that includes common pesticides (P–O bonds, phosphotriesters), phosphonofluoridates (P–F bonds – type G nerve agents),

TABLE 69.2. Substrate profile of enzymes with potential use as protective bioscavengers

Enzyme[a]	Paraoxon	Demeton-S	DFP
AchE	–	–	–
BchE	–	–	–
PON1	+	–	+
OPAA	+	–	+
OPH	+	+	+

	Tabun	Sarin	Soman	Cyclosarin	VX	RVX
AchE	–	–	–	–	–	–
BchE	–	–	–	–	–	–
PON1	+	+	+	+	–	–
OPAA	+	+	+	+	–	–
OPH	+	+	+	+	+	+

[a]Acetylcholinesterase, butyrylcholinesterase, paraoxonase, organophosphonic acid anhydrolase, organophosphate hydrolase

phosphonothioates (P–S bonds – type V CW agents), and phosphonocyanidate (P–CN – tabun). A survey of genomic and biological culture libraries, identified on the basis of either sequence homology or activity, has revealed that there is significant variation in the kinetic character of the OPAA family (Wales and Wild, unpublished). Collectively, these families of enzymes provide the opportunity to design a versatile bioscavenger technology based on a consortium of enzymes selected for maximal utility in the detoxification of chemical warfare agents (CWAs).

The kinetic efficiencies of any potential therapeutic enzyme must be considered relative to realistic exposure concentrations and the nature of the neurotoxicants (Table 69.3). Substrate saturating conditions are not likely to occur *in vivo*, and when the concentration of available substrate is at or below the K_M of the enzyme, the catalyzed reaction becomes second order with respect to substrate, and k_{cat}/K_M, the second order rate constant, is indicative of hydrolysis. The high toxicity of OP compounds sets a requirement for protection to be provided at relatively low OP vascular concentrations, well below the K_M of any hydrolyzing enzyme for the OP substrates, especially the CWAs. The half-time for reaction of a catalytic scavenger with a nerve agent can be estimated with some conservative kinetic assumptions. The expected concentration of a nerve agent in the blood at an LD_{50} dose would be approximately 8×10^{-7} M (Lenz *et al.*, 1997). If a scavenger was present in the blood at a concentration of 1 mg/ml (approximately 1×10^{-5} M), then the rate constant for the reaction of a scavenger with a toxicant would be second order with respect to substrate, and the $t_{1/2}$ for the reduction of toxicant would be ~$3–7 \times 10^{-4}$ min, assuming adequate mixing and the retention of the bioscavenger and the toxicant in the bloodstream. Where actual measurements have been made of the rate of reduction in the concentration of soman in guinea pigs in the absence of an exogenous scavenger, the concentration of a $2 \times LD_{50}$ dose of soman in circulation was reduced with a $t_{1/2}$ of 0.15 min (Langenberg *et al.*, 1997). These results support the contention that the presence of a bioscavenger in circulation at the time of exposure could provide significant prophylactic protection. The need to administer, repetitively, a host of pharmacologically active drugs with a short duration of action at a precise time following exposure would be all but eliminated if a scavenger with the appropriate kinetic characteristics were used. In order to address these issues, an *in vitro* protocol for rapidly evaluating the efficacy of enzymes and drugs to protect AChE was developed.

C. Encapsulated Enzymes as Protective Bioscavengers

Logically, since bacterially derived enzymes have no known mammalian homologs, they are likely be potent initiators of immune responses and so are deemed unlikely to be appropriate for use as a bioscavenger in humans. An enzyme

TABLE 69.3. Kinetic characterization of catalytic bioscavengers and the vascular concentration of 1 LD_{50} of the OP neurotoxicants

Enzyme/substrate	Vascular (mM)	K_m (mM)	K_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
OPH				
Paraoxon	0.017	0.12	7.8×10^3	6.46×10^7
DFP	0.32	2.8	3.7×10^3	1.3×10^6
Sarin[a]	0.0015	0.7	56.0	8×10^4
Soman[a]	0.0016	0.5	4.8	1×10^4
OPAA				
Paraoxon	0.017	4.1	1.9	4.8×10^2
DFP	0.32	15.3	3.8×10^3	2.5×10^5
Sarin[c]	0.0015	1.57	441[b]	2.8×10^5
Soman[c]	0.0016	2.48	151[b]	6.1×10^4
PON1				
Paraoxon[d]	0.017	0.5/0.3	12/33	2.4×10^4/ 1.1×10^5
DFP[e]	0.32	–	–	6.3×10^2/ nd[f]
Sarin[e]	0.0015	–	–	1.5×10^4/ 1.1×10^3
Soman[e]	0.0016	–	–	4.6×10^4/ 3.5×10^4

[a] Kolakowski *et al.* (1997)
[b] Hill *et al.* (2000)
[c] Calculated from Cheng *et al.* (1996)
[d] Smolen *et al.* (1991); the activity of each allelic form of PON1 is shown as Q191/R191
[e] Masson *et al.* (1998); the activity of each allelic form of PON1 is shown as Q191/R191
[f] Not determined

carrier or encapsulation method can be employed to present the bacterial enzymes in a manner which protects against any potential for adverse disposition and rapid clearing from the circulation. The medical value of encapsulation was first reported for enzymes and drugs successfully entrapped within resealed and annealed erythrocytes (CRBCs) (Ihler *et al.*, 1973). Since that first report, CRBs have demonstrated efficacy in both cyanide antagonism (Leung *et al.*, 1991a, b; Petrikovics *et al.*, 1994, 1995) and OP antagonism (Pei *et al.*, 1994, 1995; Petrikovics *et al.*, 2007).

In spite of their long circulation time, CRBCs are not considered a practical carrier system, as their use requires prior blood typing, and they are relatively fragile cells requiring a sophisticated encapsulation technique. Research over the years has refined the list of parameters which should be considered when selecting an encapsulation method to include: (1) circulation half-life, (2) RES (reticuloendothelial system) resistance, (3) permeability by the target toxin, (4) intrinsic toxicity, (5) encapsulation efficiency, (6) biocompatibility, (7) pharmacokinetic profile, and (8) activity of encapsulated enzyme. Most of these parameters have to be evaluated on a case-by-case basis, but liposomes are often considered the ''workhorse'' of the drug delivery industry, and dendrimers are a relative new approach.

Petrikovics *et al.* (1999a, b, 2000a, b) employed sterically stabilized liposomes (SL) as the encapsulation system to deliver OPH (SL-OPH) or OPAA (SL-OPAA) as the antagonist of the model OP compounds, paraoxon ($LD_{50} = 0.85$ mg/kg) or DFP ($LD_{50} = 4.55$ mg/kg). Liposomes are small spherical systems that are synthesized from cholesterol and nontoxic phospholipids. They can be engineered to possess different characteristics depending upon the lipid of choice in the production process. Recent studies with small, low-clearance liposomes have shown that more specialized formulations may provide benefits over simpler ''first generation'' liposomes, including prolonged residence in plasma, and increased tissue exposure. Other characteristics, such as their relatively easy preparation method, high encapsulation efficiency, favorable biodistribution/pharmacokinetic properties, and good *in vivo* tolerance, make them an attractive choice. One of the ''second generation'' systems is the ''stealth'' liposome, designed to circumvent the body's immune defenses, thereby avoiding rapid uptake by the macrophage cells of the reticuloendothelial system (Woodle and Lasic, 1992; Papahadjopoulos *et al.*, 1991). In these studies, the APRs were calculated using the mean of the LD_{50} values determined from three or more experiments, and demonstrated that while SL-OPAA was more effective than either 2-PAM or atropine, it was less effective than the combination. [The *in vivo* efficacies of the antidotal systems are expressed here as the antidotal potency ratio, or APR, which represents the ratio of the LD_{50} (mean) of the OP with antagonists and LD_{50} (mean) of OP without antagonists.] OPH, however, demonstrated not only a $33 \times LD_{50}$ protection against paraoxon poisoning when delivered as the free enzyme, but protection against 125 LD_{50} when the SL is encapsulated. This was twice the protection afforded by the 2-PAM + atropine combination (Table 69.4).

Alternatively, the *in vivo* efficacy of the nanostructures, DP-OPH and DP-OPAA, was evaluated for each encapsulated enzyme. The *in vivo* application of dendritic polymers (DP), a nanocapsule, polyoxazoline-based hyperbranched polymer (HBP) with a C18 surface modifier, was first reported in the mid-1980s (Tomalia *et al.*, 1985). The application of these dendritic polymers as drug carriers has rapidly gained favor, primarily due to their inertness relative to temperature, solvent, and pH. These polymers have attracted an interest as drug carriers which can be loaded with drugs, nucleic acid, or protein either in the interior of the dendrimers or attached to the surface groups. Due to their relatively recent appearance on the scene, the toxicity, biodegradability, and biocompatibility of dendrimers have to be explored for each system and each application. In the studies reported by Petrikovics *et al.* (2007), DP-OPH alone provides a $95.5 \times LD_{50}$ protection against paraoxon, while DP-OPAA demonstrates a more modest $2.7 \times LD_{50}$ protection against DFP (Table 69.4). The DP-OPH alone provides better protection against paraoxon than the traditional therapies of 2-PAM

TABLE 69.4. OP antidotal protection of the OPH and OPAA dendritic polymer (DP) and the sterically stabilized liposome (SL) systems

OP compound	Antidotal system	APR
DFP	Control	1.0
DFP	Free OPAA	1.6[a]
DFP	DP−OPAA	2.7[d]
DFP	SL−OPAA	2.3[a]
DFP	2-PAM	1.2[d]
DFP	Atropine	1.8[d]
DFP	2-PAM + atropine	7.8[d]
DFP	2-PAM + atropine + DP-OPAA	24.6[d]
DFP	2-PAM + atropine + SL-OPAA	23.2[a]
Paraoxon	Control	1.0
Paraoxon	Free OPH	33.5[b]
Paraoxon	DP–OPH	95.5[d]
Paraoxon	SL–OPH	125.2[c]
Paraoxon	2-PAM	4.5[d]
Paraoxon	Atropine	2.3[d]
Paraoxon	2-PAM + atropine	61.1[d]
Paraoxon	2-PAM + atropine + DP-OPH	780.0[d]
Paraoxon	2-PAM + atropine + SL-OPH	920.0[c]

[a]Petrikovics *et al.* (2000b)
[b]Pei *et al.* (1995)
[c]Petrikovics *et al.* (1999a, b)
[d]Petrikovics *et al.* (2007)

(20×) and atropine (40×). Similarly, the DP-OPAA was slightly more effective than 2-PAM (2.3×) and atropine (1.5×). DP-OPH alone was slightly better (1.5×) than the 2-PAM + atropine combination.

It is interesting to note that the co-administration of the three therapies (encapsulated enzyme, 2-PAM, and atropine) provided the most effective antidotal system. The DP-OPAA nanocapsule system enhanced the protection of the 2-PAM + atropine combination by 3.2× in DFP antagonism, while the DP-OPH nanocapsule provided a more substantial (12.8×) increase in protection with 2-PAM + atropine against paraoxon. Although the *in vivo* efficacy of the DP-OPH system was approximately 30 times greater than that of the DP-OPAA (alone or in combination with 2-PAM + atropine), both DP-enzymatic systems synergistically enhanced the traditional 2-PAM + atropine combination therapy. This was even more dramatic when SL-OPH was employed in combination with 2-PAM and/or atropine: the magnitude of prophylactic antidotal protection was an astounding 920 × LD_{50} and the therapeutic antidotal protection was 156 × LD_{50}.

III. DEVELOPMENT OF A TOXICOKINETIC PROTECTION ASSAY

A. Determining Bioscavenger Efficacy

The *in vitro* protection assay focused on the extent to which two OP hydrolyzing enzymes (OPAA and OPH) and an oxime (2-PAM), alone or in combinations, could prophylactically prevent AChE inhibition. The catalytic bioscavengers were the native forms of OPH (EC 3.1.8.1) and OPAA (EC 3.1.8.2). The effects of the regenerative oxime 2-PAM were included because its site of action is the inhibited AChE which would be expected to have an *in vitro* effect, whereas atropine, with an alternative muscarinic receptor site of action, was excluded from *in vitro* consideration (Figure 69.1). The addition of an OP hydrolyzing enzyme and the reaction of oximes with acetylthiocholine (ASCh) effected a complex, multiphasic set of reaction equations related to the analytical measurement of the activity and inhibition of AChE (Figure 69.2). The products of various interrelated reaction activities of AChE were monitored spectrophotometrically through the hydrolysis of thiocholine, the hydrolytic product of the substrate ASCh, with 5,5′-dithio-bis-2-nitrobenzoic acid, leaving a compound that absorbed at 405 nm (reactions I and VII, sequentially). Depending on the OP neurotoxin and/or the oxime, the potential existed for other reaction products to contribute to the spectrophotometric signal, thus complicating the analysis. For example, 2-PAM reacts with ASCh to produce thiocholine (reaction IV), and the enzymatic hydrolysis of paraoxon, the neurotoxic inhibitor of AChE (reaction V) or demeton-*S* produced *p*-nitrophenol, and a free thiol, respectively, both of which are typically monitored at 405 nm. In

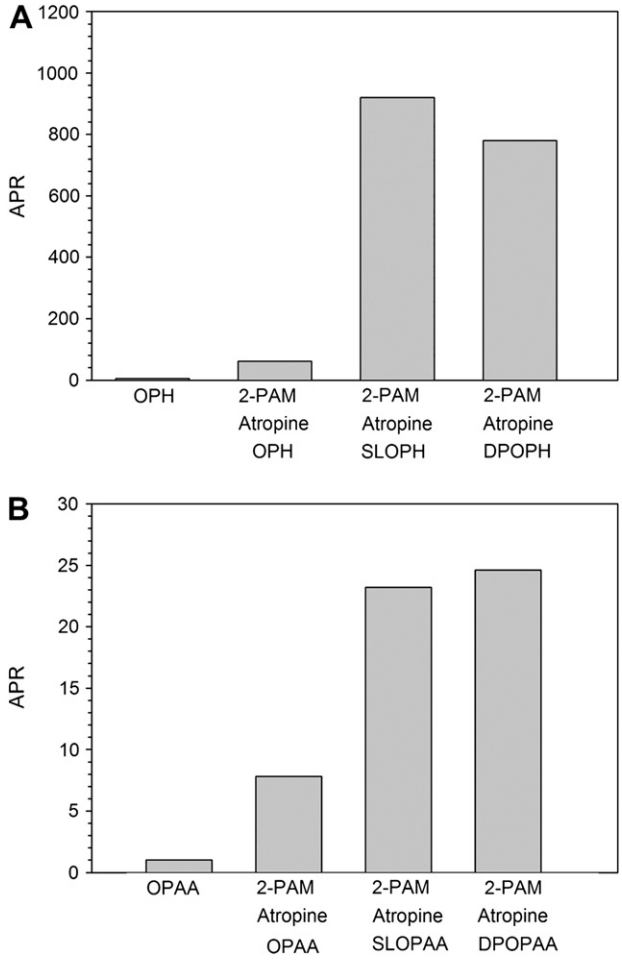

FIGURE 69.1. Bioscavenger enhancement of the protection of mice by the traditional 2-PAM + atropine therapy in mice. The *in vivo* efficacy of two different enzyme-based bioscavengers was determined by Petrokovics *et al.* (2007). Panel A summarizes the effect of OPH (EC 3.1.8.1) on paraoxon neurotoxicity, while panel B summarizes the effect of OPAA (EC 3.1.8.2) against DFP. The antidotal potency ratio, APR, is the average LD_{50} of paraoxon with and without antagonists.

order to ensure that only the catalytic AChE activity was being monitored, it was necessary to remove the remaining OP or oxime prior to spectrophotometric analysis. By working at low levels of OP neurotoxins that were just sufficient for complete AChE inhibition, the effectiveness of any added protectant could be directly evaluated by the amount of AChE activity that was protected. The potential to protect AChE with OP hydrolyzing enzymes is significant, and this would add a direct route for the elimination of the neurotoxins, complementing any regeneration of inhibited AChE that might be achieved by 2-PAM.

B. Kinetic Evaluation of the Inhibition of AChE by OP Neurotoxicants

The potential AChE activity and inhibition levels for each new preparation of AChE had to be determined empirically

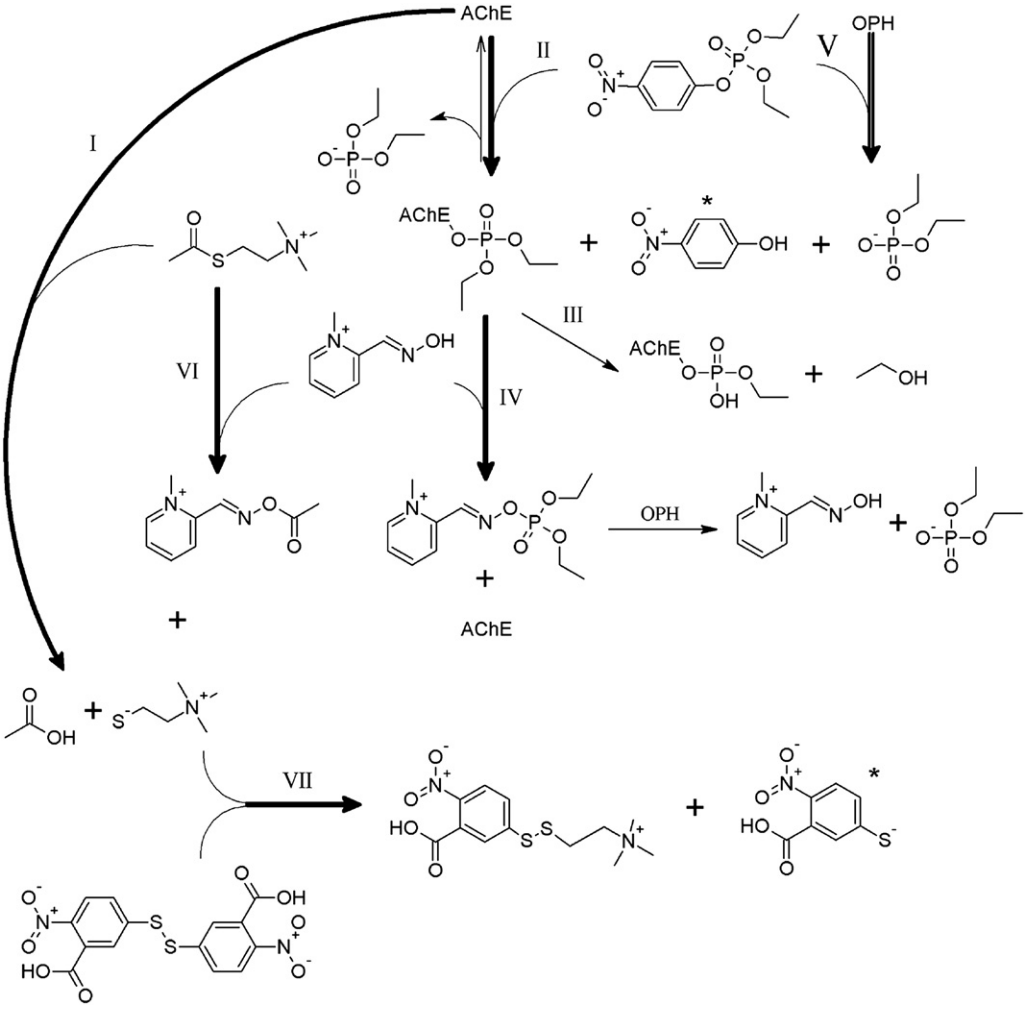

FIGURE 69.2. Reaction scheme of acetylcholinesterase inhibition, reactivation, and protection activities. OPH, 2-PAM, and paraoxon are used in the example. Reaction (I) – cholinesterase AChE reaction with ASCh; Reaction (II) – inhibition of AChE inhibition by paraoxon; Reaction (III) – aging of AChE associated with OP exposure; Reaction (IV) – reactivation of AChE by 2-PAM; Reaction (V) – hydrolysis of paraoxon by OPH; Reaction (VI) – reaction of 2-PAM oxime with ASCh; and Reaction (VII) – DTNB reaction with SCh. The * indicates a photometrically detectable metabolite.

for each of the OP compounds of interest. The AChE was resuspended to a protein concentration of 1 mg/ml (~1,000 units) and incubated for 24 h at a protein concentration of 0.1 mg/ml over a suitable concentration range determined for each neurotoxin. Following the inhibition period, a P-6 microspin column (Biorad) was used to separate the AChE and excess inhibitor. The remaining, uninhibited AChE activity was determined by measuring the rate of acetylthiocholine (ASCh) conversion by monitoring the increase in free thiol concentration at 405 nm with 1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The concentration of each of the OP neurotoxicants selected for use in the protection studies corresponded to the concentration which resulted in ~95% inhibition of AChE activity. This concentration had to be empirically determined for each preparation of AChE in order to accommodate variations in the commercial enzyme activities. The concentration ranges for the OP compounds which inhibited 95% of the AChE and the protectant concentration ranges were determined empirically (Table 69.5). The P_{50} is defined as the [protectant]/[inhibitor] molar ratio at which AChE has 50% of the activity of the no inhibitor control. As a stoichiometric protectant, one molecule of 2-PAM can reactivate one molecule of AChE and so is expected to have a relatively high P_{50} of one or greater. The P_{50}s of 2-PAM with paraoxon and DFP were 1 and 10, respectively (Figure 69.3). In the context of this protection assay, these results can be interpreted to mean that it takes one mole of 2-PAM for every mole of paraoxon to effectively protect 50% of the AChE from the inhibiting effects of paraoxon. Similarly, it requires 10 moles of 2-PAM to protect AChE from 50% inhibition by DFP. Although enzymatic bioscavengers have the potential for a much lower P_{50} due to their catalytic nature, in practice this will be related to the catalytic efficiency and binding affinity of the enzyme for the specific OP inhibitor.

The P_{50} values of OPH with paraoxon and DFP were determined to be 0.0002 and 0.0005, respectively, four and five logs more effective than the protection by 2-PAM alone. The P_{50} values of OPAA with paraoxon and DFP were 15 and 0.3, respectively. The P_{50} values for the enzymes are correlated with their catalytic efficiencies (Figure 69.4A and D). Even though the k_{cat}s of OPH and OPAA with DFP were virtually identical, $3784 \pm 370\ s^{-1}$ and $3780 \pm 560\ s^{-1}$, respectively (Figure 69.4B), the P_{50} of OPH indicated it was approximately 600 times more effective at protecting AChE

TABLE 69.5. Concentration of protectants (μM) and inhibitors (μM)

Protectant	Paraoxon (0.2–0.4)	DFP (0.6–0.8)	Demeton-S (2.0)
2-PAM	0.02–8.0	0.3–160	0.1–80.0
OPH	3.5×10^{-10}– 7.0×10^{-3}	5.3×10^{-5}– 7.0×10^{-2}	nd
OPAA	8.2×10^{-3}– 1.6×10^{1}	6.0×10^{-5}– 5.0×10^{0}	3.4×10^{-1}– 7.0×10^{-0}
H254R/H257L	nd	nd	2.0×10^{-4}– 2.0×10^{-1}
H254S/H257L	nd	nd	5.4×10^{-4}– 5.4×10^{-1}
H254R/H257F	nd	nd	2.4×10^{-4}– 2.4×10^{-1}

nd – No activity was detected

against the inhibitory effects of DFP. Thus, there appears to be a good correlation between the P_{50} (Figure 69.4A) and enzyme specificity (Figure 69.4D), but when the enzyme's binding affinity for substrate is considered a very specific aspect of AChE protection is noticed: at substrate concentrations well below the K_Ms of the bioscavenger enzymes the second order rate kinetics become an important, if not the most important, predictor of efficacy. This can be seen in the comparison of Figure 69.4A and D, where the profile of the k_{cat}/K_M values of the enzymes is inversely correlated with the P_{50} values.

IV. KINETIC RELEVENCE – EFFICACY IN PROTECTION

A. Kinetic Description of Potential Candidate Bioscavengers

Based on the concentration of an OP cholinergic neurotoxicant which is required to provide 95% inhibition of AChE, the inhibitors can be ordered by their relative toxicity, from most toxic to least toxic, as follows: paraoxon (P–O bond) > DFP (P–F bond) > demeton-S (P–S bond). The protection ratio (P_{50}) values generally correlate with the k_{cat}/K_m values of each enzyme indicating the more catalytically efficient the enzyme, the better the protection ratio. With paraoxon and DFP, both 2-PAM and OPAA require an amount approaching or above stoichiometric levels to obtain an appreciable amount of AChE protection, with P_{50} ratios from 0.3 to 15. This finding is particularly noteworthy because OPAA has significant catalytic activity with DFP ($k_{cat} = 3780\ s^{-1}$) yet its protective value ($P_{50} = 0.3$) seems to be most strongly influenced by the relatively high K_m. In contrast, OPH has a similar catalytic activity ($k_{cat} = 3784\ s^{-1}$) with a protective ratio of 5×10^{-4}, which is 600-fold more efficient. A notable characteristic which might contribute to this dissimilarity is that the K_Ms of OPAA and OPH for DFP in this study are quite different (K_M-OPAA = 15.3 mM; K_M-OPH = 2.8 mM). This observation suggests that the substrate binding affinity plays a critical role in defining the protective capacity of a bioscavenger, particularly in those cases of similar catalytic rates. In this scenario, 2-PAM and OPAA both function as a reactant. Each 2-PAM molecule reacts with a single inhibited AChE and is thus removed from further consideration as it decomposes to free AChE and phosphorylated oxime, which is no longer reactive with additional inhibited AChE. OPAA is behaving in a similarly stoichiometric fashion, with the P_{50} values suggesting that OPAA binds the OP, sequestering it and preventing AChE inhibition without evidence of catalysis. This suggests that catalytic bioscavengers with high K_m values will tend to function as stoichiometric scavengers, requiring as least one high molecular concentration to drive the second order protection reaction. This perspective provides a cautionary note with *in vivo* exposures where the LD_{50}s of the agents are so low that the apparent binding affinities of the selected bioscavenger will likely need to be in the submicromolar range to be effective.

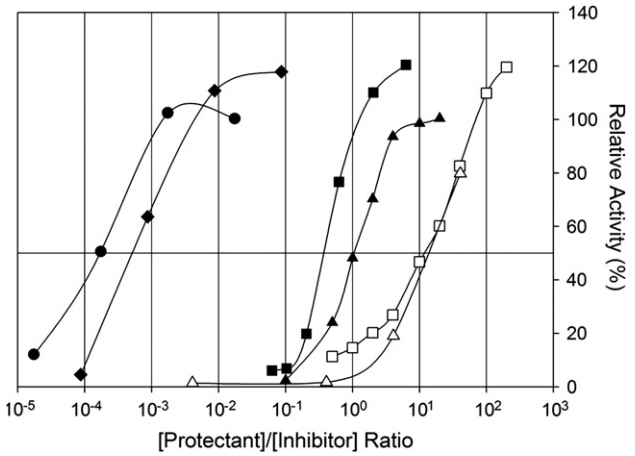

FIGURE 69.3. *In vitro* protection of AChE from inhibition by paraoxon or DFP by enzymatic bioscavengers (OPH or OPAA) and 2-PAM. The P_{50} value is the ratio at which 50% of a fixed concentration of AChE is protected from neurotoxic poisoning. From left to right, the kinetic curves are: (1) OPH and paraoxon; (2) OPH and DFP; (3) OPAA and DFP; (4) 2-PAM and paraoxon; (5) 2-PAM and DFP; (6) OPAA and paraoxon.

The broad-spectrum OP phosphonothioate (P–S bond) hydrolyzing enzyme OPH has been modified to enhance its catalytic specificity, with some of the most important modifications targeting their capability to hydrolyze

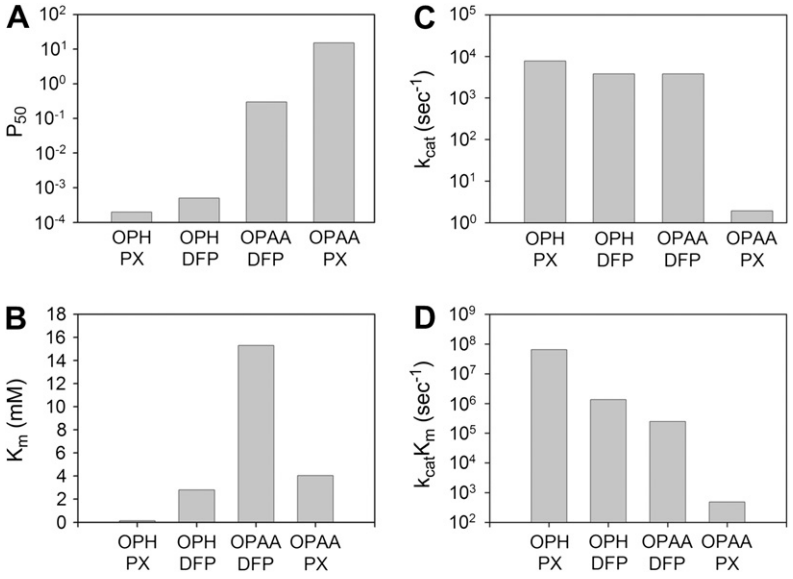

FIGURE 69.4. Comparison of the protection potential (P_{50}) and kinetic characteristics of OPH and OPAA. The P_{50}, [protectant]/[inhibitor] ratios of the enzymes are compared (Panel A), the K_Ms (mM) (Panel B), the k_{cat}s (s^{-1}) (Panel C), and the k_{cat}/K_Ms ($M^{-1}\,s^{-1}$) (Panel D) for each enzyme with both paraoxon (PX) and diisopropyl fluorophosphonate (DFP).

phosphorothioates (P–S bonds, particularly the chemical warfare V-agents). Several genetically engineered variants of OPH have been created to improve the catalytic efficiency for the VX surrogate substrate demeton-S (Reeves *et al.*, 2008). These variants have mutations near the active site that have improved k_{cat} and K_m values for the P–S bond substrates. Selected variant enzymes, including H254R/H257L (RL), H254R/H257F (RF), and H254S/H257L (SL), were evaluated to determine if the catalytic improvements were sufficient to improve protection of AChE against demeton-S over the protection provided by the native form of the bioscavenger (''WT OPH''). This was indeed the case, as the P_{50} values for the wild-type, RL, RF, and SL are 0.2, 0.015, 0.006, and 0.010, respectively. The variant enzymes demonstrated decreased P_{50} values (higher levels of protection) with demeton-S by factors of 13-, 20-, and 33-fold for RL, SL, and RF, respectively (Figure 69.5). The RF enzyme appears to be an outlier in that its K_M is higher than that of the wild-type enzyme while the other two variants selected for this study had lower apparent binding affinities. Furthermore, the enzyme specificity value (k_{cat}/K_M) is significantly lower for RL than the others. Nonetheless, its P_{50} index was virtually identical. This suggests that a larger than two-fold difference in enzyme k_{cat}s is required to observe a change in P_{50} as evidenced by the variants' k_{cat} values and their respective indistinguishable P_{50}s.

