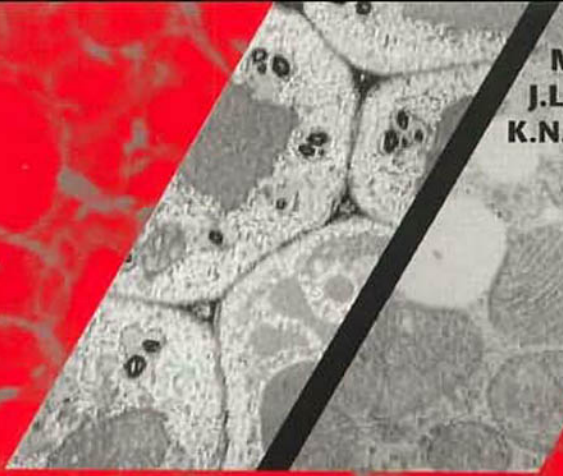
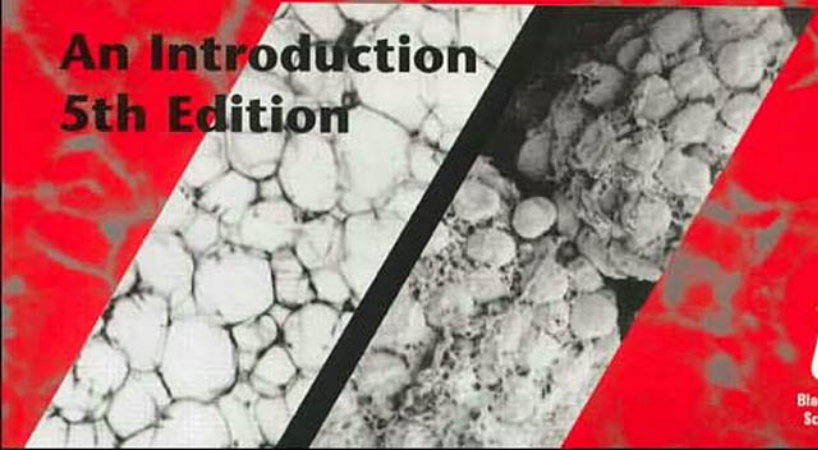
M.I. Gurr
J.L. Harwood
K.N. Frayn



LIPID BIOCHEMISTRY

An Introduction
5th Edition



Blackwell Science

Lipid Biochemistry

Lipid Biochemistry

5th Edition

by

Michael I. Gurr
St Mary's, Isles of Scilly

John L. Harwood
School of Biosciences, Cardiff University, Cardiff

and

Keith N. Frayn
Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, Oxford

Blackwell
Science

Blackwell Science Ltd, a Blackwell Publishing Company
Editorial Offices:
Osney Mead, Oxford OX2 0EL, UK
Tel: +44 (0)1865 206206
Blackwell Science, Inc., 350 Main Street, Malden, MA 02148-5018, USA
Tel: +1 781 388 8250
Iowa State Press, a Blackwell Publishing Company, 2121 State Avenue, Ames, Iowa 50014-8300, USA
Tel: +1 515 292 0140
Blackwell Science Asia Pty, 54 University Street, Carlton, Victoria 3053, Australia
Tel: +61 (0)3 9347 0300
Blackwell Wissenschafts Verlag, Kurfürstendamm 57, 10707 Berlin, Germany
Tel: +49 (0)30 32 79 060

First Edition 1971
Second Edition 1975
Third Edition 1980
Fourth Edition 1991
Fifth Edition 2002

Library of Congress Cataloging-in-Publication Data is available

ISBN 0-632-05409-3

A catalogue record for this title is available from the British Library

Set in 9/12pt Palatino
by DP Photosetting, Aylesbury, Bucks
Printed and bound in Great Britain by
MPG Books Ltd, Bodmin, Cornwall

For further information on
Blackwell Science, visit our website:
www.blackwell-science.com

Contents

Abbreviations

When a complex name is used repeatedly (e.g. triacylglycerol in Chapter 3), we have placed the abbreviation in parentheses after the first mention of the word and then used the abbreviation consistently (except in the headings and the Summary) in that chapter. We have tended to redefine the abbreviation at first mention in each chapter when that word is used repeatedly. Although our aim was to use abbreviations sparingly in this book, the list of abbreviations set out below is quite extensive. Some of the abbreviated terms are used only occasionally, but we have included them here because they may be useful to students when they come across them in other literature, where their meanings may not always be defined.

Students will note that the authors have been inconsistent in the abbreviations used for phospholipid names. This merely reflects a wider inconsistency among the research community. Thus, the system: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol is still widely used, but the alternative PtdCho, PtdEtn, PtdSer, PtdIns is becoming more common, especially in Europe.

ACAT	acyl-CoA:cholesterol acyltransferase
ACC	acetyl-CoA carboxylase
ACP	acyl carrier protein
ACS	acyl-CoA synthetase
ADD	adipocyte determination and differentiation factor
ADH	antidiuretic hormone
ADP/ATP	adenosine di-/triphosphate
(c)AMP	(cyclic) adenosine monophosphate
ARF	ADP-ribosylation factor
ASP	acylation stimulating protein
BCCP	biotin carboxyl carrier protein
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene (2,6-di-*tert*-butyl-*p*-cresol)
CDP/CTP	cytidine di-/triphosphate
CER	ceramide
CERP	cholesterol efflux regulatory protein
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
CLA	conjugated linoleic acid
CMC	critical micellar concentration
CoA	coenzyme A
COX	cycloroxygenase
CPT	carnitine palmitoyl transferase
CSF	cyanide sensitive factor
DAG	diacylglycerol
DAGAT	diacylglycerol acyltransferase
Da	dalton
DGTS	diacylglycerol trimethylhomoserine
DHA	docosahexaenoic acid (*n*-3)
(c)DNA	(complementary) deoxyribonucleic acid
EFA	essential fatty acid(s)
EPA	eicosapentaenoic acid (*n*-3)
ER	endoplasmic reticulum
ESR	electron spin resonance
FABP	fatty acid binding protein
fad genes	fatty acid degradation genes
FadR	fatty acyl-CoA responsive transcription factor
FDB	familial defective apolipoprotein-B100
FAS	fatty acid synthase
FAT	fatty acid translocase
FFA	free fatty acid(s)
FLAP	5-lipoxygenase activating protein
FMN	flavin mononucleotide
Gal	galactose
GDP/GTP	guanosine di-/triphosphate
(c)GMP	(cyclic) guanosine monophosphate

Glc	glucose
G(L)C	gas (–liquid) chromatography
GPAT	glycerol 3-phosphate acyltransferase
GPI	glycosyl phosphatidylinositol
GSH	glutathione (reduced)
GTP	guanosine triphosphate
HDL	high density lipoprotein(s)
HETE	hydroxy eicosatetraenoic acids
HIV	human immunodeficiency virus
HMG	hydroxymethylglutaryl
HNF	hepatic nuclear factor
HPETE	hydroperoxy eicosatetraenoic acids
HPLC	high performance liquid chromatography
IHD	ischaemic heart disease
IOB	immature oil bodies
KDO	3-deoxy-D-manno-octulosonic acid
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoprotein(s)
LOX	lipoxygenase
Lp(a)	lipoprotein(a)
LPAT	lysophosphatidate acyltransferase
LRP	LDL-receptor related protein
LT	leukotriene
MAP	mitogen activated protein
ME	metabolizable energy
MOB	mature oil bodies
MTP	microsomal triacylglycerol transfer protein
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	reduced nicotinamide adenine dinucleotide (phosphate)
NANA	*N*-acetyl neuraminic acid
NEFA	non-esterified fatty acid(s)
NMR	nuclear magnetic resonance
NSAID	non-steroidal anti-inflammatory drug(s)
NSF	*N*-ethylmaleimide sensitive factor
PAF	platelet activating factor
PAP	phosphatidate phosphohydrolase
PAPS	phosphoadenosine phosphosulphate
PG	prostaglandin
PH	pleckstrin homology
PLC	phospholipase C
PPAR	peroxisome proliferator activated receptor
PPRE	peroxisome proliferator response element(s)
(m)RNA	(messenger) ribonucleic acid
SCD	stearoyl-CoA desaturase
SCOT	support coated open tubular
SP(A, B, etc.)	surfactant protein (-A, -B, etc.)
SR-BI	scavenger receptor class B type I
SRE(BP)	sterol regulatory element (binding protein)
SRS	slow reacting substance
TAG	triacylglycerol
TCA	tricarboxylic acid
TLC	thin layer chromatography
TX	thromboxane
UCP	uncoupling protein
UDP	uridine diphosphate
VLDL	very low density lipoprotein(s)
VSR	variant surface glycoprotein
WCOT	wall coated open tubular

Preface

The aims of the first edition of this book in 1970 were first to aid students in learning about lipids as well as helping staff to teach the subject; secondly to encourage students to research in this field. Over 30 years, those aims have not altered but the subject itself has advanced greatly, necessitating the participation of a third author and extensive revisions of the text. Indeed, many sections and some whole chapters have been completely rewritten, but we hope that in so doing we have not decreased the readability and accessibility that we believe have been important features of this little book.

It is inevitable that addition of new material must be accompanied by some deletions if the book is to remain affordable. In general, the losses have been in certain historical material and in some detailed methods that were described in individual chapters in previous editions.

Chapter 1 now deals with definitions and routine analytical methods. A largely rewritten chapter 2 (previously chapter 3) covers current knowledge of fatty acid biochemistry including many recent advances in our understanding of the enzymes involved. This and many other changes in this edition reflect the enormous strides made in molecular biology and their applications to the study of lipids. Chapter 3 on lipids as energy stores has been updated to include the latest information on the enzymes of triacylglycerol biosynthesis and in particular the regulation of triacylglycerol metabolism and its integration into intermediary metabolism more generally. Chapter 4 on dietary lipids covers lipids in foods, including changes introduced during modern industrial processing. The second part deals with essential dietary lipids – the essential fatty acids and the fat-soluble vitamins. The subjects of lipid digestion, absorption and transport, previously included with dietary lipids, have now been allocated a separate chapter (5) called simply 'lipid transport'. This chapter focuses on current understanding of the nature and functions of the plasma lipoproteins. It includes a detailed discussion of several diseases to which aberrations in lipoprotein function make a major contribution. There have been significant advances in our understanding of these processes since the last edition and this completely rewritten chapter reflects this new knowledge. Chapter 6, as before, covers the roles of lipids in cellular structures and chapter 7 describes their metabolism.

Items in chapter 8 of the fourth edition that were then regarded as being of special interest, have now been incorporated into other chapters as appropriate. However, we realize that there are always research issues that generate debate and controversy, where there are few facts and much speculation. We have included such material where we thought it appropriate, sometimes in the form of a 'box' and have indicated where there are issues that are not yet fully resolved. We hope that these will add interest and teach students that not all material that enters a textbook is written on tablets of stone. Even when these 'hot topics' eventually become tomorrow's 'dead ducks', these features may serve to illuminate the ways in which lipid biochemistry moves forward.

We recognize that modern textbooks of general biochemistry cover the subject of lipids with a thoroughness that did not occur in 1970 when the first edition was published. We contend that in this book we bring a degree of detail and expertise to our material, to which even now the general textbooks cannot aspire. Nevertheless, such has been the progress in lipid research recently that we cannot cover all topics in the depth that we would like. Therefore an essential feature in each chapter is the list of further reading, which we hope will be useful. We regard this approach as more appro-

priate to this book than the alternative of referencing statements made throughout the text.

Lipidology has always been encumbered by complex, difficult, and sometimes obscure nomenclature. The problem has not been made easier by developments in modern molecular biology. Our philosophy has been, in general, to avoid abbreviations when they were not absolutely necessary. However, when long and complex names are repeated continually, it is practical and sensible to use abbreviations. We have been careful to define all our abbreviations and have provided a list of all those used, whether once or many times. As students will inevitably come across abbreviations in other places, sometimes with no definition, we hope our list will prove useful to students of the subject.

Finally, it is a pleasure to acknowledge the contributions of friends and colleagues in reading intermediate drafts of the text and supplying valuable information that we have incorporated.

To all our readers: happy reading! Enjoy your lipids!

M.I. Gurr, St Mary's, Isles of Scilly
J.L. Harwood, University of Cardiff
K.N. Frayn, University of Oxford

Acknowledgements

Over the years we have received invaluable assistance from many colleagues in the compilation of this book and our thanks have been recorded in previous editions. With regard to Edition 5, our special thanks are due to Dr Peter Dodds for reading and commenting upon several parts of the manuscript and to Dr Rosalind Coleman for allowing us to have access to a pre-publication copy of her excellent review: *Physiological and Nutritional Regulation of Enzymes of Triacylglycerol Synthesis* (see *Further Reading* in chapter 3). We have also received much help and encouragement from other colleagues, too numerous to mention individually, and we thank them all and hope they enjoy the product.

DEDICATION

We are honoured to dedicate this new edition of *Lipid Biochemistry: An Introduction* to the late Professor Konrad E. Bloch, who died in October 2000 and whose profound influence on the development of lipid biochemistry is amply illustrated in this book.

1 Lipids: definition, isolation, separation and detection

1.1 INTRODUCTION

Lipids occur throughout the living world in micro-organisms, higher plants and animals. They occur in all cell types and contribute to cellular structure, provide stored fuel and participate in many biological processes ranging from transcription of the genetic code to regulation of vital metabolic pathways and physiological responses. In this book, they will be described mainly in terms of their functions, although from time to time it will be convenient, even necessary, to deal with lipid classes based on their chemical structures and properties.

1.2 DEFINITIONS

Lipids are defined on the basis of their solubility properties, not primarily their chemical structure.

The word 'lipid' (in older literature spelled also as lipide or lipoid) is used by chemists to denote a chemically heterogeneous group of substances having in common the property of insolubility in water, but solubility in non-aqueous solvents such as chloroform, hydrocarbons or alcohols. It is necessary to use this definition based on physical properties since there may be little or no chemical relationship between the numerous compounds now classified as lipids, many of which are described in this book. It is not always possible to discern a clear distinction between the terms fat and lipid. The term fat is more familiar to the layman and brings to mind substances that are clearly fatty in nature, greasy in texture and immiscible with water. Familiar examples are butter and the fatty parts of meats. Fats are thought of as solid in texture as distinct from oils which are liquid at ambient temperatures. Chemically, however, there is little distinction between a fat and an oil, since the substances that the layman thinks of as edible fats and oils are composed predominantly of esters of glycerol with fatty acids. These are called triacylglycerols and are chemically quite distinct from the oils used in the petroleum industry, which are generally hydrocarbons. The term 'lipid' to the chemist embraces the wider range of fatty substances that are described in this book.

1.3 STRUCTURAL CHEMISTRY AND NOMENCLATURE

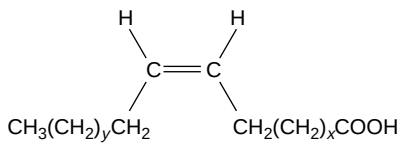
Naming systems are complex and have to be learned. The naming of lipids often poses problems. When the subject was in its infancy, research workers gave names to substances that they had newly discovered. Often, these substances would turn out to be impure mixtures and as the chemical structures of individual lipids became established, rather more systematic naming systems came into being. Later, these were further formalized under naming conventions laid down by the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB). Thus, triacylglycerol is now preferred to triglyceride, but the latter is still frequently used especially by nutritionists and clinicians and you will need to learn both (Chapter 3). Likewise, outdated names for phospholipids: 'lecithin' (for phosphatidylcholine, Chapter 6) and 'cephalin' (for an ill-defined mixture of phosphatidylethanolamine and phosphatidylserine) will be avoided in this book but you should be aware of their existence. Further reference to lipid naming and structures will be given in appropriate chapters.

The very complex naming of the fatty acids is

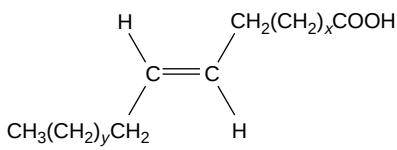
discussed in detail in Chapter 2. Their main structural features are their chain lengths, the presence of unsaturation (double bonds) and of substituent groups. In regard to chain length, it is cumbersome to have to say every time: 'a chain length of ten carbon atoms' and we shall, therefore, refer to a '10C fatty acid'. If we wish to refer to a specific carbon atom in a chain, we shall write, for example: 'the substituent at C10'. The numbering of fatty acid carbon atoms is done from the carboxyl end of the chain with the carboxyl carbon as C1. An old system of identifying carbon atoms was to give them Greek letters. Thus, C2 was the α-carbon, C3 the β-carbon and so on, ending with the ω-carbon as the last in the chain, furthest from the carboxyl carbon. Remnants of this system still survive and can sometimes be discerned in this book. However, we will routinely use the numbering, rather than the Greek lettering system. Hence, we will use 3-hydroxybutyrate, not β-hydroxybutyrate etc.

An important aspect of unsaturated fatty acids is the opportunity for isomerism, which may be either positional or geometric. Positional isomers occur when double bonds are located at different positions in the carbon chain. Thus, for example, a 16C monounsaturated fatty acid may have positional isomeric forms with double bonds at C7 or C9, sometimes written Δ7 or Δ9. (The position of unsaturation is numbered with reference to the first of the pair of carbon atoms between which the double bond occurs.) Two positional isomers of an 18C diunsaturated acid are illustrated in Fig. 1.1(c) and (d). Geometric isomerism refers to the possibility that the configuration at the double bond can be *cis* or *trans*. (Although the convention Z/E is now preferred by chemists instead of *cis*/*trans*, we shall use the more traditional and more common *cis*/*trans* nomenclature throughout this book.) In the *cis* form, the two hydrogen substituents are on the same side of the molecule, while in the *trans* form they are on opposite sides [Fig. 1.1(a) and (b)].

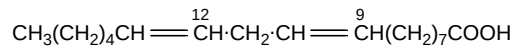
Another important feature of biological molecules is their stereochemistry. In lipids based on glycerol, for example, there is an inherent asymmetry at the central carbon atom of glycerol. Thus, chemical synthesis of phosphoglycerides yields an equal mixture of two stereoisomeric forms, whereas almost all naturally occurring phosphoglycerides have a single stereochemical configuration, much in the same way as most natural amino acids are of the L (or *S*) series. In the past, naturally occurring compounds were designated L-α- and represented by a Fisher projection (see Fig. 1.2). The glycerol derivative was put into the same category as that glyceraldehyde into which it would be transformed by oxidation, without any alteration or removal of substituents. Phosphatidylcholine was therefore named: L-α-phosphatidylcholine. The IUPAC–IUB



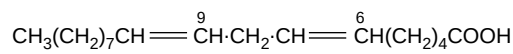
(a) *cis* (Z)



(b) *trans* (E)



(c) *cis*, *cis* –9, 12–octadecadienoic acid



(d) *cis*, *cis* –6, 9–octadecadienoic acid

Fig. 1.1 Isomerism in unsaturated fatty acids.

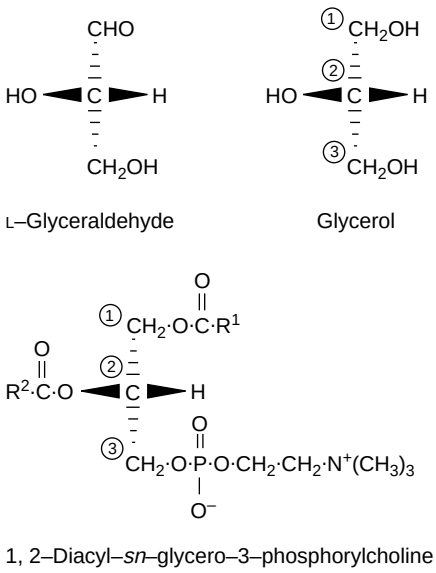


Fig. 1.2 The stereochemical numbering of lipids derived from glycerol.

convention has now abolished the DL (or even the more recent *RS*) terminology and has provided rules for the unambiguous numbering of the glycerol carbon atoms. Under this system, phosphatidylcholine becomes, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine or more shortly, 3-*sn*-phosphatidylcholine. The letters *sn* denote the *stereochemical numbering* and indicate that this system is being used. The stereochemical numbering system is too cumbersome to use routinely in a book of this type and, therefore, we shall normally use the terms 'phosphatidylcholine' etc., but introduce the more precise name when necessary.

Another field in which nomenclature has grown up haphazardly is that of the enzymes of lipid metabolism. This has now been formalized to some extent under the Enzyme Commission (EC) nomenclature. The system is incomplete and not all lipid enzymes have EC names and numbers. Moreover, the system is very cumbersome for routine use and we have decided not to use it here. You will find a reference to this nomenclature in the reading list should you wish to learn about it.

1.4 EXTRACTION OF LIPIDS FROM NATURAL SAMPLES

Since lipids are characterized generally by their ability to dissolve in water-immiscible organic solvents, advantage is taken of this property at many stages of analysis.

Extraction of lipids from natural samples, like that of many biological molecules, is best accomplished as soon as possible to minimize the degradative changes which would otherwise take place. When storage has to take place it is best done at as low a temperature as possible (say -20°C or less) and under an inert nitrogen atmosphere to minimize oxidation of groups such as double bonds. If great care is not taken during the extraction process, many lipids will be partly or completely lost. For example, the polyphosphoinositides (Sections 6.2.1, 7.1, 7.2, 7.9) are extremely labile, due to the very active degradative enzymes present in many animal tissues. Similar degradative enzymes can pose particular problems in plant tissues since they are active at very low temperatures (certainly at -20°C) and also retain activity (or may even be activated) in organic solvents! Such enzymes are obviously best inactivated as quickly as possible by, for example, brief exposure of the tissue to steam or boiling water or by a prior extraction with hot isopropanol – all measures which inactivate the lipases. In fact, these lipases may be so active that it may prove impossible to separate membrane fractions from some plant tissues because of continuous degradation during the necessary centrifugation steps!

The actual extraction method depends on the type of tissue and also the lipids it is desired to analyse. However, few lipids can be extracted by a single solvent and binary mixtures are usually used. One of the components should have some water solubility and hydrogen bonding ability because lipid–protein complexes, such as those encountered in membranes, have to be split.

Common methods are often based on the original procedure of Bligh and Dyer. The latter used a mixture of chloroform and methanol in a ratio with tissue water (1:2:0.4) to form a one-phase system. Homogenization of tissues in this mixture efficiently extracts most lipids. More chloroform

and methanol are then added to give two phases, the upper (aqueous) one containing non-lipid impurities; the lower (chloroform) phase can then be removed, washed with fresh upper phase and finally evaporated to dryness. Residual water can be removed with anhydrous sodium sulphate or by filtration through Sephadex columns. In some cases it may be desirable to use salt or dilute acid solutions in the upper phase to prevent losses of polar lipids. Finally, after removing solvent by vacuum evaporation, the crude lipid residue should be protected from oxidation by inert nitrogen gas. In fact, before storage for any length of time, lipids are best re-dissolved in a small amount of solvent containing an antioxidant such as 3,5-di-*t*-butyl-4-hydroxytoluene (BHT) before storage at $-20^{\circ}C$ or less in the dark under nitrogen.

1.5 LIKELY COMPONENTS OF THE CRUDE LIPID EXTRACT

Since the initial extraction has been based on solubility properties, the crude lipid extract will contain any molecule which also dissolves preferentially in the organic solvents used. Thus, significant quantities of non-lipids, e.g. hydrophobic proteins, may be present at this stage. The mixture of lipids (Table 1.1) will depend on the nature of the tissue extracted.

1.6 GENERAL FEATURES OF LIPIDS IMPORTANT FOR THEIR ANALYSIS

Characterization of a lipid requires separation from other components and detection based on chemical and physical features.

Most of the lipid classes cited as major components in different tissues (Table 1.1) contain esterified fatty acids. They are thus termed acyl lipids. This is important during analysis for several reasons:

(1) The nature of the fatty acids determines much of the physical and biological properties of the lipid and, therefore, it is of importance to analyse their properties.
(2) The type of fatty acid influences the stability of the sample.
(3) Because mixtures of fatty acids are found in any given lipid class, the latter contains a number of molecular species. Thus, for example, when one refers to the triacylglycerol content of a certain tissue one should say triacylglycerol*s* to show that a mixture of molecular species is present.

Table 1.1 Major components of typical lipid extracts from different tissues

Erythrocytes	Liver	Leaves	Cyanobacteria	Gram negative bacteria
Phosphoglycerides	Phosphoglycerides	Phosphoglycerides	Phosphoglycerides	Phosphoglycerides
Sphingolipids	Sphingolipids	–	–	–
–	–	Glycosyl-glycerides	Glycosyl-glycerides	–
Sterols	Sterols	–	–	–
–	Triacylglycerols	–	–	–
		Others[a,b]	Others[a]	Others[c]

[a] Pigments.
[b] Waxes, cutin.
[c] Lipopolysaccharide.

Sphingolipids and sterols are present in leaves but are minor components. Sterols in animals are predominantly cholesterol.

Total fatty acid analysis is usually conducted by forming volatile derivatives (such as methyl esters) for gas chromatography. Special techniques have to be used occasionally to avoid destroying unusual functional groups such as cyclopropene rings. Moreover, the danger of auto-oxidation means that analysis of samples containing polyunsaturated fatty acids has to be especially careful. For complete identification of individual acyl groups, degradation or derivatization usually has to be employed – for example, when double bond positions have to be assigned.

In order to determine the positional distribution of acyl groups on, say, the glycerol backbone, enzymic cleavage is usually utilized. For example, phospholipases are available which have a specificity for either the *sn*-1 or the *sn*-2 position. Use of such enzymes will release fatty acids from one position and these can be separated from the partly deacylated product. Analysis of fatty acids and deacylated lipid will then reveal which fatty acids are present at each glycerol carbon.

Molecular species of lipids can be separated on the basis of size and/or unsaturation. In the past it has often been necessary to derivatize or remove the polar part of the lipid making analyses time-consuming. For example, because the charge on the head-group of phosphatidylcholine is large in relation to differences in acyl unsaturation, it was usually necessary to degrade such phosphoglycerides to diacylglycerols before analysis by chromatography. Modern methods of *H*igh *P*erformance *L*iquid *C*hromatography (HPLC) have, however, rendered such methods unnecessary provided that adequate methods of detection are available.

Single lipid classes can be separated from each other by methods which make use of differences in their size and charge. They can often be provisionally identified by co-chromatography with authentic standards in various systems. Important constituent groups will be revealed by spectroscopic techniques or with specific colour reagents. However, unambiguous identification may require that the various products of hydrolysis be isolated, characterized and quantified. When enzymes are used to cause hydrolysis, their action (or otherwise) may also provide information about the stereochemistry of particular linkages.

1.7 CHROMATOGRAPHIC TECHNIQUES FOR SEPARATING LIPIDS

The principles of chromatography are based on distribution between two phases, one moving, the other stationary.

1.7.1 The two phases can be arranged in a variety of ways

A chromatogram (so-named by its Polish inventor, Tswett, because he used the technique to separate plant pigments) consists of two immiscible phases. One phase is kept stationary by either being held on an inert microporous support or being itself a microporous or particulate adsorbent solid; the other phase is percolated continuously through the stationary phase. Various phase pairs are possible although liquid–solid and gas–liquid are the most common.

If we take any single substance and mix it with any of these phase pairs, it will distribute itself between the two phases, the ratio of the concentrations in the two phases (at equilibrium) being known as the partition coefficient.

The partition coefficient is a physical constant dependent on the nature and magnitude of solute–solvent interactions in the two phases. Let us consider two substances A and B and imagine that, at equilibrium, substance A distributes itself between the two phases so that 90% is in the stationary phase and 10% in the moving phase. Substance B, however, distributes so that 10% is in the stationary phase and 90% in the moving phase. Then a mixture of A and B dissolved in a small volume of moving phase and applied to the chromatogram will begin to separate when the moving phase is added and washes them through the system. They will distribute themselves independently of one another: B will move as a zone at 9/10ths of the velocity of the moving phase and A at 1/10th of the velocity. Clearly the two substances will rapidly move apart. After separation, the substances can either be

visualized directly on the system by colour sprays or be eluted as pure components.

There are basically two types of chromatogram geometry:

(1) The *column* consists of a metal, glass or even plastic tube with a ratio of length to diameter of at least 10:1 and is packed with either an absorbent solid (silica gel, alumina etc.) or an inert solid, such as Kieselguhr, of large surface area, that can hold, by surface tension, a liquid as one member of the phase pair. Gas chromatograms can afford a much greater ratio of length to diameter because of their inherently lower resistance to flow than can liquid columns.
(2) The *plate* or *strip* consists of the stationary phase support arranged as a flat surface. Mixtures can be spotted and dried on the surface and when the bottom of the plate is immersed in the moving phase in a closed vessel, capillarity ensures that the liquid will move through the porous material (paper, or porous solid held to a glass or metal surface). The fact that both phases exist as relatively thin films means that the solutes have only short distances to move as they pass from phase to phase. Very refined separations can thus be obtained rapidly. The thin layer chromatogram is particularly useful for lipid separations.

In general, compounds are not eluted from flat plate chromatograms during development (i.e. movement of the liquid phase). Instead, the development is stopped just before the solvent front has reached the end of the strip which is dried to remove solvent and the position of the zones revealed by spraying. The sprays can be of a destructive type such as dilute sulphuric acid followed by heating (this produces black spots by carbonization where there is an organic material) or a non-destructive type such as dichloro- or dibromo-fluoresceins that show a changed fluorescence where there is a zone. In the latter case, the lipids can be recovered by scraping the adsorbent from the plate (wherever the spray has indicated that a compound is present) and extracting with a suitable solvent. Under standard conditions it will be found that a given substance will move relative to a standard substance to a constant ratio or relative R_f (Fig. 1.3). This relative R_f is a useful confirmation of structure of an unknown substance, but it is unwise to use it as an absolute indicator.

Only infrequently can every component of a complex mixture be resolved by one solvent system. However, by using two-dimensional development refined separations can be achieved. These involve carrying out a separation with one solvent, drying the plate, turning it at right angles and using a second solvent system for development.

1.7.2 Gas–liquid chromatography is a particularly useful method for volatile derivatives of lipids

With this method the moving phase is a permanent gas and columns contain either an inert support (e.g. Celite) on the surface of which is the stationary liquid phase or else are themselves narrow tubes on the wall of which is a thin layer of the stationary phase. The latter are known as wall-coated-open-tubular (WCOT) or capillary columns. Because no inert support is used in these columns, the flow of gas is relatively unimpeded and very long thin columns (say 100 m long and only 0.25 mm internal diameter) capable of quite remarkable separations are possible (see Fig. 1.4). Columns containing an inert support may be either packed or support-coated-open-tubular (SCOT). Packed columns are the 'work horses' of gas chromatography and glass or stainless steel columns are usual. Glass columns are better because they are almost completely inert and any breaks or deterioration in the packing material can be seen easily. Of course, they are fragile – as many students know to their cost!

In a typical gas chromatograph the column is held in an oven either at a fixed temperature (isothermal operation) or at a temperature which increases during the separation (temperature programming). The latter method is particularly useful when the mixture to be analysed contains components with a wide range of molecular weights (Fig. 1.4). Located at the end of the column is the detector

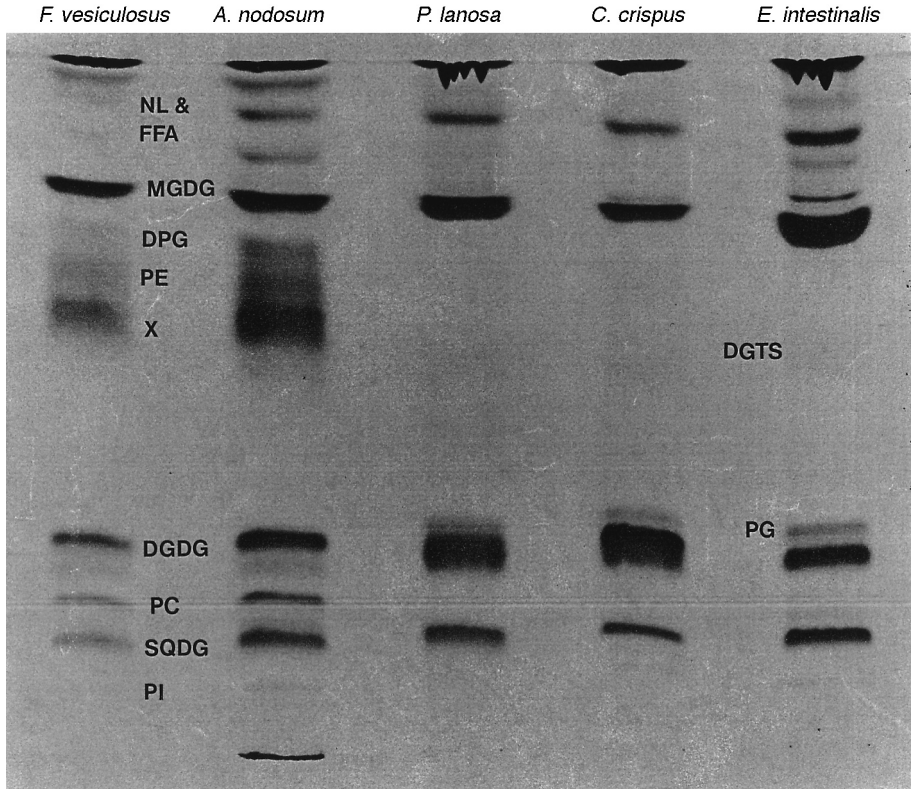
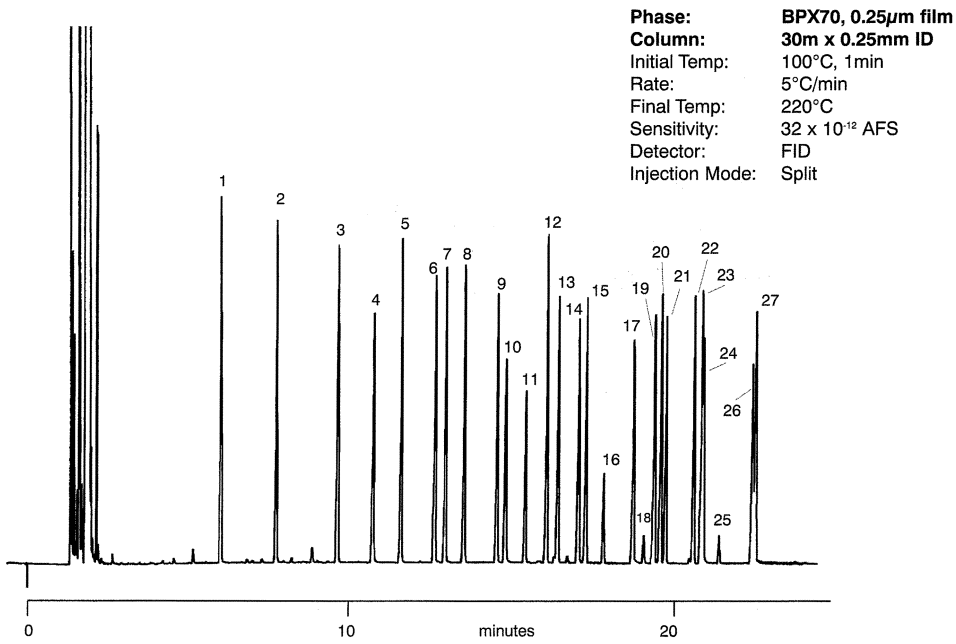


Fig. 1.3 Separation of lipid classes by thin layer chromatography (from A. L. Jones with kind permission). Total lipids from five different marine algae were run on a silica gel thin layer plate using a solvent mixture of chloroform/methanol/acetic acid/water (170:30:20:7 by volume) and the lipids revealed (non-destructively) with iodine vapour. [Abbreviations: NL, neutral lipids (e.g. triacylglycerol); FFA, (free) non-esterified fatty acids; MGDG, monogalactosyldiacylglycerol; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; X, trimethyl-β-alanine diacylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; SQDG, sulphoquinovosyldiacylglycerol; PI, phosphatidylinositol; DGTS, diacylglyceroltrimethylhomoserine; PG, phosphatidylglycerol.]

which quantifies the eluted components. For lipids the flame ionization detector is usually used. Eluates are burned in a flame of hydrogen and air to produce ions which are detected and measured by an electrical device capable of detecting about 10^{-12} g ml^{-1}. Flame ionization detectors respond to almost all organic compounds, have a good signal-to-noise ratio and are robust. If required, a T-piece can be used just before the detector so that fractions can be collected or separate radioactivity measurements (in, for example, a gas-flow proportional counter) and mass spectrometric analysis undertaken.

Individual components separated at a fixed temperature (isothermal use) and with constant gas flow can be provisionally identified by reference to standard mixtures run under the same conditions. However, because of the difficulties of keeping conditions exactly identical and also because of slow changes in the column's characteristics, it is better to use *relative retention time* to provisionally identify particular components. The comparison is usually made with a known component in the mixture or with an internal standard (which can also be used for quantitation and which, itself, is a compound not present in the original mixture). By



1. C11:0; 2. C12:0; 3. C13:0; 4. C10:0 2-OH; 5. C14:0; 6. C15:0-*i*; 7. C15:0-a; 8. C15:0; 9. C12:0 2-OH; 10. C16:0-*i*; 11. C16:0; 12. C15:1n7*cis*; 13. C12:0 3-OH; 14. C17:0-a; 15. C17:0; 16. C9, 10-methylene 16:0; 17. C14:0 2-OH; 18. C18:0; 19. C18:1n9-*trans*; 20. C18:1n9-*cis*; 21. C18:1n7-*cis*; 22. C18:2n6-*cis*; 23. C19:0; 24. C16:0 2-OH; 25. C9, 10-methylene 18:0; 26. C20:0; 27. C18:0 2-OH. a = anteiso-branched; i = iso-branched.

Fig. 1.4 Separation of fatty acid methyl esters by gas–liquid chromatography on a capillary column. The column was 30 m long and only 0.25 mm internal diameter. Initial temperature was 100°C and this was then increased at 5°C min^{-1} to 220°C. Note that some very small differences in compounds can be detected. For example, peaks 19–21 are all of octadecenoic acids that only differ in the position or configuration of their single double bond. (Reproduced with permission from SGE Europe Ltd, Products catalolgue, p. 92.)

the use of different column packings, confirmation of these identities can be made although, for complete analysis, some degradative methods must be used also. A rather convenient technique uses a combination of gas chromatography and mass spectrometry, where the eluate is split so that part goes to the detector and the remainder enters a mass spectrometer for (fragmentation) analysis.

Gas–liquid chromatography (GLC) can, of course, only be used for compounds which can be volatilized without decomposition. In practice this normally limits its application to molecules with molecular mass of less than 800 Da. Even so many lipids, such as phosphoglycerides, are not amenable to analysis as intact molecules.

Perhaps the most important use for the lipid biochemist is in the analysis of the fatty acid composition of lipids or tissues. In addition, gas chromatography may be used for steroid, glycerol, sphingosine, inositol and carbohydrate analysis. Molecular species of partial glycerides or, on highly thermostable silicone liquid phases, even triacylglycerols may be analysed also.

1.7.3 Adsorption column chromatography is used for the separation of large amounts of lipids

In this technique a moving liquid phase elutes lipids selectively from a solid support. Traditionally the most useful columns for lipid separations have been those containing silicic acid. Polar lipids tend to be tightly adsorbed onto these and can be

separated easily from less polar materials such as sterol (-esters) or triacylglycerols. By an appropriate choice of elution solvents, good separations may also be obtained within a single lipid class – say phospholipids. Two simple methods are summarized in Table 1.2. In method A, glycolipids are eluted with acetone, which although rather polar, fails to elute phospholipids because of the latter's poor solubility in this solvent. In method B, increasingly polar mixes of methanol and chloroform cause some separation between phospholipid classes. Other packing materials may be used instead of silicic acid. For example, columns containing DEAE-cellulose can be used to separate lipids carrying a net negative charge from uncharged or zwitterionic (equal numbers of positive and negative charges) molecules.

HPLC is increasingly used for lipid separations. Stainless steel columns packed with microspheres of silicic acid are most often used. Excellent separations of a host of different lipids can be made but, in the past, an insufficient number of suitable universal detectors for lipids has limited the use of HPLC. If only lipids had convenient absorbances then HPLC would become the separation method of choice for practically every occasion!

There are two solutions to the detection problem – either to use a detector which does not detect compounds by their absorbances or to derivatize the lipids for analysis so that they absorb in the UV or visible region and can, therefore, be readily measured. An example of derivatization is to convert fatty acids to their phenacyl derivatives. Fluorescent derivatives (e.g. anthrylmethyl esters) give a further increase in the sensitivity of detection.

Two general detectors are useful for lipids. The first is a differential refractometer, which senses minute differences in the refractive index of an eluant brought about by material eluting from the column. However, it can only be used easily with a solvent of constant composition. A second method is to use an evaporative light-scattering detector. In this technique the eluate is evaporated in a jet of air to produce a 'mist' of (lipid) particles which scatter light. The method is sensitive and can be used with almost any lipid. Unlike the differential refractometer, it is not affected by solvent gradients although it does need solvents that are volatile.

Table 1.2 Separation of complex lipids on silicic acid columns

Fraction	Compound eluted	Solvent	Column volume
Method A			
1	Simple lipids	Chloroform	10
2	Glycolipids (and traces of acidic phospholipids)	Acetone	40
3	Phospholipids	Methanol	10
Method B (for fraction 3 separated above)			
1[a]	DPG + PA	Chloroform/methanol (95:5)	10
2[b]	PE + PS	Chloroform/methanol (80:20)	20
3[c]	PI + PC	Chloroform/methanol (50:50)	20
4	Sphingomyelin + lyso PC	Methanol	20

[a] DPG = diphosphatidylglycerol; PA = phosphatidic acid.
[b] PE = phosphatidylethanolamine; PS = phosphatidylserine.
[c] PI = phosphatidylinositol; PC = phosphatidylcholine.

Adapted from Christie W.W. (1982) *Lipid Analysis*, 2nd edn, Pergamon Press, Oxford.

1.7.4 Thin layer adsorption chromatography can achieve very good separation of small lipid samples

In this technique a thin (usually 0.25 mm) layer is spread on a glass plate and separations are achieved by allowing appropriate solvents to rise up the plate by capillary action. Silica gel is the most usual adsorbent and samples, which are applied close to the edge of the plate, are fractionated due to their different adsorptions to the support phase and/or their solubility in the solvent. Very good separations can be achieved although the thickness of the layer means that only small amounts of lipids are usually analysed. When the plates have been run, they are dried and can be sprayed with various reagents to reveal individual lipid classes. If the spray reagent is non-destructive (e.g. 2′,7′-dichlorofluorescein, which causes all lipids to fluoresce) then the lipids can be recovered by scraping the appropriate area off the plate and eluting with a suitable solvent.

Complex lipid mixtures cannot always be separated by one-dimensional chromatography. In that case, either a preliminary fractionation on a column (to give say, neutral, glycolipid and phospholipid fractions) or two-dimensional thin layer chromatography (TLC) can be used. An example of the separation of lipid extracts from a variety of tissues by TLC is given in Fig. 1.3.

TLC offers a number of advantages over column chromatography. It is more rapid and sensitive, gives better resolution and is usually much quicker. Moreover, the apparatus required is minimal and, especially if plates are made in the laboratory, the technique is inexpensive. By incorporating various chemicals in the thin layer, special lipid separations can be made. For example, silver nitrate allows fatty acids (or more complex lipids) to be separated on the basis of their unsaturation. Silicone oil–silica gel TLC works on the basis of reverse-phase separation and can be used to fractionate fatty acid mixtures based on their hydrophobicity, with shorter chain or unsaturated components migrating faster. Boric acid impregnation allows separation of *threo*- or *erythro*-isomers of vicinal diols or fractionation of molecular species of ceramides.

A number of useful chemical methods is available for detecting or quantifying lipid classes. In some cases, the specificity of the colours developed can be used to help with identifications. Also when a non-destructive fluorescent reagent is used, lipids can be quantified on the plates by a scanning fluorimeter – though, because different lipids have different fluorescence values, calibration curves must be made.

Specific chemical methods can be used to reveal phospholipids, glycolipids, sterols or their esters as well as compounds with quaternary nitrogens or vicinal diols. Particularly useful for many membrane extracts is the reaction of ammonium molybdate with inorganic phosphate released from phospholipids. The phosphomolybdic acid so produced is then reduced to give an intense blue colour. The method can be adapted to a spray reagent or, more often, used to detect as little as 1 μg of phosphorus in scraped samples.

1.8 OTHER USEFUL METHODS

A detailed discussion of the wide variety of methods that are available to the lipid biochemist for identification and analysis is beyond the scope of this book. However, a few of the more widespread techniques are listed in Table 1.3.

1.9 SUMMARY

In contrast to carbohydrates, proteins and nucleic acids, lipids are defined on the basis of their physical properties (insolubility in water) rather than on the basis of consistent chemical features. For this reason, the student will need to learn and remember a wide range of different chemical types and their rather complex nomenclature.

Lipids can usually be extracted easily from tissues by making use of their hydrophobic characteristics. However, such extractions yield a complex mixture of different lipid classes which have to be purified further for quantitative analysis. Moreover, the crude lipid extract may be con-

Table 1.3 Some other methods for lipid analysis

Method	Use
IR spectroscopy	Identification of organic bases in phospholipids or *trans*-double bonds.
NMR spectroscopy	Widely used for lipid structure determination particularly identification and location of double bonds in fatty acids, functional groups (e.g. hydroxyl) on fatty acids and preliminary identification of glycerides, glycolipids and phospholipids. ^{1}H spectra give better quantitative data but ^{13}C spectra provide more information on functional groups. ^{31}P spectra are used often to assess solid/liquid phase ratios of lipids.
UV spectroscopy	Analysis of conjugated double bond systems, especially those formed by oxidation reactions.
Supercritical fluid chromatography	Supercritical fluid chromatography uses a compressed gas (often CO_2) above its critical temperature and pressure to elute compounds from a column. Can be used at lower temperatures than GLC and more detectors are available than for HPLC.
Mass spectrometry	Often linked to a gas chromatogram or HPLC. Has a wide variety of uses from the estimation of molecular weights (e.g. use of thermospray mass spectrometry on-line with an HPLC to quantify molecular species) to the location of double bonds in aliphatic chains or identification of functional groups. Different sample inlet systems (direct insertion, fast atom bombardment) and ion sources (electron or chemical ionization) are used depending on the lability of the sample and whether structural elucidation or detection and quantitation are the main aims.
Stable isotopes	This technique makes use of stable isotopes of H, C and O and mass spectrometry. It can be utilized for quantification of substances in mixtures (by isotope dilution), in metabolic experiments, to detect food adulteration and to determine sources of lipids in geological sediments or ecological samples.
Enzyme degradation	Used to show nature of specific bonds and substituents in lipids. For example, the positional distribution of acyl groups can be determined or the presence of individual sugars or bases revealed. The stereo-specificity of most enzymes also allows the configuration of the target linkage to be demonstrated.

taminated by other hydrophobic molecules, e.g. by intrinsic membrane proteins.

Of the various types of separation, thin layer and column chromatography are most useful for intact lipids. High performance liquid chromatography (HPLC) is becoming increasingly used. With the development of the evaporative light-scattering detector (sometimes called the 'universal' detector) its utility has been considerably enhanced and HPLC has the twin advantages that it is usually used at room temperature and that lipid oxidation is much reduced compared to thin-layer methods.

A powerful tool for quantitation of the majority of lipids is gas–liquid chromatography (GLC). The method is very sensitive and, if adapted with capillary columns, can provide information with regard to such subtle features as the position or configuration of substituents along acyl chains. By using light-scattering detectors with HPLC, this method can also be made quantitative. By coupling GLC or HPLC to a radioactivity detector, then the techniques are also very useful for metabolic measurements.

Although research laboratories use generally sophisticated analytical methods such as GLC to analyse and quantify lipid samples, chemical derivatizations are often used in hospitals. For these methods, the lipid samples are derivatized to yield a product which can be measured simply and accurately – usually by colour. Thus, total

triacylglycerol, cholesterol or phospholipid-phosphorus can be quantified conveniently without bothering with the extra information of molecular species etc. which might be determined by more thorough analyses.

FURTHER READING

Christie, W.W. (1982) *Lipid Analysis*, 2nd edn. Pergamon Press, Oxford. [A new edition of this book is in preparation and will be published by The Oily Press, Bridgwater, UK.]

Christie, W.W. (1987) *High-performance Liquid Chromatography and Lipids*. Pergamon Press, Oxford. [A new edition of this book is in preparation and will be published by The Oily Press, Bridgwater, UK.]

Christie, W.W. (1989) *Gas Chromatography and Lipids*. The Oily Press, Ayr, UK.

Christie, W.W., ed. (1997) *Advances in Lipid Methodology*, 4 vols. The Oily Press, Ayr, UK.

Gunstone, F.D., Harwood, J.L. & Padley, F.B., eds. (1994) *The Lipid Handbook*, 2nd edn. Chapman and Hall, London.

Hamilton, R.J. & Hamilton S., eds. (1992) *Lipid Analysis: a Practical Approach*. IRL Press, Oxford.

International Union of Biochemistry and Molecular Biology (1992) *Biochemical Nomenclature and Related Documents*, 2nd edn. Portland Press, London.

IUPAC–IUB Commission on Biochemical Nomenclature (1978) *Biochemical Journal*, **171**, 21–35.

IUPAC–IUB Commission on Biochemical Nomenclature (1987) *European Journal of Biochemistry*, **167**, 181–184.

IUPAC–IUB Commission on Biochemical Nomenclature (1989) *European Journal of Biochemistry*, **186**, 429–458.

Kates, M. (1986) *Techniques of Lipidology*, 2nd edn. Elsevier Science, Amsterdam. [This classic book lacks details about recent advances (e.g. HPLC) but still contains a wealth of basic information.]

Nomenclature Committee of the International Union of Biochemistry (1984) *Enzyme Nomenclature*. Academic Press, London. [The most up-to-date information on enzyme nomenclature can be found by accessing: http://www.chem.qmw.ac.uk/iubmb/enzyme/]

2 Fatty acid structure and metabolism

2.1 STRUCTURE AND PROPERTIES

Shorthand systems can help in specifying individual fatty acid structures in some detail. In lipid biochemistry – as in most other fields of science – various trivial names and shorthand nomenclatures have come into common usage. Thus, the poor student is faced with a seemingly endless series of illogical names and symbols for the various new compounds to be learnt. Fatty acid names are no exception and there are trivial names for most commonly occurring fatty acids as well as numerical symbols (as a shorthand) for given structures. The latter derive from the use of gas–liquid chromatography (Section 1.7.2) where separations are achieved due to both carbon chain length as well as unsaturation. The shorthand nomenclature consists of two numbers separated by a colon. The number before the colon gives the carbon number and the figure after denotes the number of double bonds. Thus a saturated fatty acid such as palmitic would be 16:0 while a monounsaturated acid such as oleic would be 18:1. Chain branching or substitution is denoted by a prefix, thus br-16:0 for a branched-chain hexadecanoic acid or HO-16:0 for an hydroxy-palmitic acid. This shorthand has the merit of not attributing a more precise identification than the gas chromatogram alone can give.

Where additional information such as the exact position and configuration of double bonds is known then this knowledge can be incorporated into both the shorthand nomenclature as well as the systematic names. For example, linoleic acid could be written as *cis* (Δ-) 9, *cis* (Δ-) 12-18:2 or (*cis*,*cis*) 9,12-octadecadienoic acid to indicate that it is an 18-carbon fatty acid with *cis* double bonds 9 and 12 carbons from the carboxyl end (see Chapter 1 for conventions on geometrical isomerism). Occasionally, because of their metabolic connections, it is useful to number the double bonds from the methyl end. In that case linoleic acid would become (*cis*,*cis*)*n*-6,9-octadecadienoic acid with the *n* (ω in older literature) showing that numbering has been from the methyl end.

2.1.1 Saturated fatty acids

Most saturated fatty acids are straight chain structures with an even number of carbon atoms. Acids from 2C to longer than 30C have been reported but the most common lie in the range 12–22C. Some of the more important naturally occurring straight chain saturated acids are shown in Table 2.1 together with further information. In general, fatty acids do not exist as free carboxylic acids because of their marked affinity for many proteins. (One result of this is an inhibitory action on many enzymes.) In fact, where free acids are reported as major tissue constituents they are usually artefacts owing to cell damage which allows lipases to break down the endogenous acyl lipids. Exceptions to this statement are the albumin-bound fatty acids of mammalian blood. Free acids used to be referred to as FFA (free fatty acids) but are preferably termed NEFA (non-esterified fatty acids).

The configuration of a typical saturated chain is shown in Fig. 2.1(a). Because of continuous thermal motion in living systems and the free rotation about the carbon–carbon bonds, the fatty acids are capable of adopting a huge number of possible configurations, but with a mean resembling an extended straight chain. Steric hindrance and interactions with other molecules in Nature will, of course, restrict the motion of non-esterified fatty acids and the acyl chains of complex lipids. The physical properties of acyl lipids are obviously affected by their individual fatty acids – a most obvious one being the melting point. As a general

Table 2.1 Some naturally occurring straight chain saturated acids

No. of carbon atoms	Systematic name	Common name	Melting point (°C)	Occurrence
2	*n*-Ethanoic	Acetic	16.7	As alcohol acetates in many plants, and in some plant triacylglycerols. At low levels widespread as salt or thiolester. At higher levels in the ruminant as salt.
3	*n*-Propanoic	Propionic	−22.0	At high levels in the rumen.
4	*n*-Butanoic	Butyric	−7.9	At high levels in the rumen, also in milk fat of ruminants.
6	*n*-Hexanoic	Caproic	−8.0	Milk fat.
8	*n*-Octanoic	Caprylic	12.7	Very minor component of most animal and plant fats. Major component of many milk and some seed triacylglycerols.
10	*n*-Decanoic	Capric	29.6	Widespread as a minor component. Major component of many milk and some seed triacylglycerols.
12	*n*-Dodecanoic	Lauric	42.2	Widely distributed, a major component of some seed fats (e.g. palm kernel or coconut oil).
14	*n*-Tetradecanoic	Myristic	52.1	Widespread; occasionally found as a major component.
16	*n*-Hexadecanoic	Palmitic	60.7	The most common saturated fatty acid in animals, plants and micro-organisms.
18	*n*-Octadecanoic	Stearic	69.6	Major component in animals and some fungi, minor constituent in plants (but major in a few, e.g. cocoa butter).
20	*n*-Eicosanoic	Arachidic	75.4	Widespread minor component, occasionally a major component.
22	*n*-Docosanoic	Behenic	80.0	Fairly widespread as minor component in seed triacylglycerols and plant waxes.
24	*n*-Tetracosanoic	Lignoceric	84.2	Fairly widespread as minor component in seed triacylglycerols and plant waxes.
26	*n*-Hexacosanoic	Cerotic	87.7	Widespread as component of plant and insect waxes.
28	*n*-Octacosanoic	Montanic	90.9	Major component of some plant waxes.

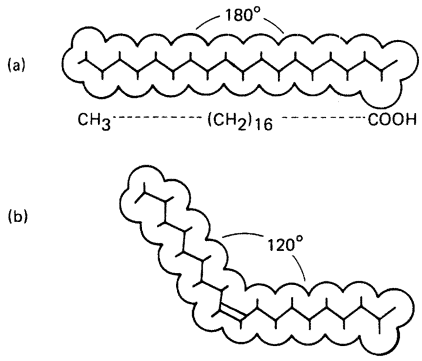


Fig. 2.1 Preferred conformation of (a) a saturated (stearic) and (b) a monounsaturated (oleic) acid.

rule, membranes are incapable of operating with lipids whose acyl chains are crystalline. Thus, for mammals, this means the acyl chains must be fluid at about 37°C and for poikilotherms (organisms unable to regulate their own temperature) at temperatures between about −10°C and over 100°C, depending on the organism. Although it is possible for the membranes of, for example, a mammal to contain lipids with acyl chains whose melting points are slightly above 37°C, the presence of other lipid types ensures that the mixture is in fact semi-liquid.

While most natural fatty acids are even-numbered, due to their mode of biosynthesis, odd-numbered acids do occur. The formation of both types is discussed in Section 2.2.3.

2.1.2 Branched-chain fatty acids

Although branched-chain fatty acids are usually also saturated, they are discussed separately here. Two distinct series, which are often found in bacteria, are the *iso*-series where the terminal group is:

$$\begin{array}{l} \quad CH_3 \\ \quad | \\ CH_3 - CH- \end{array}$$

and the *anteiso*-series where the terminal group is:

$$\begin{array}{l} \quad\quad CH_3 \\ \quad\quad | \\ CH_3 - CH_2 - CH- \end{array}$$

However, branch points can also be found in other positions. The presence of the side-chain has a similar effect on fluidity as the presence of a *cis* double bond (i.e. lowers the melting temperature). Branched-chain acids occur widely, but mainly at low concentrations in animal fats and some marine oils. They are rarely found in plant lipids. Butter fats, bacterial and skin lipids contain significant amounts. In the latter class, the uropygial (preen) gland of birds is a major source. Branched-chain fatty acids are major components of the lipids of Gram-positive bacteria and more complex structures with several branches may be found in the waxy outer coats of mycobacteria.

2.1.3 Unsaturated fatty acids

2.1.3.1 Monoenoic (monounsaturated) fatty acids

Over one hundred naturally occurring monoenoic acids have been identified but most of these are extremely rare. In general, the more common compounds have an even number of carbon atoms, a chain length of 16–22C and a double bond with the *cis* configuration. Often the *cis* bond begins at the Δ9 position. *Trans* isomers are rare but do exist, one of the most interesting being *trans*-3-hexadecenoic acid, a major fatty acid esterified to phosphatidylglycerol in the photosynthetic membranes of higher plants and algae.

The presence of a double bond causes a restriction in the motion of the acyl chain at that point. Furthermore, the *cis* configuration introduces a kink into the average molecular shape [Fig. 2.1(b)] while the *trans* double bond ensures that the fatty acid has an extended conformation and properties nearer to that of an equivalent chain length saturated acid (Tables 2.1 and 2.2). Because the *cis* forms are less stable thermodynamically than the *trans* forms, they have lower melting points than the latter or their saturated counterparts.

In addition to the normal *ethylenic* double bonds, some fatty acids possess *acetylenic* bonds. These occur in a number of rare seed oils and a few mosses.

Table 2.2 Some naturally occurring monoenoic fatty acids

No. of carbon atoms	Systematic name	Common name	Melting point (°C)	Occurrence
16	*trans*-3-hexadecenoic		53	Plant leaves; eukaryotic algae; specifically as component of phosphatidylglycerol in chloroplasts.
16	*cis*-5-hexadecenoic			Ice plant, Bacilli.
16	*cis*-7-hexadecenoic			Algae; higher plants, bacteria.
16	*cis*-9-hexadecenoic	Palmitoleic	1	Widespread: animals, plants, micro-organisms. Major component in some seed oils.
18	*cis*-6-octadecenoic	Petroselenic	33	Found in umbelliferous seed oils.
18	*cis*-9-octadecenoic	Oleic	16	Most common monoenoic fatty acid in plants and animals. Also found in most micro-organisms.
18	*trans*-9-octadecenoic	Elaidic	44	Ruminant fats, hydrogenated margarines.
18	*trans*-11-octadecenoic	*trans*-Vaccenic	44	Found in rumen fats via biohydrogenation of polyunsaturated fatty acids.
18	*cis*-11-octadecenoic	Vaccenic	15	*E. coli* and other bacteria.
20	*cis*-11-eicosenoic	Gondoic	24	Seed oil of rape; fish oils.
22	*cis*-13-docosenoic	Erucic	24	Seed oil of *Cruciferae* (rape, mustard, etc.).

2.1.3.2 Polyenoic (polyunsaturated) fatty acids

All dienoic acids are derived from monoenoic acids, the position of the second double bond being a function of the biochemical system. Thus, mammals have desaturases that are capable of removing hydrogens only from carbon atoms between an existing double bond and the carboxyl group. Because of this, further desaturations may need to be preceded by chain elongation. Higher plants on the other hand carry out desaturation mainly between the existing double bond and the terminal methyl group. In either case, the double bonds are almost invariably separated from each other by a methylene grouping (Table 2.3).

2.1.4 Cyclic fatty acids

These acids are rather uncommon but, nevertheless, examples provide important metabolic inhibitors. The ring structures are usually either cyclopropyl or cyclopentyl. Cyclopropane and cyclopropene fatty acids are produced by many bacteria and are also found in some plants and fungi (Table 2.4).

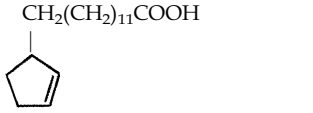
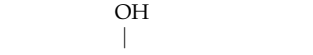
2.1.5 Oxy acids

A great range of keto, hydroxy and epoxy acids has been identified in recent years. The most widely occurring epoxy acid is vernolic acid. Hydroxy acids do not occur very extensively although they occur generally in some sphingolipids. They are

Table 2.3 Some naturally occurring polyunsaturated fatty acids

No. of carbon atoms	Systemic name	Common name	Melting point (°C)	Occurrence
Dienoic acids				
18	*cis,cis*-6,9-octadecadienoic		−11	Minor component in animals.
	cis,cis-9,12-octadecadienoic	Linoleic	−5	Major component in plant lipids. In mammals it is derived only from dietary vegetables, and plant and marine oils.
Trienoic acids (methylene interrupted)				
16	All-*cis*-7,10,13-hexadecatrienoic			Higher plants and algae.
18	All-*cis*-6,9,12-octadecatrienoic	γ-Linolenic		Minor component in animals and some algae. Important constituent of some plants.
	All-*cis*-9,12,15-octadecatrienoic	α-Linolenic	−11	Higher plants and algae, especially as component of galactosyl diacylglycerols.
Trienoic acids (conjugated)				
18	*cis*-9,*trans*-11,*trans*-13-octadecatrienoic	Eleostearic	49	Some seed oils, especially Tung oil.
Tetraenoic acids				
16	All-*cis*-4,7,10,13-hexadecatetraenoic			*Euglena gracilis*
20	All-*cis*-5,8,11,14-eicosatetraenoic	Arachidonic	−49.5	A major component of animal phospholipids. Major component of marine algae and some terrestrial species, such as mosses.
Pentaenoic acids				
20	All-*cis*-5,8,11,14,17-eicosapentaenoic			Major component of marine algae, fish oils.
22	All-*cis*-7,10,13,16,19-docosapentaenoic	Clupanodonic		Animals, especially as phospholipid component. Abundant in fish.
Hexaenoic acids				
22	All-*cis*-4,7,10,13,16,19-docosahexaenoic			Animals, especially as phospholipid component. Abundant in fish.

Table 2.4 Examples of some substituted fatty acids

Structure	Name	Notes
Cyclic fatty acids		
	Lactobacillic	Produced by Lactobacilli, *Agrobacterium tumefaciens*
	Sterculic	Found in plants of the Malvales family
	Chaulmoogric	Used for leprosy treatment
Epoxy-fatty acids		
	Vernolic	Epoxy derivative of oleate
Hydroxy-fatty acids		
	α-Hydroxy palmitate	Found in galactosyl-cerebrosides
$HOCH_2(CH_2)_{14}COOH$	ω-Hydroxy palmitate	Constituent of suberin coverings of plants
	Ricinoleic	Represents > 90% of the total acids of castor bean oil

major components of surface waxes, cutin and suberin of plants (Table 2.4).

2.1.6 Conjugated unsaturated fatty acids

The major polyunsaturated fatty acids all contain *cis*-methylene-interrupted sequences and for years it was thought that most conjugated systems were artefacts of isolation. However, many such acids have now been firmly identified and are found in sources as diverse as seed oils, some micro-organisms and some marine lipids (especially sponges). An example of one such acid would be α-eleostearic acid (Table 2.3).

2.1.7 Fatty aldehydes and alcohols

Many tissues contain appreciable amounts of fatty alcohols or aldehydes whose chain length and double bond patterns reflect those of the fatty acids from which they can be derived. Sometimes the alcohols form esters with fatty acids and these 'wax esters' are important in marine waxes such as sperm whale oil or in its plant equivalent, jojoba wax.

2.1.8 Some properties of fatty acids

The short-chain fatty acids (i.e. of chain lengths up to 8C) are poorly water-soluble although in solution they are associated and do not exist as single molecules. Indeed, the actual solubility (particularly of longer chain acids) is often very difficult to determine because it is influenced considerably by the pH and also because the tendency for fatty acids to associate leads to monolayer or micelle formation. Micelle formation is characteristic of many lipids. McBain and Salmon many years ago introduced the concept of micelles. The most striking evidence for the formation of micelles in aqueous solutions of lipids lies in extremely rapid changes in physical properties over a limited range of concentration, the point of change being known as the *critical micellar concentration* or CMC, and this exemplifies the tendency of lipids to self-associate rather than remain as single molecules. The CMC is not a fixed value but a small range of concentration and is markedly affected by the presence of other ions, neutral molecules, etc. The value of the CMC can be conveniently measured by following the absorbance of a lipophilic dye such as rhodamine in the presence of increasing 'concentrations' of the lipid. The tendency of lipids to form micelles or other structures in aqueous solution often means that study of enzyme kinetics is difficult. Thus, for example, enzymes metabolizing lipids often do not display Michaelis–Menten kinetics and phrases such as 'apparent' K_m must be used. In some cases the enzymes prefer to work at interfaces rather than with free solutions (Section 7.2.1).

Fatty acids are easily extracted from solution or suspension by lowering the pH to form the uncharged carboxyl group and extracting with a non-polar solvent such as light petroleum. In contrast, raising the pH increases solubility because of the formation of alkali metal salts, which are the familiar soaps. Soaps have important properties as association colloids and are surface-active agents.

The influence of fatty acid structure on its melting point has already been mentioned with branch chains and *cis* double bonds lowering the melting points of equivalent saturated chains. Interestingly, the melting point of fatty acids depends on whether the chain is even or odd numbered (Table 2.5).

Saturated fatty acids are very stable, but unsaturated acids are susceptible to oxidation; the more double bonds the greater the susceptibility. Unsaturated fatty acids, therefore, have to be handled under an atmosphere of inert gas (e.g. nitrogen) and kept away from (photo) oxidants or substances giving rise to free radicals. Anti-oxidant compounds have to be used frequently in the biochemical laboratory just as organisms and cells have to utilize similar compounds to prevent potentially harmful attacks on acyl chains in vivo (Sections 2.3.4 and 4.2.4.3).

Table 2.5 The melting points of a series of saturated fatty acids

Fatty acid	Chain length	Melting point (°C)
Butanoic	C4	–8
Pentanoic	C5	–35
Hexanoic	C6	–8
Heptanoic	C7	17
Octanoic	C8	13
Nonanoic	C9	32
Decanoic	C10	30
Undecanoic	C11	44
Dodecanoic	C12	42
Tridecanoic	C13	55
Tetradecanoic	C14	52
Pentadecanoic	C15	63
Hexadecanoic	C16	61

2.1.9 Quantitative and qualitative fatty acid analysis

2.1.9.1 General principles

In Chapter 1 it was pointed out that chromatographic methods cannot only be used to separate closely related compounds but may also help to define their structures and, in combination with suitable detection processes, will give quantitative data. For fatty acids, gas–liquid chromatography has proved particularly useful (Section 1.8.3).

The preparation of the samples and the choice of column packing material are rather important. For

example, it is customary to form methyl esters of fatty acids by refluxing the sample with methanolic-HCl (methylation lowers the boiling point of the derivative sufficiently for injection onto the gas chromatograph). However, if this technique is used for cyclic fatty acids they are destroyed. These acids would have to be transferred from lipids to methanol by base-catalysed methanolysis (with sodium methoxide).

No single stationary phase is capable of separating every acid – although capillary–gas chromatography (where the columns have dimensions such as 50 m by 0.25 mm interior diameter) usually separates most components including positional isomers. Generally two different types of column are necessary to resolve all the individual components.

Columns having saturated paraffin hydrocarbons (Apiezon L grease) or silicone greases separate largely on the basis of molecular weight; unsaturated acids emerge from the column earlier than the corresponding saturated acids. Branched-chain acids emerge before the saturated fatty acids of corresponding carbon number. Thus, these acids will overlap with unsaturated fatty acids.

Columns containing polar materials such as polyethylene glycol adipate give separations based on number of double bonds as well as on molecular weight (i.e. chain length). The order of emergence from this type of column will, therefore, be 16:0, 16:1, 18:0, 18:1, 18:2, etc. (Fig. 2.2). Branched-chain

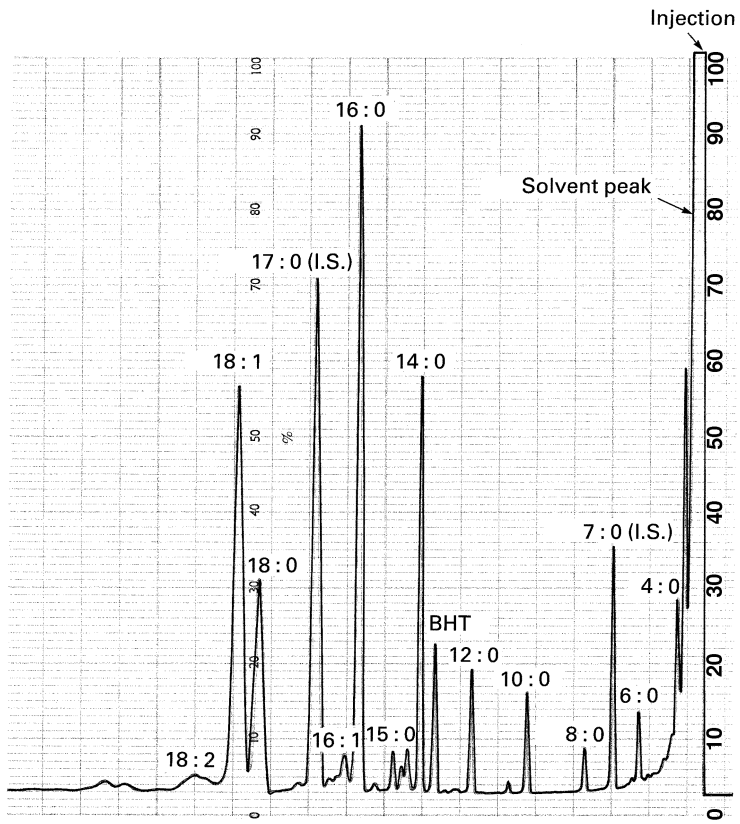


Fig. 2.2 Separation of fatty acid methyl esters by gas–liquid chromatography. The oven temperature was programmed from 65°C to 185°C at 8°C min^{-1}. The sample consists of fatty acids derived from a milk diet for calves. Heptanoic and heptadecanoic acids were added as 'internal standards' (IS). Reproduced with kind permission of Mr. J.D. Edwards-Webb.

fatty acids may, in consequence, overlap highly unsaturated fatty acids of shorter chain length.

In order to quantify the amounts of individual fatty acids in a particular sample, it is usual to include an internal standard. This will consist of a known amount of a fatty acid which does not appear in the mixture being analysed. Frequently, odd-numbered chain length acids such as 17:0 or 21:0 are used (Fig. 2.2), although again care must be taken that the peak of the internal standard does not obscure unsaturated components of the mixture.

2.1.9.2 Determination of the structure of an unknown acid

Merely running an unknown fatty acid on a column of known properties can give some information about its structure. Co-chromatography with authentic standards on different types of columns will serve to identify a fatty acid provisionally and the chain length of an unknown substance can be estimated from identified peaks because there is a simple linear relationship between the log of the retention volume (time) and the carbon number for each particular type of acid on a given column. Samples of a given component can be collected from the gaseous effluent on the column by allowing the gas stream to pass through a wide glass tube loosely packed with defatted cotton or glass wool wetted with a solvent such as methanol or petroleum ether. Such an isolated component can then be run on another type of column or subjected to specific chemical reactions. For example, the identity of an unsaturated fatty acid can be confirmed by H_2 reduction to the corresponding saturated acid and by oxidation with permanganate or ozone. With oxidation, the unsaturated acid is split into fragments at its double bonds so that analysis of the products gives information about the position of the double bond [e.g. oleic acid (*n*-9 18:1) gives oxidation products consisting of a 9C dicarboxylic acid and nonanoic (9:0) acid]. Infrared spectroscopy can be used to differentiate between *cis* and *trans* double bonds. Instead of collecting a sample of an individual component the gas chromatograph can also be connected directly to a mass spectrometer which allows the identification of many lipid components including most fatty acids. This GC–MS combination is increasingly used for fatty acid analysis.

Identification of fatty acids often also involves an initial fractionation of mixtures by $AgNO_3$–silicic acid chromatography. In this method, either on plates (TLC) or in columns, fatty acids are separated according to the number of double bonds. The mixture of, say, monounsaturated acids can then be more easily identified by GLC.

2.2 THE BIOSYNTHESIS OF FATTY ACIDS

Naturally occurring fatty acids normally, but not exclusively, have straight even-numbered hydrocarbon chains. This is explained by their principal mode of biosynthesis from acetate (2C) units.

2.2.1 Conversion of fatty acids into metabolically active thiolesters is often a prerequisite for their metabolism

For most of the metabolic reactions in which fatty acids take part, whether they be anabolic (synthetic) or catabolic (degradative), thermodynamic considerations dictate that the acids be 'activated'. For these reactions thiolesters are generally utilized. The active form is usually the thiolester of the fatty acid with the complex nucleotide, *coenzyme A* (CoA) or the small protein known as acyl carrier protein (ACP) (see Fig. 2.3). These molecules contain a thiolester and, at the same time, render the acyl chains water-soluble.

For many tissues a preliminary step involves the uptake of fatty acids. In animals, the latter are transported between organs either as NEFAs complexed to albumin or in the form of complex lipids associated with lipoproteins. NEFAs are released from the triacylglycerol component of the complex lipids by lipoprotein lipase (Sections 3.5.2 and 5.2.5). The mechanism by which NEFAs enter cells is undefined. Both protein-mediated and diffusion processes have been implicated. Certainly in artificial membrane systems, un-ionized fatty acids can move across bilayers rapidly and it has been theo-

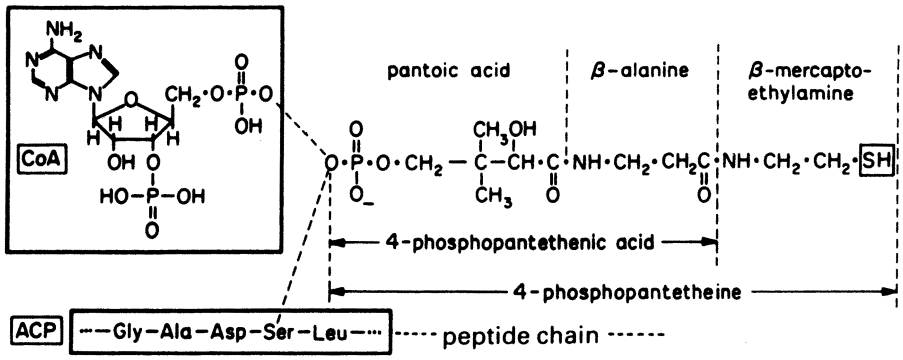


Fig. 2.3 The structures of coenzyme A and the acyl carrier protein from *E. coli*.

rized that desorption from the membrane may be helped by fatty acid binding proteins (FABPs).

In contrast to simple diffusion, there is increasing evidence for protein-mediated transport. Of the various putative transport proteins, FABPs, fatty acid translocase (FAT) and fatty acid transport protein (FATP) have been identified in a variety of cell types. All three proteins are regulated to some degree by members of the PPAR (peroxisome proliferator-activated receptor) family of transcription factors. (The PPAR transcription factors are part of the steroid hormone receptor superfamily and affect many genes of lipid metabolism, Section 5.3.2.) Several isoforms of FABPs and FATPs are also regulated by insulin. Moreover, expression, regulation and activity of the various transport proteins can often (but not always) be correlated with increased fatty acid movements in animal models of fat metabolism such as in Ob (obese) mice mutants. Null mutants, e.g. for FAT, have also been created and these show abnormal fatty acid metabolism.

Once inside cells, the fatty acids can diffuse or are transported into different organelles such as mitochrondria. The discovery of small (14–15 kDa) proteins, termed fatty acid binding proteins (FABPs, see above) in animals led to the suggestion that they are important as carriers and for intracellular storage, in addition to transport (above). Similar proteins have been identified in other organisms such as plants. Some of the FABPs will bind other ligands, in particular acyl-CoAs. The physiological significance of this is unknown because separate acyl-CoA binding proteins have also been reported.

2.2.1.1 Acyl-CoA thiolesters were the first types of activated fatty acids to be discovered

The formation of acyl-CoAs is catalysed by several acyl-CoA synthetases. These belong to a large protein family found in a wide range of, if not all, organisms, which itself is part of the superfamily of AMP-binding proteins (due to their AMP-forming reaction).

The acyl-CoA synthetase reaction occurs in two steps:

$$\mathrm{E + R-COOH + ATP \xrightarrow{Mg^{2+}} (E:R-CO-AMP) + PPi}$$
$$\mathrm{(E:R-CO-AMP) + CoASH \rightarrow E + R-CO-SCoA + AMP}$$

Both reactions are freely reversible but the reaction is driven to the right because pyrophosphate is rapidly hydrolysed. Evidence for this reaction has come mainly from work with heart acetyl-CoA and butyryl-CoA synthetases, but it is presumed that all acyl-CoA synthetases work via a similar mechanism.

The acyl-CoA synthetases differ from each other with respect to their subcellular locations and their specificities for fatty acids of different chain lengths. Their overlapping chain length specificities and their tissue distributions mean that most saturated or unsaturated fatty acids in the range 2–22C

can be activated in animal tissues, although at different rates.

Now that molecular biology can be used to identify cDNAs for acyl-CoA synthetase, we are becoming aware of just how many enzyme isoforms are likely to exist. Originally it was thought that short-, medium- and long-chain isoforms were often present. However, in oilseed rape 12 cDNAs have already been reported while in *Mycobacterium tuberculosis* (the causative agent of tuberculosis) 36 acyl-CoA synthetase genes have been identified!

The first short-chain acyl-CoA synthetase was studied in heart mitochondria. Termed acetyl-CoA synthetase, it is most active with acetate and is found in many mitochondria. A cytosolic acetyl-CoA synthetase is present in the cytosol of some tissues where it functions by activating acetate for fatty acid synthesis. Indeed, the presence of this enzyme in chloroplasts has also enabled [^{14}C]acetate to be extensively used in biochemical experiments with plants.

Acetyl-CoA synthetase (which also activates propionate at slower rates), along with other short-chain acyl-CoA synthetases, is important in ruminants. The rumen micro-organisms in these animals generate huge amounts of short-chain acids including acetate. Other sources of acetate in animals include the oxidation of ethanol by the combined action of alcohol and aldehyde dehydrogenases and from β-oxidation. Under fasting conditions or in diabetes the ketotic conditions cause a rise in the production of both ketone bodies and acetate (via the action of acetyl-CoA hydrolase). The absence of acetyl-CoA synthetase in liver mitochondria allows acetate to be transported from this tissue and to be activated and oxidized in mitochondria of other tissues.

Butyryl-CoA synthetase is active with acids in the range 3–7C and has been isolated from heart mitochondria. It is a mitochondrial enzyme in other tissues but has not been found in liver where a special propionyl-CoA synthetase has been demonstrated. The latter enzyme may be especially important in ruminants where propionate is formed by rumen micro-organisms and is an important substrate for gluconeogenesis.

The medium-chain acyl-CoA synthetase, again isolated from heart mitochondria, has its highest activity with heptanoate. It is active with acids in the range 4–12C, the lowest K_m being for octanoate. Liver mitochondria also contain a second enzyme activating medium-chain fatty acids including branched or hydroxy fatty acids, but which also functions with benzoate and salicylate. Thus, its purpose seems to lie in the formation of glycine conjugates of aromatic carboxylic acids such as benzoate and salicylate.

The long-chain acyl-CoA synthetases are firmly membrane-bound and can only be solubilized by the use of detergents. Within the cell, activity has been detected in endoplasmic reticulum and the outer mitochondrial membrane with small amounts in peroxisomes (when the latter are present). There is some dispute as to whether the activity present in mitochondrial and microsomal fractions is due to the same enzyme. Because long-chain fatty acid activation is needed for both catabolism (β-oxidation) and for synthesis (acylation of complex lipids) it would be logical if the long-chain acyl-CoA synthetases of mitochondria and the endoplasmic reticulum formed different pools of cellular acyl-CoA. This compartmentalization has been demonstrated with yeast mutants where it plays a regulatory role in lipid metabolism (Section 2.2.8.2 and Fig. 2.26).

For animals, it is thought that the mitochondrial and peroxisomal enzymes are used for fatty acid oxidation while the endoplasmic reticulum enzyme is used for lipid assembly. The original long-chain acyl-CoA synthetases that were studied in detail were those from rat liver mitochondria and endoplasmic reticulum. These were found to be very similar (if not identical) as judged by several molecular and catalytic properties. They worked efficiently for both saturated and unsaturated fatty acids in the range 10–20C. Now that cDNAs are available, however, several animal isoforms have been identified that have more selectivity – for example, at least two brain acyl-CoA synthetases have been demonstrated to show high preference for arachidonic acid.

In *Escherichia coli*, acyl-CoA synthetase activity is inducible when the bacterium is grown in a medium with long-chain fatty acids as the carbon

source. So far, only one synthetase isoform has been identified and it is localized at the inner membrane. The acyl-CoA synthetase, termed FadD in *E. coli* works co-operatively with an outer membrane fatty acid transporter, FadL, to convert transported long-chain fatty acids to substrates for β-oxidation. The fatty acid transport and activation are directly coupled to transcriptional control of the structural genes of enzymes induced in fatty acid metabolic pathways. This occurs through the fatty acyl-CoA responsive transcription factor, FadR (Fig. 2.4). In the absence of exogenous fatty acids, FadR is active in DNA binding and the *fad* (fatty acid degrading) genes, including the *fadL* and *fadD* genes, are expressed at low levels while several *fab* (fatty acid biosynthesis) genes are expressed at high levels. The FadR conformation is changed by fatty acyl-CoAs that loosen the binding to DNA. This results in derepression of the *fad* genes and reduces expression of the *fab* genes. This is a very nice example of gene regulation that fits exactly with the organism's metabolic requirements.

The *E. coli* enzyme has been studied in some detail. Site-directed mutagenesis has been used to identify seven amino acids in a highly conserved region, which are essential for catalytic activity. The motif itself functions in part to promote fatty acid chain selectivity. Moreover, the tertiary structure of *E. coli* acyl-CoA synthetase has been predicted by comparing it to known structures for other members of the AMP-binding superfamily of proteins, e.g. luciferase.

Although most acyl-CoA synthetases are ATP-dependent, some have been found that use GTP. The best known of these is succinyl-CoA synthetase which functions in the tricarboxylic acid cycle. The physiological significance of the other GTP-dependent acyl-CoA synthetases is uncertain.

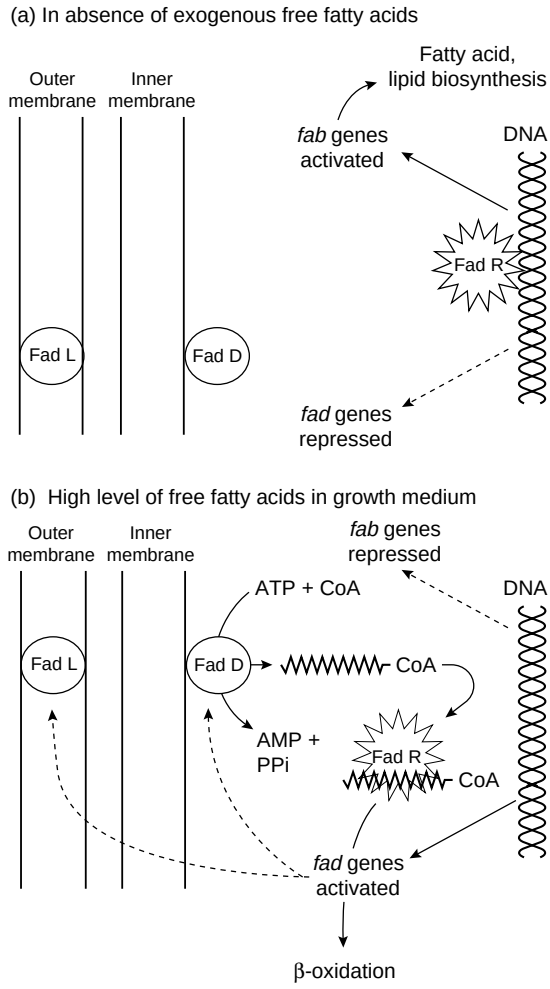


Fig. 2.4 How exogenous fatty acids control fatty acid metabolism in *E. coli*. Adapted with kind permission of C.C. DiRusso and Elsevier Science from *Progress in Lipid Research*, **38** (1999), Fig. 8, p. 162.

2.2.1.2 Acyl–acyl carrier proteins can be formed as distinct metabolic intermediates in some organisms

The other form of 'activated' fatty acid (Fig. 2.3) is that of acyl–acyl carrier protein. In these compounds the fatty acid is attached to a 4′-phosphopantetheine residue, but this in turn is connected via a serine to a peptide chain (rather than to a nucleotide as in coenzyme A). Acyl carrier proteins can be isolated from plants (including algae), cyanobacteria and most bacteria. They are small, highly stable, acidic proteins of 9–11 kDa in size. An enzyme which converts fatty acids to acyl–acyl carrier protein (acyl-ACP, Section 2.2.3.2) esters has been isolated from *E. coli* and it is presumed that a similar activity exists in plants because fatty acids up to 12C can be efficiently used by plant fatty acid

synthases which need acyl-ACPs (Section 2.2.3.4). The *E. coli* enzyme uses ATP for the activation step.

2.2.2 The biosynthesis of fatty acids can be divided into *de novo* synthesis and modification reactions

Most naturally occurring fatty acids have even numbers of carbon atoms. Therefore, it was natural for biochemists to postulate that they were formed by condensation from 2C units. This suggestion was confirmed in 1944 when Rittenberg and Bloch isolated fatty acids from tissues of rats that had been fed acetic acid labelled with ^{13}C in the carboxyl group and ^{2}H in the methyl group. The two kinds of atoms were located at alternate positions along the chain, showing that the complete chain could be derived from acetic acid.

This stimulated interest in the mechanism of chain lengthening and when the main details of the β-oxidation pathway (the means by which fatty acids are broken down two carbon atoms at a time; see Section 2.3.1) were worked out in the early 1950s, it was natural for many biochemists to ask the question, 'Can β-oxidation be reversed in certain circumstances to synthesize fatty acids instead of breaking them down?'

The study of fatty acid biosynthesis began in the laboratories of Gurin in the USA who studied liver, and Popjak in London, studying mammary gland. Several discoveries soon indicated that the major biosynthetic route to long-chain fatty acids was distinctly different from β-oxidation. In the first place, a pyridine nucleotide was involved; but the reduced form, NADPH, not NAD^+ as in β-oxidation (see below and Section 2.3.1). Second, a requirement for bicarbonate or carbon dioxide was noticed by Wakil and his colleagues who were studying fatty acid biosynthesis in pigeon liver and by Brady and Klein studying rat liver and yeast, respectively.

Fatty acid biosynthesis can be conveniently divided into *de novo* synthesis, where a small precursor molecule (usually the 2C acetyl group) is gradually lengthened by 2C units to give rise, in most systems, to 16C and 18C products and various modifications that can take place once the long-chain fatty acids have been made (Fig. 2.5). Of the

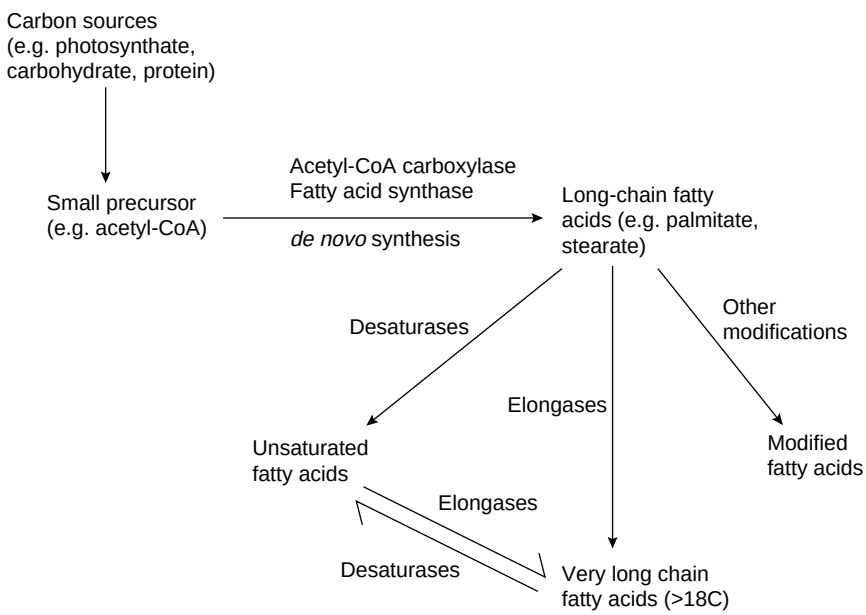


Fig. 2.5 A simplified picture of fatty acid synthesis.

modifications, elongation and desaturation are the most important.

2.2.3 *De novo* biosynthesis

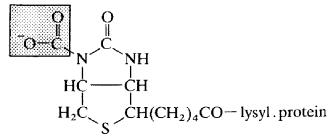
The source of carbon for fatty acid biosynthesis varies somewhat, depending on the type of organism. For mammals (especially humans!) fat deposition is a typical response to energy excess and, consequently, carbohydrate and, to some extent, amino acids may supply the carbon. Most of the carbon for *de novo* fatty acid (and lipid) formation goes through the pyruvate pool. The latter is, of course, the end-product of glycolysis.

Although pyruvate is produced in the cytosol (where the animal fatty acid biosynthesis takes place), acetyl-CoA is mainly generated from it in mitochondria. Under conditions favouring fatty acid biosynthesis, pyruvate is transported into mitochondria where pyruvate dehydrogenase is activated. The acetyl-CoA product is combined with oxalacetate to produce citrate which leaves the mitochondria via a tricarboxylate anion carrier. Back in the cytosol, acetyl-CoA is produced by ATP:citrate lyase. The NADPH needed for the reductive steps of fatty acid synthase comes from the cytosolic pentose phosphate pathway.

In plants, the acetyl-CoA needed for lipid synthesis comes ultimately from photosynthesis. How the acetyl-CoA needed for fatty acid formation – in this case within plastids – is actually generated is not clear. For some plants, all the enzymes of glycolysis (including pyruvate dehydrogenase) appear to be present in the plastid. For other plants, such as spinach, acetyl-CoA arrives via a circuitous route from the mitochondria. Thus, acetyl-CoA generated by mitochondrial pyruvate dehydrogenase is hydrolysed to free acetate. In its unionized form acetic acid can easily cross membranes and thus the 2C substrate moves to the plastid where it is activated by acetyl-CoA synthetase (see Section 2.2.1 and Fig. 2.6). It may be that some plants can use both methods.

2.2.3.1 Acetyl-CoA carboxylase

The actual process of *de novo* synthesis to produce long-chain fatty acids involves the participation of two enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Both of these are complex and catalyse multiple reactions. In early experiments on fatty acid synthesis in pigeon liver, it was found that the soluble enzymes necessary could be split into two fractions by ammonium sulphate fractionation. The cofactor biotin was bound to one of the protein fractions. At that time it was known that biotin was involved in carboxylation reactions and, when Wakil discovered that malonate was an intermediate in fatty acid biosynthesis, the essential features of the pathway began to emerge. The acetate must be in its 'activated form' as its CoA thiolester (Section 2.2.1 and Fig. 2.3). The reaction is catalysed by ACC, which could be identified in the pigeon liver fraction, where the biotin was bound. Confirmation that biotin was involved came from the inhibition of carboxylation by avidin. This protein was already known as a potent inhibitor of biotin, for it is the component of raw egg white which can bind extremely strongly to biotin thus causing a vitamin deficiency known as 'egg white injury'. The active carbon is attached to one of the ureido nitrogens of the biotin ring.



carboxyl biotinyl enzyme

ACC is a Type I biotin-containing enzyme. Such enzymes catalyse carboxylation reactions in two main steps:

1. $ATP + HCO_3^- + BCCP \xrightarrow[\text{carboxylase}]{\text{biotin}} ADP + Pi + BCCP\text{-}COO^-$

2. $BCCP\text{-}COO^- + \text{acetyl-CoA} \xrightarrow[\text{carboxyltransferase}]{} BCCP + \text{malonyl-CoA}$

Net: $ATP + HCO_3^- + \text{acetyl-CoA} \rightarrow ADP + Pi + \text{malonyl-CoA}$

(Actually, each of the main reactions takes place in steps and these are described in Knowles' 1989 review; see Further Reading.) The carboxylate group is attached to a nitrogen on the biotin moiety, which aids in its transfer to acetyl-CoA.

ACCs can have a multienzyme structure or exist as multifunctional proteins. Different examples

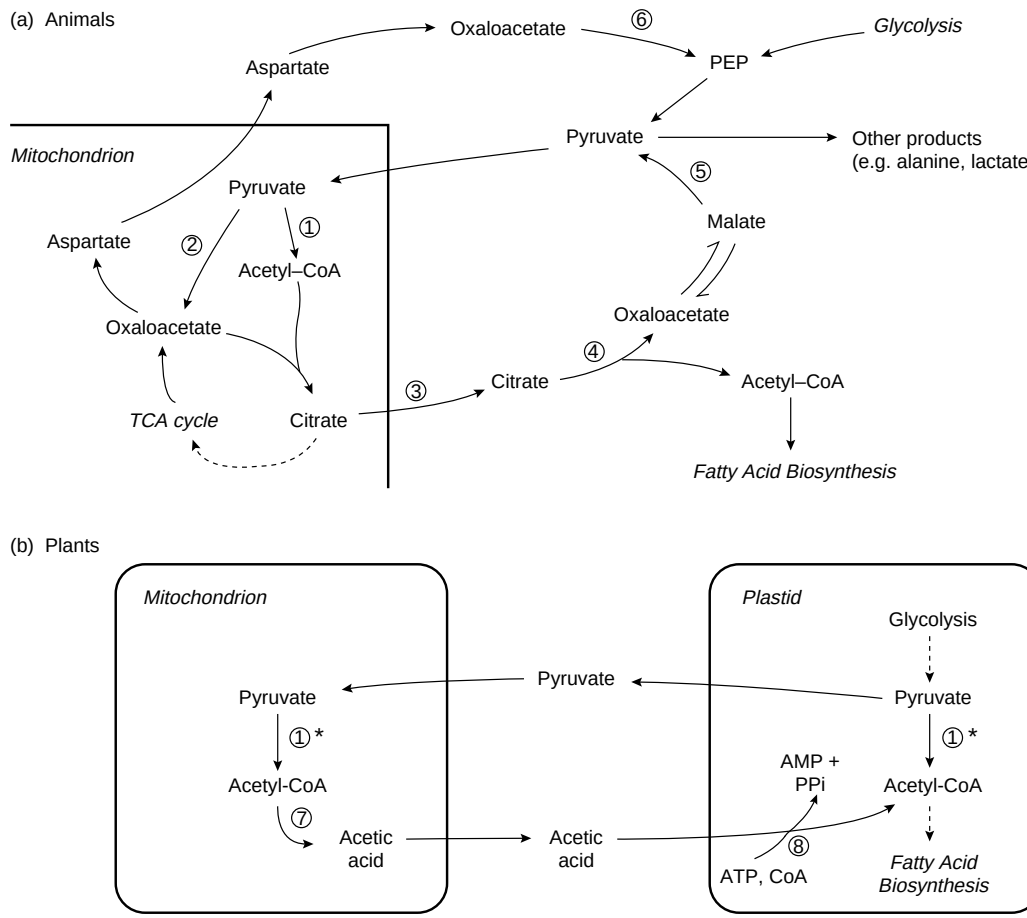


Fig. 2.6 Acetyl-CoA generation for *de novo* fatty acid biosynthesis in animals and plants. 1: Pyruvate dehydrogenase; 2: pyruvate carboxylase; 3: tricarboxylate anion carrier; 4: ATP:citrate lyase; 5: malic enzyme; 6: PEP carboxykinase; 7: acetyl-CoA thioesterase (or spontaneous cleavage); 8: acetyl-CoA synthetase. *Note: in plants, if plastid pyruvate dehydrogenase is not present then the mitochondrial enzyme must be utilized.

from the microbial, plant and animal kingdoms are shown in Table 2.6. In the case of the multi-functional proteins it is important for the biotin prosthetic group to be able to interact with the biotin carboxylase and the carboxyltransferase active sites in turn. This is permitted by the flexible nature of the protein chain around the biotin attachment site.

2.2.3.2 Fatty acid synthase

The malonyl-CoA generated by ACC forms the source of nearly all of the carbons of the fatty acyl chain. In most cases, only the first two carbons arise from a different source – acetyl-CoA – and the latter is known as the 'primer' molecule.

$$\underbrace{CH_3CH_2}_{\text{From 'primer'}}\underbrace{CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2COOH}_{\text{From malonyl-CoA}}$$

The fatty acid chain then grows in a series of reactions illustrated in Fig. 2.7. The individual enzymes for *E. coli* are listed in Table 2.7. The basic chemistry is similar in all organisms although the organization of the enzymes is not. In some cases acetyl-CoA

Table 2.6 Examples of different acetyl-CoA carboxylases

Species	Protein structure	Details
E. coli	Multiprotein complex	Four proteins: biotin carboxylase, BCCP and carboxyltransferase (a heterodimer). Transcription of all four *acc* genes is under growth rate control.
Yeast	Multifunctional protein	190–230 kDa. Activated but not polymerized by citrate.
Dicotyledon plants	Multiprotein complex	In chloroplasts. Similar properties to *E. coli* enzyme. Probably three of the subunits coded by nucleus, one by chloroplast.
	Multifunctional protein	Presumed to be cytosolic. Concentrated in epithelial cells in pea. Molecular mass 200–240 kDa. Functions as dimer. Graminicide insensitive.
Grasses (*Poaceae*)	Multifunctional proteins	Two isoforms, both 200–240 kDa, which function as dimers. Chloroplast isoform is graminicide sensitive but cytosolic form (concentrated in epithelial cells) is insensitive. Both are nuclear encoded.
Animals	Multifunctional proteins	Cytosolic. About 250 kDa but functions as polymer of up to 10^7 kDa. Aggregation increased by citrate. Also regulated by phosphorylation in response to hormones.

Adapted from Harwood (1999b) with permission from Sheffield Academic Press.

is not the usual primer. For example, in mammalian liver and mammary gland, butyryl-CoA is more active. In goat mammary gland there are enzymes that can catalyse the conversion of acetyl-CoA into crotonyl-CoA by what is, essentially, the reverse of β-oxidation (Section 2.3.1). Finally the use of propionyl-CoA or branched primers permits the formation of odd chain length or branch chain fatty acids, respectively.

The overall reaction of fatty acid (palmitate) synthesis can be summarized:

$$CH_3CO-CoA + 7HOOC.CH_2.CO-CoA + 14NADPH + 14H^+ \rightleftharpoons CH_3(CH_2)_{14}COOH + 7CO_2 + 8CoASH + 14NADP^+ + 6H_2O$$

The steps have been elucidated mainly from studies of *E. coli*, yeast, the tissues of various animals and some higher plants. The group of enzymes are known collectively as fatty acid synthase (FAS).

FASs can be divided mainly into Type I and Type II enzymes (Table 2.8). Type I synthases are multifunctional proteins in which the proteins catalysing the individual partial reactions are discrete domains. This type includes the animal synthases and those from higher bacteria and yeast. Type II synthases contain enzymes that can be separated, purified and studied individually. This system occurs in lower bacteria and plants and has been studied most extensively in *E. coli*. In addition, Type III synthases – occurring in different organisms – catalyse the addition of C2 units to preformed acyl chains and are also known as elongases (Section 2.2.3.4). Although, historically, the reactions of the yeast synthases were unravelled first, we shall start by describing the separate reactions catalysed by the enzymes of *E. coli*.

The Americans, Goldman and Vagelos, discovered that the product of the first condensation reaction of fatty acids synthesis in *E. coli* was bound to a protein through a thiolester linkage: acetoacetyl-S-protein. When they incubated this product with the coenzyme NADPH and a crude protein fraction containing the synthase, they obtained butyryl-S-protein and when malonyl-CoA was included in the reaction mixture, long-chain fatty acids were produced. Subsequently, intermediates at all steps of the reaction were shown to be

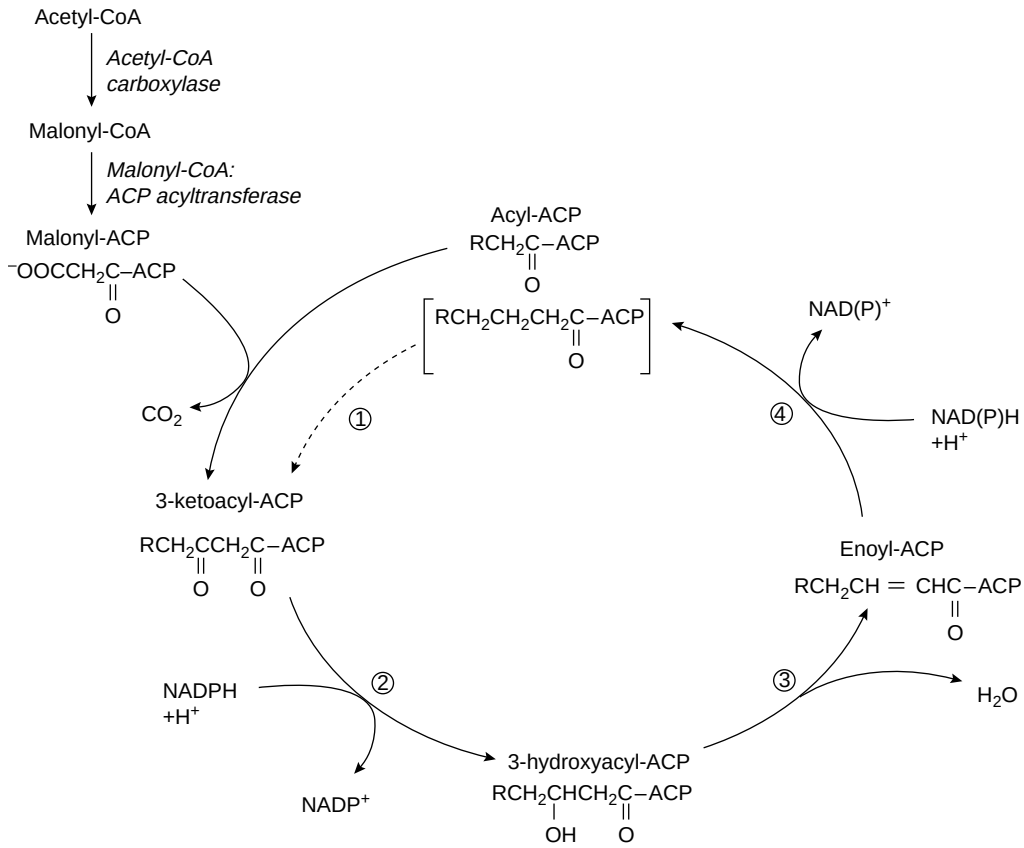
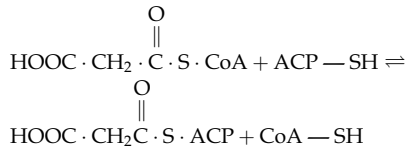
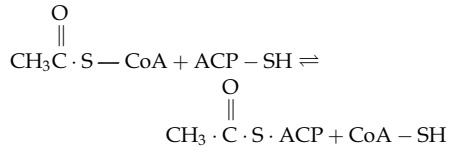
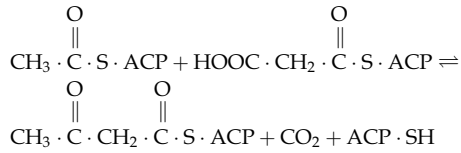
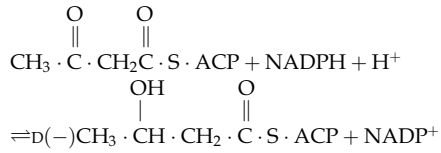
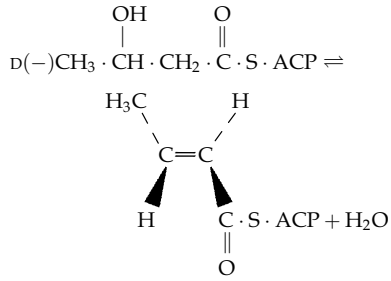
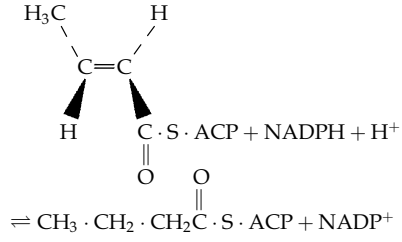
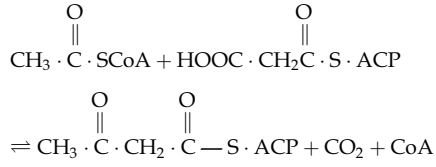


Fig. 2.7 The repeat cycle of reactions for the addition of two carbons by fatty acid synthase. Reactions of the cycle: (1) condensation (3-ketoacyl-ACP synthase); (2) reduction (3-ketoacyl-ACP reductase); (3) dehydration (3-hydroxyacyl-ACP dehydrase); (4) reduction (enoyl-ACP reductase).

attached to this protein, which was therefore called acyl carrier protein (ACP). It is a small molecular mass protein (about 8.8 kDa), has one SH group per mole of protein and is very stable to heat and acid pH. Vagelos and his colleagues worked out the structure of the prosthetic group to which the acyl moieties were attached in the following way. They made acyl-ACP derivatives in which the acyl group was labelled with radioactive carbon atoms (such as 2-[^{14}C]-malonyl-ACP) and then hydrolysed the acyl protein with proteolytic enzymes. This yielded small radioactive peptides with structures that were fairly easy to determine. The prosthetic group turned out to be remarkably similar to coenzyme A: the acyl groups were bound covalently to the thiol group of 4′-phosphopantetheine, which in turn was bound through its phosphate group to a serine hydroxyl of the protein (Fig. 2.3). To study the biosynthesis of ACP, Vagelos made use of a strain of *E. coli* that had to be supplied with pantothenate in order to grow. When the bacteria were fed with radioactive pantothenate, the labelled substance was incorporated into the cell's ACP and CoA. However, whereas the ACP level remained constant under all conditions the level of CoA was very much dependent on the concentration of pantothenate. When the cells were grown in a medium of high pantothenate concentration, the level of

Table 2.7 Reactions of fatty acid synthesis in *E. coli*

(1) Malonyl transacylase		Specific for malonate: not a saturated acyl-CoA. Malonyl-*S*-pantetheine is also a substrate. Both enzymes (1) and (2) were characterized by (i) the amount of ^{14}C-acetate or malonate transferred to ACP or (ii) paper chromatography of acetyl or malonyl hydroxamate. Intermediate is acyl-*S*-enzyme.
(2) Acetyl transacylase		There is now doubt about the importance of this reaction because acetyl-CoA can be used directly in reaction (7) to form acetoacetyl-ACP. It is also a partial reaction of enzyme (7), but a separate transacylase has been reported.
(3) 3-ketoacyl-ACP synthase		Assayed by coupling with the next reaction and following NADPH oxidation spectrophotometrically. SH-enzyme with a key cysteine at the active site inhibited by iodoacetamide. Two isoforms in *E. coli* with different substrate selectivities. Both inhibited by cerulenin.
(4) 3-ketoacyl-ACP reductase		Also reacts with acyl-CoA or pantetheine esters, but much more slowly. Specifically produces the D(−)isomer.
(5) 3-hydroxyacyl-ACP dehydrase		Measured by hydration of crotonyl-ACP accompanied by decrease in absorption at 263 nm. Does not metabolize model compounds. Stereo-specific for D(−)isomer.
(6) Enoyl-ACP reductase		Two enzymes occur: (i) NADPH-specific, short-chain acid preferred; inhibited by iodoacetamide, NEM, pCMB. Specific for ACP esters. (ii) NADH-specific, long-chain acids preferred. Uses CoA or ACP esters. NEM stimulates.
(7) Acetoacetyl-ACP synthase		Allows acetyl-CoA to be used directly and, hence, by-passes reaction (2) (acetyl transacylase). Unlike 3-ketoacyl-ACP synthase it is not inhibited by cerulenin.

Abbreviations: NEM = *N*-ethylmaleimide; pCMB = *p*-chloromercuribenzoate.

Table 2.8 Types of fatty acid synthases in different organisms

Source	Subunit types	Subunit (mol. mass)	Native (mol. mass)	Major products
Type I: Multicatalytic polypeptides				
Mammalian, avian liver	α	$220–270\times10^3$	$450–550\times10^3$	16:0 free acid
Mammalian mammary gland	α	$200–270\times10^3$	$400–550\times10^3$	4:0–16:0 free acids
Goose uropygial gland	α			2,4,6,8-tetramethyl-10:0
M. smegmatis	α	290 000	2×10^6	16:0-, 24:0-CoA
S. cerevisiae	α, β	185 000, 180 000	2.3×10^6	16:0-, 18:0-CoA
Dinoflagellates	α	180 000	4×10^5	
Type II: Freely dissociable enzymes				
Higher plant chloroplasts	Separate enzymes	–	–	16:0-, 18:0-ACP
E. gracilis chloroplast	Separate enzymes			12:0-, 14:0-, 16:0-, 18:0-ACP
E. coli	Separate enzymes	–	–	16:0-, 18:1-ACP

CoA produced was also high; but if these cells were washed free of pantothenate and transferred to a medium with no pantothenate, then the CoA level dropped rapidly while that of ACP remained constant. In other words, ACP was synthesized at the expense of CoA. It seems that ACP biosynthesis is under tight control and this may be yet another factor involved in the overall control of fatty acid biosynthesis. In fact, the ACP pool must be severely depleted in order to affect the rate of fatty acid synthesis in *E. coli*.

Two enzymes are involved in the turnover of the prosthetic group of ACP. Both enzymes are highly specific and even small modifications of the ACP structure prevents activity. The first enzyme catalyses the transfer of 4′-phosphopantetheine from CoA:

$$\text{Apo-ACP} + \text{CoA} \xrightarrow[\text{holo-ACP synthase}]{Mg^{2+}} \text{holo-ACP} + 3',5'\text{-ADP}$$

While the second causes degradation and, hence, turnover of ACP:

$$\text{holo-ACP} + H_2O \xrightarrow[\text{ACP phosphodiesterase}]{} \text{apo-ACP} + 4'\text{-phosphopantetheine}$$

In vivo, both enzymes have been demonstrated in *E. coli* and in mammalian synthases the turnover of the 4′-phosphopantetheine prosthetic group is an order of magnitude faster than the rest of the synthase, implying the presence of holo-ACP synthetase and ACP hydrolase. In plants, also, the holo-ACP synthase has been detected.

Even in Type II synthases, like that in *E. coli* the level of ACP is carefully regulated. Moreover, overproduction of ACP encoded by an inducible plasmid vector is lethal to *E. coli*. This is probably because most of the protein expressed in inducible systems is apo-ACP and the latter is a potent inhibitor of glycerol 3-phosphate acyltransferase, which is a key enzyme for lipid assembly (Sections 3.4.1 and 7.1.4).

With the discovery of ACP, the different steps in the reaction sequence were quickly elucidated, first in the yeast *Saccharomyces cerevisiae* and later confirmed with purified enzymes from *E. coli*. The availability of mutants and the ability to manipulate gene expression in *E. coli* has led to a lot of detailed knowledge of *de novo* fatty acid biosynthesis in this organism. However, *E. coli* is unusual

compared to most organisms in that its fatty acid synthase produces both saturated and unsaturated products. The main unsaturated product is *cis*-vaccenate (*cis*-11-octadecenoate) and, perhaps because *E. coli* is a facultative anaerobe, is produced without a need for oxygen – whereas the normal mechanism for fatty acid desaturation is aerobic (Section 2.2.5).

The synthesis of vaccenate and, hence the ratio of saturated to unsaturated products, is controlled by the activity of three enzymes as illustrated in Fig. 2.8.

The first enzyme is a specific dehydrase that works at the 10C stage of fatty acid synthase – 3-hydroxydecanoyl-ACP dehydrase. This enzyme can isomerize the *trans*-2 product into a *cis*-3-decenoyl-ACP. Whereas *trans*-2-decenoyl-ACP can be chain lengthened in the normal way to produce the saturated acid, palmitate, the *cis* double bond prevents the enoyl-ACP reductase working. This double bond does not, however, inhibit further addition of a two-carbon unit by 3-ketoacyl-ACP synthase activity. Nevertheless, only one of the two isomers, that coded by the *fabB* gene and designated condensing enzyme I, is active. Chain lengthening preserves the *cis* bond, but its position from the carboxyl group moves as each two-carbon addition takes place. Eventually *cis*-9-hexadecenoyl-ACP (palmitoleoyl-ACP) is formed. This can only be chain lengthened by condensing enzyme II (coded by *fabF*) to yield vaccenate. Thus, whereas both condensing enzymes are active with a variety of substrates, condensing enzyme I is the only one essential for the production of unsaturated fatty acids, but condensing enzyme II is needed for vaccenate synthesis.

Control of product quality is one of the most important aspects of microbial physiology. A good

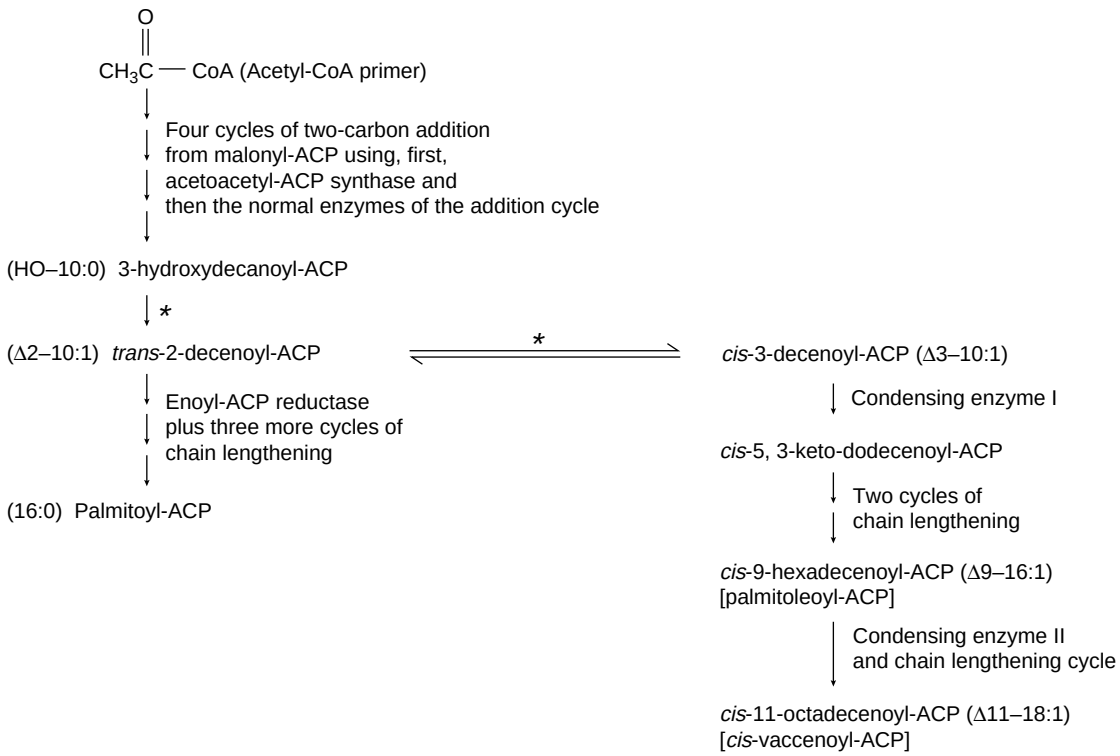


Fig. 2.8 Production of unsaturated fatty acids in *E. coli*. * Catalysed by 3-hydroxydecanoyl dehydrase, which is specific for the 10C substrate.

example is in maintenance of membrane fluidity at different temperatures (Section 6.5.9). *E. coli* is a poikilotherm (cannot adjust its own temperature) and, therefore, at lower growth temperatures it has to make more unsaturated fatty acids because these have a lower transition temperature (Section 2.1.1). Thus, the activity of condensing enzyme II in producing the main unsaturated product, vaccenate, is vital for temperature adaptation. Indeed, the observation that strains lacking condensing enzyme II also lacked thermal regulation originally demonstrated its importance. Recently, mutation of its gene (*fabF*) was shown to affect both enzyme activity and temperature regulation to prove the connection. Remarkably, *cis*-vaccenate synthesis is increased within 30 s of a temperature downshift indicating that neither mRNA nor protein synthesis is needed. It seems, therefore, that thermal regulation is normally brought about by condensing enzyme II, which is present all the time but which is much more active at low temperatures.

The discovery that the enzymes of the Type I FAS were apparently in a tight complex presented difficulties in studying the individual enzymic steps. In *E. coli*, the individual intermediates could be isolated, purified, characterized and used as substrates to study the enzymology of each reaction. In yeast this was not possible because the intermediates remained bound to the enzyme all the time. The German biochemist, Lynen, solved this problem elegantly by synthesizing model substrates – acyl derivatives of *N*-acetyl-cysteamine – in the hope that they would have enough affinity for the enzyme to be able to demonstrate the reactions. This was not always the case, but in certain reactions the model substrates had enough affinity (though very small) for the reaction to be measured. It was partly for this work that Lynen was awarded the Nobel Prize in 1964. Lynen visualized the yeast synthase as a complex of seven distinct enzymes catalysing reactions analogous to those in Table 2.7 but including a final step in which the product – a palmitoyl or stearoyl group – transferred to coenzyme A from the enzyme. However, it was perplexing that the complex could not be dissociated into functioning monomers. Studies with mutants were again responsible for a major advance in elucidating this problem. Schweizer and his colleagues in Germany found three types of mutants that required fatty acids for growth. One type lacked acetyl-CoA carboxylase whereas the other two (*fas*-1 and *fas*-2) were devoid of fatty acid synthase activity. Initial studies of these mutants showed that one mutant was deficient in protein-bound pantetheine while the second class of mutant lacked FMN. (In yeast, the enoyl reductase uses FMN and this proved a fortunate feature that allowed the easy initial classification of mutants.) Further investigation showed that three different fatty acid synthase functions, i.e. 3-ketoacyl-ACP synthase, 3-ketoacyl-ACP reductase and the binding of pantetheine (ACP function), were associated with *fas*-2. The remaining four activities were assigned to *fas*-1. It was, therefore, proposed that yeast fatty acid synthase contained two dissimilar multifunctional peptides α and β. Subsequently, these genetic results were confirmed by purifying the enzyme and dissociating it into α and β subunits by acylation with dimethyl maleic anhydride. Each subunit could then be assayed for the partial reactions in order to confirm the genetic assignments. Moreover, in vitro complementation of appropriate pairs of mutant FAS proteins has been carried out to restore activity.

	3-ketoacyl-ACP synthase	ACP	3-ketoacyl-ACP reductase
		α-chain (*fas*-2) (185 kDa)	
Acetyl transferase	Enoyl reductase	Dehydrase	Malonyl/palmitoyl (acyl) transferase
	β-chain (*fas*-1) (180 kDa)		

A model of the minimal functional entity of yeast FAS contains dimers of the two chains (i.e. α_2, β_2). The model is fully consistent with data from *fas* mutants as well as experiments with cross-linking agents in mammalian FAS where a dimer is also the minimal functional unit. The way in which yeast FAS is thought to function is shown in Fig. 2.9, where only one half of each dimer is functional at a given time. Hydrophobic interaction of the palmitoyl transferase at the end of the cycle of 2C additions causes a conformational change in the β-subunit. This facilitates transfer of the palmitoyl group to the palmitoyl transferase and, at the same time, reduces its transacylation to 3-ketoacyl-ACP synthase. Inactivation of one half of $\alpha_2\beta_2$ dimer leads simultaneously to activation of the other.

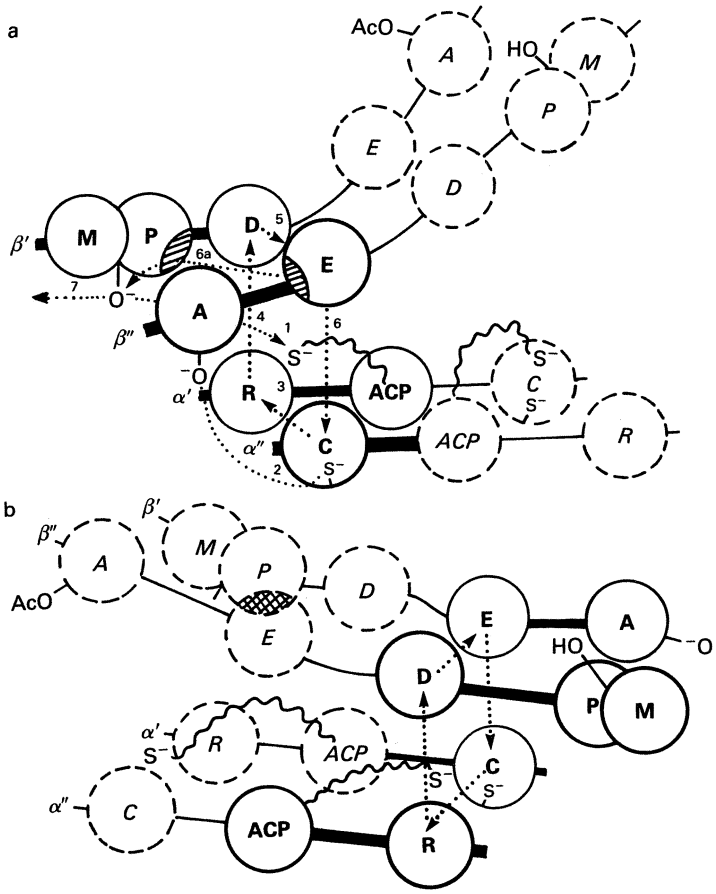


Fig. 2.9 Model of the intermolecular fatty acid synthase mechanism in the $\alpha_2\beta_2$ protomer of yeast. A: acetyl transferase; E: enoyl reductase; D: dehydratase; P: palmitoyl transferase; M: malonyl transferase; C: 3-ketoacyl synthase; R: 3-ketoacyl reductase; ACP: acyl carrier protein. Dotted lines and arrows delineate the route taken by intermediates when sequentially processed on different FAS domains. Numbers indicate the reaction sequence. Catalytically active domains, at a specific moment, are marked by bold lines. Shaded areas on E and P domains potentially interact by hydrophobic attraction in the presence of palmitate (b). On the protomer depicted in (a) fatty acyl chain elongation occurs in one-half of the $\alpha_2\beta_2$ protomer. In (b) chain termination is induced by hydrophobic interaction between E-bound palmitate and P. Subsequently, palmitate is transferred to its *O*-ester binding site on P. Inactivation of the left half of $\alpha_2\beta_2$ simultaneously activates its right half (b). Redrawn from Schweizer (1984) with the permission of the author and Elsevier Science Publishers, BV. From *Fatty Acid Metabolism and its Regulation* (1984) (ed. S. Numa), Fig. 7, p. 73.

Although the minimal functional entity of yeast FAS is $\alpha_2\beta_2$, the full yeast complex, as purified normally, has a molecular mass of about 2.2×10^6 Da and, therefore, consists of an $\alpha_6\beta_6$ structure.

Animal FASs have been purified from a variety of sources. One of the best sources is the goose uropygial gland, which is a highly specialized organ producing large amounts of fatty acids. FAS comprises up to 30% of the total soluble protein in this gland. The immunological cross-reactivity between FAS from different sources has been checked and the results indicated that there were very few common antigenic determinants on the avian and mammalian enzymes. In contrast, mammalian enzymes from various tissues and species showed some common antigenic determinants.

Animal FAS complexes consist of homodimers of native molecular mass 450–550 kDa. For some years there was considerable debate as to whether the animal enzymes were heterodimers, like yeast, or homodimers. The evidence that the latter is the case has come from several lines of research.

(1) When dissociated into monomers, only a single peptide band is obtained on electrophoresis.
(2) 4′-Phosphopantetheine was found associated with the peptide and there were approximately 2 moles per dimer.
(3) A thioesterase domain could be released by partial proteinase digestion and there were two domains per dimer.

Studies with specific ligands and inhibitors have succeeded in giving considerable information about the sites for the partial reactions along the FAS molecule. The work has been carried out in a number of laboratories but those of Kumar, Porter and Wakil made the major initial advances. It is known, for example, that there is considerable sequence homology for the ACPs from *E. coli* and barley and the ACP part of the multifunctional protein of rabbit. The sequence around the active-site serine of the acyl transferase of rabbit FAS is similar to that of the malonyl and acetyl transferases of yeast FAS and the thioesterase of goose FAS. These observations have led to the conclusion that the multifunctional forms of FAS have arisen by gene fusion. Thus, in simple terms, the genes for Type II FAS enzymes would fuse to give two genes that could code for a yeast-type FAS. The latter would then fuse to give a single gene coding for the Type I FAS enzyme. However, for mammals the fusion must have occurred by independent events (rather than by the simple scheme described above) for several reasons.

(1) The mass of the two yeast FAS proteins combined is 50% greater than the mammalian FAS.
(2) The termination mechanisms for yeast FAS (transfer to CoA) and mammalian FAS (liberation of unesterified acid) are different.
(3) The second reductase of yeast FAS is unique in using FMN.
(4) In mammalian FAS, a single active site transfers the acetyl and malonyl residues whereas in yeast FAS there are two active sites.
(5) Recent studies on the site of the partial reactions on mammalian FAS have shown how they are arranged on the dimer (Fig. 2.10). This arrangement places the enoyl reductase between 3-ketoacyl synthase and ACP in mammalian FAS whereas the reductase is on a separate protein (β-unit) from the other two functions (α-unit) in yeast FAS.

The FAS from *Mycobacterium smegmatis* may, however, have originated from fusion of the genes for the yeast subunits. It has a molecular mass of about 290 kDa (Table 2.8) and, like the yeast enzyme, uses FMN for its enoyl reductase and transfers the products to CoA.

The model of the animal fatty acid synthase, which is illustrated in Fig. 2.10, is shown in a simple diagram for one half of the dimer in Fig. 2.11. In this figure, the active site residues are indicated. Thus, the condensing enzyme has an important cysteine while the attachment of the 4′-phosphopantetheine is to a serine on the acyl carrier protein domain. Typically the thioesterase has an active-site serine (as do many hydrolytic enzymes) while both reductases have similar active-site motifs.

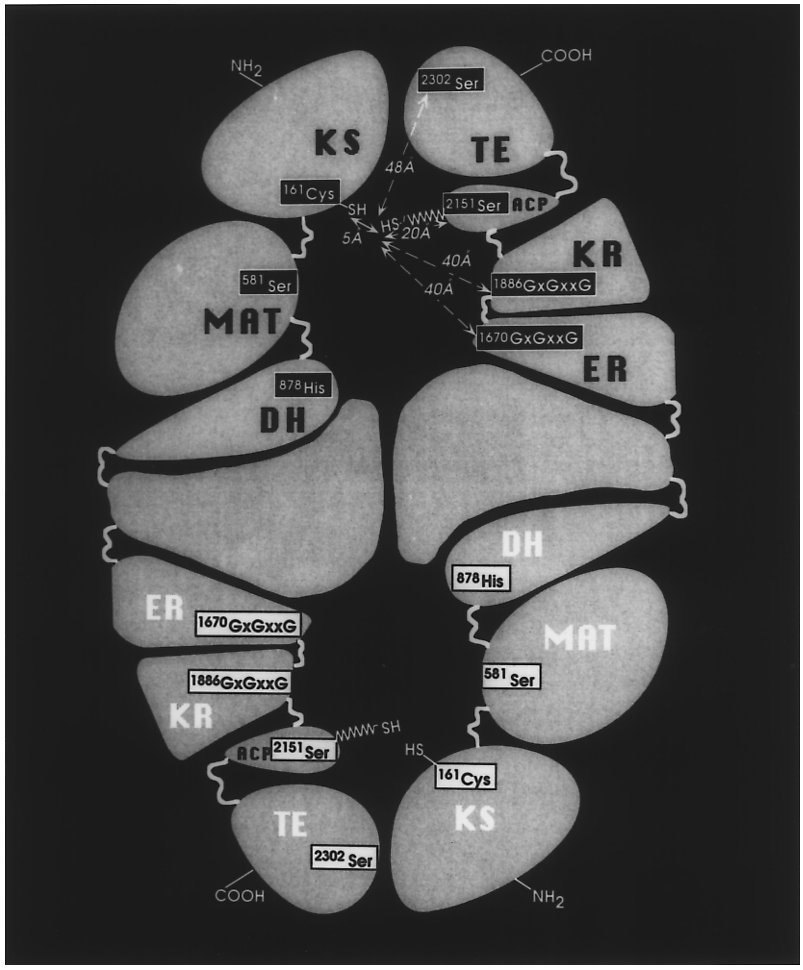


Fig. 2.10 The animal fatty acid synthase model (taken from Joshi and Smith, 1993, with kind permission of the authors and the American Society for Biochemistry and Molecular Biology). The overall shape of the protein (216 Å long, 144 Å wide, 72 Å deep) and the presence of the two 40 Å 'holes' is based on electron micrographic evidence. Two identical subunits are juxtaposed head-to-tail. Each subunit consists of two clusters of catalytic domains (KS/MAT/DH and ER/KR/ACP/TE) separated by a central structural core. The active-site residues of the various catalytic domains, the glycine-rich motifs of the NADPH-binding sites associated with the reductases and the site of attachment of the 4′-phosphopantetheine thiol are shown. A key feature of this model is that two centres for acyl chain assembly and release are formed by co-operation of three catalytic domains (KS/MAT/DH) of one subunit with four catalytic domains (ER/KR/ACP/TE) of the adjacent subunit. KS: ketoacyl synthase; MAT: malonyl/acetyltransferase; DH: dehydrase; ER: enoyl reductase; KR: ketoreductase; ACP: acyl carrier protein; TE: thioesterase. GxGxxG are the amino acids at the catalytic sites of the reductases, where G is glycine and x is any amino acid.

Stuart Smith has now studied the animal fatty acid synthase in much more detail. Various models of this FAS have been proposed but all of them involve two identical antiparallel polypeptide chains that together form two centres for palmitate synthesis at the subunit interface. Essentially in the conventional view of the FAS mechanism, a malonyl residue attached to ACP on one half of the dimer interacts with the acyl chain (originally acetyl) attached to a cysteine at the active site of the

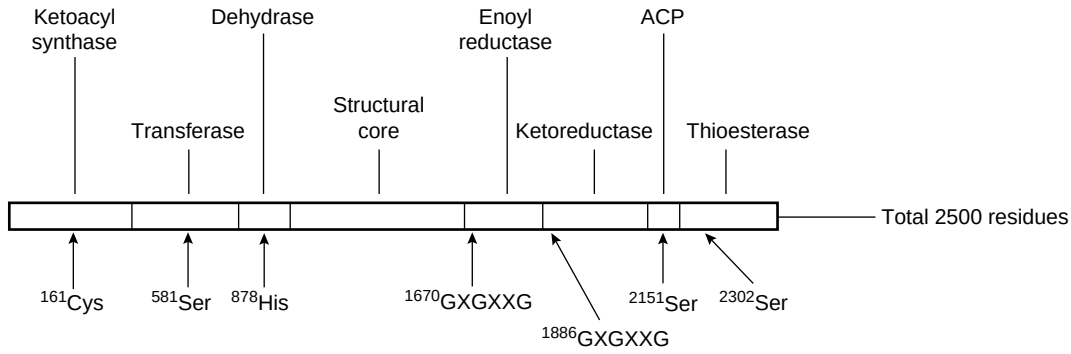


Fig. 2.11 Arrangement of the partial reactions of one monomer of the animal fatty acid synthase. Information taken from Smith (1994). The key active-site residues for each domain are indicated.

condensing enzyme on the other half of the dimer (Fig. 2.12). Other experimental evidence demonstrated that translocation of acetyl and malonyl moieties from CoA esters to the 4′-phosphopantetheine of the ACP domain also needs the dimeric form of the enzyme.

However, Smith and colleagues have produced a number of modified FASs in which the activity of one of the functional domains is specifically compromised by mutations. Heterodimers are then formed from subunits containing different single mutations to observe whether they are capable of fatty acid synthesis. Unexpectedly, Smith and colleagues found that the dehydrase and ACP domains could interact in the *same subunit*, even though they were separated by more than 1000 residues (see Fig. 2.11). Moreover, both the substrate translocation and condensation reactions could use the ACP of *either* subunit. These results led to the proposal that the two halves of the dimer are not simply arranged in an extended conformation but are folded in a manner to allow functional contacts *within* subunits in addition to *between* subunits. This allows greater flexibility for the interaction of domains (see Fig. 2.12). Based on results with cross-linking agents, it has been estimated that up to 35% of the resting, wild-type FAS adopts a conformation where the condensing enzyme active site cysteine is juxtaposed with the phosphopantetheine residues of the ACP domain of the same subunit.

2.2.3.3 Termination

The typical end-product of animal FAS enzymes is unesterified palmitic acid. The cleavage of this acid from the complex is catalysed by a thioesterase which, as discussed above, is an integral part of the enzyme. Several factors combine to achieve this normal specificity. First, the transferase that catalyses the loading of substrate moieties from CoA-esters to the enzyme-bound thiolester group has a high specificity for acetyl and butyryl groups. Thus, once the acyl chain has grown longer than 4C it cannot readily escape the FAS. Second, the condensation reactions are much faster for medium-chain acyl substrates thereby ensuring that, once elongation has started, it rapidly proceeds to long chain lengths. By contrast, chain lengths of 16C or above are not easily elongated. This is shown most convincingly in experiments where the thioesterase activity has been removed. Under these conditions, palmitate is still the main product. Moreover, chains of 16C or more tend to transfer to the 4′-phosphopantetheine of the ACP domain rather than to the condensing enzyme domain, perhaps because of steric hindrance or hydrophobicity in a similar fashion to what occurs in the yeast FAS (Fig. 2.9). This means that palmitate will dwell on the mammalian FAS until it is cleaved by the thioesterase. In addition, the thioesterase itself shows a strong preference for palmitate.

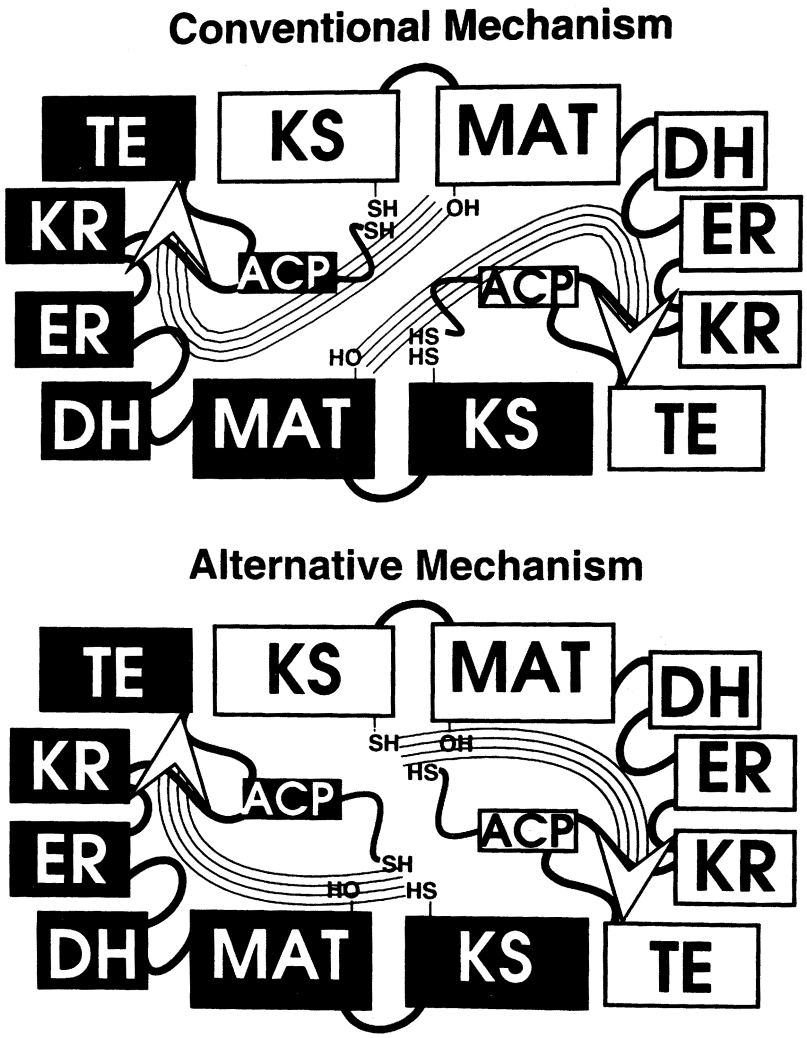


Fig. 2.12 The conventional and alternative models for the action of animal fatty acid synthase. The two-dimensional cartoon shows a simple caricature of the seven domains on each of the two identical peptides present in the dimer. For clarity a long non-catalytic domain between DH and ER (see Fig. 2.11) and extending approximately between residues 970 and 1630, has been omitted. In the conventional mechanism, the ACP of one-half of the dimer provides the 'primer' acyl chain to KS of the other half of the dimer for condensation. In the alternative mechanism, an altered three-dimensional configuration of the FAS protein allows the ACP to interact with the KS domain on the same half of the dimer. Under these circumstances, all the reactions involved in two-carbon addition take place on one-half of the dimer rather than through co-operation between catalytic domains located on both subunits. Redrawn from Joshi, A.K., Witkowski, A. and Smith, S. (1998) *Biochemistry*, **37**, 2515–2523 with kind permission of the authors and the American Chemical Society.

However, in some mammalian tissues, 4C–14C products are released. Moreover, a variety of organisms has been found that produce different types of fatty acid end-products. Several factors have been found to influence chain termination.

(1) In rat mammary gland, where the milk triacylglycerols contain large quantities of 8:0 and 10:0 acids, Smith found a second thioesterase responsible for the release of medium-chain acids. A similar thioesterase II has also been found in rabbit mammary gland. The Californian bay tree produces medium-chain fatty acids in its seed storage lipids. A specific medium-chain thioesterase has been isolated from this species and, by genetic manipulation of oilseed rape, been used to produce laurate (12:0)-enriched oils, which have industrial utility.
(2) In contrast, goat mammary gland, which also produced short- and medium-chain fatty acids, did not contain thioesterase II. In this case a transacylase was found in the FAS and, when incubated in combination with microsomes and appropriate cofactors, rapid transfer of medium-chain fatty acids into triacylglycerols is seen.
(3) The uropygial gland FAS from a number of birds produces medium branch-chain fatty acids as products. These products are released by a specific hydrolase, which is absent from those birds whose end-products are long branched-chain fatty acids.
(4) For *E. coli* early experiments showed that the specificity of the 3-ketoacyl-ACP synthase was such that palmitoyl- and vaccenoyl-ACP could not act as primers. However, additional evidence from Cronan's laboratory suggests chain elongation can continue in cells if they are starved of glycerol 3-phosphate so that the fatty acid products are not transferred into membrane phospholipids.
(5) In *Mycobacterium smegmatis* termination involves transacylation of 16C–24C fatty acids to CoA. This transacylation is stimulated by polysaccharides, which seem to act by increasing the diffusion of the acyl-CoA esters from FAS rather than promoting acyl transfer from ACP to CoA.

There may be other mechanisms for controlling the chain length of the fatty acids produced by the various FAS complexes. Certainly, there are plenty of other theories that have been proposed and evidence has been obtained, in some cases, in vitro. Moreover, there are numerous cases where unusual distributions of fatty acid products are found but about which we know very little of the mechanism of termination.

2.2.3.4 Elongation

Although, as discussed above, the major product of FAS is often palmitate, many tissues contain longer chain fatty acids in their (membrane) lipids. For example, in the myelin of nervous tissues, fatty acids of 18C or greater make up two-thirds of the total, while in many sphingolipids fatty acids of 24C are common. In plants, the surface waxes contain mainly very long chain products in the 28C–34C range.

The formation of these very long chain fatty acids is catalysed by the Type III synthases, which are commonly termed elongases because they chain lengthen preformed fatty acids (either produced endogenously or originating from the diet). Most eukaryotic cells have the capacity to carry out elongation reactions.

In liver, brain and many mammalian tissues there are two elongation systems located in the mitochondria and endoplasmic reticulum, respectively. The mitochondrial system, discovered by Wakil in rat liver, occurs by the addition of 2C units from acetyl-CoA and not malonyl-CoA. Monoenoic acyl-CoAs are generally preferred to saturated substrates. In tissues such as liver or brain, both NADPH and NADH are needed, whereas heart or skeletal muscles required only NADH. The German biochemist, Seubert, showed the virtual reversal of β-oxidation (Section 2.3.1) for mitochondrial elongation. However, the enzyme FAD-dependent acyl-CoA dehydrogenase in β-oxidation is replaced by the thermodynamically more favourable enoyl-CoA reductase. The enoyl-CoA reductase isolated

from liver mitochondria is different from that of the endoplasmic reticulum and kinetic studies suggest that its activity largely controls the speed of overall mitochondrial elongation.

The principal reactions for the elongation of longer chain fatty acids are found in the membranes of the endoplasmic reticulum that are isolated as the microsomal fraction by ultracentrifugation of tissue homogenates. The reactions involve acyl-CoAs as primers, malonyl-CoA as the donor of 2C units and NADPH as the reducing coenzyme. An example of the microsomal elongation system is in the nervous system, where large amounts of 22C and 24C saturated fatty acids are constituents of myelin sphingolipids (Chapters 6 and 7). Before myelination begins, the activity of stearoyl-CoA elongase is hardly measurable, but it rises rapidly during myelination. The mutant 'quaking mouse' is deficient in myelination and has proved to be a useful model for studies of the elongation process. The rate of elongation of 18:0-CoA to 20:0-CoA is normal and that of 16:0-CoA to 18:0-CoA is rather lower than normal in this mutant. The elongation of 20:0-CoA, however, is very much reduced, suggesting that there are at least three elongases in this tissue.

It is likely that the separate elongases referred to above (in brain) differ in the nature of their condensing enzyme rather than for the other three components (3-ketoacyl-CoA reductase, 3-hydroxyacyl-CoA dehydrase, enoyl-CoA reductase). Enzyme studies have shown more than one condensing enzyme in several tissues. All of the enzymes involved in elongation have a cytosolic orientation on the endoplasmic reticulum and, although NADPH or NADH are used by the reductases, the flow of electrons appears to be indirect. Involvement of cytochrome b_5 or of cytochrome P450 has been proposed.

One of the most important functions of elongation is in the transformation of dietary essential fatty acids to the higher polyunsaturated fatty acids. The starting point is linoleoyl-CoA, which is first desaturated to a trienoic acid. This is followed by a sequence of alternate elongations and desaturations that are described in further detail in Sections 2.2.5.3 and 4.2.3.2. Indeed, it is noticeable that, in the liver elongation system, γ-linolenic acid is the best substrate.

The end-product of the Type II FAS of plants is palmitoyl-ACP and this serves as the substrate for an elongation system (palmitate elongase). This is soluble and appears to differ from the Type II FAS forming palmitate only in having a specific condensing enzyme, 3-ketoacyl-ACP synthetase II. Because the palmitate elongase is able to chain-lengthen preformed palmitate it can be regarded as an elongase but, since it usually functions as part of the *de novo* system for fatty acid production, it can also be considered part of the Type II FAS by analogy with the two different condensing enzymes present in *E. coli*.

Very long chain fatty acids in plants are made by membrane-bound enzyme systems utilizing malonyl-CoA as the source of 2C units in similar fashion to the animal elongases. Acyl-CoAs have been shown to be the substrates in some of these systems and various elongases have been demonstrated which have different chain-length specificities. Moreover, the individual partial reactions involved have been demonstrated and some purifications achieved. Genes coding for the condensing enzymes have, for example, recently been identified. The production of very long chain (>18C) fatty acids is required for the formation of the surface-covering layers, cutin and suberin (Section 6.6.1), as well as for seed oil production in commercially important crops such as rape and jojoba (Sections 3.4.1.3 and 3.7.1).

2.2.3.5 Branched-chain fatty acids

The formation of branched-chain fatty acids by the Type I FAS of the sebaceous (uropygial) glands of waterfowl has already been mentioned (Section 2.2.3.2 and Table 2.8). These acids arise because of the use of methylmalonyl-CoA rather than malonyl-CoA, which is rapidly destroyed by a very active malonyl-CoA decarboxylase. The utilization of methylmalonyl-CoA results in the formation of products such as 2,4,6,8-tetramethyl decanoic acid or 2,4,6,8-tetramethylundecanoic acid as major products when acetyl-CoA or propionyl-CoA, respectively, are used as primers.

A high proportion of odd chain and of various polymethyl-branched fatty acids occurs in the adipose tissue triacylglycerols of sheep and goats when they are fed diets based on cereals such as barley. Cereal starch is fermented by bacteria in the rumen to form propionate, and when the animal's capacity to metabolize propionate via methylmalonyl-CoA to succinate is overloaded, propionyl- and methylmalonyl-CoA accumulate. Garton and his colleagues showed that methylmalonyl-CoA can take the place of malonyl-CoA in fatty acid synthesis and that with acetyl- or propionyl-CoA as primers, a whole range of mono-, di- and tri-methyl branched fatty acids can be produced.

The major fatty acids in most Gram-positive and some Gram-negative genera are branched-chain *iso* or *anteiso* fatty acids. The Type II FAS enzymes present in these bacteria make use of primers different from the usual acetyl-CoA. For example, *Micrococcus lysodeikticus* is rich in 15C acids of both the *iso* type, 13-methyl-C14 or *anteiso* type, 12-methyl-C14. These have been shown to originate from leucine and isoleucine, respectively (Fig. 2.13). Thus, isobutyryl-CoA is used as the primer for *iso*-branched fatty acids and 2-methylvaleryl-CoA for *anteiso* products. These branched fatty acids are used to increase membrane fluidity in those bacteria that only have low levels of unsaturated acids under most growth conditions.

Another common branched-chain fatty acid is 10-methylstearic acid, tuberculostearic acid, a major component of the fatty acids of *Mycobacterium phlei*. In this case, the methyl group originates from the methyl donor *S*-adenosyl methionine, while the acceptor is oleate esterified in a phospholipid. This is an example, therefore, of fatty acid modification taking place while the acid is in an *O*-ester rather than the *S*-esters of CoA or ACP. The formation of tuberculostearic acid takes place in two steps, the intermediate being 10-methylenestearic acid, which is then reduced to 10-methylstearic acid (Fig. 2.14).

2.2.4 The biosynthesis of hydroxy fatty acids results in hydroxyl groups in different positions along the fatty chain

Hydroxy fatty acids are formed as intermediates during various metabolic pathways (e.g. fatty acid synthesis, β-oxidation) and also because of specific hydroxylation reactions. Usually the hydroxyl group is introduced close to one end of the acyl

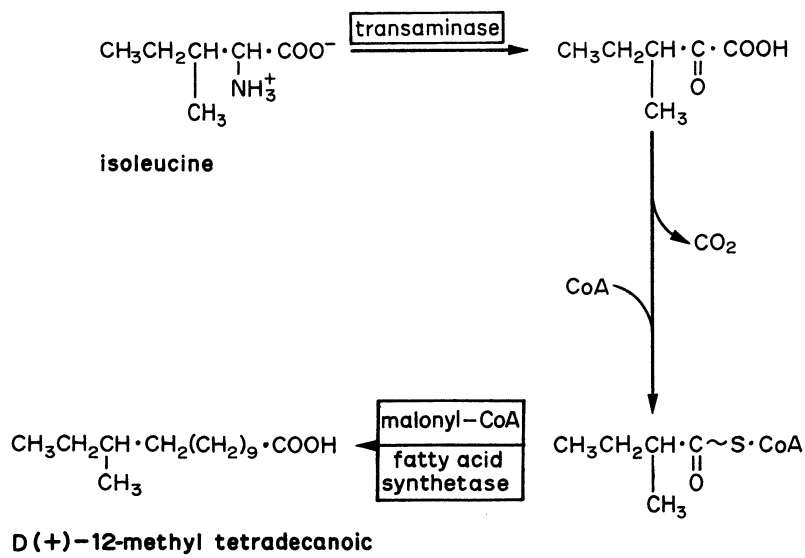


Fig. 2.13 Production of an *anteiso* branched-chain fatty acid in bacteria.

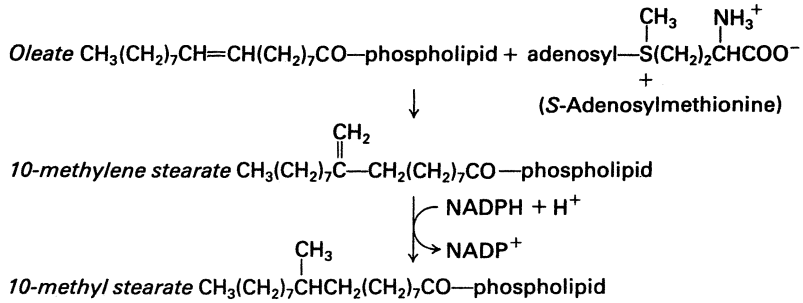


Fig. 2.14 Production of tuberculostearic acid in *Mycobacterium phlei*.

chain. However, mid-chain hydroxylations are also found – a good example being the formation of ricinoleic acid (D-12-hydroxyoleic acid; Table 2.4). This acid accounts for about 90% of the triacylglycerol fatty acids of castor oil and about 40% of those of ergot oil, the lipid produced by the parasitic fungus, *Claviceps purpurea*. In developing castor seed, ricinoleic acid is synthesized by hydroxylation of oleate while attached to position 2 of phosphatidylcholine. The Δ12-hydroxylase accepts electrons from either NADPH or NADH via a cytochrome b_5 protein and uses molecular oxygen. The gene for the enzyme shows many similarities to the fatty acid desaturases and deduced protein structures suggest a common evolutionary origin and reaction mechanism. In contrast to the method of hydroxylation in castor seed, the pathway in *Claviceps* involves hydration of linoleic acid under anaerobic conditions. Thus the hydroxyl group in this case comes from water and not from molecular oxygen.

α-Oxidation systems producing α-hydroxy (2-hydroxy) fatty acids have been demonstrated in micro-organisms, plants and animals. In plants and animals these hydroxy fatty acids appear to be preferentially esterified in sphingolipids. α-Oxidation is described more fully in Section 2.3.2.

ω-Oxidation involves a typical mixed-function oxidase. The major hydroxy fatty acids of plants have an ω-OH and an in-chain OH group (e.g. 10,16-dihydroxypalmitic acid). Their synthesis seems to involve ω-hydroxylation with NADPH and O_2 as cofactors, followed by in-chain hydroxylation with the same substrates. If the precursor is oleic acid then the double bond is converted to an epoxide, which is then hydrated to yield 9,10-hydroxy groups. These conversions involve CoA esters. ω-Oxidation is discussed further in Section 2.3.3.

2.2.5 The biosynthesis of unsaturated fatty acids is mainly by oxidative desaturation

2.2.5.1 Monounsaturated fatty acids

Unsaturated fatty acids can either be produced anaerobically or in the presence of oxygen, which acts as an essential cofactor. The anaerobic mechanism is rather rare but is used by *E. coli* (and other members of the *Eubacteriales*) as part of its FAS complex (Section 2.2.3.2). By far the most widespread pathway is by an oxidative mechanism, discovered by Bloch's team, in which a double bond is introduced directly into the preformed saturated long-chain fatty acid with O_2 and a reduced compound (such as NADH) as cofactors. This pathway is almost universal and is used by bacteria, yeasts, algae, higher plants, protozoa and animals. Apparently, the two pathways are mutually exclusive because no organism has yet been discovered that contains both the aerobic and anaerobic mechanisms of desaturation. Most of the monounsaturated acids produced have a Δ9 double bond. Exceptions are Δ7 bonds in some algae, Δ5 and Δ10 monoenoic acids in Bacilli and a Δ6 acid (petroselenic) in some plants.

The pathway was first demonstrated in yeast.

Cell-free preparations could catalyse the conversion of palmitic into palmitoleic acid (hexadec-9-enoic acid, 9-16:1) only if both a particulate fraction (microsomes) and the supernatant fraction were present. The membrane fraction alone could perform the dehydrogenation provided that the substrate was the acyl-CoA thiolester. The supernatant contained the acid:CoA ligase to activate the fatty acid. More recently another protein fraction in the soluble cytoplasm has been found to stimulate desaturation. This is probably the fatty acid binding protein, which regulates the availability of fatty acid or fatty acyl-CoA for lipid metabolizing enzymes. Bloch found that the cofactors for the desaturation were NADH or NADPH and molecular oxygen, which suggested to him a mechanism similar to many mixed-function oxygenase reactions.

It has been particularly difficult and slow to obtain a detailed understanding of the biochemistry of the desaturase enzymes. Not only are they usually located in membranes, but the substrates are micellar (Section 2.1.8) at concentrations that are suitable for studies in vitro. It was only when methods for solubilizing membranes with detergents were developed that the stearoyl-CoA desaturase enzyme was purified and a better understanding of the enzymic complex emerged. Work by Sato's group in Japan and Holloway in the USA has identified three component proteins of the complex: a flavoprotein, NADH-cytochrome b_5 reductase; a haem-containing protein, cytochrome b_5; and the desaturase itself which, because of its inhibition by low concentrations of cyanide, is sometimes referred to as the cyanide-sensitive factor (CSF). Strittmatter's group in the USA has purified this latter protein (and identified its gene), which is a single polypeptide chain of 53 kDa containing one non-haem iron atom per molecule of enzyme. The iron can be reduced in the absence of stearoyl-CoA, by NADH and the electron transport proteins, and when it is removed, enzymic activity is lost.

Although the desaturation reaction has all the characteristics of a mixed-function oxygenation, nobody has ever successfully demonstrated an hydroxylation as an intermediate step in double bond formation. In spite of our lack of knowledge of the mechanism, certain details have emerged concerning the stereochemistry of the dehydrogenation. Schroepfer and Bloch in the USA and James and coworkers in the UK have demonstrated that the D-9 and D-10 (*cis*) hydrogen atoms are removed by animal, plant and bacterial systems. Experiments with deuterium-labelled stearate substrates showed isotope effects at both the 9 and 10 positions, which are consistent with the concerted removal of hydrogens rather than a mechanism involving a hydroxylated intermediate followed by dehydration.

The three essential components of the animal Δ9-desaturase are thought to be arranged in the endoplasmic reticulum in a manner shown in Fig. 2.15. The cytochrome b_5 component is a small haem-containing protein (16–17 kDa), which has a major hydrophilic region and a hydrophobic anchor of about 40 amino acids at the carboxyl terminus. The cyanide-sensitive desaturase component is largely within the membrane with only its active centre exposed to the cytosol. Most animal Δ9-desaturases work well with saturated acyl-CoAs in the range 14C–18C. Although it was originally thought (from substrate selectivity studies) that liver contained two isoforms, isolation of genes coding for the desaturase suggests that there is only one (*SCD*-1) in liver. However, other tissues, such as lung and kidney express two genes (*SCD*-1 and *SCD*-2), while brain expresses only the *SCD*-2 gene. In some classes of tumours an increased level of desaturase mRNA has been found.

In yeast, the Δ9-desaturase is expressed by the *OLE*-1 gene. In deficient mutants, the rat liver Δ9-desaturase gene can effectively substitute for the missing activity.

A general scheme for aerobic fatty acid desaturation is shown in Fig. 2.16 and this applies to the animal Δ9-desaturase. However, although the animal enzyme clearly uses NADH and cytochrome b_5 the Δ9-desaturase of other organisms may not. Interestingly, the enzyme from plants is soluble. Moreover, it uses stearoyl-ACP as substrate instead of stearoyl-CoA and reduced ferredoxin as a source of reductant (a sensible choice of substrate for a desaturase that is located in the chloroplast).