B. Enzyme Engineering for Enhanced VX Bioscavenger Protection

The protection of AChE from demeton-S inhibition by wild-type OPH was initially very poor and approached stoichiometric levels ($P_{50} = 0.2$) with the phosphorothioate inhibitor (P–S bond; the V-agent surrogate). This can be

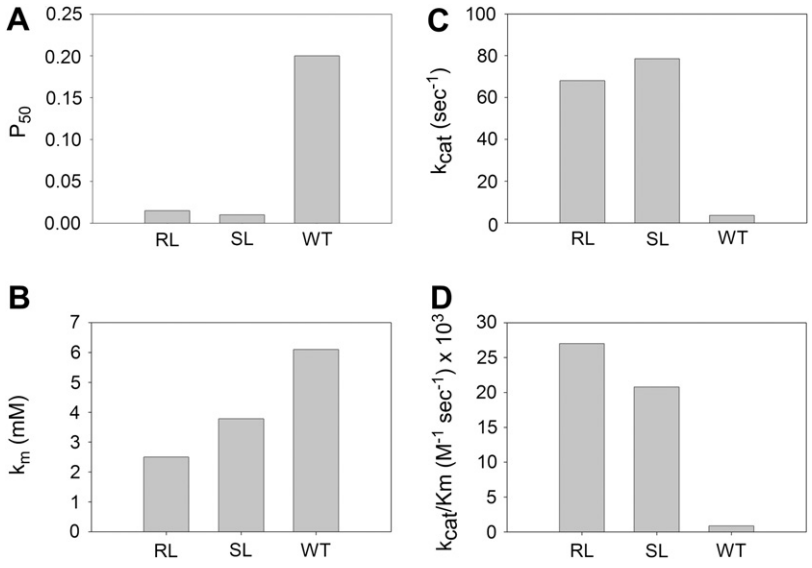

FIGURE 69.5. Comparison of the protection potential (P_{50}) and kinetic character of OPH variants with demeton-S (V-agent surrogate). The P_{50} [protectant]/[inhibitor] ratios of each enzyme are compared (Panel A), the K_M (mM) (Panel B), the k_{cat} (s^{-1}) (Panel C), and the k_{cat}/K_M ($M^{-1}\,s^{-1}$) (Panel D).

explained by the low catalytic efficiency rate ($k_{cat}/K_M = 759\ M^{-1}\ s^{-1}$) of the native enzyme toward demeton-S. Improvement in this protective ability is quite important, as there are few alternative enzymes for P–S bond hydrolysis. To address this need, OPH variants were evaluated as protectants against the inhibitory effects of demeton-S, demonstrating that stoichiometric, catalytic protectants can be designed to provide enhanced protection from OP-induced inhibition of AChE. The variant enzymes decreased the P_{50}s with demeton-S by as much as 33-fold. This finding demonstrates that enzymes with poor protection abilities can be altered to make their *in vitro* protection catalytically efficient instead of stoichiometric in nature and that the overall protection trends inversely with the catalytic efficiency. This becomes even more important when the CWAs are considered. For example, the reported LCt_{50}s of racemic sarin and soman are 70 and 100 mg/min/m^3, respectively, and the LD_{50} of VX is 0.04 mg/kg (Munro *et al.*, 1994). With the following assumptions, (1) respiration of 15 l/min, (2) a 70 kg body weight, (3) 5 liter vascular blood volume, (4) these values represent the effective dose of nerve agent, and (5) that all OP appears in the blood stream, the intravascular concentration of a lethal dose of sarin, soman, and VX can be estimated at 0.0015, 0.0016, and 0.0020 mM, respectively. Thus, the substrate binding affinity (K_M) may be the most critically important consideration for developing an intravascular treatment.

V. CONCLUDING REMARKS AND FUTURE DIRECTION

The conventional treatment for exposure to G- and V-agents involves the intramuscular injection of 2-PAM and atropine upon suspicion of neurotoxicant exposure. While this combined treatment is useful in nerve agent poisoning mitigation, it can have toxic effects of its own and is not always successful in treating extreme OP poisonings. The addition of catalytic bioscavengers to the exposure treatment procedures has proven effective at mitigating and even preventing the cholinergic effects of toxic OP exposures in mice (Petrikovics *et al*, 2007). In those studies, OPAA was able to protect mice against DFP concentrations (P–F bonds) that were 40-fold higher than the unprotected LD_{50}s, and OPH in the presence of 2-PAM and atropine. Similarly, it was possible to raise the LD_{50} for paraoxon from 400–800 LD_{50}. Furthermore, both of the OP hydrolytic enzymes demonstrated significant protective effects as determined by the AChE-protector/inhibitor:protection ratio *in vitro*. The protective effects of the enzymes as bioscavengers can be attributed to their catalytic capability, with the k_{cat}/K_Ms of the enzymes for given substrates indicative of their protective potential for those substrates. However, the K_M for a substrate becomes increasingly more important as the reaction becomes second order with low substrate concentration. It should be noted that the chemical warfare agents are chiral in nature, adding another degree of complexity in those instances in which the more toxic stereoisomers (i.e. more potent inhibitors of AChE) are poorer substrates for the hydrolytic enzymes (Hill *et al.*, 2000). While the substrates used in this study are achiral, the AChE-inhibition protection methodology described here could be easily applied to the evaluation of enzymes with the chemical warfare agents. This screening method cannot predict the bioscavenger immunogenicity or clearance rates of the therapeutics, which are important considerations with *in vivo* exposures. Enzymes can have various biodistribution and pharmacokinetic characteristics which cannot be challenged in this *in vitro* assay, yet they will be important in determining efficacy. The screening method developed here can be used as an important precursor to the costly and time-intensive *in vivo* pharmacokinetic and animal protection studies.

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CHAPTER 70

Catalytic Bioscavengers: The Next Generation of Bioscavenger-Based Medical Countermeasures

PATRICK MASSON AND DANIEL ROCHU

I. INTRODUCTION

Organophosphate esters (OPs) were discovered in the middle of the 19th century by French chemists. They are mostly pesticides (parathion, malathion, chlorpyriphos, dichlorvos); some of them have been used as drugs (echothiophate, metrifonate, cyclophosphamide); and other OPs are potent chemical warfare agents (G agents: tabun, sarin, soman, cyclohexyl-sarin, and V agents: VX, VR, VS). These compounds are irreversible inhibitors of cholinesterases (ChEs) (Figure 70.1) (Costa, 2006). There are two types of ChEs, acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8). AChE plays a major role in the cholinergic system terminating the action of acetylcholine. Inhibition of AChE leads to accumulation of acetylcholine (ACh) in synapses, and blockade of cholinergic transmissions in peripheral and central nervous systems. Inhibition of AChE is the main cause of acute toxicity of OPs (Maxwell *et al.*, 2006).

Significant progress has been made in the past 20 years in emergency treatments of acute poisoning and management of poisoned casualties (Aas, 2003; Albuquerque *et al.*, 2006; Eddelston *et al.*, 2008; Wetherell *et al.*, 2007; Eyer *et al.*, 2007; Thiermann *et al.*, 2007). However, classical pharmacological approaches are reaching their optimum limits. In addition, due to accumulation of OP in depot sites and subsequent slow release from these stocks, blood and tissue cholinesterases may remain inhibited for long periods of time. Therefore, persistence of certain OPs in the body after initial exposure complicates treatments. This is particularly well documented for severe intoxications by parathion (Willems and de Bisschop, 1993).

Endogenous enzymes are involved in natural defenses against OP toxicity. The presence of detoxifying enzymes in skin contributes to reduce the OP dose that penetrates into the body (Schallreuter *et al.*, 2007). Numerous secondary targets of OPs found in various tissues are detoxifying enzymes and they certainly play a role in the natural defenses against OPs (Wang *et al.*, 1998; Nomura *et al.*, 2005, 2008). Natural blood bioscavengers significantly contribute to reduce the amount of OP molecules reaching physiological targets. It has been shown that animal species in which the concentration of paraoxonase-1 (PON1; EC 3.1.8.1) or carboxylesterase (CaE; EC 3.1.1.1) is high are relatively resistant to OPs (Kaliste-Korhonen *et al.*, 1996). Conversely, *knockout* mice for PON1 are very sensitive to OPs (Shih *et al.*, 1998). Albumin displays a low esterase activity, and slowly reacts with carbamyl- and phosphoryl-esters. However, its concentration in blood and lymph is so high ($\approx$0.6 mM) that it likely plays a role in detoxification of carbaryl at toxicologically relevant concentrations (Sogorb *et al.*, 2007). Thus, plasma albumin could also play a role in detoxification of certain OPs (Li *et al.*, 2008; Tarhoni *et al.*, 2007).

Toxicity of OPs can be countered by reducing skin absorption and lowering OP concentration in the blood compartment, thus preventing the transfer of OP molecules towards cholinergic synapses and other biological targets (Figure 70.2). Neutralization of toxicant can be achieved by using stoichiometric traps or catalysts acting on exposed surfaces (e.g. active topical skin protectants) or in the blood stream (bioscavengers).

II. STOICHIOMETRIC SCAVENGERS

The first molecules to be studied for the purpose of making stoichiometric scavengers were cyclodextrins (Désiré and Saint-André, 1986), neutralizing antibodies (Glikson *et al.*, 1992), and activated charcoal. Though the interest of charcoal is much debated, hemodialysis on a charcoal cartridge was successfully used on a Tokyo casualty who was resistant to the classical treatment of sarin poisoning (Yokoyama *et al.*, 1995). Transfusion of human plasma has been used for treating OP poisoning. The effects of fresh frozen plasma on cholinesterase levels, and outcomes in patients

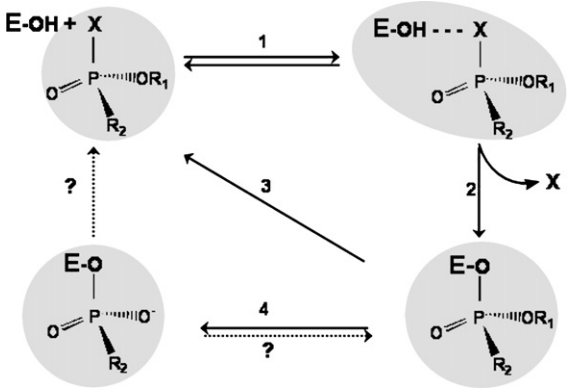

FIGURE 70.1. Mechanism of inhibition of cholinesterases by OPs. After formation of the reversible complex between ChE and OP (step 1), the active serine (esteratic site, E-OH) is phosphylated; the reaction leads to release of the leaving group X (step 2). The phosphylated enzyme can be reactivated by nucleophilic agents, such as oximes (contrathion or pralidoxime, MMB4, obidoxime, HI-6, etc.) used as antidotes in emergency treatment of OP poisoning (Lundy *et al.*, 2006; Worek *et al.*, 2007) (reaction 3); water is too weak a nucleophile for fast spontaneous reactivation of phosphylated ChE. The phosphyl–ChE conjugate may undergo a spontaneous dealkylation through alkyl–oxygen bond scission (''aging'') (Shafferman *et al.*, 1996; Viragh *et al.*, 1997; Masson *et al.*, 1999), resulting in irreversibly inactivated (''aged'') enzyme (step 4). The dealkylation reaction can be very fast ($t_{1/2} = 3$ min at 37°C for human AChE phosphonylated by soman). At the moment, drug-mediated reactivation of aged ChE is not possible.

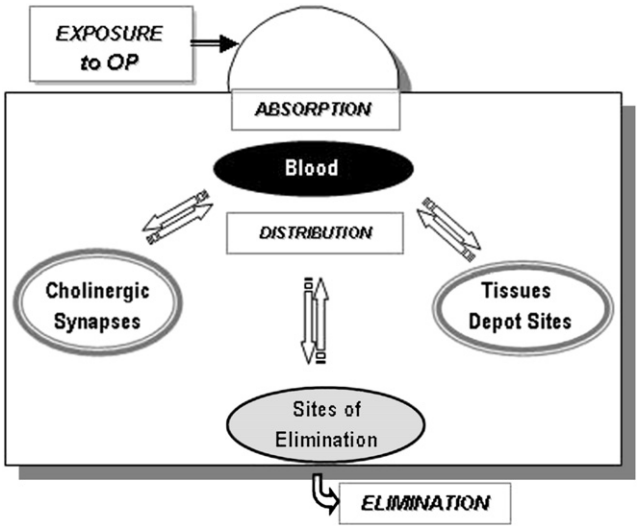

FIGURE 70.2. Biological fate of organophosphorus compounds in humans. Routes of penetration of OPs are absorption through the skin, eyes, and/or respiratory tract (nerve agents, pesticides), or ingestion (self-poisoning). OP molecules distribute from the blood compartment into tissues, including depot sites, biophase (cholinergic synapses and secondary targets) and sites of elimination (liver and kidneys). Cholinesterases are the main biological targets (acute toxicity); reaction with secondary targets (carboxylesterases, serine-amidases, peptidases, and other proteins) may be responsible for noncholinergic sublethal effects of OPs and chronic toxicity at low-dose exposure (Casida and Quistad, 2004; Costa, 2006).

with OP poisoning were evaluated (Güven *et al.*, 2004). Results suggest that plasma (fresh or freshly prepared) therapy may be an effective alternative or adjunctive treatment method. Plasma BChE, and possibly other abundant OP scavenging proteins in plasma (e.g. albumin, PON1), may have contributed to this result.

At the end of the 1980s, research on scavengers mostly focused on enzymes that specifically react with OPs. Cholinesterases (Wolfe *et al.*, 1987) and carboxylesterases (Redindo and Potter, 2005; Fleming *et al.*, 2007) have been proposed as stoichiometric scavengers. However, enzymatic stoichiometric neutralization of OP needs administration of a huge amount of enzyme, e.g. about 3 mg/kg of highly purified plasma BChE (Ashani and Pistinner, 2004). Large-scale production of enzymes under GMP conditions at a reasonable cost has been the subject of intense research over the past 10 years. However, although several thousand tons of outdated human plasma are available in the USA for preparing the enzyme, a liter of human plasma provides less than 1 mg of GMP BChE.

Today, two industrial GMP processes exist for mass production of human BChE. The first one is purification of the natural enzyme from human plasma (Cohn Fraction IV). This process has been developed by Baxter Healthcare Corporation (www.baxter.com). Human plasma derived BChE was granted Investigational New Drug (IND) status by the FDA in 2006 for protection against nerve agents (Lenz *et al.*, 2007). Clinical trials on volunteers (Phase I) started in October 2006. The second method has been developed by Nexia (www.nexiabiotech.com); it uses the recombinant human enzyme produced in milk of transgenic goats. Several grams of enzyme can be secreted in 1 liter of milk. This enzyme has been named Protexia™. Since 2005, Pharmatheme (www.pharmatheme.com) has been developing Protexia, PEGylated derivatives of this enzyme (Huang *et al.*, 2007), and fusion proteins (Huang *et al.*, 2008).

Certain secondary targets of OPs are potential bioscavengers. In particular, owing to the high number of amino acid residues in albumin that covalently bind OP molecules (5 tyrosines and 2 serines) (Ding *et al.*, 2008), it may be hypothesized that reactivity of these residues could be enhanced upon specific chemical modification. Modified albumins could lead to a new generation of stoichiometric bioscavengers. Finally, low molecular weight stoichiometric scavengers could be an economic alternative to enzyme-based stoichiometric scavengers. Several serine- and tyrosine-containing hexapeptides from a random library of peptides have been selected because they form a phosphoester bond with a fluoresent analog of sarin (Landry and Deng, 2006).

III. CATALYTIC SCAVENGERS

Catalytic scavengers are enzymes or artificial catalysts capable of degrading OPs. These catalysts detoxify OPs by hydrolyzing the phosphoester (organophosphorus acid anhydride hydrolase activity, also called phosphotriesterase activity) or lead to less toxic compounds by degrading their alkyl/aryl chains through oxidation.

The catalytic bioscavenger concept, second generation bioscavengers, is based on the idea of continuously trapping and degrading OPs in the blood stream before OP molecules reach their central and peripheral neuronal and neuromuscular targets. Then, prophylactic injection of enzymes capable of hydrolyzing OP quickly (alone or in association with current prophylactic countermeasures, i.e. pyridostigmine bromide) would allow first responders, firefighters, explosive ordnance disposal technicians, and medical personnel to operate safely in contaminated environments or with contaminated casualties. Intravenous or intramuscular administration of bioscavengers to chemical casualties is expected to greatly improve the efficacy of implemented pharmacological countermeasures (Ashani *et al.*, 1998; Saxena *et al.*, 2006).

These enzymes could be used also for protection of skin (Fisher *et al.*, 2005) and decontamination of skin, mucosa, and open wounds (Lejeune and Russell, 1999; Gill and Ballesteros, 2000). Genetically engineered bacteria producing OP hydrolases could be introduced into water effluents of decontamination units to purify contaminated water before recycling or washing up (Chen and Mulchandani, 1998).

Though research on catalytic antibodies (Amital *et al.*, 1996; Vayron *et al.*, 2000; Kolesnikov *et al.*, 2000; Jovic *et al.*, 2005) and functionalized β-cyclodextrins (Masurier *et al.*, 2005; Ramaseshan *et al.*, 2006) has made progress, engineering of enzymes capable of degrading OPs is the most promising short-term research field.

IV. REQUIREMENTS

The general requirements for the use of enzyme degrading OPs as medical countermeasures against OP poisoning are as follows.

These enzymes must have a large activity spectrum, and ideally enantioselectivity for toxic stereoisomers. Their mass production under GMP conditions must be realizable at a reasonable cost. Long-term storage without activity loss (in solution, lyophilized, or adsorbed/bound on a matrix) must be possible under field conditions. Conformational stability can be optimized by chemical modification or addition of stabilizers such as polyols. Thermostable enzymes from thermophilic bacteria (Merone *et al.*, 2005) or mutated/evolved highly stable enzymes from mesophilic bacteria (Elias *et al.*, 2008) are promising alternatives.

Other conditions depend on the method of administration, delivery system, or galenic formulation of these enzymes. For parenteral administration, the toxicant concentration in blood has to be considered. Even in the most severe case of poisoning, this concentration [OP] is always very low. For example, the sarin concentration in the serum of casualties after the Matsumoto and Tokyo chemical attacks has been estimated between 1.5 and 30 nM, 14 h post-exposure (Polhuijs *et al.*, 1997). Therefore, the [OP] in plasma is always well below the K_m of the enzyme for OP substrates. This determines first order kinetics for hydrolysis of OP in blood (Masson *et al.*, 1998) as described by Eq. (70.1):

$$v = k_{cat}/k_m \cdot [E] \cdot [OP] \tag{70.1}$$

In Eq. (70.1), the product of the bimolecular rate constant (k_{cat}/K_m) and the enzyme active site concentration ([E]) is the first order rate constant. The amount of enzyme to be injected for degradation of toxic molecules in a very short time depends on the enzyme efficiency, i.e. k_{cat}/K_m. The higher the efficiency, the lower the dose of enzyme to be administered. The enzyme concentration that reduces the OP concentration to a nontoxic concentration in time t is:

$$[E] = \frac{X}{k_{cat}/K_m.t} \tag{70.2}$$

where X is the factor by which [OP] is reduced ($X = \ln[OP]_0/[OP]_t$) (Masson *et al.*, 2008). The efficiency of a given enzyme can be increased by several orders of magnitude by mutagenesis or chemical engineering (Griffiths and Tawfik, 2003; Hill *et al.*, 2003).

The second constraint is to maintain the bioscavenger concentration, [E], in the blood as high as possible for a long time. [E] is controlled by the enzyme pharmacokinetics and/or the frequency of repeated injections. Increasing the size of the enzyme by polymerization, decreasing glycosylation microheterogeneity, and chemical modifications of the solvent-exposed surface (''capping'') improve the biological life of injected enzymes (Cohen *et al.*, 2006). Fast clearance of glycoproteins is often due to glycosylation defects. These can be corrected by chemical modifications or selection of an appropriate expression system (Chitlaru *et al.*, 1998). Immunotolerance of injected enzymes is a major issue. For instance, bacterial enzymes are not suitable for use in humans, but conjugation to dextran or polyethylene glycol is often sufficient to reduce antigenicity and slow down clearance.

Extracorporeal dialysis can be implemented for blood decontamination. Enzymes can be immobilized on dialysis cartridges (Klein and Langer, 1986). In that case, k_{cat}/K_m has to be as high as possible and the flow rate reduced to increase the efficiency of the reactor. Moreover, accessibility of OP molecules to the enzyme active center must not be altered by the immobilization method or by matrix effects. The enzyme concentration per surface unit has to be maximized to reduce diffusion constraints. First order

degradation kinetics takes place under the particular conditions of immobilized enzymes in a continuous-flow system. Immunocompatibility problems are theoretically suppressed, thus permitting the use of nonhuman enzymes.

Lastly, *in situ* transient production of enzymes, if the need arises, will be possible by gene therapy in the future. Promising results have been published with human paraoxonase (Conwan *et al.*, 2001; Fu *et al.*, 2005; Bradshaw *et al.*, 2005; Miyoshi *et al.*, 2007; Guns *et al.*, 2008) and human AChE (Li *et al.*, 2006).

Enzymes in skin and eye lotions, immobilized in foams and on tissues for skin and eye decontamination (Gordon *et al.*, 2003), or in topical skin protectants (Braue *et al.*, 2002), act under conditions where local OP concentrations can be very high. In these cases, enzyme reaction order in [OP] tends to zero, so that reaction rate is close to maximum velocity:

$$v \rightarrow V_{\max} = k_{\text{cat}} \cdot [\text{E}] \qquad (70.3)$$

The enzyme efficiency depends on its concentration and its catalytic constant k_{cat}. Thus, for external use, enzyme preparations have to be highly concentrated with high molecular catalytic activity. Co-immobilization of different enzymes could be an easy way to extend the spectra of agents to be degraded. This should allow simultaneous detoxification of G and V agents, as well as other potential chemical warfare agents. Indeed, exposure to multiple agents has to be considered. In this view, it should be remembered that during the war between Iran and Iraq, tabun and other OPs were combined to mustard gas in some attacks (UN Reports, 1984, 1987). In asymmetric conflicts, eschatological and Mafia-like terrorist activities, the most extreme scenarios have to be taken into account.

V. POTENTIAL ENZYMES

A. Cholinesterases

As seen in Figure 70.1, OPs may be regarded as pseudo-substrates of cholinesterases. When ChEs react with carboxyl-ester substrates, the acyl-enzyme intermediate is a transient, the acyl group being rapidly displaced by a water molecule. On the contrary, in the case of phosphyl-esters, the stereochemistry of the phosphyl-enzyme intermediate restricts the accessibility of water to the phosphorus atom. Thus, hydrolysis of phosphylated intermediate is very slow, and the enzyme remains inhibited (Järv, 1989). It was postulated that introduction of a second nucleophile pole in the active center could activate a water molecule. This water molecule could subsequently attack the phosphorus atom on the back face, leading to breakage of the P-serine bond.

The determination of the three-dimensional structure of AChE from *Torpedo californica* (Sussman *et al.*, 1991) opened the way to rational redesign of cholinesterases. The possibility of converting a ChE into an OP hydrolase (OPH) was demonstrated a few years later by Millard *et al.* (1995). Human BChE was chosen as the model enzyme because its active center is larger (500 $Å^3$) and less stereospecific than that of AChE (300 $Å^3$). Molecular modeling based on the structure of the *Torpedo* AChE model was used for making the first mutants of human BChE. The second nucleophile pole was created in the oxyanion hole of the active center; a glycine residue was replaced by a histidine. The first mutant, G117H, was capable of hydrolyzing paraoxon, sarin, echothiophate, and VX (Millard *et al.*, 1995; Lockridge *et al.*, 1997) (Figure 70.3). However, this mutant was irreversibly inhibited by soman because the ''aging'' process of the conjugate was faster than the dephosphorylation reaction (Figure 70.1, reaction 4). The mechanism of aging, i.e. dealkylation of an akyl chain on the adduct, is almost completely elucidated. The dealkylation reaction involves a carbocationic transient that is stabilized by active site residues E197 and W82 and water molecules (Shafferman *et al.*, 1996; Viragh *et al.*, 1997; Masson *et al.*, 1999; Li *et al.* 2007). Mutation of E197 into D, Q, or G considerably reduced the rate of aging. As expected, the double mutant G117H/E197Q was capable of hydrolyzing soman (Millard

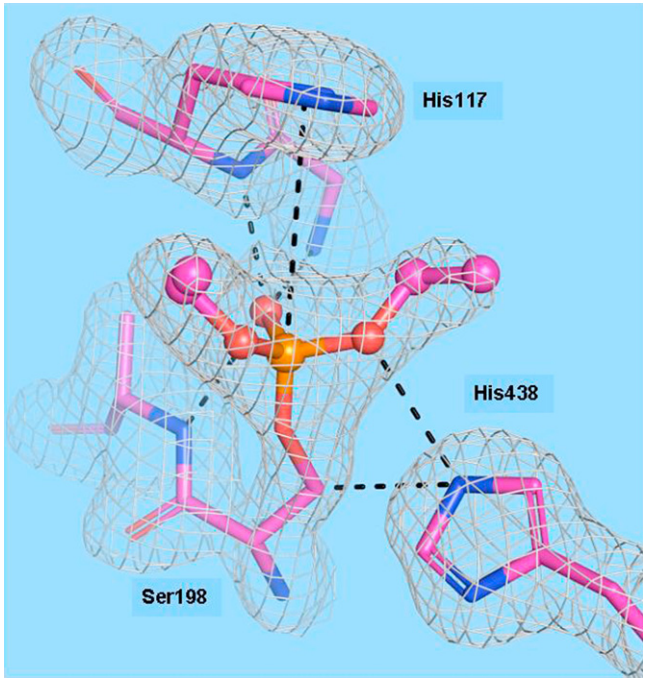

FIGURE 70.3. X-ray structure of the active center of diethylphosphorylated G117H mutant of human butyrylcholinesterase at 2.1 Å resolution (Nachon *et al.*, 2007). The enzyme was phosphorylated by soaking the crystal in 10 mM echothiophate for 5 min. X-ray data were collected at the European Synchrotron Research Facility (ESRF, Grenoble, France). Active serine (Ser198), catalytic histidine (His438), and the mutated residue (His117) in the oxyanion hole are shown as sticks. His117 is mobile enough to both activate a vicinal water molecule and stabilize the trigonal bipyramidal dephosphorylation transition state.

et al., 1999). However, the catalytic activity of this mutant was too slow to be of pharmacological interest.

The discovery of a blowfly (*Lucilia cuprina*) that is resistant to OPs because it carries a mutated carboxylesterase (CaE), G137D, at a position homologous to G117, stimulated research on G117H-based mutants of BChE. Though the OPH activity of the G137D is low, it is balanced by the abundance of the enzyme in the insect organs (Newcomb *et al.*, 1997). Transgenic mice *knockout* for AChE and carrying the G117H mutant of human BChE were found to be less sensitive to OP than wild-type animals (Wang *et al.*, 2004). Though transgenic mice expressed the G117H mutant in all organs, unlike resistance of the blowfly, their resistance to OP cannot be explained by OP hydrolysis that was too slow, but rather by hydrolysis of excess acetylcholine in cholinergic synapses.

More than 60 double or triple mutants based on G117H were made (Schopfer *et al.*, 2004), and certain mutants were eventually designed using the x-ray structure of human BChE (Nicolet *et al.*, 2003). At the same time, mutants of human AChE and *Bungarus fasciatus* AChE were made on the same principle (Poyot *et al.*, 2006). Unfortunately, none of these muteins was more active than the G117H mutants. Actually, there is evidence that mutations at position G117 cause dislocation and loss of functionality of the oxyanion hole (Masson *et al.*, 2007). For a historical review of this quest, see Masson *et al.* (2008).

Computer-assisted design of new OPH mutants of BChE is conceivable. This new approach, called ''intelligent'' directed mutagenesis design, is based on simulation of transition states. Thus, simulation of dephosphylation transition states is expected to indicate how to optimize interactions favoring productive crossing of the energetic barrier for dephosphylation. Simulation of activation transition state approach was already successfully applied to the design of BChE mutants. Using the three-dimensional structure of human BChE, molecular dynamic simulations of deacylation transition state allowed highly active mutants to be made against (−)-cocaine (Pan *et al.*, 2005; Zheng and Zhan, 2008).

Directed evolution of cholinesterases could be an alternative to computer-based methods. However, so far, functional expression of cholinesterases is difficult in yeast and has failed in bacteria.

Certain ChE mutants sensitive to OPs do not ''age'' after phosphylation; they are fully reactivatable (cf. Figure 70.1, reaction 3). Such ChE mutants when associated with oximes (e.g. 2-PAM, HI-6) act as pseudocatalysts in displacing the OP moiety bound to the enzyme. These enzyme–reactivator coupled systems could lead to a new family of pseudocatalytic bioscavengers (Kovarik *et al.*, 2007; Taylor *et al.*, 2007).