The availability of the plant Δ9-desaturase as a

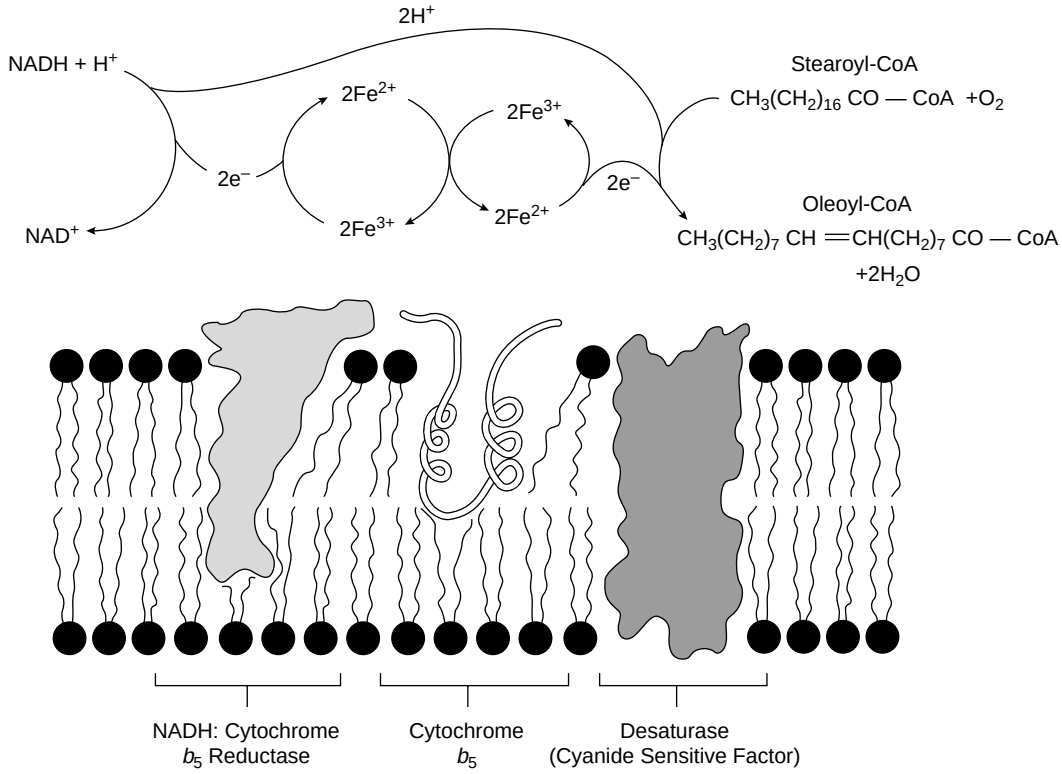


Fig. 2.15 Diagrammatic representation of the animal Δ9-fatty acid desaturase complex. Adapted from H.W. Cook (1996) with kind permission of the author and Elsevier Science. Note that the nature of the hydrogen transfer from NADH (which is depicted in the diagram as H^+) has not been proven. In some Δ5 or Δ6 polyunsaturated fatty acid desaturases, the cytochrome b_5 component is part of the same protein as the desaturase.

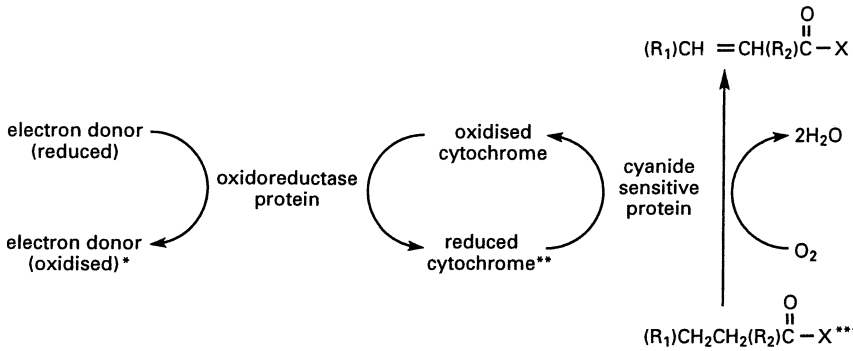


Fig. 2.16 A generalized scheme for aerobic fatty acid desaturation: *e.g. NADH, NADPH, reduced ferredoxin; **e.g. cytochrome b_5; ***e.g. acyl-ACP (stearoyl-ACP Δ9-desaturase in plants); acyl-CoA (stearoyl-CoA Δ9-desaturase in animals); oleoyl-phosphatidylcholine (Δ12-desaturase in yeast or plants); linoleoyl-monogalactosyldiacylglycerol (Δ15-desaturase in plant chloroplasts).

soluble protein allowed it to be purified in Stumpf's laboratory and, later, with the availability of a gene coding for the enzyme it has been possible to obtain a lot of important information about its reaction mechanism using point mutations. Much of this work has come from Shanklin's laboratory at Brookhaven and he and his coworkers have succeeded in obtaining structural information by X-ray studies down to about 3 Å resolution. Once sequence information was available from a number of desaturases, it was clear that they belonged to a group of proteins with di-iron centres. This di-iron cluster is also found in the reaction centre of enzymes like methane monooxygenase and, interestingly, in the oleate hydroxylase that gives rise to ricinoleate (Section 2.2.4). A computer-generated model of the plant Δ9-desaturase is shown in Fig. 2.17.

An unusual monounsaturated fatty acid, specifically linked to phosphatidylglycerol and found in chloroplasts, is *trans*-3-hexadecenoic acid (Table 2.2). Palmitic acid is its precursor and oxygen is required as cofactor. When radiolabelled *trans*-3-hexadecenoic acid is incubated with chloroplast preparations, it is not specifically esterified in phosphatidylglycerols but is either randomly esterified in all chloroplast lipids or reduced to palmitic acid. These results could be explained if the direct precursor of *trans*-3-hexadecenoic acid were palmitoyl-phosphatidylglycerol and not

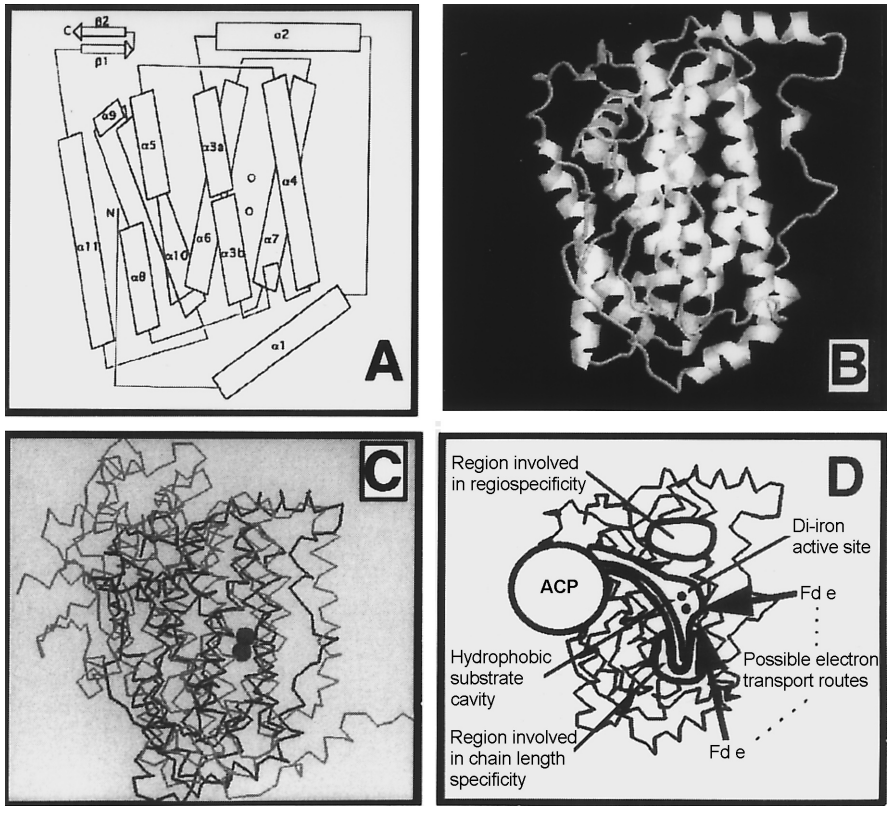


Fig. 2.17 Models of the plant Δ9-stearoyl-ACP desaturase: (A) schematic of secondary structural elements; (B) structural elements; (C) overlay of the desaturase with methane mono-oxygenase; (D) cartoon of functional regions of the desaturase. Reproduced from Fig. 1 of J. Shanklin *et al.* (1997) with kind permission of the author and Kluwer Academic Publishers.

palmitoyl-*S*-CoA or palmitoyl-*S*-ACP. This is an example of a complex lipid acting as a desaturase substrate and often such substrates seem to be important for polyunsaturated fatty acid production.

2.2.5.2 Polyunsaturated fatty acids

Although most bacteria are incapable of producing polyunsaturated fatty acids, other organisms, including many cyanobacteria and all eukaryotes, can. These acids usually contain methylene-interrupted double bonds, i.e. they are separated by a single methylene group. Animal enzymes normally introduce new double bonds between an existing double bond and the carboxyl group (Fig. 2.18); plants normally introduce the new double bond between the existing double bond and the terminal methyl group (Fig. 2.19).

We shall describe polydesaturations in plants first. The reason for this is that one of the most abundant polyunsaturated acids produced by plants, linoleic acid (*cis*,*cis*-9,12-18:2) cannot be made by animals [although it is always dangerous in biochemistry to make dogmatic statements and, indeed, some protozoa and a few species of insects are capable of forming linoleic acid] yet this acid is necessary to maintain animals in a healthy condition. For this reason linoleic acid must be supplied in the diet from plant sources, and in order to discuss adequately the metabolism of polyunsaturated fatty acids in animals, it is first necessary to understand their formation in plants. Acids of the linoleic family are known as essential fatty acids and will be discussed in later sections.

The precursor for polyunsaturated fatty acid formation in plants and algae is oleate. The next double bond is normally introduced at the 12,13 position (Δ12 desaturase) to form linoleate followed by desaturation at the 15,16 position (Δ15 desaturase) to form α-linolenic acid (all *cis*-9,12,15-18:3) as summarized in Fig. 2.19. With the exception of some cyanobacteria, α-linolenic acid is the most common fatty acid found in plants and fresh-water algae. In marine algae, highly unsaturated 20C acids are predominant, the principal of which (arachidonic and eicosapentaenoic) are made by the pathways shown in Fig. 2.19.

The possibility that desaturation could occur on fatty acyl chains esterified in complex lipids was first suggested by experiments with the phytoflagellate, *Euglena gracilis*. This organism can live as a plant or animal and can synthesize both plant and animal types of polyunsaturated fatty acids. The animal type of fatty acids accumulate in the phospholipids and the plant types in the galactolipids. It proved impossible to demonstrate plant-type desaturations in vitro when acyl-CoA or acyl-ACP thiolesters were incubated with isolated cell fractions. Either the desaturase enzymes were labile during the fractionation of the plant cells, or the substrates needed to be incorporated into the appropriate lipids before desaturation could take place. The next series of experiments was done with *Chlorella vulgaris*, a green alga that produces a very simple pattern of plant-type lipids. When cultures of the alga were labelled with ^{14}C-oleic acid as a precursor of the 18C polyunsaturated fatty acids, labelled linoleic and linolenic acids were produced and the label was only located in the phosphatidylcholine fraction. Next synthetic ^{14}C-labelled oleoyl-phosphatidylcholine was tested as a substrate for desaturation: the only product formed was linoleoyl-phosphatidylcholine. An important loophole that had to be closed was the possibility that during the incubation, labelled oleic acid might be released, activated to the CoA or ACP thiolester, desaturated as a thiolester and then re-esterified to the same complex lipid very rapidly. Appropriate control experiments eliminated this possibility.

Desaturations involving lipid-bound fatty acids have also been shown to occur in the mould *Neurospora crassa*, various yeasts such as *Candida utilis* and *Candida lipolytica*, higher plants and several animal tissues.

One of the best studied systems has been that in the leaves of higher plants. It will be recalled that synthesis of fatty acids *de novo* in plants occurs predominantly in the plastids. Fatty acid synthase forms palmitoyl-ACP, which is elongated to stearoyl-ACP and then desaturated to oleoyl-ACP. The latter can then be hydrolysed and re-esterified by the chloroplast envelope to oleoyl-CoA. Although

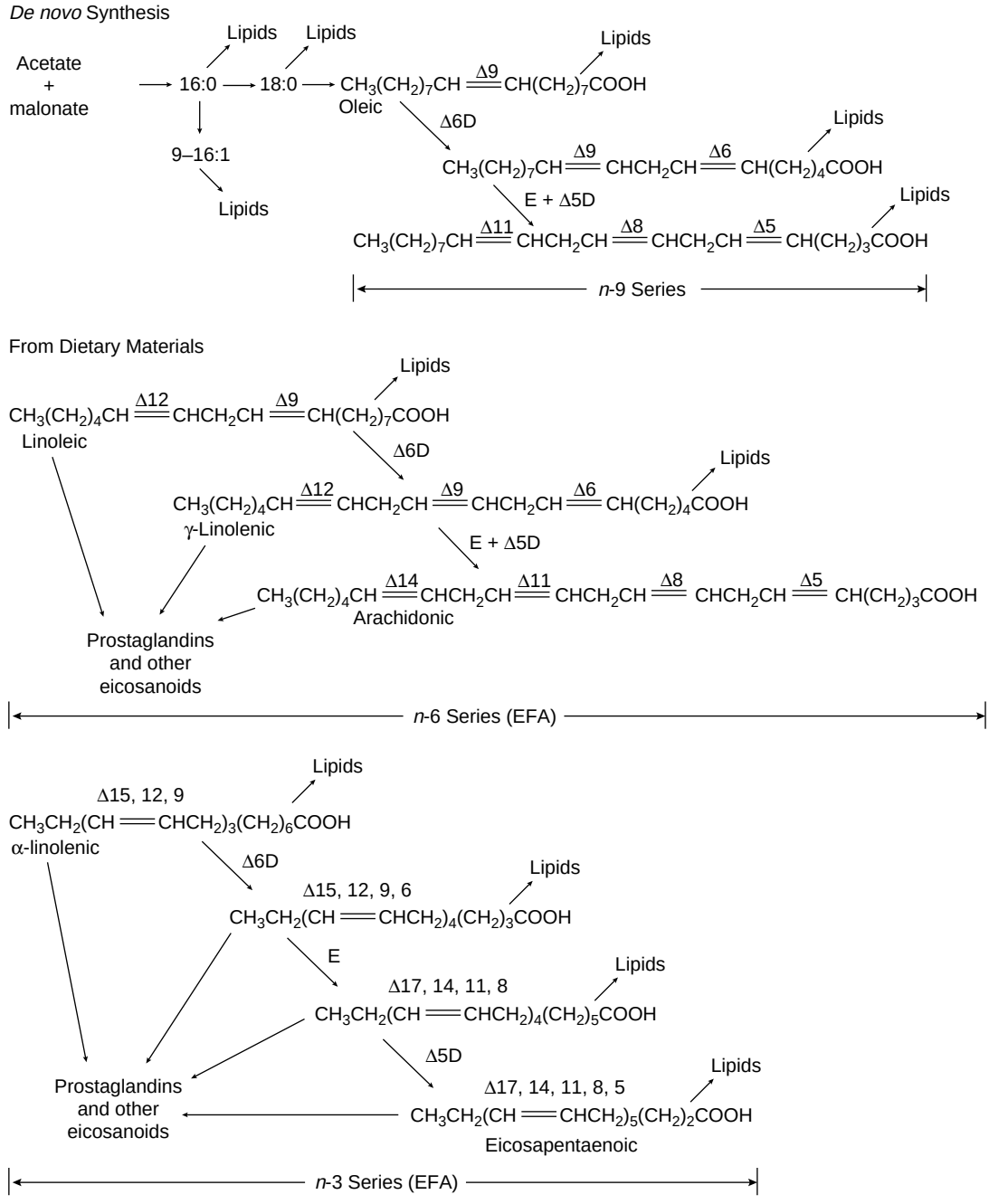


Fig. 2.18 Important pathways for unsaturated fatty acid formation in mammals. E = elongase; D = desaturase (positional specificity indicated).

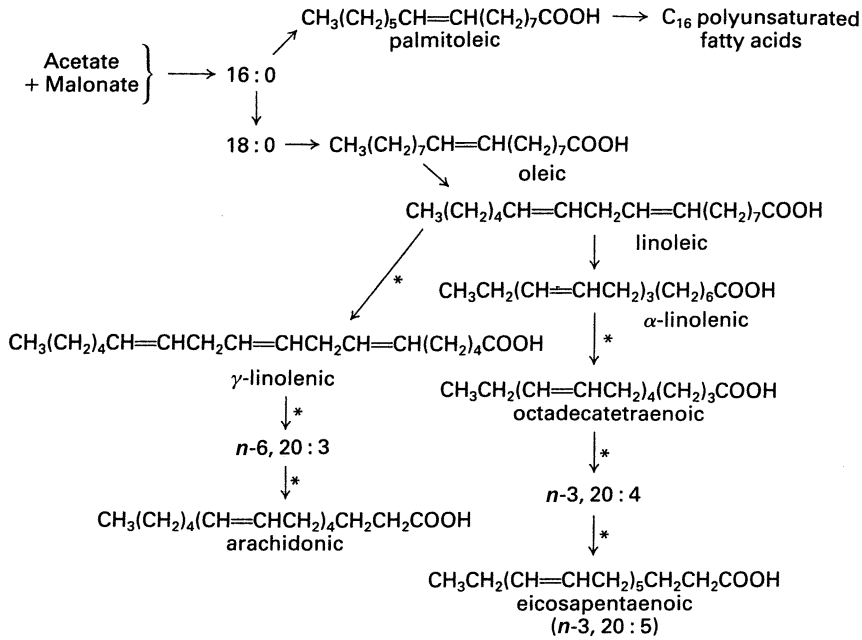


Fig. 2.19 Major pathways for polyunsaturated fatty acid synthesis in plants and algae. *Indicates a pathway found in high levels in marine algae and mosses, but less commonly in other algae or plants.

oleoyl-CoA can be used as a substrate for in vitro systems and will be desaturated rapidly to linoleate in the presence of oxygen and NADH, both substrate and product accumulate in phosphatidylcholine. Indeed, careful experiments by Roughan and Slack in New Zealand, Stymne and Stobart (Sweden, UK) and others have provided considerable evidence that the actual desaturase substrate is 1-acyl-2-oleoyl-phosphatidylcholine. Although phosphatidylcholine may also be the substrate for linoleate desaturation in a few systems, for leaf tissue the final desaturation appears usually to utilize another lipid, monogalactosyldiacylglycerol, as a substrate. There is indirect evidence in vivo for this desaturation and also a direct demonstration of the desaturation of linoleoyl-monogalactosyldiacylglycerol by chloroplasts. These pathways are indicated in Fig. 2.20.

One difficulty with the scheme is the necessity for movement of oleate out of, and linoleate back into, the plastid. The transport of oleate and its esterification into phosphatidylcholine is solved by the participation of oleoyl-CoA, which is water-soluble. In fact, acyl-CoAs are bound to a special acyl-CoA binding protein that helps to prevent any adverse effects arising from raised acyl-CoA levels having detergent activity. How the linoleate returns to the chloroplast is not known at present although the diacylglycerol part of the phosphatidylcholine molecule may be re-cycled intact. Certain phospholipid exchange proteins, which have been isolated from plants by Yamada and coworkers, may play a role here.

The pathway for polyunsaturated fatty acid formation in plants, which was discussed above (see Fig. 2.20) is probably that used by the majority of plants. It has been termed the 'eukaryotic pathway' because it involves the participation of extrachloroplastic compartments and particularly because 18C fatty acids are esterified in position 2 of participating lipids as they would be in other eukaryotes, like animals. By contrast, desaturation (and formation of chloroplast lipids) continues within the chloroplast in some plants and such

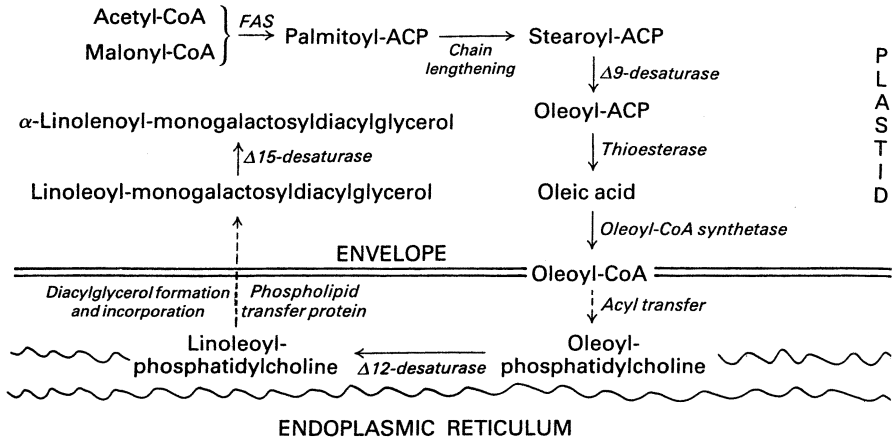


Fig. 2.20 A simplified depiction of the overall formation of α-linolenate in leaves from plants operating the 'eukaryotic pathway' of lipid synthesis. Note formation of linoleate by the Δ12-desaturase and of α-linolenate by the Δ15-desaturase is membrane-associated whereas the reactions up to oleate are all soluble (i.e. in the plastid stroma).

mechanisms are termed 'prokaryotic'. For the latter desaturations, monogalactosyldiacylglycerol is used as substrate – allowing the formation of α-linolenate and, also, hexadecatrienoate (16:3) at position 2. An example of a plant operating the prokaryotic pathway would be spinach (see Table 6.6). However, the most important point to stress is that for all plants, polyunsaturated fatty acids are made on complex lipid substrates.

A small, scruffy, plant weed named *Arabidopsis thaliana* has proven exceptionally useful to biochemists because of its small genome. Browse and Somerville made mutants of *Arabidopsis* and discovered a number with modified fatty acid and/or lipid patterns. They then used these mutants to reveal the genes coding for desaturase expression. By such experiments we now know the identity of genes for desaturations used in the prokaryotic and eukaryotic pathways referred to above.

Although complex lipid substrates have been studied best in plants, the conversion of eicosatrienoylphosphatidylcholine into arachidonoylphosphatidylcholine is an example of a similar reaction in animals (e.g. rat liver). Interestingly, some yeasts have been shown to contain oleoyl-CoA as well as oleoyl-phosphatidylcholine desaturases.

2.2.5.3 Formation of polyunsaturated fatty acids in animals

In general, double bonds are found at the Δ9, Δ6, Δ5 and Δ4 positions in animals and it has been assumed for a long time that four desaturations were involved. However, although Δ9, Δ6 and Δ5 desaturase activities have been measured in a variety of animal tissues, the Δ4-desaturase has not been convincingly demonstrated. Recently, Sprecher has found an alternative pathway to the major Δ4 polyunsaturated component (22:6, *n*-3). Thus, 22:5 (*n*-3) is elongated instead of being desaturated directly (Fig. 2.21). The 24:5 (*n*-3) product is then desaturated at the Δ6 position and shortened by two carbons through β-oxidation, probably in peroxisomes. This alternative to Δ4-desaturation is known as the Sprecher pathway and a similar mechanism is involved in the creation of 22:5 (*n*-6) as shown in Fig. 2.21.

The animal desaturases share a number of characteristics. Their reaction mechanism is that depicted in Fig. 2.16. They use molecular oxygen, reduced pyridine nucleotide and an electron transport system consisting of a cytochrome and a related reductase enzyme. Almost invariably the first double bond introduced is in the Δ9 position

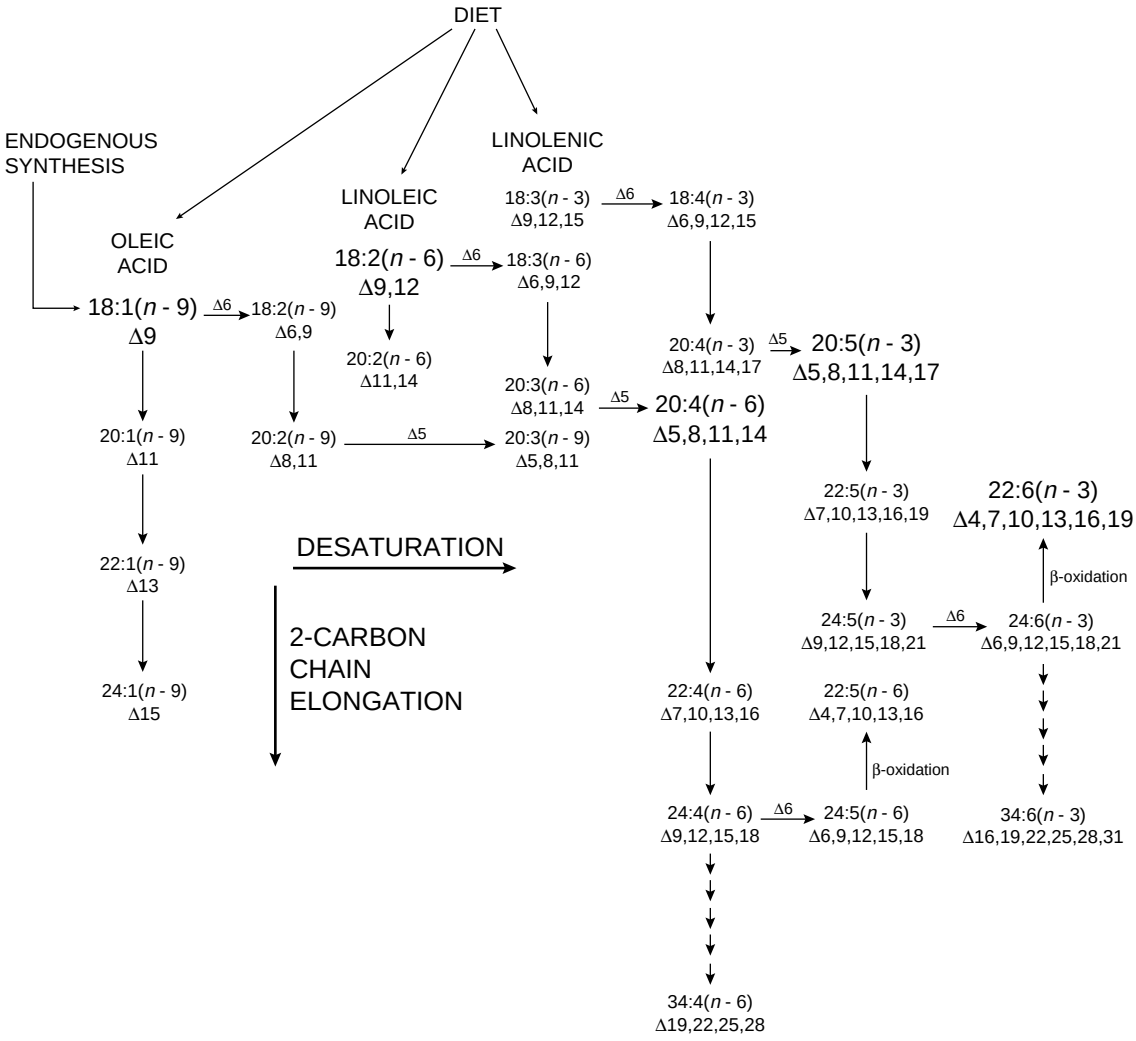


Fig. 2.21 Major pathways for polyunsaturated fatty acid synthesis in animals. Note the alternating sequence of desaturation in the horizontal direction and chain elongation in the vertical direction in the formation of polyunsaturated fatty acids from dietary essential fatty acids. Type size for individual fatty acids reflects, in a general way, relative accumulation in tissues. Adapted from H.W. Cook (1996) with kind permission of the author and Elsevier Science.

and subsequent bonds are methylene interrupted (i.e. separated by a methylene group) rather than being conjugated. They are all in the *cis* configuration.

The most important substrates for the first polydesaturation are oleic acid (either produced by the animal or coming from the diet), linoleic and α-linolenic acids (only from the diet). The structural relationships between the families of fatty acids that arise from these three precursors are most easily recognized by using the system that numbers the double bonds from the methyl end of the chain. Hence, oleic acid gives rise to a series of (*n*-9) fatty acids, the linoleic family is (*n*-6) and the α-linolenic family is (*n*-3). The first desaturation is at Δ6 and the sequence is one of alternate elongations (Type

III synthase, Section 2.2.3.4 and desaturations, Fig. 2.21).

Under some circumstances, the Δ6 desaturase may exert significant control over the process with a detectable build-up of its substrate fatty acids. This can have physiological consequences as discussed in Section 4.2.3.5. The regulation of enzymes involved in polyunsaturated fatty acid synthesis is not well defined, but it is known that development (age), diet and diabetes all affect Δ6 and Δ5 desaturase activities. The role of unsaturated fatty acid synthesis in the formation of prostaglandins, thromboxanes and prostacyclins will be discussed further in Section 2.4.

2.2.6 Biohydrogenation of unsaturated fatty acids takes place in rumen micro-organisms

Desaturation of an acyl chain is a reaction widespread in Nature. The reverse process, namely the hydrogenation of double bonds, is found in only a few organisms. These organisms are commonly found in the rumens of cows, sheep and other ruminant animals. Linoleic acid, for example, can be hydrogenated by rumen flora (anaerobic bacteria and protozoa) to stearic acid by a series of reactions shown in Fig. 2.22.

First of all the substrate fatty acids must be released from leaf complex lipids by the action of acyl hydrolases (Section 3.5.3). The first reaction of the unesterified linoleic acid involves isomerization of the *cis*-12,13 double bond to a *trans*-11,12 bond which is then in conjugation with the *cis*-9,10 double bond. The enzyme responsible for this isomerization has been partially purified from the cell envelope of *Butyrivibrio fibrisolvens* and can act on α-linolenic as well as linoleic acid. Next, hydrogen is added across the *cis*-9,10 bond to form *trans*-vaccenic acid, which is further reduced to stearic acid. Analogous reactions occur with fatty acids other than

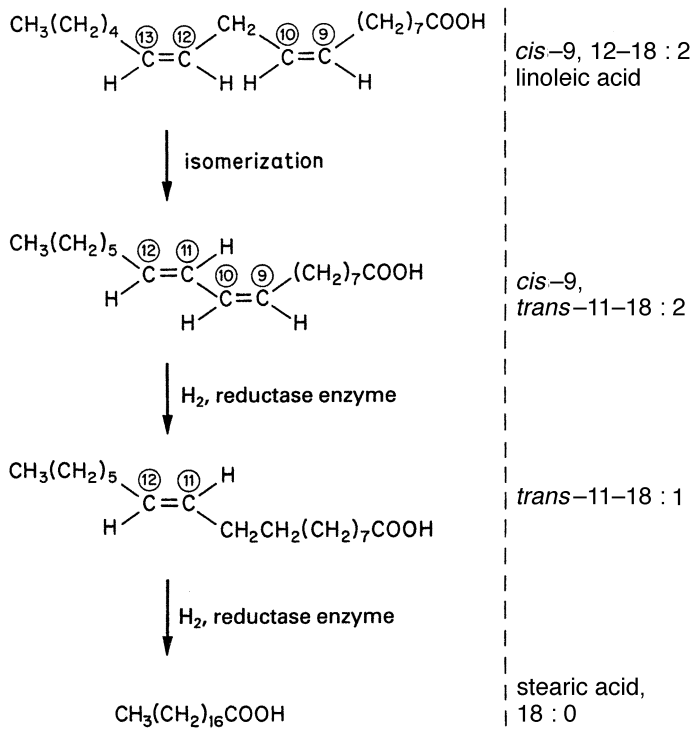


Fig. 2.22 Biohydrogenation (by rumen micro-organisms).

linoleic acid, but the positions of the *cis* and *trans* bonds will, of course, be different.

In spite of the high activity of rumen microorganisms (including the further breakdown of the fatty acids by oxidation) ruminants do not appear to suffer from essential fatty acid (EFA) deficiency. The amount of unchanged EFA passing through the rumen (up to 4% of dietary intake) is sufficient for the needs of the animal (Section 4.2.2.1). Hydrogenation can, however, be reduced by giving ruminants 'protected fats', thereby enriching their tissues with polyunsaturated fatty acids (Section 4.1.2).

An interesting example of hydrogenation occurs in *Bacillus cereus*, which can reduce oleic to stearic acid. This reductase is induced by an increase in growth temperature and seems to be involved in the overall control of membrane fluidity (Section 6.5.9).

2.2.7 The biosynthesis of cyclic acids provided one of the first examples of a complex lipid substrate for fatty acid modifications

The only ring structures we shall discuss are the cyclopropanes and cyclopropenes.

The methylene group in cyclopropane acids originates from the methyl group of methionine in *S*-adenosyl methionine ('active methionine'). This is the same methyl donor involved in the formation of 10-methylene stearic acid and 10-methyl stearic acid from oleic acid (Section 2.2.2.5). The acceptor of the methyl group is likewise an unsaturated fatty acid. Thus, *cis*-vaccenic acid gives rise to lactobacillic acid, while oleic acid yields dihydrosterculic acid, the saturated derivative of sterculic acid (see Table 2.4). These reactions occur in a number of bacteria and in certain families of higher plants, e.g. *Malvaceae* and *Sterculaceae*.

When Law and his colleagues purified cyclopropane synthase from *Clostridium butyricum*, they found that the enzyme would catalyse the formation of cyclopropane fatty acids from ^{14}C-labelled methionine only if phospholipids were added in the form of micellar solutions. They discovered that the real acceptor for the methylene group was not the unesterified monounsaturated fatty acid or its CoA or ACP thiolester, but phosphatidylethanolamine – the major lipid of the organism (see Fig. 2.23).

The biosynthesis of cyclopropane and the related cyclopropene acids in higher plants has been studied by experiments with radioactive precursors. In this method, a supposed precursor for the compounds being studied is supplied to the plant and its incorporation into more complex molecules and/or conversion to products is studied at successive time intervals. The sequence in which the radiolabel appears in different compounds can be used to deduce the pathways by which they are made. Thus it has been shown that oleic acid gives rise to the cyclopropane derivative of stearic acid

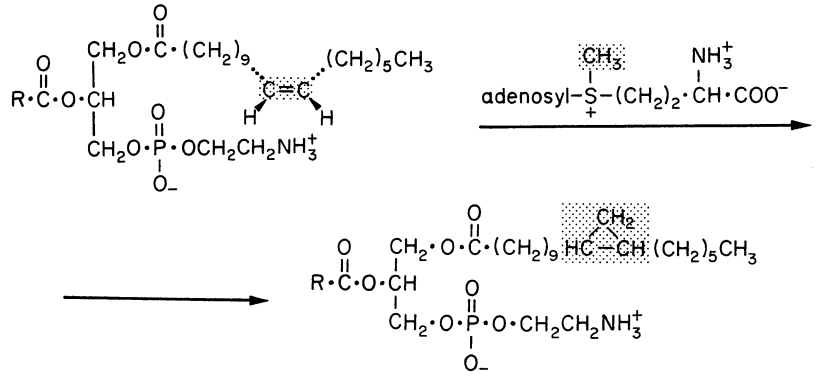


Fig. 2.23 Formation of a cyclopropane fatty acid in *Clostridium butyricum*.

(dihydrosterculic acid). The latter can either be shortened by α-oxidation or desaturated to give sterculic acid (Fig. 2.24). Desaturation of the α-oxidation product (dihydromalvalic acid) similarly yields malvalic acid (8,9-methylene-8-17:1).

2.2.8 The control of fatty acid synthesis can take place at a number of enzyme steps

2.2.8.1 Acetyl-CoA carboxylase (ACC) regulation in animals

It is often thought that enzymes near the beginning of metabolic pathways may play an important role in controlling the carbon flux down the pathway. (Note that the term 'regulatory enzyme' is incorrect since all enzymes in a pathway are potentially regulating and, therefore, all of them play a role in overall control – see Fell's (1997) book for a good discussion.) Because acetyl-CoA carboxylase (ACC) catalyses the first committed step for fatty acid (and lipid) synthesis, it has been well studied to see whether it could be important for flux control. Indeed, it is now accepted generally that ACC activity plays a key role in the overall control of fatty acid synthesis, especially in animal tissues.

Both the activity of ACC and the rate of fatty acid synthesis fluctuate in response to various internal or external factors which affect lipogenesis. These include diet, hormones and developmental or genetic factors. The major control that ACC exerts has been clearly demonstrated recently by experiments in which an ACC mRNA-specific ribozyme gene was expressed. The rate of fatty acid synthesis in these experiments was shown to be proportional to the amount of ACC mRNA and ACC protein.

Following recent demonstration of two isoforms of animal ACCs (Section 2.2.3.1), it should be borne in mind that most experiments on this enzyme's regulation were carried out before such knowledge was available. Thus, some detailed interpretations may have to be modified in the future. For the time being, however, we will assume that when control of fatty acid synthesis depends on ACC, the isoform involved is ACC-α. (The role of ACC-β in β-oxidation of fatty acids is discussed in Section 2.3.1.3.) The activity of ACC can be regulated rapidly or over a longer time course in several ways (see Table 2.9).

Activation and inhibition

Because ACC catalyses the first committed step in lipid synthesis, and because its substrate lies at a cross-roads between carbohydrate and lipid metabolism, then its acute regulation is clearly important. Hydroxytricarboxylic acids, such as citrate, have long been thought to be physiologically important because a rise in their levels could indicate a constraint in the TCA cycle and, hence, a need to convert excess carbon into lipid stores. Because citrate is a precursor of acetyl-CoA (Section 2.2.3) it also acts as a positive feed-forward activator.

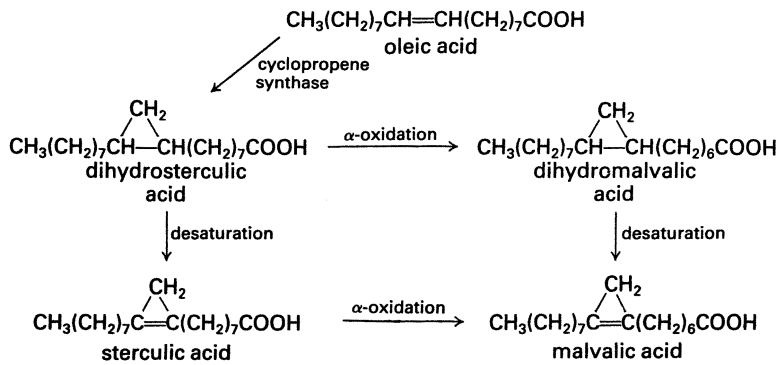


Fig. 2.24 Synthesis of sterculic and malvalic acids by plants.

Table 2.9 Methods for regulating acetyl-CoA carboxylase activity in animals

Mediator	Comments
Hydroxytricarboxylic acid (e.g. citrate)	Polymerization induced; activity much increased.
Long-chain acyl-CoAs	Negative feedback inhibitors; active at very low concentrations.
Phosphorylation/dephosphorylation	Phosphorylation by AMP-activated protein kinase or protein kinase A inactivates enzyme.
Enzyme synthesis/degradation	Used for long-term regulation. Enzyme protein amounts decreased by diabetes or by fasting and increased by re-feeding low-fat diets. Protein translation rates generally more important than catabolism.

Citrate acts to promote conversion of the inactive protomer into the catalytically active polymer. Activation by citrate is unusual in that it increases the rate of reaction (V_{max}) without affecting the K_m of its substrates. Both partial reactions are stimulated by citrate. Although (as mentioned above) citrate has a logical role in controlling carbon flux into lipid stores as opposed to ATP generation, its exact physiological role is still unclear.

Other classical effectors of ACC activity are acyl-CoAs, which can be regarded as end-products of fatty acid biosynthesis in mammals. Inhibition by long-chain acyl-CoAs is competitive towards citrate and non-competitive towards the three substrates acetyl-CoA, ATP and bicarbonate. One mole of palmitoyl-CoA was found completely to inhibit one mole of enzyme. The inhibition constant for this is 5.5 nM, which is three orders of magnitude lower than the critical micellar concentration (CMC) for palmitoyl-CoA. Saturated fatty acyl-CoAs of 16C–20C are most effective and unsaturated acyl-CoAs are considerably less inhibitory. (For a discussion of CMC and its significance, see Section 2.1.8.)

It is, of course, important to consider whether the cellular concentration of the putative regulator molecules – citrate and acyl-CoAs – are sufficient to allow the physiological control of ACC. Indeed, cellular concentrations of these molecules under various metabolic conditions are consistent with their role in regulation. For example, it has been found that in liver 50–75% of the cellular citrate is in the cytoplasm, which is where the ACC is. The estimated concentration of 0.3–1.9 mM is close to the concentration needed for half maximal activation. Furthermore, glucagon or (dibutyryl)cAMP, which lowers the rate of fatty acid synthesis, also reduces the cytoplasmic concentration of citrate. The cytoplasmic concentration of acyl-CoAs is difficult to measure because they bind to intracellular proteins and membranes. However, total cellular concentrations up to 150 μM have been reported, which are more than sufficient to inhibit the carboxylase completely.

Phosphorylation/dephosphorylation

Both isoforms of animal ACCs are phosphoproteins and control by covalent phosphorylation is well established. Purified carboxylases contain as many as nine phosphorylation sites but these are concentrated either at the N-terminus or between the biotin-binding and carboxyltransferase functional domains (Fig. 2.25).

Phosphorylation by AMP-activated protein kinase causes a large decrease in V_{max} and, of the three potential phosphorylation sites, serine 79 seemed to be critical for inactivation. In contrast, the cAMP-dependent protein kinase caused mainly an increase in the K_a for citrate and a more modest decrease in V_{max}. Serine 1200 was critical for this

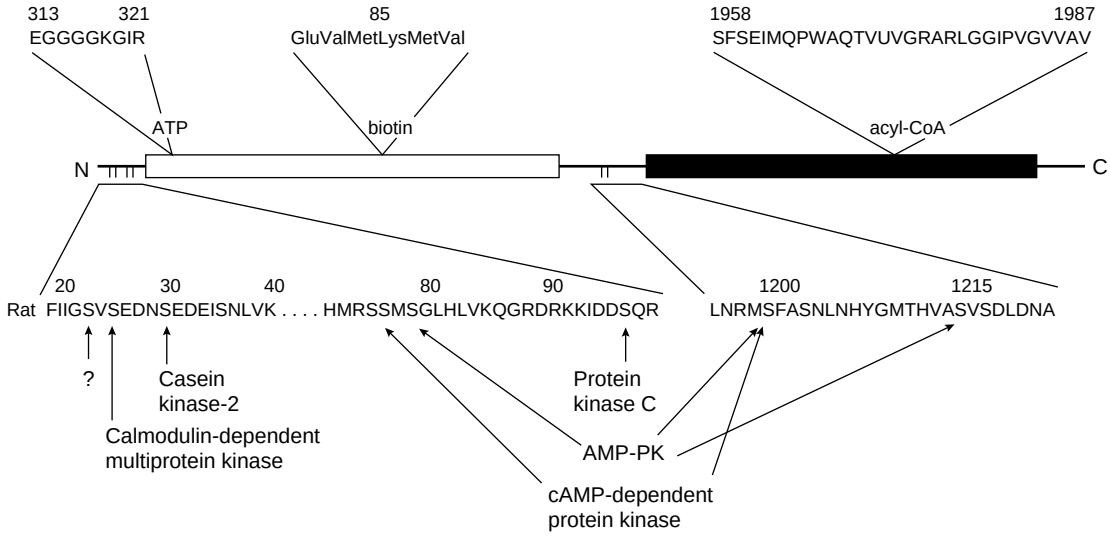


Fig. 2.25 Domain map of animal acetyl-CoA carboxylase showing locations of functional and phosphorylation sites. AMP-PK = 5′-AMP-dependent protein kinase. Taken from Kim (1997) with kind permission of the author and Annual Reviews Inc.

inactivation. Other kinases may also play a role in phosphorylating the carboxylase and, hence, inactivating it (see Fig. 2.25). Because protein kinases are affected by various signalling pathways, then indirectly the latter will influence the activity of ACC.

Synthesis and degradation

Long-term regulation of ACC in animals can be due to changes in enzyme amounts. The tissue concentration of the carboxylase protein has been shown to vary with the rate of fatty acid synthesis under a variety of nutritional, hormonal, developmental or genetic conditions. Measurements with specific antibodies have shown that, for example, fasted rats have only one-quarter the normal levels of the liver enzyme, while genetically obese mice contain four times as much compared to control animals. The amount of an enzyme protein accumulating is due to the net rates of synthesis and degradation. Depending on the specific trigger for changes in acetyl-CoA carboxylase levels, one or both of these factors may be altered. For example, the increase in enzyme content in fasted/re-fed rats is due only to changes in the rates of its synthesis (Table 2.10). By contrast, the decrease in enzyme content in fasted animals is due both to diminished synthesis and to accelerated breakdown.

Table 2.10 Relative acetyl-CoA carboxylase (ACC) levels and rate of enzyme synthesis in different conditions

	Content	Synthesis of ACC protein
Rat		
Normal	1	1
Fasted	0.25	0.50
Re-fed	4	4
Alloxan-diabetic	0.50	0.50
Mouse		
Normal	1	1
Obese mutant	4	3

Acetyl-CoA carboxylase-α mRNAs occur in a variety of forms as a result of the functioning of two independent promoters (PI and PII) and differential splicing of the primary transcripts. The different ACC mRNAs are expressed tissue specifically,

depending on the physiological state of the tissue. The generation of multiple mRNAs provides an extra dimension to control of ACC activity. For example, it is known that the 5′-untranslated regions are involved in the regulation of translation of specific mRNA species. The promoters PI and PII respond to particular physiological stimuli. Thus PII, which is constitutively expressed, contains an elaborate array of *cis*-elements (including those for insulin, cAMP and glucose) that affect its activity.

2.2.8.2 Acetyl-CoA carboxylase regulation in other organisms

The ACC from yeast is also inhibited by acyl-CoAs, but unlike the animal enzyme, is unaffected by citrate. In some mutant strains an activation by fructose-1,6-bisphosphate has been demonstrated. Like the mammalian carboxylase, the enzyme from yeast is also regulated by changes in amount. An interesting example of the role of fatty acyl-CoA in mediating the rate of synthesis of acetyl-CoA carboxylase has been demonstrated in fatty acid mutants of *C. lipolytica*. These mutants contained no apparent fatty acyl-CoA synthetase and hence, were unable to grow on exogenous fatty acids, when their own fatty acid synthesis was blocked by inhibitors. However, further examination showed that the mutants did have one type of fatty acyl-CoA synthetase (called II) in common with normal cells but lacked fatty acyl-CoA synthetase I which was needed for membrane lipid synthesis. Fatty acyl-CoA synthetase II is used for activating fatty acids destined for β-oxidation. Thus, these two acyl-CoA synthetases are responsible for generating two pools of acyl-CoAs in different parts of the cell. The acyl-CoAs formed by acyl-CoA synthetase I are in the cytosol and these cause repression of ACC (Fig. 2.26).

Regulation of *E. coli* ACC on the other hand, seems to be by a completely different system. In this bacterium (and others such as *Pseudomonas citronellolis*) highly phosphorylated guanosine nucleotides seem to be important. These compounds (guanine-5′-diphosphate-3′-diphosphate, ppGpp and guanine-5′-triphosphate-3′-diphosphate, pppGpp) act by inhibiting the carboxyltransferase partial reaction.

In plants, ACC has been identified as an important site for flux control during fatty acid synthesis in leaves. Fatty acid synthesis in such tissues is very much stimulated by light (about 20-fold) and this increase was accompanied by changes in the pool sizes of acetyl and malonyl thioesters consistent with large alterations of acetyl-CoA carboxylase activity. This was confirmed by the use of specific inhibitors that showed that most of the regulation of carbon into fatty acids and lipids was by ACC. However, the mechanism of control is poorly understood. In light stimulation experiments the activation of ACC may be secondary to stromal solute changes (ATP, ADP, Mg^{2+} and pH) that accompany photosynthesis. On the other hand, feedback control may play a role in some tissues although classical effectors such as citrate or acyl-CoAs do not seem to be effective at physiologically relevant concentrations in plants.

2.2.8.3 Regulation of fatty acid synthase

In the same way as the actual levels of ACC protein can be changed in animals under dietary influence so can that of fatty acid synthase (FAS, Table 2.11). Up to 20-fold differences have been observed in the levels of, for example, liver fatty acid synthase between starved and carbohydrate re-fed animals. Alterations in both synthesis and degradation of the enzyme seem to be involved.

It should be noted that dietary factors that affect FAS levels do not affect all tissues equally. While the liver is highly influenced by such regulation, the FAS of brain is unaffected. This is just as well for it would be extremely disadvantageous for a young animal to have its brain development influenced dramatically by its day-to-day nutritional state!

While re-feeding a high carbohydrate diet causes an increase in hepatic FAS protein levels, fat feeding reduces them. However, the effects of fat are complex with polyunsaturated fatty acids being particularly effective.

In addition, a number of hormones including insulin, glucocorticoids, glucagon, theophylline and oestradiol have also been found to produce

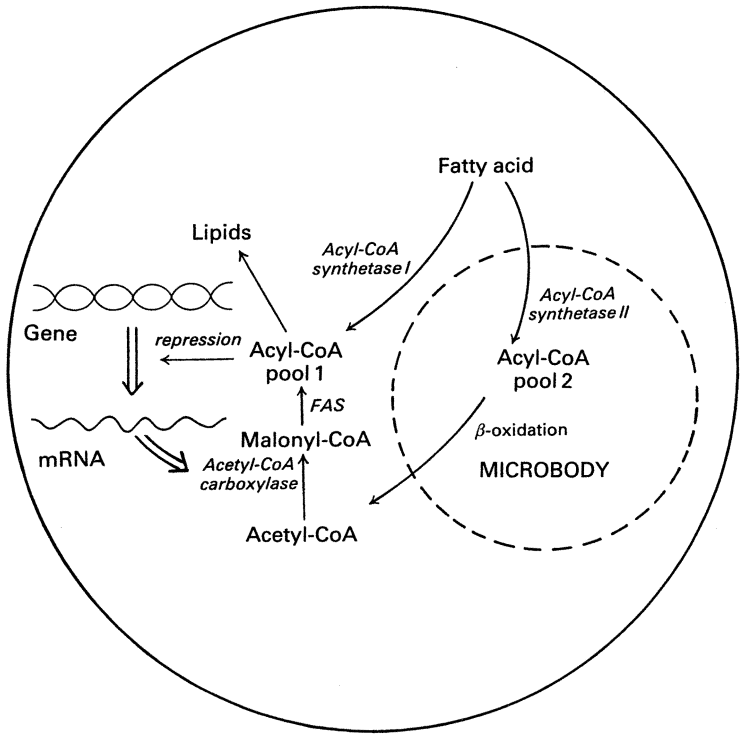


Fig. 2.26 Interaction of acyl-CoA pools with fatty acid metabolism in the hydrocarbon-utilizing yeast *Candida lipolytica*. Reproduced with kind permission of Dr. S. Numa (1981) and Elsevier Trends Journals from *Trends in Biochemical Science*, Fig. 2, p. 115.

Table 2.11 Effect of nutritional state or hormones on liver fatty acid synthase levels

Cause increase	Cause decrease
Re-feeding	Starvation
Insulin	Alloxan-diabetes
β-Oestradiol	Glucagon[a]
Hydrocortisone	
Growth hormone	

[a] Glucagon can induce enzyme synthesis in embryonic liver, but will reduce levels of fatty acid synthase in adult animals.

acute alterations in levels of various mammalian FASs (Table 2.11). Longer term factors can also play a role in determining enzyme levels. Thus, for example, the FAS increases in mammary gland during mid to late pregnancy and early in lactation. In brain development, on the other hand, the synthase is highest in foetal and neonatal rats and decreases with maturity. Like ACC, the concentration of FAS is also much higher in tissues of certain genetic mutants such as the obese hyperglycaemic mouse. The enzyme in the latter mutant is less subject to control by dietary factors, such as fasting, when compared to normal mice.

These changes in animal FAS levels have now been studied at the subcellular level. FAS mRNA has been isolated from different tissues after various hormonal (insulin, glucagon and thyroid hormone are the most important) or dietary manipulations and translated in vitro. Recombinant plasmids have been used to ascertain the size of FAS mRNA and also the amounts of this mRNA in

tissues. By these means it has been shown that differences in FAS activity are caused by changes in enzyme levels rather than its intrinsic activity. These alterations are themselves changed by the balance of enzyme synthesis and degradation.

A common way in which the rate of a particular metabolic reaction can be controlled is through the supply of substrate. In the case of animal FAS, this regulation has been examined with regard to NADPH. However, it appears that NADPH production is adjusted to cope with the altering demands of fatty acid synthesis rather than the other way around. In contrast, supply of malonyl-CoA by ACC activity (Section 2.2.8.1) is considered to be a major factor regulating overall fatty acid (and lipid) formation under many conditions.

The above mechanisms probably apply also to organisms other than animals. Thus, in developing seeds, where there is a spectacular rise in fat accumulation during a particular state of maturation (Section 3.4.1.3 and Fig. 3.7), the increase in fatty acid synthesis has been correlated with increases in levels of various synthase proteins including acyl carrier protein. Conversely, the rise in activity of FAS in photosynthetic tissues on illumination undoubtedly requires the simultaneous production of NADPH substrate by photosystem I.

The nature of plant FAS products can be altered by the relative activity of the component enzymes. Experiments in vitro with specific inhibitors have shown that the different condensing enzymes are responsible for the chain length of the final products. This fact has now been exploited in transgenic crops where the ratio of 16C to 18C products can be changed. In addition, introduction of foreign genes such as the medium-chain acyl-ACP thioesterase (from the Californian bay tree or from coconut) can be used to prematurely terminate chain lengthening and allow laurate accumulation.

E. coli adjusts its fatty acid composition in response to growth temperature. This adaptive response is the result of a change in the activity of 3-ketoacyl-ACP synthase II. Such alterations in its activity occur very rapidly (within 30 s of temperature downshift) and are therefore independent of transcriptional control. Moreover, mutants lacking synthase II (*fabF*) were unable to adapt to lower temperatures. Modifying the activity of other condensing enzymes, such as synthase I does not affect the temperature response.

2.2.8.4 Control of animal desaturases

Unsaturated fatty acids are present in all living cells and are important in regulating the physical properties of lipoproteins and membranes (Chapter 6) as well as acting as regulators of metabolism in cells (see above) or as precursors for physiologically active compounds (Sections 2.4 and 4.2.3.3). The control of their production must therefore be important, yet our knowledge of the subject is sparse. Research has lagged behind that on fatty acid synthase, because of the inability to obtain purified enzymes with which to raise antibodies to study induction of new enzyme protein or to study regulatory control by small molecules.

A good example of the importance of unsaturated fatty acids comes from experiments on temperature-induced change in membrane fluidity (see Section 6.5.9). The adaptive response of *E. coli* has already been referred to above. Some simple animals (for example the protozoa *Tetrahymena* and *Acanthamoeba*) are able to produce linoleic acid, in contrast to mammals. The Δ12-desaturase responsible in *Acanthamoeba* has been studied in some detail. When *Acanthamoeba* is cooled its membranes lose their fluidity and the organism is unable to phagocytose (it feeds on soil bacteria). The Δ12-desaturase is then induced and the membranes become more fluid, as oleate is converted to linoleate, and phagocytosis can commence again. The induction of gene expression is caused by low temperatures or, independently, by oxygen (which is more soluble at low temperatures).

Animal Δ9-stearoyl-CoA desaturase (SCD) shows extreme responses to dietary alterations. When fasted animals are re-fed a fat-free diet there can be a 'super-induction' with levels of desaturase activity increased more than 100-fold. Likewise, when rat pups are nursed by mothers on an essential fatty acid deficient diet, their *SCD*-1 mRNA is nearly 100-fold that of control levels. In contrast, brain Δ9-desaturase levels are hardly

affected in keeping with the essential nature of unsaturated fatty acids in nervous tissue development.

Dietary PUFA, particularly linoleic and arachidonic acids, inhibit Δ9-desaturase activity more than they do *de novo* fatty acid synthesis. The *SCD*-2 gene undergoes co-ordinate transcriptional down-regulation in response to these PUFAs. Hormonal regulation, by insulin in particular, has been noted but is not fully understood.

The other animal desaturases (Δ5 and Δ6-desaturases) are affected by diet and a number of hormones as noted in Section 2.2.5.3. However, in general, the effects are not as dramatic as for Δ9-desaturase.

2.3 DEGRADATION OF FATTY ACIDS

The main pathways of fatty acid breakdown involve oxidation at various points on the acyl chain or lipoxidation at certain double bonds of specific unsaturated fatty acids.

The main forms of fatty acid oxidation are termed α, β and ω. They are named depending on which carbon on the acyl chain is attacked:

$$\begin{array}{lccc} CH_3(CH_2)_nCH_2CH_2COO^- \\ \uparrow & & \uparrow & \uparrow \\ \omega & & \beta & \alpha \end{array}$$

Of the oxidations, β-oxidation is the most general and prevalent.

2.3.1 β-Oxidation is the most common type of biological oxidation of fatty acids

Long-chain fatty acids, combined as triacylglycerols, provide the long-term storage form of energy in the adipose tissues of animals. In addition, many plant seeds contain triacylglycerol stores. These fats are degraded principally by the liberation of 2C (acetyl-CoA) fragments in β-oxidation. The mechanism was originally proposed nearly a century ago (1904) by Knoop. He synthesized a series of phenyl-substituted fatty acids with odd-numbered or even-numbered carbon chains. He found that the odd-numbered substrates were metabolized to phenylacetate. Knoop was using the phenyl group in the same way that modern biochemists would use a ^{14}C-radiolabel. At around the same time the proposed intermediates were isolated by Dakin and, therefore, the basic information about β-oxidation was available 50 years before the enzymic reactions were demonstrated.

When Leloir and Munoz in 1944 showed that β-oxidation could be measured in cell-free preparations from liver, it was not long before a number of its important features was revealed. Lehninger found that ATP was needed to initiate the process, which seemed particularly active in mitochondria. Following the isolation of coenzyme A by Lipmann, Lynen was able to demonstrate that the active acetate was acetyl-CoA and Wakil and Mahler showed that the intermediates were CoA-esters. With the availability of chemically synthesized acyl-CoA substrates, Green in Wisconsin, Lynen in Munich and Ochoa in New York could study the individual enzymes involved in detail.

2.3.1.1 Cellular site of β-oxidation

Until relatively recently it had always been considered that β-oxidation was confined to mitochondria. Although animal mitochondria do contain all the enzymes necessary and are a major site for β-oxidation, other subcellular sites, such as the microbodies, are implicated. Peroxisomes or glyoxysomes together are often referred to as microbodies. They contain a primitive respiratory chain where energy released in the reduction of oxygen is lost as heat. The presence of an active β-oxidation pathway in microbodies was first detected in glyoxysomes from germinating seeds by de Duve in 1976. Since that time the various enzymes involved have been purified and characterized for microbodies from rat liver as well as from plants.

Microbodies occur in all major groups of eukaryotes including yeasts, protozoa, plants and animals. The contribution of these organelles to total β-oxidation in a given tissue varies considerably. In animals microbodies are particularly important in liver and kidney. In fact, in liver it seems that mitochondria and microbodies collaborate in

overall fatty acid oxidation. Thus, microbodies oxidize long-chain fatty acids to medium-chain products, which are then transported to mitochondria for complete breakdown. In this way very long chain fatty acids, such as erucate, which are poor substrates for mitochondria, can be catabolized. Microbodies (which also contain α-oxidation enzymes) are important for the degradation of other lipid substrates such as some xenobiotics and the eicosanoids.

In contrast, the glyoxysomes from germinating seeds are capable of the complete breakdown of fatty acids to acetyl-CoA. They also integrate this metabolism with the operation of the glyoxylate cycle, which allows plants (in contrast to animals) to synthesize sugars from acetyl-CoA. Leaf tissues also contain peroxisomes and recent work indicates that β-oxidation in leaves is always found in peroxisomes with significant activity in mitochondria in some circumstances.

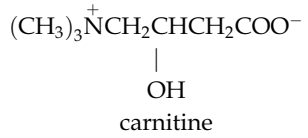
2.3.1.2 Transport of acyl groups to the site of oxidation: the role of carnitine

Fatty acids are transported between organs in animals either in the form of non-esterified fatty acids bound to serum albumin or as triacylglycerols associated with lipoproteins (especially chylomicrons and very low density lipoproteins; Section 5.2). Triacylglycerol is hydrolysed on the outer surface of cells by lipoprotein lipase and fatty acids have been shown to enter liver, adipose and heart tissue cells by saturatable and non-saturatable mechanisms.

Once inside cells, fatty acids can be activated to acyl-CoAs by various ligases. Most of the activating enzymes are ATP-dependent acyl-CoA synthetases, which act in a two-step reaction (Section 2.2.1).

Both non-esterified fatty acids and acyl-CoAs are capable of binding to distinct cytosolic proteins known as fatty acid binding proteins (FABP). The best known of these is the Z-protein of liver. These low molecular mass (about 14 kDa) FABPs have been suggested to function for intracellular transport or to provide a temporary binding site for potentially damaging compounds such as acyl-CoAs (Section 5.1.3). Specific acyl-CoA binding proteins have also been discovered recently.

Because the inner mitochondrial membrane is impermeable to CoA and its derivatives, fatty acyl-CoAs formed in the cytosol cannot enter the mitochondria directly for oxidation. The observation, by Bremer and others, that carnitine could stimulate the oxidation of fatty acids in vitro led to the idea that long-chain fatty acids could be transported in the form of carnitine esters.



carnitine

The theory really began to take shape when an enzyme (carnitine:palmitoyl transferase, CPT) was discovered, which would transfer long-chain acyl groups from CoA to carnitine. Two isoforms of the enzyme have been identified. In mitochondria CPT_1 is located on the outer mitochondrial membrane, while CPT_2 is on the inner membrane (Fig. 2.27).

Peroxisomes and the endoplasmic reticulum also have CPTs. Examination of these enzymes shows that they are identical to the mitochondrial CPT_1 and are also severely inhibited by malonyl-CoA (see below). These transferases are collectively termed CPT_o.

The acylcarnitines cross the outer mitochondrial membrane via porin and the inner membrane via a carnitine:acylcarnitine translocase, which causes a one to one exchange, thus ensuring that the mitochondrial content of carnitine remains constant (Fig. 2.27). Fatty acids of fewer than ten carbons can be taken up by mitochondria as free acids independent of carnitine. This is in spite of the fact that a third carnitine acyltransferase, which transfers acyl groups of 2–10 carbon atoms, has been found in mitochondria. The function of this enzyme is uncertain although it has been suggested to be useful in regenerating free CoA within the mitochondrial matrix.

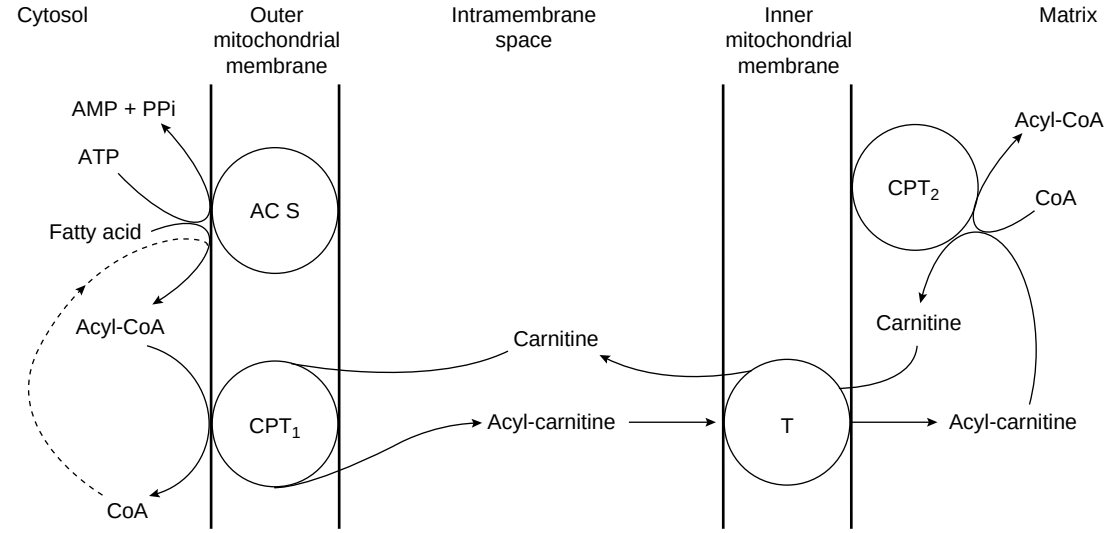


Fig. 2.27 Movement of acyl residues into mitochondria via carnitine. ACS = acyl-CoA synthetase; T = translocase; CPT = carnitine:palmitoyltransferase.

2.3.1.3 Control of acylcarnitine formation is very important

Because mitochondria, peroxisomes and the endoplasmic reticulum have very active acylcarnitine translocases this means that as soon as acylcarnitine is made by CPT_o it will be taken into these compartments. Thus, any modulation of CPT_o activity will alter rapidly the utilization of long-chain acids within these organelles or the ER lumen or, alternatively, lead to a rise in acyl-CoAs in the cytosol. If the latter occurs then a host of effects can result including for example, increased diacylglycerol or ceramide formation leading in turn to protein kinase C activation or apoptosis, respectively (Sections 7.9 and 7.12).

Activity of CPT_o is controlled by malonyl-CoA levels. These CPTs are integral membrane proteins, but both their catalytic and regulatory sites are exposed on the cytosolic side of the outer mitochondrial (peroxisomal and endoplasmic reticulum) membranes. Malonyl-CoA is generated by the newly discovered isoform of acetyl-CoA carboxylase (ACC-β). CPT_1 (the mitochondrial form of CPT_o) is extremely sensitive to malonyl-CoA: the K_i for malonyl-CoA is in the micromolar range.

The idea that ACC-β plays a key role in β-oxidation is supported by three facts. First, CPT_1, an essential component for mitochondrial β-oxidation, is very sensitive to malonyl-CoA, which is only produced by ACC. Second, tissues such as heart muscle, which are non-lipogenic, contain large amounts of ACC activity. The main form in these mitochondria-rich tissues is ACC-β. Thirdly, physiological conditions that decrease ACC activity and malonyl-CoA levels are accompanied by accelerated β-oxidation.

2.3.1.4 Enzymes of mitochondrial β-oxidation

The reactions of β-oxidation are shown in Fig. 2.28 and essentially involve four enzymes working in sequence (acyl-CoA dehydrogenase, enoyl hydratase, a second dehydrogenase and a thiolase), which results in the cleavage of two carbons at a time from the acyl chain. The 2C product, acetyl-CoA, is then used by, for example, the TCA cycle to yield energy and the acyl-CoA (two carbons shorter) is recycled.

Each of the four enzymes involved are present as isoforms that have varying chain-length specificities (see Table 2.12). Thus, for the efficient

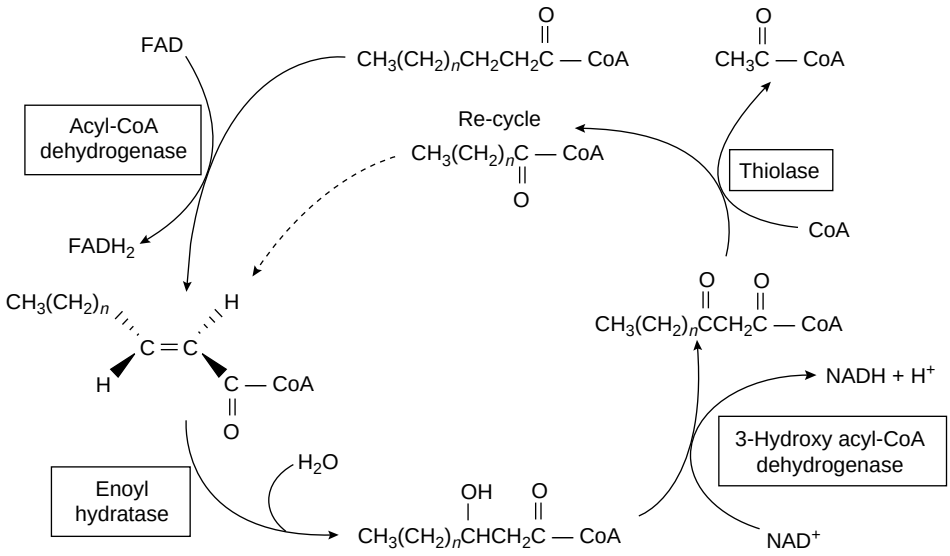


Fig. 2.28 The fatty acid β-oxidation cycle.

degradation of typical fatty acid substrates, the different isoforms must co-operate. Three of the enzymes (hydratase, 3-hydroxyacyl dehydrogenase and thiolase) have their long-chain isoforms present as a trifunctional complex on the inner mitochondrial membrane. All the other isoforms of the component enzymes are soluble, but there is evidence of metabolite channelling between the individual enzymes. This improves the efficiency of β-oxidation and prevents intermediates building up, which could be inhibitory and also lead to a greater requirement for scarce CoA. As a generalization, it is thought that long-chain acids are oxidized by the membrane-located enzymes while medium-chain substrates use the matrix enzymes.

In contrast to fatty acid biosynthesis, it should be noted that β-oxidation uses CoA-derivatives and gives rise to a 3-hydroxy intermediate with a L(+) configuration.

2.3.1.5 Other fatty acids containing branched chains, double bonds and an odd number of carbon atoms can also be oxidized

So far we have assumed that the fatty acid being oxidized is a straight chain, fully saturated compound. This is not necessarily the case and the ease with which other compounds are oxidized depends on the position along the chain of the extra group or the capacity of the cell for dealing with the end-products. From acids of odd chain length, one of the products is propionic acid and the ability of the organism to oxidize such fatty acids is governed by its ability to oxidize propionate. Liver, for example, is equipped to oxidize propionate and therefore deals with odd chain acids quite easily; heart, on the other hand, cannot perform propionate oxidation and degradation of odd chain acids grinds to a halt. The end-product of propionate oxidation, succinyl-CoA, arises by a mechanism involving the B_{12} coenzyme and the biotin-containing propionyl-CoA carboxylase:

$$\underset{\text{Propionyl-CoA}}{CH_3CH_2COSCoA} + CO_2 + ATP \xrightleftharpoons{\text{Biotin propionyl-CoA carboxylase}}$$

$$\underset{\text{Methymalonyl-CoA}}{CH_3CH(COOH)COSCoA} + ADP + Pi \xrightleftharpoons{B_{12}} \underset{\text{Succinyl-CoA}}{HOOCCH_2CH_2COSCoA}$$

Similarly, branched-chain fatty acids with an even number of carbon atoms may eventually yield propionate, while the oxidation of the odd-num-

Table 2.12 Enzymes of the mitochondrial β-oxidation cycle in animals (reaction scheme in Fig. 2.28)

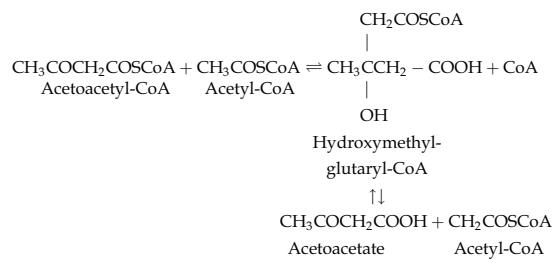
Full name	Trivial name	Description
Acyl-CoA dehydrogenase (EC 1.3.2.2)		Four acyl-CoA dehydrogenases with overlapping chain-length selectivity (short-chain, medium-chain, long-chain and very long chain). First three are soluble in mitochondrial matrix and are homotetramers. FAD is tightly but non-covalently bound. Very long chain acyl-CoA dehydrogenase is a homodimer in inner membrane. All form a *trans* 2,3-double bond.
Enoyl-CoA hydratase (EC 4.2.1.17)	Enoyl hydrase Crotonase	Two enzymes have been identified in heart mitochondria: one is crotonase or short-chain enoyl-CoA hydratase; the second is a long-chain enoyl-CoA hydratase. Crotonase activity is so high in some tissues that the second enzyme may have little function in β-oxidation there (e.g. in liver). However, in most tissues they probably co-operate in fatty acid degradation. The long-chain enoyl-CoA hydratase is a component enzyme of the trifunctional β-oxidation complex that also contains long-chain activities of L-3-hydroxyacyl-CoA dehydrogenase and thiolase. The complex is located in the inner mitochondrial membrane. Both hydratases are specific for the *trans* enoyl substrate and form the L(+) stereo-isomer.
L-3-Hydroxyacyl-CoA dehydrogenase		Three dehydrogenases have been found in mitochondria. All use NAD^+ but differ in their acyl specificities. The main enzyme is soluble and can use a variety of acyl chain lengths. However, it has poor activity for long chains, which are metabolized by a second dehydrogenase located in the inner membrane as part of the trifunctional β-oxidation complex. A third enzyme is soluble and can work with short-chain substrates with or without 2-methyl substituents but is mainly involved in leucine metabolism.
Acyl-CoA: acetyl-CoA S acyltransferase (EC: 2.3.1.16)	Thiolase	Three thiolases are present in mitochondria. The first is soluble, specific for acetoacetyl-CoA and probably mainly functions in ketone body and isoleucine metabolism. Another soluble thiolase has a broad specificity while a long-chain thiolase is part of the membrane-bound trifunctional β-oxidation complex. All the enzymes function in thiolytic cleavage: $RCH_2\overset{\overset{O}{\|}}{C}CH_2\overset{\overset{O}{\|}}{C}\text{—}SCoA + HS\text{-}E \rightleftharpoons RCH_2\overset{\overset{O}{\|}}{C} - S\text{—}E + CH_3\overset{\overset{O}{\|}}{C} - SCoA$ and in acyl transfer: $RCH_2\overset{\overset{O}{\|}}{C} - S\text{—}E + CoASH \rightleftharpoons RCH_2\overset{\overset{O}{\|}}{C} - SCoA + HS\text{-}E$ Thus the overall thiolase reaction is: $RCH_2\overset{\overset{O}{\|}}{C}CH_2\overset{\overset{O}{\|}}{C}\text{-}SCoA + CoASH \rightleftharpoons RCH_2\overset{\overset{O}{\|}}{C} - SCoA + CH_3\overset{\overset{O}{\|}}{C}\text{-}SCoA$

bered branched-chain acids proceeds by a different route involving 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (Fig. 2.29).

Most natural fatty acids are unsaturated. In addition, as discussed before (Section 2.1.3) most double bonds are *cis* and, in polyunsaturated fatty acids, are methylene interrupted (three carbons apart). When unsaturated fatty acids are β-oxidized, two problems may be encountered – the unsaturated acids have *cis* double bonds and these may be at the wrong position for β-oxidation. A typical unsaturated fatty acid might be linoleic acid and its oxidation is illustrated in Fig. 2.30. At first, β-oxidation proceeds normally but then a *cis* double bond is encountered at the wrong position (position 3). The German biochemist, Stoffel, has shown that an isomerase exists to convert the *cis*-3 compound into the necessary *trans*-2-fatty acyl-CoA. The isomerase will also act on *trans*-3 substrates, though at lower rates. Once over this obstacle, β-oxidation can again continue to eliminate a further two carbons and then dehydrogenation produces a 2-*trans*,4-*cis*-decadienoyl-CoA from linoleoyl-CoA. The discovery of a 2,4-dienoyl-CoA reductase by Kunau and Dommes showed that this enzyme could use NADPH to yield 3-*trans*-decenoyl-CoA as its product. The enoyl-CoA isomerase then moves the double bond to position 2 and β-oxidation can proceed again as normal (Fig. 2.30).

2.3.1.6 Regulation of mitochondrial β-oxidation

There are two major products of β-oxidation. Complete operation of the cycle yields acetyl-CoA, which can be fed into the TCA cycle. In some tissues, however, notably liver and the rumen epithelial cells of ruminant animals, acetoacetate accumulates. This compound, with its reduction product, 3-hydroxybutyrate, and its decarboxylation product, acetone, make up a group of metabolites known as the ketone bodies. Free acetoacetic acid may accumulate in liver in two ways. The CoA derivative may be enzymically hydrolysed to the free acid and CoA and the liver tissue lacks the thiokinase to reconvert the acid back into its thiolester. Alternatively, acetoacetyl-CoA may be converted into hydroxymethylglutaryl-CoA (HMG-CoA), which is subsequently cleaved to free acetoacetic acid:



HMG is an important intermediate in cholesterol biosynthesis and this pathway provides a link

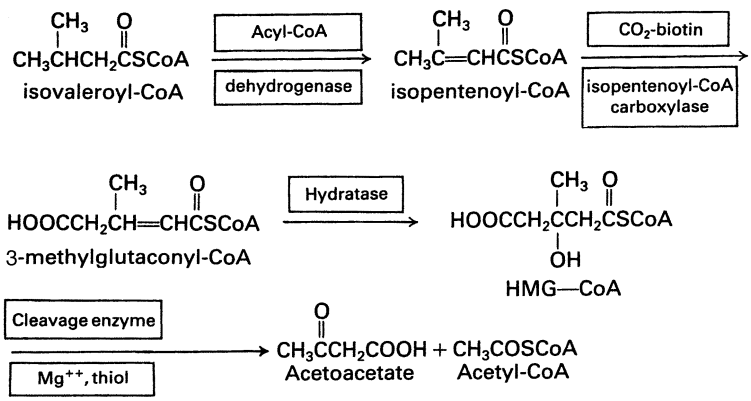


Fig. 2.29 Oxidation of odd-numbered branched-chain fatty acids.

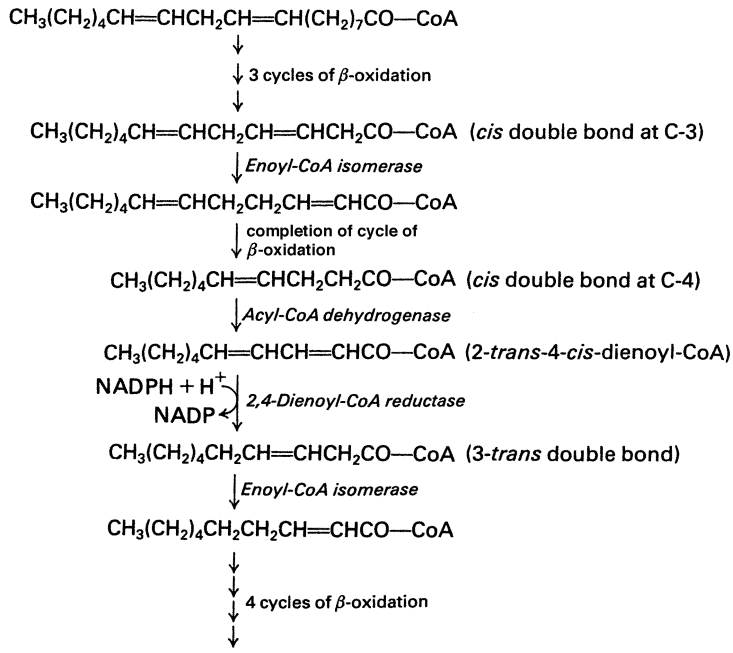


Fig. 2.30 The oxidation of linoleic acid.

between fatty acid and cholesterol metabolism. Ketone bodies are also excellent fuels for the liver and even brain during starvation, even though brain cannot utilize long-chain fatty acids as fuels.

Acetyl-CoA is therefore the substrate for two competing reactions: with oxaloacetate to form citrate or with acetoacetyl-CoA to form ketone bodies (ketogenesis). Which reaction predominates depends partly on the rate of β-oxidation itself and partly on the redox state of the mitochondrial matrix, which controls the oxidation of malate to oxaloacetate, hence, the amount of oxaloacetate available to react with acetyl-CoA. The proportion of acetyl groups entering the TCA cycle relative to ketogenesis is often referred to as the 'acetyl ratio'. The overall rate of β-oxidation may be controlled by a number of well-known mechanisms:

(1) *The availability of fatty acids*. There are two aspects to this availability. First, fatty acids have to be made available by hydrolysis of lipid stores or from the diet. Second, as discussed in Section 2.3.1.3, the entry of fatty acids into mitochondria is under careful regulation through the activity of carnitine palmitoyltransferase 1 (CPT_1).

(2) *The rate of utilization of* β*-oxidation products*, which in turn can either lead to specific inhibition of particular enzymes or to 'feedback inhibition' of the whole sequence.

The concentration of unesterified (free) fatty acids in plasma is controlled by glucagon (which stimulates) and insulin (which inhibits) breakdown of triacylglycerols in adipose tissue stores (Section 3.5.2). Once the fatty acids enter cells they can be degraded to acetyl-CoA or used for lipid synthesis. The relative rates of these two pathways depends on the nutritional state of the animal, particularly on the availability of carbohydrate.

In muscle, the rate of β-oxidation is usually dependent on both the free fatty acid concentration in the plasma as well as the energy demand of the tissue. A reduction in energy demand by muscle will lead to a build-up of NADH and acetyl-CoA. Increased $NADH/NAD^+$ ratios lead to inhibition of

the mitochondrial TCA cycle and increase further the acetyl-CoA/CoASH ratio. Kinetic studies with purified enzymes show that the major sites of the β-oxidation inhibition are 3-hydroxyacyl-CoA dehydrogenase, which is inhibited by NADH, and 3-ketoacyl-CoA thiolase, which is inhibited by acetyl-CoA (Fig. 2.31).

In the liver, because of the interaction of lipid, carbohydrate and ketone body metabolism, the situation is more complex. McGarry and Foster proposed in 1980 that the concentration of malonyl-CoA was particularly important. In the fed state, when carbohydrate (glucose) is being converted to fatty acids, the level of malonyl-CoA is raised. Malonyl-CoA is a reversible inhibitor of carnitine palmitoyltransferase I (CTP_1) and, therefore, reduced entry of acyl groups into mitochondria for oxidation. In a fasting state, lowered malonyl-CoA allows CPT_1 to function at high rates and this stimulates β-oxidation and ketogenesis (Fig. 2.32).

Malonyl-CoA itself is generated by acetyl-CoA carboxylase which is hormonally regulated (Section 2.2.8.1). As mentioned in Section 2.3.1.3, it seems that the isoform acetyl-CoA carboxylase β is particularly concerned with the control of β-oxidation through the inhibition of CPT_1, by malonyl-CoA. In the fasted condition, a high glucagon/insulin ratio elevates cellular cAMP, thus allowing short-term inhibition of acetyl-CoA carboxylase by phosphorylation. Reduction of the glucagon/insulin ratio on feeding reverses this effect. Thus, fatty acid synthesis and degradation are co-regulated by liver hormonal levels.

2.3.1.7 Fatty acid oxidation in *E. coli*

When *E. coli* is grown on a medium containing fatty acids rather than glucose, a 200-fold induction of the enzymes of β-oxidation is seen. By the use of deficient mutants, it has been possible to identify a number of genes responsible for coding the relevant enzymes. These genes are named fatty acid degradation (*fad*) genes and there are seven of them, at six different sites on the *E. coli* chromosome. Together they form a regulon, which is under the control of the *fadR* gene (Section 2.2.1; Fig. 2.4). In the presence of medium-chain or long-chain fatty acids, the *fad* regulon is co-ordinately induced as acyl-CoAs bind to a small repressor protein (produced by *fadR*) and stop it interacting with the *fad* gene promoters. All the genes except the electron transport protein (which is constitutive) are induced.

Of the different genes, one (*fadL*) codes for a permease, thus allowing rapid uptake of fatty acids

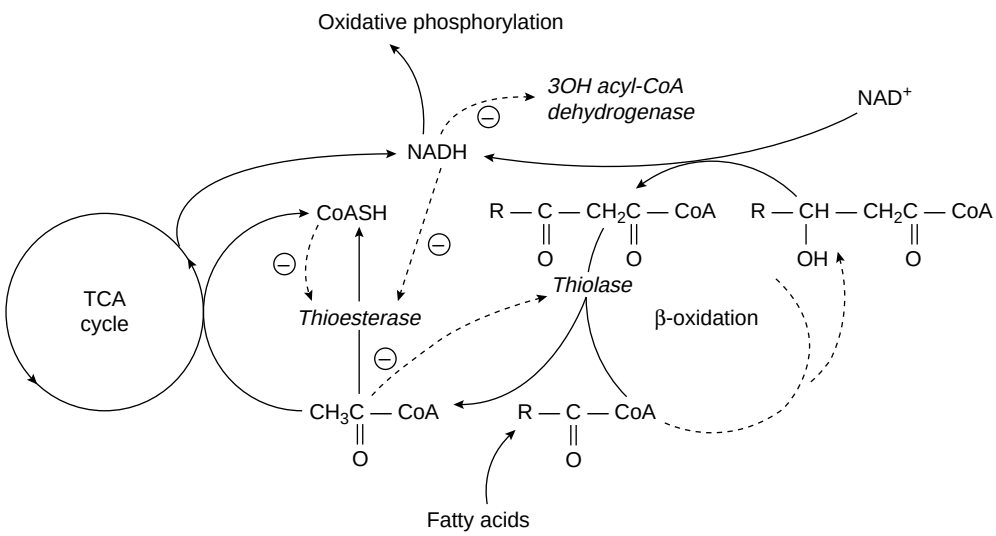


Fig. 2.31 Regulation of β-oxidation in muscle. Inhibition of enzymes is indicated.

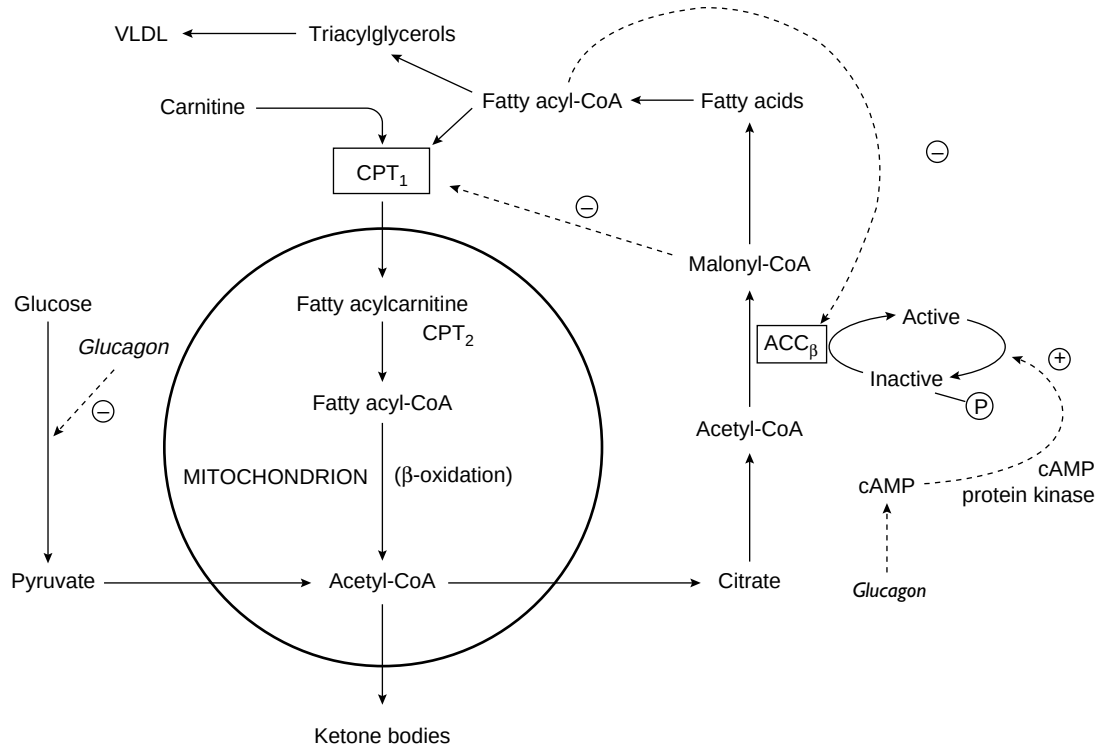


Fig. 2.32 Regulation of fatty acid metabolism in liver. CPT_1, CPT_2 = carnitine palmitoyltransferase 1 and 2; ACC = acetyl-CoA carboxylase; VLDL = very low density lipoproteins.

into *E. coli*. *FadD* codes for a single acyl-CoA synthetase while there are two genes for acyl-CoA dehydrogenases (*fadF*, *fadG*).

Perhaps the most interesting feature of β-oxidation in *E. coli* was the discovery of a homogeneous protein that contained five enzyme activities (crotonase, 3-hydroxyacyl-CoA dehydrogenase, thiolase, Δ^3,Δ^2-enoyl-CoA epimerase, 3-hydroxyacyl-CoA epimerase). The protein is coded by the *fadBA* operon. The protein exists as an $\alpha_2\beta_2$ heterotetramer. The β chain is smaller, contains the thiolase and is coded for by the *fadA* gene. The α chain contains the other activities and is coded by the *fadB* gene.

2.3.1.8 β-Oxidation in microbodies

It was mentioned in Section 2.3.1.1 that microbodies are an important (in plants main) site of fatty acid oxidation. Microbodies do not have an electron transport system coupled to energy production as in mitochondria. Moreover, the flavin-containing oxidase, which catalyses the first reaction, transfers electrons to oxygen to produce H_2O_2 (which is rapidly destroyed by catalase). Thus, this energy is lost as heat. Nevertheless, microbodies produce chain-shortened acyl-CoAs, acetyl-CoA and NADH all of which can exit the organelle. The initial uptake of fatty acids into the organelle is most probably via a malonyl-CoA-sensitive carnitine palmitoyl transferase (CPT_o; Section 2.3.1.2) or, possibly, unesterified fatty acids or acyl-CoAs can cross the microbody membrane.

As mentioned above, β-oxidation in microbodies differs from that in mitochondria (Fig. 2.33). The first step, catalysed by acyl-CoA oxidase, introduces a *trans*-2 double bond and produces H_2O_2. In some tissues (e.g. rat liver) there are several oxi-

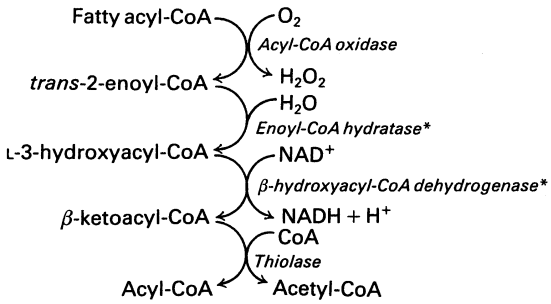


Fig. 2.33 Peroxisomal β-oxidation of fatty acids. *Catalysed by a trifunctional protein in liver and a bifunctional protein in yeast or fungi.

dases with different chain-length selectivities. The next two reactions are catalysed by a trifunctional protein (which also contains the Δ^3,Δ^2-enoyl-CoA epimerase). NADH is a product of the dehydrogenase reaction. No epimerase is contained in yeast and fungi, so the protein is *bifunctional*. The last reaction uses a thiolase and, in liver, there are two enzymes one of which is constitutively expressed and the other is induced by peroxisomal proliferators.

As with entry of fatty acids into microbodies (above) there is controversy about the exit of chain-shortened acyl-CoAs from animal microbodies. Although it was believed previously that such compounds could cross the membrane relatively easily, it is now increasingly thought that a carnitine transport system is used. Because of the absence of an electron transport chain in microbodies there is no internal means of regenerating NAD^+. The reoxidation of NADH is thought to occur either by a glycerol phosphate shuttle or by movement of NADH to the cytosol and NAD^+ back.

In animal tissues, such as liver, the microbody system seems incapable of oxidizing long-chain acyl-CoAs completely, owing to the limited substrate specificity of the acyl-CoA oxidases. The medium-chain products are transferred to carnitine by a peroxisomal medium-chain carnitine acyltransferase. The acylcarnitines can then be moved to the mitochondria for further oxidation. Studies on this system and on peroxisomal β-oxidation in general have been facilitated by the fact that hypolipidemic drugs like clofibrate, high fat diets, starvation or diabetes all increase peroxisomal oxidation considerably. Under average conditions peroxisomes are thought to contribute up to 50% of the total fatty acid oxidative activity of liver. A disease known as adrenoleukodystrophy is characterized by the accumulation of very long chain fatty acids in the adrenals. It may be due to a defect in peroxisomal β-oxidation.

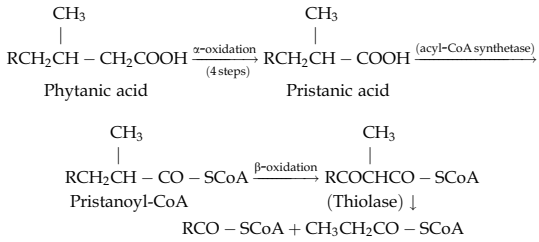
In contrast to the chain length limitation of β-oxidation in mammalian peroxisomes, the glyoxysomes of fatty seedlings and peroxisomes of plant leaves, fungi or yeast are capable of completely oxidizing fatty acids to acetate. As mentioned before, no co-operation with mitochondria is necessary (apart from regeneration of NAD^+) and, indeed, many plant mitochondria do not appear to contain β-oxidation enzymes.

2.3.2 α-Oxidation of fatty acids is important when structural features prevent β-oxidation

α-Oxidation is important in animals for the formation of α-hydroxy fatty acids and for chain shortening, particularly in regard to catabolism of molecules that cannot be metabolized directly by β-oxidation. Brain cerebrosides and other sphingolipids (Section 6.3) contain large amounts of α-hydroxy fatty acids and a mixed-function oxidase has been identified in microsomal fractions that requires O_2 and NADPH. Breakdown of α-hydroxy fatty acids also takes place in such fractions, but is probably localized in peroxisomes.

The peroxisomal α-oxidation system has been well-characterized recently, partly because it is very important for the breakdown of branched-chain fatty acids – in particular, phytanic acid (3,7,11,15-tetramethyl palmitic acid), which is formed in animals from phytol, the side-chain of chlorophyll. In phytanic acid the methyl group at C3 prevents β-oxidation. The acid is activated to its CoA ester, hydroxylated at the 2 position (to give an α-hydroxy intermediate), followed by cleavage to yield pristanal (an aldehyde) with the release of formyl-CoA. The latter gives rise to CO_2. Pristanal is then oxidized (using NAD) to pristanic acid,

which as pristanoyl-CoA, is a substrate for β-oxidation, which continues with the release of propionyl-CoA. This is summarized:



An inborn error of metabolism (Refsum's disease), due to a failure of α-oxidation, is described in Section 7.8.

Plants have an active α-oxidation system that is concerned, amongst other functions, with the turnover of the phytol moiety of chlorophyll as described above. The mechanism has been studied in several tissues. Although there is still some controversy as to whether O_2 or H_2O_2 is the substrate and which cofactors are involved, it seems probable that O_2 is activated by a reduced flavoprotein and that a hydroperoxide intermediate is the active species that reacts with the fatty acid.

Until 1973 it seemed that there must be at least two different pathways for α-oxidation depending on the source of the enzymes. The pathways that had been studied in pea leaves and in germinating peanut cotyledons apparently had different cofactor requirements and different intermediates seemed to be involved. The discrepancies have now been resolved and a unified pathway has been proposed by Stumpf and his team in California (Fig. 2.34).

These researchers showed that molecular oxygen was a requirement and, in the peanut system, that a hydrogen peroxide-generating system was involved. Enzymes catalysing the reduction of peroxides, such as glutathione peroxidase, reduced α-oxidation and increased the production of D-hydroxypalmitate. This pointed to the existence of a peroxide (2-hydroperoxypalmitate) intermediate. Accumulation of hydroxyacids in experiments was due to the intermediates being channelled into a dead-end pathway.

After the hydroperoxy intermediate, CO_2 is lost and a fatty aldehyde produced. This is oxidized by an NAD^+-requiring system to yield a fatty acid, one carbon less than the original (palmitic acid) substrate. α-Oxidation in plants is extra-mitochondrial and may be in the cytoplasm or associated with the endoplasmic reticulum (depending on the tissue).

2.3.3 ω-Oxidation uses mixed-function oxidases

ω-Oxidation of straight-chain fatty acids yields dicarboxylic acid products. Normal acids are only slowly catabolized by such a system since β-oxidation is usually highly active. However, in substituted derivatives ω-oxidation is often an important first step to allow subsequent β-oxidation to take place. ω-Oxidation has an ω-hydroxy fatty acid as an intermediate and the enzyme is a mixed-function oxidase. The enzyme is very similar to, or identical with, the drug-hydroxylating enzyme system and involves cytochrome P450. Molecular oxygen and NADPH are co-substrates.

An ω-hydroxylase has been studied in *Pseudomonas* by Coon and coworkers. They showed that the enzyme had non-haem iron as, apparently, the only prosthetic group involved directly in the hydroxylation reactions. The system was fractionated into three components: a non-haem iron protein (similar to rubredoxin), a flavoprotein and a final component needed for hydroxylase activity.

In plants, the ω-hydroxylase system is responsible for synthesis of the ω-hydroxy fatty acid components of cutin and suberin (Section 6.6.1). Kolattukudy has studied the reactions in preparations from *Vicia faba*. NADPH and O_2 were cofactors and the enzyme showed typical properties of a mixed-function oxidase. However, the involvement of P450 in the plant system is unproven since, although the hydroxylation is inhibited by CO, the inhibition is not reversed by 420–460 nm light (a property typical for cytochrome P450 systems). Where dihydroxy fatty acids are being synthesized for cutin or suberin, the ω-hydroxy fatty acid is the substrate for the second hydroxylation. Like ω-oxidation, mid-chain hydroxylation also requires

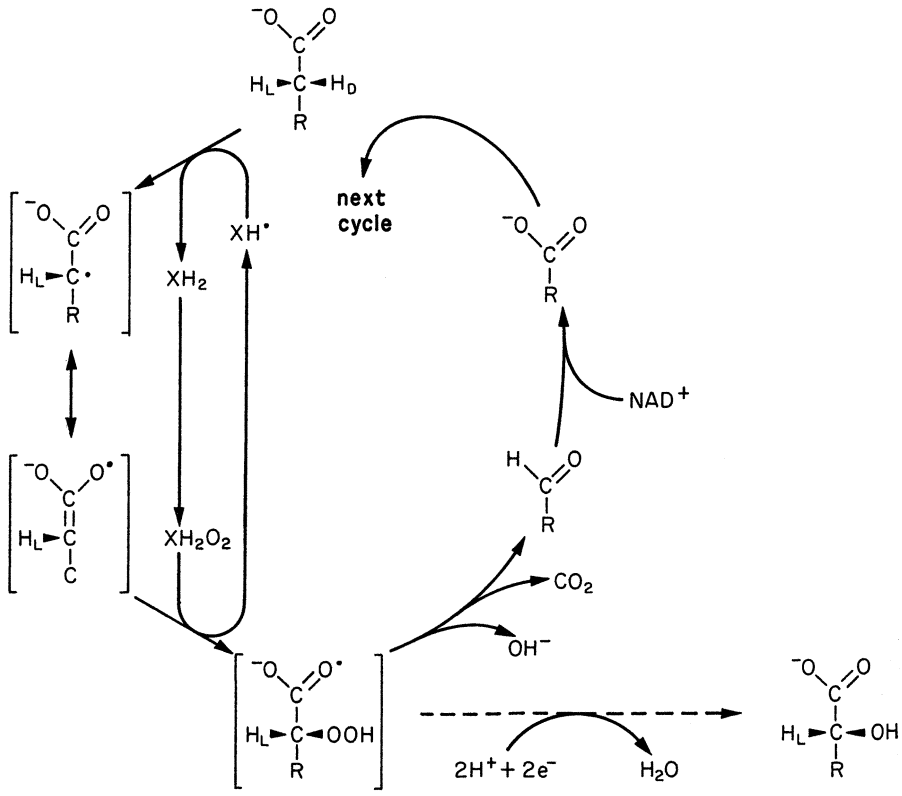


Fig. 2.34 Proposed α-oxidation pathway in plants.

NADPH and O_2 and is located in the endoplasmic reticulum.

2.3.4 Chemical peroxidation is an important reaction of unsaturated fatty acids

One of the characteristic reactions of lipids that are exposed to oxygen is the formation of peroxides. Indeed, among non-enzymic chemical reactions taking place in the environment at ambient temperatures, the oxidation of unsaturated compounds is, perhaps, the most important both from an industrial and a medical point of view. In biological tissues, uncontrolled lipid peroxidation causes membrane destruction and is increasingly regarded as an important event in the control or development of diseases (Sections 4.3 and 5.4.1). In food, oxidation (either enzymically or chemically catalysed) can have desirable as well as adverse consequences (Sections 4.2.5 and 4.3).

In common with other radical chain reactions, lipid peroxidation can be divided into three separate processes – initiation, propagation and termination. During initiation a very small number of radicals (e.g. transition metal ions or a radical generated by photolysis or high-energy irradiation) allow the production of $R^\bullet$ from a substrate RH:

$$X^\bullet + RH \rightarrow R^\bullet + XH$$

Propagation then allows a reaction with molecular oxygen:

$$R^\bullet + O_2 \rightarrow ROO^\bullet$$

and this peroxide radical can then react with the original substrate:

$$ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$$

Thus, the events form the basis of a chain-reaction process.

Free radicals such as $ROO^{\cdot}$ (and $RO^{\cdot}$, $OH^{\cdot}$, etc., which can be formed by additional side-reactions) can react at random by hydrogen abstraction and a variety of addition reactions to damage proteins, other lipids and vitamins (particularly vitamin A). Compounds that react rapidly with free radicals can be useful in slowing peroxidation damage. Thus, naturally occurring compounds such as vitamin E are powerful anti-oxidants and tissues deficient in such compounds may be prone to peroxidation damage. Formation of lipid hydroperoxides can be readily detected by a number of methods of which the absorption of conjugated hydroperoxides at 235 nm is particularly useful.

Termination reactions may lead to the formation of both high and low molecular mass products of the peroxidation reactions. Depending on the lipid, some of the low molecular mass compounds may be important flavours (or aromas) of foods (Section 4.2.5). For example, short- to medium-chain aldehydes formed from unsaturated fatty acids may give rise to rancidity and bitter flavours on the one hand or more pleasant attributes such as those associated with fresh green leaves, oranges or cucumbers on the other hand. Fish odours are attributed to a ketone. Some relevant changes in quality or nutritional value of food are indicated in Chapter 4 (Fig. 4.11).

In general, it is considered desirable to reduce the initiation reaction as a means of controlling peroxidation. Apart from natural anti-oxidants, BHT (3,5-di-*t*-butyl-4-hydroxytoluene) is often used as a food additive. Metal binding compounds and phenolic compounds may also be inhibitory as well as the endogenous superoxide dismutase and glucose oxidase–catalase enzyme systems.

2.3.5 Peroxidation catalysed by lipoxygenase enzymes

The second kind of peroxidation is catalysed by the enzyme lipoxidase (or lipoxygenase). The enzyme was originally thought to be present only in plants, but it has now been realized that it catalyses very important reactions in animals (Section 2.4.6). The chief sources of the enzyme are peas and beans (especially soybean), cereal grains and oil seeds. It was originally detected by its oxidation of carotene and has been used extensively in the baking industry for bleaching carotenoids in dough.

All known lipoxygenases catalyse the following reaction:

$$R-\underset{cis}{CH=CH}-CH_2-\underset{cis}{CH=CH}-R_1 + O_2 \rightarrow R-\underset{cis}{CH=CH}-\underset{trans}{CH=CH}-\underset{\displaystyle\overset{|}{O}\overset{|}{H}}{CH}-R_1$$

That is, they catalyse the addition of molecular oxygen to a 1,4-*cis*,*cis*-pentadiene moiety to produce a 1-hydroperoxy-2,4-*trans*,*cis*-pentadiene unit.

When Theorell and his colleagues in Sweden first purified and crystallized soybean lipoxygenase in 1947, they reported that it had no prosthetic group or heavy metal associated with it. Such a situation would make lipoxygenase unique among oxidation enzymes. However, Chan in England and Roza and Franke in The Netherlands demonstrated the presence of one atom of iron per mole of enzyme by atomic absorption spectroscopy. The product of the enzymic reaction – a hydroperoxide – is similar to the products of purely chemical catalysis, but the lipoxidase reaction has a number of distinguishing features. The activation energy is smaller than that for chemical reactions, and the enzyme has very specific substrate requirements. In order to be a substrate, the fatty acid must contain at least two *cis* double bonds interrupted by a methylene group. Thus, linoleic and α-linolenic acids are good substrates for the plant enzymes while arachidonic acid, the major polyunsaturated fatty acid in mammals, is attacked by different lipoxygenases in their tissues. Like chemically catalysed peroxidation, the lipoxidase reaction involves free radicals and can be inhibited by radical trapping reagents such as the tocopherols. The reaction sequence shown in Fig. 2.35 represents the currently accepted pathway.

Lipoxygenases are widespread in plants. One effect of the enzymes in plant tissues is to yield

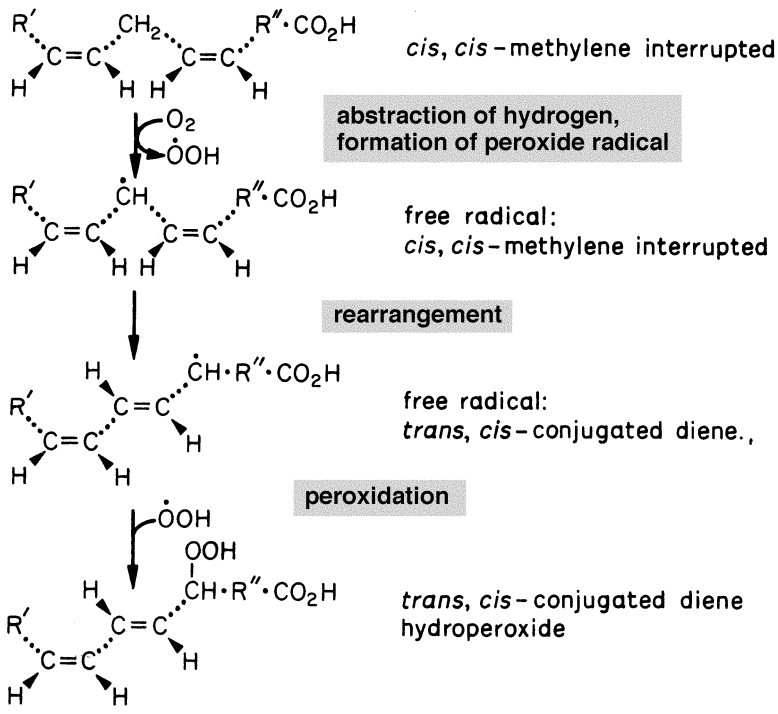


Fig. 2.35 Mechanism of lipoxygenase enzymes.

volatile products with characteristic flavours and aromas – either desirable or undesirable. This aspect is dealt with in more detail in the next section, where the physiological importance of plant lipoxygenases is covered. In general it seems that plant lipoxygenases work best with unesterified fatty acid substrates. These are released from storage triacylglycerols by lipases or, more importantly, from membrane lipids by non-specific acylhydrolases or phospholipase As.

Several lipoxygenases are found in mammals, being distinguished from one another by the position of oxygen insertion. Their role in producing leukotrienes and other oxy-lipids is discussed in Sections 2.4.6 and 2.4.8. As for the plant enzymes, animal lipoxygenases are non-esterified fatty acids that must first be released from complex lipids to provide sufficient substrate.

2.3.6 Lipoxygenases are important for stress responses and development in plants

Whereas in animals, arachidonate is the major polyunsaturated fatty acid and, hence, the most important substrate for lipoxygenase attack, in plants α-linolenate is the main acid. Breakdown of this fatty acid is known as the α-linolenic acid cascade and the pathways give rise to a variety of important molecules (Figs 2.36 and 2.37).

The fatty acid hydroperoxides produced by lipoxygenases can be converted into three main products (Fig. 2.36) with important known functions:

(1) Co-oxidative reactions with peroxygenase give a mixture of epoxy and hydroxy fatty acids (depending on the nature of the acceptor fatty acid for the mono-oxygenation). The

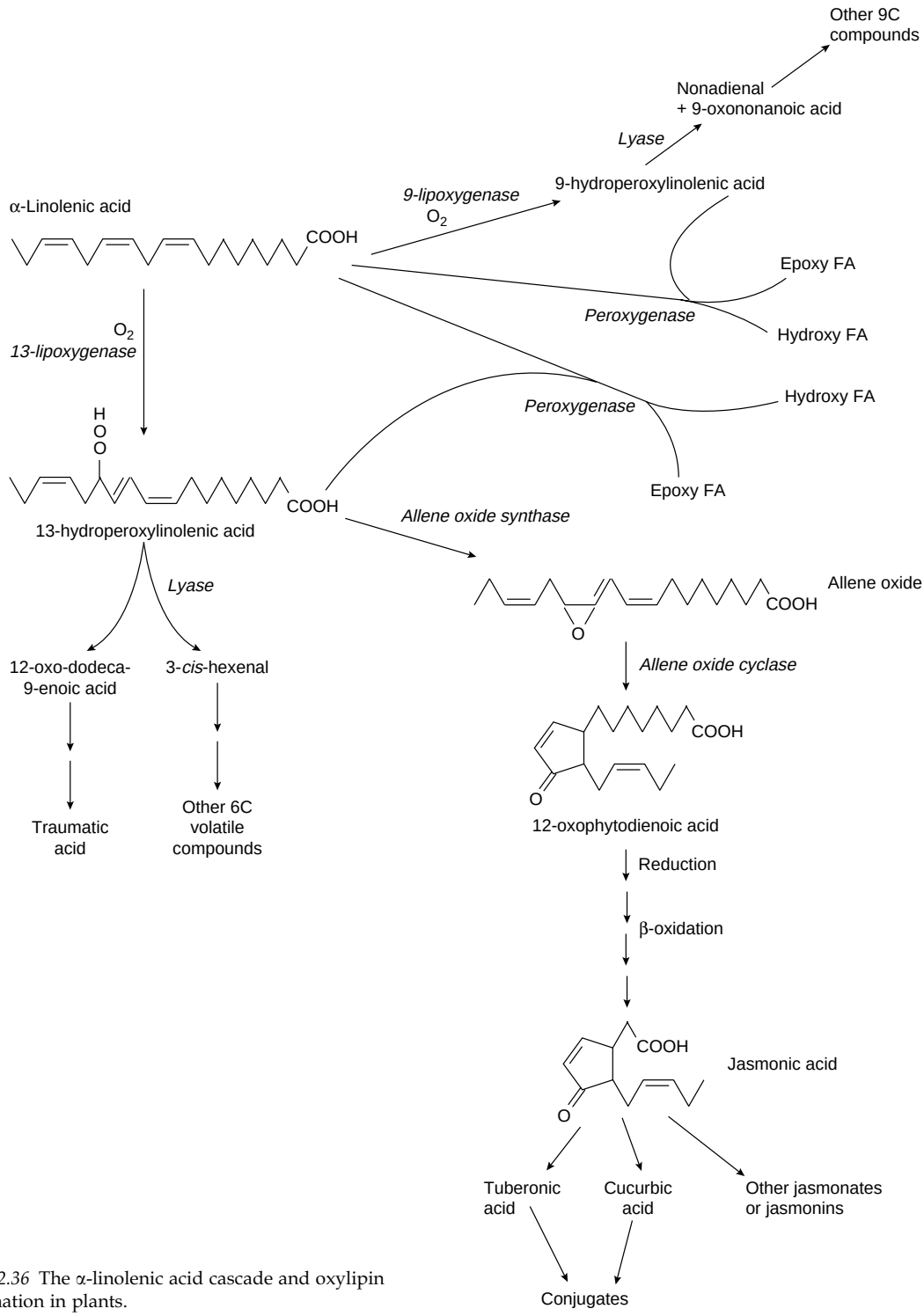


Fig. 2.36 The α-linolenic acid cascade and oxylipin formation in plants.

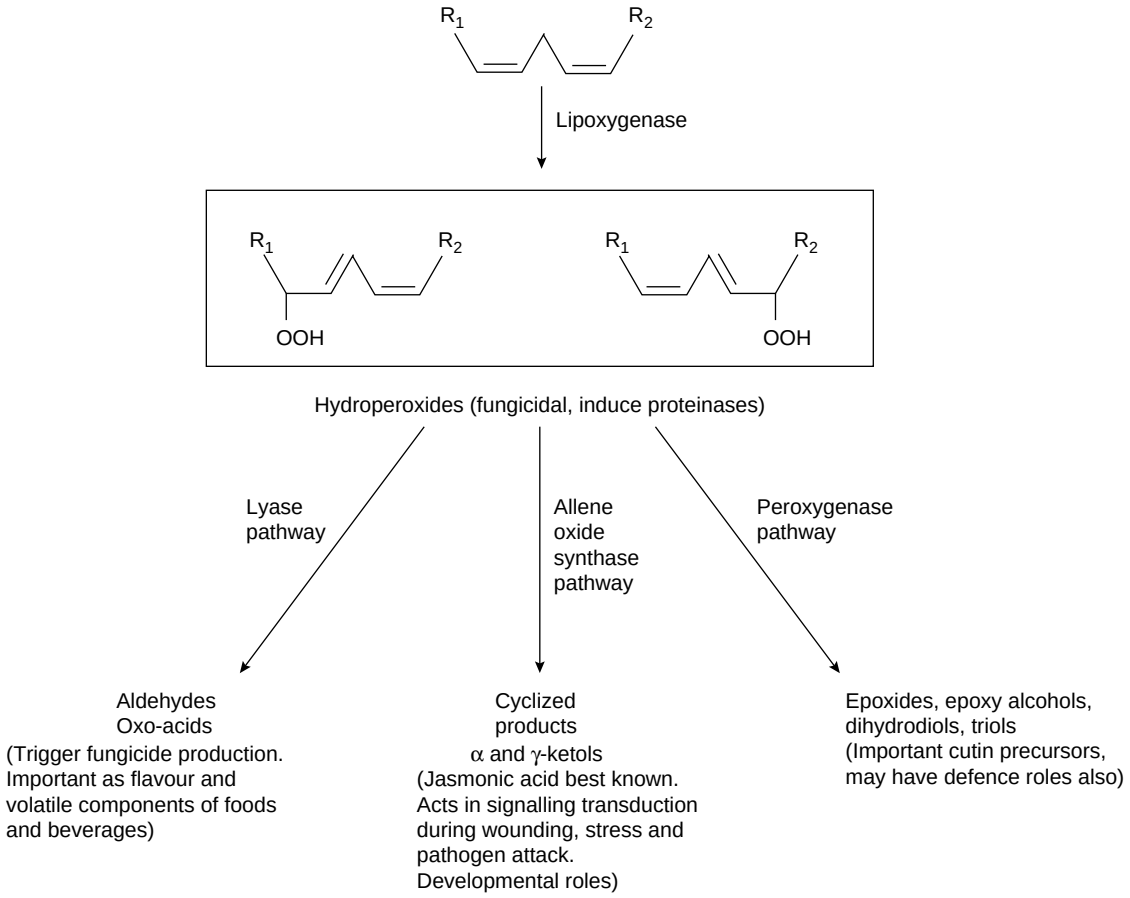


Fig. 2.37 Lipoxygenase products and physiological roles in plants.

products have roles in cutin (the surface covering of leaves) biogenesis and in pest attack.

(2) Hydroperoxide lyase cleaves the hydroperoxide into an aldehyde and an oxo-unsaturated fatty acid. The products have a role in pest defence and appear to act as pollinator and herbivore attractants, especially for flowers and fruits. They are also important flavour and aroma components of foods, drinks and perfumes.

(3) Allene oxide synthase gives rise to the precursor of jasmonic acid. The latter and associated derivatives are commonly known as the jasmonates although Hildebrand has proposed the term 'jasmonins' to differentiate from jasmonic acid esters. The jasmonins have important effects on plant growth, development and senescence.

Some of the important roles for lipoxygenase-derived plant products are listed in Table 2.13.

Under item (2) above, hydroperoxide lyase cleavage is listed. This usually occurs with the 13-hydroperoxide derivative thus giving rise to a 6C aldehyde. Particularly, if the aldehyde is the unsaturated hexenal, then the products will be rather volatile. The aldehyde can be converted to its alcohol (by alcohol dehydrogenase), the double bond isomerized (3-*cis* to 2-*trans*) and acetate (or other short-chain acid) esters formed. The combi-

Table 2.13 Some physiological roles for lipoxygenase-derived products in plants

Phenomenon	Notes	Compounds involved
Plant resistance to pathogens	Activation of several defence systems in 'hypersensitive response' lipoxygenase expression rapidly induced following infection.	Jasmonic acid; phytoalexins (e.g. hexenal); eicosanoids from pathogen arachidonate
Mobilization of storage lipids	13-Lipoxygenase induced, translocates to lipid body and dioxygenates storage lipids, which are then hydrolysed and the oxygenated fatty acid catabolized.	13-Hydroperoxygenated lipids
Drought stress	Widespread phenomenon in plants is the drought-induced accumulation of 9-hydroperoxy derivatives of membrane lipids. Function unknown.	9-Hydroperoxygenated lipids
Senescence	Lipoxygenase may be involved directly in photosystem inactivation and chlorophyll oxidation. Jasmonate as senescence-promoting substance, by inducing production of certain proteins. Has complementary effects with ABA (abscisic acid) and may influence ethylene production.	Jasmonins
Fruit ripening	Differential effects of jasmonates on ethylene formation. Several fruit-specific lipoxygenase genes identified.	Jasmonins
Tuber induction	Stimulate a number of associated phenomena such as cell expansion, cytoskeleton structure and carbohydrate accumulation.	Tuberonic acid and other jasmonins

nation of volatiles thus produced are effective attractants to both pollinators and herbivores and smells like 'cut grass', 'cucumber' and various fruits derive from them. It is interesting that the different aromas and tastes of the best quality olive oils, for example, can be identified either by human tasters or via sophisticated gas chromatographic analysis of the lipoxygenase-derived volatiles.

2.4 ESSENTIAL FATTY ACIDS AND THE BIOSYNTHESIS OF EICOSANOIDS

Some types of polyunsaturated fatty acids, the so-called essential fatty acids, are converted into oxygenated fatty acids with potent physiological effects. These include effects on muscle contraction, cell adhesion, immune function and vascular tone.

In Section 2.2.5 we described the biosynthesis of the polyunsaturated fatty acids and indicated that mammals lack the $\Delta 12$ and $\Delta 15$-desaturases. They cannot, therefore, synthesize linoleic or α-linolenic acids. It turns out, however, that these polyunsaturated fatty acids are absolutely necessary for the maintenance of growth, reproduction and good health and must, therefore, be obtained in the diet from plant foods. They are called essential fatty acids and their role in nutrition and health will be described in detail in Section 4.2.3. They are precursors of a variety of oxygenated 20C fatty acids with potent biological activities now generally known collectively as the eicosanoids.

As early as 1930, two American gynaecologists, Kurzrok and Lieb, reported that the human uterus, on contact with fresh human semen, was provoked into either strong contraction or relaxation. Both von Euler in Sweden and Goldblatt in England

subsequently discovered marked stimulation of smooth muscle by seminal plasma. Von Euler then showed that lipid extracts of ram vesicular glands contained the activity and this was associated with a fatty acid fraction. The active factor was named prostaglandin and was shown to possess a variety of physiological and pharmacological properties.

In 1947, the Swede, Bergström, started to purify these extracts and soon showed that the active principle was associated with a fraction containing unsaturated hydroxy acids. The work then lapsed until 1956, when with the help of an improved test system (smooth muscle stimulation in the rabbit duodenum) Bergström isolated two prostaglandins in crystalline form (PGE_1 and $PGF_{1\alpha}$). Their structure, as well as that of a number of other prostaglandins, was elucidated by a combination of degradative, mass spectrometric, X-ray crystallographic and NMR studies (Fig. 2.38). The nomenclature is based on the fully saturated 20C acid with C_8 to C_{12} closed to form a 5-membered ring; this is called prostanoic acid. Thus PGE_1 is designated 9-keto-11α,15α-dihydroxyprost-13-enoic acid. The 13,14 double bond has a *trans* configuration; all the other double bonds are *cis*. Figure 2.38 clearly brings out the difference between the 'E' and

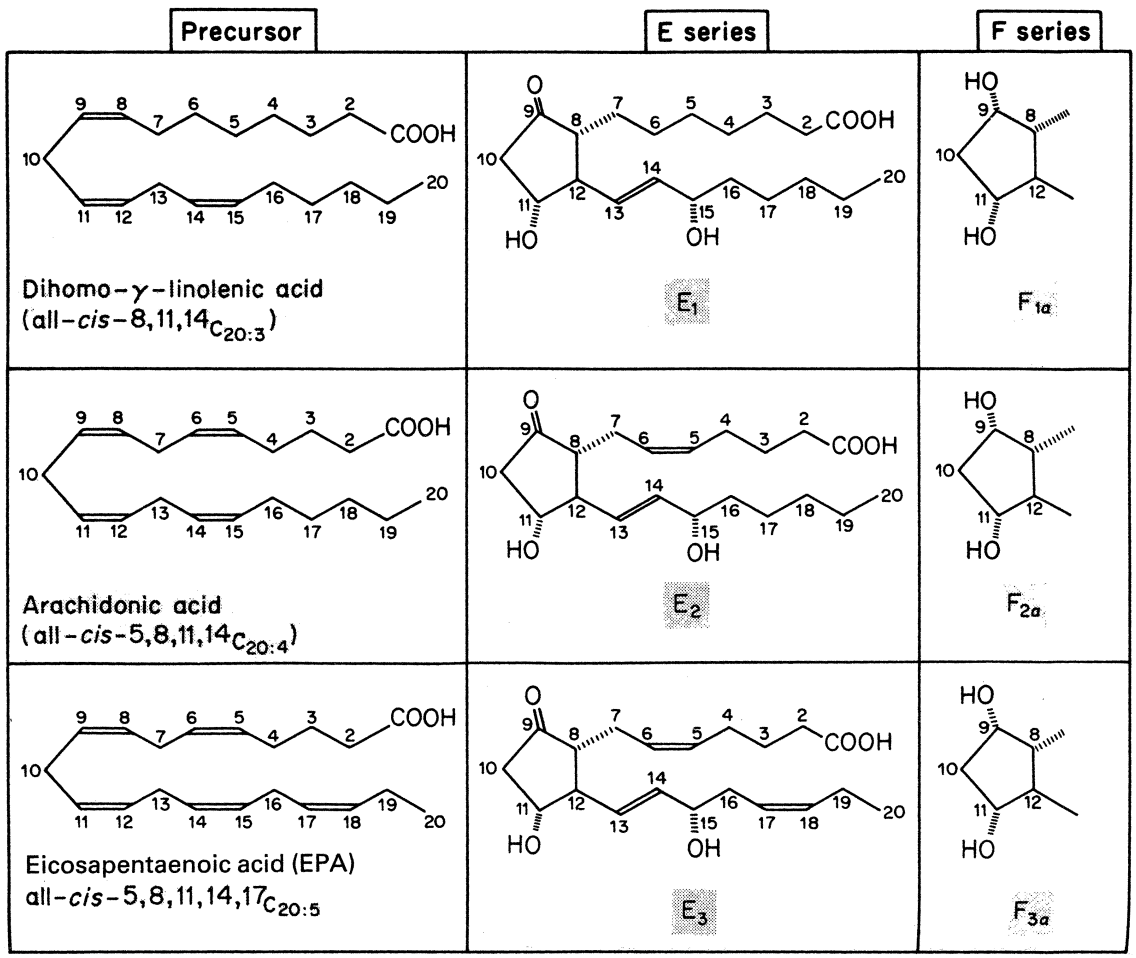


Fig. 2.38 Structures of prostaglandins E and F and their precursors.

'F' series, which have a keto and hydroxyl group at position 9, respectively; 'α' refers to the stereochemistry of the hydroxyl, and the suffix 1, 2 or 3 is related to the precursor fatty acid from which they are derived. That is, the 1, 2 or 3 refer to how many double bonds are contained in the prostaglandin structure. The name prostaglandin (and the related prostanoic acid structure) derives from the fact that early researchers believed that the prostate gland was the site of their synthesis.

2.4.1 The pathways for prostaglandin synthesis are discovered

Although prostaglandins were the first biologically active eicosanoids to be identified, it is now known that the essential fatty acids are converted into a number of different types of eicosanoids. (Eicosanoid is a term meaning a 20C fatty acid derivative.) The various eicosanoids are important examples of local hormones. That is, they are generated in situ and, because they are rapidly metabolized, only have activity in the immediate vicinity. A summary of the overall pathway for generation of eicosanoids is shown in Fig. 2.39. Essential fatty acids can be attacked by lipoxygenases, which give rise to leukotrienes or hydroxy fatty acids and lipoxins. Alternatively, metabolism by cyclooxygenase gives cyclic endoperoxides from which the classical prostaglandins or thromboxanes and prostacyclin can be synthesized. A third possibility is via cytochrome P450 oxygenation where atomic oxygen is introduced leading to fatty acid hydroxylation or epoxidation of double bonds. Whereas both the lipoxygenase and cyclooxygenase reactions arise from the formation of a fatty acid radical, in P450-oxygenation activation of atmospheric molecular oxygen is involved. After this, one oxygen is transferred to the fatty acid substrate and one is reduced forming water. Because, historically, prostaglandin synthesis was elucidated before that of the other eicosanoids, we shall describe prostaglandin formation first.

After the structures of PGE and PGF had been defined, the subsequent rapid exploitation of this field, including the unravelling of the biosynthetic pathways, was done almost entirely by two research teams led by van Dorp in Holland and by Bergström and Samuelsson in Sweden. Both realized that the most likely precursor of PGE_2 and $PGF_{2\alpha}$ was arachidonic acid. This was then demonstrated by both groups simultaneously (with the same preparation of tritiated arachidonic acid) by incubation with whole homogenates of sheep

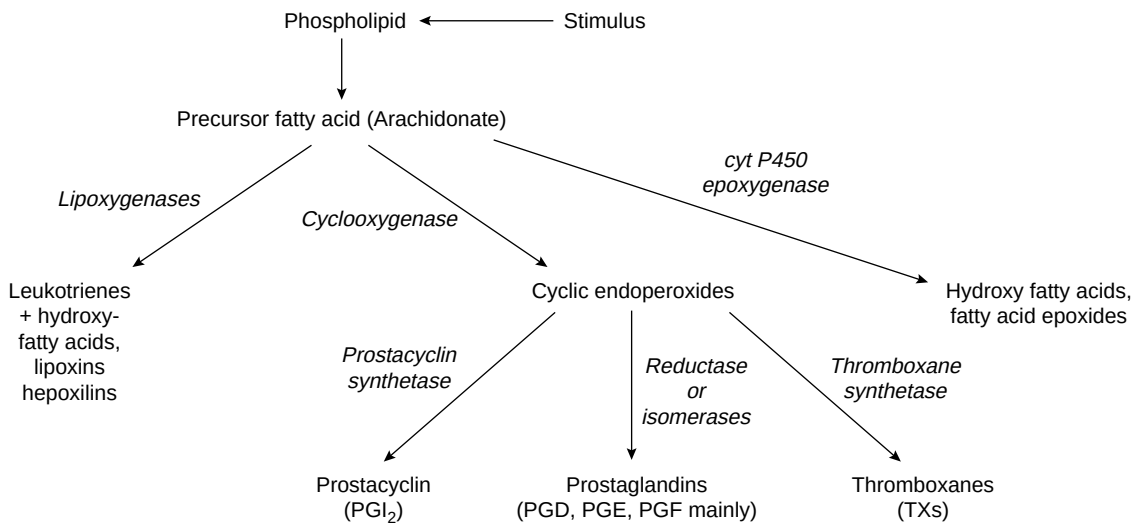


Fig. 2.39 Overall pathway for conversion of essential fatty acids into eicosanoids.

vesicular glands. An important point that was noted, was that the unesterified fatty acid was the substrate and not an activated form (see later for discussion about release of free fatty acids in tissues). The ability of the arachidonic acid chain to fold allows the appropriate groups to come into juxtaposition for the ring closure to occur (Fig. 2.38 and Fig. 2.40). The reactions take place in the microsomal fraction, but a soluble heat-stable factor is required. This cofactor can be replaced by reduced glutathione. The reaction also requires molecular oxygen. Labelling with $^{18}O_2$ demonstrated that all three oxygen atoms in the final prostaglandin (Fig. 2.40) are derived from the gas.

This key initial reaction in prostaglandin formation is catalysed by the enzyme prostaglandin endoperoxide synthase. For simplicity this is usually known as cyclooxygenase (COX; Figs 2.39 and 2.40). There are two major isoforms and crystal structures for both have been obtained. The cyclooxygenases are haemoproteins and they exhibit both cyclooxygenase and peroxidase activities. Cyclooxygenase-1 is constitutively expressed in many mammalian cells and tissues, and appears to be responsible for the formation of prostaglandins involved in the general regulation of physiological events. On the other hand, cyclooxygenase-2 is present at low basal levels in inflammatory cells. It is strongly induced by inflammatory stimuli such as cytokines, endotoxins, tumour promoters and some lipids. Both isoforms have similar V_{max} and K_m values for arachidonate, undergo suicide inactivation and their reactions are initiated by hydroperoxide. However, COX-2 needs much lower levels of hydroperoxide and has somewhat different substrate selectivity than COX-1. Both enzymes will, nevertheless, utilize a broad spectrum of polyunsaturated fatty acid substrates such as linoleate, γ-linolenate, α-linolenate and arachidonate. Thus, they can give rise to a variety of endoperoxides and, therefore, to a host of different prostaglandins.

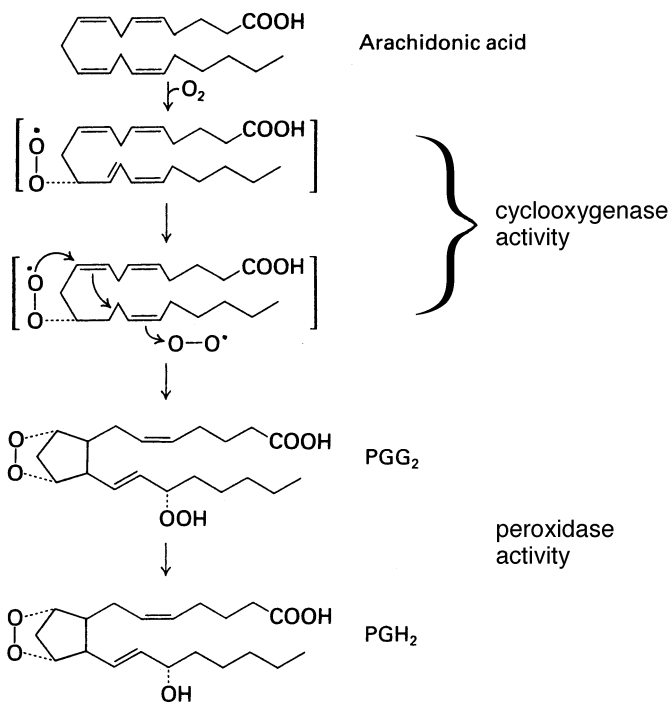


Fig. 2.40 Mechanism of biosynthesis of the cyclic endoperoxide, PGH_2.

The overall reaction is shown in Fig. 2.40. First, prostaglandin endoperoxide synthase (PES) inserts two molecules of oxygen to yield a 15-hydroperoxy-9,11-endoperoxide with a substituted cyclopentane ring (PGG; Fig. 2.40). This is the cyclooxygenase activity of the enzyme. The peroxidase activity then reduces PGG to its 15-hydroxy analogue, PGH.

As mentioned above, the cyclooxygenase activity requires a hydroperoxide activator to remove a hydrogen atom from position 13 on the incoming fatty acid, and therefore allow attack by oxygen. This is an unusual mechanism because, usually, oxygenases work by activating the oxygen substrate. There are other interesting features of the enzyme. For example, the free radical intermediates generated can also inactivate the enzyme. This self-deactivation of the cyclooxygenase occurs in vivo as well as for purified preparations. It may ensure that only a certain amount of endoperoxide is generated even when large quantities of precursor fatty acid are available.

Both the cyclooxygenases have a dual localization in the cell, being present in both the endoplasmic reticulum and the nuclear envelope. A classical inhibitor of the cyclooxygenases is aspirin and, indeed, much of our knowledge of these enzymes has come from studies with the non-steroidal anti-inflammatory drugs (NSAIDs). In addition, gene disruption and overexpression of COX isoforms have confirmed that, as a generalization, the therapeutic anti-inflammatory action of the NSAIDs is due to inhibition of COX-2 while simultaneous inhibition of COX-1 causes most of the unwanted side-effects such as gastric ulceration.

Of the various non-steroidal anti-inflammatory drugs (such as aspirin, ibuprofen, indomethacin and diclofenac) aspirin is the best known. Aspirin competes with arachidonate for binding to the cyclooxygenase active site. Although arachidonate binds about 10 000 times better than aspirin, once bound aspirin acetylates a serine residue (serine 530) at the active site to cause irreversible cyclooxygenase inactivation (Fig. 2.41). Thus, the cell can only restore COX activity by making fresh enzyme. In COX-1 the serine 530 residue, although at the active site, is not needed for catalysis and acetylation by aspirin results in steric hinderance to prevent arachidonate binding. For COX-2, acetylation by aspirin still permits oxygenation of arachidonate, but the usual product PGH_2 is not made.

Not only is aspirin used for pain relief but low-dose therapy is used for a selective inhibition of platelet thromboxane formation, which reduces platelet aggregation and, hence, blood clotting.

Although the other non-steroidal anti-inflammatory drugs inhibit cyclooxygenase activity, most of them cause reversible enzyme inhibition by competing with arachidonate for binding. A well-known example of a reversible NSAID is ibuprofen. The use of NSAIDs is a huge part of the pharmaceutical market and currently accounts for over £3 billion in annual sales – or to put it in another way, approximately 15×10^{12} tablets of aspirin are consumed annually! Because NSAIDs inhibit both cyclooxygenases, there has been active development of COX-2-specific drugs, which should pos-

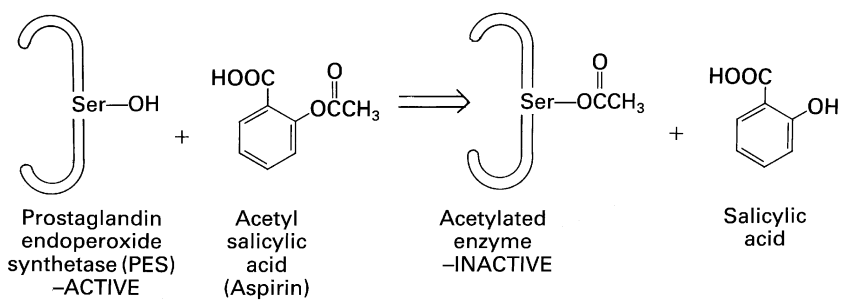


Fig. 2.41 Mechanism for the inactivation of the cyclooxygenase-1 by aspirin.

sess anti-inflammatory properties without the unwanted side-effects of common NSAIDs. The first such drugs have recently become available for patients.

Prostaglandin H is the key intermediate for conversion to various active eicosanoids. The enzymes responsible for its further metabolism are present in catalytic excess to COX and, hence, are not regulatory except in the sense that the balance of their activities determines the pattern of prostaglandins and thromboxanes, which are formed in a given tissue. Thus, although Fig. 2.42 depicts some possible conversions of PGH_2 into various biologically active eicosanoids, prostanoid synthesis is cell-specific. For example, platelets form mainly the thromboxane TxA_2; endothelial cells produce PGI_2 (prostacyclin) as their major prostanoid; while kidney tubule cells synthesize predominantly PGE_2.

2.4.2 Cyclic endoperoxides can be converted into different types of eicosanoids

The various eicosanoids produced from PGH_2 (Fig. 2.42) have a remarkable range of biological activities. Moreover, the effect of a given eicosanoid varies from tissue to tissue. In the absence of

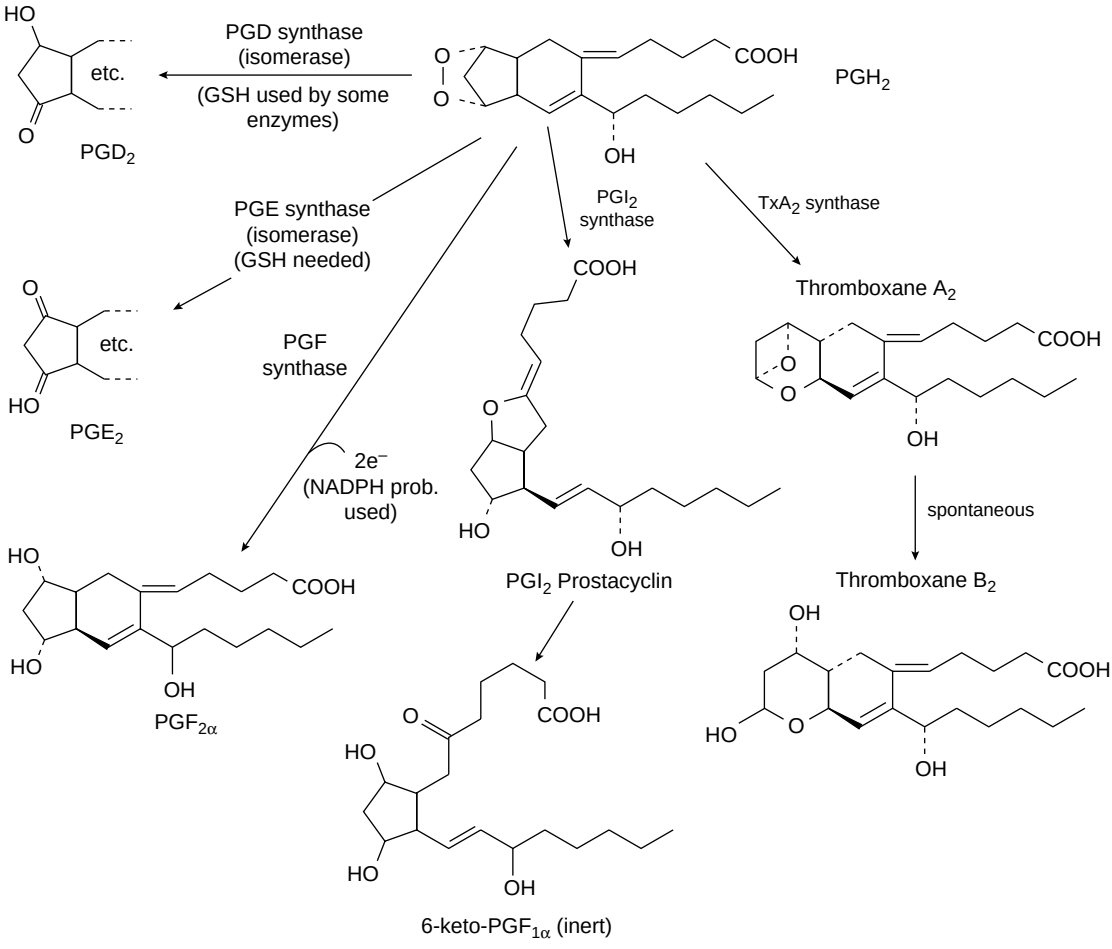


Fig. 2.42 Conversion of PGH_2 into various eicosanoids.

specific inhibitors of their formation, and given the interacting effects of many eicosanoids, it has proved rather difficult to elucidate all their biological functions.

The classic prostaglandins are PGD, PGE and PGF. These, and other prostanoids, bind to cell receptors and there are pharmacologically distinct receptors for each of the known prostanoids. Moreover, there may be multiple receptors for a given prostaglandin. For example, in the case of PGE_2, four distinct receptors have been identified. Several receptors have been cloned and shown to have typical membrane-spanning regions. So far they all appear to be of the G protein-linked receptor family and will, therefore, modify cellular reactions through classical G-protein signalling pathways.

PGD synthase (which is an isomerase) has high activity in brain and the spinal cord and PGD_2 is also the main cyclooxygenase product of mast cells. Both glutathione-dependent and -independent forms of PGD synthase have been found (Fig. 2.42). PGD_2 inhibits platelet aggregation, increases platelet cAMP content and has a membrane receptor distinct from that for PGI_2 (Section 2.4.3). It can act as a peripheral vasoconstrictor, pulmonary vasoconstrictor and bronchoconstrictor. The latter activity can be demonstrated when PGD_2 is inhaled. It is thought to have various neuromodulatory actions (e.g. it can decrease noradrenaline release from adrenergic nerve terminals) and overproduction of PGD_2 may be involved in the hypotensive attacks of patients with mastocytosis.

PGE_2 is the main arachidonate metabolite in kidneys where it reduces ADH (antidiuretic hormone)-induced water reabsorption and may help to control renin release. It may also help to mediate the metabolism and interaction of macrophages with other cells. PGE synthases (again isomerases) are unique in that all of them use glutathione as a cofactor.

$PGF_{2\alpha}$ seems to be the so-called luteolytic factor produced by mammalian uteri. When the compound is injected into cows it causes regression of the corpus luteum and induces ovulation and is used commercially as a regulator of ovulation in dairy cows. $PGF_{2\alpha}$ is useful for the induction of abortions in women in midtrimester. In addition, if injected as a slow-releasable form it will act as a birth control agent probably by inhibiting implantation of the fertilized ovum in the uterine wall. In contrast to the formation of PGD or PGE, the biosynthesis of PGF is a two-electron reduction. A PGF synthase has been purified from lung and uses NADPH as reductant.

2.4.3 New eicosanoids are discovered

In 1973 the Swedes Hamberg and Samuelsson were studying the role of prostaglandins in platelet aggregation. They discovered two new cyclooxygenase products, one of which was highly active in stimulating aggregation. As little as $5\,ng\,ml^{-1}$ caused platelets to aggregate and the aorta to contract. They called the substance *thromboxane* because of its discovery in thrombocytes. Thromboxane A_2 (Fig. 2.42) is an extremely labile substance and is rearranged with a half-life of about 30 s to a stable and physiologically inert derivative, thromboxane B_2 (Fig. 2.42). Because of the role of thromboxane A_2 in platelet aggregation, considerable effort has been devoted to trying to find inhibitors of thromboxane synthase. Imidazole and pyridine or imadazole derivatives seem to be the most effective. In addition to causing platelet aggregation, thromboxane A_2 induces smooth muscle contraction (vasoconstriction) and cell adhesion to the vessel wall. Thus, TxA_2 is synthesized by platelets when they bind to subendothelial collagen exposed by microinjuries. The newly synthesized thromboxane promotes subsequent adherence and aggregation of circulating platelets to the damaged vessel wall and constriction of the vascular smooth muscle. Usually this is part of a necessary repair process but, where vessel damage is chronic (or extensive), blood clots can result and cause arteriovascular crises such as strokes or heart attacks.

In 1976, Needleman and coworkers found an enol-ether (prostacyclin, PGI_2) that was produced in vascular endothelial cells and which would lead to coronary vasodilation. At the same time, Vane showed that prostacyclin had an antiaggregatory action on platelets. In fact, it is the most powerful

inhibitor of platelet aggregation known and concentrations of less than 1 ng ml^{-1} prevent arachidonate-induced aggregation in vitro. The physiological effects of prostacyclin are essentially the opposite to those of thromboxane (Table 2.14). Thus, for healthy blood vessels, production of prostacyclin by the vascular endothelial cells counteracts the effects of thromboxane on platelet aggregation, etc. Like thromboxane, PGI_2 has a short half-life and rearranges to a stable and physiologically inert compound, 6-keto-$PGF_{1\alpha}$ (Fig. 2.42). PGI_2 synthase is inactivated by a variety of lipid hydroperoxides and this explains why free radical scavengers (like vitamin E) serve to protect the enzyme. Vasodepressor substances such as histamine or bradykinin along with thrombin all stimulate PGI_2 synthesis in cultured endothelial cells and may serve physiologically to limit the area of platelet deposition about a site of vascular injury. In general, PGI_2 elevates cAMP levels in responsive cells (platelets, vascular smooth muscle cells) and activation of adenylate cyclase through PGI_2 binding to its membrane receptor may represent its mechanism of action.

In summary, the actions of thromboxanes and prostacyclins are antagonistic and their ratio is important for systemic blood pressure regulation and for the pathogenesis of thrombosis (Section 5.4.1).

2.4.4 The cyclooxygenase products exert a range of activities

The eicosanoids produced by the cyclooxygenase reaction exert a range of profound activities at concentrations down to 10^{-9} g·g^{-1} of tissue. These are now known to include effects on smooth muscle contraction, inhibition or stimulation of platelet stickiness, bronchoconstriction/dilation and vasoconstriction/dilation with a consequent influence on blood pressure. The effect on smooth muscle contraction was the earliest to be recognized and has been much used as a biological assay for eicosanoids. This use has been largely replaced by modern analytical methods such as gas chromatography–mass spectrometry or a combination of HPLC separations followed by radioimmunoassay.

The effects of prostaglandins, in particular on smooth muscle contraction, are utilized in both medicine and agriculture. PGE_2 is known to have a cytoprotective effect in the upper gut while PGI_2 (prostacyclin) is used in dialysis and the treatment of peripheral vascular disease. Continuous perfusion of very small amounts (e.g. 0.01 μg PGE_1 min^{-1}) into pregnant women causes uterine activity similar to that encountered in normal labour without any effect on blood pressure. Prostaglandins and synthetic analogues are now being used for induction of labour and for therapeutic abortion in both humans and domestic animals. The exact time of farrowing of piglets, for example, can be precisely controlled by prostaglandin treatment. For abortions in women, a solution of PGF_2 at a concentration of 5 μg ml^{-1} is administered by intravenous infusion at a rate of 5 μg min^{-1} until abortion is complete (about 5 h). The method appears, so far, to be safe with few side-effects and no further surgical intervention is required.

PGE is also an effective antagonizer of the effect of a number of hormones on free fatty acid release from adipose tissue (Section 3.6.3). It is likely that its mode of action, and that of thromboxanes and prostacyclin, in smooth muscle and platelets involves interaction with the adenyl cyclase

Table 2.14 Opposing effects of prostacyclin and thromboxanes on the cardiovascular systems

Thromboxanes produced in platelets	Physiological effect	Prostacyclin synthesized in arterial wall
Stimulates	Platelet aggregation	Inhibits
Constricts	Arterial wall	Relaxes
Lowers	Platelet cAMP levels	Raises
Raises	Blood pressure	Lowers

enzyme in the cell membrane, so that their physiological activities are largely expressed through regulation of cellular cAMP levels. The sequence of events is probably:

(1) release of arachidonic acid from a membrane phospholipid by the activation of phospholipase A (Section 2.4.9);
(2) the formation of the active compound via the cyclic endoperoxide;
(3) the reaction of the active molecules with specific receptor sites on the target cell membrane;
(4) the positive or negative regulation of adenyl cyclase.

In fat cells, PGE acts to reduce cAMP levels; in platelets prostacyclins tend to raise cAMP levels, while thromboxanes have the reverse effect, thereby initiating aggregation (Table 2.14). Ca^{2+} is required for the aggregation process and there is some evidence that thromboxane A_2 or other cyclooxygenase products can act as Ca^{2+} ionophores. The interactive effects of thromboxane A_2 and prostacyclin with regard to platelet cAMP levels are depicted in Figure 2.43.

In healthy blood vessels prostacyclin is produced, which counteracts the thromboxane effect on cAMP levels and, therefore, reduces platelet adhesion. In artificial or damaged vessels the thromboxane effect is unopposed and adhesion takes place.

Another area of prostaglandin action that has been studied in detail is their role in water retention by the kidney. The final event in urine production occurs in the terminal portion of the renal tubule where ADH (antidiuretic hormone) interacts with receptors on the tubule to cause movement of water back into the tissue. ADH does this by activating adenylate cyclase and increasing cellular cAMP levels. (cAMP itself has been shown to have a hydro-osmotic effect also.) It has been known for some time that low concentrations of PGE_2 inhibit the ADH-induced cAMP formation and, hence, the hydro-osmotic effect. The mechanism for this antagonism is unclear but it has been suggested that PGE_2 may prevent ADH activating the adenylate cyclase by modifying the properties of one of the GTP-binding proteins at the receptor.

2.4.5 Prostaglandins and other eicosanoids are rapidly catabolized

We have already mentioned that thromboxane A_2 and prostacyclin have very short half-lives in vivo. In addition, it was shown by Vane and Piper in the late 1960s that prostaglandins like PGE_2 or $PGF_{2\alpha}$ were rapidly catabolized and did not survive a

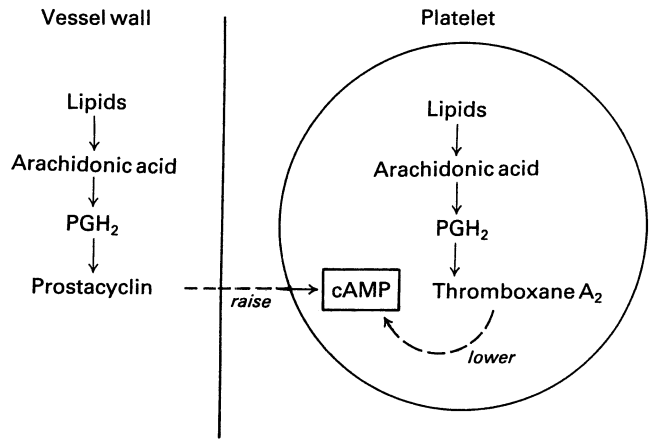


Fig. 2.43 Interaction of thromboxanes and prostacyclins in regulating platelet cAMP levels.

single pass through the circulation. The lung plays a major role in this inactivation process, which is usually initiated by oxidation of the hydroxyl group at C_{15}. The $\Delta 13$ double bond is next attacked and further degradation involves β- and ω-oxidation (Sections 2.3.1 and 2.3.3) in peroxisomes. Concentrations of major active prostaglandin products in blood are less than 10^{-10} M and because of their rapid catabolism they can only act as local hormones or *autocoids*, which modify biological events close to their sites of synthesis. Moreover, in contrast to typical circulating hormones, prostanoids are produced by practically every cell in the body. They exit the cell via carrier-mediated transport before being inactivated rapidly in the circulation.

2.4.6 Instead of cyclooxygenation, arachidonate can be lipoxygenated or epoxygenated

For over 40 years it has been known that plant tissues contain lipoxygenases, which catalyse the introduction of oxygen into polyunsaturated fatty acids (Section 2.3.6). In 1974 Hamberg and Samuelsson found that platelets contained a 12-lipoxygenase and since that time 5-, 8- and 15-lipoxygenases have also been discovered. The immediate products are hydroperoxy fatty acids, which for the arachidonate substrate are hydroperoxy eicostatetraenoic acids (HPETEs).

The HPETEs can undergo three reactions (Fig. 2.44). The hydroperoxy group can be reduced to an alcohol, thus forming a hydroxyeicosatetraenoic acid (HETE). Alternatively, a second lipoxygenation elsewhere on the chain yields a dihydroxyeicosatetraenoic acid (diHETE) or dehydration produces an epoxy fatty acid.

Epoxy fatty acids, such as leukotriene A_4 (Fig. 2.44) can undergo non-enzymic reactions to various diHETEs, can be specifically hydrated to a given diHETE or can undergo ring opening with GSH to yield peptide derivatives. Epoxy eicosatrienoic acids and their metabolic products are called leukotrienes – the name being derived from the cells (leukocytes) in which they were originally recognized.

As mentioned above, four lipoxygenases (5-, 8-, 12- and 15-) have been found in mammalian tissues. The 5-lipoxygenase is responsible for leukotriene production and is important in neutrophils, eosinophils, monocytes, mast cells and keratinocytes as well as lung, spleen, brain and heart. Products of 12-lipoxygenase activity also have biological activity, e.g. 12-HPETE inhibits collagen-induced platelet aggregation and 12-HETE can cause migration of smooth muscle cells in vitro at concentrations as low as one femtomolar (10^{-15} M). In contrast, few biological activities have been reported for 15-HETE, although this compound has a potentially important action in inhibiting the 5- and 12-lipoxygenases of various tissues. The physiological role of the 8-lipoxygenase, which has only recently been discovered, is also obscure.

Arachidonic acid and other oxygenated derivatives of the arachidonate cascade can also be metabolized by cytochrome P450-mediated pathways of which the most important in animals appears to be the epoxygenase pathway.

The opening of the epoxy group of leukotriene LTA_4 by the action of glutathione S-transferase attaches the glutathionyl residue to the 6 position (Fig. 2.44). Unlike most glutathione S-transferases, which are soluble, the LTC_4 synthase is a microsomal protein with a high preference for its LTA_4 substrate. The product (LTC_4) can then lose a γ-glutamyl residue to give LTD_4 and the glycyl group is released in a further reaction to give LTE_4 (Fig. 2.45). Prior to their structural elucidation, leukotrienes were recognized in perfusates of lungs as slow-reacting substances (SRS) after stimulation with cobra venom or as slow-reacting substances of anaphylaxis (SRS-A) after immunological challenge. LTC_4 and LTD_4 are now known to be major components of SRS-A.

2.4.7 Control of leukotriene formation

Unlike the cylcooxygenase products, the formation of leukotrienes is not solely determined by the availability of free arachidonic acid in cells (Section 2.4.9). Thus for example, 5-lipoxygenase requires activation in most tissues and various immunological or inflammatory stimuli are able to cause this.

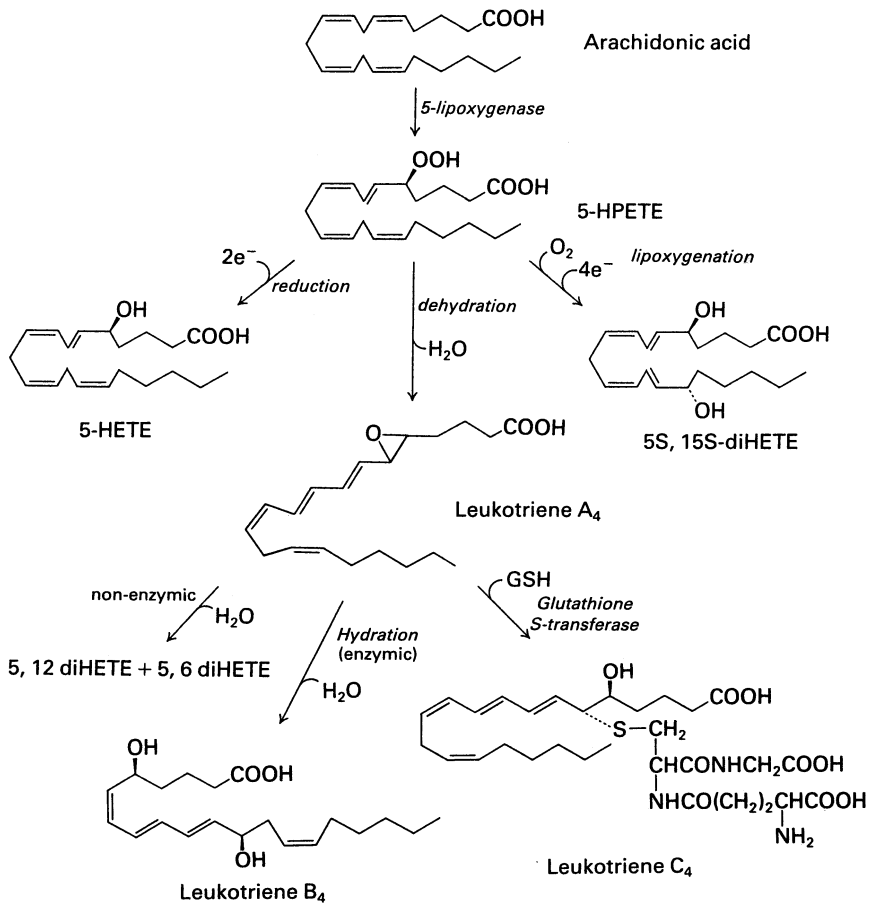


Fig. 2.44 Formation of leukotrienes from arachidonic acid.

In some cells both cyclooxygenase and lipoxygenase products are formed from arachidonic acid. It is possible that the arachidonic acid substrate comes from separate pools. For example, macrophages release prostaglandins but not leukotrienes when treated with soluble stimuli. In contrast, when insoluble phagocytic stimuli (such as bacteria) are used, both prostaglandin and leukotriene formation is increased.

We have already mentioned that 15-HETE, a 15-lipoxygenase product, can inhibit 5- and 12-lipoxygenases. Conversely, 12-HPETE has been shown to increase 5-HETE and LTB_4 formation. Thus, the various lipoxygenase pathways have interacting effects as well as the interdependent actions of cyclooxygenase and lipoxygenase products.