Bioavailability and biological stability of mutated ChEs for injection is an important issue. First, pharmacokinetic studies of highly purified human BChE injected into the rat showed that the half-time ($t_{1/2}$) of enzyme in the blood stream depends on sialylation of enzyme carbohydrate chains (Douchet *et al.*, 1982). It is well known that rapid elimination of asialoglycoprotein from the circulation is due to their capture by specific receptors located on the surface of hepatocytes. These receptors recognize galactosyl residue, the carbohydrate that precedes sialic acid at the terminus of complex glycans. Studies with other natural and recombinant ChEs confirmed the importance of sialic acid (*N*-acetylneuraminic acid) residues ending glycans (Kronman *et al.*, 1995, 2007; Saxena *et al.*, 1998; Cohen *et al.*, 2007). It was found that $t_{1/2}$ is inversely proportional to the number of unoccupied attachment sites of sialic acid (Kronman *et al.*, 2000). To increase $t_{1/2}$ of administered recombinant ChEs, all galactosyl residues have to sialylated. Full sialylation of recombinant enzymes can be achieved using an expression system capable of synthesizing glycans similar to natural human glycoprotein glycans and adding inhibitors of sialidase in the cell culture medium. Co-expression of the enzyme of interest and sialyltransferase in HEK 293 cells was found to lead to fully sialylated recombinant human AChE (Kronman *et al.*, 2000). Alternatively, *in vitro* sialylation of purified enzymes is possible with a sialyltransferase or using a chemical method (Gregoriadis *et al.*, 1999). PEGylation has also been proven to be an effective chemical modification for increasing circulatory half-life of administered recombinant ChE (Huang *et al.*, 2007; Cohen *et al.*, 2007; Kronman *et al.*, 2007). Recently, a 150 kDa recombinant fusion protein human albumin–human BChE showed a substantially improved pharmacokinetics when administered to juvenile pigs, $t_{1/2} \approx 32$ h against ≈ 3 h for recombinant 70% tetrameric BChE (Huang *et al.*, 2008).

B. Phosphotriesterases

1. Bacterial Phosphotriesterases

The bacterial phosphotriesterases (PTEs; EC 3.1.8.1) are encoded by the organophosphate degradation (*opd*) gene found in species of *Pseudomonas diminuta*, *Flavobacterium* sp., and *Agrobacterium radiobacter*, and genes similar to *opd* were also located in Archaea (Merone *et al.*, 2005). PTEs belong to a superfamily of amidohydrolases (Holm and Sander, 1997).

Pseudomonas diminuta PTE is a 72 kDa dimeric bimetallic enzyme with Zn^{2+} involved in the catalytic process (Carletti *et al.*, 2009, in press). Substitution of the native Zn^{2+} ions in the active site with Mn, Co, Ni, or Cd ions results in the almost full retention of catalytic activity. Following the first determination of the three-dimensional structure of *P. diminuta* PTE (Benning *et al.*, 1994), a series of crystal structures, kinetic, and spectroscopic experiments were described. Nevertheless, the enzyme mechanism is still debated and the functional roles of divalent metal cations and amino acids in the active centre are not yet fully understood (Aubert *et al.*, 2004; Samples *et al.*, 2007; Chen *et al.*, 2007; Wong and Gao, 2007; Jackson *et al.*, 2008). No natural substrate has yet been identified (Ghanem and

Raushel, 2005) and PTE is suspected to have evolved from a bacterial lactonase (Afriat *et al.*, 2006), the phosphotriesterase activity being a promiscuous activity (Elias *et al.*, 2008). Whereas the catalytic efficiency of PTE for paraoxon, the best substrate identified so far, is approaching the diffusion-controlled limit, it is slowest against OP nerve agents (Table 70.1). Meanwhile, directed evolution of PTE showed that only three amino acid changes dramatically enhanced the catalytic efficiency for an analog of soman by ~3 orders of magnitude (Hill *et al.*, 2003).

There have been numerous studies highlighting the potential of this enzyme for decontamination or skin protection in addition to OP detection (Lejeune and Russell, 1999; Gill and Ballesteros, 2000; Létant *et al.*, 2005; Ghanem and Raushel, 2005; Karnati *et al.*, 2007). Administration of PTE before or after OP exposure was shown to improve pretreatment and current treatment of OP intoxication (Doctor and Saxena, 2005). However, in order to prevent abnormally fast pharmacokinetics and/or immunological response due to injection of a bacterial enzyme, PTE could be PEGylated (Jun *et al.*, 2007) or encapsulated. First attempts at using PTE encapsulated within sterically stabilized liposomes were promising, providing protection of rats from multiple LD_{50}s of OP pesticides (Petrikovics *et al.*, 2004). An alternative route could be the blood detoxification by extracorporeal circulation through a cartridge containing PTE immobilized in hollow fibers (unpublished results). PTEs could possibly also be used for skin protection as active components of topical skin protectants (TSPs) or covalently coupled to the cornified layer of the epidermis (Parsa and Green, 2001).

PTE was also entrapped in additives for latex coating of biodefensive surfaces. Such PTE-based additives for paints and coatings were shown to retain catalytic parameters and stability of the enzyme (McDaniel *et al.*, 2006). For decontamination of OPs in the environment and remediation, an alternative approach, phytodegradation by transgenic plants (e.g. tobacco) expressing a bacterial PTE, has been considered as a potentially low-cost, effective, and environmentally friendly method (Wang *et al.*, 2008).

2. Human Paraoxonase (PON1)

The human paraoxonase-1 (PON1) is a 45 kDa calcium-dependent enzyme bound to high-density lipoprotein (HDL) particles, in association with other apolipoproteins. PON1 shows a genetic polymorphism; the most prominent determines the Q192R allozyme, which can have a substantial impact on PON1 activity against OPs and arylesters (Smolen *et al.*, 1991) (Table 70.1). The enzyme was shown to be involved in the protection against atherosclerosis (Shih

TABLE 70.1. Catalytic efficiency (k_{cat}/K_m $10^6 \cdot M^{-1}$ min^{-1}) of different natural and engineered OP hydrolases towards different OPs

Enzyme	Paraoxon	DFP	Soman	Sarin	Echothiophate	VX
Human Q192 PON1	0.68[a]	0.04[b]	2.8[c]	0.91[c]		+[d]
Human R192 PON1	2.4[a]		2.1[c]	0.07[c]		+[d]
Human in 293T rPON1			0.62–4.13[e]			
Mammalian G3C9 rPON1	0.72[f]					
Human G117H BChE	0.0057[g]	0.0052[g]	–	0.00016[h]	0.0101[g]	0.0015[h]
Blowfly G117D CaE	0.2[i]					
B. fasciatus HQT AChE	0.00006[g]	0.00076[g]			0.000024[g]	
Loligo vulgaris DFPase		78[j]	2.4[j]	2.4[j]		0[j]
P. diminuta OPAH	2000[k]	580[l]	0.6[m]	4.8[m]		0.04[n]
Alteromonas OPAA		46[o]				

[a] Smollen *et al.*(1991)
[b] Masson *et al.* (1998)
[c] Davis *et al.* (1996)
[d] C.A. Broomfield, unpublished result
[e] Yeung *et al.* (2008), with the four soman stereoisomers
[f] Harel *et al.* (2004)
[g] Poyot *et al.* (2006)
[h] Lockridge *et al.* (1997)
[i] Newcomb *et al.* (1997)
[j] Hartlieb and Ruterjans (2001)
[k] Kuo *et al.* (1997)
[l] Lai *et al.* (1995)
[m] Dumas *et al.* (1990)
[n] Rastogi *et al.* (1997)
[o] Cheng *et al.* (1999)

et al., 1998; Watson *et al.*, 1995) and thus became a player in vascular physiology. Although its primary function is likely to be a lipophilic lactonase (Khersonsky and Tawfik, 2005), PON1 displays promiscuous enzyme activities.

Abundant biochemical, biological, and toxicological information has been collected for a decade, leading to partial characterization of the enzyme function (Costa and Furlong, 2002; Mackness *et al.*, 2008), but recurrent attempts at solving the structure of human PON1 failed. Through chemical modification and site-directed mutagenesis studies, some amino acid residues have been identified as essential for activity (Josse *et al.*, 1999). Finally, molecular modeling (Fokine *et al.*, 2003; Yeung *et al.*, 2004) and crystal structure of a hybrid rPON1 (a synthetic construct issued from shuffling of rabbit, mouse, rat, and human PON1 genes expressed in *E. coli*) (Harel *et al.*, 2004) showed that human PON1 is a six-bladed β-propeller protein with a structure very similar to that of *Loligo vulgaris* DFPase (Katsemi *et al.*, 2005). The phosphotriesterase mechanism of this eukaryotic calcium-dependent enzyme was recently described (Blum *et al.*, 2006). Confirmation of a similar mechanism for human PON1 activity would considerably help in designing more active PON1 mutants.

As a naturally occurring enzyme present in plasma, human PON1 is the most promising catalytic scavenger candidate for pretreatment and therapy of poisoning by OP (La Du, 1996; Rochu *et al.*, 2007a). Thus, the enzyme is the focus of intensive research to improve its efficacy and functionalization. To provide a valuable medical countermeasure against intoxication by nerve agents, the catalytic efficiency of PON1 has to be enhanced by only one or two orders of magnitude. New rPON1 mutants obtained by directed evolution and exhibiting enhanced OP-hydrolase activity (Amitai *et al.*, 2006) suggest this goal could be achieved reasonably soon. However, instability of the mutants of interest could impinge on their biotechnological development. Indeed, as an HDL-bound protein, PON1 requires association to apolipoprotein partners to retain its stable active conformation (James and Deakin, 2004; Gaidukov and Tawfik, 2005).

Human phosphate binding protein (HPBP), an apolipoprotein that binds inorganic phosphate in blood, was serendipitously discovered. Its three-dimensional structure and complete amino acid sequence were solved (Morales *et al.*, 2006; Diemer *et al.*, 2008). The conditions found to separate HPBP and PON1 *in vitro* indicated that HPBP is strongly associated with PON1 (Renault *et al.*, 2006). Moreover, the stabilization of the active form(s) of human PON1 by HPBP suggests that HPBP could be a functional chaperone for PON1 (Rochu *et al.*, 2007b, c).

Our strategy is now to attempt co-crystallization of the PON1/HPBP complex. For this, the first step will be to construct a hybrid gene allowing co-expression of HPBP and PON1 in *E. coli*. This approach needs to have the gene coding for HPBP, up to now not found in the human genome. To overcome the difficulty of this absence, we made a synthetic gene issued from amino acid sequence data (Diemer *et al.*, 2008). Afterwards, co-expression of the PON1/HPBP complex will be attempted. Co-expression, aimed to favor correct folding of active PON1 and stabilization of the active functional conformation of the enzyme, is expected to provide crystallizable PON1/HPBP complex. Finally, diffractable crystals of the complex are assumed to provide the three-dimensional structure of natural human PON1. This crucial phase will be the first step on the staircase leading to the development of stable human PON1 mutants with enhanced catalytic efficiency against OPs.

Gene therapy could also be considered to challenge OPs by using a mutated PON1 gene coding for an enzyme with high OPH activity against nerve agents. Several approaches with different gene delivery vectors in mice showed increased PON1 level serum that reduced or even prevented the entry of OP into the brain, and reduced atherosclerosis signs (Conwan *et al.*, 2001; Fu *et al.*, 2005; Bradshaw *et al.*, 2005; Guns *et al.*, 2008). Local delivery of PON1 gene using Sendai virus vector inhibited neonatal hyperplasia after arterial balloon injury in rabbits fed a high-fat diet (Miyoshi *et al.*, 2007). Thus, enhanced expression of PON1 by gene therapy could be beneficial for the different functions of the enzyme. Meanwhile, the complex and defectively identified PON1 activities make it apparent that strategies for repetitive administration of high concentrations in humans must be undertaken cautiously.

C. Other Enzymes

Other enzymes are involved in biodegradation of OPs, e.g. hydrolases such as prolidases; others are oxidases such as cytochrome P450s, glutathione *S*-transferases, laccases, and peroxidases. Prolidases (EC 3.4.13.9) were first isolated from halophilic bacteria (*Alteromonas haloplanktis* and *Alteromonas* sp. JD6.5). Prolidase from *Alteromonas* sp. JD6.5 is an OPAA that displays the highest known activity against soman ($k_{cat} = 3{,}100\ s^{-1}$), but it is inactive against VX (Cheng *et al.*, 1999). Prolidase was also isolated from human liver. This enzyme displays a catalytic activity against soman (Wang *et al.*, 1998) and exhibits sequence homology with the *A. haloplanktis* prolidase (Wang *et al.*, 2006). Glutathione *S*-transferases (GST; EC 2.5.1.18) are 20–30 kDa enzymes that catalyze glutathione conjugation (nucleophilic attack of the thiol group) to electrophilic substrates. They are involved in cellular detoxification processes of endogenous compounds and of numerous xenobiotics, and their role in resistance to insecticide of insects is well established. OP detoxification by GSTs results from a regioselective dealkylation of alkyl or aryl side chain (Maturano *et al.*, 1997). These enzymes exhibit wide genetic polymorphism. Some GST allelozymes from the flies *Drosophila melanogaste*r and *Musca domestica*, highly active against OP insecticides, have been cloned and expressed in *E. coli* (Fournier *et al.*, 1992). There is evidence that GSTs contribute to OP detoxification in

humans (Fujioka and Casida, 2007). Laccases (EC 1.10.3.2) are fungal phenol oxidoreductases that have been used for detoxification of numerous xenobiotics, including dyes and pesticides (Richardt and Blum, 2008). The laccases from *Pleurotus ostreatus* and *Chaetomium thermophilium* were found to rapidly degrade VX and VR in the presence of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as a mediator (Amitai *et al.*, 1998). In our laboratory, we found that laccases from *Trametes versicolor* and *Coriolopsis polyzona* with ABTS display similar properties against V agents (Trovaslet *et al.*, unpublished results). The heme-containing chloroperoxidase (EC 1.11.1.X) from *Caldaromyces fumago*, with peroxide as co-substrate, is another efficient VX-degrading enzyme (Amitai *et al.*, 2003).

These enzymes are promising for destruction of chemical weapons stockpiles, soil remediation, decontamination of materials, protective equipment, and water polluted by pesticides and nerve agents (Russel *et al.*, 2003). In particular, phosphorothiolates such as VX are relatively resistant to PTE. Thus, oxidative cleavage of the P–S bond could be achieved by oxidases like laccases. These enzymes could be used in association with other OP-degrading enzymes for skin decontamination or in topical skin protection formulations. Though no work has been performed on combined action of oxidases and hydrolases, oxidation of P-bonded alkyl/aryl chains by oxidases is expected to alter enantioselectivity of PTE for parent OPs, and therefore to improve the efficiency of catalytic bioscavengers.

VI. CONCLUDING REMARKS AND FUTURE DIRECTION

Enzymes that neutralize or degrade OPs can be purified from natural sources, e.g. human plasma. Recombinant enzymes can be produced using procaryotic expression systems (*E. coli*) or eucaryotic expression systems (yeast, insect, mammalian cell cultures), or transgenic animals (worm, rabbit, goat), or transgenic plants (tomato, potato, tobacco), and also acellular biosynthetic systems. The goal of current research in protein engineering is to improve mass production of stable muteins at low cost. OP hydrolases improvement of *in vitro* and *in vivo* catalytic properties toward nerve agents and pesticides is still the main issue. In addition, improvement of thermodynamic stability (storage stability in solution or in dry forms) and *in vivo* operational stability, improvement of immunotolerance and biodisponibility are other goals. For this purpose, the different strategies of enzyme engineering have been implemented. They consist of research into new natural enzymes, in particular in extreme environments (Feerer *et al.*, 2007) – potential extremozymes have been discovered in halophilic, thermophilic, and piezophilic bacteria – in insects resistant to OP pesticides, and among secondary targets of OPs in humans. Molecular modeling and transition state simulations, site-directed mutagenesis and directed evolution approaches in combination with chemical modifications and medium manipulations have been used with success to improve the desired properties of selected enzymes (Bershtein and Tawfik, 2008). Lastly, pharmacokinetic, toxicokinetic, and immunological studies on animal models allow the validation of enzymes of interest.

Catalytic bioscavengers will be part of the arsenal of medical countermeasures for prophylaxis and treatments of OP poisoning in the very near future. Next, gene therapy will offer the possibility of transitory production of humanized OP-degrading enzymes in the body. Multiple enzyme associations will be part of active topical skin protectants and decontamination tools.

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CHAPTER 71

Rapid Decontamination of Chemical Warfare Agents

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I. INTRODUCTION

Chemical warfare agents are some of the most lethal and sinister substances manufactured (organophosphates) and the most psychologically threatening (vesicants). Organophosphate nerve agents include tabun (GA), sarin (GB), and soman (GD) and the low volatile compound VX, some of which were incorporated into munitions during World War II. Sarin was used by terrorists in the mid-1990s in Japan. The chemical nerve agents irreversibly inhibit the enzyme acetylcholinesterase (AChE) in both the central nervous system (CNS) and peripheral nervous system (PNS). When AChE is inhibited, it cannot hydrolyze the neurotransmitter acetylcholine (ACh). Excess endogenous ACh produces characteristic signs of nerve agent poisoning and cholinergic overload, such as hypersecretion and respiratory distress. When the nerve agent accesses the CNS, symptoms such as convulsions appear and lead to coma and death. In contrast, vesicants, such as sulfur mustard used in World War I and the Iran–Iraq War in the 1980s, are known not for lethality, but for the resulting burns and blisters that incapacitate military personnel. In contrast to nerve agents, where symptoms are observed in minutes, sulfur mustard presents clinical symptoms hours after exposure, although tissue damage by alkylation is rapid. Sulfur mustard alkylates peptides, proteins, and nucleic acids, and perturbs other cellular components. It is documented that to reduce the effects of chemical warfare agents, decontamination of the skin soon after exposure is the best post-medical countermeasure against chemical warfare agents. Thus, the decontamination product should be lightweight for incorporation into the limited space in a soldier's pack and easily used in the field under harsh and likely confusing conditions. Personal wipes, pads, and sponges have been developed to facilitate this goal. These products should exhibit long-term stability and be nontoxic and environmentally friendly. In addition to removing these toxic agents from skin, increased effectiveness can be obtained by detoxification of the chemical agents both on and in the skin and upon removal to protect the environment. Detoxification of the agent will also prevent secondary contamination of medical personnel or a soldier's buddy. The different approaches to solving field decontamination and detoxification are explored in this chapter. The importance of continued improvement of personal decontamination products cannot be underestimated when one considers today's constant threat of chemical warfare, terrorist acts, and pesticide spills.

II. THE NATURE OF HUMAN SKIN

Human skin, the largest human organ, developed as a physical barrier to the environment (to keep things out) but also maintains the aqueous nature of the human body (to keep things in). Mammalian skin consists of three major layers: stratum corneum, epidermis, and dermis. The stratum corneum, the thin outer layer of keratin-filled dead cells (corneocytes) bounded by densely crosslinked protein and embedded in crystalline lamellar lipids, represents the major barrier protecting the body from loss of internal components and entry of undesirable external materials. The layer underneath the stratum corneum, the epidermis, contains cells that differentiate from viable keratinocytes to corneocytes during their migration from the dermis to the stratum corneum. It also contains a large number of specialized dendritic cells. Smaller amounts of specialized cells are integral to the epidermis, including the pigmentation melanocytes, the immunological Langerhans cells, and the sensory Merkel cells. Throughout the epidermis sebaceous glands, sweat glands, and hair can be found. The next inward layer, the dermis, contains hair follicles with associated sebaceous glands, ecrine sweat glands and ducts, dendritic cells, and a vascular network including subepidermal capillaries, vascular plexi associated with the sweat glands, and dermal papillae associated with the hair follicles. Capillaries are responsible for transporting any chemicals that enter the skin systemically. Recent reviews of the skin structure and permeation are available for further reading (Menon, 2002; Hadgraft and Lane, 2005; Godin and Touitou, 2007; Wester and Maibach, 2000).

The stratum corneum, composed of keratinized dead cells that are continually being replaced, is the first major barrier to chemical agents. The barrier qualities of the stratum corneum depend on a number of factors, including its location on the body, which affects thickness, and how much hair is present. Thus, hair follicles and sweat glands can either provide channels through the stratum corneum, and thereby bypass its barrier attributes, or at least provide increased surface area for penetration of compounds, since a number of compounds were shown to penetrate faster in hair follicle-rich areas (Illel *et al.*, 1991). Maibach studied three radiolabeled pesticides – parathion, malathion, and carbaryl – for their permeability at 13 different anatomical sites in humans (Maibach *et al.*, 1971). Variations in percutaneous penetration were observed; higher penetration of the pesticides occurred at the abdomen and dorsum of the hand.

The lipid matrix is another feature important for barrier function in the epidermis. The arrangement of lamellar-like sheets yields a barrier to hydrophilic compounds and transcutaneous water transport. Extraction of those lipids from skin with organic solvents reduces barrier function (Hadgraft, 2001). The lamellae, which have few phospholipids as they are catabolized, ultimately contain mainly ceramides, cholesterol, and fatty acids (Wertz and Downing, 1989; Bouwstra and Ponec, 2006). The resulting matrix is composed of nonpolar compounds enriched in cholesterol that are adapted to protect from water loss. While extraction of these lipids may increase the penetration of aqueous moieties, in the case of organophosphates (note the organic-like nature of the chemical warfare agents, as described below), the hydrophobic nature of skin likely facilitates partition of these chemical agents through the lipid matrix, which then enter the subepidermal capillaries for dissemination throughout the body. Further insight into the barrier properties of skin can be observed in disease states including psoriasis, where there is an increase in epidermal cell replication yielding an irregularly stacked stratum corneum and abnormal capillaries in the dermis. This leads to an increase in drug penetration such as hydrocortisone (Kranz *et al.*, 1977). No studies have evaluated pesticide or chemical warfare agent penetration in psoriasis.

Aging contributes to decreased lipid barrier protection, decreased intercellular cohesion and increased absorption of toxic material. This barrier is also complicated by environmental effects such as exposure to sun, disease, and other aging processes that include many changes to the structure of the skin. Examples of such changes are decreased amounts of collagen, loss of melanocytes, decreased number of glands and hair follicles, reduced blood flow (Yates and Hiley, 1979), and the loss of lipid content in the stratum corneum (Elias and Ghadially, 2002). Another study found that 11 of 14 pesticides showed different rates of skin penetration in aged rats compared to young rats (Shah *et al.*, 1987). Generally, decreased absorption occurred in studies of aged skin (Fisher *et al.*, 1992; Farage *et al.*, 2007).

Percutaneous absorption *in vivo* leads to the occurrence of chemical or drug delivery to the microcirculation in the dermis. The period of time that it takes for entrance to the blood supply and circulation throughout the body depends on the diffusion parameters and the interaction with the lipid matrix (Roberts, 1997). Thus, chemicals exhibiting a longer lag time through the skin should be less toxic if quickly removed compared to rapidly penetrating compounds. Another aspect of percutaneous absorption is whether there are single or multiple exposures to the chemical. Some chemicals, such as azone (1-dodecylazacylohepan-2-one), alter the organization of the skin so that there is an increase of absorption or synergistic effect observed with each exposure (Ademola *et al.*, 1993). Chemicals that do not alter the skin's structure would not be likely to increase their bioavailability and absorption, but rather provide an additive response (Bucks *et al.*, 1985).

III. ORGANOPHOSPHATE NERVE AGENTS

Nerve agents are among the most toxic of the known chemical agents. Nerve agents are organophosphates (OPs) that bind irreversibly to acetylcholinesterase (AChE) (Taylor *et al.*, 1999), and to the bioscavenger butyrylcholinesterase (BChE) (Wolfe *et al.*, 1992) in both the peripheral and central nervous system. AChE is responsible for terminating the action of the neurotransmitter acetylcholine by hydrolysis. OP-inhibited AChE results in an excess of acetylcholine and the overstimulation of muscarinic and nicotinic receptors. Characteristic signs of nerve agent poisoning and cholinergic overload include hypersecretion and respiratory distress. When the nerve agent is transported past the blood–brain barrier, convulsions can lead to coma and death. OPs pose a hazard in both their vapor and liquid states. Notably, AChE inhibitors are used as both a therapy for treating glaucoma, myasthenia gravis, Alzheimer's disease or atropine poisoning and for more sinister reasons, e.g. as pesticides to kill insects and as chemical warfare agents by terrorists and in warfare to kill humans (Sidell, 1977; Leikin *et al.*, 2002; Martin and Lobert, 2003).

The G-nerve agents include GA (tabun, ethyl *N*,*N*-dimethyl-phosphoramidocyanidate), GB (sarin, isopropyl-methylphosphonofluoridate), GD (soman, 1,2,2-trimethylpropyl methylphosphonofluoridate), and VX (*o*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothiolate). The V-type nerve agents are several orders of magnitude less volatile than the G-type agents and act primarily as a liquid via the percutaneous route; for example, VX is several orders of magnitude more lethal percutaneously than sarin (Reutter, 1999).

Log P data (octanol:water partition coefficients and a reflection of lipid solubility) of nerve agents were used to both predict absorption through the skin and determine the distribution of OP compounds in tissues, and then correlated with toxicity as measured by the onset of fasciculation in

guinea pigs. An excellent correlation ($r = 0.95$) was established between the measured log P value and the rate of onset of local fasciculations, reflecting absorption in the skin and penetration to blood and dissemination to muscle tissue throughout the animal (Czerwinski *et al.*, 2006).

Maxwell and Lenz (1992) reported that, in general, AChE and BChE are more reactive with cationic nerve agents such as VX, while neutral agents that contain less than two bulky groups (e.g. GA, GB, and GD) were equally reactive with the cholinesterase enzymes. Since AChE has a smaller active site than BChE, the size and ionic character of the active sites determine the specificity of these esterases for the agents. The estimated ranked percutaneous nerve agent LD_{50}s are VX > GD > GA > GB, which reflect their volatility. The ranked volatility for these agents is VX < GA < GD < GB.

Using parathion as a model simulant for the nerve agent VX, the *in vitro* percutaneous absorption through unprotected human skin and clothed and uniformed skin was determined. The percent parathion dose absorbed through the unprotected skin was significantly greater than that observed through dry uniformed skin, while absorption was higher through the wet (sweat) uniform. These results suggested that military uniforms and public clothing provide protection to this stimulant and by analogy to VX; but absorption through cloth and skin quantitatively occurred more readily with wet clothing than dry. Thus, even with clothing, immediate responses and decontamination of skin and clothing are required (Wester *et al.*, 2000).

In conclusion, due to the extreme toxicity of nerve agents, the search for medical decontamination countermeasures to OPs is of paramount importance. Rapid removal from the skin would prevent penetration to the general circulation and the resulting decrements of cholinergic toxicity, which ultimately leads to seizure and/or death in untreated individuals. In the development of medical decontamination countermeasures to nerve agent poisoning, it is acknowledged that different nerve agent administration routes are likely to have different requirements for effective treatment. There is a limited window of opportunity for decontamination treatment following agent exposure. The signs of poisoning develop within minutes, and if decontamination is delayed, it is likely that toxic levels of the nerve agents will be disseminated via the blood stream after the agent has been absorbed. Decontamination will prevent continued absorption of the agent, reducing the need for further medical management (Table 71.1) (Hamilton *et al.*, 2004; Clarkson *et al.*, 2004).

TABLE 71.1. VX applied to pig skin (ear)

Decontamination	
Delay[a] (min)	**Signs**
0	–
15 (no decontamination)	+++
15 (decontamination)	+

[a]Delay in decontamination
Hamilton *et al.* (2004)

IV. VESICATING AGENTS (DISTILLED SULFUR MUSTARD, HD; IMPURE SULFUR MUSTARD, H; LEWISITE, L)

Sulfur mustard (HD), a synthetic vesicating agent, was a major chemical warfare agent during World War I and continues to be a modern-day threat (Reutter, 1999; Ghanei and Harandi, 2007; Bismuth *et al.*, 2004). Sulfur mustard's simple and cheap chemical synthesis makes it readily accessible to terrorists and use by the military. Sulfur mustard is an alkylating agent that causes its damage by disrupting nucleic acids and proteins, impairing cell homeostasis and eventually causing cell death, although the significance of the multiple pathways is unclear (Smith *et al.*, 1995). Whole-body exposure results in cutaneous (liquefaction necrosis of the epidermis), respiratory (injures the laryngeal and tracheobronchial mucosa), and ocular effects (severe conjunctivitis). In contrast to H agents, there is no delay with lewisite, which produces immediate burning of the skin and eyes. Compared with the G-nerve agents, sulfur mustard has a relatively low acute lethal toxicity, that is, its toxicity as an incapacitating agent is of much greater concern than its capacity to kill. Furthermore, mustard is persistent in the soil and other materials for hours to weeks (Devereaux *et al.*, 2002).

The skin is an important port of entry for vesicating agents. The agent's lipophilic nature, and the propensity of skin to exclude aqueous compounds but not lipophilic substances, make the skin an unwitting transport system. An increase in ambient temperature causes increased penetration (which was used effectively in World War I, where it was disseminated at night and warmed in the early morning sun). It has been estimated that 80% of liquid mustard evaporates, and 20% penetrates the skin. Of the 20%, 12% is retained in the skin matrix, while 8% is absorbed systemically, so only large dosages of mustard will produce significant systemic toxicities (Cullumbine, 1947; Dacre and Goldman, 1996).

Mustard skin lesions first present erythema followed by blisters (Somani and Babu, 1989). Erythema usually begins 2–24 h after contact, followed by acute itching, which diminishes as the characteristic blisters appear. Blisters initially appear 18 h after contamination as small vesicles within the area of erythema, which then coalesce to form the characteristic pendulous blisters containing large volumes of clear but yellow fluid. Blisters are not painful *per se* but they may be uncomfortable and may feel tense. Warm, moist areas such as genitalia and axilla are more likely to exhibit bullous lesions. By 48 h post-exposure, blistering is clearly evident and a new round of blisters appears. Due to the disruption of the skin layer, the large blisters break leading to erosions and full thickness skin loss and

ulceration, necrosis, and 72 h post-exposure, formation of an eschar. The eschar sloughs in a 4–6 day time period, finally leaving a pigmented scar (Reid *et al.*, 2000, 2007). The burn caused by blister agents is much slower to heal in comparison with a thermal burn, likely due to the multiple mechanisms by which the agent affects biological tissue, as known from World War I and reestablished in Iranian casualties. The site of healed mustard burns is hypersensitive to mechanical trauma. In a comparison of cutaneous lesions in 500 mustard-exposed Iranian veterans and 500 unexposed veterans, a correlation was observed between exposure and skin lesions such as severe dry skin, hyper- and hypopigmentation, local hair loss, eczema, and chronic urticaria. Histopathological examination of skin biopsies has revealed nonspecific findings including epidermal atrophy, keratosis, and basal membrane hyperpigmentation (Balali-Mood and Hefanzi, 2006).

V. MODEL SYSTEMS TO MEASURE ABSORPTION, REMOVAL, AND DECONTAMINATION

A. Rats

There are many different animal models that have been used to assess the percutaneous absorption of toxic chemicals. There is little question that while *in vivo* human studies are best for predicting the absorption of percutaneous applied chemical warfare agents, ethics preclude conducting such studies. Rats have been widely used in the study of skin contamination, wounds, and healing and the efficacy of different decontamination modalities (Wester and Maibach, 2000; Shah *et al.*, 1987; Baynes *et al.*, 1997).

B. Guinea Pigs

While rats are often selected for their availability, low cost, small size, and thorough biological characterization, they are not the ideal chemical warfare agent model because they contain a high amount of carboxylesterase, a potential hydrolytic enzyme for OPs (Sweeney and Maxwell, 2003). Unlike rats, humans have small amounts of this enzyme relative to acetylcholinesterase and butyrylcholinesterase. To overcome this limitation, the guinea pig, which exhibits low carboxylesterase, has been developed as a model for chemical warfare agent exposure (Fonnum *et al.*, 1985). Guinea pigs have been evaluated for skin damage due to burns, often used as a wound healing model for sulfur mustard (Ramos *et al.*, 2008), and for skin irritation to toxic industrial chemicals (Kennedy, 2007; Weaver *et al.*, 2003). Guinea pigs have also been used to study absorption of chemical warfare agents through the skin (Dalton *et al.*, 2006; Wormser *et al.*, 2002), uptake of radioactive sulfur mustard through guinea pig skin (Logan *et al.*, 1999), as animal models for pretreatment regimens to protect against chemical warfare agents (Wetherell *et al.*, 2006), and as a model of cholinesterase activity assessments (Haigh *et al.*, 2005) to GD exposure and OP-induced seizure (Harrison *et al.*, 2004).

For evaluating the decontamination of guinea pig skin, typically, sedated and shaved guinea pigs were cutaneously exposed to neat soman on their sides. One minute after exposure, a sponge wrapped around a pair of forceps was moved across the guinea pig's side; then the forceps were rotated 180 degrees, so that the clean surface of the sponge was pointed at the animal. Three more passes were taken from the rear towards the front. An identical procedure was used when the protocol required an additional second sponge to decontaminate the animal. Similarly, guinea pigs were used for decontamination of sulfur mustard. In this case, 24 h post-neat HD exposure and decontamination, animals were injected with trypan blue and then euthanized. The skin covering the backs of the animals was removed. In addition, skin punches were taken from each of the exposure sites (control, exposed, and decontaminated sites) (Gordon *et al.*, 1999; Gordon and Doctor, 2003).

C. Swine

Pig skin has long been a valuable model for human skin (Meyer *et al.*, 1978; Riviere and Monteiro-Riviere, 1991) since pig skin expresses a sparse hair covering, epidermis, and a similar arrangement of dermal collagen and elastic fibers to human skin. Again, many investigations have used the porcine skin model to study cutaneous toxicology of sulfur mustard (Gold *et al.*, 1994). Pig skin, because of its similarity to human skin with respect to hair covering, apocrine sweat glands, and other morphological similarities (Reifenrath *et al.*, 1991), is an attractive model for cutaneous absorption and toxicology studies of OP nerve agents (Hamilton *et al.*, 2004). Cutaneous absorption studies show that pig skin permeability, compared to rat and rabbit, most closely resembles that of human skin (Bartek *et al.*, 1972) with a variety of test agents. Therefore the pig represents a good model to assess the effects of introduction of extraneous material or chemicals on the early events of exposure. The downside is that pigs are large animals, difficult to house, are more costly, and require special cages to maintain them in comparison to rodents.

VI. DECONTAMINATION REQUIREMENTS

Medical decontamination executes removal and/or neutralization of chemical warfare agents, which, upon penetration of the skin, produces vesication, or for OPs, penetrates to the systemic circulation and inactivates ChEs. The most important process for the exposed soldier or civilian is to remove the chemical agent from the skin as quickly as possible. The soldier, under harsh conditions, must use the

product quickly to minimize transdermal penetration. A decontaminant that inactivates the chemical agent prevents its penetration through the skin and potentially protects a medical worker or buddy from suffering a second hand exposure.

Other criteria for the decontaminating system and reagents are that they are as universal as possible and protect against the various classes of chemical agents (as well as radiochemicals and biological agents, although the latter compounds will not be discussed). In other words, the soldier has a limited amount of space and weight to carry, and cannot carry multiple decontamination schemes. Furthermore, it is unlikely that a soldier would be able to determine the type of agent with which he is contaminated in the absence of symptoms.

In addition, proven efficacy of a decontamination product would have to meet Federal Drug Administration (FDA) guidelines and approval, assuring safety of the product for the soldier. The product should be environmentally safe to use by itself and render the chemical/biological agent environmentally safe, to prevent cross-contamination. Logistics would preclude decontamination products that require freezing or refrigeration, since they would not be available in the field. Lastly, the product needs to be simple and easy to use by the soldier under stressful conditions. Complicated decontamination procedures are unacceptable, since they increase the probability of failure during the stressful decontamination procedure. Products like the M291 kit (see below) are used by simply wiping the contaminated skin, and do not require significant training. However, the M291 kit has some drawbacks, such as a black offensive dust that is precluded from the eye. Thus, a decontaminating product should not have offensive odor (as some potential mercapto compounds exhibit) (Shi *et al.*, 2008) and be nonirritating and nonallergenic, or the product will be hesitantly used.

Methods for decontamination, neutralization, and removal of chemicals, such as OP and organosulfur compounds, herbicides and insecticides, are known in the literature (Hurst, 1977; Houston and Hendrickson, 2005; Rosenberg, 2005; Baker, 2004). The compositions and devices utilized for medical purposes are markedly different from nonmedical devices; the latter are not compatible with the skin or other sensitive tissues, having undesirable properties such as corrosiveness, flammability, toxicity, difficulty in making and storing, two component system composition, and limited shelf-life. For example, DS2, a standard decontamination agent, is comprised of 70% diethylenetriamine, 28% ethylene glycol monomethyl ether, and 2% NaOH by weight (Modec, 2003). Although DS2 is effective, it is corrosive upon exposure to air. DS2 and any matter resulting from its use are classified and regulated as hazardous material. After an application, the DS2 must stand for 30 min before rinsing the treated area with water. Additionally, DS2 comprises a teratogen. Clearly, this is not a better method for neutralizing, detoxifying, decontaminating, and cleaning personnel exposed to chemical warfare compounds.

VII. DECONTAMINATION SCHEMES

OP nerve agents are a serious threat to military and civilian personnel. Another serious problem that may be encountered while caring for personnel contaminated with OP nerve agents is the possibility that there will be cross-contamination to the medical personnel treating affected personnel. During combat or terrorist acts, individuals might be exposed to chemical toxins before they don their protective gear. Several schemes, each exhibiting their own advantages and disadvantages as medical decontaminants, are described below. While the ideal candidate does not exist, the final product must be fieldable for the individual – that is, for personal use in a rapid, deployable manner. Simple materials such as bleach and complex products such as OP-degrading immobilized enzymes sponges are described. Inexpensive and readily available household materials were tested for pesticide decontamination of fabric materials over 20 years ago (Easter and De Jonge, 1985). The household products provided only marginal decontamination efficacy.

A. Classical Liquid–Sodium Hypochlorite (Bleach)

Decontamination methods employing hypochlorite formulations have some corrosive and toxic side effects. A Chlorox® (hypochlorite) solution is composed of household bleach, which is about 5% sodium hypochlorite. Thus, for a 0.5% solution, bleach is mixed with 9 parts water, although even this diluted solution is contraindicated for use in or on a number of anatomical areas including the eye.

Undiluted bleach is not used because it is toxic to the skin and may create more damage than no decontaminant. Hairless guinea pigs were exposed to sulfur mustard in wounds and the surrounding intact skin, and then decontaminated with water, 0.5 or 2.5% bleach. No significant differences were observed among wounds decontaminated with the three solutions. Unexpectedly, the skin surrounding nondecontaminated (but exposed) control animals showed the least visual pathology. The lesions observed after decontamination might be due to the mechanical flushing of sulfur mustard onto the perilesional skin, by chemical damage of the skin induced by the solution, enhanced penetration of the agent, or interaction of sulfur mustard with the decontaminating solutions (Gold *et al.*, 1994). In a study evaluating decontamination of GB from skin, it was observed that rabbits receiving GB had no convulsions or deaths without decontamination. In contrast, when decontaminated with 5% bleach, there were increased symptoms and death, suggesting that 5% bleach perturbed the protective barrier of the skin or facilitated GB transport through the skin (Kondritzer *et al.*, 1959). Since diluted bleach

(0.5%) is a nonirritant to human skin, it is preferred (Racioppi *et al.*, 1994).

In contrast, the effectiveness of diluted bleach has been demonstrated. This study measured the rate of sulfur mustard disappearance from the skin after topical application of the vesicant, which rapidly penetrates the skin due to its hydrophobicity. Three swabbing treatments of undiluted HD-exposed skin with gauze pads soaked in 0.5% hypochlorite caused 68% reduction in skin HD content and a 64% reduction when hypochlorite was replaced by water (Wormser *et al.*, 2002). The effectiveness of 0.5% hypochlorite with water for decontaminating sulfur mustard on guinea pigs was also evaluated. However, the gauze pads soaked with the bleach contained microgram quantities of HD when water was used but no detectable HD levels when 0.5% bleach was used. Thus, the neutralizing effect of 0.5% bleach occurred after the agent was removed from the skin and away from the lipophilic structures of the skin where the 0.5% hypochlorite could react with and reduce levels of the agent.

Similar to bleach's oxidative iodine properties, topical povidone–iodine at 15 and 30 min post-exposure to sulfur mustard exhibited protective effects. Severity of the dermal parameters, acute inflammation and dermal necrosis was significantly reduced, and reduced skin damage was observed in areas adjacent to treated sites (Brodsky and Wormser, 2007).

B. Powder Decontamination Material: M291 Skin Decontamination Kit

The current individual product provided to the US soldier for use in the field is the M291 personal skin decontamination kit (Figure 71.1). There are three main components incorporated into individual pouches: a fiber pad (six to a pouch), an absorbent activated charcoal, and a reactive resin (Ambergard XE-555). Each component serves a unique purpose. First, the cotton pad provides structural integrity for use on a finger. Second, the carbon incorporated into the pad absorbs organic material such as OPs and HD. Third, the ion-exchange resin binds chemical agents and very slowly detoxifies them. The soldier takes the M291 pad and rubs the area that needs to be decontaminated. The goal is to rapidly bind chemical agents still on the surface of the skin and prevent their penetration through the stratum corneum. The M291 kit is precluded from use in the eye because its particulate nature is irritating; but can otherwise be used on the face and around wounds. Much of the black powder from the M291 kit remains on the skin, and has been a deterrent to its use. However, the black powder provides the soldier with an indication that the material still remains and should be brushed off for maximum effectiveness.

Efficacy of the M291 kit has been evaluated in a number of animal models for OP poisoning (DO49 Technical Report, 1987). In an early report, rabbit skin was shaved (to mimic human skin without fur) and then the skin exposed to

FIGURE 71.1. M291 kit (www.defenselink.mil).

GD and VX for 2 min. Decontamination with the M291 kit yielded higher LD_{50}s (of more than ten- and 20-fold, respectively) (Hobson *et al.*, 1985), in comparison to control (not decontaminated) animals. In another study, with rabbits under similar conditions, the penetration of the organophosphate VX was measured by red blood cell AChE inhibition. The M291 kit increased the amount of VX required to inhibit AChE by 50% (Joiner *et al.*, 1988). Thus, the M291 kit neutralized and/or removed VX so that less penetrated through the skin to be systemically delivered as indicated by RBC AChE inhibition. In another animal model, the guinea pig, decontamination with the M291 kit after 1 min of neat exposure to GD increased the LD_{50} from 9.9 to 17.7 mg/kg, yielding a protective ratio of 1.8. Some of the differences in protection can be related to the animal model (rabbit in comparison to the guinea pig as described above) and likely methods of decontamination (number of wipes with the M291 pad). The M291 kit is also efficacious in rabbits against the vesicating agents HD and L. Shaved dorsal skin of rabbits was exposed to neat HD and then decontaminated after 1 min. Vesicant-induced histopathology determined after tissue staining was reduced over 20-fold compared to nondecontaminated control rabbits.

C. Liquid Decontamination Material – Sandia Foam

Sandia National Laboratories decontaminating foam (licensed to Modec Inc., Denver, Colorado) is a solution (MDF-100) composed of two parts: part a: a solution of 6.6% *N*,*N*,*N*,*N*′′,*N*′-penta-methyl,-*N*′-tallow alkyl 1,3-propanamine diammonium; 2.6% tallow pentamethyl propane quaternary ammonium compounds; benzyl-C12-18 alkyl dimethyl; 1% isopropyl alcohol, and part b: a solution of 8% hydrogen peroxide. Mixing the two parts results in a foam-like product which lasts for up to 30 min (Figure 71.2).

The mixture was tested in the guinea pig model, where fur was shaved on the side of the animal one day before exposing the skin of the anesthetized animals to neat GD or VX (Lukey *et al.*, 2004). The animals were decontaminated 1 min later with sterile gauze soaked in the combined solution in a defined manner: the contaminated side was wiped across the exposure site in the direction of the shaved fur once and then rotated so that a clean surface of the gauze could be used to wipe the skin for three additional passes. Next, the OP-exposed area was similarly dried with a second piece of gauze. The exposed area was wiped a total of eight times. Twenty-four hours later, survival was determined. Control non-decontaminated animals yielded an LD_{50} of 11.3 mg/kg for the OP GD, while animals decontaminated with the mixture yielded an LD_{50} of 400 mg/kg, a 35-fold protective ratio. For the OP VX, cutaneous neat exposure and decontamination with Sandia foam yielded an LD_{50} of 10.1 mg/kg compared to the control animals' LD_{50} of 0.14 mg/kg, a 72-fold protective ratio.

FIGURE 71.2. Sandia foam.

Despite its efficacy, Sandia foam has a number of drawbacks for field use by the soldier. First, it must be stored as separate components, which would require a rapid, personal, on-site mixing chamber for combining the two solutions. Second, the presence of hydrogen peroxide, a strong oxidizing agent, precludes its use near the eye, and would create much discomfort if used in a wound (Watt *et al.*, 2004). To partially address these concerns, Sandia developed the formulation DF-200, which in part contains less hydrogen peroxide and surfactant.

D. Liquid Decontamination Material – Diphotérine®

Diphotérine is a product for chemical spatters on the eye and skin. Prevor Laboratory in France manufactures this odorless, colorless liquid dispensed as an eye wash or skin decontamination spray. It is composed of an aqueous solution to wash many chemical families and pull hydrophilic chemical agents away from the surface of tissues, an amphoteric solution that acts on acids and bases and restores the tissue physiological pH, and a hypertonic solution that stops penetration of corrosive chemicals into tissues. The pH is slightly alkaline (pH 7.2–7.7) and is sterile. Although not classified as such in the USA, it is classified as a medical device in Europe, Canada, Australia, and Brazil (www.prevor.com).

Diphotérine's action on more than 600 chemical compounds was reviewed (Hall *et al.*, 2002). These chemicals included acids, alkalis, oxidizing and reducing agents, irritants, lacrimators, solvents, alkylating agents, and radionuclides. In the literature, there is one abstract describing the decontamination of sulfur mustard (Gerasimo *et al.*, 2000). In this report, radiolabeled sulfur mustard was placed on human skin for 5 min *in vitro*. The skin was then treated with Diphotérine, water and soap, or saline at different time periods after sulfur mustard exposure and Diphotérine was reported to be significantly better at removing sulfur mustard. No reports could be found in the literature for organophosphate decontamination, although Hall *et al.* (2002) also state that Diphotérine is suitable for decontamination of OP pesticides.

The bulk of evaluation of Diphotérine has occurred in the European workplace. There, it has been reported not to be irritating to normal human eyes or skin, and is essentially

nontoxic in guinea pigs and does not sensitize their skin (Mathieu *et al.*, 2007). The product has prevented or decreased severity of chemical eye/skin burns with 96% sulfuric acid, 100% acrylic acid, 50% acrylamide, solid sodium hydroxide flakes, and dimethylethylamine; no eye/skin burns developed and there was no necessity for further medical or surgical burn treatment in a German metallurgy facility where 24 workers were exposed to weak or strong acids and bases and obtained immediate Diphotérine decontamination (Nehles *et al.*, 2006). Clearly, Diphotérine's potential is intriguing and needs to be critically evaluated for decontamination and detoxification of chemical warfare agents.

E. Liquid and Sponges: Reactive Skin Decontamination Lotion

Reactive skin decontamination lotion (RSDL) was developed for cutaneous decontamination of chemical warfare agents after exposure to a chemical warfare agent (Figure 71.3). It was developed for topical use by the Defense Research Establishment in Suffield, Canada, with broad spectrum decontamination properties for chemical agent cutaneous threats. The RSDL solution is composed of 1.25 M potassium 2,3-butanedione monoximate in polyethylene glycol monoethyl ethers of average molecular weight 550 daltons ($MPEG_{550}$) with 10% w/w water (pH 10.6). The pads consist of a sponge-like plastic foam, Opcell®, which is lightweight and easy to store. It is used instead of a cotton pad, and the Opcell holds more of the decontaminating solution for spreading on the skin.

The toxicological profile of this formulation was determined prior to FDA (Food and Drug Administration) clearance by the US military (Tonucci *et al.*, 2004). The Army tested the product's safety by conducting skin irritation, sensitization, and photoirritation studies in more than 300 people. It also tested its effectiveness by treating animals that had been exposed to chemical agents. On March 28, 2003, the product was approved by the FDA to remove or neutralize chemical warfare agents and T-2 fungal toxin from the skin (but not other biological threat agents or radiological contaminants).

The efficacy of reactive skin decontamination lotion has been demonstrated. The high efficacy of this lotion in inactivating OPs was measured in rats. In addition, primary cultures of chick embryo neurons were used to test the efficacy of the RSDL. By relating the anticholinesterase activity in these cultures of the OP/RSDL mixture to that of pure OP standards, a sensitive measure of the value of the RSD in inactivating tabun, sarin, soman, and VX was obtained. Experiments with all four nerve agents in this *in vitro* system provided a good correlation with the *in vivo* data, and also indicated that the inactivation process was time and agent dependent and also related to the ratio of OP to RSDL. RSDL is also effective in decontaminating GD and VX cutaneously in exposed guinea pigs (Table 2, Gordon, unpublished observations).

FIGURE 71.3. RSDL reactive skin decontamination lotion (www.nbcdefence.net).

The product was also compared to Fuller's earth in a pig model. The potency of the RSDL/sponge was statistically better than Fuller's earth against skin injury induced by sulfur mustard, observed 3 days post-exposure. RSDL was more efficient than Fuller's earth in reducing the formation of perinuclear vacuoles and inflammation processes in the epidermis and dermis. The potencies of the RSDL/sponge and Fuller's earth were similar to severe inhibition of plasma cholinesterases induced by VX poisoning. Both systems completely prevented cholinesterase inhibition, which indirectly indicates a prevention of toxic absorption through the skin (Taysse *et al.*, 2007).

However, there are some caveats for the use of RSDL. The application of RSDL directly to open wounds impaired wound strength and decreased collagen content in the early phases of wound healing. This may have clinical implications for the treatment and outcomes of chemical casualty combat trauma (Walters *et al.*, 2007). RSDL is also reported to be flammable.

F. Polyurethane Sponge

At the Walter Reed Army Institute of Research, an enzyme immobilized polyurethane foam sponge to decontaminate the skin of chemical warfare agents in a wide variety of

environmental conditions is being developed (Munnecke, 1979; Wood *et al*., 1982; Havens and Rase, 1993) (Figure 71.4). A porous polyurethane foam formed *in situ* from water-miscible hydrophilic urethane prepolymers has been combined with enzymes such as ChEs, producing immobilized enzyme sponges (Gordon *et al.*, 1999; Gordon and Doctor, 2003; Ember, 1997; Medlin, 1998). In this method, the enzyme becomes an integral part of the solid support. Some of the advantages of this technique include retention of similar kinetic characteristics as the soluble form of the enzyme. Most important, the immobilized enzyme retains high activity after prolonged storage, and it is resistant to the detrimental effects of low and high temperatures and long exposure to the environment. In addition, the enzymes are covalently attached to the polyurethane so they will not leach from this polymer support.

In order to increase the OP/enzyme stoichiometry, polyurethane immobilized enzymes were combined with oximes (enzyme reactivators such as HI-6; Peter *et al.*, 2007) so that the catalytic activity of OP-inhibited AChE (or BChE) is rapidly and continuously restored before irreversible aging of the enzyme–OP complex can occur. The OP is continuously detoxified. Thus, a reusable immobilized enzyme sponge of ChEs and oximes for OP decontamination is the envisioned product. Next, it was demonstrated that the OPs diisopropyl fluorophosphate (DPF) or 7-(methyl-ethoxyphosphinyloxy)-1-methylquinolinium iodide (MEPQ) inhibited the activity of ChE sponges, as was observed for non-immobilized ChE in solution. The oxime HI-6 restored activity of the AChE sponge until the molar concentration of MEPQ reached approximately 1,000 times that of the cholinesterase active site. However, the AChE sponge could be recycled many times by rinsing the sponge with HI-6 in the absence of OP. In this case, most of the original ChE activity could then be restored to the sponge. Therefore, the bioscavenger approach can be used externally: the sponge would soak up OP decontaminating the OP contaminated skin (Caranto *et al.*, 1994). Then the ChE sponge and oxime would detoxify the OP in the sponge. We have found that the ability of the immobilized enzymes and HI-6 to detoxify the OP MEPQ was dependent upon the efficiency of the sponge in decontaminating particular surfaces including plastic or guinea pig skin.

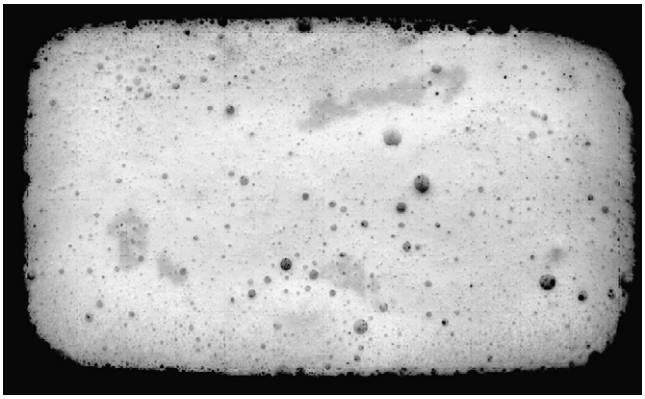

FIGURE 71.4. Polyurethane sponge.

Characteristics of polyurethane immobilized enzymes are as follows. The longevity of sponges composed of immobilized ChEs is greater than 5 years at room temperature (not shown). The immobilized enzymes are also very stable in aqueous environments. One significant difference and advantage the immobilized enzymes have compared to the soluble ChEs is that immobilized enzymes do not dissociate (leach) from the sponge. Therefore, the immobilized enzymes can be left in the liquid or other environments. For instance, the AChE activity in the immobilized sponge was stable for more than 60 days in continuous immersion in aqueous samples including Allegheny River fresh water or brackish water (Gordon *et al.*, 2002). Since the results were identical for autoclaved and untreated water, the immobilized enzymes were also resistant to microbial-induced proteolytic degradation. Also note that the same sponge was assayed multiple times over many days, so it is evident that the immobilization process confers dramatic stability to covalently coupled ChEs.

OP removal by the sponge and formulation is as follows. The capacity of sponges to remove GD or other OPs from guinea pig skin was determined using a back-titration method, where removed OP in the sponge was added to a known amount of ChE. Inhibition of the exogenously added ChE permits quantitation of the OP concentration. From these studies, it was determined that additional components when added to the sponge improved the sponge's efficacy by leaching out the chemical warfare agents. In this case, we settled on tetraglyme, which has a propensity to dissolve organic-like materials, including OPs and sulfur mustard (see below). In part, the inability of sponge–tetraglyme to remove all the GD likely reflects the rapid penetration of GD through skin and that tetraglyme cannot extract this fraction. It also points to the requirement for as rapid decontamination as possible. These results clearly demonstrate that the sponge not only removed OP from the skin of the guinea pigs, but in the presence of oxime effectively and completely detoxified the OPs within hours. Thus, these sponges would not pose any additional cross-contamination hazard.

The protective ratios of the sponge are as follows. It proved to be impossible to modify the prepolymer since currently there is no formulation with an increased hydrophobic nature that might be expected to absorb the OP more effectively. Instead, we utilized additives described above to provide the additional ability to remove soman from the skin, protecting guinea pigs significantly better than the M291 kit (Table 71.2). A comparison with RSDL is also shown. Compared to LD_{50} values of 9.9 and 17.7 mg/kg for untreated animals (not decontaminated) and the M291 kit, respectively, the sponge provided an LD_{50} of 290. This combination is also effective against VX contaminated guinea pigs: the sponge increased the LD_{50} from 0.14 mg/kg to 21, yielding a protective ratio of 150. RSDL provided a protective ratio of 137.

TABLE 71.2. Decontamination of OP-exposed guinea pigs

OP/treatment	LD_{50} (mg/kg)	PR
Soman		
M291 kit	17.7	1.8
RSDL	240	24
Sponge	290	29
None	9.9	n/a
VX		
M291 kit	0.14	n/a
RSDL	19.1	137
Sponge	21.0	150
None	0.14	n/a

PR – protective ratio

Sulfur mustard decontamination and formulation are as follows. The sponge was used to wipe guinea pig skin contaminated with neat sulfur mustard. The following day, the animals were injected with trypan blue. Those areas representing vesicant injury take up the dye. It was observed that the neat HD-exposed tissue (positive control) has a significant dye uptake, while the area decontaminated by the sponge has only a slight uptake of the dye. The control has no dye uptake. In addition, the amount of HD taken up and removed by the sponge was measured over time. While neat mustard remained after 15 min in water, the corresponding amounts are destroyed in the matrix of the sponge and additives. Histopathology of the HD-exposed skin specimens after 24 h demonstrated microvesicles, coagulation at the dermal interface, and in the most severe cases, dermal coagulation. Overall, sponge decontamination of the HD-exposed area exhibited characteristics associated with reduced exposure (microvesicles). Thus, these sponges could reduce the damage that HD produced. ChEs may provide sinks as alkylation sites for the sulfur mustard, and account for some of the reduced toxicity of the agent upon sponge decontamination. Another feature demonstrated was that the tetraglyme leaches from skin not only OPs, but also the organic-like sulfur mustard, thereby reducing its ability to alkylate skin proteins. Finally, the formulation of the sponge was modified to include nucleophilic additives to act as a reactive moiety for the sulfur mustard in place of skin proteins. Taken together, the polyurethane sponge was shown to decontaminate and detoxify guinea pig skin exposed to two classes of chemical warfare agents: OPs such as GD and alkylating compounds such as HD.

G. Immobilized Enzyme Badges

The sponge can incorporate a detection system for OPs and alkylating agents – a unique attribute not present in current decontamination methods (Gordon *et al.*, 2002). The immobilized enzymes provide a detector and a rapid field system capable of identifying the type of OP. In addition to OP detection, a coupled enzyme reaction provides a rapid colorimetric or electrochemical indication of mustard. With the constant threat of chemical warfare or terrorist acts, the development of alternative means of protecting and decontaminating individuals from exposure to CWA agents is critical.

Like Diphotérine, which has been evaluated only for sulfur mustard decontamination, the polyurethane enzyme immobilized sponge is a novel technology that has demonstrated efficacy for OP and sulfur mustard cutaneous poisoning, but is only now undergoing evaluation for decontamination of biological warfare agents (T-2 mycotoxin, botulinum toxin) and radionucleotides (Gordon *et al.*, 2006). Another advantage of ChEs over general reacting additives is that the ChEs are the direct target for current or future warfare agents, and therefore should not require major reformulation. These new technologies likely will provide more efficacious solutions for the soldier in the future.

VIII. CONCLUDING REMARKS AND FUTURE DIRECTION

OP nerve agents are a serious threat to military and civilian personnel. These agents are some of the most potent toxic agents and are specific inhibitors of ChEs. OP nerve agents can exist as a vapor and be inhaled; as a liquid they can contaminate skin, or can be ingested if food or water is contaminated. Vesicating agents such as sulfur mustard cause irreversible cell damage as a result of rapid alkylation, and were an agent of terror during World War I and more recently in Iran and Iraq. Another serious problem that may be encountered while caring for personnel contaminated with chemical warfare agents is the possibility that there will be cross-contamination to the medical personnel. In addition, during combat or terrorist acts, individuals might be exposed to chemical toxins before they don their protective gear. Decontamination post-exposure has the potential to be an important, integral, and therefore necessary step for medical countermeasures against chemical warfare agents. The products described here must meet several criteria to be effective personal decontaminants and detoxifiers of chemical warfare agents for the soldier, although there is room for improvement, thus novel technologies have been discussed. However, any product must be lightweight for individual use, yet be shelf-stable under environmental conditions found in the field. The importance for readily available and rapid use of a decontamination and detoxification product is due to the rapid damage caused by CWAs – OPs penetrate skin in less than 5 min and mustard produces irreversible cell damage as a result of alkylation equally rapidly. The product should also be environmentally friendly. In the future, one product should incorporate chemical, biological, and radiological decontamination and,

when possible, detoxification. With the constant threat of chemical warfare, terrorist acts, or spillage of pesticides, the development of alternative means of protecting and decontaminating individuals from exposure is critical.

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CHAPTER 72

Detoxification of Arsenic

KIRAN KALIA AND DHAVAL N. JOSHI

I. INTRODUCTION

Arsenic, which is ubiquitous in the environment, has become a worldwide public health problem. Every organism, from bacteria to mammals, has a mechanism for transport and detoxification of arsenicals (Rosen, 2002a). It has become evident that increasing use of arsenic-containing insecticides, herbicides, fungicides, pesticides and wood preservatives, and through the mining and burning of coal, have modified the global cycle of arsenic (Luo *et al.*, 2007; Smedley and Kinniburgh, 2002). Arsenic (As) ranks first on the US government's Priority List of Hazardous Substances, because of both its toxicity and its prevalence in the environment (Chou and De Rosa, 2003). The health of millions of people worldwide is at risk from drinking arsenic-contaminated well water (Milton and Rahman, 2002). Increased arsenic mobility in natural environments is a major concern in the creation of new wells and water supply systems in areas that are rich in arsenic. A large-scale shift in water resource allocation from surface water to groundwater in West Bengal, India, and Bangladesh has resulted in very extreme environmental health effects (Mukherjee *et al.*, 2006). The toxic effects of arsenic are related to its oxidation state (Thomas *et al.*, 2007), therefore, the examination of factors affecting the speciation of arsenic has become an important issue.