Genes for various animal lipoxygenases have been isolated and their primary sequences compared. Various *cis*-acting elements have been found in the 5′-flanking regions and all the genes contain multiple GC boxes but no typical TATA boxes in their promoter regions. They can, therefore, be considered as housekeeping genes. Apart from various putative transcriptional regulatory elements, the lipoxygenases themselves can be subject to activation. For the 5-lipoxygenase, ATP, Ca^{2+} and various leukocyte stimulatory factors have been demonstrated. Moreover, in the absence of phos-

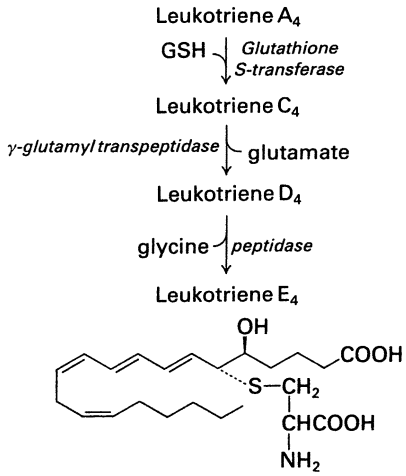


Fig. 2.45 Formation of peptidoleukotrienes.

pholipid the purified enzyme has poor activity suggesting that it may need to act at a membrane interface.

Whereas, the key event for prostaglandin production is the release of substrate-free fatty acid by phospholipase A_2 (Section 2.4.9), leukotriene synthesis only occurs in intact cells following exposure to certain stimuli of which the most important is a rise in intracellular calcium. Moreover, activation of 5-lipoxygenase seems to involve translocation of the enzyme from the cytosol to membrane surfaces, where it becomes active, makes leukotrienes and undergoes suicide inactivation.

Studies with a highly potent inhibitor of leukotriene biosynthesis, MK-886, in intact cells led to the discovery of 5-lipoxygenase activating protein (FLAP). Although the details of FLAP's function are still being elucidated it seems most likely that the protein makes the 5-lipoxygenase reaction much more efficient by containing a binding site for arachidonic acid and facilitating transfer of the substrate to the enzyme. A possible model for FLAP-dependent synthesis and release of LTC_4 is shown in Fig. 2.46.

Because both FLAP and 5-lipoxygenase are needed for leukotriene synthesis it might be expected that their genes would be regulated together. Indeed, this has been shown to be the case in some cells. However, the mechanism of regulation is distinctly different with the FLAP gene containing a TATA box and other regulatory motives not found on the 5-lipoxygenase gene.

Because of the important role of FLAP in 5-lipoxygenase activity and, hence, leukotriene biosynthesis, pharmaceuticals that bind to FLAP have been developed in addition to those that inhibit the catalytic reaction. Many of the latter are redox-active and, although very effective 5-lipoxygenase inhibitors, they often show unwanted side-effects. Two separate classes of drugs, indoles (like compound HK-886) and quinolines, bind to FLAP and several members have been developed and tested for medical use.

2.4.8 Physiological action of leukotrienes

Leukotrienes have potent biological activity. A summary of some of their more important actions is provided in Table 2.15. The peptidoleukotrienes contract respiratory, vascular and intestinal smooth muscles. In general LTC_4 and LTD_4 are more potent than LTE_4. By contrast, LTB_4 is a chemotactic agent for neutrophils and eosinophils. Although it can cause plasma exudation by increasing vascular permeability, it is less potent than the peptidoleukotrienes. These actions of the leukotrienes have implications for asthma, immediate hypersensitivity reactions, inflammatory reactions and myocardial infarction.

The action of leukotrienes at the molecular level first of all involves binding to specific high-affinity receptors. Receptors for LTB_4, LTC_4, D_4 and E_4 have been characterized. The detailed mechanism has been studied best in neutrophils where binding of LTB_4 involves a G-protein sensitive to pertussis toxin. An inositol lipid-specific phospholipase C (Sections 7.2.4 and 7.9) is then activated and intracellular Ca^{2+} is elevated both by increased influx and by release from stores.

The function of the 12- and 15-lipoxygenases is much less clear than that of the 5-lipoxygenase. Nevertheless, it is thought that the 15-lipoxygenase products (15-HETE) may play a role in endocrine (e.g. testosterone) secretion. A build-up of 12-HETE

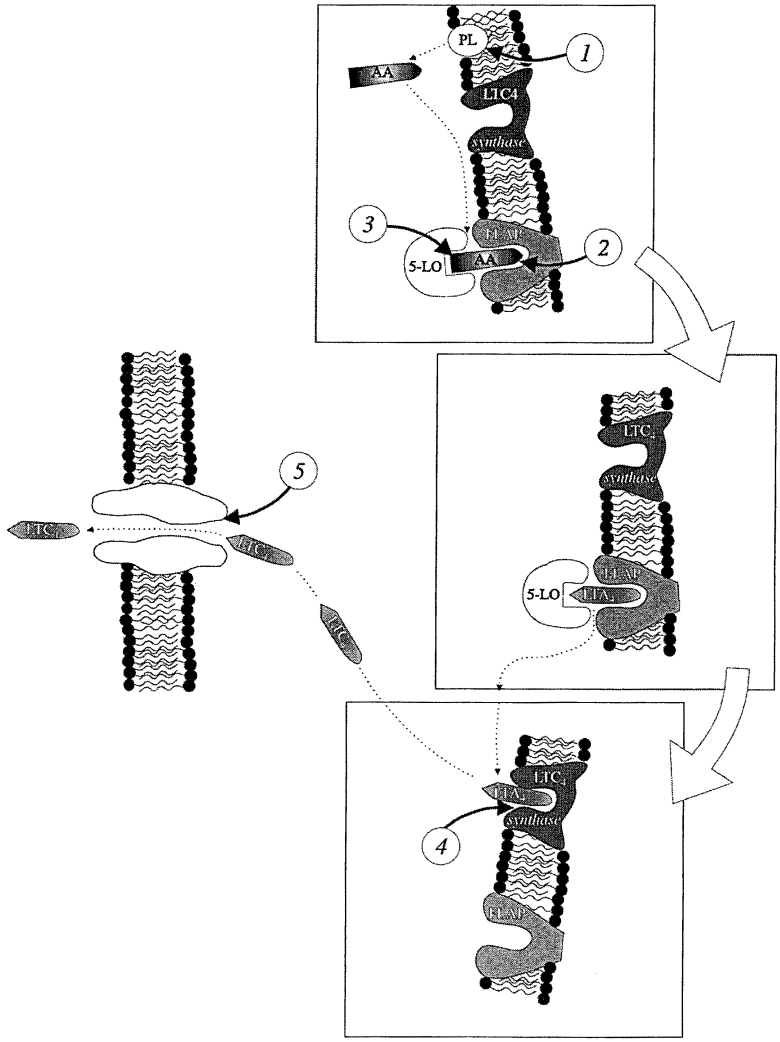


Fig. 2.46 A model for the synthesis and release of leukotriene LTC_4. FLAP and LTC_4 synthase are shown as integral nuclear membrane proteins, with a phospholipase (PL) associating with this membrane to release AA①. Following the release of AA, 5-LOX (5-LO) translocates to the nuclear membrane③ in a process regulated by FLAP②. 5-LOX then converts AA into LTA_4, which is subsequently converted into LTC_4 by LTC_4 synthase④. LTC_4 is exported from the cell by a membrane carrier⑤. Points at which this process can be inhibited are indicated (1–5; blockade of PL, FLAP, 5-LOX, LTC_4 synthase and the carrier protein, respectively). Reproduced from Vickers (1998) with kind permission of the author and Portland Press.

(46 × normal) has been reported in lesions of patients suffering from the skin disease psoriasis. This compound is know to be a chemoattractant and topical application of 12-HETE causes erythema (similar to sunburn). Interestingly, one of the most effective anti-erythema treatments is etretinate, which inhibits the 12-lipoxygenase. Etretinate has been used for psoriatic patients, but it is particularly effective for anti-sunburn treatment.

Table 2.15 Biological effects of leukotrienes

Effect	Comments
Respiratory	Peptidoleukotrienes cause constriction of bronchi especially smaller airways and increase mucus secretion
Microvascular	Peptidoleukotrienes cause arteriolar constriction, venous dilation, plasma exudation
Leukocytes	LTB_4 chemotactic agent for neutrophils, eosinophils, e.g. increase degranulation of platelets, cell-surface receptors and adherence of polymorphonucleocytes to receptor cells
Gastrointestinal	Peptidoleukotrienes cause contraction of smooth muscle (LTB_4 no effect)

2.4.9 For eicosanoid synthesis an unesterified fatty acid is needed

A necessary prerequisite for eicosanoid formation is the availability of an appropriate unesterified fatty acid. In animals, this is most commonly arachidonic acid, which is the dominant polyunsaturated fatty acid in most membrane phospholipids. The concentration of unesterified arachidonic acid in cells is well below the K_m for prostaglandin H synthetase. Thus, the first stage for eicosanoid formation will normally be an activation of the release of arachidonate from position 2 of phosphoglycerides that contain it. Two classes of phospholipids are thought to play major roles as sources of arachidonate in cells: phosphatidylcholine (the major membrane constituent) and the phosphoinositides (by virtue of the high enrichment of arachidonate at position 2). Thus, hydrolysis of phosphoinositides not only produces two second messengers directly (Section 7.9) but may also initiate an arachidonate cascade. Other potential sources of arachidonic acid are the plasmalogens (Section 6.2.5) that also have a high enrichment at the *sn*-2 (acyl) position. Because plasmalogens are poor substrates for phospholipase A_2, they are hydrolysed by a plasmalogenase first. Thus, release of arachidonic acid from plasmalogens could be controlled independently from that for diacylphosphoglycerides.

Two main types of stimuli increase arachidonate release. These can be called physiological (specific) and pathological (non-specific). Physiological stimuli, such as adrenaline, angiotensin II and certain antibody–antigen complexes, cause the selective release of arachidonic acid. In contrast, pathological stimuli, such as mellitin or tumour promoters like phorbol esters, have generalized effects on cellular membranes and promote release of all fatty acids from position 2 of phosphoglycerides.

Of the various cellular lipases stimulated by hormones or other effectors and which, in theory, could give rise to arachidonate hydrolysis, cytosolic phospholipase A_2 and the non-pancreatic secretory phospholipase A_2 are the most important. Cytosolic phospholipase A_2 is stimulated by Ca^{2+} and hormonal-induced mobilization of Ca^{2+} leads to movement of the enzyme from the cytosol to the ER and nuclear envelope. It is relatively specific for arachidonate and is stimulated by phosphorylation.

Secretory phospholipase A_2 is also stimulated by Ca^{2+}, but at the higher concentrations found outside the cell. It is relatively non-specific towards different phospholipids and towards the fatty acid at the *sn*-2 position. Its involvement in prostaglandin synthesis has been shown in endothelial cells by the use of antibodies that prevent it binding to the cell surface. It has been suggested that cytosolic phospholipase A_2 produces the initial burst of prostaglandin synthesis whereas the secretory enzyme is involved in late-phase prostaglandin formation after cells have been stimulated further by cytokines, inflammatory mediators or growth factors.

It is also of interest that phospholipase A_2, the activity of which is needed to initiate eicosanoid production, is also needed to produce another type of biologically active lipid: platelet activating factor (Section 7.1.10).

2.4.10 Essential fatty acid activity is related to double bond structure and to the ability of such acids to be converted into a physiologically active eicosanoid

Work from the Dutch school and from Holman's laboratory at the Hormel Institute, Minnesota, originally showed that only those fatty acids (including new synthetic odd-numbered acids) that act as precursors for biologically active eicosanoids have essential fatty acid (EFA) activity. This and other results superseded the old dogma that essential fatty acids all had the *n*-6, *n*-9 double bond system. Indeed, some synthetic acids without this structure had EFA activity (Table 2.16). Van Dorp and his colleagues at the Unilever Laboratories at Vlaardingen have made the postulate that only those fatty acids capable of being converted into the Δ5,8,11,14-tetraenoic fatty acids of chain lengths 19C, 20C and 22C will show EFA activity because only these tetraenoic acids can give rise to physiologically active eicosanoids. However, it is now known that columbinic acid (*trans*-5, *cis*-9, *cis*-12-18:3), when given to EFA-deficient rats, normalizes growth and cures the dermatitis yet it is unable to form an eicosanoid.

Moreover, although there is a correlation between EFA activity and the potential to be converted to eicosanoids, one cannot cure EFA deficiency by infusion of eicosanoids because they are rapidly destroyed and because different cells produce their own special pattern of eicosanoids. However, one of the first organs to show EFA deficiency is the skin, whose water permeability is very much increased in this condition. Topical application of EFA to skin can reverse the deficiency symptoms and there is now evidence that topical application of prostaglandins can also be very effective. (See Sections 4.2.3.4 and 6.6.6 for other comments about lipids and skin diseases.)

We are still a long way from being able to account for the fate of all the EFA that enter the body and the fate of the eicosanoids that may be formed from them. Eicosanoids are metabolized very rapidly and their metabolites excreted in the urine or bile. The detection, isolation and analysis of such metabolites is, therefore, one approach to studying the daily eicosanoid production. In this way it has been estimated that 1 mg of prostaglandin metabolites is formed in 24 h in man – considerably less than the 10 g of EFA, which are thought to be necessary daily. However, the demonstration that EFA like linoleic acid may have additional functions (Sec-

Table 2.16 Relationship between fatty acid structure and EFA activity

	Position of double bonds		
Fatty acid chain length	From carboxyl end (Δ)	From methyl end (ω)	EFA potency (unit g^{-1})
18:2	9, 12	6, 9	100
18:3	6, 9, 12	6, 9, 12	115
18:3	8, 11, 14	4, 7, 10	0[a]
18:4	6, 9, 12, 15	3, 6, 9, 12	34
18:4	5, 8, 11, 14	4, 7, 10, 13	0[a]
19:2	10, 13	6, 9	9
20:2	11, 14	6, 9	46
20:3	8, 11, 14	6, 9, 12	100
20:3	7, 10, 13	7, 10, 13	0[a]
20:4	5, 8, 11, 14	6, 9, 12, 15	139
21:3	8, 11, 14	7, 10, 13	56
22:3	8, 11, 14	8, 11, 14	0[a]
22:5	4, 7, 10, 13, 16	6, 9, 12, 15, 18	139

[a] Cannot give rise to any prostaglandins.

tions 4.2.3 and 6.6.6) apart from being eicosanoid precursors may account for this apparent discrepancy and raises the possibility that new roles for EFAs may be discovered in the future.

2.5 SUMMARY

Many hundreds of different fatty acid structures have been found in Nature but only a few such compounds occur commonly. These are usually even chain fatty acids of 16–22 carbons in length. The most common saturated fatty acids are palmitic and stearic with oleic acid the most prevalent monounsaturated molecule. When polyunsaturated fatty acids occur, their double bonds are almost invariably separated by three carbons (i.e. methylene interrupted). Linoleic and α-linolenic acids are found in plants, arachidonic acid is common in animals while marine organisms contain significant amounts of 20C or 22C polyunsaturates.

Fatty acids are made initially by a combination of the action of acetyl-CoA carboxylase and fatty acid synthase. The acetyl-CoA carboxylase enzymes from different organisms vary in their structure with multifunctional proteins being found in mammals while *E. coli* uses a multienzyme complex. Most plants have both types of acetyl-CoA carboxylase, present in isoforms in different parts of their cells, but grasses have two multifunctional protein isoforms. The mechanism of acetyl-CoA carboxylase is, however, broadly similar and takes place in two main stages. First, biotin is carboxylated by an ATP-dependent reaction. Second, the carboxyl group is transferred to acetyl-CoA thus producing malonyl-CoA.

Acetyl-CoA carboxylase can be regarded as the first committed step of fatty acid synthesis. It is not surprising, therefore, that the enzyme is under careful regulation. The control mechanisms have been studied best in mammals where allosteric regulation takes place through several mechanisms including phosphorylation. Long-term regulation by diet involves changes in the amount of carboxylase protein. Different regulatory mechanisms are used by *E. coli* and by higher plants.

Fatty acid synthases are divided into Types I, II and III. Type I enzymes are multifunctional proteins containing covalently bound acyl carrier protein (ACP) and release products as unesterified fatty acids (mammals) or transfer them to CoA (yeast). In animals the fatty acid synthase is a homo dimer with each chain containing all of the partial reactions. By contrast, yeast fatty acid synthase contains two dissimilar peptides and operates as an $\alpha_6\beta_6$ complex.

Type II fatty acid synthases are found in bacteria such as *E. coli*, cyanobacteria and the chloroplasts of algae, higher plants and *Euglena*. They are multienzyme complexes that comprise a number of dissociable proteins responsible for acyl transfer, condensation, dehydration and reduction reactions. ACP can also be isolated as a small molecular mass acidic protein. In *E. coli* the fatty acid synthase has the additional feature that it produces monounsaturated fatty acids (mainly *cis*-vaccenate) in addition to long-chain saturated products.

Type III synthases are responsible for the chain lengthening of preformed fatty acids and are also called elongase enzymes. Most fatty acid elongases are membrane-bound enzyme complexes located on the endoplasmic reticulum. Like the Type I and Type II fatty acid synthases they use malonyl-CoA as the source of 2C addition units. However, some elongation activity occurs in mitochondria where acetyl-CoA is used for what is, essentially, a reversal of β-oxidation.

Once fatty acids have been made *de novo* they can either be modified by elongation (above), by desaturation or by other reactions. Desaturation usually takes place by an aerobic mechanism – an exception being in the *E. coli* fatty acid synthase. Aerobic desaturases differ from each other by the nature of the acyl substrate they use, the type of reduced cofactor and the position at which the double bond is introduced into the acyl chain. Particularly notable are the desaturases that produce the polyunsaturated linoleic and α-linolenic acids. These enzymes use complex lipid substrates rather than acyl-thioesters.

Like acetyl-CoA carboxylase, the different fatty acid synthases and desaturases are under careful metabolic regulation. Various mechanisms are used to control their activity, depending on the organism.

Fatty acids are oxidized by chemical or enzymic mechanisms. Chemical oxidation is particularly important for polyunsaturated fatty acids and gives rise to the formation of free radicals which can, in turn, damage tissues. Similar peroxidation reactions can also be catalysed by lipoxygenase enzymes.

Oxidation of saturated and unsaturated fatty acids can take place by α-, β- or ω-oxidations. α-Oxidation is common in plants and brain tissue and causes the release of CO_2 and formation of a new fatty acid, one carbon shorter. The more important β-oxidation mechanism causes the release of acetyl-CoA. The process requires a cycle of four reactions (oxidation, hydration, oxidation, thiolysis) and is localized in mitochondria and microbodies in eukaryotes, the proportional distribution varying with the organism. β-Oxidation is the most important process by which energy is released from fat stores in animals and plants.

Polyunsaturated fatty acids can be converted, via various oxidations, into biologically active metabolites. In plants, lipoxygenase activity gives rise to the lipoxygenase pathway, which generates many important food flavours and aromas. In addition, products like jasmonic acid are very important signalling and plant defence molecules.

In animals, eicosanoids (20C and 22C biologically active compounds) are derived from various polyunsaturated fatty acids, particularly arachidonate. The latter can be converted into a cyclic endoperoxide and this, in turn, can form prostaglandins, thromboxanes or prostacyclins. In contrast, if arachidonate is acted upon by a lipoxygenase then the leukotrienes or hydroxy fatty acids can be formed. A third series of compounds is produced via cytochrome P450-mediated oxidase reactions. All these eicosanoids have very potent, and often interacting, biological actions and affect almost every tissue in mammals.

FURTHER READING

General

Gunstone, F.D., Harwood, J.L. & Padley, F.B., eds. (1994) *The Lipid Handbook*, 2nd edn. Chapman and Hall, London.

Harwood, J.L., ed. (1998) *Plant Lipid Biosynthesis*. Cambridge University Press, Cambridge. [Contains chapters specific to topics on Fatty Acid Synthesis.]

Ratledge, C. & Wilkinson, S.G., eds. (1989) *Microbial Lipids*, 2 Vols. Academic Press, London.

Vance, D.E. & Vance, J.E., eds. (1996) *Biochemistry of Lipids, Lipoproteins and Membranes*. Elsevier, Amsterdam. [Contains chapters specific to topics in the sections on Fatty Acid Synthesis, Fatty Acid Breakdown and Eicosanoids.]

Fatty Acid Synthesis

Bernlohr, D.A., Simpson, M.A., Hertzel, A.V. & Banaszaz, L.J. (1997) Intracellular lipid-binding proteins and their genes. *Annual Review of Nutrition*, **17**, 277–303.

Browse, J. & Somerville, C.R. (1991) Glycerolipid metabolism, biochemistry and regulation. *Annual Reviews of Plant Physiology and Plant Molecular Biology*, **42**, 467–506.

Cahoon, E., Lindqvist, Y., Schneider, G. & Shanklin, J. (1997) Redesign of soluble fatty acid desaturases from plants for altered substrate specificity and double bond position. *Proceedings of the National Academy of Sciences, U.S.A.*, **94**, 4872–4877.

Cinti, D.L., Cook, L., Nagi, M.N. & Suneja, S.K. (1992) The fatty acid elongation system of mammalian endoplasmic reticulum. *Progress in Lipid Research*, **31**, 1–51.

Fell, D. (1997) *Understanding the Control of Metabolism*. Portland Press, London.

Frohnest, B.I. & Bernlohr, D.A. (2000) Regulation of fatty acid transporters in mammalian cells. *Progress in Lipid Research*, **39**, 83–107.

Girard, J., Farré, P. & Foufelle, F. (1997) Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes. *Annual Review of Nutrition*, **17**, 325–352.

Glatz, J.F.C. & van der Vusse, G.J. (1996) Cellular fatty acid-binding proteins: their function and physiological significance. *Progress in Lipid Research*, **35**, 243–282.

Harwood, J.L. (1996) Recent advances in the biosynthesis of plant fatty acids. *Biochimica et Biophysica Acta*, **1301**, 7–56.

Joshi, A.K. & Smith, S. (1993) Construction, expression and characterisation of a mutated animal fatty acid synthase deficient in the dehydrase function. *Journal of Biological Chemistry*, **268**, 22508–22513.

Joshi, A.K., Witkowski, A. & Smith, S. (1998) The malonyl/acetyl transferase and β-ketoacyl synthase domains of the animal fatty acid synthase can cooperate with the acyl carrier protein domain of either subunit. *Biochemistry*, **37**, 2515–2523.

Kim, K.-H. (1997) Regulation of mammalian acetyl-CoA carboxylase. *Annual Review of Nutrition*, **17**, 77–99.

Knowles, J.R. (1989) The mechanism of biotin-containing enzymes. *Annual Review of Biochemistry*, **58**, 195–221.

Ntambi, J.M. (1995) Regulation of stearoyl-CoA desaturase. *Progress in Lipid Research*, **34**, 139–150.
Ohlrogge, J.B. (1999) Plant metabolic engineering: are we ready for phase two? *Current Plant Biology*, **2**, 121–122.
Ohlrogge, J.B. & Jaworski, J.G. (1997) Regulation of fatty acid synthesis. *Annual Review of Plant Physiology and Plant Molecular Biology*, **48**, 109–136.
Semenkovich, C.F. (1997) Regulation of fatty acid synthase. *Progress in Lipid Research*, **36**, 43–54.
Smith, S. (1994) The animal fatty acid synthase: one gene, one polypeptide, seven enzymes. *FASEB Journal*, **8**, 1248–1259.
Tocher, D.R., Leaver, M.J. & Hodgson, P.A. (1998) Recent advances in the biochemistry and molecular biology of fatty acid desaturases. *Progress in Lipid Research*, **37**, 73–118.
Voelker, T.A., Worrelle, A.C., Anderson, L., Bleibaum, J., Fan, C., Hawkins, D.J., Radke, S.E. & Davies, H.M. (1992) Fatty acid biosynthesis redirected to medium chains in transgenic oilseed plants. *Science*, **257**, 72–73.
Watkins, P.A. (1997) Fatty acid activation. *Progress in Lipid Research*, **36**, 55–83.
Zammit, V.A. (1999) The malonyl-CoA–long chain acyl-CoA axis in the maintenance of mammalian cell function. *Biochemical Journal*, **343**, 505–515. [See also Zammit (1999) under Fatty Acid Breakdown.]

See also specific chapters in multiauthor volumes in the *General* section above.

Fatty Acid Breakdown

Eaton, S., Bartlett, K. & Pourfarzam, M. (1996) Mammalian mitochondrial β-oxidation. *Biochemical Journal*, **320**, 345–357.
Gerhardt, B. (1992) Fatty acid degradation in plants. *Progress in Lipid Research*, **31**, 417–446.
Kunau, W.-H., Dommes, V. & Schulz, H. (1995) β-Oxidation of fatty acids in mitochondria, peroxisomes and bacteria: a century of continued progress. *Progress in Lipid Research*, **34**, 267–342.
McGarry, J.D. (1995) The mitochondrial carnitine palmitoyltransferase system: its broadening role in fuel homeostasis and new insights into its molecular features. *Biochemical Society Transactions*, **23**, 321–324.
Tanaka, K. & Coates, P.M., eds. (1990) *Fatty Acid Oxidation: Chemical, Biochemical and Molecular Aspects*. Liss, New York.
Zammit, V.A. (1999) Carnitine acyltransferase: functional significance of subcellular distributions and membrane topology. *Progress in Lipid Research*, **38**, 199–224. [See also Zammit (1999) under Fatty Acid Synthesis.]

See also specific chapters in multiauthor volumes in the *General* section above.

Eicosanoids and Related Compounds

Blée, E. (1998) Phytooxylipins and plant defense reactions. *Progress in Lipid Research*, **37**, 33–72.
Diczfalusy, U. (1994) β-Oxidation of eicosanoids. *Progress in Lipid Research*, **33**, 403–428.
Gaffney, B.J. (1996) Lipoxygenase: structural principles and spectroscopy. *Annual Review of Biochemistry*, **25**, 431–459.
Grechkin, A. (1998) Recent developments in biochemistry of the plant lipoxygenase pathway. *Progress in Lipid Research*, **37**, 317–352.
Hammerstrom, S. (1983) Leukotrienes. *Annual Review of Biochemistry*, **52**, 355–377.
Marnett, L.J. & Kalgutkar, A.S. (1999) Cyclooxygenase 2 inhibitors: discovery, selectivity and the future. *Trends in Pharmaceutical Science*, **20**, 465–469.
Oliw, E.H. (1994) Oxygenation of polyunsaturated fatty acids by cytochrome P450 monooxygenases. *Progress in Lipid Research*, **33**, 329–354.
Piazza, G., ed. (1996) *Lipoxygenase and Lipoxygenase Pathway Enzymes*. American Oil Chemists Society, Champaign, IL.
Rowley, A.F., Kuhn, H. & Schewe, T., eds. (1998) *Eicosanoids and Related Compounds in Plants and Animals*. Portland Press, London.
Ryan, C.A., Lamb, C.J., Jagendorf, A.T. & Kolattukudy, P.E. (1995) Self-defense by plants: induction and signalling pathways. Colloquium papers. *Proceedings of the National Academy of Science, U.S.A.*, **92**, 4075–4205.
Sugimoto, Y., Narumiya, S. & Ichikawa, A. (2000) Distribution and function of prostanoid receptors: studies from knockout mice. *Progress in Lipid Research*, **39**, 289–314.
Vane, J.R., Bakhle, Y.S. & Botting, R.M. (1998) Cyclooxygenases 1 and 2. *Annual Review of Pharmacology & Toxicology*, **38**, 97–120.
Yamamoto, S. & Smith, W.L., eds. (1995) *Molecular Biology of the Arachidonate Cascade*. Elsevier, Amsterdam.
Yamamoto, S., Suzuki, H. & Ueda, N. (1997) Arachidonate 12-lipoxygenases. *Progress in Lipid Research*, **36**, 23–42.

See also specific chapters in multiauthor volumes in the *General* section above.

3 Lipids as energy stores

3.1 INTRODUCTION

Many plants and animals need to store energy for use at a later time. Lipid fuel stores are mainly triacylglycerols or wax esters.

All living organisms require a form of energy to sustain life. Whereas the basic mechanisms for powering the life-sustaining anabolic chemical reactions through the high-energy bonds of ATP and similar molecules are common to animals and plants, the primary sources of energy are very different. Plants use sunlight as the primary energy source to enable them to synthesize carbohydrates. They are then able to synthesize fatty acids from the degradation products of carbohydrates as described in Section 2.2. Animals do not have the facility to use sunlight directly, but must take in their fuel in the diet as lipid or carbohydrate from plants or from other animals.

Energy needs are of two main types. One is a requirement to maintain, throughout life, on a minute-to-minute basis, the organism's essential biochemistry. Either carbohydrates or fatty acids can fulfil this function, although tissues do vary in their ability to use different fuels: the mammalian brain, for example, is normally reliant on glucose as a fuel source, since it cannot use fatty acids directly. (However, during prolonged starvation, the brain can make use of metabolites of fatty acids called 'ketone bodies'. These were described in detail in Section 2.3.1.6.) The second type of energy requirement is for a more intermittent and specialized store of fuel that can be mobilized when required. Thus, animals may need to use such reserves after a period of starvation or hibernation or for the energy-demanding processes of pregnancy and lactation, while plants need energy for seed germination. Animals and plants each make use of different types of fuels that may be stored in different tissues. Thus, practically all animals use lipids as a long-term form of stored energy and almost all use triacylglycerols as the preferred lipid. Some marine animals, however, use wax esters as their energy store. Most animals store their energy in a specialized tissue, the adipose tissue, but some fish use their flesh or the liver as a lipid store. In general, plants store the fuel required for germination in their seeds either as lipid or polysaccharide. Oil-bearing seeds usually contain triacylglycerols, but wax esters are used as fuel by some desert plants, such as jojoba.

This chapter covers storage lipids in general, but is mainly about triacylglycerols because of their predominance as a fuel and their general importance in biochemistry.

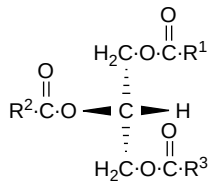
3.2 THE NAMING AND STRUCTURE OF THE ACYLGLYCEROLS (GLYCERIDES)

Acylglycerols are esters of the trihydric alcohol called glycerol, in which one, two or three hydroxyls may be esterified with fatty acids.

3.2.1 Triacylglycerols are the major components of natural fats and oils; partial acylglycerols are usually intermediates in the breakdown or synthesis of triacylglycerols

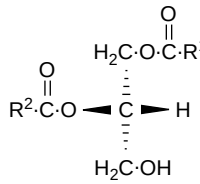
The preferred term for esters of glycerol (a trihydric alcohol) with fatty acids is now acylglycerols, although you will certainly come across the synonym glycerides in other literature. Triacylglycerol, diacylglycerol and monoacylglycerol (Fig. 3.1) will be used here for the specific compounds in which

Triacylglycerols

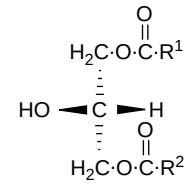


(a) 1, 2, 3–Triacyl-*sn*-glycerols

Diacylglycerols

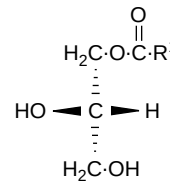


(b) 1, 2–Diacyl-*sn*-glycerols

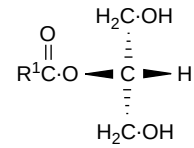


(c) 1, 3–Diacyl-*sn*-glycerols

Monoacylglycerols

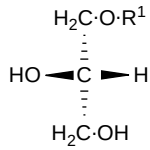


(d) 1–Monoacyl-*sn*-glycerols



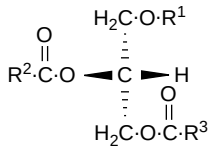
(e) 2–Monoacyl-*sn*-glycerols

Alkylglycerols

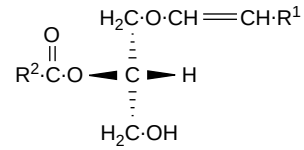


(f) 1–Alkyl-*sn*-glycerols

Alkylacyl- and Alkenylacyl-glycerols



(g) 1–Alkyl, 2, 3-diacyl-*sn*-glycerols



(h) 1–Alkenyl-2-acyl-*sn*-glycerols

Fig. 3.1 Structures of acylglycerols and alkylglycerols. To emphasize the stereochemistry of the central carbon atom, imagine that this carbon is in the plane of the paper. The groups linked by dotted lines are to be thought of as behind, and those linked by solid lines in front of the plane of the paper. R represents a long hydrocarbon chain. Examples (a)–(e) illustrate structure of acylglycerols. In (f)–(h), one of the groups has an alkyl (or 'ether') linkage. In example (f), when R^1 is derived from long-chain alcohols corresponding to 16:0, 18:0 and 18:1, the glyceryl monoethers are named chimyl, batyl and selachyl alcohols, respectively. In example (h) the group containing a *cis* double bond, $-O-CH{=}CHR^1$ is sometimes called a 'vinyl ether' and is also found in the class of phospholipids called plasmalogens (see Chapter 7).

three, two or one of the glycerol hydroxy groups are esterified, rather than the older triglyceride, diglyceride and monoglyceride.

Triacylglycerols (TAG) are the chief constituents of natural fats (solids) and oils (liquids), names that are often used synonymously for them, although it is important to remember that natural fats and oils also contain minor proportions of other lipids, for example steroids and carotenoids. The most abundant fatty acids in natural acylglycerols are palmitic, stearic, oleic and linoleic; plant acylglycerols usually have a relatively higher proportion of the more unsaturated fatty acids. Coconut and palm oils are exceptions in having a predominance of medium chain (8–12C) saturated fatty acids (Section 4.1.2). Milk fats have a much higher proportion, depending on species, of short-chain (2–6C) and medium-chain (8–10C) fatty acids than other animal fats (Section 4.1.2). Odd-chain or branched-chain fatty acids are only minor constituents of acylglycerols. Some seed oils contain a variety of unusual fatty acids with oxygen-containing groups and ring systems (Section 2.1). Some acylglycerols that contain ricinoleic acid (Sections 2.1.5 and 2.2.4) may have further fatty acids esterified with the hydroxy group of the ricinoleic acid. Thus, tetra-, penta-, and hexa-acid acylglycerols occur in some plant oils.

Alkylglycerols contain hydrocarbon chains linked to glycerol by an ether rather than an ester linkage. Chimyl, batyl and selachyl alcohols are glyceryl monoethers derived from long-chain alcohols corresponding to 16:0, 18:0 and 18:1, respectively [Fig. 3.1(f)]. These are the major naturally occurring glyceryl ethers and may be esterified in addition with one or two fatty acids [Fig. 3.1(g) and (h)]. Usually both alkyl and alkenyl ethers occur together in varying proportions. They are abundant in the liver oils of fish such as sharks and in the central nervous system. Only very small quantities of ether glycerides are found in plants.

3.2.2 All natural oils are complex mixtures of molecular species

Natural acylglycerols contain a vast range of different fatty acids. Even if the number of fatty acids was restricted to three, it would be possible to have 18 different arrangements of these fatty acids within the TAG molecule. There are, however, many hundreds of fatty acids and therefore the potential number of possible molecular species of TAG is enormous.

The pioneering work that provided the solid basis for a knowledge of natural TAG structure was that of Hilditch and his school beginning in 1927. They had to rely on the painstaking use of fractional crystallization, low-pressure fractional distillation and countercurrent distribution. At that time they assumed that fatty acids were randomly distributed among the three positions of TAG. Modern procedures of lipase hydrolysis and separations by different types of chromatography (see Section 1.8 and Box 3.1) have revealed that the fatty acids are not normally distributed in a random fashion. In most TAG, there is a stereo-specific arrangement of fatty acids at the three positions. Box 3.1 illustrates how such a stereo-specific structure may be determined.

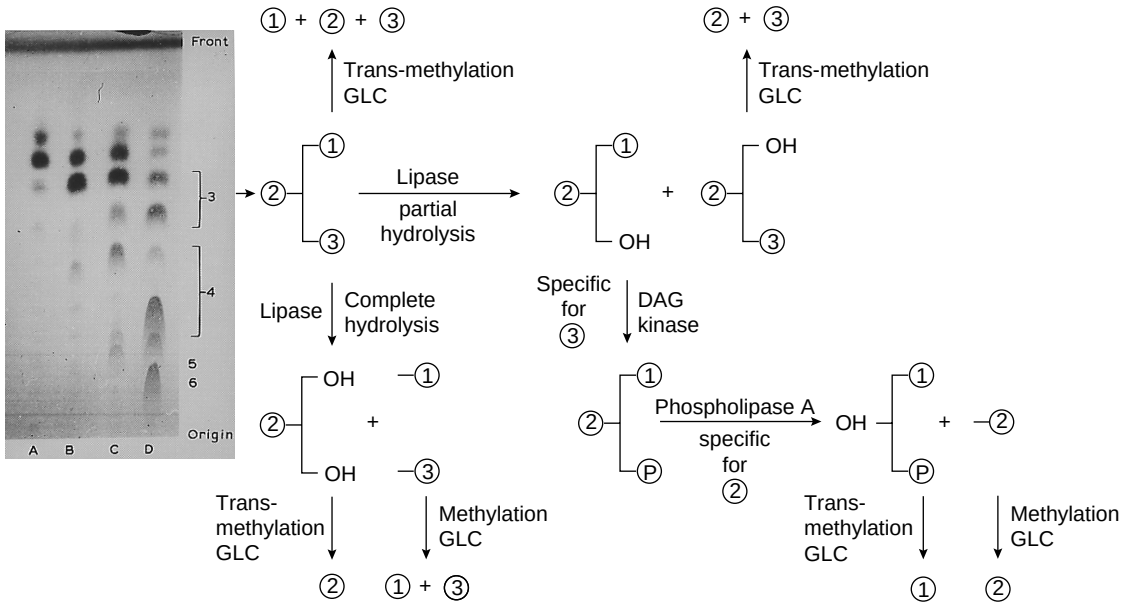
Examples of common fats with important stereo-specific fatty acid distributions are:

(1) Milk fats, in which the characteristic short-chain fatty acids accumulate at position 3 (for example, butyrate (4:0) in cow's milk fat). Long-chain saturated fatty acids tend to occur at position 1 and unsaturated acids at position 2. A notable exception is human milk fat in which palmitic acid occurs mainly at position 2.
(2) Animal depot fats have saturated fatty acids at position 1, short-chain and unsaturated fatty acids at position 2. A notable exception is lard (pig adipose tissue) in which palmitic acid is mainly located at position 2. Position 3 seems to have a more random population, although polyenoic acids tend to concentrate at position 3 in mammals, but in position 2 in fish and invertebrates.
(3) Certain seed oils contain acetate residues that occur only at position 3. This inherent asymmetry may lead to optical activity, which although extremely small, is measurable, especially in the extreme cases of acylglycerols containing acetate.

Box 3.1 Stereo-specific structural analysis of triacylglycerols

By combining chromatographic separations with specific enzymic hydrolyses of the separated triacylglycerol components, it is possible to identify the fatty acids on each of the three positions, *sn*-1, -2 and -3, which are stereochemically distinct. An initial separation by argentation TLC (see diagram below and Chapter 1) separates triacylglycerol species according to the total degree of unsaturation of the molecule. A more recent development is argentation HPLC, which is capable of some extremely good resolutions and lends itself more readily to quantitative analysis than TLC.

In the example shown, palm (A), olive (B), groundnut (C) and cottonseed (D) oils are separated on silica gel impregnated with silver nitrate and developed in isopropanol:chloroform, 1.5:98.5 (v/v). Spots in marker lanes are located by spraying with 50% sulphuric acid and charring. The numbers along the right-hand side indicate the total number of bonds per molecule in each separated species. Areas of gel corresponding to each molecular species are removed from lanes *that are not sprayed with sulphuric acid* and the triacylglycerols eluted with diethyl ether. They are then treated with lipases to liberate fatty acids from specific positions as shown in the diagram. In this example, the fatty acid composition at position 3 is determined by difference. A slight disadvantage of methods involving lipase hydrolysis is that they may not always yield a random sample of fatty acids from any one position. Thus very long chain polyunsaturated fatty acids characteristic of fish oils (e.g. 20:5, 22:6) are more slowly hydrolysed, whereas short- and medium-chain fatty acids (4–10C) are more rapidly hydrolysed than normal chain length fatty acids (12–18C).



(4) Older varieties of rapeseed oil, which contain the unusual fatty acid erucic acid (22:1*n*-9), have this fatty acid exclusively at positions 1 and 3, but none at position 2.

There are many other examples of stereo-specific distribution arising because of the specificity of the various transacylase enzymes responsible for the esterification of fatty acids at each of the gly-

cerol hydroxyl groups as discussed in Section 3.4.1.

3.3 THE STORAGE OF TRIACYLGLYCEROLS IN ANIMALS AND PLANTS

Both animals and plants can store TAG in specialized tissues as long-term fuel reserves.

3.3.1 Adipose tissue depots are the sites of TAG storage in animals

As discussed in more detail in Section 4.2.1, TAG provide a concentrated form of metabolic energy, having a metabolizable energy value of $38\,kJ\,g^{-1}$. When the energy supply from the diet exceeds the energy demands of the body, TAG molecules are deposited in adipose tissue. They have the advantage that they can be stored in anhydrous form and represent more energy for less bulk than the glycogen stored in the liver or muscle, which is heavily hydrated. The glycogen store, too, has little capacity to expand, whereas the adipose tissue is seemingly capable of enormous expansion.

There are two types of adipose tissue in the body, known as 'brown' and 'white'. White adipose tissue is the more abundant and is the main tissue involved in the storage of body fat. Brown adipose tissue has a more specialized function in energy metabolism (see below). White adipose tissue is widely distributed throughout the body. In human beings, a large proportion is located just beneath the skin (subcutaneous adipose tissue) and is the tissue that influences the contours of the body. It also provides an insulating and protective layer. Fat contributes a larger proportion of the body weight in women than in men and their subcutaneous adipose tissue is correspondingly more abundant. Four-fifths or more of the mammary gland in non-lactating premenopausal women, for example, may be adipose tissue. The contribution of subcutaneous adipose tissue to body mass is particularly noticeable in overweight or obese individuals (Section 5.4.3). The tissue is also located internally, for example surrounding the kidneys (perirenal adipose tissue), along the intestinal tract (mesenteric adipose tissue) and in the omentum.

Although adipose tissue contains many types of cells, the ones responsible for fat storage are the adipocytes (Fig. 3.2), which are bound together with connective tissue and supplied by an extensive network of blood vessels. Adipocytes are unusual in being able to expand to many times their original size by increasing their content of stored fat. When the fat content of the diet is low, fat cells can synthesize their lipid *de novo* from glucose, which is transported into the cell from the bloodstream (Section 3.4). When fat makes a large contribution to dietary energy, the adipocytes can take in fat from circulating lipoproteins. This involves hydrolytic breakdown of the TAG in the lipoproteins and release of fatty acids catalysed by the enzyme lipoprotein lipase (Sections 3.5.2 and 5.2.5). The fatty acids are transported into the cell and incorporated back into TAG (Section 3.4).

Because circulating lipoproteins are influenced by the fat content of the diet, the composition of the adipose tissue can provide a good indication of the type of dietary fat eaten. This is illustrated for pig adipose tissue in Table 3.1 and for human adipose tissue in Table 4.7.

When there is a demand for fatty acids elsewhere in the body, they can be mobilized by breakdown of the TAG in the lipid globule. This is catalysed by the enzyme hormone-sensitive lipase (Section 3.5.2), followed by transport out of the cell. The integration of the systems for synthesis and breakdown, storage and mobilization is orchestrated by a variety of hormones and is geared to the needs and nutritional status of the animal (Section 3.6.3).

White adipose tissue is also a secretory organ, producing proteins that are liberated in the circulation. These factors include hormones, which then act as signals to other tissues including the brain, and proteins that themselves regulate lipid metabolism. This aspect of adipose tissue will be covered in Section 5.3.4.

Brown adipose tissue is found in small animals such as rodents and in new-born larger mammals,

Fig. 3.2 White adipose tissue as seen by scanning electron microscopy. The bar indicates the scale: 1 cm represents 50 μm. Reproduced with kind permission of The Society of Chemical Industry, from *Chemistry and Industry*, 18 September, 1976, p. 768, figure 1.

but is almost absent from large adult mammals such as humans. Its role is the generation of heat. Therefore it is important to smaller animals and neonates that have a large body surface area (through which heat is lost) in relation to their body mass (in which heat is generated). It plays an important role in hibernating animals, generating heat to allow them to warm up from their hibernating state. The heat is generated by oxidation of fatty acids, which are mainly obtained from the tissue's own stores of TAG. The TAG is stored in multiple, small lipid droplets, 1–3 μm in diameter. The cells also contain a large number of specialized mitochondria, adapted for oxidizing the fatty acids from the oil droplets that they surround. Release of noradrenaline from sympathetic nerve terminals in the tissue leads to activation of β-adrenergic receptors, which are coupled to both TAG hydrolysis (as in white adipose tissue; Section 3.6.3) and regulation of blood flow. The latter is important as the heat generated from fatty acid oxidation needs to be transported out of the brown adipose tissue depot to the rest of the body.

The unique feature of brown adipose tissue is the presence, in its mitochondrial membrane, of a protein known as uncoupling protein (UCP) or thermogenin. Normally the operation of the electron transport chain (carrying reducing equivalents from the oxidation of fatty acids or other substrates) leads to the pumping of protons across the inner mitochondrial membrane. The 'proton gradient' that is generated provides the energy needed for ATP synthesis by ATP synthase. In the mitochondria of brown adipose tissue, UCP 'short-circuits' this mechanism by allowing the protons to leak back into the mitochondrial matrix. Thus, the

Table 3.1 The fatty acid composition of some animal storage fats* (g per 100 g total fatty acids)

	4:0–12:0	14:0	16:0	16:1	18:0	18:1	18:2 *n*-6	20:1+22:1	20:5 *n*-3	22:6 *n*-3	total
Adipose tissue											
Cow		3	26	9	8	45	2				93
Human (1)		2	19	7	3	48	13				92
Human (2)		2	20	4	5	39	24				94
Lamb		3	21	4	20	41	5				94
Pig (1)		1	29	3	14	43	9				99
Pig (2)		1	21	3	12	46	16				99
Poultry		1	27	9	7	45	11				94
Milk											
Cow	10	12	26	3	11	29	2				93
Goat	20	11	26	3	10	26	2				98
Egg yolk											
Hen			29	4	9	43	11				96
Fish oils											
Cod (liver)		6	8	10	3	17	3	25	10	11	93
Mackerel (flesh)		8	16	9	2	13	1	26	8	8	91
Herring (flesh)		9	15	8	1	17	1	39	3	3	96
Sardine (flesh)		8	18	10	1	13	1	7	18	9	85

* The figures are rounded to the nearest whole number; therefore the content of an individual fatty acid of less than 0.5% is not recorded. The figures across a row rarely sum to 100% because each sample contains a large number of components, each contributing less than 1%. Thus, samples for ruminants (cow, goat, lamb) contain a wide variety of odd- and branched-chain fatty acids. Hen's egg yolk contains about 1% 20:4*n*-6, which is not normally found in other storage fats and is not listed individually in this table. Data on human milk composition are presented in Table 4.6. Human adipose tissue (1) is a sample from British adults, whereas human adipose tissue (2) is a sample from Israeli adults, whose diet contains more linoleic acid than the British diet. Pig adipose tissue (1) is a sample taken from pigs given a normal cereal-based diet, whereas pig adipose tissue (2) is a sample from pigs given diets to which have been added oilseeds rich in linoleic acid. Contrast the linoleic acid content from storage fats of simple-stomached animals (pig, poultry, human) with those from ruminants in which much of the polyunsaturates in the animals' diets have been reduced by biohydrogenation in the rumen. Note also that whereas fish oils are mainly of interest because of their content of *n*-3 polyunsaturates, cod, herring and mackerel are particularly rich in long-chain monoenes.

energy liberated by oxidation of substrates is not used for chemical work and is released instead as heat.

The UCP of brown adipose tissue is a member of a family of mitochondrial transporter proteins. It is now known as UCP1 as several closely related proteins have been found in animals and plants, and it is suggested that they may also play a role in energy dissipation as heat. The mammalian proteins are known as UCP2 (expressed in many tissues), UCP3 (expressed mainly in skeletal muscle) and UCP4 (expressed only in brain). The closely related protein found in some plants may play a role in warming up tubers for sprouting.

3.3.2 Milk triacylglycerols provide a supply of energy for the needs of the new-born

Milk produced in the mammary gland can be regarded as a store of energy for the new-born animal. The cream fraction of milk consists of milk fat globules composed primarily of triacylglycerols, with smaller amounts of cholesterol, fat-soluble vitamins and other hydrophobic lipids, some of which may be environmental contaminants. The milk fat globule is surrounded by a unit membrane containing mainly protein, phospholipid and cholesterol and has an average diameter of 1–2 μm,

although some may attain sizes up to 10 μm. The fat droplets found in the mammary cell have no discernible membrane. They are formed within the endoplasmic reticulum membranes and migrate to the apical regions of the mammary secretory cell where they are enveloped in the plasma membrane. A neck of membrane is pinched off and the resulting vesicle is expelled into the lumen as a milk fat globule. Like the triacylglycerols in adipose tissue, their synthesis and breakdown in the mammary gland is controlled by hormones the secretion of which is in part governed by the nutritional status of the animal (Section 3.6).

3.3.3 Some plants use lipids as a fuel, stored as minute globules in the seed

There are many hundred varieties of plants known to have oil-bearing seeds, but only a few are significant commercially. These are listed in Table 3.2 with their fatty acid compositions. Most are important sources of edible oils for human foods or animal feeds, but some are used for other industrial purposes such as paints, varnishes and lubricants. Although the seed is the most important organ for the storage of triacylglycerols, some species store large quantities of oil in the mesocarp or pericarp of the fruit surrounding the seed kernel. The avocado is a familiar example of a plant with an oily mesocarp and has been the subject of extensive studies of fatty acid biosynthesis. The mesocarp of the oil palm is a commercially important source of palm oil, used in soap and margarine making, which is quite distinct in chemical and physical properties from the seed oil of the same plant: palm kernel oil (Fig. 3.3 and Table 3.2). Droplets of triacylglycerols are also present in the seeds of plants that store predominantly carbohydrates (starch) as the primary source of fuel for seed germination. These include legumes such as peas or beans and cereals like wheat or barley.

Microscopic examination of a mature oil seed or one in the active phase of oil accumulation reveals a cytoplasm packed with spherical organelles that consist mainly of triacylglycerols, called oil bodies (Fig. 3.4). Unfortunately, the nomenclature has not been standardized and you may also encounter terms such as lipid bodies, storage oil bodies, storage oil droplets, oleosomes, lipid-containing vesicles and reserve oil droplets, all of which are synonymous. Some authors have used the term spherosomes to refer to the same organelle, but it should strictly be used to describe another particle in plant cells that has a high content of phospholipids.

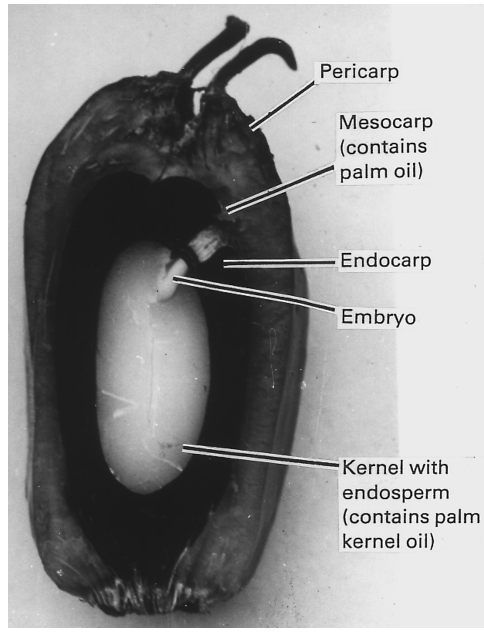


Fig. 3.3 Section through an oil palm fruit. Note the location of the two quite distinct types of oil, palm oil and palm kernel oil. Reproduced with kind permission of Dr L.H. Jones.

The core of the oil body is composed mainly of triacylglycerols, with minor quantities of other hydrophobic lipids such as sterols, hydrocarbons and carotenoids. The oil body is surrounded by a half-unit membrane of protein and phospholipids. The origin of the oil body continues to be controversial and Murphy's review (see Further Reading) refers to several early theories. A current view is illustrated in Fig. 3.5. TAG are synthesized in the endoplasmic reticulum as described in Section 3.4.1.3. Accumulation of oil in the space between the membrane bilayers results in mem-

Table 3.2 The fatty acid composition of some vegetable oils* (g per 100 g of total fatty acids)

	8:0	10:0	12:0	14:0	16:0	18:0	18:1 *n*-9	18:2 *n*-6	18:3 *n*-3	20:1+ 22:1	total
A. Major edible oil crops											
Cocoa butter					26	34	35	3			98
Coconut (3.4)	8	7	48	18	9	3	6	2			99
Corn (1.9)					13	3	31	52	1		100
Cottonseed (4.0)				1	24	3	19	53			100
Groundnut[a] (4.2)					13	3	37	41		2	96
Olive (2.7)					10	2	78	7	1		98
Palm (17.6)				1	43	4	41	10			99
Palm kernel (2.2)	4	4	45	18	9	3	15	2			100
Rape (Canola) (11.8)					4	2	56	26	10	2	100
Sesame (0.8)					9	6	38	45	1	1	100
Soybean (20.8)					11	4	22	53	8	1	99
Sunflower (9.3)					6	6	18	69			99
B. Major industrial oils											
Castor[b] (0.5)					1		3	4			98[b]
Linseed (0.7)					6	3	17	14	60		100
Rape (high erucic)					3	1	16	14	10	55	99
C. Other oils and fats of interest											
Avocado					20	1	60	18			99
Cuphea viscosissima	9	76	3	1	3		2	5			99
Evening primrose[c]					7	2	9	72			90[c]
Rice bran					16	2	42	37	1		98
Safflower (high oleic)					6	2	74	16			98
Safflower (high linoleic)					7	3	14	75			99

[a] Also called peanut.
[b] Castor oil contains 90% of ricinoleic acid (see Table 2.4).
[c] Evening primrose oil contains about 10% of γ-linolenic acid (see Table 2.3).

* The vegetable oils are listed according to whether their main commercial use is in (A) foods, (B) chemicals. Some crops mainly used in foods also contribute to the chemical industry (e.g. use of palm oil in making 'biodiesel', a vehicle fuel made from fatty acid esters). Category (C) includes the fat-rich fruit, avocado, two varieties of safflower, which have been much used in nutrition studies and three crops for which there is increasing specialist interest: evening primrose for its high γ-linolenic acid content, *Cuphea* species for their high content of medium-chain fatty acids and rice bran for its good balance of oleic and linoleic acids. The numbers in parentheses after each crop name represent world production in 1997 in millions of tonnes.

brane distension and eventually immature oil bodies (IOB) bud off. These IOB are bounded by a stabilizing phospholipid monolayer derived from the endoplasmic reticulum. The immature oil bodies subsequently become encircled by rough endoplasmic reticulum and begin to acquire a surface coat of proteins called 'oleosins', leading finally to the formation of mature oil bodies (MOB) with a monomolecular oleosin layer.

Oleosin is the name given to proteins that are specific to seeds and exclusively associated with the surfaces of oil bodies in plants. They are made up of three similarly sized but structurally distinct domains comprising a relatively polar C-terminal region, a central hydrophobic domain and an amphipathic N-terminal fragment. The two outer regions are located on the surface of the oil body while the central β-strand domain intrudes through the phospholipid monolayer into the triacylglycerol core. The relatively small size and great abundance of oleosins in oilseeds, coupled with their structural amphipathic characteristics should make them useful as model systems for structural studies, once they can be removed and purified from their

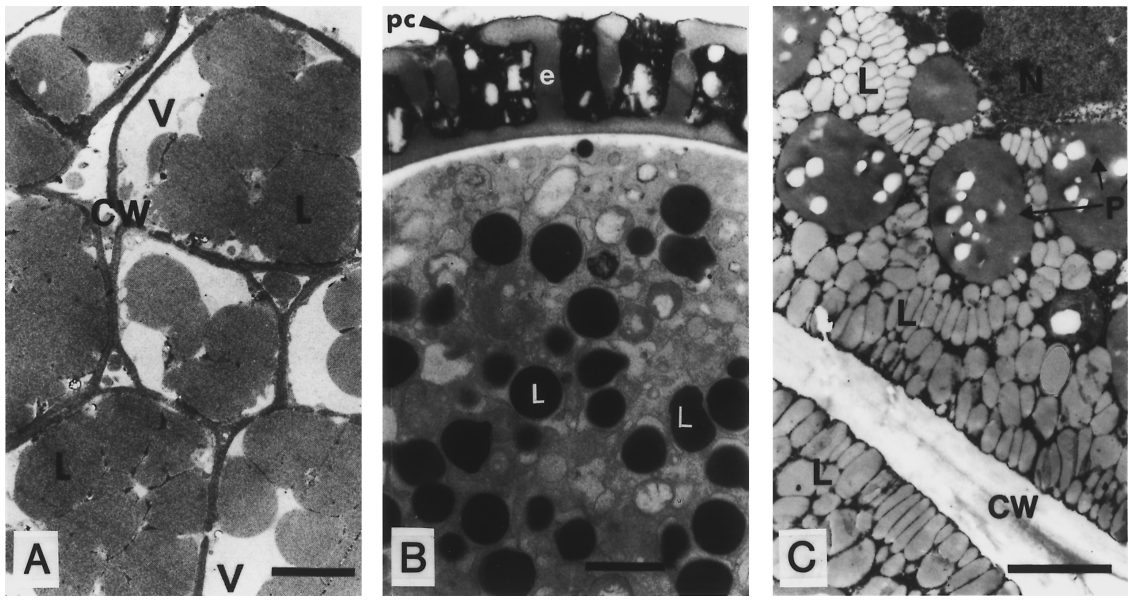


Fig. 3.4 Ultrastructure of oil bodies in plant tissues. The figure shows transmission electron micrographs of sections from (A) mesocarp of a mature avocado pear (*Persea americana*); (B) mature pollen of rape (*Brassica napus*); (C) mature seed of rape. **L**, oil (lipid) bodies; **PB**, protein bodies; **V**, vacuoles; **PC**, pollen coat; **CW**, cell wall. Note that, whereas the pollen and seed oil bodies are about 0.3–0.5 μm in diameter the fruit oil bodies are 10–15 μm in diameter. Reprinted from *Progress in Lipid Research*, **32**, 247–280, 1993 with permission from Elsevier Science.

natural milieu without denaturation. The model proposed in Fig. 3.5 is based on the finding that oleosin gene expression occurs relatively late in embryo development compared with genes for lipid biosynthesis.

It is notable that the morphology of oil bodies in seeds is quite different from that in fruit mesocarp (e.g. palm, olive, avocado). The diameters of oil bodies in seeds tend to be small (often less than 1 μm), whereas fruit oil bodies often have diameters exceeding 20 μm. Although the pathways of lipid biosynthesis in both types of oil storage body appear to be identical, fruits lack oleosins, again underlining the principle that oil body synthesis can proceed independently of oleosin formation. The function of oleosins does not seem, therefore, to be in the initial formation of oil bodies but in the stabilization of small oil bodies in seeds and pollen. Without oleosins the immature oil bodies in fruit mesocarp coalesce until the cells contain just a few large oil droplets.

In the animal kingdom, there is a close parallel to seed oil bodies, namely the cells of the brown adipose tissue which, as described earlier (Section 3.3.1), are packed with small oil droplets. These cells also contain a large number of specialized mitochondria, adapted for oxidizing the fatty acids from the oil droplets that they surround. Similarly, seed oil bodies are surrounded by glyoxysomes. During seed germination, these receive fatty acids from the oil bodies for oxidation prior to the synthesis of carbohydrates, which occurs actively at this time.

3.4 THE BIOSYNTHESIS OF TRIACYLGLYCEROLS

There are several different routes to TAG. Animals and plants can esterify glycerol-3-phosphate. The product is then dephosphorylated and the remaining hydroxyl is acylated. Another pathway in animals resynthesizes TAG from the absorbed digestion products of dietary TAG.

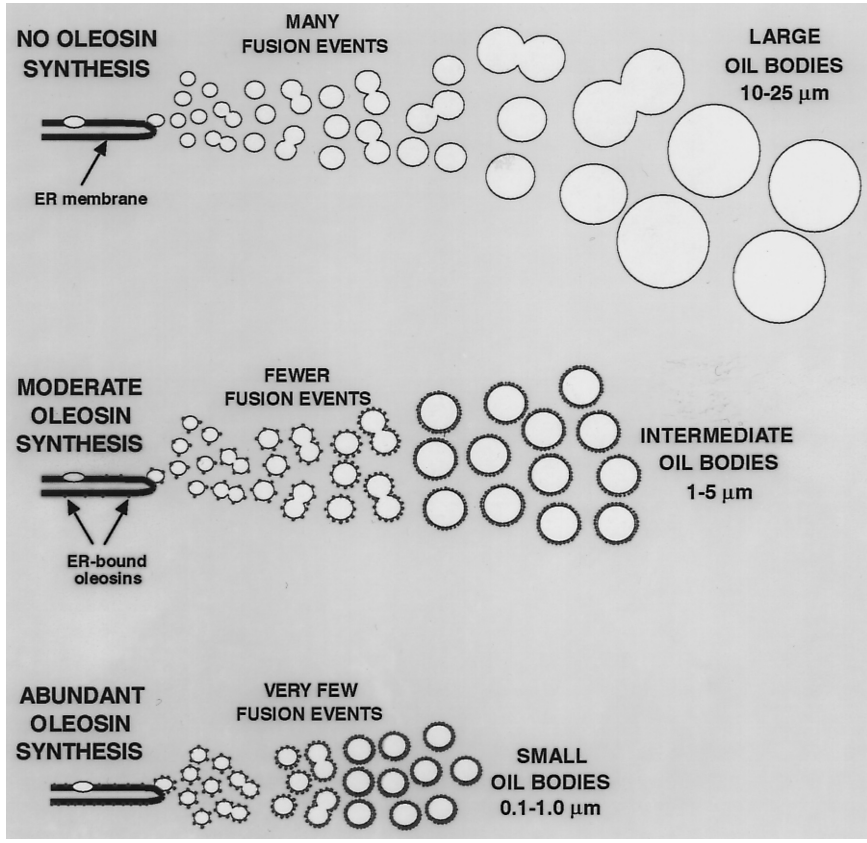


Fig. 3.5 A proposed scheme for the formation of oil bodies. Oil bodies probably bud off from the ER as small TAG droplets of 60–100 nm diameter. Such small oil droplets are unstable in aqueous media and would tend to coalesce, rapidly at first and then more slowly until reaching a stable size. (A) In the absence of oleosins and in non-desiccating tissues like palm mesocarp, the final oil body size is about 10–25 μm. (B) Directly after their synthesis, oleosins are inserted into the ER membrane, from where they may diffuse into nascent oil bodies, probably undergoing a conformational change. Alternatively, oleosin-poor oil bodies may re-fuse with ER to undergo TAG turnover and acquire more oleosins. If the ratio of oleosin:TAG synthesis is only low to moderate, the nascent oil bodies will need to undergo many fusion events before acquiring a complete oleosin monolayer. In oilseed that has oil bodies of 1–4 μm, approximately 9–14 fusions will be required to get from a diameter of 100 nm to 1–4 μm. (C) With much higher ratios of oleosin: TAG synthesis, fewer fusions are required before the oleosin monolayer is complete, which results in smaller oil bodies. Therefore in rapeseed, mature oil body sizes of 0.2–1.0 μm can be achieved by only 3–8 fusion events from 100 nm oil bodies. Reprinted from Fig. 1 in D.J. Murphy, C. Sarmiento, J.H.E. Ross & E. Herman, Oleosins: their subcellular targeting and role in oil-body ontogeny, in *Physiology, Biochemistry and Molecular Biology of Plant Lipids* (eds J.P. Williams, M.U. Khan & N.W. Lem) with kind permission of Kluwer Academic Publishers.

There is an important difference between animals and plants with respect to TAG composition and metabolism. Plants must of necessity synthesize their TAG from simple starting materials according to their requirements, since they have no dietary source of preformed lipids. Unlike animals, they have the ability to synthesize the linoleic acid that they normally possess in abundance: animals rely on plants for this essential nutrient (Section 4.2.3). The fatty acid composition of animal TAG is greatly influenced by their diet and, therefore, ultimately by the plant materials they eat. The way in which

dietary TAG are modified by animals may differ between species and from organ to organ within a species. Such modifications are not necessarily always effected by the animal's own cells. For example, in ruminants such as cows, sheep or goats, the micro-organisms present in the rumen hydrogenate the double bonds of dietary polyunsaturated fatty acids like linoleic and α-linolenic acids to form a mixture of mainly saturated and *cis* and *trans* monoenoic acids (Section 2.2.5). An outstanding and significant feature of TAG fatty acid composition is that it is quite distinctly different from that of the phospholipids or the non-esterified fatty acid (NEFA) pool. An understanding of this cannot be obtained simply from analyses of fatty acid distributions but must depend upon the study of different metabolic pathways, and of the individual enzymes in those pathways that are involved in the biosynthesis of each lipid class.

3.4.1 Pathways for complete (*de novo*) synthesis build up TAG from small basic components

3.4.1.1 The glycerol 3-phosphate pathway in mammalian tissues provides a link between TAG and phospholipid metabolism

Historically, the *de novo* pathway, now usually known as the glycerol phosphate pathway for TAG biosynthesis (Fig. 3.6), was worked out first. It was first proposed by the American biochemist, Kennedy, based on the earlier work of Kornberg and Pricer, who first studied reactions 1 and 4 (Fig. 3.6), the formation of phosphatidic acid by stepwise acylation of glycerol 3-phosphate.

Kennedy also demonstrated the central role of phosphatidic acid in both phospholipid (Section 7.1.2) and TAG biosynthesis and one of his outstanding contributions was to point out that diacylglycerols derived from phosphatidic acid form the basic building blocks for triacylglycerols as well as phosphoglycerides.

It is now known that steps 1 and 4 in Fig. 3.6, the stepwise transfer of acyl groups from acyl-coenzyme A to glycerol 3-phosphate, are catalysed by two distinct enzymes specific for positions 1 and 2. The enzyme that transfers acyl groups to position 1 (acyl-CoA:glycerol phosphate 1-O-acyltransferase, GPAT) exhibits marked specificity for saturated acyl-CoA thiolesters whereas the second enzyme (acyl-CoA:1-acyl glycerol phosphate 2-O-acyltransferase, sometimes referred to as lysophosphatidate acyltransferase, LPAT) shows specificity towards mono- and dienoic fatty acyl-CoA thiolesters. This is in accord with the observed tendency for saturated fatty acids to be found in position 1 and unsaturated ones in position 2 in the lipids of most animal tissues.

GPAT, which transfers an acyl group to position 1, has been cloned and sequenced. There are two isoforms in mammals, one associated with the endoplasmic reticulum, the other with the outer mitochondrial membrane. The active sites of both isoforms face the cytosol. In the liver of most mammals studied so far, there is a similar activity at both subcellular locations, whereas in other tissues, the microsomal enzyme has about ten times the activity of the mitochondrial isoform. It is not yet known whether the two isoforms have different functions but there is experimental evidence to link changes in activity of both enzymes with changes in overall TAG biosynthesis. For example, when cultured 3T3-L1 cells differentiate into mature adipocytes, the activity of microsomal GPAT increases about 70-fold and the mitochondrial activity (and the amount of its mRNA) increases about tenfold. It is interesting to speculate whether these questions might be resolved by manipulating 3T3-L1 cells to produce lines in which the mitochondrial GPAT gene is either deleted or over-expressed.

GPAT was first purified from *E. coli* membranes and more recently a rat liver mitochondrial enzyme has also been purified. The purified *E. coli* enzyme, with a molecular mass of 83 kDa, proved to be inactive, but activity was restored by reconstitution with phospholipid preparations, principally cardiolipin and phosphatidylglycerol. A 90 kDa protein ('p90'), which has about 30% identity with the *E. coli* GPAT, has been cloned in mice. When the mice were fasted and then given a diet rich in carbohy-

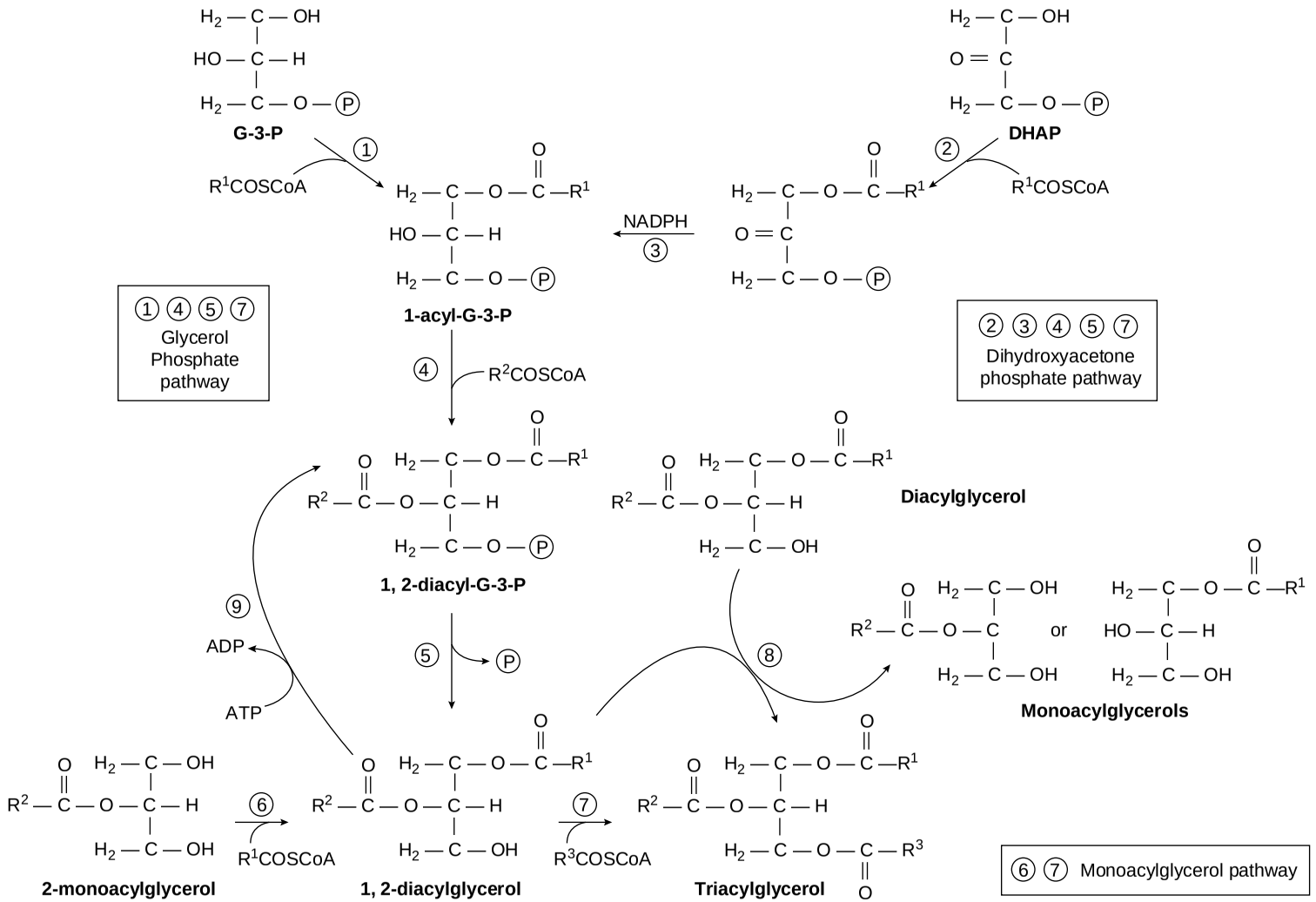


Fig. 3.6 The biosynthesis of triacylglycerols in mammals. The figure illustrates the three main pathways: the glycerol 3-phosphate (G3P) pathway (reactions 1, 4, 5, 7), the dihydroxyacetone phosphate (DHAP) pathway (reactions 2, 3, 4, 5, 7) and the monoacylglycerol pathway (reactions 6, 7). The enzymes involved are: (1) glycerol 3-phosphate acyltransferase (GPAT); (2) dihydroxyacetone phosphate acyltransferase; (3) acyl-dihydroxyacetone phosphate reductase; (4) lysophosphatidate acyltransferase (LPAT); (5) phosphatidate phosphohydrolase (PAP); (6) monoacylglycerol acyltransferase; (7) diacylglycerol acyltransferase (DAGAT). A minor route from diacylglycerols proceeds via reaction 8: diacylglycerol transacylase. In some tissues, diacylglycerol can be reconverted into phosphatidic acid by diacylglycerol kinase (9).

drate, the mRNA for p90 was rapidly induced at high levels in liver, muscle and kidney and at relatively low levels in brain. Such induction was up-regulated by insulin and down-regulated by cyclic AMP. This protein has also been detected in fully differentiated adipocytes but not pre-adipocytes.

LPAT, which transfers an acyl group to position 2 of 1-acylglycerol 3-phosphate to form phosphatidic acid, is also found in both mitochondrial and microsomal fractions, but predominantly the latter. It has proved more difficult to purify this activity but two human isoforms, α and β with 46% homology, have been cloned. LPAT-α is present in all tissues (but predominantly skeletal muscle), whereas the β-isoform is found primarily in heart, liver and pancreas. It is localized in the endoplasmic reticulum. (A third protein with LPAT activity has been identified. This is called endophilin-I and is involved, not in TAG biosynthesis, but in the formation of synaptic vesicles, during which process arachidonate is transferred to lysophosphatidic acid.)

When mitochondria are incubated with glycerol 3-phosphate and acyl-CoA, the major product is phosphatidic acid. However, if a liver fatty acid binding protein is present as a lysophosphatidic acid acceptor, lysophosphatidate accumulates. It has been shown that lysophosphatidate synthesized in the mitochondria is transferred to endoplasmic reticulum and is there converted into phosphatidic acid. More research is required into the co-operativity between intracellular sites in the process of TAG assembly and the role of fatty acid binding proteins (or 'Z' proteins; Sections 2.2.1 and 5.1.3) in this process.

It should be noted that phosphatidic acid, the product of the esterification of the two hydroxyls of glycerol 3-phosphate, can also be formed by phosphorylation of diacylglycerols by diacylglycerol kinase in the presence of ATP. The contribution of this reaction to overall biosynthesis of phosphatidic acid is unknown but it is unlikely to be important in TAG biosynthesis. Its importance is more likely to be in the phosphorylation of diacylglycerols as part of the signal transduction pathway involving phosphoinositides, described in Section 7.9.

The next step of the glycerol 3-phosphate pathway (step 5 in Fig. 3.6) is catalysed by phosphatidate phosphohydrolase (PAP). This enzyme is found in both membrane-bound and soluble forms, which may be relevant to the regulation of its activity (Section 3.6.2).

The final step in the pathway (step 7, Fig. 3.6) is catalysed by diacylglycerol acyltransferase (DAGAT), an enzyme unique to TAG biosynthesis. The enzyme has recently been cloned and sequenced but is membrane-bound and insoluble and has long eluded traditional methods of purification. The enzyme accepts a wide variety of acyl groups for transfer to diacylglycerols formed from phosphatidic acid by the action of PAP. Its activity is highest in tissues specialized in TAG biosynthesis: adipose tissue, liver, lactating mammary gland, small intestinal mucosa and adrenal gland. In mammalian cells, DAGAT forms a family with acyl-CoA:cholesterol acyltransferases-1 and -2. Yeast cells contain an acyl-CoA:sterol acyltransferase, which has been well characterized. Because of considerable homology between this yeast enzyme and mammalian DAGAT, it has now been possible to clone human and mouse DAGAT. The concentration of DAGAT mRNA increases eightfold during differentiation of 3T3-L1 adipocytes in culture. However, because enzyme activity increases nearly 60-fold at this time, the enzyme may also be regulated post-transcriptionally.

In this section, much has been said about the specificity of acyltransferases that leads to characteristic distributions of fatty acids on the three positions of the glycerol backbone. Despite this sometimes seemingly tight specificity, numerous examples have been found of TAG that contain unnatural or 'xenobiotic' acyl groups. Xenobiotic carboxylic acids may arise from many sources including herbicides, pesticides and drugs of various kinds. Many are substrates for acyl-CoA synthetase and the resulting xenobiotic acyl-CoAs may be incorporated into TAG, cholesteryl esters, phospholipids and other complex lipids. An example of a xenobiotic TAG is the one formed from ibuprofen, a commonly used non-steroidal anti-inflammatory drug. Such compounds are normally stored in adipose tissue where they may

have relatively long half-lives since the ester bonds of many xenobiotic acyl groups are poor substrates for lipases. Much needs to be learned of their metabolism and potential toxicity.

3.4.1.2 The dihydroxyacetone phosphate pathway in mammalian tissues is a slight variant to the main glycerol 3-phosphate pathway and provides an important route to ether lipids

In the 1960s the American biochemists Hajra and Agranoff discovered that radio-phosphorus was incorporated from ^{32}P[ATP] into a hitherto unknown lipid, which they identified as acyl dihydroxyacetone phosphate. Further research demonstrated that dihydroxyacetone phosphate could provide the glycerol backbone of TAG without first being converted into glycerol 3-phosphate (Fig. 3.6). The first reaction of this so-called 'dihydroxyacetone phosphate pathway' is the acylation of dihydroxyacetone phosphate at position 1, catalysed by the enzyme dihydroxyacetone phosphate acyltransferase. Although first studied in a microsomal fraction, the activity has also been demonstrated in mitochondria and in peroxisomes. Peroxisomes (sometimes called microbodies) are subcellular organelles specialized for the oxidation of small molecules by hydrogen peroxide. They also contain many enzymes of lipid metabolism including those of the dihydroxyacetone phosphate pathway. Whereas it is now established that the principal location of dihydroxyacetone phosphate acyltransferase is in peroxisomes, there is still debate about whether the activity in microsomal and mitochondrial fractions is due to contamination by peroxisomal enzymes or to non-specificity of the GPAT in those organelles. Evidence from molecular mass of the purified enzymes and the differential effects of diet on the activities suggests that glycerol 3-phosphate and dihydroxyacetone phosphate acyltransferases may be separate enzymes.

It now seems certain that dihydroxyacetone phosphate acyltransferase has two separate roles. The first is in providing an alternative route to TAG as illustrated in Fig. 3.6. (The second role, in ether lipid synthesis is described below.)

Dihydroxyacetone phosphate acyltransferase has been purified after detergent treatment of peroxisomal membranes from guinea pig liver and human placenta. The purified protein has a molecular mass of 65–69 kDa. Certain chemical substances, including the hypolipidaemic drug clofibrate, cause peroxisomes to proliferate. Under such conditions, the activity of dihydroxyacetone phosphate acyltransferase increases 2–3-fold.

The next step in the pathway is the reduction of the keto group in 1-acyl-dihydroxyacetone phosphate to form 1-acylglycerol 3-phosphate, linking once more into the main triacylglycerol biosynthetic pathway. This enzyme is located on the cytosolic side of the peroxisomal membrane and is notable in that it requires NADPH rather than NADH. NADPH is normally associated with reactions of reductive synthesis such as fatty acid biosynthesis. There is some evidence to support the view that the activity of the dihydroxyacetone phosphate pathway is enhanced under conditions of increased fatty acid synthesis and relatively reduced in conditions of starvation or when the animal is fed a relatively high fat diet (particularly unsaturated fat). Evidence from Amiya Hajra's laboratory in Ann Arbor, published in early 2000, suggests that the DHAP pathway may indeed make a significant contribution to TAG accumulation when 3T3-L1 preadipocytes differentiate in culture. However, we do not yet have a clear picture of the quantitative significance of the dihydroxyacetone phosphate pathway in overall triacylglycerol assembly.

Peroxisomes do not contain the enzymes catalysing the final steps of acylglycerol biosynthesis. The end-products of peroxisomal biosynthesis, acyl or alkyl (see below) dihydroxyacetone phosphate or 1-O-acyl- or 1-O-alkylglycerol 3-phosphate must be exported to the endoplasmic reticulum before acylation at position 2, dephosphorylation to the diacylglycerol or its ether analogue and the final acylation at position 3 by diacylglycerol acyltransferase can occur. The details of the transport mechanism from peroxisomal to microsomal membranes are unclear, but

there is some evidence for the participation of a fatty acid binding protein.

It is now generally agreed that the other and most important role for dihydroxyacetone phosphate acyltransferase is to catalyse the first step in the biosynthesis of ether lipids, a reaction that occurs on the luminal side of peroxisomal membranes. The peroxisome is now regarded as the principal site of biosynthesis of the alkyl (ether) lipids. The mechanism of formation of the alkyl linkage at position 1 of dihydroxyacetone phosphate is described in more detail in Section 7.1.9 because the alkyl phospholipids are more widespread and of greater physiological importance than the neutral alkyl acylglycerols. However, as described in Section 3.2.1, the neutral alkyl lipids are found in significant quantities in the liver oils of sharks. The vital importance of ether lipids is illustrated by a number of serious neurological diseases resulting from lack of peroxisomes or peroxisomal enzymes. Thus, one piece of research, in which human cDNA for dihydroxyacetone phosphate acyltransferase was cloned, showed that absence of the enzyme causes severe neurological impairment and skeletal deformities, but no alteration in overall TAG biosynthesis.

3.4.1.3 Formation of triacylglycerols in plants involves the co-operation of different subcellular compartments

The primary pathway for the biosynthesis of TAG in plants that use lipids as their major energy store, is the glycerol 3-phosphate ('Kennedy') pathway. However, there are sufficient differences from animals in terms of subcellular location and sources of substrates to merit a separate discussion of plant TAG biosynthesis.

To study the different enzymes in the pathway, seeds or fruits need to be harvested at the time when the rate of lipid accumulation is most rapid, since there are distinct phases of development as illustrated in Fig. 3.7. In phase 1, cell division is rapid but there is little deposition of storage material, whether it be protein, lipid or carbohydrate. In phase 2, there is a fast accumulation of storage material. Moreover, if the TAG in the lipid stores contain unusual fatty acids, the special enzymes needed for synthesis are active only at this stage. Finally, in phase 3, desiccation takes place with little further metabolism.

Fatty acid biosynthesis *de novo* is concentrated in the plastid of the plant cell (Section 2.2.3). By a

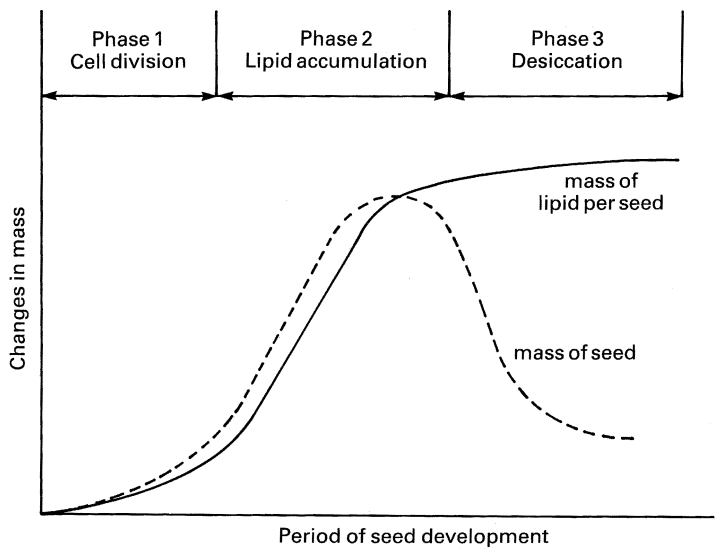


Fig. 3.7 The accumulation of lipid during seed development.

combination of acetyl-CoA carboxylase and fatty acid synthase, palmitoyl-acyl carrier protein (ACP) is produced. This is chain-lengthened by a specific condensing enzyme to stearoyl-ACP, which is desaturated to oleoyl-ACP by a Δ9-desaturase (Section 2.2.4.2). Under most conditions, palmitate and oleate are the main products of biosynthesis in plastids (chloroplasts in leaves). These acyl groups can be transferred to glycerol 3-phosphate within the organelle or hydrolysed, converted into CoA-esters and exported outside the plastid (Fig. 3.8). If fatty acids are esterified to glycerol 3-phosphate within the plastid, then 16C acids (mainly palmitate) are attached at position 2, whereas 18C acids (mainly oleate) are attached at position 1.

The biosynthesis of TAG for energy storage purposes takes place in the endoplasmic reticulum, not in the plastid. Therefore, during the relatively short period of oil accumulation (a few days only) mechanisms must be in place to export acyl groups from plastid to endoplasmic reticulum membranes. The first requirement is a thiolesterase to hydrolyse ACP derivatives to free fatty acids. During oil accumulation, the activity of this enzyme needs to be much higher than that of plastid GPAT and PAP (thought to be rate limiting for TAG biosynthesis)

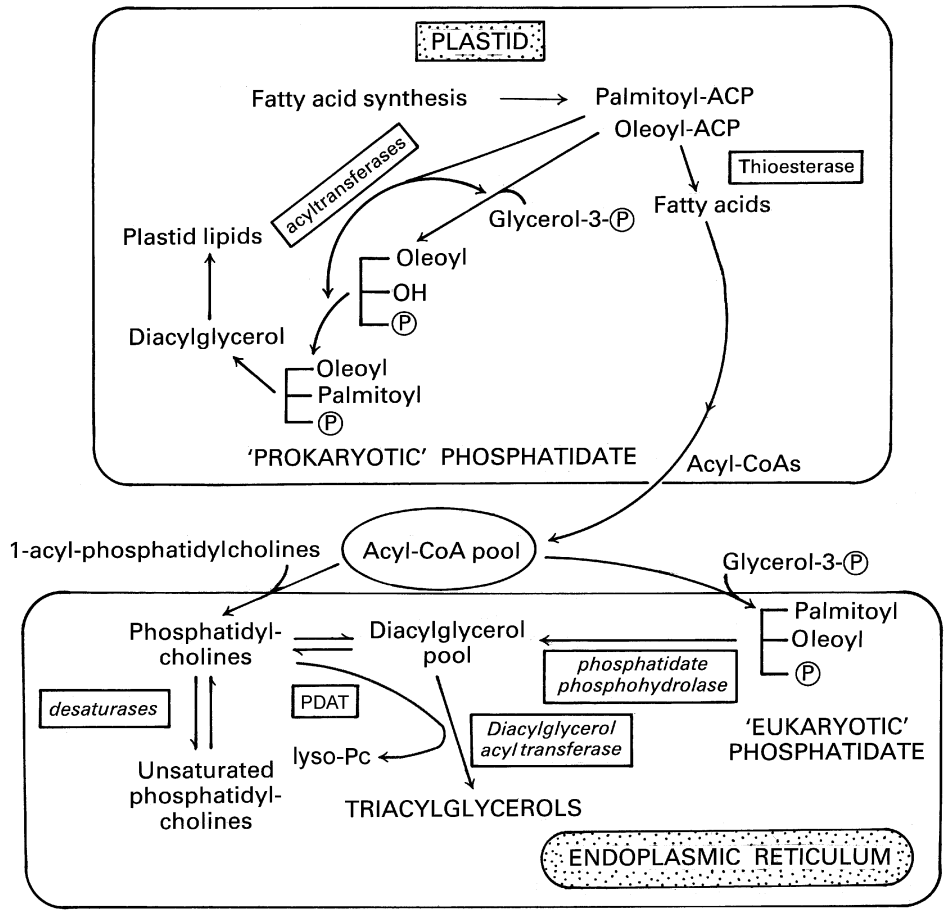


Fig. 3.8 The 'plant pathway' for triacylglycerol biosynthesis. lyso-Pc, 1-acyl phosphatidylcholine; PDAT, phospholipid: diacylglycerol acyltransferase. This newly discovered enzyme probably plays an important role in channelling unusual fatty acids out of membrane phosphoglycerides into TAG stores.

as there is no need for a rapid production of diacylglycerols in plastids when the carbon flux is mainly to endoplasmic reticulum.

After non-esterified fatty acids are attached to CoA in the plastid envelope they move rapidly to the endoplasmic reticulum (Fig. 3.8). It is not known how this transport takes place, although acyl-CoA-binding transport proteins have been purified from a number of plant tissues. It is clear, however, that acyl-CoAs (rather than acyl-ACPs as in the plastid) serve as substrates for acyltransferases in the endoplasmic reticulum.

GPAT, the enzyme that transfers an acyl group to position 1, generally has broad specificity and accepts both saturated and unsaturated fatty acids. The acyl group at position 1 should therefore broadly represent the distribution of acyl groups in the acyl-CoA pool. In some species that produce unusual seed-specific fatty acids (e.g. *Cuphea*) GPAT may exhibit particularly high specificity for that fatty acid. 'Unusual' fatty acids are almost invariably concentrated in TAG, little being esterified in membrane phospholipids. There must, therefore, be mechanisms for channelling 'unusual' acyl groups away from membrane lipids (where they might adversely influence membrane function) into storage lipids. One possible mechanism is that there are isoforms, specific to storage lipids, of each of the Kennedy pathway enzymes in the endoplasmic reticulum, although this remains to be demonstrated unequivocally. Microsomal fractions isolated from *Cuphea*, a plant whose seed oil contains high levels of medium-chain fatty acids, incorporate those fatty acids into TAG, actively excluding them from membrane lipids. Even seeds that do not normally synthesize 'unusual' fatty acids have a mechanism for excluding such fatty acids from their membrane lipids.

The enzyme LPAT, which catalyses the acylation at position 2, usually has a stricter specificity for unsaturated fatty acids. Thus, the action of GPAT and LPAT results in a fatty acid distribution in diacylglycerols produced in the endoplasmic reticulum that is the reverse of that in diacylglycerols produced in plastids (Fig. 3.8). For example, if a microsomal fraction of developing safflower seeds is incubated with 16:0-CoA alone, lysophosphatidate accumulates, with little formation of phosphatidic acid. Incubation with 18:1-CoA or 18:2-CoA, which are accepted efficiently by both GPAT and LPAT, results in the formation of phosphatidic acid. Likewise, accumulation of lysophosphatidate is also seen when a microsomal fraction from developing rapeseed is incubated with erucoyl-CoA. In rapeseed oil, erucic acid is exclusively located at positions 1 and 3. As erucic acid is a valuable industrial commodity (for use in lubricating oils) there is currently much interest in either modifying the substrate specificity of LPAT or introducing into rapeseed a gene for the enzyme from another plant species that does not discriminate against erucic acid.

A PAP located in the endoplasmic reticulum then acts to generate diacylglycerols. PAP may regulate carbon flux through the TAG biosynthesis pathway. As in mammalian cells, it exists in two separate forms, one associated with the endoplasmic reticulum membranes, the other in the cytoplasm. The amount of the enzyme attached to membranes is influenced by the local concentration of non-esterified fatty acids, thereby allowing feed-forward control over carbon flux to TAG.

The diacylglycerol products of PAP can be further acylated at position 3 with acyl-CoA, catalysed by the enzyme DAGAT to complete the Kennedy pathway. This enzyme usually has less specificity than the acyltransferases that esterify positions 1 and 2. Thus, in many plants, the fatty acids that accumulate at the *sn*-3 position depend upon the composition of the acyl-CoA pool. Nevertheless, in a few tissues, the substrate specificity of the DAGAT may also have an important role in determining the nature of the final stored lipid. From measurements of enzyme activities in vitro and because diacylglycerol accumulates during lipid deposition, it is often considered that DAGAT may have a regulatory role in the rate of TAG biosynthesis. It may also be involved in channelling 'unusual' fatty acids away from membrane phospholipids and into triacylglycerols as discussed above. Thus, the DAGAT of castor bean, when presented with a mixture of di-ricinoleoyl and dioleoyl species in vitro, specifically selects the di-ricinoleoyl species.

During active oil accumulation, carbon from diacylglycerols is preferentially channelled into TAG biosynthesis. However, at other phases of the seed's life cycle, the utilization of diacylglycerols for the biosynthesis of membrane phospholipids, such as phosphatidylcholine, catalysed by the enzyme CDPcholine:diacylglycerol choline phosphotransferase, is more important. In some plants, the latter enzyme may also play a role in TAG biosynthesis, however, as illustrated in Fig. 3.8. The reaction catalysed by this enzyme is approximately in equilibrium and can, therefore, allow the rapid exchange of diacylglycerols between their pool and that of the newly synthesized phosphatidylcholine. Since phosphatidylcholine is the substrate for oleate (and linoleate) desaturation in seeds, the reversible nature of the choline phosphotransferase allows the diacylglycerol pool to become enriched in polyunsaturated fatty acids. This process has been studied in particular detail in safflower (which has an oil rich in polyunsaturated fatty acids, comprising 75% linoleate). It has also been shown that the acyl-CoA pool can also be utilized through the activity of an acyl-CoA:lysophosphatidylcholine acyltransferase. In contrast, for seeds where less unsaturated oils accumulate (e.g. avocado) the subsidiary flux of diacylglycerols through phosphatidylcholines and their consequent desaturation, is much less important.

In some plants, diacylglycerol molecules may have yet another fate. In their studies with developing safflower seed microsomal fractions, Stobart and Stymne made a puzzling observation. Apparently TAG could be formed from diacylglycerols in the absence of acyl-CoA. The most likely explanation is a transacylase reaction in which two molecules of diacylglycerol can form one of monoacylglycerol and one of triacylglycerol (Fig. 3.8). It is further proposed that the monoacylglycerols thus formed are rapidly converted first into diacylglycerols and then into TAG. Details of the transacylation mechanism are still to be elucidated and its significance is a matter of speculation. It may be involved in 'retailoring' molecular species of TAG or of regulating the size of the diacylglycerol pool and protecting phospholipid biosynthesis at a period of rapid oil accumulation.

From this discussion it will be apparent that the plastid and endoplasmic reticulum compartments of the plant cell have to integrate their metabolism during lipid storage (Fig. 3.8). Questions remaining to be answered include the mechanism by which acyl groups are exported from plastid to endoplasmic reticulum, how specific fatty acids are channelled into storage TAG and excluded from membrane phospholipids (Fig. 3.8), how switching between phospholipid and TAG biosynthesis is regulated during seed development and what, if any, role the transacylation of diacylglycerols plays in regulating these processes.

3.4.2 The monoacylglycerol pathway is important mainly in rebuilding TAG from absorbed dietary fat

The main function of the monoacylglycerol pathway (Fig. 3.6) is to resynthesize TAG from the monoacylglycerols formed during the digestion of fats in the small intestine. Therefore, this mechanism is one by which existing TAG are modified rather than one by which new fat is formed (Section 5.1.3). During the hydrolysis of dietary TAG in the intestinal lumen by pancreatic lipase, the fatty acids in positions 1 and 3 are preferentially removed. The remaining 2-monoacylglycerols are relatively resistant to further hydrolysis. When 2-monoacylglycerols, radiolabelled in both fatty acid and glycerol moieties, were given in the diet, the molecules were absorbed intact, reacylated and secreted into the lymph without dissociation of the label. The pathway involves a stepwise acylation first of monoacylglycerols, then diacylglycerols and was first discovered by Georg Hubscher's research team in Birmingham, UK in 1960.

The reactions are catalysed by enzymes in the endoplasmic reticulum of the enterocytes of many species of animals. There is some controversy about the exact location of the enzymes and this is described in some detail in the review by Lehner and Kuksis (see Further Reading). The 2-monoacylglycerols are the preferred substrates compared with 1-monoacylglycerols and the rate of the first esterification is influenced by the nature of the fatty

acid esterified at position 2. Monoacylglycerols with medium-chain saturated or longer chain unsaturated fatty acids are the best substrates. However, it is worth stressing, as we do so frequently in this book, that such generalizations are made almost entirely from the results of studies with subcellular preparations in vitro, which may not necessarily obtain in vivo. Differences in solubility of the added substrates may limit the proper interpretation of the results. If the reaction of monoacylglycerol acyltransferase were completely stereospecific, it would be expected that the reaction products would be entirely either *sn*-1,2- or *sn*-2,3-diacylglycerols. In fact, several studies, employing different analytical methods, have indicated that the reaction mixture contains about 90% 1,2- and about 10% 2,3-diacylglycerols, so the reaction may not be completely stereospecific.

Diacylglycerol acyltransferase is specific for 1,2-*sn*-diacylglycerols and will not acylate the *sn*-2,3- or *sn*-1,3-isomers. Diunsaturated or mixed-acid diacylglycerols are better substrates than disaturated compounds. Again, we have to be cautious when we interpret results of this kind. Lipids containing unsaturated fatty acids are more easily emulsified than saturated ones, so that we may not be observing differences in specificity of the enzyme for fatty acid composition, but differences in solubility of the substrates when the enzyme assays are performed in vitro. There is little information on whether diacylglycerol acylating activity in the monoacylglycerol pathway is identical to that in the glycerol phosphate pathway or whether different isoforms of the enzyme are involved.

Attempts to purify the individual enzymes involved in the monoacylglycerol pathway have met with only partial success. Frequently (but not always!) the partly purified preparation has contained all three enzyme activities: monoacylglycerol and diacylglycerol acyltransferases and acyl-CoA synthetase. This has led to the concept that there is a complex of enzymes acting in concert, now generally referred to as 'triacylglycerol synthase' or 'triacylglycerol synthase complex'. A molecular mass of 350 kDa has been proposed by the Canadian, Kuksis. A more purified preparation that migrated as a 37 kDa band on SDS-PAGE had monoacylglycerol acyltransferase activity, but whether it was a genuine subunit of the complex or a proteolytic fragment was not clear.

The monoacylglycerol pathway has also been demonstrated in the liver and adipose tissue of the hamster and the rat and these enzymes have also been partly purified. The activity is particularly high in pig liver. Under certain conditions it appears to compete with the glycerol phosphate pathway for acyl groups and may serve to regulate the activity of the latter pathway. The origin of the monoacylglycerol substrate in tissues other than intestine is not known and the role of the pathway in these other tissues is far from clear.

The liver and intestinal enzymes differ in substrate specificity, thermolability and response to different inhibitors, suggesting the existence of separate isoforms. Because the liver isoform has a preference for 2-monoacylglycerols that contain polyunsaturated fatty acids, it has been suggested that one of its roles may be to prevent excessive degradation of polyunsaturates under conditions of high rates of β-oxidation. Consistent with this proposal is the high activity of liver monoacylglycerol acyltransferase in neonatal life and in hibernating animals. The adipose tissue isoform is particularly active in birds during migration. The normal neonatal rise in monoacylglycerol acyltransferase is attenuated in rat pups given an artificial high-carbohydrate diet compared with those sucking mother's milk.

An alternative route to TAG assembly in intestinal tissue was discovered by Lehner and Kuksis in the early 1990s. This involves the transfer of acyl groups between two diacylglycerol molecules without the intervention of acyl-CoA, catalysed by diacylglycerol transacylase (Fig. 3.6). The activity is located in endoplasmic reticulum and has been solubilized and purified to homogeneity as a 52 kDa protein. Its precise function is unknown but because its activity can be as much as 15% of that of diacylglycerol acyltransferase, it could supply significant amounts of TAG. The substrates are 1,2- and 1,3-diacylglycerols and the monoacylglycerol product can also be fed into the monoacylglycerol pathway.

3.5 THE CATABOLISM OF ACYLGLYCEROLS

This section is concerned entirely with the hydrolysis of fatty acids from one or more positions on the glycerol backbone of acylglycerols, catalysed by enzymes termed lipases, which act mainly at the surfaces of large fat particles.

Catabolism refers to the metabolic breakdown of complex biological molecules. One of the main themes running through this book is that, with very few exceptions, lipids in biological tissues are in a dynamic state: they are continually being synthesized and broken down. This is known as turnover. Complete catabolism of acylglycerols takes place in two stages. Hydrolysis of the ester bonds that link fatty acyl chains to the glycerol backbone is brought about by enzymes known as lipases. Following the action of a lipase, releasing fatty acids, these may be further catabolized by oxidation (Section 2.3). Acylglycerols themselves are not substrates for oxidation. Alternatively, however, the fatty acids released from acylglycerols may follow other metabolic pathways, including re-esterification with glycerol to make new acylglycerols. Therefore the lipases may be seen as 'gatekeepers', releasing fatty acids from the acylglycerol energy stores for oxidation or for further metabolism.

There is a large number of lipases, some related in families, and they differ in respect to their substrates and the positions in substrates of the bonds that they hydrolyse. There are also many enzymes called esterases that hydrolyse ester bonds in general, but lipases form a distinct class and the distinction lies in the physical state of their substrates. The milieu in which a lipase acts is heterogeneous: the lipid substrate is dispersed as an emulsion in the aqueous medium, or is present as a fat droplet (e.g. within the mammalian adipocyte), and the enzyme acts at the interface between the lipid and aqueous phases. If, by some means, a single-phase system is obtained, for example when the triacylglycerol contains short-chain fatty acids (as in triacetin) or when a powerful detergent is present, then the lipid may be hydrolysed by an esterase, not a lipase.

Our understanding of lipase action has increased markedly in recent years with the use of X-ray crystallography to determine the three-dimensional structure of one such enzyme, pancreatic lipase (Section 5.1.1). This work, which has involved the crystallization of pancreatic lipase in the presence of emulsified fat, has provided a mechanistic basis for the phenomenon of interfacial activation, the activation of the enzyme that occurs on the surface of an oil-in-water droplet. Most importantly, in the presence of emulsified lipid, conformational changes in the enzyme lead to the opening of a 'lid' that allows access of the substrate to the active site. This lid-opening mechanism appears to be common to other members of the same family of lipases.

3.5.1 The nature and distribution of lipases

Lipases are widespread in nature and are found in animals, plants and micro-organisms. The initial step in the hydrolysis is generally the splitting of the fatty acids esterified at positions 1 and 3 (see Box 3.1). TAG lipases in general are ineffective in hydrolysis of the 2-position ester bond. Therefore 2-monoacylglycerols tend to be produced, at least in artificial models of lipase action. In vivo these may be removed by cellular uptake, by non-enzymic isomerization to the 1(3)-form so that complete hydrolysis can occur, or by the action of a more specific monoacylglycerol lipase.

Some lipases not only hydrolyse fatty acids on the primary positions of acylglycerols, but will also liberate the fatty acid esterified at position 1 of phosphoglycerides. In most cases, the rate of hydrolysis is independent of the nature of the fatty acids released. There are, however, several exceptions to this rule. The ester bonds of fatty acids with chain lengths less than 12 carbon atoms, especially the very short chain lengths of milk fats, are cleaved more rapidly than those of the normal chain length fatty acids (14C–18C), while the ester bonds of the very long chain polyenoic acids (20:5 and 22:6) found in the oils of fish and marine mammals are more slowly hydrolysed. The lipases in some micro-organisms have fatty acid specificity. For example, the fungus *Geotrichum candidum* possesses

a lipase that seems to be specific for oleic acid in whichever position it is esterified.

3.5.2 Animal triacylglycerol lipases play a key role in the digestion of food and in the uptake and release of fatty acids by tissues

Animal TAG lipases may be broadly classified into extra-cellular and intracellular. Extra-cellular lipases are secreted from their cells of synthesis and act upon TAG that are present in the extra-cellular environment, releasing fatty acids that may be taken up into cells. Therefore, they are concerned mainly with cellular uptake of fatty acids. There are four major extra-cellular TAG lipases (in adults), which are members of one family and have sequence and structural similarities to each other. Pancreatic lipase is, as its name suggests, secreted from the exocrine tissue of the pancreas into the small intestine, where it acts to hydrolyse dietary TAG so that the constituent fatty acids and monoacylglycerols may be absorbed from the intestine into enterocytes. Its action is described in more detail in Section 5.1.1.

Within the body, TAG circulates in the plasma in the form of macromolecular complexes, the lipoproteins (Section 5.2). Some lipoprotein TAG is taken up directly into cells that express receptors that bind and internalize complete particles, but this is mainly a route for the clearance of 'remnant' particles, once they have lost most of their TAG. Most of the TAG is hydrolysed within the vascular compartment by the enzyme lipoprotein lipase, to release fatty acids that may again be taken up into cells. (This enzyme is called clearing factor lipase in older literature, because of its ability to clear the turbidity of the plasma caused by the presence of large TAG-rich lipoprotein particles.) Lipoprotein lipase is related to pancreatic lipase. It is synthesized within the parenchymal cells of tissues (e.g. adipocytes, muscle cells, milk-producing cells of the mammary gland), but then exported to the endothelial cells that line blood capillaries. Here it is bound to the luminal membrane of the endothelial cell (facing into the blood) where it can act upon the TAG in lipoprotein particles as they pass by. The fatty acids it releases may then diffuse into the adjacent cells, for re-esterification and storage (e.g. in adipose tissue) or for oxidation (in muscle).

Hepatic lipase is the third member of this family. As its name suggests, it is synthesized within the liver and, just like lipoprotein lipase, exported to bind to the endothelial cells that line the hepatic sinusoids – the tiny vessels that are the liver's equivalent of capillaries. It plays a role particularly in hydrolysis of TAG in smaller lipoprotein particles, and also assists in binding these particles to the receptors that may remove them from the circulation.

The fourth member of the family is known as endothelial lipase. It was discovered only in 1999 and its function is not yet clear. It is expressed by the endothelial cells in several tissues including liver, lung, kidney and placenta, and some endocrine tissues including thyroid, ovary and testis. It is more active as a phospholipase (with A1-type activity, Section 7.2) than as a TAG lipase. It has been suggested that it plays a role in lipoprotein metabolism and vascular biology.

Of the intracellular lipases, one has been particularly well studied: the so-called hormone-sensitive lipase expressed in adipocytes and in some cells with an active pattern of steroid metabolism (adrenal cortex cells, macrophages, testis). Its name reflects the fact that its activity is rapidly regulated by a number of hormones (Section 3.6.3) although this can be confusing because other lipases, especially lipoprotein lipase, are also regulated by hormones. Hormone-sensitive lipase acts on the surface of the triacylglycerol droplet stored within adipocytes, to release fatty acids that may be delivered into the circulation for transport to other tissues where they may be substrates for oxidation or for re-esterification to glycerol. Hormone-sensitive lipase is also a cholesteryl esterase and acts with equal efficiency to hydrolyse cholesteryl esters: this is presumably its role in cells that metabolize steroids. Adipose tissue also contains a lipase that is much more active on monoacylglycerols than is hormone-sensitive lipase.

Lipases are still being discovered and it may be a long time before the whole complex jigsaw puzzle of glyceride breakdown can be pieced together. For

instance, it is known that an intracellular lipase is involved in hydrolysis of triacylglycerol within hepatocytes, releasing fatty acids that are then re-esterified before incorporation into very low density lipoprotein particles (the lipoprotein particles secreted by the liver; see Section 5.2.3). This lipase has not yet been characterized.

3.5.3 Plant lipases break down the lipids stored in seeds in a specialized organelle, the glyoxysome

Seeds that contain lipid may have as much as 80% of their dry weight represented by triacylglycerols. Plants such as soybean face two particular problems in using such energy reserves. First, these plants have to mobilize the lipid rapidly and break it down to useful products. This overall process involves the synthesis of degradative enzymes as well as the production of the necessary membranes and organelles that are the sites of such catabolism. Secondly, plants with lipid-rich seeds must be able to form water-soluble carbohydrates (mainly sucrose) from the lipid as a supply of carbon to the rapidly elongating stems and roots. Animals are unable to convert lipid into carbohydrate (Fig. 3.9) because of the decarboxylation reactions of the Krebs (tricarboxylic acid) cycle (isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase). Thus, for every two carbons entering the Krebs cycle from lipid as acetyl-CoA, two carbons are lost as CO_2. In plants, these decarboxylations are avoided by a modification of the Krebs cycle, which is called the glyoxylate cycle. This allows lipid carbon to contribute to the synthesis of oxaloacetate, which is an effective precursor of glucose and hence, sucrose.

When water is imbibed into a dry seed, there is a sudden activation of metabolism once the total water content has reached a certain critical proportion. So far as lipid-storing seeds are concerned, TAG lipase activity is induced and studies by Huang in California and others suggest that this lipase interacts with the outside of the oil droplet through a specific protein (an oleosin; Section 3.3.3) that aids its binding. This interaction has some similarities to the binding of lipoprotein lipase to very low density lipoproteins in animals (Sections 3.5.2 and 5.2.5). The lipases in seeds hydrolyse the 1 and 3 positions of TAG and because the acyl group of the 2-monoacylglycerol products can migrate rapidly to position 1, the lipases can completely degrade the lipid stores.

The fatty acids that are liberated are activated to CoA-esters and broken down by a modification of β-oxidation, which takes place in specialized microbodies. Because these microbodies also contain the enzymes of the glyoxylate cycle (see above), they have been termed glyoxysomes by the Californian biochemist, Harry Beevers. Beevers and his group worked out methods for the isolation of

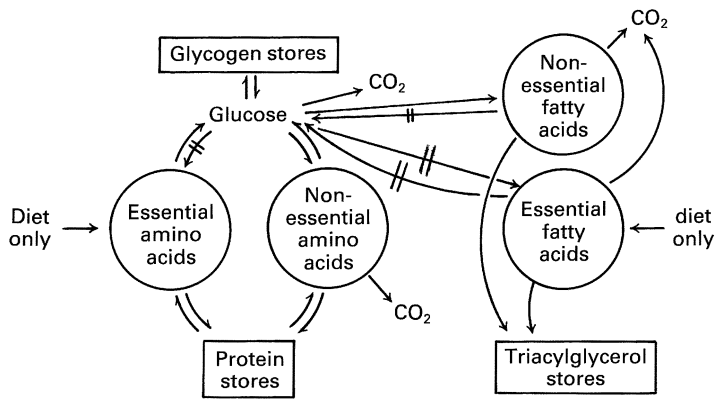


Fig. 3.9 Permitted and forbidden interconversions of fats, carbohydrates and proteins in animals.

glyoxysomes from germinating castor bean seeds and showed by careful study that fatty acid β-oxidation was confined to this organelle. The glyoxysomes have only a temporary existence. They are formed during the first two days of germination and, once the lipid stores have disappeared (after about 6 days), they gradually break down. Nevertheless, in leaf tissues, the role of glyoxysomes in the β-oxidation of fatty acids is replaced by other microbodies (Section 2.3.1.1).

Most plant lipases appear to be membrane-bound although soluble enzymes are present in some tissues. There has been some interest in using the latter in the food industry in order to carry out the transesterification of triacylglycerols and, thus, modify their composition for certain purposes [e.g. to make fats that resemble cocoa butter ('cocoa butter equivalents') for chocolate]. These transesterification reactions are favoured by very low water contents (usually less than 1%).

Plant lipase activities also have implications for food quality, when the release of unesterified fatty acids can cause deterioration. For example, during olive oil production, significant endogenous fatty acid levels, themselves, determine whether an oil can be considered high quality ('extra virgin') or not. Alternatively, in poorly-stored wholemeal flour, endogenous lipases give rise to fatty acids that can be broken down by lipoxygenases (Sections 2.3.5 and 2.3.6) and cause off-flavours and a reduction in baking quality.

3.6 THE INTEGRATION AND CONTROL OF ANIMAL ACYLGLYCEROL METABOLISM

The major function of TAG in animals is as a source of fatty acids to be used as metabolic fuel. A full description of the overall fuel economy of the body requires an understanding of the metabolism (storage, transport, synthesis, oxidation) of the fatty acids as well as of the integration of fat and carbohydrate metabolism.

3.6.1 Fuel economy: the interconversion of different types of fuels is hormonally regulated to maintain blood glucose concentration within the normal range and ensure storage of excess dietary energy in triacylglycerols

The maintenance of fuel reserves within fairly narrow limits is referred to as fuel homeostasis. Glycogen is stored within muscle and liver cells (and in small amounts elsewhere, e.g. in the brain). Because glycogen is stored in a hydrated form (Section 3.3.1) the amount that can be stored in a cell is limited, and also it would be disadvantageous for the body to carry around excessive amounts of this fuel source. In adult humans the liver glycogen store is typically about 100 g, and it is probably no coincidence that this is approximately the amount of glucose needed by the brain in 24 h. Glycogen stores are for immediate, 'emergency' use in maintaining carbohydrate supply for tissues that require it, such as the brain, and their total amount is held within fairly narrow limits. Protein is potentially a major energy source (the adult human body has about 12.5 kg of protein). However, there is no specific storage form of protein, and so all body protein plays some role (enzymes, structural, etc.). Therefore, it is not generally used as a fuel store, and indeed its degradation during starvation is specifically spared. Again, therefore, its amount appears to be controlled within fairly narrow limits. In contrast, humans and other mammals appear to have an almost infinite capacity for storage of TAG, achieved both by expansion of existing adipocytes (so that a mature, fat-filled human adipocyte may be 0.1 mm in diameter) and by an increase in the number of adipocytes.

The various fuels can be interconverted to some extent. Excess carbohydrate and protein can be converted into fat, and amino acids from protein can be converted into carbohydrate (as happens during starvation as a means of supplying new glucose). However, fatty acids cannot be converted into carbohydrate (Section 3.5.3; Fig. 3.9) and do not appear to be significant precursors of amino acids.

The constituent glycerol from triacylglycerols that have been hydrolysed to release fatty acids may, however, be an important precursor of glucose during starvation.

When the energy in the diet exceeds immediate requirements, excess carbohydrate is preferentially used to replenish glycogen stores. Excess protein tends to be oxidized after satisfying the tissues' needs for protein synthesis. Any remaining excess of either fuel, or of fat, then tends to be converted into triacylglycerol for storage in adipose tissue. Adipose tissue TAG is the ultimate repository for excess dietary energy.

The control of these interconversions is mediated by the amount of energy in the diet, the nature of the dietary constituents and the concentrations of the relevant hormones in the blood. A dominant role is played by insulin. Its concentration in the blood helps to co-ordinate the flow of fuel either into storage or from the stores into various tissues as required. High concentrations of insulin characterize the fed state when ample fuel is available from the diet; low levels signal the starved state when the animal's own reserves need to be called upon. However, it has recently become clear that dietary fatty acids themselves may play an important role in regulating the expression of various genes involved in these processes (Section 5.3).

3.6.2 The control of acylglycerol biosynthesis is important, not only for fuel economy, but for membrane formation, requiring close integration of storage and structural lipid metabolism

An important concept of metabolism is that of turnover, which envisages the continual renewal, involving biosynthesis and breakdown, of body constituents. The rates of turnover may differ widely between different biological molecules in different tissues. The rates of forward and backward reactions may also differ. Thus, when there is net synthesis of the tissue component, the rate of synthesis is faster than the rate of breakdown, but both may be proceeding simultaneously. When metabolic control is exercised, by whatever means, it may affect the rate of synthesis, the rate of degradation, or both. Turnover allows a fine degree of control of metabolic pathways. Control may be exercised on the synthesis or degradation of enzymes catalysing the metabolic reactions at the level of gene transcription or translation, or on the allosteric control of enzymes by small molecules or cofactors.

In animals, net TAG synthesis occurs when energy supply exceeds immediate requirements. Most diets contain both fat and carbohydrates. When there is an excess of energy as carbohydrates, the body switches mainly to a pattern of carbohydrate oxidation so that these are used as the preferential metabolic fuel. When there is a considerable excess of carbohydrates for some time, then the tissues convert them into fatty acids that are esterified into acylglycerols. Conversely, when there is a preponderance of fat in the diet, fat synthesis from carbohydrate is depressed in the tissues and fat oxidation and fat storage will tend to predominate. This involves conversion of the products of fat digestion into lipoproteins (Section 5.2), which circulate in the bloodstream. When the lipoproteins reach the tissues, fatty acids are released from the acylglycerols at the endothelial lining of the capillaries, a process catalysed by the enzyme lipoprotein lipase (Section 3.5.2). The fatty acids are taken up into the cells (Fig. 3.10). Once inside, they are either oxidized or esterified into acylglycerols. In discussing the control of acylglycerol synthesis, we shall be discussing the esterification of fatty acids synthesized *de novo* or released from circulating lipoproteins. The control of fatty acid synthesis itself is discussed in Section 2.2.8.

Although acylglycerols may be synthesized in many animal tissues, the most important are: the small intestine, which resynthesizes TAG from components absorbed after digestion of dietary fats; the liver, which is concerned mainly with synthesis from carbohydrates and redistribution; adipose tissue, which is concerned with longer term storage of fat; and the mammary gland, which synthesizes milk fat during lactation. There is also turnover of a relatively smaller pool of TAG within muscle cells.

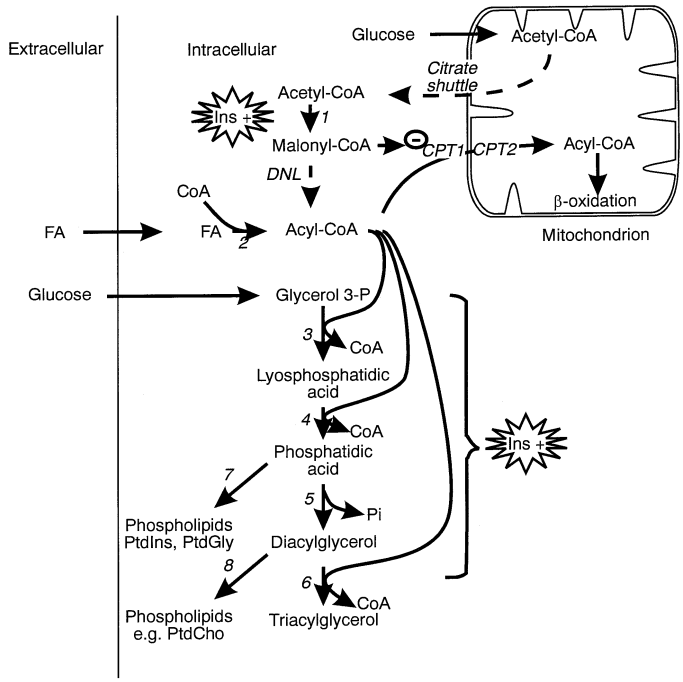


Fig. 3.10 Central role of acyl-CoA in hepatic lipid metabolism. Acyl-CoA is formed either from esterification (sometimes called activation) of a fatty acid with CoA, or from the pathway of *de novo* lipogenesis (DNL). It may be utilized for β-oxidation in the mitochondria (catabolism) or for glycerolipid biosynthesis. The relative rates of these pathways are determined by the nutritional state, mediated largely by variations in plasma insulin concentration. When insulin concentrations are high (well-fed state) (shown as Ins +), generation of malonyl-CoA through the activity of acetyl-CoA carboxylase (1) inhibits entry of acyl-CoA into the mitochondrion by inhibition of carnitine palmitoyl transferase-1 (CPT_1). At the same time, the pathway of glycerolipid synthesis is stimulated by insulin. Other enzymes/pathways shown are: (2) acyl-CoA synthase (also known as acid:CoA ligase); (3) acyl-CoA:glycerol phosphate 1-O-acyl transferase (GPAT); (4) acyl-CoA:1-acylglycerol phosphate 2-O-acyl transferase (LPAT); (5) phosphatidate phosphohydrolase (PAP); (6) diacylglycerol acyltransferase (DGAT); (7) pathway of phospholipid biosynthesis via CDP-DAG (see Figs 7.1 and 7.3) leading to phosphatidylinositol (PtdIns), phosphatidylglycerol (PtdGly) and others; (8) 1,2-diacylglycerol:choline phosphotransferase, leading to phosphatidylcholine (PtdCho) and other phospholipids (see Figs 7.1 and 7.2). Glucose metabolism is also shown in outline; glucose (via glycolysis) leads to the provision of glycerol 3-phosphate, and via pyruvate dehydrogenase in the mitochondrion to acetyl-CoA. For acetyl-CoA produced by this route to be a substrate for *de novo* lipogenesis, it must be exported to the cytoplasm using the citrate shuttle.

In the enterocytes of the small intestine, the rate of influx of dietary fatty acids appears to be the main factor controlling the rate of TAG synthesis via the 2-monoacylglycerol pathway. The glycerol 3-phosphate pathway seems to supply a basal rate of TAG synthesis between meals in the enterocyte. In other tissues, in which the glycerol 3-phosphate pathway is predominant, TAG synthesis is more tightly regulated by the prevailing nutritional state.

The nutritional regulation of acylglycerol synthesis has been mostly studied in liver and adipose tissue. In neither tissue, however, is our understanding complete, mainly because of the difficulty of isolating the relevant enzymes, which are insoluble and membrane-bound. Attempts to purify the enzyme diacylglycerol acyltransferase, for example, have been described as 'masochistic enzymology'. This has changed as modern methods of cloning by homology searching (screening cDNA libraries for related sequences) have been applied.

The cellular concentrations of the substrates for the acyltransferases that catalyse acylglycerol esterification, acyl-CoA and glycerol 3-phosphate, are influenced by nutritional status. Since glycerol 3-phosphate is produced from dihydroxyacetone phosphate, an intermediate in the pathways of glycolysis and gluconeogenesis, the main factors influencing the amounts of glycerol 3-phosphate available for acylglycerol synthesis are those that regulate the levels and activities of the enzymes of glucose metabolism. Starvation reduces the intracellular concentration of glycerol 3-phosphate severely, whereas carbohydrate feeding increases it. Intracellular concentrations of acyl-CoA increase during starvation.

In adipose tissue there is evidence that the supply of glycerol 3-phosphate may regulate TAG synthesis. Insulin stimulates glucose uptake by adipocytes and, by implication, glycerol 3-phosphate production. Certainly TAG synthesis in adipose tissue increases in the period following a meal, as is necessary to accommodate the influx of fatty acids from lipoprotein lipase (itself stimulated by insulin) acting on circulating TAG in the capillaries. This might equally, though, reflect stimulation of the enzymes of TAG synthesis by insulin and other factors. One such factor is the 76-amino acid peptide known as acylation stimulating protein (ASP). The production of ASP from adipocytes will be described in more detail in Section 5.3.4.

In the liver, however, there is little evidence that intracellular concentrations of glycerol 3-phosphate or acyl-CoA are important in regulating acylglycerol synthesis. Instead, the rate of acylglycerol synthesis in the liver appears to reflect the relative activities of the competing pathways for fatty acid utilization, acylglycerol synthesis and β-oxidation (Fig. 3.10). Entry of fatty acids into the mitochondrion for oxidation is mediated by carnitine palmitoyl transferase 1 (CPT_1; Section 2.3.1), and this enzyme is powerfully suppressed by an increase in the cytosolic concentration of malonyl-CoA, the first committed intermediate in fatty acid synthesis (Section 2.2.3). The formation of malonyl-CoA is stimulated under well-fed conditions (when insulin levels are high; Section 2.2.8) and so fatty acid oxidation will be suppressed and acylglycerol synthesis favoured under these conditions. The opposite will be true in starvation or energy deficit. An important question is, whether there is also coordinated regulation of the enzymes of acylglycerol synthesis. The answer appears to be yes, but pinpointing the locus of control has proved difficult.

The enzymes that may be involved in regulation of mammalian acylglycerol biosynthesis include the acyltransferases that link successive molecules of acyl-CoA to the glycerol backbone, and phosphatidate phosphohydrolase (PAP) (Fig. 3.6). The first of these enzymes is GPAT (Section 3.4.1). It has been suggested that GPAT and CPT_1 (see above), both expressed on the outer mitochondrial membrane, represent an important branch point in fatty acid metabolism, leading acyl-CoA into esterification or oxidation, respectively. GPAT expression is generally regulated in parallel with the rate of TAG synthesis in different nutritional states. This regulation is brought about by a number of factors including insulin, for which there is a specific response element in the promoter region of the mitochondrial GPAT gene. GPAT expression is regulated almost exactly in parallel with the expression of fatty acid synthase.

Nutritional and hormonal factors appear to influence the mitochondrial GPAT activity more than the microsomal. Thus, when fasted rats are given a diet low in fat and rich in carbohydrate, mitochondrial GPAT activity increases sixfold with little change in the microsomal activity. Similarly in perfused rat liver, inclusion of insulin in the perfusion fluid increases the mitochondrial GPAT activity four times more than the microsomal. During starvation, hepatic mitochondrial GPAT activity decreases, but the overall capacity for TAG biosynthesis in the liver remains unchanged provided that β-oxidation is inhibited. It seems that in starvation, the decrease in GPAT activity is due primarily to competition by CPT_1 for acyl-CoA (Section 2.3.1.6).

The activity of the next enzyme in the pathway, LPAT, is increased 2.5-fold in liver post-natally and about 60-fold during the differentiation of 3T3-L1 adipocytes. Changes in mRNA for the enzyme in response to dietary changes have not been reported.

The activity of PAP, like that of GPAT, generally runs parallel to the potential for overall acylglycerol synthesis in that tissue, and it has been suggested that PAP is the major locus for regulation of TAG biosynthesis. However, it now appears more likely that control is 'shared' by a number of enzymes. PAP activity in liver is increased by high levels of dietary sucrose and fat, by ethanol and by conditions, such as starvation, that result in high concentrations of plasma non-esterified fatty acids. It is also increased in obese animals. It is decreased in diabetes and by administration of drugs that result in a reduction of circulating lipid concentrations. The factors that tend to increase the activity of PAP are also those that result in an increased supply of saturated and monounsaturated fatty acids to the liver, namely those fatty acids normally esterified in simple acylglycerols. If PAP activity is low, the substrate for the enzyme – phosphatidic acid, the central intermediate in lipid metabolism – does not accumulate, but becomes a substrate for the biosynthesis of acidic membrane phospholipids such as phosphatidylinositol (Sections 7.1.6 and 7.9). Phospholipid metabolism makes more extensive demands on a supply of unsaturated fatty acids than does simple acylglycerol metabolism and the activities of enzymes that divert phosphatidic acid into phospholipid rather than TAG metabolism tend to be elevated in conditions where unsaturated fatty acids predominate (Fig. 3.10).

PAP exists in the cytosol and in the endoplasmic reticulum. There is another isoform associated with the cell membrane, but this is thought to be involved more with signal transduction than TAG biosynthesis. The cytosolic enzyme is physiologically inactive, but translocates to the membranes of the endoplasmic reticulum on which phosphatidate is being synthesized. The translocation process seems to be regulated both by hormones and substrates. Cyclic-AMP (cAMP), for example, displaces the enzyme from membranes, whereas increasing concentrations of non-esterified fatty acids and their CoA esters promote its attachment. Insulin, which has the effect of decreasing intracellular concentrations of cAMP, ensures that the translocation is more effective at lower fatty acid concentrations. The mechanisms that cause these changes are not yet established but may involve the reversible phosphorylation of PAP. It seems, therefore, that the membrane-associated PAP is the physiologically active form of the enzyme and the cytosolic form represents a reservoir of potential activity. This phenomenon of translocation is seen with some other enzymes involved in lipid metabolism. Enzymes that exist in different locations in the cell and can regulate metabolism by moving from one location to another are called ambiquitous enzymes. Other examples in lipid metabolism are the CTP:phosphorylcholine cytidylyl transferase, which regulates the biosynthesis of phosphatidylcholine (Section 7.1.5), and hormone-sensitive lipase in adipocytes (Section 3.6.3).

The diacylglycerol formed by the action of PAP can be used either for TAG synthesis (Fig. 3.6), or for glycerophospholipid synthesis (Fig. 7.1). The biosynthesis of phospholipids takes precedence over that of TAG when the rate of synthesis of diacylglycerol is relatively low. This ensures the maintenance of membrane turnover and bile secretion, which are more essential processes in physiological terms than the accumulation of TAG. The precise mechanisms for the preferred synthesis of phosphoglycerides are not certain, however. Among the factors involved is probably a relatively low K_m of choline phosphotransferase for diacylglycerol. A factor that may eventually limit the biosynthesis of phosphatidylcholine is a limitation in the supply of choline and it is well recognized that a major effect of choline deficiency is fatty liver indicating a diversion away from phospholipid biosynthesis into TAG biosynthesis.

The last enzyme in the biosynthesis of TAG, DAGAT, is highly expressed in tissues that have a high rate of TAG synthesis, including small intestine and adipocytes. However, the level of expression is relatively low in the liver, which is surprising and leaves open the possibility that yet another enzyme is still awaiting identification. As yet, little is known of its regulation, although it has been claimed to be an important locus of control of TAG synthesis by acylation stimulating protein (ASP; Section 5.3.4) in adipocytes. There is evidence for short-term regulation of its activity by reversible

phosphorylation, but this has not yet been shown conclusively.

Further research into the hormonal and nutritional control of these processes may give insights into how to control the common diseases of lipid metabolism (Section 5.4).

3.6.3 Mobilization of fatty acids from the fat stores is regulated by hormonal balance, which in turn is responsive to nutritional and physiological states

In physiological states demanding the consumption of fuel reserves, the resulting low concentrations of insulin turn off the biosynthetic pathways and release the inhibition of hormone-sensitive lipase within adipocytes. The activity of hormone-sensitive lipase is regulated in the short term by a cascade mechanism illustrated in Fig. 3.11. In the longer term it is also regulated by control of transcription; its expression is up-regulated, for instance, during prolonged fasting.

Short-term regulation is brought about by reversible phosphorylation. The phosphorylated enzyme is active and the dephosphorylated form inactive. Changes in activity in vivo, or in intact cells, are much greater than can be achieved when the purified enzyme is phosphorylated in vitro and acts on a synthetic lipid emulsion. This has led to

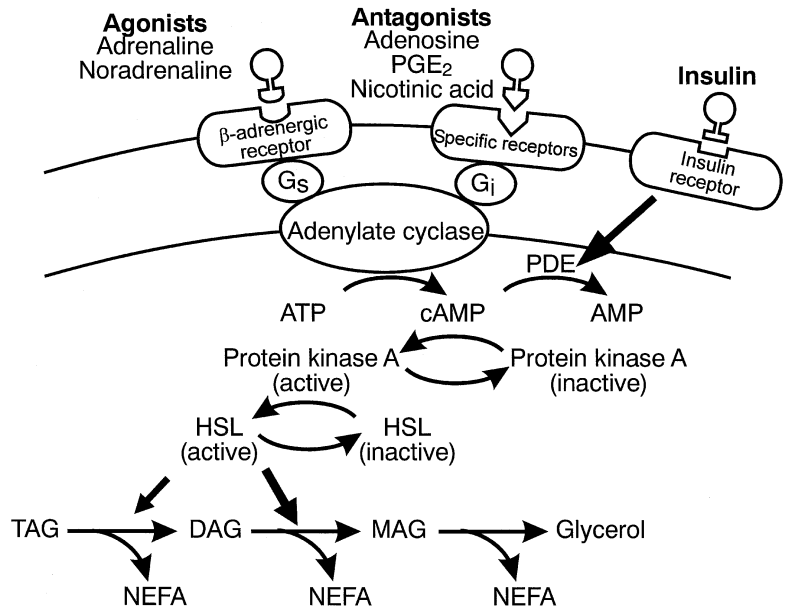


Fig. 3.11 Short-term regulation of the activity of hormone-sensitive lipase (HSL) in adipocytes. HSL is regulated in the short term by reversible phosphorylation brought about by the enzyme protein kinase A (also known as cyclic AMP-dependent protein kinase). Protein kinase A is activated by the binding of cyclic AMP (cAMP), generated by the action of adenylate cyclase on ATP. In turn, adenylate cyclase is regulated by membrane-associated heterotrimeric guanine-nucleotide binding proteins, known as G-proteins. These link cell-membrane hormone receptors with adenylate cyclase. There are stimulatory (G_s) and inhibitory (G_i) G-proteins that link with the appropriate receptors. Insulin suppresses the activity of HSL by causing its dephosphorylation. This reflects activation of a particular phosphodiesterase (PDE), which breaks down cAMP and therefore reduces the activity of protein kinase A. There are constitutively expressed protein phosphatases that return HSL to its inactivated state under these conditions.

HSL is active against the triacylglycerols (TAG) and diacylglycerols (DAG), but less so against monoacylglycerols (MAG). There is a specific, highly active MAG lipase in adipose tissue. The overall reaction is the liberation from stored TAG of three non-esterified fatty acids (NEFA) and a molecule of glycerol.

the realization that phosphorylation of hormone-sensitive lipase is associated with translocation of the enzyme from a cytosolic location, to the surface of the intracellular lipid droplet. This may involve 'docking' with a protein, perilipin, that is associated with the lipid droplet surface, and is itself a substrate for phosphorylation under similar conditions to hormone-sensitive lipase.

Phosphorylation of hormone-sensitive lipase is mediated by the enzyme protein kinase A, or cAMP-dependent protein kinase. This in turn is activated by the binding of cAMP when cellular cAMP concentrations are elevated through increased activity of the enzyme adenylate cyclase. The activity of the cyclase is under the control of the catecholamines adrenaline (a true hormone, released from the adrenal medulla) and noradrenaline, a neurotransmitter released from sympathetic nerve terminals in adipose tissue. Binding of catecholamines to β-adrenergic receptors in the cell membrane activates adenylate cyclase through the intermediary G-proteins (proteins that bind GTP, and couple receptors to adenylate cyclase). The situation is complex because catecholamines can also bind to α-adrenergic receptors that act through inhibitory G-proteins to reduce adenylate cyclase activity, although the overall effect in vivo is usually towards activation. There is also a number of locally produced mediators, such as adenosine and prostaglandins (formed from dietary essential fatty acids; Section 2.4), that inhibit adenylate cyclase activity, again via specific cell-surface receptors and inhibitory G-proteins. This complex system undoubtedly exists to regulate fat mobilization extremely precisely.

Counter-regulation is achieved mainly by insulin, which acts via cell-surface insulin receptors, to activate a specific form of phosphodiesterase that catalyses the breakdown of cAMP. Under these conditions, protein phosphatases in the cell, which do not appear to be regulated but are present in high activity, dephosphorylate hormone-sensitive lipase, which is thus inactivated. Presumably hormone-sensitive lipase in vivo is continually cycling between phosphorylated and dephosphorylated states, with the balance determined by cellular cAMP concentrations. The phosphodiesterase can be inhibited by methylxanthines, such as caffeine, therefore leading to activation of hormone-sensitive lipase and fat mobilization. This is one reason why some athletes claim that drinking strong coffee before an endurance event improves their performance.

Hormone-sensitive lipase is most active against tri- and diacylglycerols, but relatively inactive against monoacylglycerols. There is a highly active monoacylglycerol lipase expressed in adipocytes that leads to the complete hydrolysis of stored TAG with release of three molecules of fatty acid and one of glycerol, which leave the cell. There is currently great interest in the question of whether the exit of fatty acids occurs by diffusion across the cell membrane or via a specific transport protein. A number of putative fatty acid transport proteins has now been identified, although it is not clear whether this step might be open to regulation. The non-esterified fatty acids (often referred to as free fatty acids, or FFA) are bound to plasma albumin in the circulation (Fig. 3.11). They may then be taken up by tissues such as muscle that utilize fatty acids as a major source of fuel, and by the liver where they may be used for oxidation or for the synthesis of new acylglycerols.

It has become clear recently that hormone-sensitive lipase is also responsible for TAG hydrolysis within skeletal muscle. The lipid droplets that are seen within muscle fibres on electron microscopy provide a local supply of fatty acids during intense exercise. The mechanisms for activation of muscle hormone-sensitive lipase may be similar to those within adipocytes, except that muscle contraction is also a stimulus to TAG hydrolysis. The mechanism by which contraction activates hormone-sensitive lipase is not yet known.

The co-ordination of TAG synthesis and breakdown in adipose tissue is illustrated in Fig. 3.12).

3.7 WAX ESTERS

These esters of long-chain fatty acids and long-chain fatty alcohols provide energy sources, as an alternative to TAG, in some animals, plants and micro-organisms.

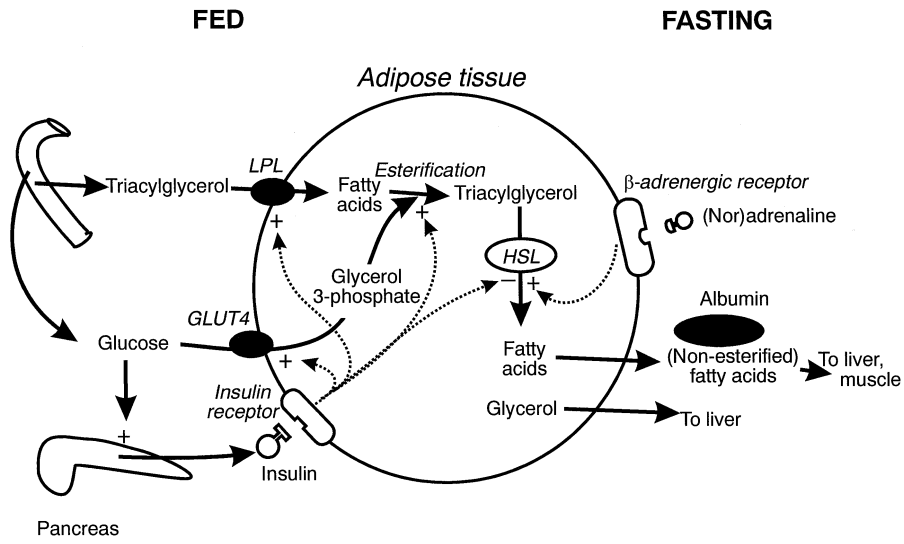


Fig. 3.12 The co-ordination of triacylglycerol synthesis and breakdown in adipose tissue. In the fed state, dietary fatty acids are delivered as chylomicron-triacylglycerol, which is hydrolysed by lipoprotein lipase (LPL) in adipose tissue capillaries to liberate fatty acids, that may be taken up into adipocytes and esterified for storage as triacylglycerol. Glycerol 3-phosphate for this process is provided by glycolysis, following glucose uptake by the insulin-regulated glucose transporter GLUT4. High circulating glucose concentrations in the fed state stimulate pancreatic secretion of insulin, which stimulates all aspects of the pathway of triacylglycerol storage via the cell-surface insulin receptor. In the fasted state or during exercise, low insulin concentrations remove the suppression of hormone-sensitive lipase (HSL), which may be stimulated by catecholamines (adrenaline and noradrenaline) acting through cell-surface β-adrenergic receptors. This process delivers non-esterified fatty acids to the circulation, for use as a fuel by other tissues including liver and skeletal muscle.

3.7.1 Occurrence and characteristics

Wax esters have the general formula R^1COOR^2 and are esters of long-chain fatty acids (R^1COOH) with long-chain fatty alcohols (R^2OH). They occur in some species of bacteria, notably the mycobacteria and corynebacteria. However, they are mainly important for providing a form of energy reserve that is an alternative to triacylglycerols in certain seed oils (e.g. jojoba), the oil of the sperm whale, the flesh oils of several deep-sea fish (e.g. orange roughy) and in zooplankton. Thus, a major part of jojoba oil consists of wax esters, 70% of sperm whale oil and as much as 95% of orange roughy oil. In global terms, however, the zooplankton of the oceans may be of greatest importance. At certain stages of their life cycles, the zooplankton may synthesize and store massive amounts of wax esters, as much as 70% of their dry weight.

The component fatty acids and alcohols of wax esters have an even number of between 10 and 30 carbon atoms. Normally, they are straight-chain saturated or monounsaturated chains of 16C–24C. Branched and odd-numbered chains are rare in both constituents except for the bacterial waxes. Wax esters from cold-water organisms exist as oils down to about 0°C and their physico-chemical properties differ from those of the TAG in that they are more hydrophobic and less dense, which may help to provide greater buoyancy for marine animals.

3.7.2 Biosynthesis of wax esters involves the condensation of a long-chain fatty alcohol with fatty acyl-CoA

It is concluded that zooplankton synthesize their own wax esters *de novo* since their primary food, the

phytoplankton, are devoid of these lipids. Indeed, all marine organisms so far studied, mammals and fish as well as crustaceans, can synthesize wax esters *de novo*.

The fatty acyl components of wax esters are synthesized by the malonyl-CoA pathway (Section 2.2.3.4), although they can also be derived from dietary lipids. Because wax esters are often characterized by very long chain acyl groups, further elongation of the products of the fatty acid synthetase may be necessary, as described in Section 2.2.3.4. Waxes sometimes contain one or more keto groups that have escaped removal at the condensation stage of fatty acid synthesis (Section 2.2.3.2).

The fatty alcohol components are formed from acyl-CoAs by reduction, first by acyl-CoA reductase to form a fatty aldehyde, and then by an aldehyde reductase to form the alcohol (Fig. 3.13). The enzymes are membrane-bound and require NADPH in the reduction; the aldehyde is normally a transient intermediate and does not accumulate. The final esterification is catalysed by acyl-CoA alcohol transacylase (Fig. 3.13).

3.7.3 Digestion and utilization of wax esters is poorly understood

The wax esters of fish and of jojoba seed oils are poorly hydrolysed by the pancreatic lipases of the human digestive system so that these lipids have poor nutritive value for man. Fish, such as salmon and herring grow rapidly when feeding on zooplankton rich in wax esters, yet do not contain these lipids themselves. Their digestive systems are adapted to the efficient hydrolysis of wax esters, most of the products being absorbed and resynthesized into TAG. Tissue breakdown of wax esters so that the products can be utilized as metabolic fuels presumably involves lipases or esterases analogous to the hormone-sensitive lipases in adipose tissue of organisms that store TAG, but these reactions have been little studied.

Some authorities now believe that wax esters have to be considered as key compounds in the transmission of carbon through the marine food chain. Wax esters seem to be found in greatest amounts in regions where animals experience short periods of food plenty followed by long periods of food shortage, for example in polar regions, where

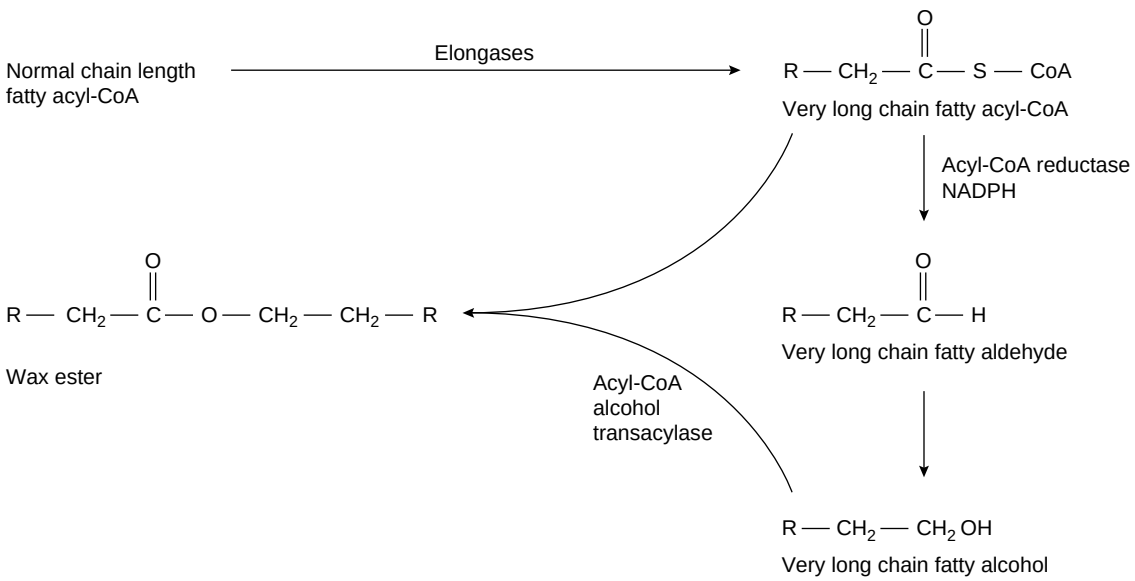


Fig. 3.13 The biosynthesis of wax esters.

the short summer limits the period of phytoplankton growth, or in deep waters with a low biomass. On a weight basis, more energy can be stored as wax ester than as TAG because the oxygen content of wax esters is proportionately less.

3.7.4 Surface lipids include not only wax esters but a wide variety of lipid molecules

Although true waxes are esters of long-chain fatty acids and fatty alcohols, the term wax is often used to refer to the whole mixture of lipids found on the surfaces of leaves, or on the skin or the fur of animals. These surface lipids comprise a complex mixture of wax esters, long-chain hydrocarbons, non-esterified long-chain fatty acids, alcohols and sterols. The surface lipids are responsible for the water-repellent character of the surface and are important in conserving the organism's water balance and providing a barrier against the environment. Some components, notably the long-chain non-esterified fatty acids, have anti-microbial properties and this also contributes to their protective role in a physiological as well as a physical way.

The hydrocarbon components of the surface lipids (Sections 6.6 and 2.2.3.4) have very long chain lengths (around 30C) and are formed from long-chain fatty acids (Section 2.2.3.4) by two sorts of mechanisms. Early experiments suggested that fatty acids could be decarboxylated to yield hydrocarbons. Now it is thought that odd-chain alkanes are derived from aldehyde intermediates by aerobic decarboxylation or by a novel decarboxylation reaction (CO released).

3.8 SUMMARY

Animals and plants require long-term reserves of metabolic fuels that can, at times of need, be mobilized and oxidized to drive vital metabolic processes. Thus, in times of starvation or strenuous exercise, animals can utilize fuel reserves to maintain basal metabolism or sustain muscular activity, respectively. Plants may use their reserves to fuel the energy-demanding process of seed germination. Triacylglycerols are the most common forms of energy reserve on account of the high amount of energy stored per gram of fuel compared with carbohydrates, and because they can be stored in a compact, unhydrated form. Wax esters, however, have some advantages as reserve fuels for certain desert plants and marine animals that live in relatively hostile conditions. Animals store triacylglycerols in adipose tissue, although some marine species utilize the flesh or liver as storage organs. Plants store triacylglycerols (or wax esters) in the seed or sometimes in the fleshy fruit mesocarp.

In both animals and plants, the predominant pathway for the biosynthesis of triacylglycerols is the glycerol 3-phosphate pathway. Glycerol 3-phosphate is acylated at positions 1 and 2 to form phosphatidic acid, which is then dephosphorylated to a diacylglycerol by phosphatidate phosphohydrolase. This step provides a branch point in metabolism since the diacylglycerols can be either channelled into phospholipid biosynthesis or acylated to form triacylglycerols. Several of the enzymes in this pathway appear to be regulated by nutritional state, although the molecular details of their activation are generally not known. Plants differ from animals, not in the enzymic steps involved in triacylglycerol biosynthesis, but in the subcellular organization of those enzymes. A variant of the glycerol 3-phosphate pathway is the dihydroxyacetone phosphate pathway. In some circumstances this pathway may merely serve as an alternative route to triacylglycerols, but its main function is now thought to provide the first step in the biosynthesis of alkyl (or 'ether') lipids. In the mammalian intestine, the components of digested and absorbed dietary fat are resynthesized into triacylglycerols via the stepwise acylation of monoacylglycerols.

Breakdown of triacylglycerols in the animal gut and in the tissues of animals and plants is catalysed by a range of hydrolytic enzymes termed lipases. There are extracellular and intracellular lipases, involved predominantly in uptake of triacylglycerol fatty acids into cells, and mobilization of triacylglycerol fatty acids from tissues, respectively.

Detailed studies of the enzymes of triacylglycerol

metabolism are difficult because many enzymes are firmly membrane-bound and have resisted attempts to isolate and purify them. However, application of molecular biology techniques has now allowed the cloning and sequencing of several important enzymes of lipid metabolism and this is opening the way for better understanding of the mechanisms of regulation of metabolism. Although the need for metabolic fuels is important, other requirements of lipids, such as membrane function, may take precedence, requiring close integration of storage and structural lipid formation. Fuel homeostasis in animals is under complex dietary and hormonal control. Conditions resulting in high circulating concentrations of insulin favour triacylglycerol synthesis and fuel storage, while utilization is stimulated when insulin concentrations are low, and by release of stress hormones.

Our understanding of the regulation of triacylglycerol metabolism in plants is more rudimentary. In preparation for germination, lipases are induced that are capable of complete degradation of the stored fat. Specialized organelles, the glyoxysomes, are then formed to utilize the fatty acids and convert them into simple carbohydrates needed for stem and root formation. The conversion of fatty acids into carbohydrates is not possible in animals.

In some organisms (including several deep-sea fishes, sperm whale and some seed oils), wax esters provide the main store of metabolic energy, rather than triacylglycerols. They are formed by the esterification of long-chain alcohols with very long-chain fatty acids. These compounds are also found in the surface lipids of some plants, animals and bacteria.

FURTHER READING

Coleman, R.A., Lewin, T.M. & Muoio, D.M. (2000) Physiological and nutritional regulation of enzymes of triacylglycerol synthesis. *Annual Review of Nutrition*, **20**, 77–103.

Dircks, L. & Sul, H.S. (1999) Acyltransferases of de novo glycerophospholipid biosynthesis. *Progress in Lipid Research*, **38**, 461–479.

Dodds, P.F. (1995) Xenobiotic lipids: the inclusion of xenobiotic compounds in pathways of lipid biosynthesis. *Progress in Lipid Research*, **34**, 219–247.

Frayn, K.N., Coppack, S.W., Fielding, B.A. & Humphreys, S.M. (1995) Coordinated regulation of hormone-sensitive lipase and lipoprotein lipase in human adipose tissue *in vivo*: implications for the control of fat storage and fat mobilization. *Advances in Enzyme Regulation*, **35**, 163–178.

Gibbons, G.F., Islam, K. & Pease, R.J. (2000) Mobilisation of triacylglycerol stores. *Biochimica et Biophysica Acta*, **1483**, 37–57.

Hajra, A.K. (1995) Glycerolipid biosynthesis in peroxisomes (microbodies). *Progress in Lipid Research*, **34**, 343–364.

Langin, D., Holm, C. & Lafontan, M. (1996) Adipocyte hormone-sensitive lipase: a major regulator of lipid metabolism. *Proceedings of the Nutrition Society*, **55**, 93–109.

Lehner, R. & Kuksis, A. (1996) Biosynthesis of triacylglycerols. *Progress in Lipid Research*, **35**, 169–201.

Murphy, D.J. (1993) Structure, function and biogenesis of storage lipid bodies and oleosins in plants. *Progress in Lipid Research*, **32**, 247–280.

Stobart, A.K., Stymne, S., Shewry, P.R. & Napier J. (1998) Triacylglycerol biosynthesis. In: *Plant Lipid Biosynthesis: Fundamentals and Agricultural Applications*, Society for Experimental Biology Seminar Series 67 (ed. J.L. Harwood). Cambridge University Press, Cambridge.

Sul, H.S. & Wang, D. (1998) Nutritional and hormonal regulation of enzymes in fat synthesis. Studies of fatty acid synthase and mitochondrial glycerol-3-phosphate acyltransferase gene transcription. *Annual Review of Nutrition*, **18**, 331–351.

Vance, D.E. (1996) Glycerolipid biosynthesis in eukaryotes. In: *Biochemistry of Lipids, Lipoproteins and Membranes* (ed. D.E. Vance & J.E. Vance), pp. 153–181. Elsevier Science, Amsterdam.

Vance, J.E. (1998) Eurkaryotic lipid biosynthetic enzymes: the same but not the same. *Trends in Biochemical Sciences*, **23**, 423–428.

Von Wettstein-Knowles, P. (1995) Biosynthesis and genetics of waxes. In: *Waxes: Chemistry, Molecular Biology and Functions* (ed. R.J. Hamilton). The Oily Press, Bridgwater.

Zammit, V.A. (1999) The malonyl-CoA-long-chain acyl-CoA axis in the maintenance of mammalian cell function. *Biochemical Journal*, **343**, 505–515.

4 Dietary lipids

The main emphasis of this chapter is on lipids in the human diet, their metabolism in the healthy body and their relationship to disease processes. Lipid metabolism in other animals will be touched on, however, for two main reasons. First, a great deal of the food we eat is supplied by farm animals and the types of lipids we consume from this source are determined by the animals' own metabolism, their diets and by farming practices. Second, rigorously controlled studies of human metabolism are difficult and expensive to achieve and scientists are, therefore, frequently obliged to make inferences about the metabolic effects of lipids in man from research with experimental animals, generally laboratory rodents.

4.1 LIPIDS IN FOOD

Whereas food lipids originate from biological matter, they may be modified by processing before they are eaten.

4.1.1 The fats in foods are derived from the structural and storage fats of animals and plants

With few exceptions, all food originates from living matter; therefore the lipids in the diet of humans are the structural and storage lipids of animals and plants. Animal storage fats (mainly triacylglycerols with some dissolved cholesterol and fat-soluble vitamins) are present in meat that retains parts of the adipose tissue. Lard, for example, is a cooking fat derived from the adipose tissue of the pig. Milk fat occurs in full fat milk, cheese, cream, full fat yoghurt, butter and low fat dairy spreads. Plant storage fats (mainly triacylglycerols with some fat-soluble vitamins) are present in their original form in nuts (e.g. peanuts), cereal grains and some fruits such as the avocado. They are also familiar in cooking oils, salad oils and mayonnaise. Both animal and plant storage fats are used in the manufacture of spreading fat. Eggs are rich sources of fat. The lipid is present as lipoproteins in which triacylglycerols, cholesterol and phospholipids are associated together with proteins. The lipoproteins are either in a high-density form ('lipovitellin', 78% protein, 12% phospholipid, 9% triacylglycerol) or a low-density form (18% protein, 22% phospholipid, 58% triacylglycerol). They are somewhat analogous to the plasma lipoproteins described in Section 5.2.

The predominance of fat in foods like cooking oils, margarines, butter and meat fat is obvious: we call these the visible fats. Where fat is incorporated into the structure of the food, either naturally (e.g. in peanuts or cheese), or when it is added in the cooking or manufacturing process (e.g. in cakes, pastries, meats), it is less obvious to the consumer and we call these hidden fats.

Because most food derives from biological tissues and because all tissues contain biological membranes, all foods contain, to a greater or lesser extent, structural fats. These are predominantly phospholipids and glycolipids (Chapter 6) with, in animal tissues, cholesterol and in plant tissues, the plant sterols. Thus, the lean part of meat contains the structural lipids, phospholipids and cholesterol of the muscle membranes and minor quantities of glycolipids. Consumption of tissues like brain would introduce larger quantities of animal sphingolipids into the diet. Dairy products contribute small amounts of structural lipids because of the presence of the milk fat globule membrane. The structural lipids of plants that are important in the diet are those in green leafy vegetables, which are predominantly galactolipids (Section 6.2.3) and the membrane lipids of cereal grains, vegetables and fruits.

4.1.2 The fatty acid composition of dietary lipids depends on the relative contributions of animal and plant structural or storage lipids

The most significant aspect of lipids in the human diet is their content of different types of fatty acids: saturated, monounsaturated and polyunsaturated (Section 2.1). It is important to mention at the outset that all natural fats contain complex mixtures of all three types of fatty acids. It is incorrect, therefore, to describe a fat as saturated or polyunsaturated: only the constituent fatty acids can be so described. Yet fats in which the saturated acids form the largest single fraction are frequently categorized as saturated fats to contrast them with fats in which polyunsaturated fatty acids (PUFA) predominate. As an example of where this can be misleading to a layman, pig fat (lard) is frequently categorized as a saturated fat, yet over half (about 55%) of the fatty acids are unsaturated (see Table 3.1). Similarly, among vegetable oils, palm oil is frequently categorized as 'saturated' even though 50% of its fatty acids are unsaturated.

In general, the animal storage fats are characterized by a predominance of saturated and monounsaturated fatty acids (Table 3.1). Storage fats from ruminant animals tend to have a greater proportion of saturated fatty acids because of the extensive hydrogenation of the animal's dietary fats in the rumen (Section 2.2.6). Nevertheless, ruminant fats contain a substantial proportion of monounsaturated fatty acids because of the presence in mammary gland and in adipose tissue of desaturases (Section 2.2.5). Milk fat is unusual among animal fats in containing significant amounts of saturated fatty acids with chain lengths of 12 carbon atoms or less. The proportions of 4:0, 6:0, 8:0, 10:0 and 12:0 are highly dependent on species (Tables 3.1 and 4.6). These acids are synthesized in the mammary gland itself (Section 2.2.3). The fatty acid composition of simple-stomached animals is more dependent on their diet since they do not extensively hydrogenate the dietary unsaturated fatty acids, but can incorporate them directly into the adipose tissue. Thus pig adipose tissue can be significantly enriched in linoleic acid, simply by feeding the animals rations supplemented with soybean oil. Even ruminant fats can be enriched with polyunsaturated fatty acids, but in order to do so the dietary PUFA have to be protected from hydrogenation in the rumen. This can be achieved by treating the oilseeds used to supplement the feed with formaldehyde, which cross-links the protein and renders the seed oil unavailable to the rumen micro-organisms. When the food reaches the acid environment of the true stomach, the cross-links are digested and the PUFA are available for absorption just as in simple-stomached animals.