The threat of environmental pollution from the release and dispersal of naturally occurring and anthropogenic arsenic has stimulated extensive research on the properties of the metalloid and its interaction with biological systems. Arsenic is lethal to most microorganisms, yet certain bacteria and phytoplankton are known to survive arsenic exposure. Microorganisms have evolved a number of mechanisms to tolerate high levels of arsenic in their environment, including transformation of arsenic species by oxidation (Fisher *et al.*, 2008), reduction (Mukhopadhyay *et al.*, 2002), and methylation (Bentley and Chasteen, 2002). Arsenic detoxification has been reported in a number of bacterial species such as *Escherichia coli*, *Bacillus* sp., *Staphylococcus aureus*, *Staphylococcus xylosis, Chromobacterium violaceum* and *Pseudomonas* sp. (Patel *et al.*, 2007; Mateos *et al.*, 2006; Rosen, 2002a) which select variants possessing genetic resistance determinants which confer the ability to tolerate the higher levels of arsenic in their environment. It has been well documented that bacteria can use arsenic as electron donors, electron acceptors, or possess arsenic detoxification mechanisms to confer resistance against arsenic toxicity. In general, metals in contaminated water are removed by methods such as chemical precipitation, ion exchange, or adsorption (Schmidt *et al.*, 2008). However, these methods have disadvantages such as high cost and the generation of secondary contaminants. Recent recognition of the need to develop low-cost environmentally friendly technologies for water treatment has stimulated interest in studies in the bioremediation of metals (Mondal *et al.*, 2008; Patel *et al.*, 2007; Mateos *et al.*, 2006). The bacterial cells capable of removing arsenic from their surroundings could be ideal candidates for bioremediation, and could be used as an alternative or to supplement existing physicochemical methods of arsenic removal. Although some genetically engineered microorganisms have been developed (Kostal *et al.*, 2004) their potential use for the removal of arsenic is not yet established (Takeuchi *et al.*, 2007).

II. ARSENIC IN THE ENVIRONMENT

Arsenic enters to biosphere primarily by leaching from geological formations. Its ubiquity in the environment has led to the evolution of arsenic defense mechanisms in every organism studied, from *Escherichia coli* to humans. Arsenic ranks 20th in natural abundance, consisting about 0.00005% of the earth's crust, 14th in seawater, and 12th in the human body (Mandal and Suzuki, 2002). Arsenic is positioned in Group 15 in the Periodic Table below phosphorus, and is a silver–gray crystalline metallic material that melts at 817°C, sublimates at 613°C, and has a density of 5.72 g/cm^3 (Eisler, 1994). Due to its complex chemistry and ability to form many different compounds arsenic is an intricate element to understand. Arsenic is most commonly found in two valence states, As(III) and As(V). The most common inorganic As(III) compounds found are arsenic trioxide, sodium arsenate, and arsenic trichloride. As(V) compounds such as arsenic pentoxide, arsenic acid, and arsenates are also quite common. Arsenic is methylated by microorganisms,

but neither group is considered as toxic as inorganic As(III) and As(V) compounds (Islam *et al.*, 2005).

Arsenic in the atmosphere comes from various natural sources such as volcanoes (gas fumes), microorganisms, and human release (mainly by burning fossil fuels) (Figure 72.1). Under anoxic conditions, arsenite can be reduced by microorganisms in soil to the volatile compounds arsine (AsH_3) and methyl arsines, these compounds being the most toxic form of arsenic. In addition, the anthropogenic sources have broadened the arsenic cycle and large amounts of arsenic are found in the environment and in living organisms. About 90% of arsenic compounds are used as wood preservatives and the remaining 10% as insecticides, anthropogenic fertilizers, weedkillers, fungicides, semiconductors, and in the production of glass and metal alloys. In most cases, arsenic occurs naturally within subsurface aquifers. This mobilization of arsenic into the aqueous phase serves as the first crucial step in eventual human arsenicosis (Oremland and Stolz, 2005). Worldwide, arsenic in soil ranges from 0.1 to 40 mg/kg[1] with a median concentration of 5–6 mg/kg[1] (Smedley and Kinniburgh, 2002). Arsenic in seawater averages 1.7 µg/l with a relatively homogeneous range from 1.5 to 5 µg/l. In contrast, freshwater from lakes and rivers varies widely in arsenic concentration and is dependent upon the minerals subject to transport. Arsenate is more predominant in oxygenated water while arsenite is more common under reduced anaerobic conditions. The total arsenic influx into oceans is estimated at 246,110 metric tons/year. Of this total 62,900 metric tons is dissolved arsenic, 178,900 metric tons is sediment suspended arsenic, and 4,310 metric tons is from the atmosphere per year (Smedley and Kinniburgh, 2002).

Arsenic has been detected in groundwater in several countries of the world, with concentration levels exceeding the World Health Organization (WHO) drinking water guideline value of 10 µg/l as well as the national regulatory standards of 50 µg/l in India and Bangladesh (Mukherjee *et al.*, 2006). In groundwater it is often associated with geological sources, but in some locations anthropogenic inputs can be extremely important. Arsenic in groundwater has emerged as the largest environmental health disaster putting at least 100 million people at risk of cancer and other arsenic-related diseases. In Asia, the impact of arsenic toxicity is particularly alarming predominantly in the Bengal Basin of Bangladesh and West Bengal, India. Recent studies indicate the occurrence of geogenic arsenic in the Central Gangatic Plains of Uttar Pradesh, Bihar, Jharkhand and the Brahmaputra valley in Assam, and several regions of Madhya Pradesh and Chattisgarh, India (Mukherjee *et al.*, 2006).

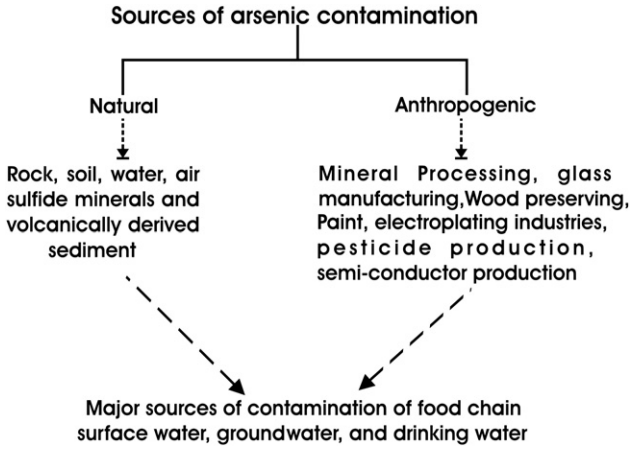

FIGURE 72.1. Arsenic contamination in the environment through natural and anthropogenic sources.

III. BIOLOGICAL PROPERTIES OF ARSENIC

Arsenic occurs in four oxidation states: As^{5+}, As^{3+}, As^{0}, and As^{3-}. The two highest oxidation states are the most common biologically important redox states, which are arsenite [As (III)], the reduced form and the oxidized form, arsenate [As (V)], whereas the other two are rare. The ionic forms, arsenate and arsenite, are interconvertible and pH dependent, hence at physiological pH, As(V) is dominant as $H_2AsO_4^{-1}$ $(2.5 < pH < 7)$ and $HAsO_4^{-2}$ $(7 < pH < 12)$, while As(III) is dominant as H_3AsO_3 $(pH < 9.3)$ (Inskeep *et al.*, 2002). Arsenic also exists in organic forms, which are often found in association with biological systems. These include the methylated arsenicals such as monomethylarsonate (MMA) and dimethylarsonate (DMA), which are common arsenic metabolites found in most environmental compartments, and other arsenicals such as arsenobetaine, arsenocholine, and tetramethylarsonium ion, which are common species in marine animals (Geiszinger *et al.*, 2002). In the natural environment, both arsenate and arsenite are subjected to chemically and microbiologically mediate oxidation, reduction, and methylation reactions. The oxyanion form as pentavalent arsenate (As^{5+}) is an analog of phosphate and is a potent inhibitor of oxidative phosphorylation, the key reaction of energy metabolism in metazoans, including humans, the most toxic form to algae (Knauer *et al.*, 1999). The arsenite has strong affinity to form bonds with functional groups such as the thiolates of cysteine residues and the imidazolium nitrogens of histidine, thus it binds readily to many enzymes, including those involved in respiration (Knauer *et al.*, 1999). In solution at neutral pH, arsenic acid exists as the arsenate anion. The pKa of arsenous acid is 9.2, so that, at neutral pH, arsenic would be present in solution as neutral $As(OH)_3$ (Rosen, 2002a). In natural waters, arsenic is mostly found in inorganic form as oxyanions of trivalent arsenite (As^{3+}), which is the most toxic form to humans and other vertebrates. In soils, the most often encountered arsenic forms are inorganic As(III) and As(V) (Cullen and Reimer, 1989).

IV. MICROBIAL TRANSFORMATIONS OF ARSENIC

The ecology of arsenic is strongly dependent on microbial transformations which affect the mobility and bioavailability as well as the toxicity of arsenic in the environment. Bacteria implicated in the processes of biotransformation readily metabolize arsenic, participating in various metabolic functions including detoxification, anaerobic respiration, assimilation, and methylation (Figure 72.2). Arsenic speciation and mobility are affected by microbes through oxidation/reduction reactions as part of resistance and respiratory processes. The *arr* and *ars* encode two different arsenate reductases associated with cellular respiration and the detoxification mechanism, respectively, whereas *aox* and *aso* coding for arsenite oxidase is responsible for its oxidation to gain energy. Respiratory arsenate reductases encoded by *arr*, arsenic methyltransferases encoded by *arsM* and some new components in arsenic resistance have been recently described. To date, the best studied mechanism of transformation is the reduction of arsenate As(V) to arsenite As(III) mediated by *ars* operon. On the other hand, the physiology, enzymology, and genetics of the oxidation of arsenite as well as the underlying regulatory processes remain largely unknown at present.

A. Dissimilatory Reduction of Arsenate

Microbial arsenate respiration contributes to the mobilization of arsenic from the solid to the soluble phase in various locales worldwide. In particular, microbial respiratory reduction of arsenate, As(V), to arsenite, As(III), is thought to prevent the readsorption of arsenic onto sediment surfaces once it is transported away from iron-rich environments (Ahmann *et al.*, 1994). Dissimilatory arsenate-respiring prokaryotes including archea and bacteria capable of using arsenate as the terminal electron acceptor in anaerobic respiration of arsenate could provide enough energy for microbial growth (Ahmann *et al.*, 1994; Laverman *et al.*, 1995). Arsenate reduction may be an important process in an environment where arsenic concentrations are too high. Arsenate reduction by bacteria can significantly enhance the arsenic mobility in the environment. Two different arsenate reduction pathways exist in microorganisms encoded by the *ars* and *arr* systems, known to specifically reduce As(V) (Figure 72.3). The arsenate reductase encoded by the *arr* genes is associated with cellular respiration, whereas *ars* genes encode arsenate reductase associated with detoxification mechanisms (Saltikov *et al.*, 2005). The *arr* system is required for As(V) reduction under environmentally relevant conditions and is highly conserved among diverse bacterial species. Respiratory arsenate reductase (Arr) has been purified from anaerobic organism *Chrysiogenes arsenatis* (Krafft and Macy, 1998) and *Bacillus selenitireducens* strain MLS10 (Afkar *et al.*, 2003). It has been reported that an enzyme from *Bacillus selenitireducens* (Afkar *et al.*, 2003) was membrane associated, whereas an enzyme from *Chrysiogenes arsenatis* was found in periplasm and is different from the nonrespiratory arsenate reductases of *Escherichia coli* and *Staphylococcus aureus* (Krafft and Macy, 1998).

Recently, the respiratory arsenate reductase was characterized from *Shewanella* sp. strain ANA-3 (Malasarn *et al.*, 2008), which was initially identified in the same strain

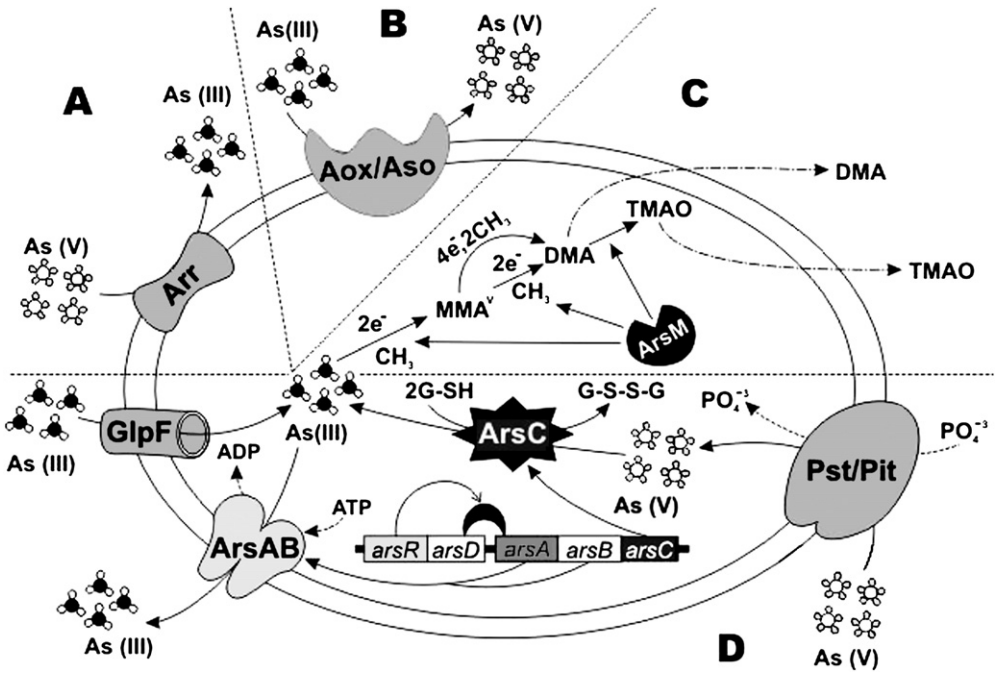

FIGURE 72.2. Arsenic detoxification mechanisms (reduction, oxidation, methylation, and resistance) in prokaryotes. (A) Respiratory arsenate reductase (Arr) is involved in the reduction of As(V) by the dissimilatory arsenate respiring organisms. (B) Arsenite oxidase (Aox/Aso) is responsible for oxidation of As(III) by chemoautotrophic or heterotrophic arsenite oxidizers. (C) *S*-adenosylmethyltransferase (ArsM) is responsible for methylation of As(III) to produce trimethylarsine as the end product. (D) Genes (*arsRDABC*) and proteins involved in the uptake of As(III) and As(V) (GlpF and Pst/Pit transporter), reduction of As(V) (ArsC), extrusion of As(III) (ArsAB), and regulation (ArsRD) by arsenic resistant organisms.

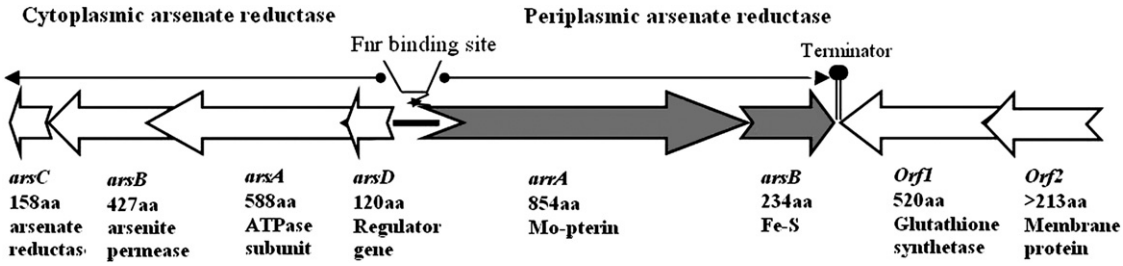

FIGURE 72.3. Molecular organization of *arrAB* (periplasmic arsenate reductase) and *arsDABC* (cytoplasmic arsenate reductase) of *Shewanella* strain ANA-3 (from GenBank Accession no. AY271310). *arrAB* gene cluster is located upstream in the opposite orientation to an *arsDABC*. aa–amino acid (modified from Saltikov and Newman, 2003; Silver and Phung, 2005).

through mutagenesis studies (Saltikov and Newman, 2003). The *Shewanella* sp. contains two systems for reducing As(V), one is similar to the well-conserved *ars* detoxification system of *Escherichia coli* plasmid R773, which is advantageous, but not required for respiratory As(V) reduction mediated by *arr* operon. The *arr* operon in *Shewanella* sp. lies immediately downstream of the *ars* operon and contains only two genes, *arrA* and *arrB*. Sequence analysis and biochemical studies of respiratory arsenate reductase from various sources revealed that it is a heterodimer $\alpha_1\beta_1$ with a native molecular mass of ≈123–131 kDa and consists of larger subunits with molecular masses of ArrA ≈ 87–95 kDa, molybdenum-containing enzyme and smaller subunit ArsB ≈ 27–26 kDa, containing several Fe–S clusters (Krafft and Macy, 1998, Malasarn *et al.*, 2008 Afkar *et al.*, 2003; Sargent *et al.*, 2002). The N-terminus of ArrA was similar to a number of prokaryotic molybdenum-containing polypeptides (e.g. the formate dehydrogenases H and N of *Escherichia coli*) and the N-terminus of ArrB is similar to iron–sulfur proteins (Krafft and Macy, 1998). Both the proteins belong to the enzymes of the dimethyl sulfoxide reductase family (Krafft and Macy, 1998; Afkar *et al.*, 2003). It has been reported that ArrA could be the subunit that binds As(V) and reduces it to As(III), whereas ArrB serves as a channel for electrons stemming from c-type cytochromes in the respiratory chain (Macy *et al.*, 2000). Because the *arrA* is well conserved among almost all of the isolated bacterial As(V) respires, several research groups have used a set of degenerate PCR primers to amplify and sequence a major portion of *arrA* from other bacterial species including *Bacillus arseniciselenatis*, *Chrysiogenes arsenatis*, and the *Desulfoporosinus* sp. strain Y5 (Malasarn *et al.*, 2004; Perez-Jimenez *et al.*, 2005). The expression dynamics of As(V) respiration and detoxification showed that at very low concentrations of arsenate and under anaerobic conditions the *Shweanella* sp. strain ANA-3 was primed to activate *arr* transcription and during the early phases of growth couples the reduction of As(V) to growth instead of detoxification by *arsC*, whereas increased intracellular As(III) concentration initiates production of its detoxification machinery by turning on the transcription of *ars* operon (Saltikov *et al.*, 2005). Recently, respiratory arsenate reductase has been identified from *Alkaliphilus oremlandii* through biochemical and Western blot analysis and confirmed by cloning and sequencing of the gene encoded for the structural subunit ArrA (Fisher *et al.*, 2008). It has been suggested through genomic analysis and growth experiments that *Alkaliphilus oremlandii* couples the reduction of the nitro group of the organoarsenical to the oxidation of either lactate or fructose in a dissimilatory manner, generating ATP via a sodium-dependent ATP synthase (Fisher *et al.*, 2008).

Gram-negative bacterial strains MIT-13 and SES-3 were capable of obtaining energy from arsenate respiration (Ahmann *et al.*, 1994; Laverman *et al.*, 1995) and classified as two species of *Sufurospirillum* based on 16S rRNA sequence analysis and DNA–DNA hybridization, designated as *Sufurospirillum arsenophilum* and *Sufurospirillum barnesii* with 97% sequence homology with each other and differing slightly in metabolism (Stolz *et al.*, 1999). Both the strains were able to grow using arsenate, nitrate, thiosulfate, or sulfur as the terminal electron acceptor in anaerobic respiration and micro-aerobically with low levels of oxygen (Stolz *et al.*, 1999; Laverman *et al.*, 1995). The *Desulfomaculum auripigmentum*, a sulfate-reducing organism capable of respiring lactate when using either arsenate or sulfate as the terminal electron acceptor, was isolated and characterized, which was phylogenetically affiliated to MIT-13 and SES-3 (Newman *et al.*, 1997). Gram-positive *Bacillus arsenicoselenatis* and *Bacillus selenitireducens*, with the low G + C content isolated from an alkaline, arsenic-rich, saline lake, which can specifically recognize arsenate as electron acceptor, showed a novel mechanism of arsenate reduction (Switzer Blum *et al.*, 1998). Dissimilatory arsenate-respiring prokaryotes characterized at the molecular level are shown in Table 72.1. *Pyrobaculum arsenaticum* is capable of respiratory growth using arsenate, sulfur, or selenate (Huber *et al.*, 2000). 16S rRNA gene sequence analysis showed that this organism belongs to the *Thermoproteales* order of archea, which can also grow anaerobically in the presence of arsenate or selenate (Huber *et al.*, 2000). The strain GBFH of *Desulfitobacterium* and strain HR13 of *Deincoccus* have been isolated from arsenic-contaminated sites (Niggemyer *et al.*, 2001; Gihring and

TABLE 72.1. Dissimilatory arsenate-respiring prokaryotes characterized at molecular level

Ac. No.	Organisms	Opero/Gene	Reference
AY271310	*Shewanella* sp. ANA-3	*arrAB, arsDABC*	Saltikov and Newman (2003)
AY660883X81319	*Chrysiogenes arsenatis* strain BAL-1	*arrA*16S rRNA	Krafft and Macy (1998); Macy *et al.* (1996)
AJ307028	*Desulfitobacterium* sp. strain GBFH	16S rRNA	Niggemyer *et al.* (2001)
DQ220794	*Desulfoporosinus* sp. strain Y5	*arrA*	Perez-Jimenez *et al.* (2005)
U40078	*Desulfitobacterium frappieri*	16S rRNA	Niggemyer *et al.* (2001)
X94975	*Desulfitobacterium hafniense*	16S rRNA	Niggemyer *et al.* (2001)
AF131233	*Desulfomicrobium* sp. str. Ben-RB	16S rRNA	Macy *et al.* (2000)
U85624	*Desulfosporosinus auripigmenti*	16S rRNA	Newman *et al.* (1997)
DQ250645	*Alkaliphilus oremlandii* OhILAs	16S rRNA	Stolz *et al.* (2007)
U85964	*Sufurospirillum arsenophilum* MIT-13	16S rRNA	Ahmann *et al.* (1994)
AY660884U41564	*Sufurospirillum barnesii* SES-3	*arrA*16S rRNA	Malasarn *et al.* (2004); Stolz *et al.* (1999)
AY032601	*Bacillus* sp. str. JMM-4	16SrRNA	Santini *et al.* (2004)
AF064705	*Bacillus arsenicoselenatis* str. E1H	16SrRNA	Switzer Blum *et al.* (1998)
AY283639AF064704	*Bacillus selenitireducens* strain MLS10	*arrA*16SrRNA	Afkar *et al.* (2003); Switzer Blum *et al.* (1998)
L07510	*Pyrobaculum aerophilum* str. IM2	16SrRNA	Huber *et al.* (2000)
AJ277124	*Pyrobaculum arsenaticum* str. PZ6	16SrRNA	Huber *et al.* (2000)
AF384168	*Thermus* sp. str. HR13	16SrRNA	Gihring and Banfield (2001)
AF463533	*Citrobacter* sp. str. TSA-1	16SrRNA	Herbel *et al.* (2002)
AF463534	*Wolinella* sp. str. BSA-1	16SrRNA	Herbel *et al.* (2002)

Banfield, 2001), having the ability to grow through dissimilatory reduction. Indeed, evidence is mounting that dissimilatory arsenate reduction may be an important process in an environment where arsenic concentrations are too high. It is suggested that arsenic may inhibit a variety of ecologically important anaerobic respiratory processes as arsenic was shown to inhibit denitrification in subsurface aquifer sediments. It is possible that the overall toxicity of arsenic has limited the distribution of this process among bacteria. However, very little information is available about regulatory genes and details of the mechanisms involved in the dissimilatory process.

B. Oxidation of Arsenite

Microbial As(III) oxidation is considered to be the mechanism of detoxification because the toxicity of arsenic is dependent on its oxidation state; As(III) is 100 times more toxic than the As(V) for most biological systems (Cervantes *et al.*, 1994). In contrast to the dissimilatory arsenate reducers, which use arsenate as the terminal electron acceptor in anaerobic respiration, some bacteria are capable of using arsenite as the electron donor and hence gain energy for growth by oxidizing As(III) to As(V). This was first reported in 1918 and since then organisms representing over 30 strains of at least nine genera including α, β and γ proteobacteria, *Deinocci* (*Thermus*) and *Crenarcheota* have been shown to oxidize As(III) to As(V). These include *Thermus aquaticus* and *Thermus thermophilus*, which have been found to rapidly oxidize As(III) to As(V) (Gihring *et al.*, 2001). The investigation into structural genes coding for arsenite oxidase in various organisms indicates that *aox* and *aso* genes are responsible for the activity of arsenite oxidase in *Cenibacterium arsenoxidans* and *Alcaligens faecalis*, respectively (Rhine *et al.*, 2007; Muller *et al.*, 2003).

Alcaligenes faecalis and five members of the β *Proteobacteria* are heterotrophic arsenite oxidizers, whereas *Pseudomonas arsenitoxidans* and ''NT-26'' grew anaerobically through chemoautotrophic oxidation (Oremland and Stolz, 2005; Santini *et al.*, 2000). However, six members of α *Proteobacteria* (Ben-5, NT-3, NT-4, NT-2, NT-26, and NT-25) and one member of γ *Proteobacteria* (MLHE-1) were known chemolithoautotrophic arsenite oxidizers (Oremland *et al.*, 2002). The best characterized and probably most studied of all arsenite oxidizers is *Alcaligenes faecalis*, a heterotrophic arsenite oxidizer (Osborne and Enrlich, 1976). The arsenite oxidase from *Alcaligenes faecalis* has been purified and structurally characterized (Ellis *et al.*, 2001). A similar enzyme has also been purified from the heterotrophic arsenite oxidizers *Hydrogenophaga* sp. strain NT-14 (Vanden Hoven and Santini, 2004) and the chemolithoautotrophic *Rhizobium* sp. strain NT-26 (Santini and Vanden Hoven, 2004), which indicate that the arsenite oxidase enzyme is also a member of the DMSO reductase family of molybdenum enzymes, similar to the respiratory arsenate reductases (Arr). The arsenite oxidase heterodimer comprises an 88 kDa catalytic subunit encoded by the *aoxB* gene that contains a [3Fe–4S] cluster and molybdenum bound to the pyranopterin cofactor and a 14 kDa subunit

encoded by the *aoxA* gene with a Rieske 2Fe–2S cluster motif (Ellis *et al.*, 2001) which is a unique feature among all arsenite oxidizers. The genes encoding the two subunits have been cloned and sequenced by different groups and unfortunately were given different names; the gene designation *aox* is synonymous with *aro* and *aso* (Stolz *et al.*, 2006). It has been reported that Na^+:H^+ antiporter and a molybdate transporter play an important role in arsenite oxidation in *Agrobacterium tumafaciens* (Kashyap *et al.*, 2006). Recently, over 160 diverse *aroA*-like sequences from arsenic-contaminated sites and from 13 different arsenite-oxidizing organisms have been characterized by using degenerate primers suggesting that genes for aerobic arsenite oxidation are widely distributed in the bacterial domain. These bacteria are widespread in soil–water systems containing arsenic and play a critical role in the biogeochemical cycling of arsenic (Inskeep *et al.*, 2007). However, despite progress characterizing the metabolism of arsenic in various pure cultures, no functional gene approaches have been developed to determine the importance and distribution of arsenite-oxidizing genes in soil–water–sediment systems (Table 72.2).

C. Methylation of Arsenic

In the 19th century, the inhabitants of many houses who had wallpaper decorated with green arsenical pigments suffered illness and sometimes death caused by certain fungi that grew on the wallpapers – these fungi formed a toxic, garlic-odor gas which was actually used to develop a delicate microbiological test for arsenic. Microbial methylation plays important roles in the biogeochemical cycling of arsenic and possibly in their detoxification. Although biomethylation of arsenic is very widespread, occurring not only in microorganisms but also in algae, plants, animals, and humans, it is not universal. The mechanism for trimethylarsine formation was originally described on the basis of work done with the fungus *Scopulariopsis brevicaulis* (Challenger, 1945), which involves a series of steps, in which the reduction of arsenate was followed by oxidative addition of a methyl group by *S*-adenosylmethionine as the usual methyl donor. Thiol groups have important roles in the reductions. In anaerobic bacteria, methylcobalamin may be the donor. A similar pathway has also been suggested for prokaryotes, although the formation of arsine is a feature more common in bacteria (Bentley and Chasteen, 2002).

The widely used wood preservative chromated copper arsenate is converted to trimethylarsine by the yeast *Candida humiculus* (Cullen *et al.*, 1984). Chromated copper arsenate is quite similar to the arsenical wallpaper pigments that had triggered Gosio's work.

The methylation of As(III) has been observed in a number of organisms, including humans. For example, higher eukaryotes and bacteria have been reported to produce monomethylarsine or dimethylarsine, fungi which produce trimethylarsine (Bentley and Chasteen, 2002; Dombrowski *et al.*, 2005), and methanogens and aerobic eubacteria which produce methylated arsines (Honschopp *et al.*, 1996).

TABLE 72.2. Arsenite oxidizing prokaryotes characterized at molecular level

Ac. No.	Organisms	Operon/Gene	Reference
DQ412673	*Achromobacter* sp. strain NT10	*aroA*	Inskeep *et al.* (2007)
DQ412678	*Achromobacter* sp. strain NT10	*aroA*	Inskeep *et al.* (2007)
AF509588	*Cenibacterium arsenoxidans*	*aoxABCD*	Muller *et al.* (2003)
AAQ19838	*Alcaligens faecalis*	*arsO*	
AY345225 DQ412672	*Hydrogenophaga* sp. strain NT-14	*aroAB* *aroA*	Santiniand Vanden Hoven (2004) Inskeep *et al.* (2007)
DQ412676	*Hydrogenophaga* sp. strain WA13	*aroA*	Inskeep *et al.* (2007)
AAN05581	*Herminiimonas arsenicoxydans* strain ULPAs1	*aoxB*	Muller *et al.* (2003)
DQ380569	*Variovorax* sp RM1	*aoxB*	Inskeep *et al.* (2007)
DQ412675	*Agrobacterium* sp. strain Ben5	*aroA*	Inskeep *et al.* (2007)
DQ412674	*Sinorhzobium* sp. strain NT4	*aroA*	Inskeep *et al.* (2007)
ABB51928	*Abrobacterium tumefaciens* strain 5A	*arsO*	Kashyap *et al.* (2006)
AY345225 AAR05656	*Rhizobium* sp. strain NT-26	*aroAB* *aroA*	Santiniand Vanden Hoven (2004)
DQ380570	*Mesorhizobium* sp. DM1	*aoxB*	Inskeep *et al.* (2007)
ABB17183	*Thermus* sp. HR13	*aroB*	
BAD71923	*Thermus thermophilus* HB8	*arsO*	
AM502288	*Thiomonas* sp. 3As	*aoxAB*	Duquesne *et al.* (2008)
AM503077	*Burkholderia* sp. 2As	16S rRNA	Duquesne *et al.* (2008)

Cornybacterium sp., *Escherichia coli*, *Flavobacterium* sp., *Proteus* sp., and *Pseudomonas* sp. transform arsenate to arsenite, and they all produce dimethylarsine, whereas *Pseudomonas* sp. forms all three of the methylated arsines from arsenic-containing pesticides (Shariatpanahi *et al.*, 1981). Six bacterial species (*Achromobacter* sp., *Aeromonas* sp., *Alcaligenes* sp., *Flavobacterium* sp., *Nocardia* sp., and *Pseudomonas* sp.) produce both mono and dimethylarsine from methylarsonate and only two of them produce trimethylarsine, whereas *Nocardia* sp. was the only organism to produce all of the methylarsines from arsenical herbicides (Shariatpanahi *et al.*, 1983).

It has been reported that soil bacteria *Pseudomonas* sp. and *Alcaligenes* sp. incubate under anaerobic conditions, producing arsine, but only in the presence of nitrate and nitrite. In order to explore the formation of trimethylarsine by the bacteria, human skin anaerobic and aerobic cultures were isolated, which showed a reduction of trimethylarsine oxide to trimethylarsine with the production rate of 3–10 nmol/min/g of cells (wet weight) (Cullen and Reimer, 1989). Three bacterial spp., *Veillonella alcalescus*, *Streptococcus sanguis* and *Fusobacterium nucleatum*, which were isolated from dental plaque also showed transformation of trimethylarsine oxide to trimethylarsine (Cullen and Reimer, 1989). *Staphylococcus aureus* has shown transformation of trimethylarsine oxide to trimethylarsine with a very high production rate, i.e. 208 nmol/min/g of cells (wet weight), which was almost 20–60 times higher than the value reported in anaerobic bacteria (Cullen and Reimer, 1989). *Pseudomons* sp., isolated from the marine environment, has shown a production rate of 585 nmol/min/g of cells (wet weight) which was the highest production rate for the formation of trimethylarsine in aerobic bacteria (Bentley and Chasteen, 2002).

The enzymology of arsenic biomethylation is complicated because of its many oxidation states, its propensity to react with sulfur compounds, and low concentrations of arsenic compounds in biological specimens. The chemical intermediates and reactions in the metabolism of arsenate are similar in microorganisms and animals. However, in microorganisms, the reactions tend to proceed to methylarsines, whereas in mammalian species the major urinary metabolite is generally dimethylarsinate and only a very small amount of it is reduced further. The arsenate reductase and methylarsonate reductase were thought to play an important role in arsenic biomethylation; however, with the exception of arsenate reductase most of the enzymatic experiments involved mammalian systems.

Arsenate reductase can reduce arsenate to arsenite, and is involved in bacterial resistance to arsenic; however, the reduction can also be mediated nonezymatically by reduced glutathione. The arsenate reductase utilizes thioredoxin and glutaredoxin rather than glutathione which is usually postulated to play a role in biomethylation. The cytosolic and periplasmic, two different arsenate reductases, exist in microorganisms encoded by *ars* and *arr* systems. *Escherichia coli* converts dimethylarsinate to trimethylarsine oxide, however, both *Staphylococcus aureus* and *Escherichia coli* has shown biotransformation of trimethylarsine oxide to trimethylarsine (Bentley and Chasteen, 2002).

Monomethylarsonate reductase was expressed in *Escherichia coli* and purified by Ni–agarose affinity chromatography (Schmuck *et al.*, 2005). The same reductase was also identified in human liver, indicating that this enzyme is a member of the glutathione-*S*-transferase superfamily (Zakharyan *et al.*, 2001). Methylarsonate reductase purified from rabbit liver required GSH. The dithiothreotol did not substitute glutathione; however, dithiothreotol almost doubled the formation of methylarsonite in the presence of glutathione (Zakharyan and Aposhian, 1999). This enzyme was rate-limiting in arsenic biotransformation; however, no bacterial methylarsonate reductase has been reported to date.

In general, methylation of inorganic arsenic has been regarded as a detoxification mechanism as monomethylarsonic acid and dimethylarsinic acid are less toxic forms than inorganic As(V) and As(III) because of their low solubility and reduced affinity. In recent years, however, this interpretation has been questioned and it has become evident that methylation increases the arsenic toxicity, because the trivalent species are more toxic than inorganic As(III) (Suwalsky *et al.*, 2008; Styblo *et al.*, 2000). It has been reported that *arsM* [As(III) *S*-adenosylmethyltransferase] contributes to global cycling of arsenic through methylation of As(III) (Qin *et al.*, 2006; Wang *et al.*, 2004). The *arsM* was found to be adjacent to *arsR* (transacting repressor) for *ars* operon, suggesting that *arsM* plays an important role in arsenic detoxification. The mammalian Cyt19 catalyzes the arsenic *S*-adenosylmethyltransferase activity and has shown homology with *arsM* found in bacteria and archea, suggesting that this enzyme may evolve for arsenic detoxification. In bacteria and fungi, *arsM* homologs catalyze the formation of a number of methylated intermediates from As(III), with trimethylarsine as the end product (Bentley and Chasteen, 2002). This biogenic source of gaseous arsenic has been estimated to produce eight-fold more atmospheric arsenic than does continental dust (Tamaki and Frankenberger, 1992). Because *arsM* homologs are widespread in nature, this microbial-mediated transformation is proposed to have an important impact on the global arsenic cycle (Qin *et al.*, 2006). The *arsM* [As(III) *S*-adenosylmethyltransferase] from arsenite methylating prokaryotes is characterized at molecular level as shown in Table 72.3.

D. Resistance Mechanisms for Arsenic

Cellular arsenic uptake in the form of As(V) is mediated via phosphate transporters and in the form of As(III) via glyceroporin membrane proteins (Mukhopadhyay *et al.*, 2003) or hexose transporters (Liu *et al.*, 2004) or glucose permease GLUT1 (Liu *et al.*, 2006). The best characterized arsenic

TABLE 72.3. Arsenite methylating prokaryotes characterized at molecular level

Ac. No.	Organisms	Operon	Reference
NP_948900	*Rhodopseudomonas palustris* CGA009	*arsM*	Larimer *et al.* (2004)
NP_618654	*Methanosarcina acetivorans* C2A	*arsM*	Galagan *et al.* (2002)
YP_076198	*Symbiobacterium thermophilum* IAM 14863	*arsM*	Ueda *et al.* (2004)
NP_046066	*Halobacterium* sp. NRC-1	*arsMarsADRC-R2M*	Ng *et al.* (2000) Wang *et al.* (2004)
YP_146445	*Geobacillus kaustophilus* HTA426	*arsM*	
YP_182128	*Dehalococcoides ethenogenes* 195	*arsM*	Seshadri *et al.* (2005)

Symbiobacterium thermophilum is an uncultivable bacterium isolated from compost that depends on microbial commensalism. The 16S ribosomal DNA-based phylogeny suggests that this bacterium belongs to an unknown taxon in the Gram-positive bacterial cluster (Ueda *et al.*, 2004), low G + C Gram-positive bacteria

resistance mechanisms in bacteria involve reduction of As(V) to As(III), which is either extruded from the cells or sequestered in intracellular compartments.

The transport of arsenate into bacterial cells comparable to those of phosphate is carried out by phosphate transport membrane systems because of the structural similarity of phosphate and arsenate ions; for example, the arsenate oxyanions in water show three pKa values, 2.2, 7.0, and 11.5 (Ni Dhubhghaill and Sadler, 1991), comparable to 2.1, 7.2, and 12.7 for phosphate. In bacteria, arsenate As(V) enters the periplasmic space through the outer membrane porin, the PhoE protein, and is transported into the cytoplasm via two phosphate uptake systems, inorganic Pi transport (Pit) and phosphate-specific transport (Pst). The constitutive Pit system does not discriminate between phosphate and arsenate, having a K_m value for phosphate equal to the K_i for arsenate (25 μM). Under phosphate abundant conditions, the V_{max} is high, but the less specific Pit system fulfills the phosphate's need for the cell and leads also to arsenate accumulation (Elvin *et al.*, 1987), whereas under phosphate starvation conditions, the more specific Pst system is induced (Surin *et al.*, 1987). Eukaryotic microbes *Saccharomyces cerevisae* express a phosphate transporter similar to the low-affinity transporter of the prokaryotes. Their expression is regulated via a feedback mechanism. Members of the low-affinity Pit family are expressed predominantly during high phosphate concentration in the environment. These transporters belong to the permease transporter channels. Pst is the transporter which discriminates between phosphate and arsenate 100-fold better than Pit (Rosenberg *et al.*, 1987). Thus, one way for the cell to adapt to arsenate stress is to inactivate the Pit system by a pit mutation, which leads to moderate arsenate tolerance due to the discrimination between arsenate and phosphate by the Pst system. During periods of phosphate starvation or arsenate toxicity the Pst system is activated and despite having an identical K_i for arsenate, the higher affinity for phosphate leads to the reduction in cellular arsenic. The K_m of the Pst system for phosphate is 0.25 pM showing 100 times higher affinity than the Pit system (Rosenberg *et al.*, 1987). Thus the activation of the Pst system during the arsenic stress confers higher levels of arsenate resistance by virtue of reduced uptake of arsenate (Silver and Nakahara, 1983). Arsenite, in contrast, appears mostly unionized as $As(OH)_3$ at neutral pH, with a pKa of 9.3 for dissociation to $H_2AsO_3^{3-}$ (Ni Dhubhghaill and Sadler, 1991).

$As(OH)_3$ is transported into cells at neutral pH by aqua-glyceroporins (AQP) (Stahlberg *et al.*, 2000) in bacteria (Meng *et al.*, 2004), yeast (Liu *et al.*, 2006; Wysocki *et al.*, 2001), and mammals (Liu *et al.*, 2002). $As(OH)_3$ resembles the inorganic equivalent of a polyol, GlpF, which was originally identified as the glycerol facilitator in *Escherichia coli*, and is a member of the aqua-glyceroporin family. Transporters of this kind are involved in the osmoregulation of every cell. The ability of glycerol transporters to transport arsenite is probably a result of the similarity of arsenite to other polyols. It seems that $As(OH)_3$ is also a GlpF substrate. The structure of the *Escherichia coli* GlpF protein has been solved at 6.9 Å resolution (Stahlberg *et al.*, 2000) and showed a tetrameric assembly of subunits, comparable to aquaporins, but with a larger central channel in each subunit. Deletion of the gene for yeast aqua-glyceroporin Fps1p confers resistance to As(III) (Wysocki *et al.*, 2001). However, the Fps1v strain is still relatively sensitive to As(III), suggesting that the yeast cell also has an additional As(III) uptake pathway (Mukhopadhyay *et al.*, 2002). High osmolarity, which closes the Fps1p aqua-glyceroporin channel, provides resistance to As(III) and mutants with a constitutively open glycerol channel protein, hypersensitive to As(III). It was reported that the mammalian homologs of Fps1p, AQP7 and AQP9, functionally substitute for Fps1p in yeast strain Fps1v (Liu *et al.*, 2002). Thus, it is probable that As(III) also enters mammalian cells via aqua-glyceroporins. In order to understand the role of AQP in arsenic resistance, the *aqpS* gene of *Sinorhizobium meliloti* was disrupted resulting in increased tolerance to arsenite and indicating that AqpS facilitates transport of arsenite in *Sinorhizobium meliloti* (Yang *et al.*, 2005).

Once inside the cell, there is a problem involving arsenate detoxification due to the structural similarity with

phosphate; it would be difficult to export arsenate effectively in the presence of a higher intracellular phosphate concentration (Rosen, 1999). Thus, arsenate detoxification has to follow arsenate transformation mechanisms by discriminating it with phosphate, which is mainly by the reduction of arsenate to arsenite using arsenate reductase and/or other detoxification mechanisms such as methylation and sequestration (Patel *et al.*, 2007; Mateos *et al.*, 2006; Ji and Silver, 1992).

The arsenate reductase (ArsC) was found to have unrelated sequences and structural folds, divided into three different families based upon their structure, reduction mechanism, and location of catalytic cysteine residues. The first type, based on the product of the *arsC* gene from the *Escherichia coli* plasmid R773, uses glutaredoxin as a source of reducing equivalents, and was present in several Gram-negative bacteria (Jackson and Dugas, 2003). The second type of arsenate reductase from *Staphylococcus aureus* pI258 and *Bacillus subtilis* was related to low molecular weight tyrosine phosphatase and uses thioredoxin as the source of reducing equivalent, which did not show significant sequence similarity with the R773-encoded enzyme. The third type of arsenate reductase represented by the Acr2p enzyme from *Saccharomyces cerevisiae*, which was a homolog of CDC25 phosphatase, uses glutaredoxin as the source of reducing equivalents (Mukhopadhyay *et al.*, 2003).

The structure of the bacterial ArsC from *Escherichia coli* plasmid R773 has been solved at 1.65 Å resolution, and revealed that arsenate reductase (ArsC) has only one cysteine residue (Cys-12) in the active site, surrounded by an arginine triad composed of Arg-60, Arg-94, and Arg-107 (Mukhopadhyay *et al.*, 2002). However, the arsenate reductase from *Staphylococcus aureus* has three cysteine residues (Cys-10, Cys-82, and Cys-89). The biochemical and mutational studies established that the arsenate binds to the triad of arginine (Arg-60, Arg-94, and Arg-107) residues and forms a covalent bond with the cysteine (Cys-12) residue near the N-terminus at the active site of arsenate reductase and/or participates in catalysis (Rosen, 2002a; Silver and Phung, 2005; Shi *et al.*, 2003; Martin *et al.*, 2001).

V. GENES ASSOCIATED WITH ARSENIC RESISTANCE MECHANISM

The arsenic resistance mechanism conferred by *ars* operon, which is located on plasmid or chromosome, has been extensively studied in various microorganisms (Silver and Phung, 2005; Stolz *et al.*, 2006; Mukhopadhyay *et al.*, 2002; Rosen, 2002a). As more and more bacterial genomes are sequenced, it has become clear that arsenic resistance operon is a very ancient system (Qin *et al.*, 2007; Mukhopadhyay *et al.*, 2002) which must have evolved early after the origin of life because of the wide range of toxic inorganic chemicals that were likely present at that time. The gene organization of the *ars* operons from Gram-negative and Gram-positive bacteria is shown in Figure 72.4. The observed different but comparable arsenic operons have evolved through convergent evolution (Silver and Phung, 2005). These arsenic resistance determinants, isolated from both Gram-positive and Gram-negative bacterial species, have been found to be very homologous and each system has essential components, the arsenate reductase (*arsC*, *acr2*), an arsenic-specific efflux pump (*arsB*, *acr3*), and the trans-acting repressor (*arsR*, *acr1)* which senses environmental arsenic trioxide and controls the expression of *arsB* and *ars C* (Stolz *et al.*, 2006; Silver and Walderhaug, 1992). The *arsC* encoded small molecular weight protein (13–15 kDa) related to the class of tyrosine phophatase mediates the reduction of As(V) to As(III) in the cytoplasm (Rosen, 2002a), while *arsB* encoded arsenite permease extrudes As(III) out of the cells by functioning as an $As(OH)_3/H^+$ antiporter (Meng *et al.*, 2004), therefore expression of both *arsB* and *arsC* provides resistance to both As(III) and As(V), which are controlled by *arsR*. The appearance of arsenate-resistance microorganisms, however, is surely more recent, and probably evolved after the appearance of atmospheric oxygen, which created pressure for the evolution of arsenate reductase (*arsC*) from the protein tyrosine phosphatase (Mukhopadhyay *et al.*, 2003).

In some of the organisms, the *ars* operon (*arsRDABC*) consists of five genes with two additional components *arsA* and *arsD* which code for two additional proteins. *arsD* exhibits weak As(III)-responsive transcriptional repressor activity (Lin *et al.*, 2007; Chen and Rosen, 1997). The *arsA* codes for intracellular ATPase proteins which bind as a dimer to the arsenic membrane efflux pump coded by *arsB* to provide the higher levels of As(III) resistance (Rosen, 2002b). The arsenite membrane efflux pump is unique in that it can function either chemiosmotically (alone) or as an ATPase (dimer complexes).

The chromosomal locus of *Escherichia coli* contains only *arsRBC* (Martin *et al.*, 2001), whereas *ars* operon in *Escherichia coli* plasmid R733 (Accession no. J02591), R46 (Accession no. U38947), and *Acidiphilium multivorum* plasmid KW301 (Accession no.AB004659) comprises five mentioned genes, *arsRDABC*. The *Staphylococcus* plasmid pI258 (Accession no. M86824), pSX267 (Accession no. M80565), chromosome of *Pseudomonas stutzeri* (Accession no. AY702476), and chromosome of *Pseudomonas aeruginosa* (Accession no. X80057) contains only three genes, *arsRBC*. The *ars* operon in the skin element of *Bacillus subtilis* contains *arsR*, *orf2*, *arsB*, and *arsC*; however, *orf2* homologs are not established enough to play an essential role in the resistance mechanism (Sato and Kobayashi, 1998). The multiple *arsC* genes were found in the plasmid YS314 *Cornybacterium efficiens* (Accession no. NC_004369). Arsenite and arsenate resistance has been identified in *Cornybacterium glutamicum* resulting in the finding that resistance is mediated by two operons *ars1* and *ars2* located some distance from each other in the bacterial chromosome. Although both the operon *ars1* and *ars2* contain *arsR*, *arsB*,

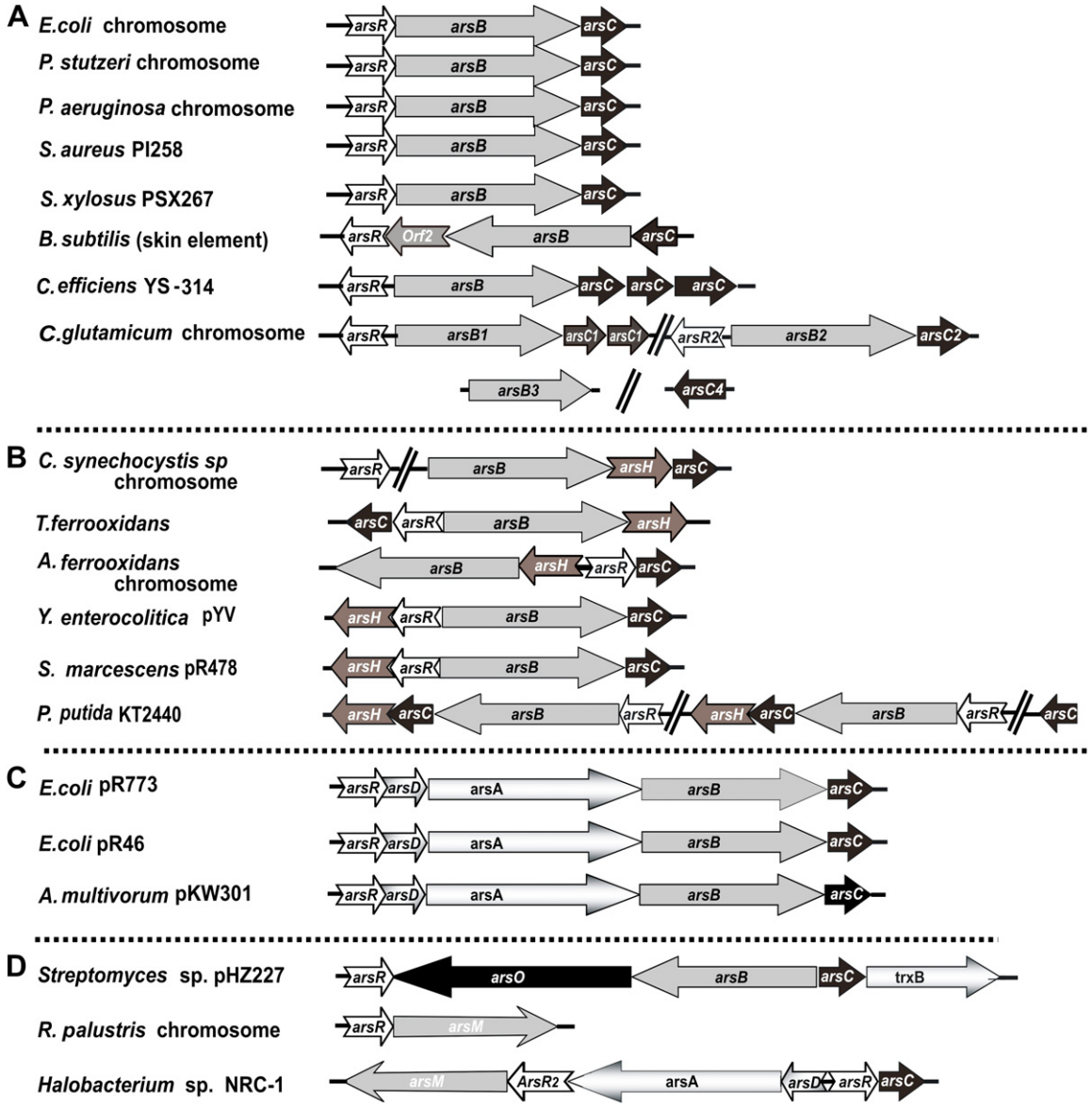

FIGURE 72.4. Schematic representation of genes involved in arsenic resistance/detoxification. Arrows represent open reading frames. Dashed boxes indicate the internal fragments of desired genes. (A) Three gene operon (*arsRBC*) encoding transcriptional repressor (*arsR*), an arsenite permease (*arsB*), and arsenate reductase (*arsC*) present in the *E. coli* chromosome (Accession number X80057), *P. stutzeri* chromosome (Accession number AY702476), *P. aeruginosa* chromosome (Accession number AF010234), *Staphylococcus* plasmid I258 (Accession number M86824), and SX267 (Accession number M80565), *Bacillus subtilis* skin element (Accession number D84432), *C. efficiens* plasmid YS314 (Accession number NC_004369), *C. gutamicum* chromosome (Accession number NC_003450). (B) The four gene operon (*arsRBHC*) has an additional protein of unknown function (*arsH*) in chromosome C, S*ynechocystis* sp. (Lopez-Maury *et al.*, 2003), in chromosome from *T. ferooxidans* (Butcher *et al.*, 2000), in chromosome from *A. ferooxidans* (Accession number AF173880), plasmid R478 from *S. marcescens* (Accession number AJ288983), plasmid YV from *Yerisiniae enterocolitica* (Accession number U58366), and plasmid *P. putida* (Accession number NC_002947). (C) The five gene operon (*arsRDABC*) encoding arsenite inducible repressor (*arsR*), negative regulatory protein (*arsD*), an oxyanion-protruding ATPase, an arsenite efflux pump (arsA and *arsB*, respectively), and arsenate reductase (*arsC*) present in *E. coli* plasmid R773 (Accession number J02591) and R46 (Accession number U38947) and in plasmid KW301 from *A. multivorum* (Accession number AB004659). (D) The five gene operon (*arsROBCT*) encoding arsenite inducible repressor (*arsR*), putative monoxygease (*arsO*), an arsenite permease (*arsB*), arsenate reductase (*arsC*), and putative thioredoxin reductases (*arsT*) present in plasmid HZ227 of *Streptomyces* sp. (Accession number DQ231520). Additional arsM [putative arsenite (III)] methyl transferase regulated by arsR type repressor from *R. palustris* (Qin *et al.*, 2006) and the arsADRC-R2M-type arsenic gene cluster from *Halobacterium* sp. strain NRC-1 (Wang *et al.*, 2004) (modified from Ordonez *et al.*, 2005, Mateos *et al.*, 2006).

and *arsC* genes, the operon *ars1* contains an additional arsenate reductase gene (*arsC1*), located immediately downstream from *arsC1*, arsenite permease (*arsB3*) and arsenate reductase gene (*arsC4*), scattered on the bacterial chromosome (Ordonez *et al.*, 2005).

In addition to the above-mentioned arsenic resistance operons, a broad diversity of four gene *ars* operons has been described in different species. An additional regulatory gene *arsH* has been identified which regulates the arsenic resistance operon. The *arsH* was originally identified in the *ars* cluster of a Tn2502 transposon of *Yersinia enterocolitica*, and appears to be necessary for arsenic resistance (Neyt *et al.*, 1997). The chromosomal arsenic resistance genes, including *arsH* of the acidophilic, chemolithoautotrophic, biomining bacterium *Thiobacillus ferrooxidans*, were cloned and sequenced and showed an unusual arrangement of *ars* genes where the putative *arsR* and *arsC* genes and the *arsBH* genes were translated in opposite directions (Butcher *et al.*, 2000). The complete genome sequence and comparative analysis of *Pseudomonas putida* KT2440 revealed the presence of additional regulatory genes *arsH1* and *arsH2* (Canovas *et al.*, 2003). In *Pseudomonas putida* both *arsH1* and *arsH2* were very similar to their counterparts in *Yersinia enterocolitica* and *Thiobacillus ferrooxidans* (over 74%). The genetic organization of the *ars* operon in *Pseudomonas putida*, *Yersinia enterocolitica*, and *Thiobacillus ferrooxidans* is different. In *Pseudomonas putida arsH* is placed downstream of *arsC* and transcribed in the same orientation, whereas *Yersinia arsH* is located upstream of *arsR* and transcribed divergently. The *arsBHC* operon in the *Cyanobacterium synechocystis* strain PCC 6803 was elucidated where the *arsH* gene was required for arsenic resistance, although the function of the *arsH* product was not explained (Lopez-Marury *et al.*, 2003). The *arsH* protein was annotated as a member of the NADPH-dependent FMN reductase family. Recently, the crystal structure of an *arsH* protein from *Shigella flexneri* refined at 1.7 Å resolution revealed that the *arsH* protein has an alpha/beta/alpha-fold typical for FMN binding proteins (Vorontsov *et al.*, 2007).

The arsenic resistance systems are found in many prokaryotes and there can be many variations in gene number and order (Butcher *et al.*, 2000). Some species have multiple *ars* operon and tandem *arsC* genes (Qin *et al.*, 2006). The legume symbiont *Sinorhizobium meliloti* possesses a unique *aqpS–arsC* detoxification system, the AqpS is the primary transport system associated with arsenite import, which was found in place of ArsB and functioned in arsenite efflux (Yang *et al.*, 2005). The *ars* operons have also been found in arsenate-respiring bacteria though arsenic resistance is not directly involved in arsenic respiration (Saltikov *et al.*, 2005). Many arsenite-oxidizing bacteria also have an *ars* operon, providing them with the ability to both oxidize and reduce arsenic (Macur *et al.*, 2004). Other genes that have also been found in *ars* operons are rhodanese (Bordo and Bork, 2002) and *arsO* and *arsT* (Wang *et al.*, 2006) but their functions have not been established in the arsenic resistance mechanism. New arsenic resistance gene clusters continue to emerge, and varied mechanisms seem to occur in diverse biological systems. The *ars* operon in the *Rhodopseudomonas palustris* contains *arsM* [As(III) *S*-adenosylmethyltransferase] to confer the arsenic resistance (Qin *et al.*, 2006). The *Halobacterium* sp. strain NRC-1 possesses an *arsADRC-R2M*-type arsenic resistance gene cluster involving a putative arsenite (III)-methyl-transferase (Wang *et al.*, 2004). The circular plasmid pWCFS103 in *Lactobacillus plantarum* carries a uniquely organized *arsRDDB*-type arsenic resistance gene cluster (Kleerebezem *et al.*, 2003) that lacks the arsenate reductase gene *arsC* and contains two copies of the *arsD* regulatory gene. In the arsenic resistance gene cluster from the large linear plasmid pHZ227 in *Streptomyces* sp. strain FR-008, two novel genes, *arsO* (putative flavin-binding monooxygenase) and *arsT* (putative thioredoxin reductase), were coactivated and cotranscribed with *arsR1–arsB* and *arsC*, respectively (Wang *et al.*, 2006). The *arsO* was homologous to flavin-binding monooxygenases, as it was 57% identical to putative monooxygenase (ZP_00420634) from *Burkholderia vitenaminesis* G4 and 48% identical to putative monooxygenase (NP_744078) from *Pseudomonas putida* KT2440. The *arsT* showed extensive homology to numerous thioredoxin reductases, such as thioredoxin reductases from *Streptomyces coelicolor* A3 (71% amino acid identity to CAA63076) and *Streptomyces clavuligerus* (73% amino acid identity to CAA79940).