Such feeding methods are theoretically useful for modifying the fatty acid composition of food products, if higher contents of polyunsaturated fatty acids are required. Polyunsaturated ruminant products are not generally available, however: they are expensive and subject to oxidative deterioration (Section 2.3.4), which may result in poor taste and appearance. Table 3.1 illustrates the wide range of fatty acid compositions that can occur in animal storage fats and the changes in PUFA content that can be achieved by dietary manipulation. Examples of animal storage fats naturally rich in PUFA of the *n*-3 family are those of many species of fish (Table 3.1) and marine animals.

Seed oils contain a wide variety of fatty acids, the composition of which is characteristic of the family to which the plant belongs. Generally one fatty acid predominates (Table 3.2). It may either be one of the normal fatty acids, palmitic, oleic, or linoleic, as exemplified by palm oil, olive oil and sunflower seed oil or it may be an unusual acid, for example erucic acid in older varieties of rapeseed oil. Coconut oil and palm kernel oil are unusual among seed oils in having a preponderance of medium chain length saturated fatty acids. It is therefore an unjustified generalization to characterize all vegetable oils as unsaturated.

The fatty acid composition of the structural lipids of the animal tissues used as food is remarkably uniform irrespective of the animal's diet or whether it is simple-stomached or a ruminant (Table 4.1), although there are clearly some species differences, and diet can influence membrane composition in

Table 4.1 The fatty acid composition of some structural lipids important in foods

	Fatty acid (g per 100 g total fatty acids)*								
	16:0	16:1	18:0	18:1	18:2	18:3	20:4	LCPUFA[a]	total
Foodstuff									
Beef: muscle	16	2	11	20	26	1	13		89
Lamb: muscle	22	2	13	30	18	4	7		96
Lamb: brain	22	1	18	28	1		4	14	88
Chicken: muscle	23	6	12	33	18	1	6		99
Chicken: liver	25	3	17	26	15	1	6		99
Chicken: egg yolk	29	4	9	43	11				96
Pork: muscle	19	2	12	19	26		8		86
Green leaves	13	3		7	16	56			95

[a] Longer chain polyunsaturates, mainly 20:5*n*-3 and 22:6*n*-3.

* The figures are rounded to the nearest whole number. Therefore the content of an individual fatty acid of less than 0.5% is not recorded. The figures across a row rarely sum to 100% because each sample contains a large number of components each contributing less than 1%. Thus the samples for ruminants contain a wide variety of odd and branched-chain fatty acids.

subtle ways (Sections 4.2.2, 4.2.3 and 4.3). An important PUFA in animal structural lipids is arachidonic acid, a member of the *n*-6 family, and meat provides most of our dietary supply of this fatty acid. The flesh of fish, however, contains relatively less arachidonic acid and more of the longer chain PUFA of the *n*-3 family. Plant structural fats are similarly dominated by PUFA and supply mainly α-linolenic acid, a member of the *n*-3 family.

4.1.3 Industrial processing may influence the chemical and physical properties of dietary fats either beneficially or adversely

The fatty acid composition of animal food lipids can be influenced by animal feeding practices as discussed in Section 4.1.2. Likewise, the fatty acid composition of plant fats can be modified by breeding. The best example of this is the change that was brought about by breeding the erucic acid out of rapeseed in exchange for its metabolic precursor, oleic acid (Section 4.1.4). By far the greatest influence on food fatty acid composition, however, is industrial processing.

4.1.3.1 Catalytic hydrogenation

The objective of industrial hydrogenation is to reduce the degree of unsaturation in fatty acids. There are two main reasons for this. The first is to improve oxidative stability. The more highly unsaturated a fat, the greater is its susceptibility to oxidation (Section 2.3.4), resulting in poor flavour and colour and even generating toxic compounds (Section 4.1.4). The second is to improve the physical properties of the fat. Highly unsaturated oils have low melting points and as such are unsuitable for many food uses where a solid texture is desired. Industrial hydrogenation (hardening) raises the melting point, thereby giving better textural properties to many food fats. By careful choice of catalyst and temperature, the oil can be selectively hydrogenated so as to achieve the desired characteristics. Indeed, the process is seldom taken to completion, since fully saturated fats, especially those that would be derived from the very long chain fatty acids of fish oils, would have melting points that were too high.

Hydrogenation is carried out in an enclosed tank in the presence of 0.05–0.20% of a finely powdered

catalyst (usually nickel) at temperatures up to 180°C after which all traces of the catalyst are removed by filtration. Chemically, there are three main results of hydrogenation:

(1) the total number of double bonds in the fatty acid molecule is reduced;

(2) some of the double bonds that are present in the original oil in the *cis* geometric configuration are isomerized to the *trans* form; and

(3) the double bonds may be shifted along the hydrocarbon chain from their original positions in the natural fat to yield a range of positional isomers (Table 4.2; Fig. 4.1).

Table 4.2 Changes in the fatty acid composition of a vegetable oil during hydrogenation (g per 100 g total fatty acids)

Fatty acid		Soybean oil	Hydrogenated soybean oil
16:0		11	11
18:0		4	7
18:1	*cis*	22	33
	trans	0	12
18:2	*cis*-9, *cis*-12	54	22
	cis-9, *trans*-12 } *trans*-9, *cis*-12 }	0	8
	cis-9, *trans*-12 } *trans*-8, *cis*-12 }	0	4
18:3	all isomers	8	2

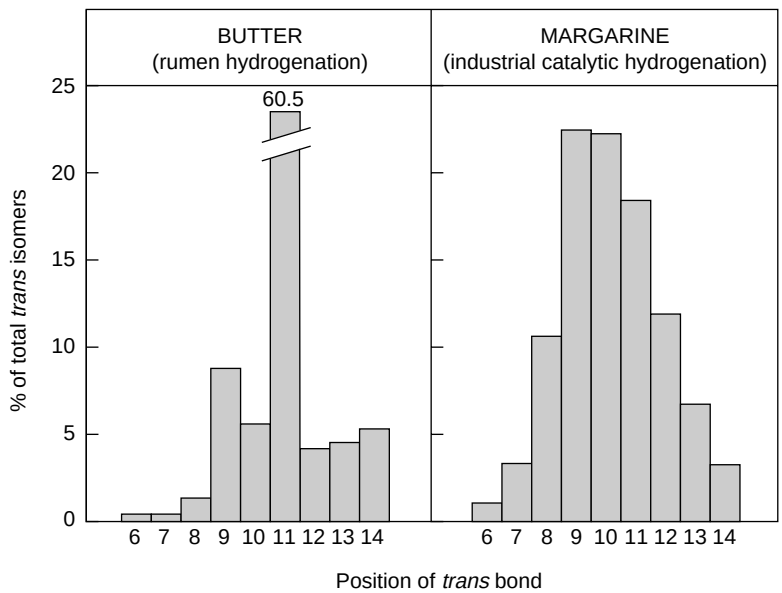


Fig. 4.1 The positional distribution of *trans* double bonds in butter and margarine. Adapted from M.I. Gurr (1996), *Nutrition Research Reviews*, **9**, 259–279. Note the sharp peak at position 11 for butter, resulting from biohydrogenation by anaerobic rumen bacteria, compared with the broad distribution in the industrially hydrogenated product.

It is important to note that *trans* double bonds do occur in natural fats, as well as in industrially processed fats, but generally much less abundantly than *cis* bonds. Thus some seed oils have a significant content of fatty acids with *trans* unsaturation (see Table 2.3) although these are not important sources of fat in the human diet. All green plants contain small quantities of *trans*-3-hexadecenoic acid (see Table 2.2). The most important naturally occurring isomeric fatty acids in human foods are those in ruminant fats. In principle, the chemical outcome of rumen biohydrogenation (Section 2.2.6) is similar to that of industrial hydrogenation. However, whereas in ruminant fats there is a preponderance of the monoene with the *trans* bond at position 11 (*trans*-vaccenic acid), in industrially hydrogenated fats, monoenes with double bonds at positions 8, 9, 10, 11 and 12 make similar contributions (Fig. 4.1). Whereas *trans*-monoenes usually predominate, PUFA with *trans* unsaturation also occur in both natural and processed fats and these can have *cis* and *trans* bonds within the same molecule. Since the early 1990s, interest has been growing in a group of such isomers given the general description 'conjugated linoleic acid' (CLA). It was discovered that some components of the mixture, notably the *cis*-9, *trans*-11 isomer, could inhibit the growth of cancer cells in vivo or in vitro (Sections 4.4 and 5.4.2).

The fatty acid structural changes that occur as a result of industrial hydrogenation may have nutritional significance as discussed in Sections 4.1.4.3 and 5.4.2.

4.1.3.2 Heating

Either industrially or in the home, heating may be used to make food palatable and to make it safe by killing micro-organisms. Risk of reduction of heat-labile and oxygen-sensitive nutrients requires a balance to be struck between microbiological safety and nutritive value. Heating fat that has little contact with air (e.g. in a deep fat fryer) gives rise to gradual accumulation of polymeric products. The first substances to be formed are cyclic monomers of triacylglycerols followed by polymeric products. As long as oils are not reused excessively, such polymeric material does not significantly reduce the functional properties of the oil, nor is it digested and absorbed.

More important changes in fats are brought about by heating in the presence of oxygen and trace metal catalysts (e.g. iron, copper). The major pathway is attack by reactive oxygen radicals at the double bonds of unsaturated fatty acids with the initial formation of a lipid peroxide (Section 2.3.4). The more highly unsaturated the fatty acid, the greater the susceptibility to oxidation. If allowed to go too far, lipid peroxidation can significantly reduce concentrations of essential fatty acids, vitamin A and vitamin E in foods and may also result in damage to proteins (including enzymes) and DNA. Some products may be carcinogenic. Lipid peroxidation may be held in check by the presence of lipid-soluble antioxidants, either natural (e.g. vitamin E, carotenoids) or synthetic (butylated hydroxytoluene, BHT, or butylated hydroxyanisole, BHA). Cholesterol in foods can also auto-oxidize, a process that is accelerated in heating or drying. Thus, 25-dihydroxycholesterol, 7-ketocholesterol, 7-hydroxycholesterol, 5- and 7-hydroperoxycholesterol and cholesterol-3,5,6-triol have been detected in products such as dried egg powder. These components are known to initiate arterial disease in rabbits when they are given in low quantities in the diet, where cholesterol itself has no effect.

4.1.3.3 Irradiation

Irradiation, used to kill pathogens in some types of foods, may generate lipid radicals and thus cause peroxidative damage. Vitamins E and K are particularly susceptible to irradiation damage but not, surprisingly, carotenes. In practice, however, very little direct nutritional damage occurs as a result of food irradiation because of its limited applications.

4.1.3.4 Interesterification

The process of interesterification involves treatment of a fat or fat blend with a catalyst (e.g. sodium methoxide) at an elevated temperature so as to randomize the fatty acids among the

triacylglycerol molecules, as distinct from their natural stereo-specific distribution (Section 3.2.2). Interesterification is used to change physical properties of lipids (crystal structure, melting point) resulting in a changed food texture. The process is now being used more extensively as an alternative to catalytic hydrogenation because of concerns about potential adverse metabolic effects of overconsuming *trans*-unsaturated fatty acids (Sections 4.1.3.1 and 5.4.2). Some evidence has been presented that interesterified fats are metabolized differently from their natural counterparts. For example, work in the 1960s showed that butter that had been subjected to interesterification to randomize its fatty acids had little influence on the concentration of cholesterol in plasma when given in the diet, in contrast to the generally observed cholesterol-raising effect of natural butter. However, these and other similar observations have not been confirmed by more recent research.

4.1.3.5 Fractionation

All natural fats and oils are mixtures of triacylglycerols with different fatty acid distributions (Section 3.2.2) and it is possible to effect partial or almost complete separations (fractionation) with various physical methods. Thus fractions enriched in more highly saturated or unsaturated molecular species can be obtained, yielding products with desirable physical, textural and metabolic properties. For example, fractions of triacylglycerols enriched in medium-chain fatty acids (8:0, 10:0) can be obtained from coconut oil (Table 3.2). These find clinical use for patients with fat malabsorption (Section 5.1.4). A recent development has been the production of spreadable butters by selective removal of higher melting triacylglycerols. Reduced cholesterol foods can now be produced by differential extraction with, for example, carbon dioxide. (Alternatively, the same result has also been achieved by treatment with cholesterol degrading enzymes.)

4.1.3.6 Structured fats

'Structured fats' is the name now generally given to semi-synthetic mixed-acid triacylglycerols containing both very long and very short chain fatty acids. It is proposed that they may find applications in reduced energy foods. There are two main principles here. First, the short-chain acids have an intrinsically lower energy value than the longer chain fatty acids and are, in any case, metabolized in the liver in such a way that they are not deposited in adipose tissue (Section 5.1.3). Second, the very long chain acids are less efficiently absorbed. The combination of reduced gross energy and poorer absorption reduces the overall metabolizable energy of the fat. This is a relatively new concept and there is as yet no convincing evidence that such products contribute effectively to weight-reducing diets in the long term. Another type of structured triacylglycerol, which contains palmitic acid at position *sn*-2 to mimic the structure of a major molecular species in human milk triacylglycerols, has been introduced commercially for use in infant feeds. Molecules with this structure are digested and absorbed more efficiently by the human infant (Section 5.1.1).

Other products that are aimed at reducing dietary energy, including sucrose polyesters, are also now approved in the USA for limited food use. These are not triacylglycerols, but have fat-like texture. They are not attacked by pancreatic lipase (Section 5.1.1) and so are not absorbed and contribute no energy value to the diet. Other applications of such products are discussed in Section 5.1.4. Much discussion has taken place about potentially harmful effects of ingesting such compounds. These may include significant reductions in the absorption of fat-soluble vitamins (which can be overcome by fortification) or gastrointestinal discomfort and fatty stools.

4.1.4 A few dietary lipids may be toxic

Much of the discussion in this chapter is concerned with the positive contributions made by dietary lipids, especially fatty acids, to nutrition and health. It is equally important to consider whether dietary lipids can also have adverse or toxic effects.

4.1.4.1 Cyclopropenes

Of the fatty acids, those containing a cyclopropene ring (Section 2.1.4) have been considered toxic as a result of their ability to inhibit the Δ9-desaturase. One result of this is to alter membrane permeability as seen in 'pink-white disease'. If cyclopropene fatty acids are present in the diet of laying hens, the permeability of the membrane surrounding the yolk is increased, allowing release of pigments into the yolk. Rats given diets containing 5% of dietary energy as sterculic acid (see Table 2.4) died within a few weeks and at the 2% level, the reproductive performance of females was completely inhibited. Cottonseed oil is the only important oil in the human diet that contains cyclopropene fatty acids. However, their concentration in the natural oil is low (0.6–1.2%) and is reduced still further to harmless levels (0.1–0.5%) by processing. There has been no evidence that consumption of cottonseed oil in manufactured products has had any adverse nutritional effects.

4.1.4.2 Long-chain monoenes

The long-chain monoenoic acid, erucic acid (13*c*-22:1*n*-9; see Table 2.2) is present in high concentration (up to 45% of total fatty acids) in the seed oil of older varieties of rape (*Brassica napus*). When young rats were given diets containing more than 5% of dietary energy as high erucic rapeseed oil, their heart muscle became infiltrated with triacylglycerols. After about a week on the diet, the hearts contained three to four times as much lipid as normal hearts and although, with continued feeding, the size of the fat deposits (referred to as a lipidosis) decreased, other pathological changes were noticeable. These included the formation of fibrous tissue in the heart muscle. The biochemistry of the heart muscle was also affected. Mitochondrial oxidation of substrates, such as glutamate, was reduced and the rate of ATP biosynthesis was impaired. The degradation of triacylglycerols that contain erucic acid is somewhat slower than with fatty acids of normal chain length and this may have contributed to the accumulation of lipid deposits.

Despite lack of evidence for harmful effects in man, an extensive breeding programme was undertaken to replace older varieties of rape with new 'zero-erucic' varieties (see Table 3.2). The use of low erucic varieties in manufactured products is now mandatory in most industrial countries, but high erucic varieties continue to be used in countries such as China.

High concentrations of long-chain monoenoic fatty acids also occur in some fish oils and therefore contribute to the diets of people consuming these fish, as well as to diets containing certain fat spreads that incorporate hardened fish oils (see Table 3.1). The nutritional and toxicological consequences of long-term consumption of marine long-chain monoenes have been less extensively studied than have those of high erucic acid-containing rapeseed oil.

4.1.4.3 *Trans*-unsaturated fatty acids

Concerns about possible toxic effects of fatty acids with *trans*-unsaturation began with the publication of results of experiments with pigs given diets containing hydrogenated vegetable fat for 8 months. They had more extensive arterial disease than those given otherwise equivalent diets devoid of *trans*-unsaturated fatty acids. Subsequently, numerous animal feeding trials, epidemiological studies of human populations and controlled dietary experiments with human subjects have been reported. Some, but not all, epidemiological studies have shown an apparent statistically significant correlation between intakes of certain types of *trans*-unsaturated fatty acids and increased risk of coronary heart disease. Critical evaluation of all such studies leads to the conclusion that an association between *trans*-unsaturated fatty acid consumption and coronary heart disease has not been demonstrated; nor is there any reliable evidence linking *trans*-unsaturated fatty acid intake with cancer or other chronic diseases.

A theoretical basis for some of the claimed effects of *trans*-unsaturated fatty acids on heart disease has been discussed in terms of their influence on plasma lipoproteins and on components of the blood-clotting system. While there is no conclusive

evidence for the latter, there is an effect of *trans*-unsaturated fatty acids in raising LDL-cholesterol and decreasing HDL-cholesterol concentrations in the blood. This is further discussed in Section 5.4.2.

High concentrations of *trans*-unsaturated fatty acids may adversely affect the metabolism of the parent essential fatty acids, linoleic and α-linolenic acids to their long-chain metabolites by inhibiting desaturase activity or competing with normal substrates for desaturases (Section 4.2.3.5). In this way, they may raise the dietary requirement for essential fatty acids as explained later. This kind of 'nutritional imbalance' must be distinguished, however, from direct and specific toxic effects. It is worth noting that the hydrogenated fats used in many experiments designed to investigate potential adverse effects of dietary *trans*-unsaturated fatty acids, contain many unusual *cis* as well as *trans* isomers and their potential toxicity has been less extensively researched.

4.1.4.4 Lipid peroxides

Lipid peroxides may be formed from polyunsaturated fatty acids when they are in contact with oxygen. As described in Sections 2.3.4 and 4.1.3, the reaction is accelerated in the presence of catalysts such as transition metal ions or haem compounds and by heating. When lipid hydroperoxides are ingested, they are rapidly metabolized in the mucosal cells of the small intestine to various oxyacids that are then rapidly oxidized to carbon dioxide. High concentrations of lipid peroxides in the gut may damage the mucosa and potentiate the growth of tumours. However, there is little evidence for the absorption of unchanged hydroperoxides nor for their incorporation into tissue lipids. Hydroperoxyalkenals, lower molecular weight breakdown products of lipid hydroperoxides, are absorbed and may be toxic. Rats given diets enriched in these compounds manifested increased liver weight, increased concentrations of malondialdehyde, peroxides and other carbonyl compounds and decreased concentrations of α-tocopherol and linoleic acid in tissues. The toxicity of peroxidized cholesterol has been referred to in Section 4.1.3.

4.2 ROLES OF DIETARY LIPIDS

Lipids contribute to intakes of energy, structural components of cells, essential fatty acids and fat-soluble vitamins as well as enhancing the palatability of foods.

4.2.1 Triacylglycerols provide a major source of metabolic energy especially in affluent countries

More than 90% of dietary lipids are triacylglycerols, which originate from the adipose tissue or milk of animals or from plant seed oils, mainly in the form of manufactured products. Some 35–45% of dietary energy in industrialized countries comes from triacylglycerols. World-wide, however, the contribution that fat makes to the diet can vary enormously. For example, the Ho tribe in India makes little or no use of fat and their fat intake has been estimated to be no more than 2–4 g day^{-1} or 2% of dietary energy. Certain Inuit communities, by contrast, may consume as much as 80% of their energy as fat. Whereas the presence of fat can contribute substantially to the palatability of the diet (Section 4.2.5), the main nutritional contribution of triacylglycerols is to supply metabolic energy. The useful energy available to the body (metabolizable energy, ME) depends mainly on the digestibility of the fat (Section 5.1) and the chain length of the constituent fatty acids. Most common food fats are efficiently digested by most people with insignificant differences in their digestible energies. As discussed in Section 4.1.3, some practical use has been made of differences in chain length as reflected in their metabolism. Useful metabolic energy is derived from triacylglycerols mainly through β-oxidation of the constituent fatty acids (Section 2.3.1). At each step of the cycle, acetyl-CoA is generated and fed into the tricarboxylic acid cycle, where the end-products of oxidation are carbon dioxide and water. The reduced pyridine nucleotides generated in the tricarboxylic acid cycle enter the mitochondrial electron transport chain to fuel the generation of ATP as a source of metabolic energy. One mole of palmitic acid (16:0) yields 129 moles of ATP (Table 4.3).

Table 4.3 The ATP yield of palmitate oxidation

Reaction	ATP yield
A. Beta-oxidation $CH_3(CH_2)_{14}COSCoA + 7FAD + 7H_2O + 7NAD^+ + 7CoA$ $\longrightarrow 7FADH_2 + 7NADH + 7H^+ + 8CH_3COSCoA$ (B) (C) (D)	
B. Oxidation of $FADH_2$ in the mitochondrial electron transport chain $14H^+ + 7FADH_2 + 14Pi + 14ADP + 3.5O_2$ $\longrightarrow 7FAD + 21H_2O + 14ATP$	14
C. Oxidation of NADH in the mitochondrial electron transport chain $7NADH + 28H^+ + 21Pi + 21ADP + 3.5O_2$ $\longrightarrow 7NAD^+ + 28H_2O + 21ATP$	21
D. Oxidation of acetyl-CoA in the tricarboxylic acid cycle $96H^+ + 8CH_3COSCoA + 16O_2 + 96Pi + 96ADP$ $\longrightarrow 8CoA + 104H_2O + 16CO_2 + 96ATP$	96
Overall equation $131H^+ + CH_3(CH_2)_{14}COSCoA + 131Pi + 131ADP + 23O_2$ $\longrightarrow 131ATP + 16CO_2 + 146H_2O + CoA$	131
Subtract 2ATP required for initial activation of palmitate (formation of CoA thiolester)	–2 **129**

4.2.2 Lipids supply components of organs and tissues for membrane synthesis and other functions

Some lipids that are needed for tissue growth and development can be synthesized by the cells themselves. However, the fatty acids linoleic (18:2 *n*-6) and α-linolenic (18:3*n*-3) cannot be made by animal cells and these so-called 'essential fatty acids' must be supplied by the diet. Once incorporated into cells they may be further elongated and desaturated to longer chain polyunsaturated products. These structural and metabolic relationships were described in Section 2.2.5 and the student is recommended to cross-refer to the earlier section. Section 4.2.3 will describe the nature and function of essential fatty acids and their dietary requirements, but first it will be useful to discuss more general needs for lipids in growth.

4.2.2.1 Foetal growth

From conception, the cells of the growing foetus need to incorporate lipids into their rapidly proliferating membranes. The foetus is totally dependent on the placental transfer of substrates from the mother's circulation, which are then elaborated into lipids. Glucose is a major substrate for the foetus, which possesses the enzymic activities for its conversion into (non-essential) fatty acids and for the production of the glycerol moiety of glycerides via glycerol phosphate. A crucial question in developmental biology is how essential fatty acids are acquired and metabolized at this early stage.

Placental transfer of fatty acids

Figure 4.2 illustrates several pathways by which the foetus could obtain the polyunsaturated fatty acids that it requires for growth. The placenta of most mammals, including human beings, is permeable to non-esterified fatty acids although there are large differences between animal species in rates of transfer. There are also large species differences in the lipid biosynthetic capacity of the placenta and of foetal tissues, so that generalizations cannot be made from research on any one species. It is well

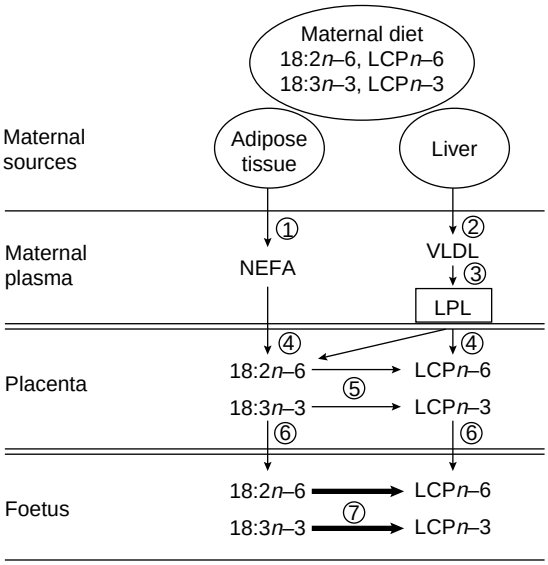


Fig. 4.2 Origins and metabolism of polyunsaturated fatty acids in the foetus. The maternal diet contains a mixture of *n*-6 and *n*-3 parent and long-chain polyunsaturated fatty acids (LCP), which will vary widely among species and individuals. After digestion and absorption of the dietary lipids, the re-esterified fatty acids are transported in the maternal plasma as lipoproteins (Chapter 5) and incorporated into maternal tissues, e.g. adipose tissue and liver. Non-esterified fatty acids (NEFA) are released from adipose tissue stores (stage 1 in the figure; also see Chapter 3). The liver exports fatty acids as triacylglycerols in very low density lipoproteins (VLDL; stage 2 in figure) into the maternal plasma. The maternal face of the placenta contains a lipoprotein lipase (LPL) (stage 3; see also Chapters 3 and 5), which releases NEFA from VLDL. The placenta is permeable to NEFA (stage 4). The human placenta contains minimal desaturase activity and stage 5 is probably not a significant source of LCP. Parent polyunsaturates and LCP are transported to the foetal circulation (stage 6) and the foetus can convert a proportion of the parent polyunsaturates into LCP (stage 7).

established that the amounts of 18:2*n*-6 and 18:3*n*-3 and of their elongation/desaturation products in the mother's diet influences the fatty acid composition of the developing foetus. Desaturation activity is present in developing foetal brain and liver (Fig. 4.2, reaction 7), so that the foetus may not be totally dependent on a supply of long-chain PUFA (LCPUFA) from the mother (Fig. 4.2, reaction 6). There is, however, little evidence for placental desaturase activity in simple-stomached animals, so that reaction 5 in Fig. 4.2 is unlikely to provide significant amounts of LCPUFA.

Concentrations of all lipoprotein classes increase in the maternal circulation during pregnancy, a process that is mediated by the sex hormones. A lipoprotein lipase has been discovered in human placenta. This is consistent with the hypothesis that during hyperlipidaemia of pregnancy, release of fatty acids by placental lipoprotein lipase could generate substrates for synthesis of lipids by the foetus. The transfer of intact lipoproteins by a receptor-mediated pathway has not been demonstrated but cannot be ruled out. Placental lipoprotein lipase is present on the maternal, but not on the foetal, side of the placenta and hydrolyses very low density lipoproteins, but apparently not chylomicrons. Perhaps this constitutes a mechanism for ensuring a flow of fatty acids from mother to foetus, rather than in the reverse direction. It may also allow the mother to control the fatty acids available to the placenta since VLDL are supplied by synthesis in the mother's liver, whereas chylomicrons derive from dietary fat (Section 5.2.3).

In simple-stomached animals, arachidonic and some other LCPUFA are present in higher concentration in foetal than in maternal plasma and in foetal tissues than in foetal plasma. Arachidonic acid seems to be selectively incorporated and trapped into placental phospholipids for export to the foetal circulation. The term 'biomagnification' has been coined for a process in which the proportion of long-chain polyunsaturates increases in phospholipids progressing from maternal blood, to cord blood, foetal liver and foetal brain. Such a biomagnification process probably incorporates the combined effects of placental fatty acid uptake, selective protection of specific fatty acids against β-oxidation and selective direction of fatty acids into membrane or storage lipid biosynthesis.

The study of foetal conservation of essential fatty acids in ruminants is extremely rewarding since the availability of essential fatty acids to the mother is severely limited by rumen hydrogenation (Section 2.2.6). The ratio of 20:3*n*-9/20:4*n*-6 in foetal lamb tissues is 1.6. In simple-stomached animals, a value

of this ratio above 0.4 is a biochemical marker of essential fatty acid deficiency, as will be explained in Section 4.2.3.2. By 10 days after birth, the ratio falls to 0.4 and by 30 days to 0.1. These values are well within the normal range despite the extremely low concentration of linoleic acid in ewe's milk (0.5% of energy). Ruminants are, therefore, able to conserve linoleic acid with supreme efficiency. Sheep placenta transfers linoleic acid at a relatively slow rate, but has a very high Δ6-desaturase activity by comparison with non-ruminants, providing the major source of arachidonic acid for the foetus. This metabolite is concentrated into glycerophospholipids whereas the linoleic acid precursor is in higher concentration in the triacylglycerols. This molecular compartmentation has the effect of conserving arachidonic acid and directing it into membranes.

Storage in adipose tissue

In some species, reserves of fatty acids are already being built up in the foetus. For example, the development of human fat cells begins in the last third of gestation and at birth a baby weighing 3.5 kg has, on average, 560 g of adipose tissue. Guinea pigs are also born with a large amount of adipose tissue whereas, in contrast, pigs, cats and rats are born with little or none. There is a remarkable parallelism between the placental permeability to non-esterified fatty acids and the tendency to accumulate adipose tissue by the foetus, suggesting that an important source of foetal fat reserves is derived from circulating maternal lipids. The adipose tissue composition of the foetal and new-born fat will, therefore, reflect the fatty acid composition of the maternal diet as illustrated in Table 4.4.

Brain development

A large proportion of the long-chain polyunsaturated fatty acids synthesized or accumulated during the perinatal period is destined for the growth of the brain, of which 50% of the acyl groups may consist of 20:4*n*-6, 22:4*n*-6, 22:5*n*-6 and 22:6*n*-3. As with adipose tissue, there are large species differences in the time at which birth occurs in relation to the extent of brain development. The peak rate of brain development occurs in guinea-pigs in foetal life; in the rat, post-natally; while in man and pig it reaches a peak in late gestation and continues after birth. It has been suggested that transfer from the placenta is the major source of LCPUFA for the human foetal brain, but the experimental difficulties of demonstrating this in man are enormous. Because of their remarkable similarity to man in the timing of brain development and its lipid composition, pigs have been used as models. Long-chain derivatives of linoleic acid increase in brain from mid-gestation to term. Little linoleic acid, however, accumulates until birth,

Table 4.4 Influence of the fatty acid composition of the mother's dietary fat on the fatty acid composition of adipose tissue in new-born guinea-pigs (g per 100 g total fatty acids)

	Maize oil		Beef tallow	
Fatty acid	Maternal diet	Adipose tissue	Maternal diet	Adipose tissue
16:0	13	23	25	26
18:0	3	6	18	9
18:1	26	28	36	42
18:2	54	33	7	16
18:3	2	5	2	6
Others	2	5	12	1

when its concentration increases threefold while the concentrations of its elongation/desaturation products remain constant. By labelling experiments with [1-^{14}C]linoleic acid in vivo, it has been shown that linoleic acid is metabolized to LCPUFA by piglet brain and liver throughout the perinatal period. The contribution of the liver was many-fold greater than the brain at all stages. Whether foetal tissues can supply all the needs of the nervous system for LCPUFA without the need for the maternal transfer of intact LCPUFA is still to be resolved. In regard to maternal transfer, it has been proposed that an intracellular α-fetoprotein, derived from maternal plasma α-fetoprotein may play a role in delivering LCPUFA to foetal brain. It binds fatty acids avidly, especially the LCPUFA such as 20:4*n*-6 and 22:6*n*-3. Indeed plasma non-esterified 22:6*n*-3 seems to be almost exclusively transported on α-fetoprotein despite the quantitative predominance of albumin.

An outstanding feature of the composition of brain phospholipid is its remarkable consistency, irrespective of species or diet. The concentrations of the parent essential fatty acids are extremely low (18:2*n*-6, 0.1–1.5% and 18:3*n*-3, 0.1–1.0%) while arachidonic (20:4*n*-6) and docosahexaenoic (22:6*n*-3) acids predominate at 8–17% and 13–29%, respectively in all species (Table 4.5).

Table 4.5 Principal polyunsaturated fatty acids of the liver and brain phospholipids of zebra and dolphin

	Zebra	Dolphin
Food fatty acids		
n-6/*n*-3	1:3	1:20
Liver fatty acids (g per 100 g total fatty acids)		
18:2*n*-6	47	2
20:4*n*-6	4	13
18:3*n*-3	2	
20:5*n*-3	1	8
22:6*n*-3		11
Brain fatty acids (g per 100 g total fatty acids)		
18:2*n*-6		1
20:4*n*-6	10	7
18:3*n*-3		
20:5*n*-3		1
22:6*n*-3	18	27

Data are taken from Crawford, M.A., Casper N.M. and Sinclair A.J. (1976) *Biochem. Physiol.*, **54B**, 395–401. The zebra's food polyunsaturates, both *n*-6 and *n*-3 are mainly 18C and the dolphin's 20C and 22C. Of the liver and brain fatty acids, only the principal polyunsaturates are shown for clarity. Figures are rounded to the nearest whole number so that components contributing less than 0.5% of total are not recorded. Note the preponderance of *n*-6 polyunsaturates in the liver lipids of both species despite the dietary excess of *n*-3 polyunsaturates and a preponderance of long-chain *n*-3 polyunsaturates in the brain, with virtually none of the parent acids (18:2*n*-6, 18:3*n*-3).

Liver lipids

In contrast to the conservative lipid composition of brain, the liver lipids exhibit much greater variation. The parent essential fatty acids are present in much greater concentrations than they are in brain and there are major differences in the elongation/desaturation products. For example, 22:5 is the major *n*-3 fatty acid in the liver lipids of ruminants and other herbivores while 22:6*n*-3 predominates in the carnivores and omnivores. Fatty acids of the *n*-6 family usually predominate in liver phosphoglycerides, even when the dietary intake is in favour of the *n*-3 fatty acids. Thus, zebra and dolphin, both species that have an overwhelming excess of *n*-3 fatty acids in the diet, attain a preponderance of *n*-6 acids in the liver phosphoglycerides (Table 4.5).

4.2.2.2 Post-natal growth

At birth, quite large changes in lipid metabolism occur. Whereas the foetus had relied extensively on glucose from which to synthesize (non-essential) fatty acids and triacylglycerols, the sole source of nutrition for the new-born is milk from which about 50% of energy comes from fat. The enzymes of fatty acid synthesis are suppressed and the baby's metabolism becomes geared to using fat directly from the diet.

Milk composition

The lipid composition of human milk is therefore the main factor that determines the availability of lipids for the breast-fed baby's development, especially in respect of the essential fatty acids. Triacylglycerols comprise about 98% of the total lipids in milk and provide most of the required

linoleic and α-linolenic acids. Although the glycerophospholipids represent only about 1% of total milk lipids, they provide about 50% of the long-chain *n*-3 and *n*-6 PUFA in milk. The fatty acid composition of human milk (and indeed that of any simple-stomached animal) is highly variable since it is strongly influenced by the fatty acid composition of the mother's diet. However, the milk and dietary fatty acid compositions are not related in a linear manner because the maternal adipose tissue stores also make a contribution to milk fatty acid composition. Table 4.6 illustrates this variability and also compares the composition of human milk with that of cow's milk, which provides the base for most commercial infant formulas. Women eating a vegetarian diet tend to have higher concentrations of 18:2*n*-6 and 18:3*n*-3 in their milk. Concentrations of 20:5*n*-3 and 22:6*n*-3 are increased many-fold when the diet is supplemented with fish oils. The increase in *n*-3 LCPUFA is not, however, accompanied by a reduction in 20:4*n*-6.

Table 4.6 The fatty acid composition of human and cow's milk fat (g per 100 g total fatty acids)

	Cow's milk	Human milk	
		Vegans	Omnivores
Fatty acid			
4:0	3		
6:0	2		
8:0	1		
10:0	3		
12:0	4	4	3
14:0	12	7	8
16:0	26	16	28
16:1	3	1	4
18:0	11	5	11
18:1	28	31	35
18:2	2	32	7
18:3	1	1	1
20:4		1	1
Total	96	98	98

The data for human milk are adapted from the review by Jensen *et al*. (1990) and those for cow's milk from Gurr (1991). Figures are rounded to the nearest whole number. Note the high proportion of saturated fatty acids in cow's milk fat and especially those with short- and medium-chain lengths. The components not listed are mainly odd-chain and branched-chain fatty acids, individually in very small amounts. Note the high proportion of linoleic acid in the milk of human vegans, which mainly replaces palmitic acid. The components not listed are mainly longer chain polyunsaturated fatty acids of the *n*-3 and *n*-6 families each in very small amounts.

The fatty acid composition of the milk feed given to new-born infants is quickly reflected in the composition of the adipose tissue, as illustrated in Table 4.7.

Brain development

A dietary supply of 18:2*n*-6, 18:3*n*-3 and their long-chain metabolites is important because, as discussed earlier, human brain development continues after birth. Data on the sequential development of human brain and nervous system are difficult to collect, but such data as exist, together with information from pigs, suggest a preferential accumulation of *n*-6 fatty acids in early foetal development, followed by an accumulation of *n*-3 acids later in gestation. The deposition of myelin occurs late in the development of the central nervous system, predominantly after birth in human babies. The fatty acid composition of immature myelin reflects the composition of the plasma membrane of the oligodendrocyte from which it is made. The high lipid content of the myelin sheath (Section 6.5) and the rapid rate of myelin synthesis in early post-natal life put great demands on the supply of milk LCPUFA. Later, there is a marked decrease in the LCPUFA content of myelin and a concomitant increase in monoenoic and saturated fatty acids, thus reducing the demands on the milk PUFA supply.

Infant formulas traditionally contained cow's milk fat having a low concentration of essential fatty acids. A major development was replacement of the cow's milk fat by vegetable oils providing high intakes of 18:2*n*-6. More recent developments have been to supply a higher content of *n*-3 PUFA, but the optimal ratio of 18:3*n*-3 to its longer chain metabolites is still debated. Limited data suggest that a dietary supply of preformed long-chain *n*-3 PUFA results in significantly higher deposition of these acids in developing organs than when dietary 18:3*n*-3 alone is supplied and the practice of adding fish oils to infant formulas is becoming more

Table 4.7 Influence of the type of fat in infant formulas on adipose tissue composition of babies (g per l00 g total fatty acids)

Fatty acid	British[a]			Dutch[b]		
	Fat in formula	Adipose tissue		Fat in formula	Adipose tissue	
		Birth	6–12months		Birth	6–12months
14:0	11.5	3.8	8.5	0	3.3	4.8
16:0	30.0	48.9	32.0	10.7	48.5	29.7
18:0	14.3	4.1	5.6	2.0	3.8	2.9
16:1	2.0	12.6	7.0	0	15.2	13.8
18:1	31.1	29.6	43.4	27.2	29.0	40.8
18:2	1.8	1.0	3.5	58.2	2.9	8.0

[a] British babies were given a formula in which the fat component came from cow's milk.
[b] Dutch babies were given a formula in which the fat component was provided by a vegetable oil.

widespread. Reasons for the superiority of the LCPUFA may be the significantly higher β-oxidation of 18:3*n*-3 compared with LCPUFA and limited activity in the elongation/desaturation pathway.

An adequate supply of LCPUFA in milk is even more important in infants born prematurely, since these acids would normally have been supplied to the foetus from the mother's blood or metabolized from parent acids in foetal tissues. There is limited evidence that giving formula enriched with fish oil improves the 22:6*n*-3 status and visual function of premature babies, but their 20:4*n*-6 status deteriorates. Thus, the optimum amounts of parental essential fatty acids and their elongation/desaturation products, and the optimal ratios between *n*-3 and *n*-6 families remain crucial topics in paediatric nutrition research. Box 4.1 illustrates some of the debate about the appropriate fatty acid composition of infant formulas in more detail.

4.2.3 Dietary lipids supply essential fatty acids that are essential to life but cannot be made in the animal body

4.2.3.1 Historical background: discovery of essential fatty acid deficiency

In 1929 the Americans Burr and Burr described how acute deficiency states could be produced in rats by feeding fat-free diets and that these deficiencies could be eliminated by adding only certain specific fatty acids to the diet. Until that time it had been thought that fat was important only in so far as its energy contribution was concerned. It was shown that linoleic and later arachidonic acid were responsible for this effect and the term vitamin F was coined for them. They are now known universally as 'essential fatty acids' (EFA).

EFA deficiency can be produced in a variety of animals, including man, but data for the rat are the best documented (Table 4.8). The disease is characterized by skin symptoms, such as dermatosis and the skin becomes more 'leaky' to water. Growth is retarded, reproduction is impaired and there is degeneration or impairment of function in many organs of the body. Biochemically, EFA deficiency is characterized by changes in the fatty acid composition of many tissues, especially their biological membranes, whose function is impaired and, in the mitochondria, the efficiency of oxidative phosphorylation is much reduced. Well-documented EFA deficiency in man is rare, but was first seen in children fed virtually fat-free diets. Four hundred infants were fed milk formulas containing different amounts of linoleic acid. When the formulas contained less than 0.1% of the dietary energy as linoleic acid, clinical and chemical signs of EFA deficiency ensued. The skin abnormalities were very similar to those described in rats and these and other signs of EFA deficiency disappeared when linoleic acid was added to the diet.

Box 4.1 Are long-chain *n*-3 polyunsaturates necessary constituents of infant feeds?

Long-chain polyunsaturated fatty acids (LCPUFA) such as 20:4*n*-6 (AA) and 22:6*n*-3 (DHA) make up about a third of brain lipids. DHA seems to have a key role in the structure of retinal, neural and synaptic membranes and AA may have a neurotransmitter role. Human brain development begins in foetal life and continues for some months after birth. The mother's circulation supplies the foetus with preformed LCPUFA or parent acids that the foetus can desaturate and elongate. After birth, breast milk supplies parent acids and small amounts of LCPUFA. Infant formula milks normally supply parent acids (albeit in widely differing amounts depending on the brand), but not LCPUFA. During the latter part of the 1990s some manufacturers started to provide products containing LCPUFA

The need for a supply of LCPUFA has been a topic of controversy mainly due to lack of sufficient information. Limited data suggest that:

- visual and neural abnormalities develop in infant monkeys given feeds with a low concentration of 18:3*n*-3;
- similar deficiencies have been observed in premature human infants;
- infants randomly allocated to formula containing DHA displayed even better visual development than those whose sole source of *n*-3PUFA was 18:3.
- *n*-3PUFA may suppress growth in premature babies.

Furthermore, there is substantial evidence that breast feeding confers significant advantages compared with normal formula in respect of neural development. However, such findings are almost impossible to interpret because breast milk differs from formula milk in many aspects other than its LCPUFA content. Nevertheless, a consensus seemed to have developed that the addition of LCPUFA to infant formula was beneficial, if not essential.

A study published in December 1999 has caused this view to be less secure. Its authors concluded that:

> There was no evidence of a beneficial or adverse effect on cognitive or motor development ... Our trial does not provide support for the addition of LCPUFA to standard infant formula but we are now doing further follow-up of this cohort. (A. Lucas *et al*. (1999) *Lancet*, **354**, 1948–1954).

The study was a double-blind, randomized, controlled trial of formula with (*n*=154) and without (*n*=155) LCPUFA and a reference group that was breast-fed (*n*=138). The outcome measures were several tests of mental and psychomotor development.

The authors were careful also to include tests that would assess the safety of consuming feeds rich in LCPUFA, since there had been previous evidence of adverse effects on growth. These included tests for allergic reactions, infections, growth and gastrointestinal tolerance. There was no evidence of adverse effects.

What can we conclude? Although addition of LCPUFA to infant formula at a concentration of $0.2\,g\,l^{-1}$ (the amount used by Lucas and colleagues) can be regarded as safe, there is insufficient evidence to conclude that the development of infants is improved by LCPUFA supplements. Any benefits are likely to be modest and smaller than the advantage of breast milk over formula feeds.

In 1971, the first unequivocal case of EFA deficiency in an adult was reported. The patient, a man of 44, had all but 60 cm of his bowel surgically removed. He was then given intravenous feeding only, with preparations containing no fat, and after 100 days he developed scaly dermatitis. In a London hospital in 1974, three patients with chronic disease of the small bowel, who had been treated with low fat diets, but not given intravenous feeding, were suspected of having EFA deficiency. They responded successfully to the application to the skin of lipids containing a high proportion of linoleic acid, demonstrating that EFA need not necessarily be absorbed through the conventional route to be effective.

4.2.3.2 Biochemical basis for EFA deficiency

Once EFA deficiency had been demonstrated, the hunt was on to discover which fatty acids had EFA

Table 4.8 Major effects of *n*-6 essential fatty acid deficiency in rats

Skin	Dermatosis, water permeability increased Sebum secretion decreased Epithelial hyperplasia
Body weight	Decreased
Circulation	Heart enlargement Capillary resistance decreased
Kidney	Enlargement; intertubular haemorrhage
Lung	Cholesterol accumulation
Endocrine glands	Adrenals: weight decreased in females, increased in males Thyroid: weight increased
Reproduction	Females: irregular oestrus; impaired lactation, reproduction Males: degeneration of the seminiferous tubules
Metabolism	Changes in tissue fatty acid composition (see Table 4.9) Reduced cholesterol concentration in plasma Increased cholesterol concentration in liver, adrenals and skin Mitochondrial swelling and uncoupled oxidative phosphorylation Increased triacylglycerol output by liver

activity. The effects of different acids were first compared by measurement of growth response. Later, the team at the Unilever Laboratories in The Netherlands used a test based on disturbances in water metabolism, since an important result of EFA deficiency is an altered permeability of biological membranes. In this way they were able to rank fatty acids according to their 'EFA activity' and to correlate such activity with molecular structure. Linoleic (all-*cis*-9,12-18:2), γ-linolenic (all-*cis*-6,9,12-18:3) and arachidonic (all-*cis*-5,8,11,14-20:4) acids (all in the *n*-6 family) had a similar order of activity whereas that of α-linolenic acid (all-*cis*-9,12,15-18:3, *n*-3) was much lower. Work with a wide range of synthetic unsaturated fatty acids demonstrated that virtually all acids with significant activity were polyunsaturated with a *cis,cis* methylene-interrupted double bond system.

An important observation that gave further clues about which fatty acids were 'essential' was that in the tissues of animals that developed EFA deficiency, there was an accumulation of a PUFA that was either present in extremely small concentration or undetectable. This fatty acid is all-*cis*-5,8,11-eicosatrienoic acid (20:3*n*-9). Among the first biochemists to appreciate its significance was the American, James Mead, and it is now frequently referred to as the 'Mead acid'. Since that time, the ratio of the concentration of the Mead acid to arachidonic acid in tissues or plasma phospholipids (the 'triene/tetraene ratio') has been used as a biochemical index of essential fatty acid deficiency. In health, the ratio is about 0.1 or less, rising to 1.0 in severe EFA deficiency. A ratio of 0.4 has been used conventionally as indicating EFA deficiency, although more recently some authors have argued that it might be prudent to revise this figure downwards to nearer 0.2. The relationship between the proportion of linoleic acid in the diet and the magnitude of the triene/tetraene ratio in plasma phospholipids is similar in all species and all tissues examined (Fig. 4.3). It is a sensitive indicator since it increases significantly before overt EFA deficiency signs are apparent.

The explanation for the elevation of the concentration of 20:3*n*-9 in EFA deficiency can be readily appreciated from Fig. 4.4. During the course of evolution, animals lost the ability (retained by plants) to insert double bonds in positions 12 and 15. Each 'family' of unsaturated fatty acids (*n*-3, *n*-6, *n*-9) is biochemically distinct and its members cannot be interconverted in animal tissues. The 'parent' or 'precursor' fatty acid of the *n*-9 family, oleic acid, can originate from the diet or from biosynthesis in the body, whereas linoleic and α-linolenic acids, the precursors of the *n*-6 and *n*-3 families, respectively, must be obtained from the diet. They are thus essential nutrients. All three precursors can compete for desaturation by the same Δ6-desaturase enzyme as illustrated in Fig. 4.4. The affinity of the substrates for the Δ6-desaturase is in the order 18:3 > 18:2 > 18:1. In humans and many other mammals, quantitatively

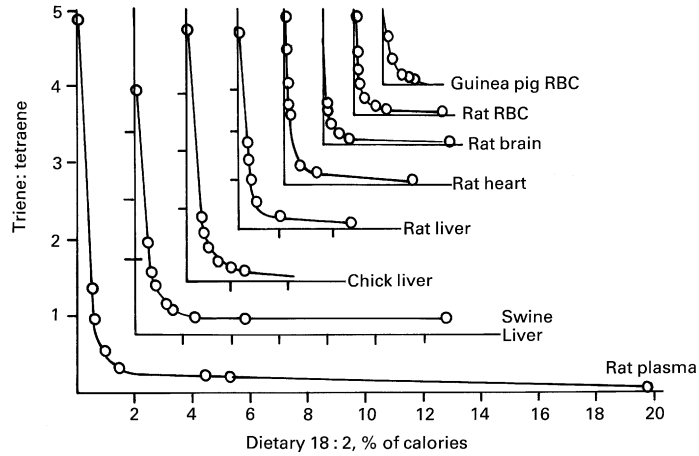


Fig. 4.3 Influence of dietary linoleic acid on the ratio of 20:3*n*-9 to 20:4*n*-6 (the triene/tetraene ratio) in different species. The relative scales are similar in all plots; the inset plots indicate the similarity in the relationship over a wide range of tissues and species. Reproduced with kind permission of Professor R.T. Holman (1970), and Pergamon Press Ltd, from *Progress in the Chemistry of Fats and Other Lipids*, **9**, 607–682.

the most important metabolic pathway is the one in which linoleic acid is converted into arachidonic acid. Normally, the diet contains sufficient linoleic acid for this pathway to be able continually to supply the quantity of arachidonic acid needed by the membranes of body tissues. When the amount of linoleic acid in the diet is very low, however, the *n*-9 pathway takes precedence. Hence, there is a relative accumulation of the Mead acid and a relative depletion in arachidonic acid. In practice, although a relatively large proportional increase in 20:3*n*-9 may be observed early in the progression to EFA deficiency, noticeable depletion of arachidonic acid stores requires long-term consumption of diets containing little or no linoleic acid.

It will be clear from Fig. 4.4 that there is also potential for competition for the Δ6-desaturase between 18:2*n*-6 and 18:3*n*-3 as well as the competition between the *n*-6 and *n*-9 substrates discussed above. Furthermore, there may also be competition at the level of the Δ5-desaturase, although this has been less well researched. Notwithstanding the high affinity of α-linolenic acid for the Δ6-desaturase, the flux through the *n*-3 pathway is generally low because of the small proportion of α-linolenic acid in most diets compared with linoleic acid. The consequences of this for health are discussed in more detail in Section 4.2.3.5.

4.2.3.3 Functions of essential fatty acids

Two distinct roles, to account for their essentiality, have been discussed for the EFA. These are as membrane components and as precursors for biologically active metabolites, the eicosanoids. Each will be discussed in turn, followed by a discussion of the links between the two roles. Students are advised to read the membrane section in conjunction with Section 6.5 and the eicosanoid section in conjunction with Section 2.4. A specific role for linoleic acid in skin function is discussed in Sections 4.2.3.4 and 6.6.6.

Essential fatty acids in membranes

Among the results of EFA deficiency are changes in the properties of biological membranes of which PUFA are major constituents of the structural lipids. These changes in properties, for example the permeability of the membrane to water and small molecules such as sugars and metal ions, can be correlated with changes in the fatty acid composi-

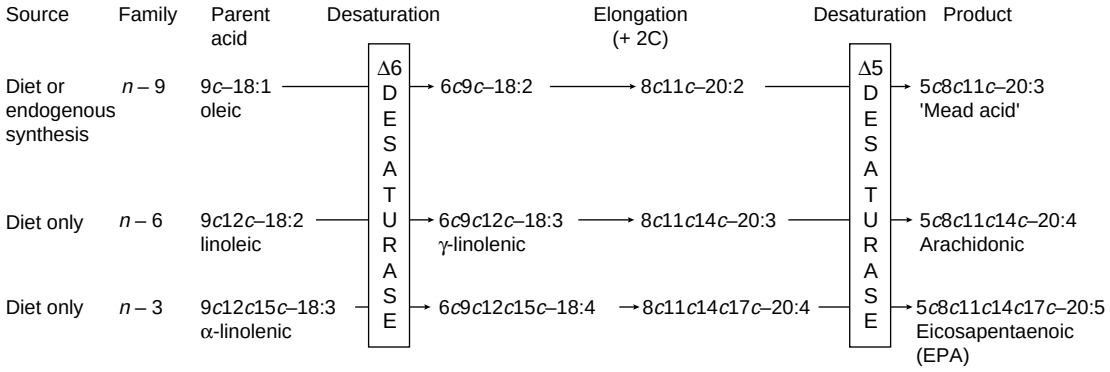


Fig. 4.4 Principal metabolic pathways for desaturation and elongation of 'parent acids' to long-chain polyunsaturated fatty acids in the *n*-9, *n*-6 and *n*-3 families. A minor family, *n*-7 (whose 'parent' acid is palmitoleic acid, 9*c*-16:1) has been omitted for simplicity. As far as we know it has little nutritional significance. The figure aims mainly to illustrate the sequence of alternate desaturations and elongations of the parent acids in each family and the competition between families for the Δ5 and Δ6-desaturases. For example, it should be readily apparent how the Mead acid accumulates in preference to arachidonic acid when there is a dietary deficiency of 18:2*n*-6 and an excess of 18:1*n*-9. An important continuation of the *n*-3 pathway via further elongation to C22 and further metabolism to form docosahexaenoic acid (DHA, 22:6*n*-3) has also been omitted. It is important to note that a 4-desaturase has not been identified and current understanding of the pathway to 22:6*n*-3 is discussed in Section 2.2.5.3.

tion of the membrane. The permeability of the skin to water is particularly sensitive to the amount of linoleic acid in the epidermis as described later. Membranes of liver mitochondria from EFA-deficient animals have smaller proportions of linoleic and arachidonic acids and larger proportions of oleic acid and all-*cis*-5,8,11-eicosatrienoic acid than those of healthy animals (Table 4.9). β-Oxidation and oxidative phosphorylation (Section 2.3.1) are less efficient. These changes at the molecular and cellular levels are reflected in the animal's poorer performance in converting food energy into metabolic energy for growth and in the maintenance of body function.

Table 4.9 Changes in the fatty acid composition of the phospholipids of mitochondrial membranes in *n*-6 essential fatty acid deficiency (g per 100 g total fatty acids)

Fatty acid	*n*-6 EFA sufficiency	*n*-6 EFA deficiency
16:0	31	31
18:0	10	9
18:1*n*-9	13	32
18:2*n*-6	21	10
20:3*n*-9	0.6	3.7
20:3*n*-6	1.7	1.0
20:4*n*-6	14	7
20:3*n*-9/20:4*n*-6	0.04	0.53

Organs and tissues performing functions such as storage (e.g. adipose tissue), chemical processing (e.g. liver), mechanical work (e.g. muscle) and excretion (e.g. kidney) tend to have membranes in which the *n*-6 fatty acids predominate, with arachidonic acid as the major component. In contrast, nervous tissue, reproductive organs and the retina of the eye have a greater proportion of the longer chain acids with 5 or 6 double bonds, predominantly of the *n*-3 family. It has been assumed that fatty acids of the *n*-3 family have specific roles in vision, reproduction and nerve function and there is some evidence in rats to show that when the ratio of *n*-3 to *n*-6 acids is modified experimentally, some changes do occur in the electroretinograms and in behavioural responses. We still do not have sufficient evidence, however, to pinpoint the specific functions for these fatty acids.

Essential fatty acids as precursors for eicosanoids

In Section 2.4 we described the biosynthesis of eicosanoids from polyunsaturated fatty acids of the *n*-3 and *n*-6 families and indicated in Section 2.4.4 that the balance of eicosanoids produced was important in, for example, maintaining normal vascular function. Several studies have demonstrated that altering the amounts and types of *n*-6 and *n*-3 fatty acids in the diet can influence the spectrum of eicosanoids produced. For example, substitution of fish oils, in which *n*-3 polyunsaturated fatty acids predominate, for diets in which linoleic acid (*n*-6) is the main polyunsaturated fatty acid (as typified by the UK and US diets) results in significant changes in plasma and platelet fatty acid profiles. Arachidonic (*n*-6) gives way to eicosapentaenoic (*n*-3) acid as the predominant polyunsaturated fatty acid and there is a reduction in the formation by platelets of thromboxane A_2, an eicosanoid that stimulates their aggregation (Table 4.10).

The physiological effects of eicosanoids are so potent in such minute quantities that they need to be generated locally and destroyed immediately after they have produced their effect by enzymes that convert them into inactive metabolites. These properties are typical of 'local chemical mediators' or 'autocoids', which distinguish them from hormones; the latter are produced in specific glands and act at more distant sites. The excretion of these metabolites in the urine has been used as a quantitative method for estimating the daily production of eicosanoids and assessing the quantities required by the body. The mechanism that ensures efficient local production is the release of the precursor polyunsaturated fatty acids from membrane phospholipids by specific phospholipases (Sections 2.4.9 and 7.2) and transfer to the enzymes of eicosanoid biosynthesis, which are also located within the membrane. The membrane phospholipids can therefore be regarded as a vast body store of essential fatty acids that are immediately available for eicosanoid biosynthesis when required. As they are depleted, they must be replaced by new parent acids of dietary origin or LCPUFA biosynthesized by the pathways illustrated in Figs 2.18, 2.21 and 4.4. This in part explains the dynamic turnover of fatty acids in biological membranes, although eicosanoid formation represents a very small proportion of fatty acid turnover.

Several aspects of eicosanoid metabolism and function, however, need further research. These include: the mechanism by which the relative proportions of the different eicosanoids are regulated; the significance of changes of dietary fatty acid composition for whole-body eicosanoid production; the quantitative significance of the different pathways and sites of synthesis; and the quantitative relationships between the requirements for EFA, which are measured in grams and the daily production of eicosanoids, which is measured in micrograms.

Table 4.10 Influence of dietary fatty acid composition on eicosanoid production by cardiac tissue

Animal species	Eicosanoid	Dietary fat (pg mg^{-1} dry weight)		
		Sheep fat	Sunflower seed oil	Tuna fish oil
Rat (*n*=12)	PGI_2	122[a]	104[a]	61[a]
	TXA_2	36[a]	19[a]	9[a]
Marmoset (*n*=6)	PGI_2	167[a,b]	114[a]	88[b]
	TXA_2	69[a]	24[a]	12[a]

Data from Abeywardena *et al.* (1992) in *Essential Fatty Acids and Eicosanoids* (eds A. Sinclair and R. Gibson), p 258, American Oil Chemist's Society, Champaign, IL. Animals were given the diets for 12 months (rats) or 24 months (marmosets). Minced heart tissue was incubated in buffer. Eicosanoids were determined by radioimmunoassays. Common superscripts within a row indicate a statistically significant difference at $P < 0.05$. PGI_2 is prostacyclin; TXA_2 is thromboxane A_2.

4.2.3.4 Which fatty acids are essential?

The precursor fatty acids

Historically, most research attention has been devoted to linoleic acid. As far as we know, linoleic acid is essential for almost every animal species at a dietary level of about 1% of energy, although there is evidence that some insects and protozoa can produce this fatty acid.

Linoleic acid seems to have a specific role in the skin epidermis that cannot be substituted by arachidonic acid, suggesting that the classic skin symptoms of EFA are not due to lack of eicosanoids. This conclusion is strengthened by the fact that ingestion of aspirin, which would inhibit eicosanoid formation from linoleic acid (Section 2.4.1), has no adverse effect on epidermal function. In EFA (linoleic acid) deficiency, the linoleate of several specific epidermal glycosphingolipids (see Section 6.6.6 and Fig. 6.28) is replaced by oleate, a change that is associated with a loss of barrier function. Moreover, when EFA deficiency is cured by giving a mixture of linoleic and arachidonic acids in the diet, only linoleate appears in the epidermal sphingolipids. These results suggest a biochemical mechanism for some of the EFA defects in skin whereby linoleate *per se* is an essential part of the human diet.

There has always been less certainty about α-linolenic acid. Fish, whose lipid metabolism is geared to processing a high dietary intake of *n*-3 fatty acids, seem to have a definite and high requirement (3% of energy) for fatty acids of this family. The brain and retinal rods of most species, including the rat, are characterized by a high proportion of the *n*-3 fatty acids and we can infer that, since these acids cannot be synthesized in the body, there is some dietary requirement for them, however small. Capuchin monkeys maintained on a diet containing adequate linoleic acid, but little or no α-linolenic acid, suffered symptoms closely resembling those of classical EFA deficiency. These were cured by the addition of linseed oil (which contains appreciable amounts of α-linolenic acid) to the diet. In rodents, there is documented evidence that α-linolenic cannot substitute for linoleic acid in reproduction in male and female rats. It has not been possible, however, to demonstrate unambiguously a function for α-linolenic acid that could not be replaced by linoleic acid.

In humans, the essentiality of α-linolenic acid remained in doubt until Holman and his colleagues in 1982 described a case of a young girl who displayed neurological symptoms 4–5 months after being on total parenteral nutrition. The fat component of the parenteral feed contained mainly linoleic acid and only a minute amount of α-linolenic acid. When safflower oil was replaced by soybean oil containing much more α-linolenic acid, the neurological symptoms disappeared. Later, a Norwegian group provided evidence for α-linolenic acid deficiency in elderly patients fed by gastric tube. It seems quite certain that only very small amounts of this nutrient are needed in human diets to prevent overt deficiency signs, although there is much debate about the amounts required to normalize functions that may not be manifested by classic deficiency signs.

Cats are unusual among mammals in that neither linoleic nor α-linolenic acid alone is sufficient to protect against the effects of fatty acid deficiency: these animals require arachidonic acid in their diets because they lack the Δ6-desaturase as well as the Δ12-desaturase.

The desaturation/elongation products

In studies of physiological processes such as spermatogenesis and parturition, it is rarely clear whether the EFA are functioning mainly or solely through their contribution to membrane structure or as precursors of one of the eicosanoids. If the latter, then it is likely that elongation/desaturation of the precursor acid is a critical process, since most important eicosanoids are derived from long-chain polyunsaturated fatty acids rather than their precursors (Section 2.4). Students who read further in this field of research (see Further Reading) may be confused to find that different authors use the term 'essential fatty acids' in apparently different ways. To nutritionists, there are only two essential fatty acids, linoleic and α-linolenic acids. They have to be supplied in the diet and are *essential nutrients*. The longer chain derivatives, it is argued, can be formed

in the body. They are '*essential metabolites*' but not essential nutrients. (Cholesterol provides a similar example in the steroid field.) Others have argued that all these structures, whether precursors or long-chain products, are 'essential fatty acids' because they are essential to life and are precursors of eicosanoids. Both schools of thought may be right to some extent if there are limitations on the supply of the elongation/desaturation products as discussed in the next section.

4.2.3.5 What are the quantitative requirements for essential fatty acids in the diet?

It seems possible that there are different levels of requirements for EFA for their different functions. For example, there may be a relatively low level of requirement (as for most vitamins), which is necessary to prevent overt signs of EFA deficiency such as skin conditions and growth retardation. This may be around 1–3% of dietary energy as linoleic acid and about one-fifth of this amount of α-linolenic acid. The upper figures for 18:2*n*-6 have been suggested as the increased requirement in pregnancy (to supply the additional needs of the foetus) and in lactation (for milk fat). However, little is known about the ability of women to adapt their metabolism to 'spare' their essential fatty acids in times of high demand. Intakes of EFA may be low enough to induce deficiency signs when hospital patients are given poorly designed enteral or parenteral feeds, when babies are given inadequate artificial formulas, in cases of malabsorption (when the amount of the EFA in the diet itself may be adequate) and in general malnutrition,

Over and above the requirement to satisfy classic EFA deficiency, there may be a larger but quantitatively less easily definable quantity required for such functions as maintaining an optimal balance of membrane fatty acids, preserving a reservoir for eicosanoid formation and maintaining optimal concentrations of the different plasma lipoproteins. The absolute amounts required at this level are likely to be influenced by the amounts and types of other fatty acids in the diet because of the competition for metabolic pathways between EFA and non-EFA as well as competitions between the EFA families. Because primary EFA may be only slowly converted into their long-chain polyunsaturated fatty acid derivatives, there may sometimes be a dietary requirement for the latter. These may be termed 'conditionally essential fatty acids', which have their counterparts in the conditionally essential amino acids.

Long-chain polyunsaturated fatty acids downstream of linoleic and α-linolenic acids may become conditionally essential in the following circumstances.

Desaturase abnormalities

Low activity or complete absence of a specific desaturase may result from single or multiple gene defects. The American biochemist Ralph Holman, who was a pioneer in our understanding of the essential fatty acids, has used the technique of 'plasma fatty acid profiling'. This involves comparison of the detailed fatty acid composition of normal plasma with the plasma profile of patients with a disease to pinpoint aberrations in the pathways of PUFA metabolism, which might be due to defects in desaturases. Such aberrations have been found in Sjörgren–Larsson, Reyes and Prader–Willi syndromes and in cystic fibrosis. Several desaturases have now been cloned and this will allow more rapid progress in pinpointing the nature of the metabolic abnormalities than was possible using these indirect techniques.

Δ6-Desaturase activity decreases with age and is also depressed by the so-called 'stress hormones', adrenaline and cortisol, by alcohol, by fatty acids of the *n*-3 family and in the condition of diabetes mellitus. One strategy to overcome the problem of low or absent Δ6-desaturase activity in the *n*-6 pathway is to provide γ-linolenic acid (*n*-6) in the diet, thereby by-passing the faulty Δ6-desaturase. The main dietary sources of γ-linolenic acid are evening primrose, borage and blackcurrant oils. The literature is replete with claims for the therapeutic benefits of 18:3*n*-6 and disorders for which its efficacy has been tested in controlled clinical trials include: atopic dermatitis; rheumatoid arthritis; diabetic neuropathy; multiple sclerosis; ulcerative colitis; hypertension; premenstrual

syndrome; schizophrenia; hyperactivity; and several cancers. In almost all categories the results have been inconsistent. There are preliminary indications that evening primrose oil may be more effective in some of the physiological effects described above than the other oils in which 18:3*n*-6 occurs. One explanation that has been offered is that 18:3*n*-6 is present in evening primrose oil almost entirely as molecular species of triacylglycerol in which one 18:3*n*-6 is combined with two linoleic acid molecules: dilinoleoyl-monogammalinolenoyl-glycerol. Another possibility is that minor components of evening primrose oil, not 18:3*n*-6, are responsible for some of the effects.

Metabolic competition

The competition between the *n*-9 and *n*-6 pathways that gives rise to an accumulation of 20:3*n*-9 in linoleic acid deficiency has already been discussed. There may, however, be more subtle changes in concentrations of 20:3*n*-9 and other polyunsaturated fatty acids without concomitant overt EFA deficiency signs, when intakes of 18:2*n*-6 are on the low side of normal and intakes of 18:1 (which is normally the most abundant dietary fatty acid) are exceptionally high. One of the arguments for limiting the amounts of *trans*-unsaturated fatty acids in the diet has been that high levels may result in changes in fatty acid profiles indicative of 'covert' EFA deficiency even when intakes of 18:2*n*-6 are apparently adequate. However, despite much debate, there is as yet no consensus about the biological significance of such metabolic changes.

During the 1990s, the question of appropriate dietary intakes of the *n*-3 family has been hotly debated. It is fair to say that, hitherto, this fatty acid family had been somewhat neglected. In Western industrialized countries, intakes of *n*-3 PUFA are generally very low compared with intakes of the *n*-6 PUFA. The parent acid, α-linolenic is obtained from green vegetables, rapeseed and soybean oils and products manufactured from them. The only significant sources of the longer chain *n*-3 fatty acids are certain fish oils (see Table 3.1). Populations consuming large amounts of fatty fish (for example the Inuit and many Japanese) tend to have much higher intakes of *n*-3 PUFA than in the West.

Increases in daily consumption of fatty fish are quickly reflected in elevated concentrations of these fatty acids, mainly 20:5*n*-3 (EPA) and 22:6*n*-3 (DHA), in the plasma and membranes of red and white blood cells. A major effect of such membrane changes is to alter the composition of eicosanoids formed through the action of the enzymes cyclooxygenase and lipoxygenase (Section 2.4). As well as increased production of weakly inflammatory prostanoids and leukotrienes from EPA, there is also inhibition of the strongly inflammatory eicosanoids from arachidonic acid (Fig. 4.5).

Some authors (for example, see the review by Okuyama *et al* in the Further Reading section) have argued a detailed case that when the ratio of dietary *n*-6 to *n*-3 fatty acids is too high, there is an increased risk of certain cancers, allergic diseases – including asthma – and thrombosis, all of which are prevalent in Western countries (Sections 4.3, 4.4 and 5.4). Other authors have discussed evidence that certain depressive conditions may also result from chronic dietary intakes with a high *n*-6/*n*-3 ratio. They cite evidence for disruption of neurotransmitter systems, defective signal transduction pathways, reduced insulin receptor sensitivity, and enhanced calcium channel activity, all of which are a consequence of the relative depletion of *n*-3 in nervous tissue membranes.

Developmental immaturity

At certain critical periods in the life-cycle (for example, pregnancy/foetal life or lactation/neonatal life), the high demands of tissues for particular LCPUFA may outstrip their availability, even though the supply of precursors would normally be adequate and there are no specific enzyme abnormalities. The flux along the metabolic pathway may simply be inadequate for the task. Under these conditions, the LCPUFA may become conditionally essential and need to be supplied in the diet (or in foetal development, from the mother's blood) for a limited period of time.

Current best estimates for human require-

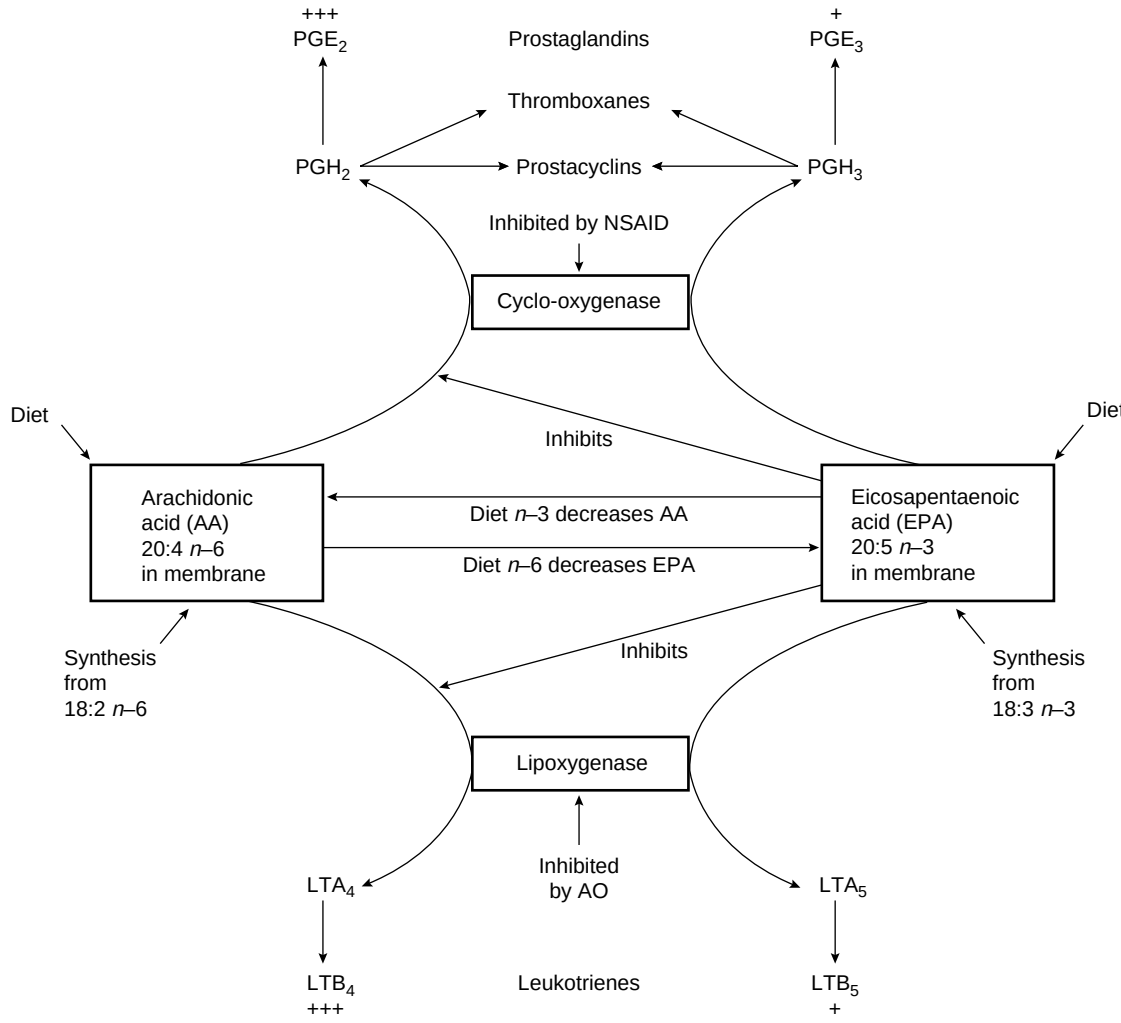


Fig. 4.5 Influence of dietary *n*-3/*n*-6 polyunsaturated fatty acids and drugs on inflammatory eicosanoid production. NSAID: non-steroidal anti-inflammatory drugs; AO: antioxidants; +: signifies weakly inflammatory; +++: signifies strongly inflammatory.

ments for essential fatty acids are summarized in Box 4.2.

Finally, our discussion of 'conditional essentiality' has followed the generally made assumption that the body's requirements for saturated and monounsaturated fatty acids can always be met entirely by endogenous biosynthesis; in other words there is never a dietary requirement for them. There is evidence, albeit limited, that fatty acid biosynthesis is a slow process in human tissues even when people are consuming diets with a low fat content. It is worth considering, therefore, whether there may be circumstances of high demand for saturated fatty acids (for example in brain or lung development), during which even saturated fatty acids become conditionally essential. Such a concept has not been actively researched.

Box 4.2 Views on human requirements for essential fatty acids

An answer to the apparently simple question, 'What are the requirements of human beings for essential fatty acids (EFA)?' is in fact not at all simple for several reasons.

- We must first define whether we mean EFA of the *n*-6 or *n*-3 families. As discussed in the text, needs for the *n*-6 family seem to be much greater than those for the *n*-3 family.
- Within each family, we must define whether we mean the parent acids (18:2*n*-6 or 18:3*n*-3) or their long chain polyunsaturated (LCP) metabolites. When the desaturation/elongation enzymes are fully active, then there may be a strict requirement only for the parent acids; when the activity of these enzymes is limiting, a requirement for the LCP may be defined (conditional essentiality).
- We must understand how members of the *n*-3 and *n*-6 families interact to influence each other's requirements. For example, because of competition for desaturases, a large excess of dietary *n*-6 over *n*-3 acids (which is usually the case in industrialized countries) may increase the requirement for the *n*-3 family.
- We must be clear what the requirement is for:
  - at the lowest level, there is a minimum requirement for either *n*-3 or *n*-6 acids to prevent the onset of overt deficiency signs;
  - over and above this level of intake, although no deficiency signs are seen, there may be public health recommendations for intakes to, for example, maintain low plasma concentrations of LDL (*n*-6 acids) or VLDL (*n*-3 family), to stimulate anti-inflammatory eicosanoid formation or limit platelet aggregation tendency etc.;
  - intermediate levels of intake may be required to optimize visual and other functions that are not necessarily easy to quantify.

Recommendations to prevent EFA deficiency

Each country tends to make slightly different recommendations, based on consensus views of its own scientists. In the UK, for example, recommended intakes to avoid deficiency are not less than 1% of dietary energy of 18:2*n*-6 (approximately 1–2.5 g day^{-1}) and 0.2% of dietary energy for 18:3*n*-3 (approximately 0.4–0.5 g day^{-1}). Some authorities may recommend intakes of up to 3% of dietary energy as *n*-6 polyunsaturated fatty acids during pregnancy and lactation to account for increased needs of the foetus or milk for the new-born. However, this assumes that there is no metabolic adaptation. For example stores of EFA in adipose tissues (which are very large) could be mobilized for this purpose.

Recommendations to maintain optimum blood lipid concentrations

There is clearly less precision in such recommendations because of differing views on what is 'optimal'. In the UK, the recommended intake is 6% of dietary energy as *n*-6 polyunsaturates, or approximately 12–15 g day^{-1}. No recommendation has been made for the *n*-3/*n*-6 ratio in this regard, although it is clearly an important issue to be resolved. Most authorities now set an upper limit for *n*-6 polyunsaturates intake of about 10% of dietary energy. The reasons include concern for the effects of peroxidative damage (see Box 4.3) and to avoid inhibiting pathways of *n*-3 polyunsaturated fatty acid metabolism.