VI. ENHANCED ARSENIC ACCUMULATION IN BACTERIAL CELLS

Biological methods are gaining momentum because of their potential in providing a cost-effective technology for heavy metal remediation. One more emerging technology that has received more attention in recent years is the development of biosorbents with high affinity and specificity. Unfortunately, very few reports for arsenic removal have been demonstrated except for nonspecific binding to the cell walls. These biosorbents, however, generally lack the high affinity and specificity. Although arsenic hyperresistant and hypertolerant organisms have been isolated from arsenic-contaminated sites (Joshi *et al.*, 2008; Canovas *et al.*, 2003), their use for arsenic bioremediation has not been reported. The selective removal of arsenic and mercury was reported by generating microbial biosorbence with surface exposed *arsR* and *merR*. The phytochelatins play a central role in heavy metal tolerance and detoxification in plants. The phytochelatins (PC), with good binding affinities for a wide range of heavy metals (Schmoger *et al.*, 2000), were exploited to develop microbial sorbents for removal of cadmium and arsenic by cloning PC synthase genes from *Arabdiposis thalina* in *Escherichia coli* and resulted in increased cellular content of copper and arsenic (Li *et al.*,

2004; Sauge-Merle *et al.*, 2003). The transgenic plant *Brassica juncea* expressing an *Arabidopsis* PC synthase exhibited enhanced arsenic tolerance without increasing arsenic accumulation (Gasic and Korban, 2007). Recently, PCs were introduced by expressing *Arabidopsis thaliana* PC synthase in the yeast *Saccharomyces cerevisiae* for enhanced arsenic accumulation and removal, resulting in six times higher arsenic accumulation as compared to the control strain under a wide range of arsenic concentrations (Singh *et al.*, 2008).

The metalloregulatory protein *arsR* was overexpressed in *Escherichia coli* and resulted in both elevated levels of arsenite bioaccumulation leading to severe reduction in cellular growth (Kostal *et al.*, 2004), and the efficacy of this strain at low arsenic levels. Equivalent strains overexpressing PC synthase genes and *arsR* could be developed to have arsenic hypertolerant strains with higher bioaccumulation, valuable for the bioremediation of arsenic.

VII. PROTEINS INVOLVED IN ARSENIC RESISTANCE

Arsenite exposure affects the transcription of gene-encoding functions related to protein biosynthesis, arsenic detoxification, oxidative stress defense, redox maintenance, and proteolytic activity. Importantly, it was observed that nearly all the components of the sulfate assimilation and glutathione biosynthesis pathways were induced at both gene and protein levels in *Saccharomyces cerevisiae*. The arsenite-exposed cells channel a large part of assimilated sulfur into glutathione biosynthesis, controlled by the transcriptional regulators Yap1p and Met4p (Thorsen *et al.*, 2007).

One of the most striking mechanisms by which bacteria combat arsenic stress is through internal metal sequestration. However, it can be assumed that proteins within the cells may provide a mechanism for sequestering metal ions and conferring a degree of resistance. In the prokaryotes, metal ion sequestration within the cell is performed by a well-characterized family of metal binding proteins called metallothioneins. Arsenic binding to *Fucus vesiculosus* metallothionein has been reported; however, the mechanism and character of metallothionein induction by arsenicals is unknown (Merrifield *et al.*, 2004). Exposure to arsenicals either *in vitro* or *in vivo* in a variety of model systems has been shown to cause the induction of a number of major stress protein families such as heat shock proteins. Among them are members with low molecular weight, such as metallothionein and ubiquitin, with masses of 27, 32, 60, 70, 90, and 110 kDa. It has been reported that a 72 kDa stress protein (Hsp72) was induced in cultured human pulmonary (L-132) cells by exposure to dimethylarsinic acid (DMA) and was accumulated specifically in the cell nuclei (Kato *et al.*, 2000). One of the most important heat shock proteins, GroEL, has been shown to be an essential component for maintaining viability with changes in temperature (Susin *et al.*, 2006; Fayet *et al.*, 1989). Recently, it has been reported that arsenate stress leads to induction of 18 unique proteins belonging to a group of phosphate transporters, heat-shock proteins involved in protein refolding, and enzymes participating in carbon and energy metabolism in the *Comamonas* sp. strain CNB-1 (Zhang *et al.*, 2007). Although very little information is available regarding arsenic stress proteins, detailed studies would enable researchers to explore the role of these proteins in arsenic resistance mechanisms, which will further open up a new avenue in the rapidly growing field of environmental bioremediation.

VIII. POTENTIAL APPLICATION FOR BIOREMEDIATION OF CONTAMINATED SITES

The discharge of heavy metals due to industrial, agricultural, and military operations has serious adverse effects on the environment (Ji and Silver, 1995). In recent years, there has been dynamic growth in the understanding of arsenic as a result of teamwork by a worldwide community of researchers working on arsenic speciation, transformations, transport kinetics, seasonal cycling, accumulation, biochemistry, molecular biology, geochemistry, and toxicology. New developments in arsenic biological and geochemical behavior will engender a better understanding of the development of new, safer, and cheaper technology to clean up the arsenic contaminated sites and polluted drinking water.

A wide variety of fungi, algae, and bacteria are now under study or are already in use as biosorbents for arsenic remediation (Deng and Ting, 2007; Macaskie and Dean, 1990). Metal binding by biomolecules of structural components or excreted polymers is fortuitous, and relative efficiencies depend on attributes of the metal ion, as well as on the reactivity of the provided ligands. The macromolecular composition of a biosorbent could be manipulated by cultivation conditions, e.g. stress-inducible fungal melanins (Macaskie and Dean, 1990) to improve its metal binding properties.

During the last few decades extensive attention has been paid to the hazards arising from contamination of the environment by arsenic. Decontamination of heavy metals in the soil and water around industrial plants has been a challenge for a long time. The use of microorganisms for the recovery of metals from waste streams (Joshi *et al.*, 2008; Patel *et al.*, 2006, 2007; Macaskie and Dean, 1990), as well as the employment of plants for landfill application (Tripathi *et al.*, 2007), has received increasing attention. Recent developments and improvements have resulted in the construction of bioreactors (Oehmen *et al.*, 2006) that have a smaller footprint, and treat the metals more effectively. Many studies have demonstrated primary removal mechanisms for the metals by arsenate-reducing bacteria, which transform arsenate to arsenite (Cohen, 2006; Afkar *et al.*, 2003; Mukhopadhyay *et al.*, 2002). Plants have been

shown to remove metals by uptake or oxidative precipitation near the roots (Shi *et al.*, 2008; Tripathi *et al.*, 2007). Plants seem to account for only a small percentage of the metal removal capacity of the wetland treatment systems. Moreover, the possibility of altering the properties of living species used in heavy metal remediation or constructing chimeric organisms possessing desirable features using genetic engineering is now under study in many laboratories. Many scientists have sought microbial community members responsible for arsenate reduction. It was found that, in the anoxic water of Mono Lake (California), two subgroups of the proteobacteria lineage *Sulfurospirillium* and *Desulfovibrio* were present and most likely using arsenate as an electron acceptor for growth (Hoeft *et al.*, 2002). They have found an interesting cycling of arsenic by rapid reoxidation of arsenate in the presence of nitrate. Thus, in some environments, both oxidation and reduction of arsenic may occur. In another study of aerobic contaminated mine tailings, it was found that members of the *Caulobacter*, *Sphingomonas*, and *Rhizobium* families may be responsible for the reduction and mobilization of arsenic (Macur *et al.*, 2001). These studies provide encouraging information on the interaction of living organisms and their constituents with arsenic, which will be useful for the bioremediation of polluted sites.

When organisms are confronted with sudden changes such as exposure to potentially toxic substances (heavy metal ions) or the onset of starvation, these stimulations induce the production of the so-called stress responsive proteins. The most thoroughly studied stress proteins include the heat-shock proteins (HSP), the induction of which is a highly conserved response across genera.

Naturally occurring microbial consortia have been utilized in a variety of bioremediation processes. Recent developments in molecular microbial ecology offer new tools that facilitate molecular analyses of microbial populations at contaminated and bioremediated sites. Information provided by such analyses aids in the evaluation of the effectiveness of bioremediation and the formulation of strategies that might accelerate bioremediation.

IX. CONCLUDING REMARKS AND FUTURE DIRECTION

The relative ubiquity of arsenic operon and the processes involved in arsenic detoxification and resistance indicate that there have been important selective factors in microbial evolution. These taxonomically diverse and metabolically versatile microorganisms have evolved a number of mechanisms to gain energy for their growth from this toxic element in their environment, and developed detoxification mechanisms including oxidation, reduction, and methylation for increased tolerance and resistance to high levels of arsenic in their environment. Through a variety of detoxification and respiratory mechanisms, microorganisms have the ability to greatly influence the speciation of arsenic within the environment and thus play a significant role in the arsenic cycle and impact of arsenic toxicity. The extensive database for arsenic resistance genes in bacterial genomes indicates that arsenic resistance operon must have evolved due to the presence of arsenic in the natural environment. New arsenic resistance gene clusters continue to emerge, and varied mechanisms seem to occur in diverse biological systems. The increasing availability of genome data and proteome analysis will help researchers to explore metabolic diversity, which may possibly lead to the discovery of new pathways and regulatory elements. Additional key areas for further investigation are regulatory genes and mechanisms involved in the dissimilatory process and the processes and enzymes involved in methylation and demethylation of arsenic which are the main processes to regulate gaseous forms of this toxic metalloid. With a better understanding of the biochemical processes involved in bacterial arsenic uptake, transport, accumulation and resistance, systematic improvements in bioremediation using molecular genetic approaches are suggested which can develop arsenic hyperaccumulating microorganisms for developing the environmental cleanup processes However, long-term efforts should be directed towards the development of a ‘‘molecular toolbox’’ composed of genes valuable for bioremediation and the systematic screening of bacterial species, and genotypes for arsenic accumulation and resistance which will broaden the spectrum of genetic material available for optimization and transfer. Mutagenesis of selected bacterial genes may also produce improved strains for the potent bioremediation processes.

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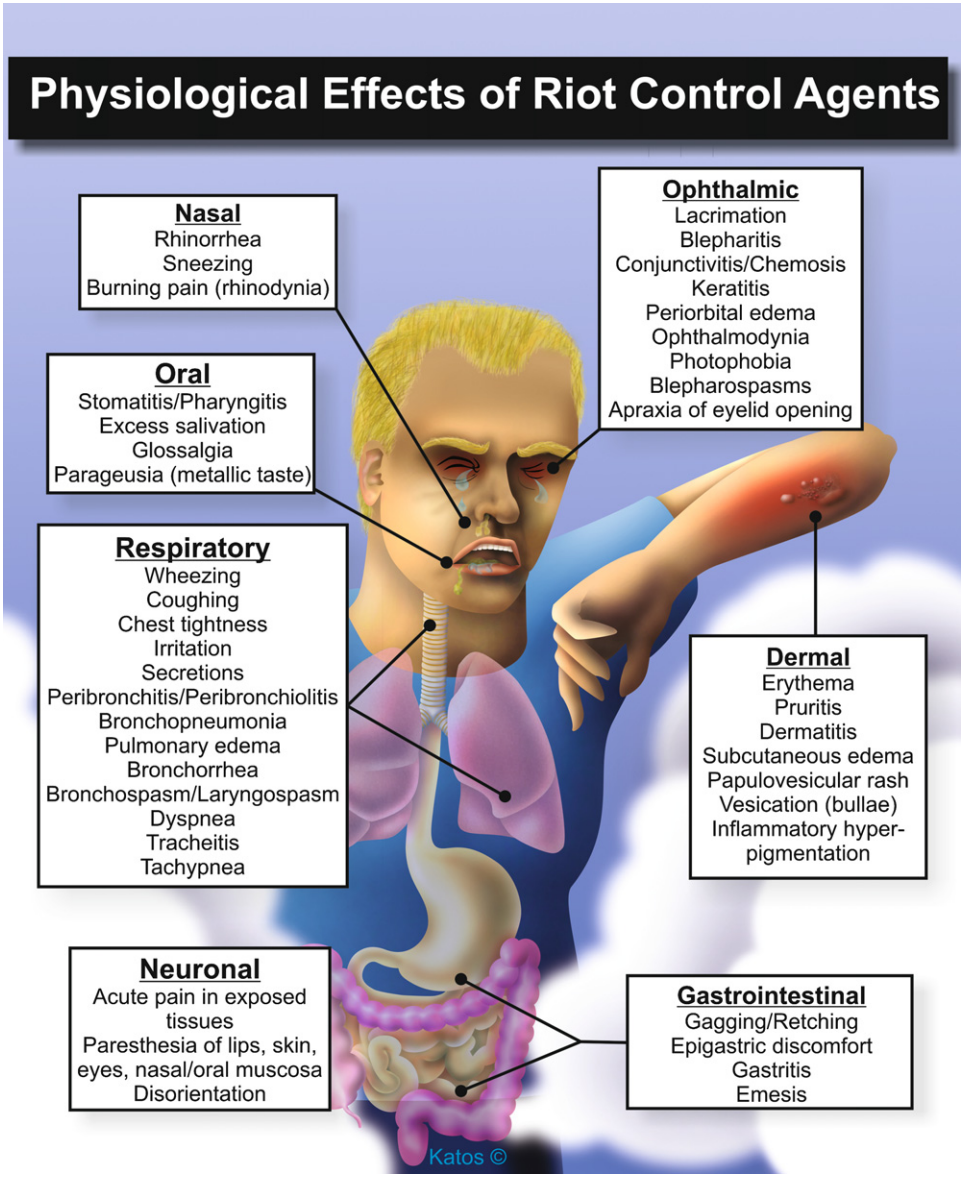

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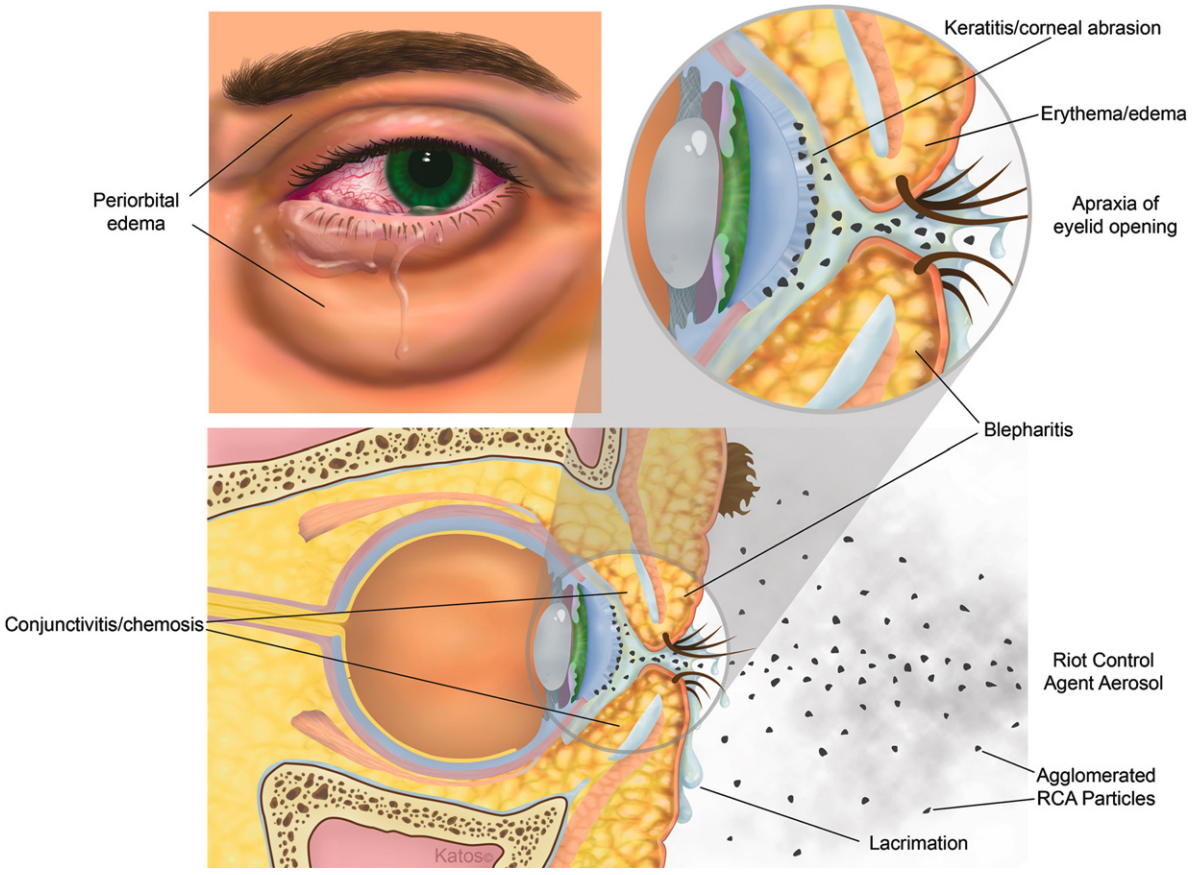

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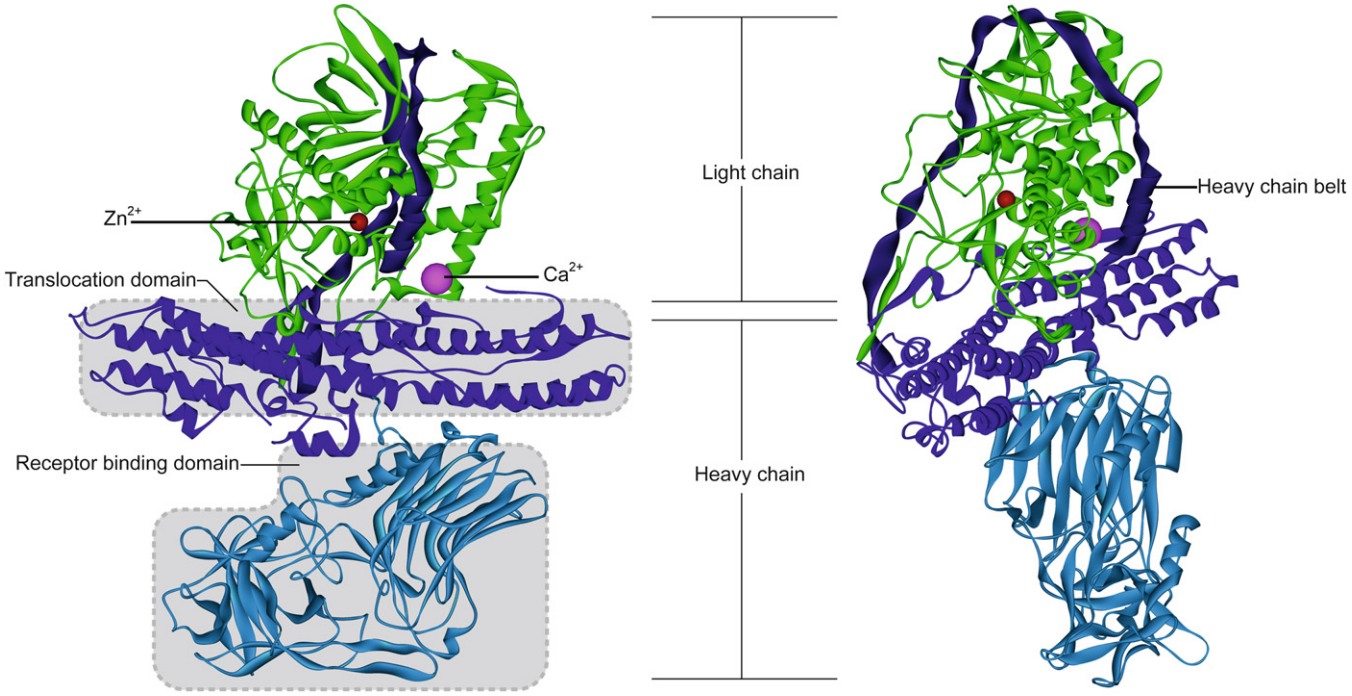

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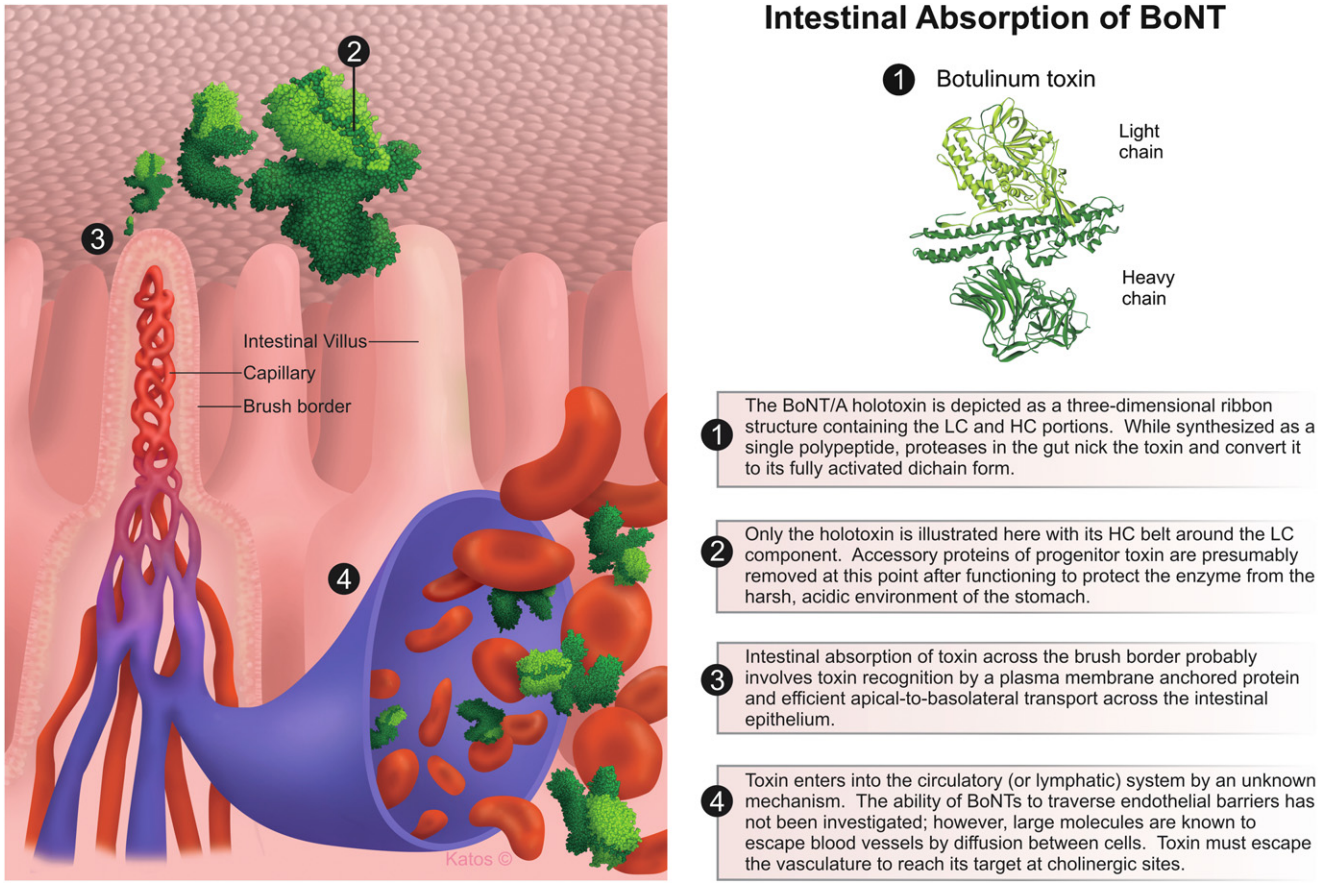

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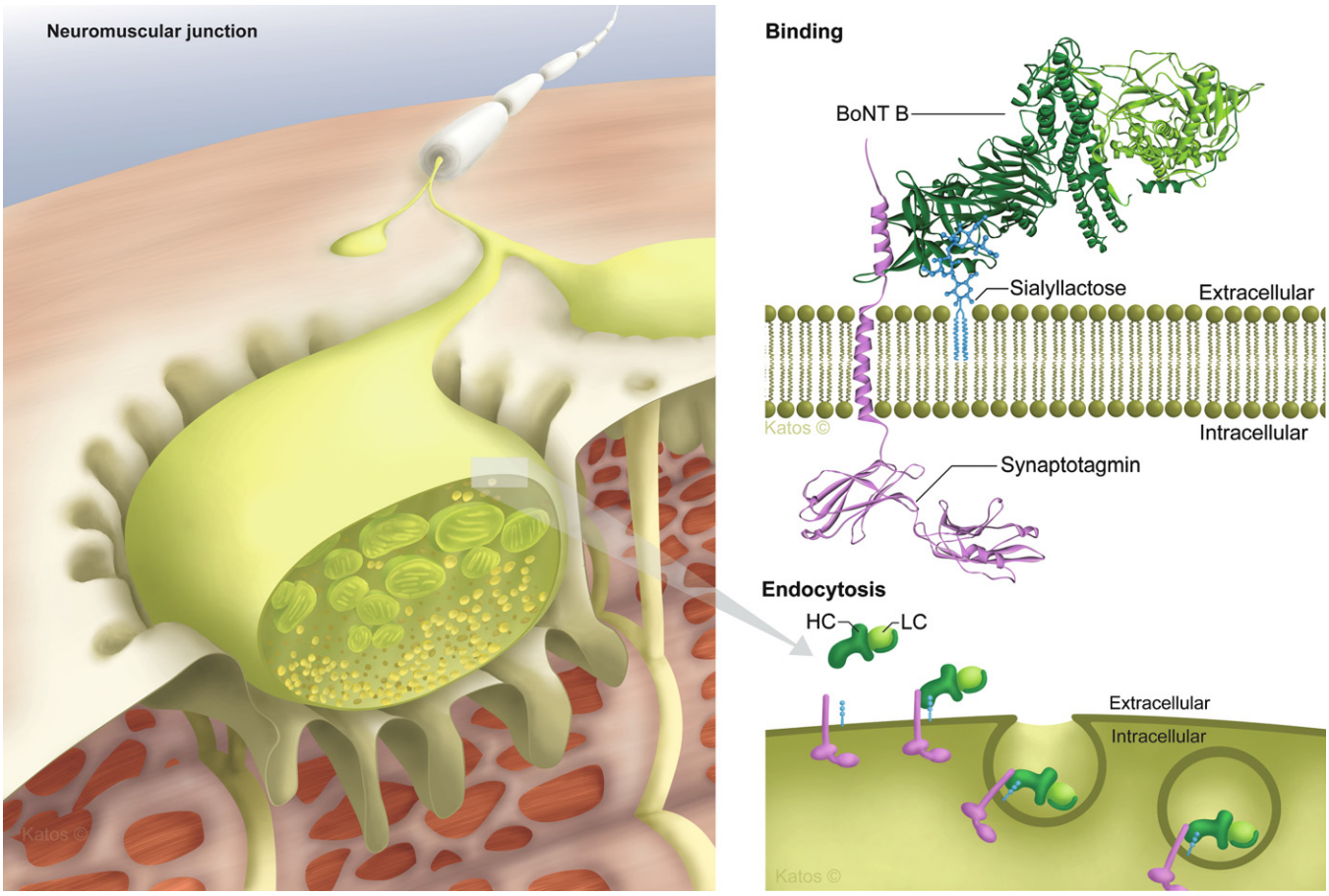

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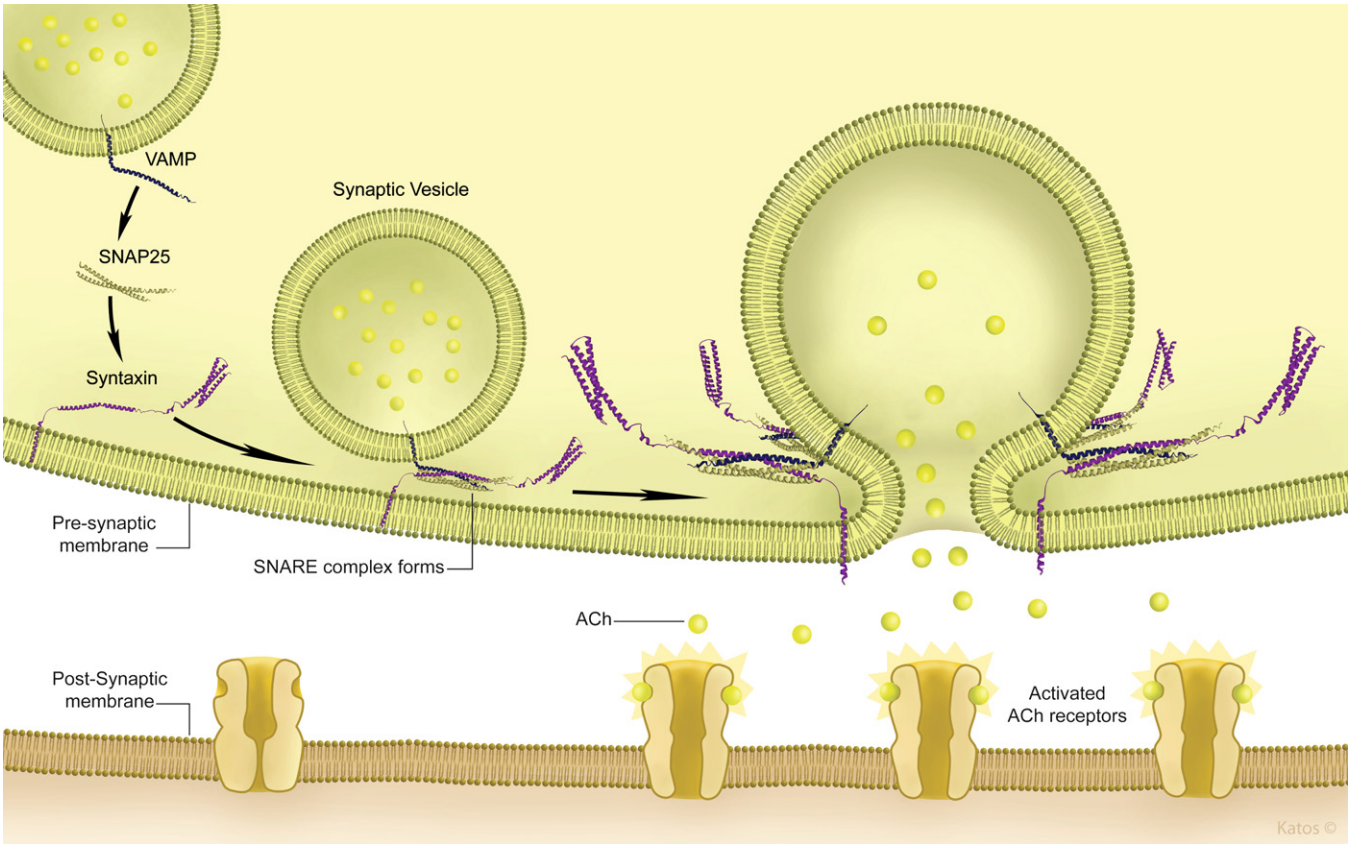

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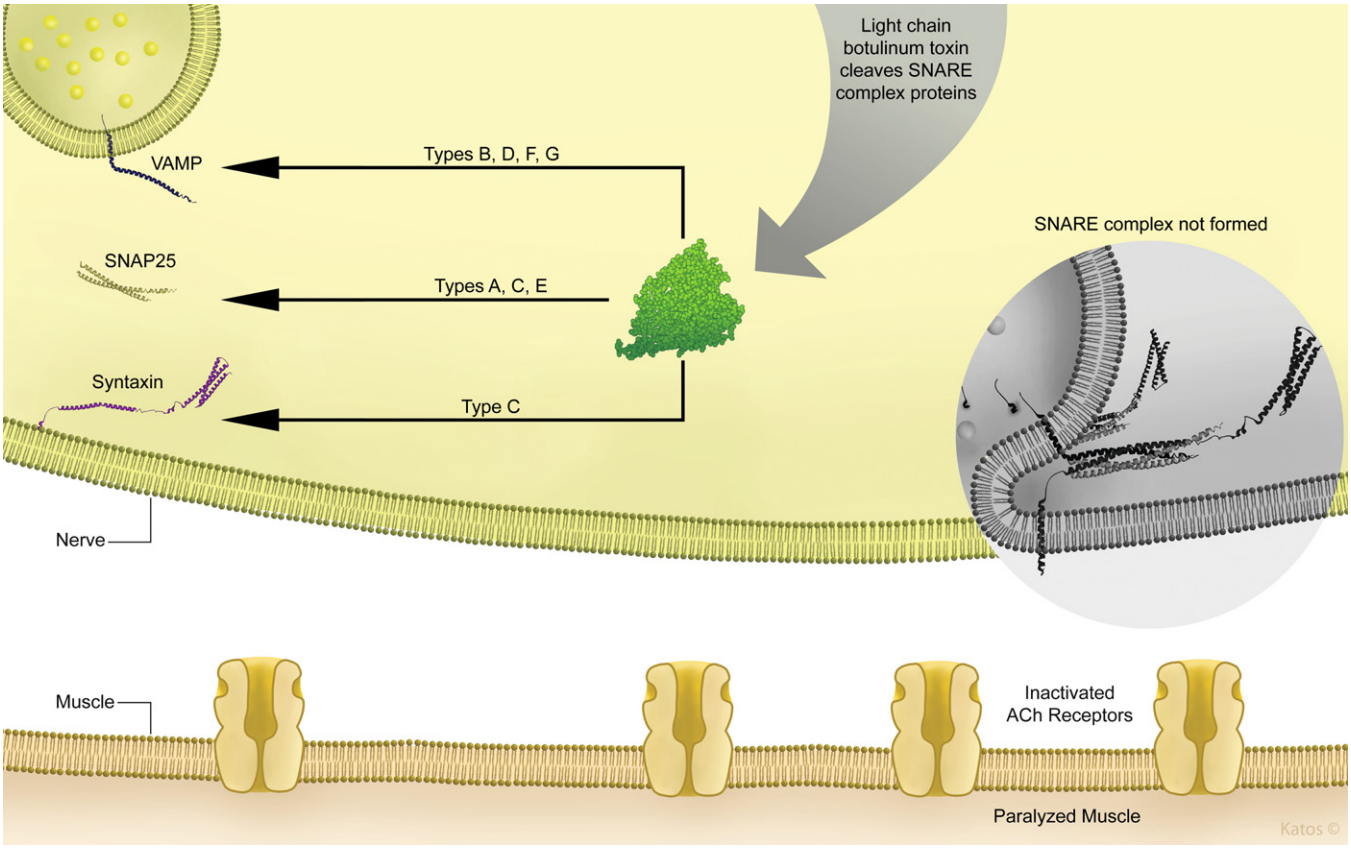

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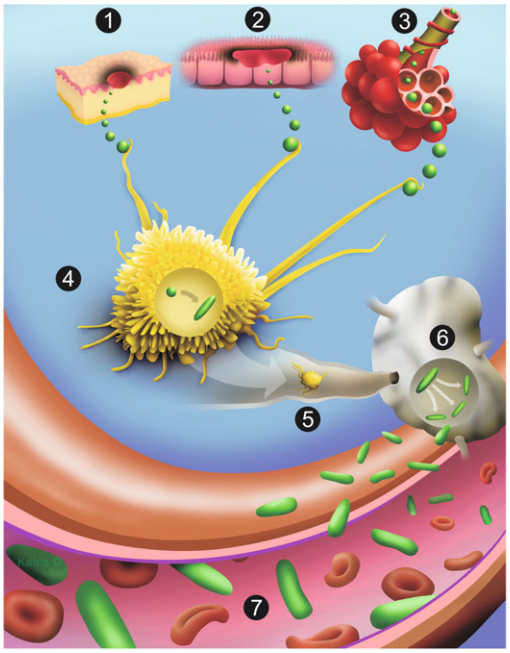
Summary of Anthrax Pathogenesis

Cutaneous 1
Spores gain access to subepidermal structures in the host through an abrasion of the skin, followed by uptake via resident macrophages.

Gastrointestinal 2
Spore uptake by phagocytes occurs after ingestion of contaminated food. Germination follows soon after entry in host immune cells.

Inhalational 3
Alveolar macrophages take up inhaled spores from the alveoli and repiratory tract. A small fraction of spores will evade destruction in the phagolysosome. Lung lesions are not found after inhalational anthrax.



4 In each case, spore germination into mature *B. anthracis* bacilli takes place in the macrophages at the primary site of infection. In the case of inhalational anthrax, germination occurs later upon arrival at the local lymph node.

5 After spore uptake into phagolysosomes by tissue macrophages, the bacilli are transported via lymphatic channels to local and regional lymph nodes.

6 Final germination takes place in the lymph nodes draining the primary site of infection. Through an unclear mechanism, mature bacilli escape from macrophages and multiply systemically.

7 Bacilli spread through the circulatory system, causing septicemia and infection of other target organs.

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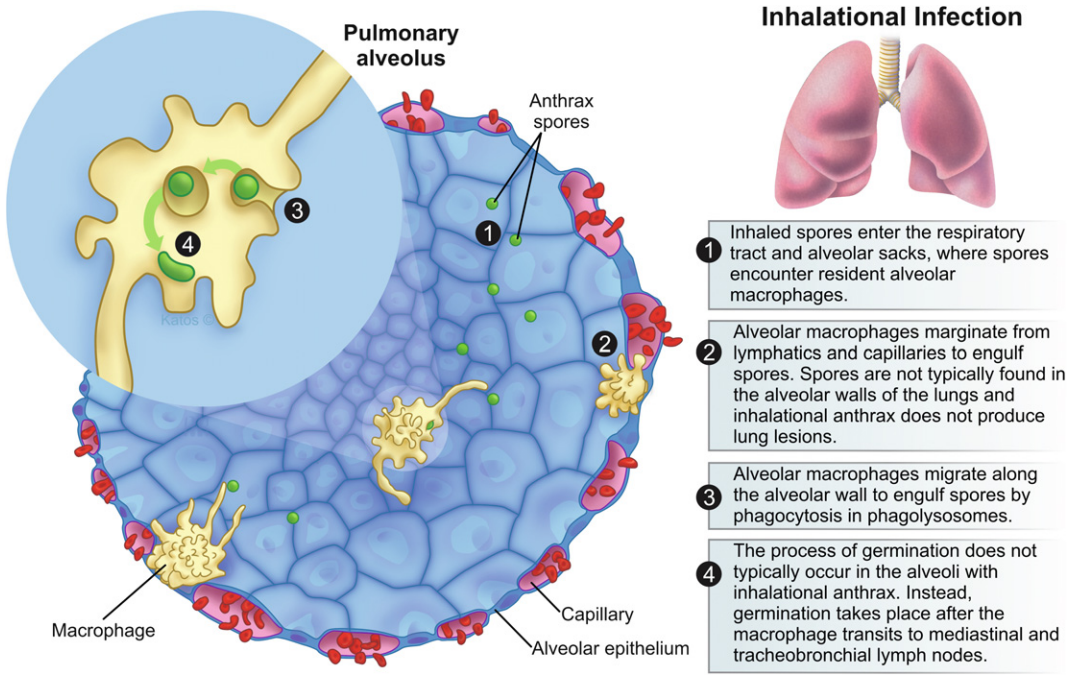

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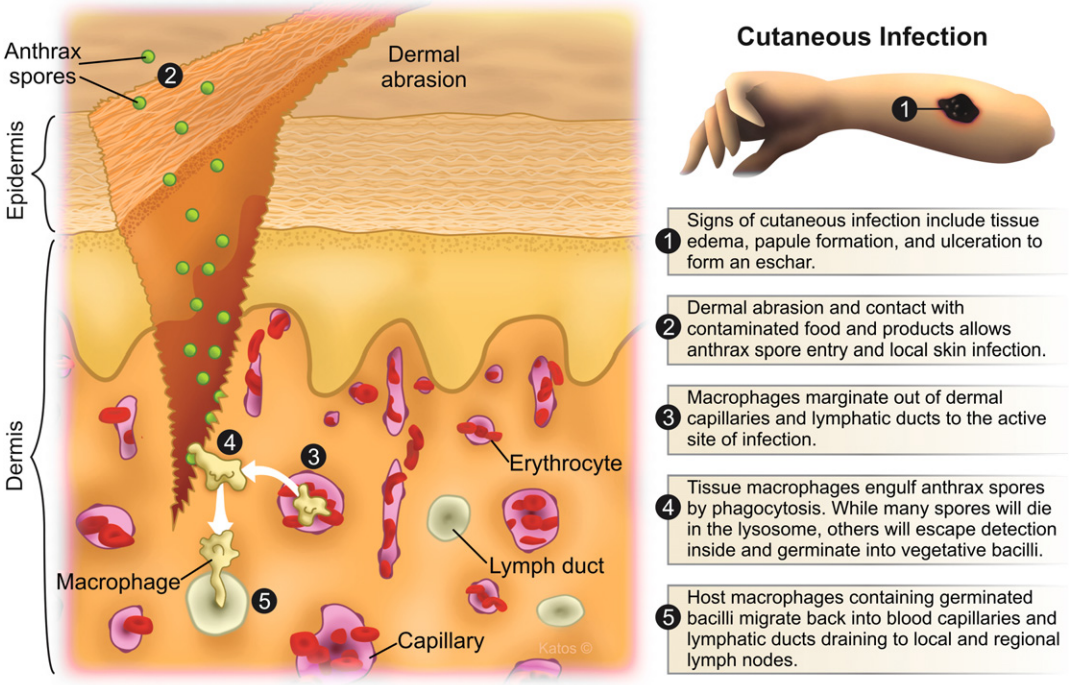

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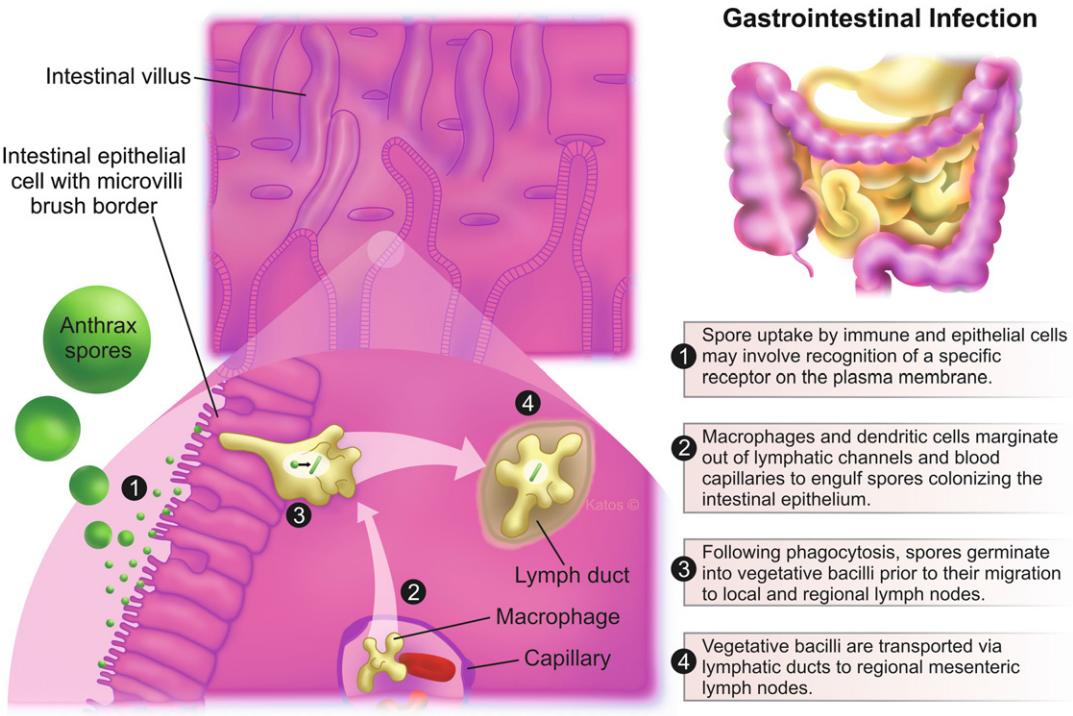

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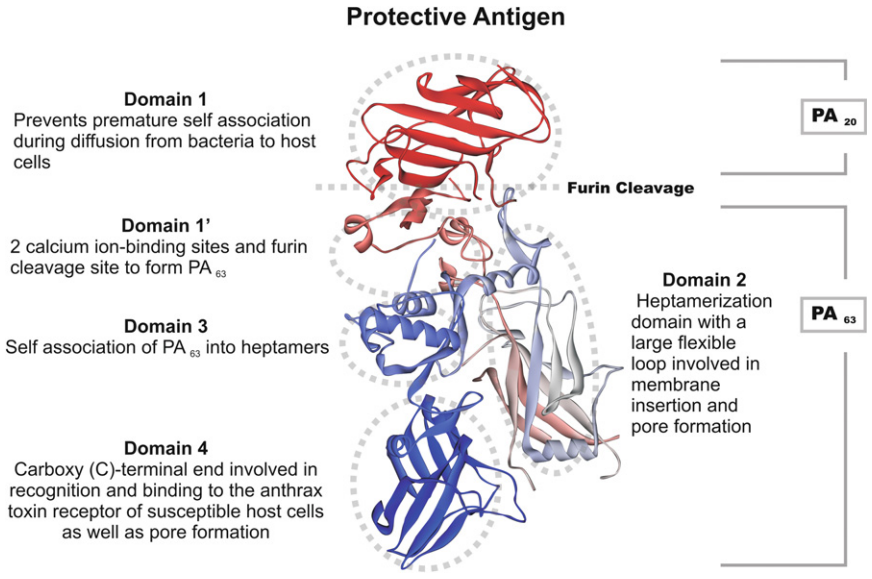

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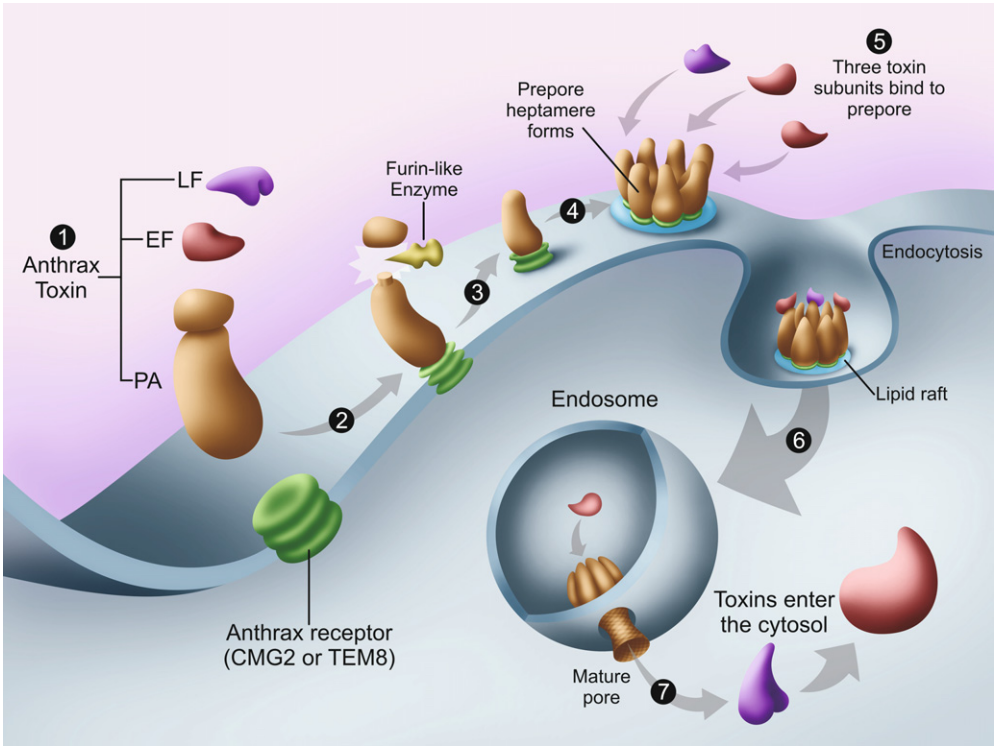

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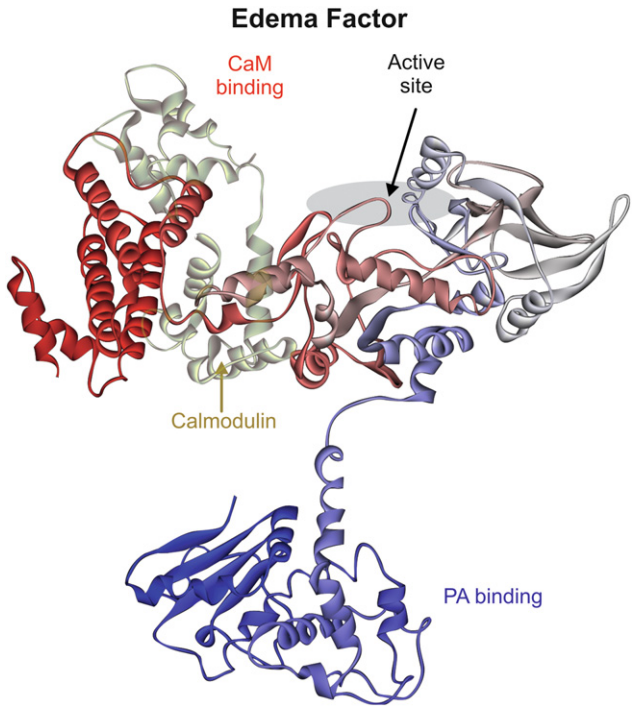

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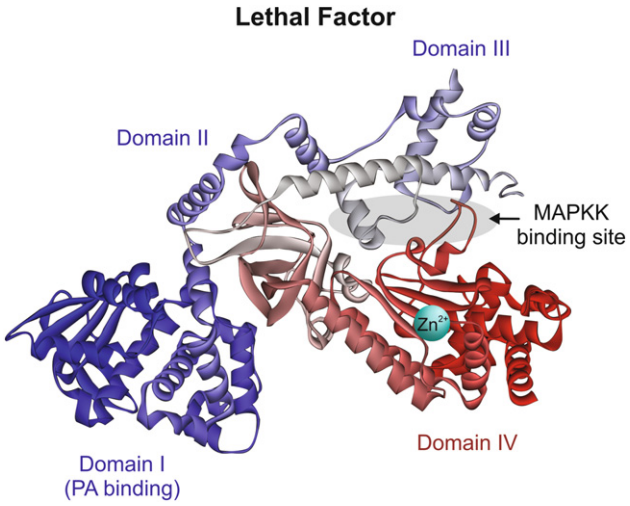

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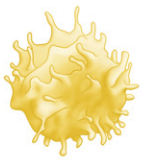
Effects of Anthrax Bacilli and Toxins on Various Cell Types

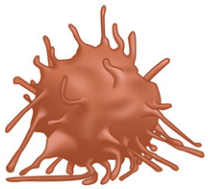
Macrophage



Suppresses cytokine production (LT)
Cell death (ET)
Increased apoptosis
Inhibits:
- Proliferation (LT)
- Differentiation (LT)

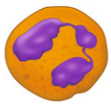
Decreases:
- ROS
- NF-κB
- IRF-3
- TNF-α
- IL-1β

Dendritic Cell



Causes cell death(Immature Dendritic) (LT)
Suppresses cytokine production (LT, ET)
Co-stimulatory molecule expression (LT)
Co-stimulatory T cell stimulation (LT)
Decreases:
- TNF-α
- IL-1β
- IL-12
- IL-10
- CD40
- Cd80
- Cd86

Neutrophil



Inhibits:
- Mobility (LT)
- Phagocytosis (ET)

T Cell

Inhibits:
- Activation (LT, ET)
- Proliferation (LT, ET)
- Surface-molecule expression (LT, ET)
- Cytokine expression (LT, ET)

Erythrocyte

Cell death

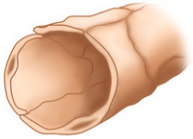
Platelet

Coagulopathy (LT, ET)

B Cell

Lowers:
- Proliferation
- IgM production
- IgG production

Endothelial Cells



Apoptosis
Leaky blood vessels
Hemorrhages

Melanocyte

Increased melanin production (LT, ET)

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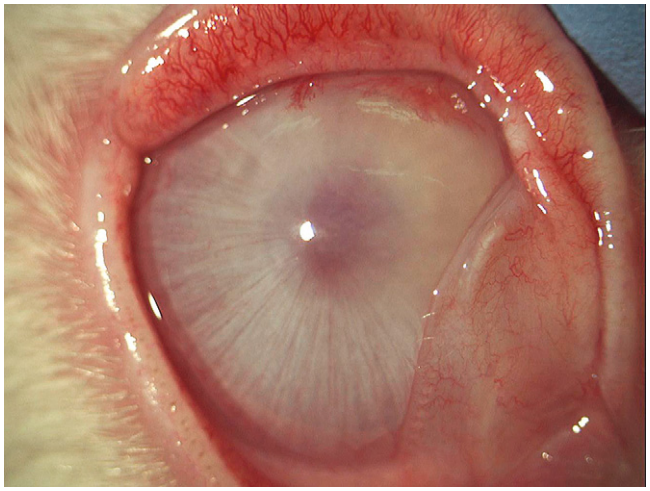

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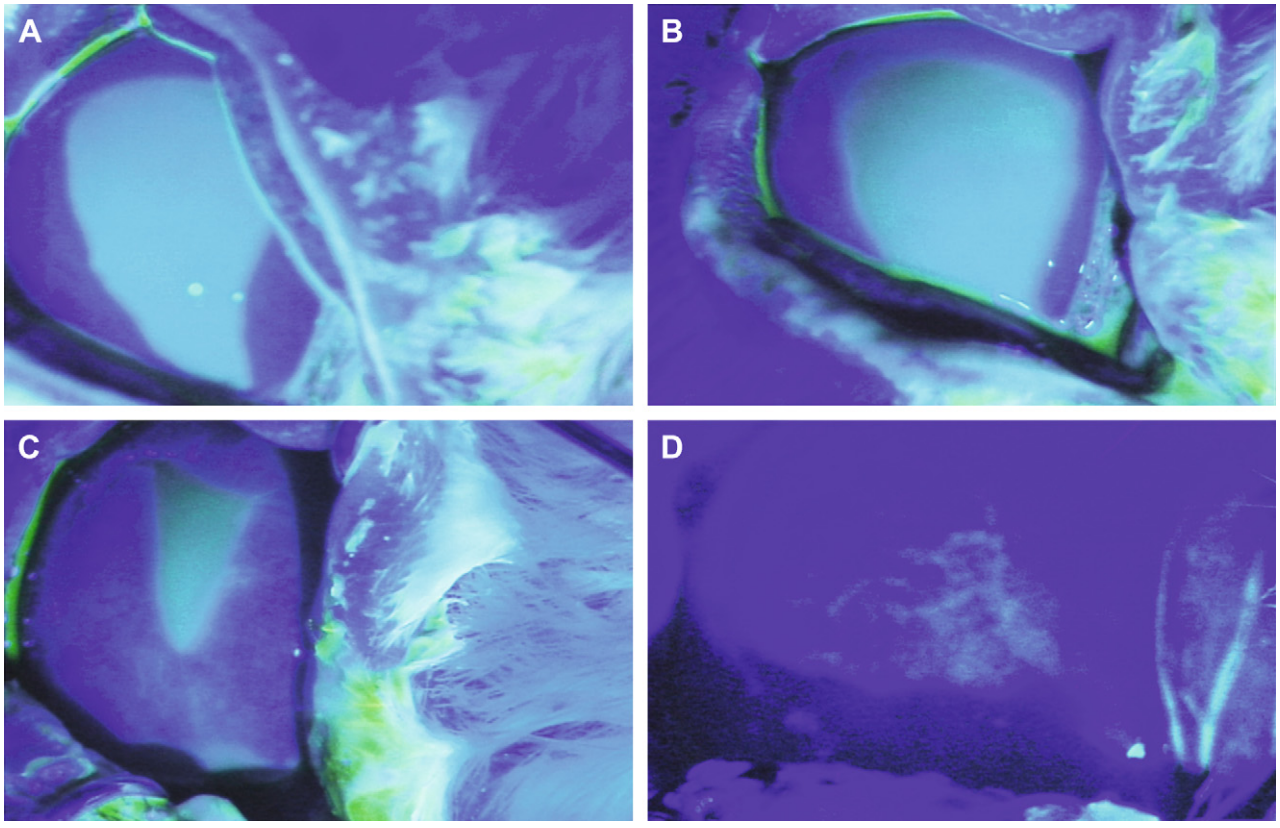

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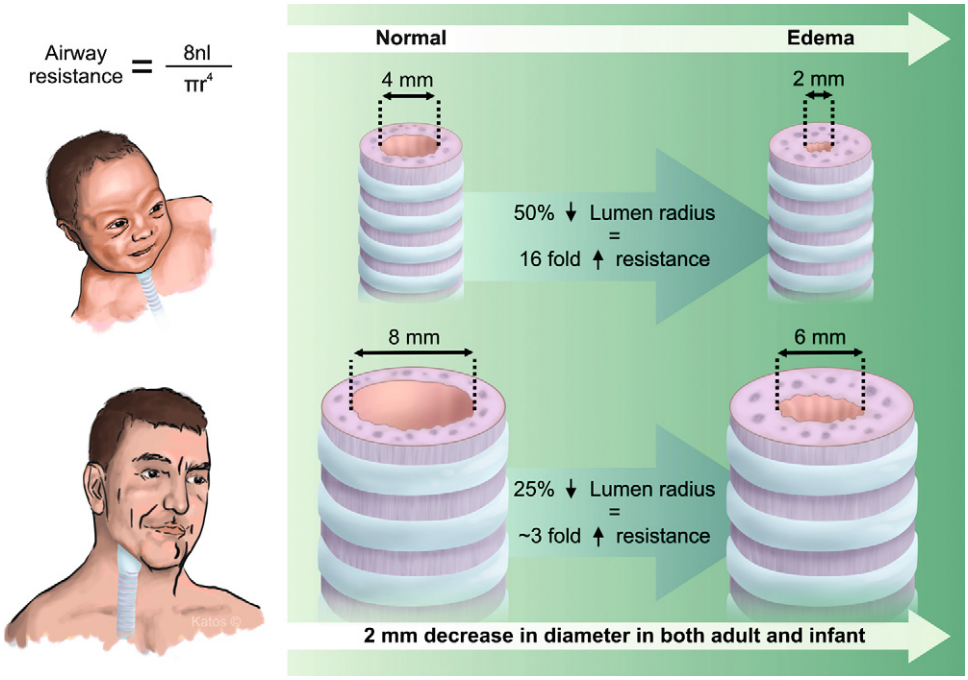
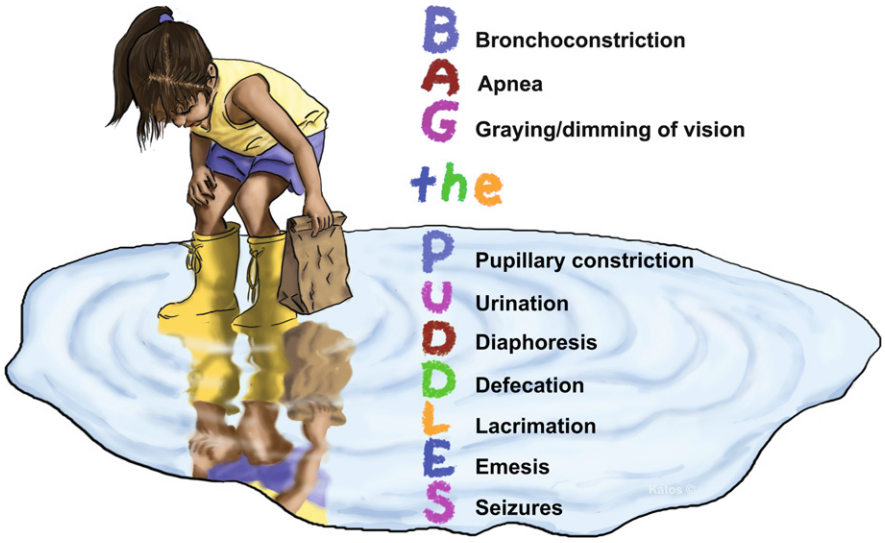
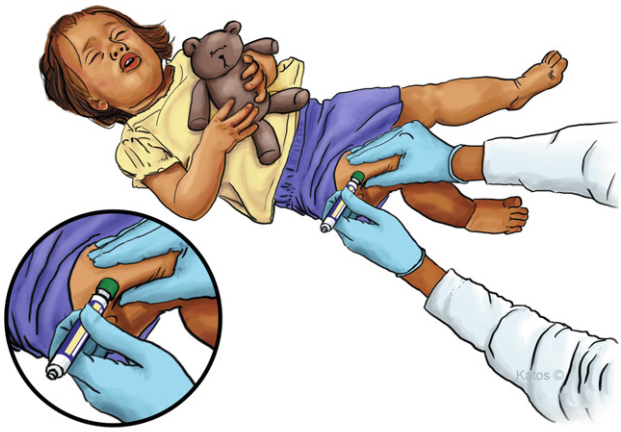


Chapter 61, Figure 61.2 (See Page 923 of this volume).



Chapter 61, Figure 61.3 (See Page 927 of this volume).



Chapter 61, Figure 61.7 (See Page 929 of this volume).

Chapter 71, Figure 71.1 (See Page 1074 of this volume).