4.2.4 Dietary lipids supply fat-soluble vitamins

It was realized early in this century that minute amounts of substances other than proteins, carbohydrates, fats and minerals are needed in the diet to sustain growth and reproduction and to maintain health. These accessory food factors appeared to be either fat-soluble or water-soluble and were given the name vitamins. They were divided into fat-soluble A and water-soluble B, but eventually it was realized that both these classes were mixtures of chemically unrelated compounds. The fat-soluble vitamins, found in mainly fatty foods, are now classified as vitamins A, D, E and K. In contrast to EFA (Section 4.2.3), which are required in gram

quantities, fat-soluble vitamins are required in microgram quantities per day. Although some fat is necessary in the diet to improve the absorption and utilization of fat-soluble vitamins, there is little evidence that, within the normal range of fat intakes, the amount of dietary fat significantly affects the utilization of fat-soluble vitamins. However, when fat absorption is impaired, insufficient fat-soluble vitamins may be absorbed, leading to a deficiency state. This could occur when the secretion of bile salts is restricted (as in biliary obstruction), when sections of the gut have been removed or damaged by surgery or in diseases, such as tropical sprue and cystic fibrosis, all of which are associated with poor intestinal absorption. When people have access to varied diets, there is little likelihood of dietary deficiencies of fat-soluble vitamins. However, some fat-soluble vitamins are present in a limited number of foods; inevitably, when food supplies are limited by economic conditions, deficiency diseases can then become a significant problem.

4.2.4.1 Vitamin A

The chemical name for vitamin A is all-*trans*-retinol [Fig. 4.6(A)]. Retinol itself is found only in animal fats. Plant materials, such as dark green leaves and vegetable oils, like palm oil, contain a precursor, β-carotene (provitamin A), which can serve as a source of retinol in the body. Dietary β-carotene [Fig. 4.6(B)] is split by an enzyme in the small intestinal mucosa, β-carotene 15,15′-oxygenase, yielding two molecules of all-*trans*-retinal (Fig. 4.6, reaction 1), which are then oxidized to all-*trans*-retinol (vitamin A) by an NADH-dependent reaction (Fig. 4.6, reaction 8). After absorption, or conversion from carotene, retinol is esterified mainly with palmitic acid (irrespective of the composition of the dietary esters) in the enterocytes in a reaction catalysed by one of two microsomal enzymes. The first is phosphatidylcholine:retinol acyltransferase, which transfers palmitic acid from position *sn*-1 of phosphatidylcholine (Fig. 4.6, reaction 2). The second type of reaction uses the CoA thiolester as a substrate and is catalysed by acyl-CoA:retinol acyltransferase.

Retinyl esters are the main storage forms of vitamin A in the body, mainly in the liver. They can be hydrolysed by a specific retinyl palmitate esterase (Fig. 4.6, reaction 3), the activity of which can increase some 100-fold during vitamin A deficiency. Transport in the blood requires hydrolysis of the ester followed by binding of retinol to a specific transport protein, retinol binding protein. Retinol is delivered to target tissues (e.g. visual cells) by interaction of the retinol binding protein to cell-surface receptors.

Vitamin A, with its analogues and metabolites, has several distinct functions: in the visual process, in cellular differentiation, embryogenesis and in the immune response.

Vision

This is the best-defined function in molecular terms. The reactions involved in the visual process take place in the outer segment of rod cells and in the colour-sensing cone cells of the retina. All-*trans*-retinol, carried in the circulation on retinol binding protein, is discharged to the visual cells and converted into 11-*cis*-retinol. The reaction is complex, since it involves first the esterification of retinol to retinyl palmitate by transfer of an acyl group from phosphatidylcholine (Fig. 4.6, reaction 2) in the pigment epithelium at the back of the eye. Then retinyl palmitate breaks down in a concerted reaction that couples isomerization of the retinol and release of a free fatty acid (Fig. 4.6, reaction 4). 11-*cis*-Retinol is oxidized to the aldehyde, 11-*cis*-retinal (Fig. 4.6, reaction 5), which then reacts with a lysine residue in the protein, opsin, through a Schiff base linkage to form rhodopsin (Fig. 4.6, reaction 6). This complex can absorb a photon of visible light (reaction 7), converting 11-*cis*-retinal into all-*trans*-retinal and triggering a conformational change in the protein. The resulting change in membrane potential is transmitted to the brain and perceived as sight. Following the conformational change, all-*trans*-retinol is discharged from the protein and the active 11-*cis* form may be regenerated by the reaction sequence 8, 3, 4, 5 shown in Fig. 4.6.

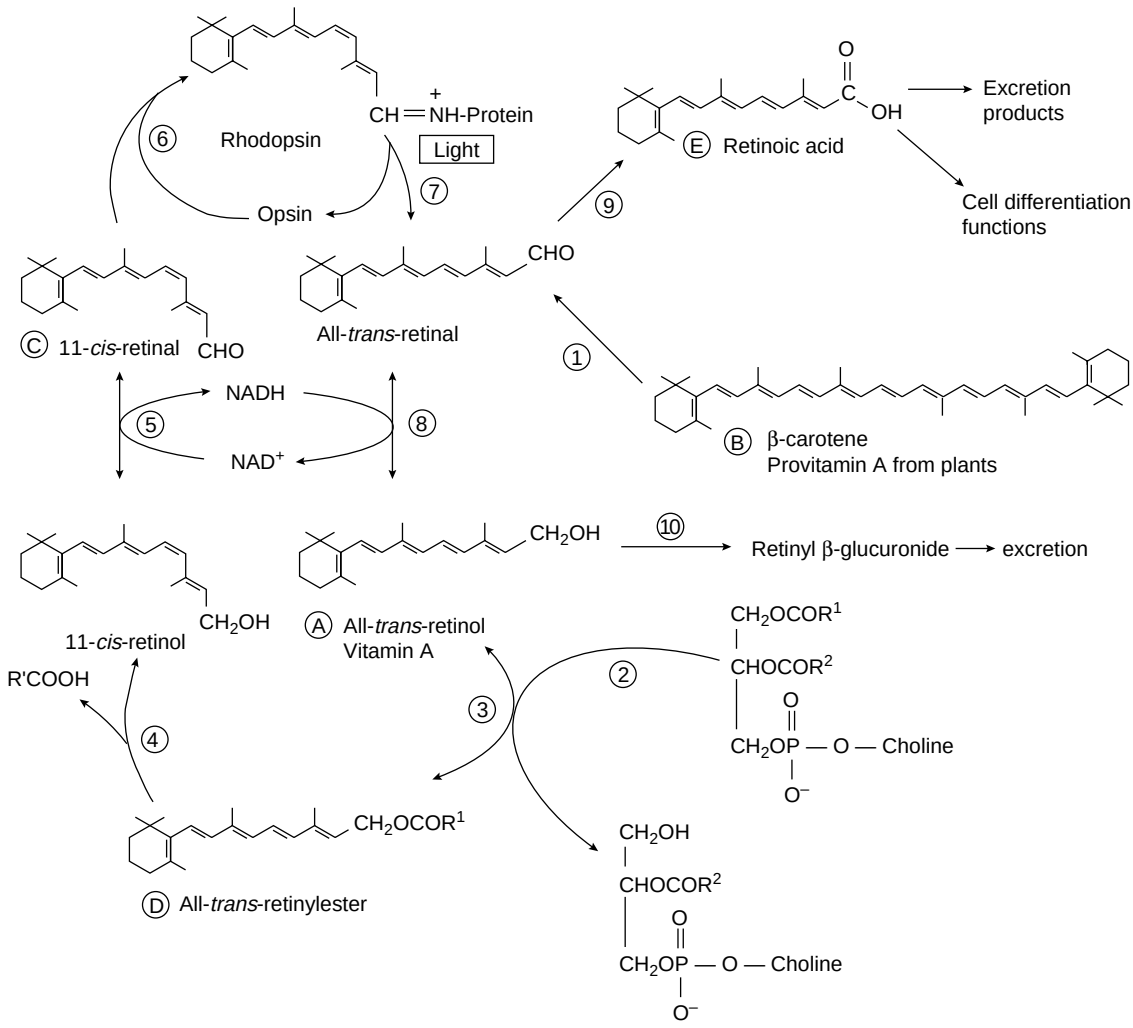


Fig. 4.6 Some important metabolic transformations of vitamin A. The parent compound of the vitamin A group is all-*trans*-retinol (A). It can be obtained directly from the diet or formed from the breakdown of dietary β-carotene (provitamin A) from plants (B). The active form of vitamin A in the visual process is 11-*cis*-retinal (C). Retinyl esters (mainly palmitate) provide a storage form of the vitamin in tissues, especially the visual pigment at the back of the eye (D). All-*trans*-retinal can be converted into retinoic acid (E), which has no visual function, but is involved in processes of cellular differentiation. Vitamin A can be excreted via the formation of glucuronides from retinoic acid or from vitamin A itself. Reactions 1–10 are described in further detail in the text.

Cellular differentiation

Just as 11-*cis*-retinal is the analogue of vitamin A involved in vision, so 9-*cis*-retinoic acid is the analogue mainly involved in differentiation [Fig. 4.6(E), reaction 9]. The cell nucleus contains two principal types of high-affinity receptor proteins for retinoic acid, designated RAR and RXR. Each may be present in α, β or γ isoforms. Furthermore, each set of receptors has six different domains that are involved in the transcription of genes. The receptors are coded by three different genes expressed at

different times and places during differentiation. RAR bind either all-*trans*- or 9-*cis*-retinoic acids, whereas RXR bind only the 9-*cis* isomer. Retinoic acid isomers, together with their nuclear (or 'intracellular') receptors can both stimulate and inhibit gene expression, depending on the types of interactions described above. RXR appears to interact with a number of other intracellular receptors such as the vitamin D receptor and the PPAR. This raises the possibility of interesting interactions between vitamin A and other chemical messengers (see the later section on vitamin D).

Retinoic acid can also form covalent bonds with certain proteins. These retinoylated proteins are similar in size to the nuclear retinoic acid receptors and may play roles similar to those of lipid-anchored proteins described in Section 6.5.14.

Immune response

Risk of infection is increased markedly in vitamin A deficiency and both humoral and cell-mediated immune responses are impaired. A major site of vitamin A action in the immune response is thought to be the T-helper cell and there seems to be a requirement for a specific form of vitamin A – 14-hydroxyretroretinoic acid – rather than retinoic acid. Understanding of the mechanisms by which vitamin A is involved in immunity at the molecular level, as well as in embryogenesis, spermatogenesis and taste perception is still in its infancy.

Nutritional aspects

Major sources of vitamin A are green vegetables and carrots (provitamin A), liver (especially fish), milk and some fat spreads. No more than 750 μg vitamin A is required by the average person daily. Compare this with the gram quantities required for EFA intake (Section 4.2.3.5).

The most tragic manifestation of vitamin A deficiency is blindness in young children. The first effects are seen as severe eye lesions, a condition known as xerophthalmia, which is eventually followed by keratomalacia with dense scarring of the cornea and complete blindness. Xerophthalmia is considered to be one of the four commonest preventable diseases in the world. Although there are large-scale programmes for the supplementation of children's diets with vitamin A, these are difficult to implement successfully and the World Health Organization considers that if the consumption of green leafy vegetables and suitable fruits by young children could be substantially increased, there is every reason to believe that the problem would be solved. There might be a case for trying to increase the overall fat content of the diet, too. Here, we are more concerned with solving problems of economics and distribution and with changing local eating habits than with biochemistry and nutrition, where the knowledge is already to hand.

Epidemiological evidence shows that people with above-average blood retinol concentrations or above-average β-carotene intakes have a lower than average risk of developing some types of cancer (Section 4.4.1).

In contrast to most water-soluble vitamins, excessive intakes of fat-soluble vitamins can be harmful, since in general they accumulate in tissues rather than being excreted. Thus, vitamin A, if taken in excess, accumulates in the liver. Chronic over-consumption may then cause not only liver necrosis, but also permanent damage to bone, vision, muscles and joints. Vitamin A can be excreted via its conversion into glucuronides formed from retinoic acid (Fig. 4.6, reaction 9) or from all-*trans*-retinol itself (Fig. 4.6, reaction 10).

4.2.4.2 Vitamin D

Vitamin D is the generic name for two sterols with the property of preventing the disease rickets. Ergocalciferol (vitamin D_2) is formed by irradiation of the plant sterol, ergosterol, and is the main dietary source of vitamin D. Cholecalciferol (vitamin D_3) is produced in the skin by ultraviolet irradiation of 7-dehydrocholesterol present in the skin surface lipids. It is the main source of the vitamin for most human beings. Dietary sources are fish liver oils (e.g. cod), eggs, liver and some fat spreads.

The parent forms of vitamin D are biologically inactive. The active metabolites are formed in the liver and kidneys. After absorption from the diet or

formation in the skin, vitamin D is carried in the circulation bound to a specific transport protein of the α-globulin class. In the liver, cholecalciferol is first hydroxylated to 25-hydroxycholecalciferol (Fig. 4.7). The 25-hydroxylase requires NADPH, molecular oxygen and Mg^{2+} ions. It is the main transport form of vitamin D and its concentration in plasma is used as an indicator of vitamin D status. Although this metabolite has modest biological activity, it is carried to the kidney, also attached to the transport protein, where it is further hydroxylated either to 1,25-dihydroxycholecalciferol (sometimes called calcitriol) or 24,25-dihydroxy derivative (Fig. 4.7) by enzymes similar to the liver 1-α-hydroxylase. 1,25-Dihydroxycholecalciferol is the most biologically active vitamin D metabolite, some 100 times more active than the 25-hydroxy metabolite. Its circulating concentration is tightly controlled by the plasma level of parathyroid hormone and the body's overall vitamin D status.

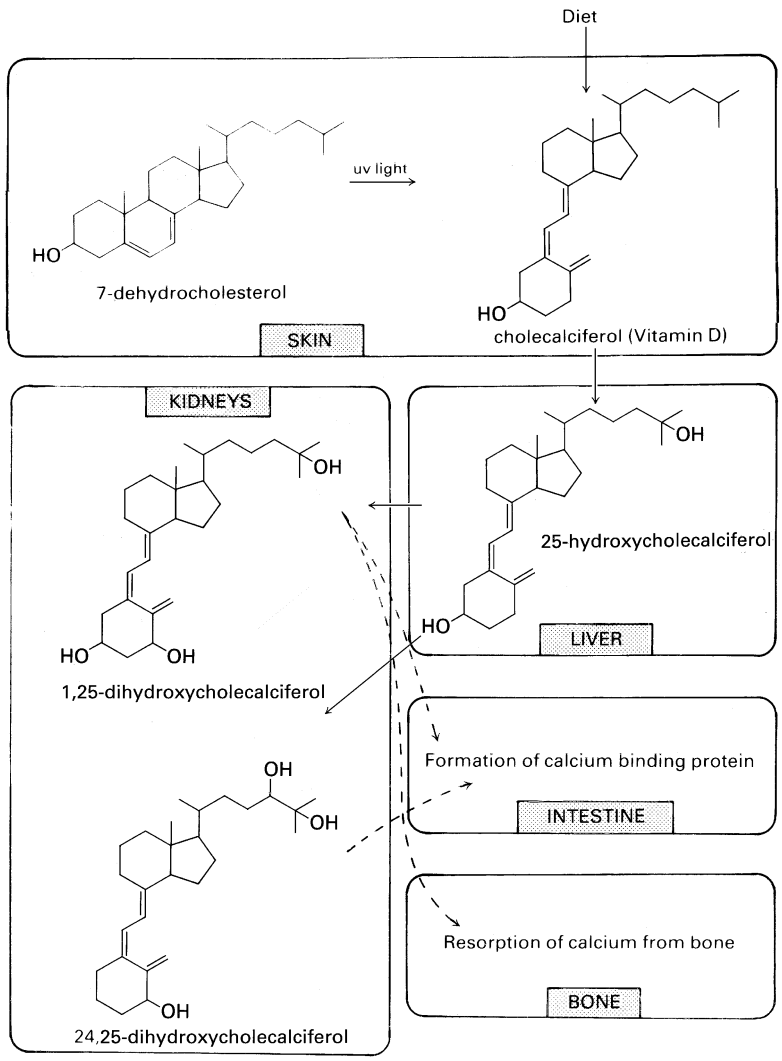


Fig. 4.7 Metabolism of vitamin D.

Thus, in relative vitamin D deficiency, the circulating concentration of 1,25-dihydroxycholecalciferol is high as a result of the stimulation of the liver 1-α-hydroxylase by parathyroid hormone, whereas that of the 24,25-dihydroxy metabolite is low. Calcitriol functions as a hormone in several distinct ways.

Calcium homeostasis

The best-studied function of calcitriol is in calcium homeostasis. Several mechanisms seem to be involved. The first is to increase the absorption efficiency of calcium by increasing its transport across the enterocyte brush border membrane and its subsequent export from the enterocyte into the blood. Calcitriol, a lipid-soluble compound, easily passes through the enterocyte cell membrane, binds to a specific receptor and is translocated to the nucleus where it binds to the DNA of specific response genes. Loops in the receptor, known as 'zinc fingers' enable the receptor to interact with DNA to induce the transcription of mRNA for a specific Ca-binding protein that aids active transport of Ca from the gut to maintain blood concentrations within the narrow range of 2.1–2.6 mM. A second role for calcitriol, in the presence of parathyroid hormone, is to activate bone osteoclasts to resorb calcium from bone, again to maintain an appropriate plasma calcium concentration. However, the role of calcitriol in bone is more complex than implied here, since it can also stimulate bone formation by osteoblasts, thus participating in the regulation of the overall process of bone turnover.

Other functions

The discovery of receptors for 1,25-dihydroxycholecalciferol in many tissues other than those involved in calcium and bone metabolism (e.g. pancreatic islet cells, skin keratinocytes, mammary epithelium and some neurones) suggests a wider involvement in aspects of tissue development. Indeed, calcitriol seems to be a general developmental hormone, inhibiting proliferation and promoting differentiation in many tissues. Present knowledge suggests functions that include regulation of gene products associated with mineral metabolism, differentiation of cells in skin and in the immune system and regulation of DNA replication and cell proliferation. A recent finding is that when 1,25-dihydroxycholecalciferol binds to a response element in the promoter region of its target gene, it requires an accessory protein. It is now thought that this protein may be identical to the retinoic acid X receptor discussed in the previous section, suggesting an interesting convergence of vitamin A and D functions that is yet to be properly elucidated.

Nutritional aspects

It is difficult to decide upon a precise dietary requirement for vitamin D because much is derived from the skin lipids rather than the diet. Thus the UK committee that advises on dietary requirements set no dietary reference value for vitamin D for people between the ages of 4 and 50 years. Two groups of people, however, may have a special need to obtain vitamin D from the diet. In the first group are children and pregnant and lactating women whose requirements are particularly high. In the second group are people who are little exposed to sunlight, such as the housebound elderly and people in far northern latitudes or those who wear enveloping clothes. Dark-skinned immigrants to Northern Europe are especially vulnerable. Infants and children who obtain too little vitamin D develop rickets, with deformed bones that are too weak to support their weight. The reason why, in the UK and some other countries, vitamin D preparations are provided for children and pregnant women, and margarine is fortified with it, is because these degenerative changes soon become permanent if supplementation is not started early enough.

Like vitamin A, vitamin D is toxic in high doses. Even amounts that are no more than five times the normal intake can be toxic. Too high an intake causes more calcium to be absorbed than can be excreted, resulting in excessive deposition in, and damage to, the kidneys.

4.2.4.3 Vitamin E

Vitamin E activity is possessed by eight tocopherols and tocotrienols (Fig. 4.8). α-Tocopherol is the most potent of these, the other compounds having between 10% and 50% of its activity. α-Tocopherol is also the most abundant form of vitamin E in animal tissues, representing 90% of the mixture. The form used in commercial preparations is synthetic racemic α-tocopherol (the natural compounds have optical activity), often in the acetylated form as a protection from oxidation.

In 1922, the Americans, Evans and Bishop, discovered that vitamin E prevented sterility in rats reared on fat-deficient diets fortified with vitamins A and D. Because vitamin E is so widespread in foods and like all other fat-soluble vitamins is stored in the body, deficiency states are rarely if ever seen, possible exceptions being in premature infants with low fat stores and in people with severe malabsorption. The richest sources are vegetable oils, cereal products and eggs.

Vitamin E is carried in the blood by the plasma lipoproteins (Section 5.2). There is evidence for specific tissue vitamin E binding proteins, but research into this topic is still in its infancy.

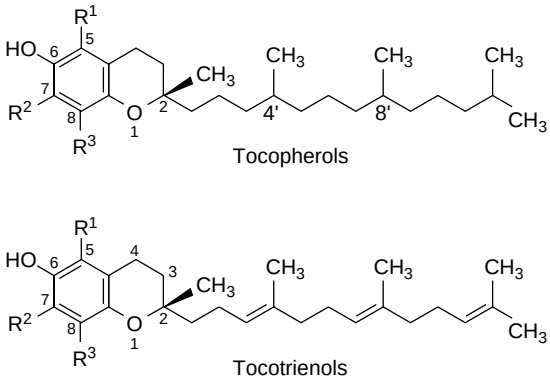


Fig. 4.8 Structures of compounds with vitamin E activity. All molecules comprise a chromanol ring system and a 16C phytyl side chain. Tocopherols have a saturated side chain; tocotrienols contain three unsaturated bonds in the side chain. Analogues are named α, β, γ and δ-tocopherols or tocotrienols as follows:

α: $R^1 = CH_3$; $R^2 = CH_3$; $R^3 = CH_3$
β: $R^1 = CH_3$; $R^2 = H$; $R^3 = CH_3$
γ: $R^1 = H$; $R^2 = CH_3$; $R^3 = CH_3$
δ: $R^1 = H$; $R^2 = H$; $R^3 = CH_3$

Vitamin E as an antioxidant

Until the last decade of the twentieth century, almost all interest in vitamin E was concerned with its putative role as a natural antioxidant. α-Tocopherol is present in the lipid bilayers of biological membranes (Section 6.5) and may play a structural role there. However, its main function is thought to be the prevention of the oxidation of the unsaturated lipids present in the membrane (Section 2.3.4 and Box 4.3). The products of lipid peroxidation can cause damage to cells if the oxidative process is not kept in check (Box 4.3). Such damage appears to be exacerbated in animals fed diets deficient in vitamin E. α-Tocopherol primarily acts as a terminator of the lipid peroxidation chain reactions by donating a hydrogen atom to a lipid radical (Section 2.3.4). The resulting tocopheryl radical is relatively unreactive and unable to attack adjacent unsaturated fatty acids because the unpaired electron becomes delocalized in the aromatic ring structure. Experiments in vitro demonstrate that ascorbic acid (vitamin C) is capable of regenerating the antioxidant form of vitamin E. If this were to occur in vivo, it would allow one vitamin E molecule to scavenge many lipid radicals, but such a process has not been demonstrated conclusively in vivo.

It is a generally held view that dietary requirements should be considered in relation to the PUFA content of the diet rather than in absolute amounts. A ratio of vitamin E to linoleic acid of about $0.6\,mg\,g^{-1}$ is generally recommended. In general, those vegetable oils that contain high concentrations of PUFA are sufficiently rich in vitamin E to give adequate protection.

Other functions

There is now accumulating evidence that vitamin E may also play other biological roles that do not necessarily involve its antioxidant function. A possible structural role in the maintenance of cell membrane integrity has already been mentioned. Some observations imply diverse roles in immune

Box 4.3 The far-reaching effects of lipid peroxidation

The process of lipid peroxidation has such wide ramifications that it is discussed in several chapters of this book. The purpose of this box is to bring together and summarize these diverse effects and underline their importance.

Reactive oxygen species

- Oxygen is essential to life but paradoxically, reactive forms of oxygen or 'ROS', which are produced during normal metabolism, have the potential to cause extensive damage in the body.
- ROS, which include hydroxyl and superoxide radicals and the non-radicals hydrogen peroxide and singlet oxygen, are not always harmful. For example they are involved in the destruction of pathogens by phagocytes.
- In the absence of adequate defence mechanisms (see below), ROS can attack DNA, proteins and lipids in the body; food lipids may also be oxidized.

Initiation, propagation and termination of lipid peroxidation

- Lipid peroxidation begins by ROS attack on the carbon atoms between the double bonds in polyunsaturated fatty acids and the lipid radicals so formed can propagate chain reactions that generate yet more lipid radicals (Section 2.3.4).
- Peroxidation is accelerated by heating and by catalysts such as transition metal ions (e.g. Cu^{2+}, Fe^{3+}) and haem compounds (Section 4.1.3.2).
- Chain reactions are terminated when antioxidant radicals combine with lipid radicals to halt further propagation (Section 2.3.4). Vitamin E is an example of a natural lipid-soluble antioxidant with this function (Section 4.2.4.3).

Links to food palatability

- Lipid radicals undergo degradation to smaller molecular weight compounds that are associated with changes (either desirable or adverse) in flavour, aroma, colour and texture of foods (Fig. 4.11).
- Controlled oxidation is capable of generating compounds associated with very specific flavours or aromas (Fig. 4.10).
- Unchecked peroxidation can reduce food palatability quite severely.
- Some products of lipid peroxidation can be toxic (Section 4.1.4.4) although food normally is rendered inedible long before the concentration of such compounds has reached toxic levels. Long-chain lipid peroxides are not well absorbed, but lower molecular weight compounds are absorbed and potentially harmful (Section 4.1.4.4).

Links to disease

- The membranes of organs and tissues are packed with polyunsaturated fatty acids. Integrated antioxidant systems (see below) are normally adequate to prevent peroxidation and membrane disruption.
- Disruption of cellular structures may cause antioxidant systems to be ineffective.
- Changes to proteins and DNA resulting from lipid radical attack can initiate or exacerbate disease processes, for example oxidation of LDL fatty acids and apolipoprotein-B in atherosclerosis (Section 5.4.1) and DNA changes in cancer (Section 4.4).

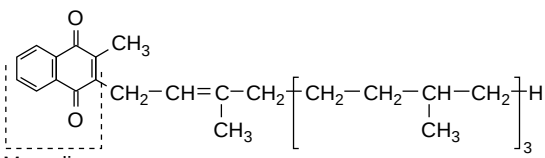
Antioxidant defences

- One type of antioxidant defence is through enzymes that destroy radicals (e.g. superoxide dismutase, glutathione peroxidase) or non-radicals (e.g. catalase).
- Another type of defence is through consumption of antioxidant nutrients in the diet. These may act as radical scavengers (e.g. vitamin E, carotenoids, Section 4.2.4). A large number of diverse compounds with antioxidant properties exists in foods. Little is known about their mechanism of action.
- Some dietary minerals are essential for the function of antioxidant enzymes (e.g. the various isoforms of superoxide dismutase use copper and zinc or manganese as cofactors, whereas an isoform of glutathione peroxidase uses selenium).

function. Vitamin E has anti-inflammatory effects, possibly by interacting with the prostaglandin synthetase that produces inflammatory eicosanoids (Section 2.4) and also appears to stimulate the immune response. α-Tocopherol seems to be involved in the regulation of intercellular signalling and cell proliferation through the modulation of protein kinase C activity. Finally, it may have a role in DNA biosynthesis. The details of each of these 'other functions' are still far from clear and this should provide a fascinating area of future research.

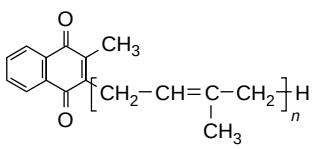
4.2.4.4 Vitamin K

Vitamin K is the generic name given to a group of compounds, having in common a naphthoquinone ring system (menadione) with different side chains (Fig. 4.9). Plants synthesize phylloquinone (vitamin K_1) with a phytyl side chain identical to that in chlorophyll. Bacteria synthesize menaquinones (vitamin K_2) the side chains of which comprise 4–13 isoprenyl units (Sections 7.5.2 and 7.5.3). Major dietary sources of vitamin K_1 are fresh green leafy vegetables, green beans and some seed oils. Cereals are poor sources and, of animal foods, only beef liver is a significant source. Vitamin K_2 is found in fermented foods, such as cheeses and yoghurt and in ruminant liver. Bacteria in the human gut synthesize menaquinones, but there is uncertainty about their bioavailability and hence their significance as a source of vitamin K.



Menadione

Vitamin K_1, Phylloquinone



Vitamin K_2, Menaquinones

Fig. 4.9 Structures of compounds with vitamin K activity.

Free phylloquinone is absorbed from the small intestine with about 80% efficiency, but only about a tenth of the vitamin K_1 present in dark green leafy vegetables is absorbed. Vitamin K is carried in chylomicrons (Section 5.2.3) and delivered mainly to the liver, in chylomicron remnants (Section 5.2.5). About two-thirds of the vitamin K intake is soon excreted from the body (into the faeces via the bile) and as there is no evidence of an entero-hepatic circulation, it is presumed that a constant intake is essential.

Function in enzymic carboxylation reactions

Like many water-soluble vitamins, but in contrast to other fat-soluble vitamins, vitamin K has a specific function as an enzyme cofactor. The enzyme, present in the endoplasmic reticulum of liver cells and in some other tissues, is γ-glutamyl- (or vitamin K-dependent-) carboxylase. It requires both molecular oxygen and carbon dioxide and inserts a carboxyl group into the glutamyl residue of several specific calcium-binding proteins. The reaction is a post-translational modification of the protein. The active form of vitamin K in this reaction is the reduced quinol form, which donates hydrogen to glutamic acid, being transformed in the process to an epoxide. The 2,3-epoxide is then transformed to the quinone form by vitamin K epoxide reductase in a dithiol-dependent reaction and the quinone can be further reduced to the quinol, thus completing the so-called vitamin K epoxide cycle.

Role in blood coagulation

The best-studied role of vitamin K has been in relation to the blood coagulation cascade. Four of the procoagulant proteins of the cascade depend on the presence of vitamin K for their formation (factor II, or prothrombin, and factors VII, IX and X). The formation of γ-carboxyglutamate residues in these protein factors provides efficient chelating sites for Ca ions, enabling ion bridges to be made between the factor and the surface phospholipids of platelets and endothelial cells. Anticoagulants, such as

warfarin, inhibit the γ-carboxylation reaction, so preventing clotting.

Role in bone metabolism

It was only in 1975 that it was discovered that bone tissue contains a γ-carboxyglutamyl protein, osteocalcin, which accounts for up to 15% of non-collagenous proteins in bone. Fully carboxylated osteocalcin is able to form a strong complex with hydroxyapatite, the calcium phosphate mineral component of bone. Much circumstantial evidence suggests a key role for vitamin K in bone health. For example, treatment of pregnant mothers with vitamin K anticoagulants, such as warfarin, can lead to bone defects in their infants. Measurement of 'undercarboxylated' species of osteocalcin has been explored as an index of vitamin K deficiency. Yet in practice, there is no direct evidence that vitamin K deficiency has long-term adverse effects on bone health. This apparent paradox is probably due to insufficient detailed information and this area promises to be an important growth point in bone research.

Nutritional aspects

In vitamin K deficiency, the time taken for blood to clot is prolonged and the activities of factors II, VII, IX and X are reduced. It is rare to see vitamin K deficiency in an adult except when fat absorption is impaired. Deficiency signs occur more frequently in infants. This is because they have an immature liver that synthesizes prothrombin only slowly. They also have an undeveloped intestinal flora, that might otherwise provide some menaquinones and, finally, human milk contains only small amounts of vitamin K. Dietary amounts needed to satisfy most individuals' requirements are thought to be about $10\,\mu g\,day^{-1}$ for infants and between 40 and $80\,\mu g\,day^{-1}$ for adults. In some new-born infants, insufficient intakes of vitamin K give rise to bleeding into tissues, known as haemorrhagic disease of the new-born. In some countries (e.g. the UK) vitamin K is given routinely at birth, but this is not universally the practice.

There is little evidence of harm from high intakes of natural vitamin K, although high intakes of menadione can lead to haemolysis and liver damage in infants.

4.2.5 Lipids play an important role in enhancing the flavour and texture and therefore the palatability of foods

Whatever its nutrient composition might be, food has no nutritive value if it is not eaten. In countries where choice of food is abundant, palatability is a major factor in determining food choice and, therefore, nutrient intakes.

Fats contribute to palatability mainly in two ways. Firstly by responses to their texture in the mouth (sometimes called mouthfeel) and secondly by olfactory responses, namely taste in the mouth and aroma or odour in the nose; together, these are called flavour. Fat is, on the one hand, a source of taste and aroma compounds and, on the other, a medium that regulates the distribution of these compounds between water, fat and vapour phases, which influence their perception by the sense organs. Some flavour compounds result from the decomposition of lipids by lipolysis, oxidation (Section 2.3.4), and microbial or thermal degradation. These processes may produce free fatty acids, aldehydes, ketones, lactones and other volatile compounds (Fig. 4.10). PUFA play a dominant role because of their susceptibility to oxidation. Figure 4.10 illustrates the generation of a specific odour compound, whereas Fig. 4.11 summarizes the different aspects of food quality that may be influenced by oxidative metabolites of PUFA.

4.2.5.1 Odour

When low molecular weight volatile compounds interact with receptors in the nose, they give rise to the sensation of odour or aroma. This sensation depends on features of the molecular structure of the odour compounds and on their partitioning between the fat and the vapour phases. Thus, short-chain length fatty acids (e.g. in butter) have a more intense odour than longer chain acids; those with chain lengths greater than about ten carbon atoms

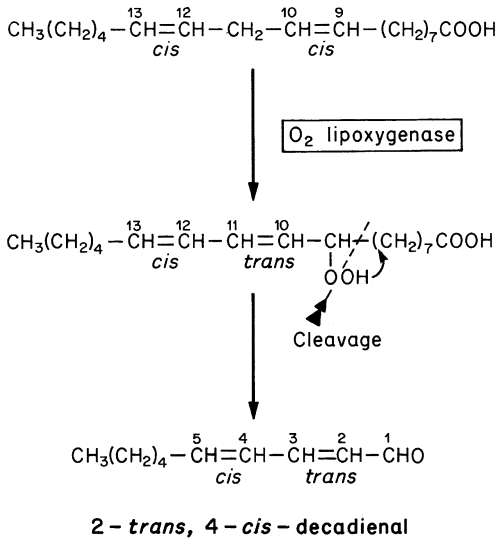


Fig. 4.10 The formation of an odour compound. 2-*trans*, 4-*cis*-Decadienal is responsible for some of the flavour of oxidized soybeans.

have little odour because they are not sufficiently volatile. The fat content of the food has an important influence on aroma. A high fat content favours partition into the fat phase and may slow down the loss of volatiles, which are generally hydrophobic. Sometimes an advantage of a high fat content may be in lowering the intensity of the sensation of an unpleasant odour. Frequently the perception of a particular odour is quite different (and often more unpleasant) at high compared to low concentrations and the effective concentrations will be much influenced by the fat content of the foods. If food products do not contain much lipid, then there will be an early release of aroma compounds as the food is eaten. The nature and time-scale of the perceived sensation will be quite different, and possibly less pleasant, than when a fatty food is eaten.

4.2.5.2 Taste

As food is eaten, taste compounds, which may elicit acid, sweet, salty or bitter tastes, must pass into the aqueous phase before they are sensed by the taste receptors in the mouth. The aqueous phase is

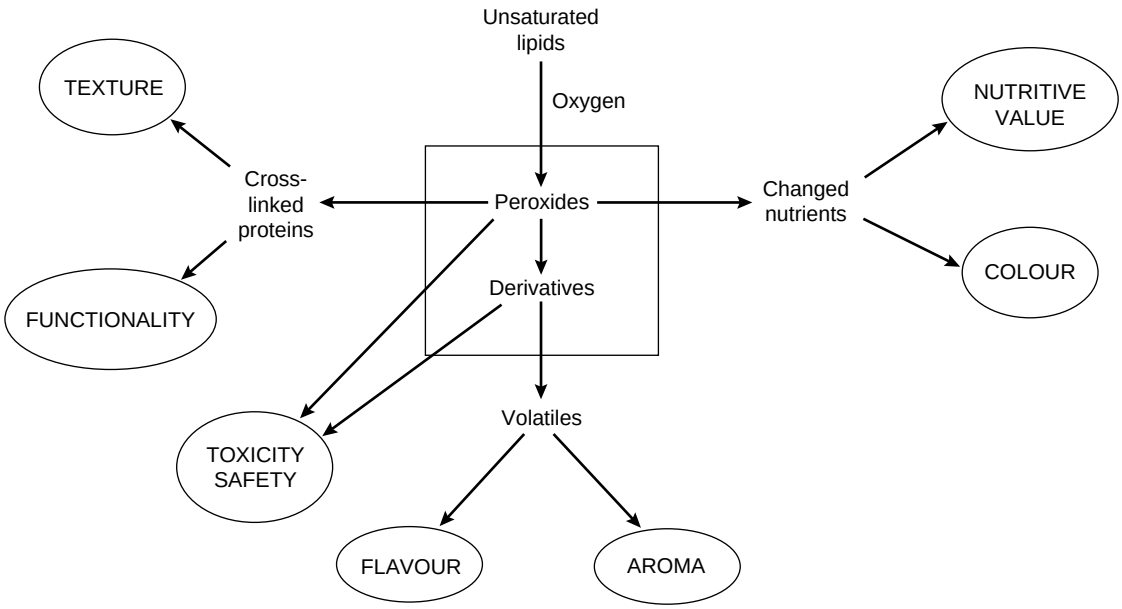


Fig. 4.11 Reactions of polyunsaturated fatty acids leading to changes in food quality.

composed of the water from the food mixed with saliva in the mouth. Thus, factors such as water solubility, pH and salt formation, which influence the partitioning of taste compounds between aqueous and fat phases, influence flavour perception. For example, the presence of fat may retard their passage into saliva and thus limit the perception of taste. The way in which fat and water are distributed in a food, and the proportions of each, influence flavour perception. Many foods have a poor flavour if they contain too little fat (cheese is a good example). The effect, while very obvious to the consumer, is not fully understood scientifically but may be related to the state of dispersion of the fat.

4.2.5.3 Texture

Fat may also have an important effect on the texture of food as perceived during eating. The physical state of the fat is important. Pure oil is unpleasant to swallow for most people, while an emulsion may be perceived as pleasantly creamy. An oil-in-water emulsion gives quite a different sensation from a water-in-oil emulsion of the same chemical composition. Thus, full fat milk, cream and butter each has its individual sensory characteristics. A high content of solid fat (e.g. in chocolate) gives a cooling effect on the tongue during eating because of the heat needed for melting. If the fat does not melt completely during eating, however, an impression of stickiness results. Firmness and graininess are other attributes affected by solid fat content. People vary greatly in their appreciation of fat in relation to palatability. In this respect, composition and state of dispersion of the fat may be crucial.

4.3 DIETARY LIPIDS IN RELATION TO IMMUNE FUNCTION

Lipids influence immunity in several ways. Some glycolipids are components of cell-surface antigens. Dietary fat enhances immunity in malnourished people through its energy contribution. Individual lipids have more specific roles through effects on membrane structure, eicosanoid production, peroxidation and gene expression.

4.3.1 Components of the immune system and their functional assessment

It is well established that diet has an important influence on immunity. Energy malnutrition and deficiencies in specific nutrients result in increased susceptibility to infection and increased mortality, especially in children.

Lipids may be involved in immunity in several ways. The role of some glycosphingolipids in cell-surface antigens is discussed later in Section 4.4.2. The following deals only with the contributions of dietary lipids. Because of their importance as a source of dietary energy, triacylglycerols may contribute to the enhancement of immune function in malnourished individuals. Individual lipids, including some fat-soluble vitamins and different types of fatty acids, play more specific roles through their contribution to membrane structure and integrity (Section 6.5), their metabolism to eicosanoids (Section 2.4), their participation in processes of lipid peroxidation (Box 4.3), or their influence on gene expression (Section 5.3).

The immune system has evolved to protect the body from constant attack by infectious organisms and other non-self molecules that could have detrimental effects on the host. It involves complex interactions between different types of cells, which produce substances that are toxic to invading pathogens. Lymphocytes are key players in the immune system. B-lymphocytes produce antibodies against specific antigens located on the surface of invading organisms (humoral immunity). T-lymphocytes do not produce antibodies. They recognize peptide antigens attached to major histocompatibility complex proteins on the surfaces of so-called antigen-presenting cells. In response to antigenic stimulation, they secrete cytokines (e.g. interleukins, interferon, transforming growth factor, etc.), whose function is to promote the proliferation and differentiation of T-lymphocytes as well as other cells of the immune system. These include B-lymphocytes, monocytes, neutrophils, natural killer cells and macrophages. Whereas life would hardly be possible without this well-integrated system, failure adequately to regulate these

processes can lead to damage to the body's own tissues as seen in severe inflammation and in autoimmune diseases.

In the 1970s it was discovered that diets rich in PUFA could prolong the survival of skin allografts in mice and such diets were subsequently employed as adjuncts to conventional immunosuppressive therapy to reduce rejection of human kidney grafts. Such diets also appeared beneficial in treating patients with multiple sclerosis, an autoimmune disease in which an inappropriate immune response to one of the body's own proteins causes damage to the myelin membrane. These findings, and others over the intervening years, strongly suggested that PUFA were acting to suppress the immune system. Recent research has been directed to better understanding the mechanisms of the action of PUFA at the cellular and molecular levels.

Much of the research into the effects of dietary fatty acids on aspects of immune function has been done with lymphocytes 'ex vivo'. The cells are isolated from the blood of animals (including human subjects) given diets differing in fatty acid composition. Commonly used tests are (a) rates of cell division (measured by incorporation of tritiated thymidine into DNA) of lymphocytes stimulated with specific antigens or non-specific mitogens; (b) macrophage migration in an electric field; (c) cytokine production by different cell types; (d) expression of lymphocyte cell-surface molecules; (e) lymphocyte-mediated cytolysis; (f) cytotoxic T-lymphocyte activity; (g) natural killer cell activity. Such ex vivo tests have been supplemented by assessment of immune function in vivo by measuring (a) delayed-type hypersensitivity reactions; (b) graft versus host responses; (c) organ transplantation and (d) antibody production. It is not possible to describe these methods here and interested students should consult the reference lists in appropriate publications listed under Further Reading at the end of this chapter.

Interpretation of the results of all these studies is difficult for several reasons. Each of the aforementioned tests measures a different aspect of immune function and dietary lipids may not affect all functions in the same way. Results of tests involving lymphocytes in culture are often critically dependent on the type of serum used in the culture medium. When animal models are used, results may differ between species and may not always be relevant to man. Very importantly, dietary experiments have normally used natural fats and oils to represent different classes of fatty acids. For example beef tallow, olive oil, sunflower oil and fish oils have been given to provide dietary fats dominated by saturated, monounsaturated, *n*-6 polyunsaturated and *n*-3 PUFA, respectively. Of course all these fats contain mixtures of fatty acids, as well as many minor components, such as fat-soluble vitamins and sterols. Consequently, it can never be certain that the effects observed were due to the fatty acid of interest, even though it may be the major component of the mixture. Some authors have tried to overcome this problem by giving synthetic diets containing the ethyl esters of specific fatty acids. However, with this approach, potential problems of nutrient balance need to be addressed.

4.3.2 Summary of lipid effects on different components of immunity

Bearing in mind all the reservations expressed above, the main findings may be summarized in the following sections.

4.3.2.1 Influence on target cell composition

(1) Giving diets enriched with specific fatty acids generally leads to a corresponding enrichment of that fatty acid in the different cells of the immune system.
(2) It is frequently assumed that the observed changes occur in membrane lipids. However not all studies specifically isolated membrane phospholipid fatty acids. Some of the cell types of interest contain small stores of triacylglycerols, which are more susceptible to modification by diet.
(3) Membrane fatty acid changes do occur, however, especially after giving dietary *n*-3 PUFA, which tend to replace membrane arachidonic acid (*n*-6).

4.3.2.2 Influence on lymphocyte functions ex vivo

(1) Animal studies indicate that high fat diets in general suppress lymphocyte proliferation compared with low fat diets.
(2) Among high fat diets the order of potency is fats rich in saturated fatty acids < oils rich in *n*-6 PUFA < olive oil < linseed oil < fish oil.
(3) Some, but by no means all, human studies replicate these results.
(4) Cytotoxic T-lymphocytes and natural killer cell activities also tend to be suppressed by high fat diets in a manner similar to the lymphocyte proliferation.
(5) Fewer studies have described effects on cytokine production or surface-molecule expression and these have mainly focused on *n*-3 PUFA. For example, when diets of healthy human subjects have been supplemented with fish oils, neutrophil leukotriene B_4 (LTB_4, Section 2.4.8) production was suppressed, concomitantly with an increase in neutrophil EPA and a decrease in arachidonic acid content.
(6) At the same time neutrophil chemotactic responses to LTB_4 were also suppressed, as well as the expression of some surface molecules.
(7) Similar changes were found in subjects with rheumatoid arthritis.

4.3.2.3 Influence on antibody production

(1) Animal studies have found suppression of IgG and IgM antibody production following an antigenic challenge, when diets rich in *n*-6 PUFA were compared with a low fat diet or diets rich in SFA.
(2) Enhanced IgE production to ovalbumin has been reported when rats were given diets enriched with fish oil compared with SFA.
(3) The few human studies that have been reported found minimal effects of dietary fat on antibody production.

4.3.2.4 Influence on delayed-type hypersensitivity

(1) Delayed-type hypersensitivity is the result of a cell-mediated response to challenge with an antigen to which the individual has already been primed.
(2) In general, animal studies indicate that high fat diets reduce this type of response compared with low fat diets.
(3) Among high fat diets, the order of potency is SFA-rich fats < *n*-6 PUFA-rich oils < fish oils.
(4) Again there are few studies with human subjects and these reveal minimal effects of dietary fats.

4.3.2.5 Graft versus host and host versus graft reactions and organ transplants

(1) A limited number of animal studies suggests that high fat diets reduce graft versus host and host versus graft responses compared with low fat diets.
(2) Among high fat diets the order of potency is similar to that seen with delayed hypersensitivity.
(3) Diets rich in unsaturated lipids tend to prolong transplant survival, the effect being greatest with fish oils.

4.3.2.6 Survival after infection

Given the general conclusion that unsaturated fatty acids suppress immune responsiveness, an important question arises. Are animals that habitually consume diets rich in unsaturated lipids less able to combat infection? A small number of animal studies suggests that this is so but there have been no reports of compromised ability to fight infection in human subjects supplementing their diets with different kinds of unsaturated fatty acids.

4.3.2.7 Influence on autoimmune and inflammatory disease processes

(1) In general, studies have demonstrated a pro-inflammatory effect when diets were rich in *n*-6 PUFA.

(2) Meta-analyses of studies on the effect of supplementing the diets of rheumatoid arthritis patients with fish oils for several months confirmed statistically significant improvements in tender joint and morning stiffness symptoms.
(3) There is accumulating evidence for amelioration by fish oils of other inflammatory disease processes including psoriasis, asthma, Crohn's disease and ulcerative colitis.
(4) The substantial weight loss that occurs in patients with pancreatic cancer has been alleviated by daily supplements of fish oils.

4.3.3 Mechanisms

4.3.3.1 Membrane properties

The observed enrichment of specific dietary unsaturated fatty acids in the membranes of cells of the immune system immediately suggests the possibility that membrane physical properties could be affected (Section 6.5.7). Changes in so-called membrane fluidity may modify activities of membrane-bound enzymes (Section 6.5.8). For example, G-protein activity changes can result in changes in adenylate cyclase, phospholipase A_2 and phospholipase C activities. Alterations in membrane phospholipids can influence the production of lipid mediators such as diacylglycerols (Section 7.9).

4.3.3.2 Availability of eicosanoid precursors

The pattern of production of eicosanoids can be modified not only by changes in the nature of cyclooxygenase substrates (e.g. arachidonic acid replaced by EPA), but also in their availability via changes in the activity of phospholipase A_2, which releases these cyclooxygenase substrates from membrane phospholipids. A general observation is that eicosanoids formed from arachidonic acid are pro-inflammatory, whereas those formed from PUFA of the *n*-3 family reduce inflammation.

4.3.3.3 Availability of vitamin E

As discussed in Section 4.2.4.3 and Box 4.3, PUFA are highly susceptible to peroxidation unless adequate vitamin E is available as an antioxidant. When diets that contain high levels of *n*-3 PUFA from fish oils are given, some diminution in the concentration of plasma vitamin E may be observed. This is because such diets contain relatively low levels of vitamin E. Concomitantly, some increase in the concentration of the products of lipid peroxidation may be measured in the plasma. Such changes do not occur when the sources of *n*-3 PUFA are plant oils, because they are richer in vitamin E than fish oils. There is limited evidence that the marked suppression by *n*-3 PUFA of lymphocyte proliferation in response to antigens or non-specific mitogens, does not occur when the diet is supplemented with vitamin E. Functions such as neutrophil phagocytosis are suppressed by vitamin E deficiency and enhanced by vitamin E supplementation. However, vitamin E supplementation decreases bactericidal activity, probably because such activity is promoted by reactive oxygen species, which are suppressed by vitamin E. Many authors have routinely supplemented diets with vitamin E, whether they contained high levels of *n*-3 PUFA or not, whereas in other studies there has been no supplementation. The evidence cited above suggests that functions such as lymphocyte proliferation are inhibited by peroxidation damage when vitamin E is limiting. Therefore, many of the results of studies on the effects of PUFA on immune function need to be interpreted with caution until the contribution of reactive oxygen species has been fully elucidated.

4.3.3.4 Gene expression

PUFA of the *n*-3 family appear to be able to modify gene expression directly or indirectly, depending on the gene in question. Direct effects are most probably mediated by their ability to bind to positive and/or negative regulatory transcription factors (Section 5.3), whereas indirect effects seem to be mediated through alterations in the generation of intracellular lipid second messengers such as diacylglycerols (Section 7.9) or ceramides (Section 7.12).

4.3.3.5 Implications for dietary advice

In many industrial countries, large increases in intakes of linoleic acid (*n*-6) have occurred mainly as a result of advice that such dietary changes are likely to result in lowering of plasma LDL-cholesterol concentration (Section 5.4.2) and a general reduction in coronary heart disease incidence and mortality. At the same time, intakes of *n*-3 PUFA have not increased, and may even have decreased, resulting in significant reductions in the ratio of dietary *n*-3/*n*-6 PUFA.

Many people have pointed out the correlation between the fall in the *n*-3/*n*-6 PUFA ratio and the concomitant rise in the incidence of inflammatory diseases such as asthma. Whereas such statistical correlations do not show that one is the result of the other, the foregoing section has illustrated how there may be some biological basis for such an association. Many nutritionists are now recommending increases in consumption of *n*-3 PUFA and advising against further increases in the *n*-6 family. Some of them are coupling their advice on *n*-3 PUFA supplementation with advice on vitamin E intakes, but others are not. It is clear that immune suppression may be advantageous in some conditions and hazardous in others. Moreover, different dietary components have different effects on immunity depending on the immune function under consideration and their relationship with other components of the diet. The current scientific debates about the appropriate inclusion of different fatty acids in the diet in relation to optimum health are important in reminding us that it is unwise to view the effects of a single nutrient in isolation. Interactions between a whole host of nutrients have to be taken into account.

4.4 LIPIDS AND CANCER

Lipids may influence processes leading to cancer development by affecting membrane properties, cell signalling pathways, gene expression and immune function. Some dietary lipids may exacerbate carcinogenesis, others retard or prevent it. Liposomes may be used to target drugs in cancer therapy.

Cancer represents the uncontrolled proliferation of cells. It arises from mutations in genes that normally control cell division or promote programmed cell death (apoptosis). The development of cancer involves a number of stages. Initiation is the initial process of DNA mutation; promotion is a stage at which a potentially cancerous cell, through accumulation of further mutations, becomes a fully cancerous cell. Then there is a stage of progression, or development by cell division, following which the cancerous cells may spread and invade other tissues (metastasis).

Lipids are involved in cancer in many ways. Dietary lipids, including micronutrients such as fat-soluble antioxidants, may play a role in predisposing to, or protecting from cancer. The development of cancer involves cellular changes that include alterations to lipid components of the cell (and this may be relevant to the design of new treatments). Finally, there may be a role for certain dietary lipids in the control or treatment of certain cancers or aspects of cancer.

4.4.1 Dietary lipids and cancer

There is a large body of evidence on the role of dietary lipids in cancer. It is based on epidemiological studies of various sorts in humans, and on feeding studies in animals. It should be stressed that the evidence is in all cases somewhat conflicting. In 1997–1998, two major reports on diet and cancer were published in which all existing evidence was reviewed (see Further Reading). The expert groups who compiled these reports assessed the literature and graded the evidence for associations between dietary components and specific cancers on a scale from 'convincing' to 'insufficient'. For dietary fat, no association was found to be convincing, and a few 'possible'. There was considered to be strong evidence, however, for a relationship between obesity (particularly central fat deposition) and risk of both endometrial cancer and breast cancer in post-menopausal women.

Ken Carroll, a Canadian biochemist who studied this subject extensively, wrote two thorough review articles shortly before his death in 1998, ironically from stomach cancer. In those reviews, Carroll

distinguished indirect from direct effects. Indirect effects are mainly related to dietary fat as a form of energy, leading to overweight or obesity.

As reviewed above, the evidence is strong for a link between adiposity and breast cancer and for endometrial cancer, both in post-menopausal women. The most consistent theory implicates the production of oestrogens by the excess adipose tissue. Adipose tissue is an important site for the conversion of androgens (particularly androstenedione, secreted from the adrenal cortex) to oestrogens, oestradiol and oestrone. The development of some forms of breast and endometrial cancer is stimulated by oestrogens, and hence the association with dietary fat or obesity. The intake of some antioxidants, including tocopherols and lycopene (the red carotenoid pigment of tomatoes), has been linked to protection against certain cancers, including prostate. Even if these findings should be confirmed, it should be emphasized that cancer prevention may not be as simple as supplementation with the antioxidant vitamins at high doses. A number of supplementation studies has been carried out, none so far with positive results, and in one study of β-carotene supplementation, there was an excess of mortality from lung cancer (in those already at high risk).

Cancer of the colon is common, but its incidence varies widely between countries. In comparisons between countries, colon cancer incidence is correlated with meat and fat consumption. However, the interpretation of such studies needs caution as many other factors may be different between countries. The evidence from other types of study, such as case–control studies (where each 'case', or person suffering from the disease, is matched with a similar non-sufferer), for the link between meat consumption and colon cancer is very conflicting, with some studies showing clear positive links and others the opposite. There is moderately consistent evidence, however, for a protective effect of dietary fibre intake against colon cancer. Whilst dietary fibre (or non-starch polysaccharides) is not a dietary lipid, this association is relevant to this book. The mechanism responsible is believed by most to be that fermentation of non-starch polysaccharides by bacteria in the colon produces the short-chain fatty acids, acetic, propionic and butyric. Butyric acid is an important and preferred fuel for the colonic mucosa, but there is now also strong evidence that butyric acid exerts anti-proliferative effects on the mucosal cells.

Evidence for direct effects of dietary fat on cancer is based mainly on animal studies. Ken Carroll and other workers have consistently found that *n*-6 PUFA in the diet act as mammary tumour promoters when tumours are induced by a carcinogen such as 7,12-dimethylbenz(α)anthracene. Saturated fatty acids in the diet had no such effect, and *n*-3 PUFA have been found to inhibit tumorigenesis. However, the relevance to human cancer remains unclear. Likewise, the therapeutic usefulness of CLA (Section 4.1.3.1) for human cancer patients remains to be demonstrated.

4.4.2 Cellular lipid changes in cancer

Cells express a number of carbohydrate and lipid derivatives on their surfaces. Amongst these are glycosphingolipids, which are involved in cellular recognition, cell adhesion and regulation of cell growth. In 1968 the Japanese biochemist Hakomori observed that transformed tissues (i.e. cancer cells) expressed variant forms of glycosphingolipids, often in larger amounts than normal. These glycosphingolipids may play a role in the uncontrolled cell growth of cancer. There are characteristic changes in glycosphingolipids in different cancers. In general, there is a reduction in the more complex glycosphingolipids and an over-expression of some of the more unusual glycosphingolipids that may normally only be present in trace amounts.

The more complex glycosphingolipids act as cell-surface antigens; that is, they may be recognized by specific antibodies. The blood group antigens, which cause an immune reaction if blood of one group is infused into someone of another blood group, are of this type. These cell-surface antigens are released to some extent into the circulation and may therefore provide a means of diagnosis of particular cancers. Equally exciting is the idea that specific antibodies against these antigens might be used to target drugs to the cancer cells. Either drugs could be coupled to the antibodies, or the presence

of the antigens might be used as the basis for development of a specific vaccine. These ideas have been promoted consistently by Hakomori, who is still very active in the field. Amongst Hakomori's suggestions is the idea that the glycosphingolipids of cancer cells are involved in tumour cell adhesion and in signal transduction, and that it may be possible to develop therapeutic agents that disrupt adhesion ('anti-adhesion therapy') or normalize signalling pathways ('ortho-signalling therapy'). Although trials in cell culture have demonstrated the possibility of antibody-directed tumour killing, these ideas have yet to be developed to a clinically useful stage.

4.4.3 Lipids and the treatment of cancer

The expression of variant forms of glycolipids on cell surfaces may offer a means of directing specific drugs to kill the tumour cells, as discussed above. In addition, several potential new treatments based on phospholipid analogues have been developed and in some cases tested in clinical trials. These compounds are intended to incorporate into target membranes, either the cell membrane or intracellular membranes such as the endoplasmic reticulum. Thus, their mode of action differs from conventional anti-cancer drugs that target DNA replication. Several types of compounds have been tested, although most are analogues of phosphatidylcholine or of lysophosphatidylcholine, e.g. with a sugar molecule ether-linked at the *sn*-2 position. They are believed to act in diverse ways, all of which could be seen as targeting cells with a high rate of cell division. They may act by disrupting signal transduction and signalling pathways, e.g. inhibition of the phosphoinositide-specific phospholipase C with suppression of the diacylglycerol–protein kinase C pathway (Section 7.9), or inhibition of phosphocholine cytidylyltransferase leading to inhibition of phosphatidylcholine biosynthesis *de novo* (Section 7.1.5). These compounds have shown great promise in cellular systems, but their clinical application has been limited by problems of toxicity to the intestinal tract (whose cells are also characterized by a high rate of division). They have found some application as topical therapies (direct application, e.g. to skin lesions).

Cancer causes death through a number of mechanisms including direct invasion of critical organs. However, a common and life-threatening feature of many cancers is a marked loss of body fat, cachexia. There have been many investigations of the cause of cancer cachexia. One theory is that a circulating factor, known as cachectin, leads to inhibition of adipose tissue lipoprotein lipase and consequent failure of fat storage (so there is continuous net fat loss). Cachectin is now known to be identical to tumour necrosis factor-α, a cytokine produced by many cell types including macrophages and adipocytes. The evidence for this mechanism has largely been based on animal studies. In humans, measurements of energy intake have shown that the loss of body fat is mainly a problem of energy balance. Patients lose appetite and in many cases involving cancers of the gastrointestinal tract have difficulty eating. There have been some interesting developments in the treatment of cancer cachexia by the oral administration of relatively large amounts of *n*-3 PUFA (1–2 g day^{-1} of eicosapentaenoic acid, 20:5*n*-3), together with a conventional nutritional supplement. Patients who were previously losing weight have shown an increase in body weight during this treatment. It is likely that the mechanism relates to a general anti-inflammatory effect of large doses of the *n*-3 PUFA, mediated in part by their role as precursors of eicosanoids of the 3-series rather than the 2-series (Section 2.4). The *n*-3 PUFA also tend to reduce the production of pro-inflammatory cytokines including tumour necrosis factor-α and interleukin-6.

4.5 SUMMARY

Lipids are important constituents of the diet, although the proportion of energy provided by dietary lipids varies widely between species, between different human societies and between individuals. Quantitatively, the most important lipids in the human diet are the triacylglycerols that are provided mainly by the adipose tissue of farm animals, the oils of fish and the seed oils of plants. Their fatty acids may be predominantly saturated

and monounsaturated, as in ruminant fats, coconut and palm oils, di- and triunsaturated as in many seed oils or highly polyunsaturated as in many marine oils. Their physical form and chemical composition is usually modified during food manufacture and processing, for example in the hydrogenation employed in the manufacture of margarine to reduce the degree of unsaturation.

Lipids confer palatability to the diet through the textural properties they give to foods and through low molecular weight flavour and odour compounds produced by enzymic oxidation, heating and fermentation. Modern industrial techniques now allow the production of dietary fats with reduced or even zero energy value and of different textures. Triacylglycerols provide a concentrated form of dietary energy and act as vehicles for the fat-soluble vitamins. They also provide the fatty acids required for cellular structures such as biological membranes. Whereas the human body has the capacity to synthesize for itself saturated and monounsaturated fatty acids and cholesterol, it is unable to synthesize linoleic and α-linolenic acids (essential fatty acids) and vitamins A, E and K. The amounts required daily in the diet can be assessed, albeit rather imprecisely. Requirements vary between individuals and depend on the rate of growth, age and physiological states such as puberty, pregnancy and lactation.

Linoleic and α-linolenic acids are converted in many tissues into two series of longer chain, more highly polyunsaturated fatty acids, classified as the *n*-6 and *n*-3 families, respectively. These are important in contributing to the structural integrity of membranes and in acting as precursors for a range of oxygenated fatty acids, the eicosanoids. The latter are produced locally, have short half-lives and exert powerful physiological activities such as muscle contraction and platelet aggregation at extremely low concentrations. Although many human tissues contain the enzymes for converting the parent fatty acids of the *n*-6 and *n*-3 families (linoleic and α-linolenic) into longer chain, more highly polyunsaturated fatty acids by a series of desaturations and elongations, the measured activities of these enzyme systems are often very weak. There is uncertainty, therefore, as to whether essential fatty acid requirements can always be satisfied by dietary consumption of the parent fatty acids alone, or whether there may sometimes be a specific requirement for the long-chain polyunsaturates. This additional requirement may be particularly acute in times of high demand, for example during brain development, especially in premature infants. There is continuing debate about the potential requirements for long-chain polyunsaturates (Box 4.1) which can only be resolved by further research.

The metabolism of essential fatty acids is influenced by the amounts and types of other fatty acids in the diet as a result of competition for the elongases and desaturases. Consequently, the ratio between the different families of unsaturated fatty acids in the dietary lipids may be as important in nutrition as the absolute amounts of dietary linoleic and α-linolenic acids.

Lipids influence immunity in several ways. Some glycolipids are components of cell-surface antigens. Dietary fat enhances immunity in malnourished people through its energy contribution. Individual lipids have more specific roles through effects on membrane structure, eicosanoid production, peroxidation and gene expression.

Lipids may influence processes leading to cancer development by affecting membrane properties, cell signalling pathways, gene expression and immune function. Some dietary lipids may exacerbate carcinogenesis, others retard or prevent it. Liposomes may be used to target drugs in cancer therapy (see also Section 6.5.13).

FURTHER READING

British Nutrition Foundation Task Force (1992) *Report on Unsaturated Fatty Acids: Nutritional and Physiological Significance*. Chapman & Hall, London.

Calder, P.C., *et al*. (1998) Symposium of The Nutrition Society comprising twelve reviews on 'Lipids and the Immune System'. *Proceedings of the Nutrition Society*, **57**, 487–585.

Chow, C.K., ed. (1992) *Fatty Acids in Foods and Their Health Implications*. Marcel Dekker, New York.

Forsyth, J.S. (1998) Lipids and infant formulas. *Nutrition Research Reviews*, **11**, 255–278.

Fredman, P. (1993) Glycosphingolipid tumor antigens. *Advances in Lipid Research*, **25**, 213–234.

Garrow, J.S., James, W.P.T. & Ralph, A., eds. (2000) *Human Nutrition and Dietetics*. Churchill Livingstone, Edinburgh.

Gunstone, F.D. (1998) Movements towards tailor-made fats. *Progress in Lipid Research*, **37**, 277–305.

Gunstone, F.D., Harwood, J.L. & Padley, F.B., eds. (1994) *The Lipid Handbook*, 2nd edn. Chapman & Hall, London.

Gunstone, F.D. & Padley, F.B., eds. (1997) *Lipid Technologies and Applications*. Marcel Dekker, New York.

Gurr, M.I. (1996) Dietary fatty acids with *trans* unsaturation. *Nutrition Research Reviews*, **9**, 259–279.

Guthrie, N. & Carroll, K.K. (1999) Specific versus nonspecific effects of dietary fat on carcinogenesis. *Progress in Lipid Research*, **38**, 261–271.

Hakomori, S. (1996) Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. *Cancer Research*, **56**, 5309–5318.

HMSO (1998) Nutritional aspects of the development of cancer. *Report of the working group on diet and cancer of the Committee on Medical Aspects of Food and Nutrition Policy*. The Stationery Office, London.

Hunt, S.M. & Groff, J.L. (1990) *Advanced Nutrition and Human Metabolism*. West Publishing, St Paul, MN.

Innes, S.M. (1991) Essential fatty acids in growth and development. *Progress in Lipid Research*, **30**, 39–103.

Jensen, R.G. (1996) The lipids in human milk. *Progress in Lipid Research*, **35**, 53–92.

Lermer, C.M. & Mattes, R.D. (1999) Perception of dietary fat: ingestive and metabolic implications. *Progress in Lipid Research*, **38**, 117–128.

Pond, C.M. (1999) Physiological specialisation of adipose tissue. *Progress in Lipid Research*, **38**, 225–248.

Wang, X. & Quinn, P.J. (1999) Vitamin E and its function in membranes. *Progress in Lipid Research*, **38**, 309–336.

Wieder, T., Reutter, W., Orfanos, C.E. & Geilen, C.C. (1999) Mechanisms of action of phospholipid analogs as anticancer compounds. *Progress in Lipid Research*, **38**, 249–259.

World Cancer Research Fund (1997) *Food, Nutrition and the Prevention of Cancer: a Global Perspective*. World Cancer Research Fund in association with the American Institute for Cancer Research, Washington, DC.

5 Lipid transport

5.1 DIGESTION AND ABSORPTION

To be usable by the body, dietary fats must be digested in the lumen of the small intestine. The digestion products pass through the gut wall and are resynthesized in the intestinal epithelial cells and packaged for transport in the bloodstream.

5.1.1 Intestinal digestion of dietary fats involves breakdown into their component parts by a variety of digestive enzymes

Typically 90–95% of fat in the human diet is provided by triacylglycerols, with smaller contributions from phospholipids and cholesterol. The dietary triacylglycerols must be partially hydrolysed before the body can assimilate them.

At birth, the new-born animal has to adapt to the relatively high fat content of breast milk after relying mainly on glucose as an energy substrate in foetal life. It is presented with two major problems in fat digestion: the pancreatic secretion of lipase is rather low and the immature liver is unable to provide sufficient bile salts to solubilize the digested lipids. These problems are even more acute in the premature infant. Yet the new-born baby can digest fat, albeit less efficiently than the older child or adult. There are two mechanisms that allow this to occur. Breast milk contains a lipase that is stimulated by bile salts, known as bile-salt stimulated lipase (BSSL). Milk also contains lipoprotein lipase, although this is not thought to play a role in fat digestion. BSSL may assist the neonate in digesting the milk triacylglycerols. In addition, the neonate secretes a triacylglycerol lipase from glands in the stomach. This is an acid lipase, with a pH optimum of around 4–6, although it is still active even at a pH of 1. In other mammals a homologous lipase may be secreted higher up the gastrointestinal tract – from the serous glands of the tongue in rodents (lingual lipase) and from the pharyngeal region in ruminants. The realization that humans (neonatal and adult) secrete a gastric lipase is relatively recent, and there are still references to human lingual lipase in the literature.

The products of these lipases are mainly 2-monoacylglycerols, diacylglycerols and non-esterified fatty acids, the latter being relatively richer in medium-chain length fatty acids than the original acylglycerols. The milk fat of most mammals is relatively rich in medium-chain length fatty acids (8C–12C) rather than the usual 16C and 18C fatty acids. The relative ease with which lipids containing medium-chain fatty acids can be absorbed certainly helps lipid uptake in babies. Human milk triacylglycerols (along with those of some other mammals including pigs) have a predominance of palmitic acid esterified at position 2. This will lead to the production of 2-palmitoylglycerol, which is readily absorbed (see below). Since the absorption of saturated (non-esterified) fatty acids (which are solid at body temperature unless emulsified, Section 2.1.1) may be less efficient that that of unsaturated fatty acids, the structure of human milk triacylglycerols seems to be a way of optimizing absorption of palmitic acid.

As the baby is weaned onto solid food, the major site of fat digestion shifts from the stomach to the duodenum. Gastric lipase continues to play a role, however, even into adulthood. It has been estimated that gastric lipase is responsible for 25% of the partial triacylglycerol hydrolysis necessary for absorption to occur. In addition, the action of gastric lipase seems to produce lipid droplets that are better substrates for the later action of pancreatic lipase. The stomach also plays a role with its churning action, creating a coarse oil-in-water

emulsion, stabilized by phospholipids. Also, proteolytic digestion in the stomach serves to release lipids from the food particles where they are generally associated with proteins. The acidic fat emulsion that enters the duodenum from the stomach is neutralized and modified by mixing with bile and pancreatic juice. Bile supplies bile salts that in humans are mainly the glycine and taurine conjugates of tri- and di-hydroxycholanic acids (usually known as cholic and chenodeoxycholic acid), formed from cholesterol in the liver (Section 7.5.6) and phospholipids. Much of the intestinal phospholipid in humans is of biliary origin and is estimated at between 7 and 22 g day^{-1} compared with a dietary contribution of 4–8 g day^{-1}. Biliary secretion is enhanced by the enzyme cholecystokinin, secreted from the duodenal mucosa, in response to entry of the acidic mixture from the stomach (chyme) into the duodenum. Pancreatic juice, whose secretion is also stimulated by cholecystokinin, supplies bicarbonate to neutralize the acidic chyme, and enzymes that catalyse the hydrolysis of fatty acids from triacylglycerols, phospholipids and cholesterol esters (Fig. 5.1).

In most adults the process of fat digestion is very efficient and the hydrolysis of triacylglycerols is mainly accomplished in the small intestine by a triacylglycerol lipase secreted from the pancreas, pancreatic lipase. This enzyme is related to lipoprotein lipase and hepatic lipase (Section 3.5.2). Pancreatic lipase attacks triacylglycerol molecules at the surface of the large emulsion particles (the oil–water interface), but before lipolysis can occur, the surface and the enzyme must be modified to allow interaction to take place. Firstly, bile salt molecules accumulate on the surface of the lipid droplet, displacing other surface-active constituents. As amphipathic molecules they are

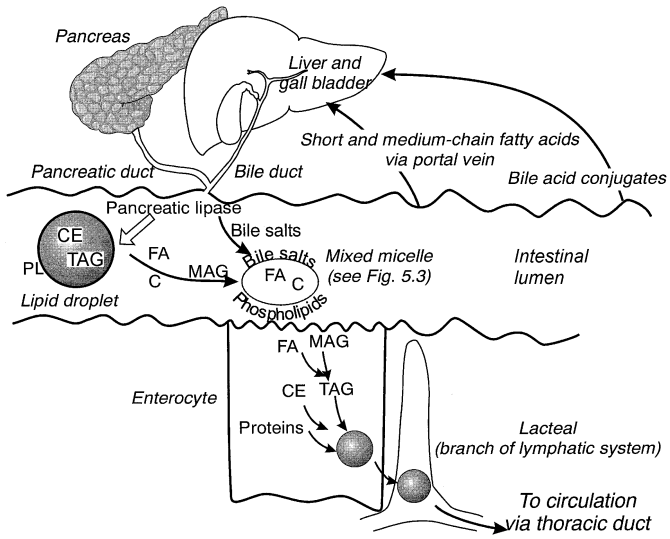


Fig. 5.1 The digestion and absorption of dietary fat in the small intestine. Lipid droplets entering the small intestine from the stomach are subjected to the action of pancreatic lipase, phospholipase A_2 and cholesterol esterase, which hydrolyse triacylglycerol (TAG) to produce monoacylglycerols (MAG) and fatty acids (FA), phospholipids (PL) to produce lysophospholipids and fatty acids; and cholesterol esters (CE) to liberate cholesterol (C) and fatty acids. These are emulsified with bile salts (from the gall bladder) to produce a micellar suspension (the mixed micelles) from which components are absorbed across the epithelial cell (enterocyte) membranes. Short- and medium-chain fatty acids pass through into the circulation (into the hepatic portal vein), and bile salts are reabsorbed, along with further cholesterol, in the lower part of the small intestine. Within the enterocyte, the components are reassembled, and packaged into chylomicrons, the largest of the lipoprotein particles (see Fig. 5.4 for further details of this process). The chylomicrons are secreted into small branches of the lymphatic system, the lacteals.

adapted for this task since one side of the rigid planar structure of the steroid nucleus is hydrophobic and can essentially dissolve in the oil surface. The other face contains hydrophilic groups that interact with the aqueous phase (Fig. 5.2). The presence of the bile salts donates a negative charge to the oil droplets, which attracts a protein to the surface. This protein has a molecular mass of 10 kDa and is called colipase. Colipase, also secreted from the pancreas, is essential for the activity of pancreatic lipase, which is otherwise strongly inhibited by bile salts. Thus, bile salts, colipase and pancreatic lipase interact in a ternary complex, which also contains calcium ions that are necessary for the full lipolytic activity.

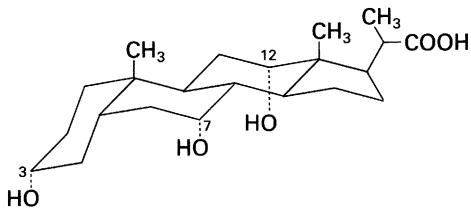


Fig. 5.2 The structure of a bile acid (cholic acid).

Pancreatic lipase catalyses the hydrolysis of fatty acids from positions 1 and 3 of triacylglycerols to yield 2-monoacylglycerols. There is very little hydrolysis of the ester bond at position 2 and very little isomerization to the 1(3)-monoacylglycerols, presumably because of rapid uptake of the monoacylglycerols into epithelial cells. Phospholipase A_2 (Section 7.2.2) hydrolyses the fatty acid at position 2 of phospholipids, the most abundant being phosphatidylcholine. The enzyme is present as an inactive proenzyme in pancreatic juice and is activated by the tryptic hydrolysis of a heptapeptide from the N-terminus. The major digestion products that accumulate in intestinal contents are lysophospholipids. Any cholesteryl ester entering the small intestine is hydrolysed by a pancreatic cholesteryl ester hydrolase.

As these enzymes act upon the contents of the large emulsion particles, which may be around 1 μm in diameter, the products of their hydrolytic action disperse and form multimolecular aggregates called mixed micelles (Fig. 5.3), typically 4–6 nm in diameter. Lysophospholipids and monoacylglycerols are highly amphipathic substances and, with bile salts, stabilize these aggregates. In the more neutral environment of the small

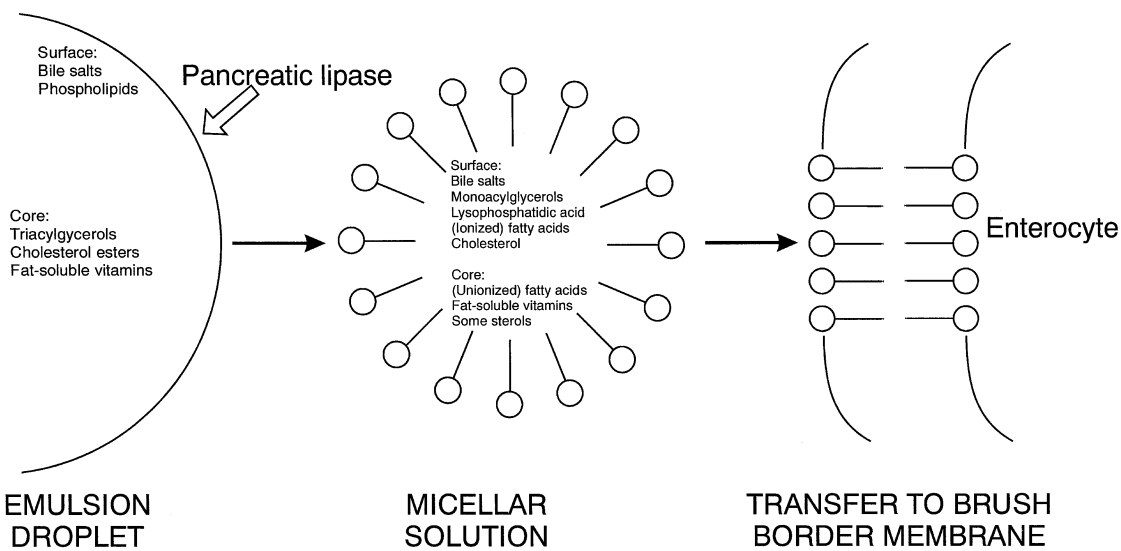


Fig. 5.3 Role of the mixed micelle in fat absorption.

intestine, fatty acids are found largely in the ionized form and are therefore also amphipathic. The presence of these amphiphiles helps to incorporate insoluble non-polar molecules like cholesterol and the fat-soluble vitamins into the micelles and aid their absorption.

In ruminant animals, the complex population of micro-organisms contains lipases that split triacylglycerols completely to glycerol and non-esterified fatty acids. Some of these are fermented to acetic and propionic acids, which are absorbed directly from the rumen and carried to the liver, where they are substrates for gluconeogenesis. A proportion of the remaining long-chain unsaturated fatty acids undergoes several metabolic transformations catalysed by enzymes in the rumen micro-organisms (Fig. 2.22) before passing into the small intestine where they are absorbed. Principal among these is hydrogenation, in which double bonds are reduced by a process that is strictly anaerobic (Section 2.2.6). During hydrogenation, some double bonds are isomerized from the *cis* to the *trans* geometrical configuration. Positional isomerization also occurs, in which the double bonds, both *cis* and *trans*, migrate along the carbon chain. The result is a complex mixture of fatty acids, generally less unsaturated than the fatty acids in the ruminant's diet and containing a wide spectrum of positional and geometrical isomers. Because there is very little monoacylglycerol present in the digestion mixture, the mixed micelles in ruminants are composed largely of non-esterified fatty acids, lysophospholipids and bile salts

5.1.2 The intraluminal phase of fat absorption involves passage of digestion products into the absorptive cells of the small intestine

Lipid absorption in humans begins in the distal duodenum and is completed in the jejunum. The principal molecular species passing across the brush-border membrane of the enterocyte are the monoacylglycerols and non-esterified long-chain fatty acids. The bile salts themselves are not absorbed in the proximal small intestine, but pass on to the ileum where they are absorbed and recirculated in the portal blood to the liver, from whence they are re-secreted in the bile. This is also true for some cholesterol that is a constituent of bile. The recirculation of bile constituents in this way is referred to as the entero-hepatic circulation. It may be interrupted by resins (given by mouth) that bind cholesterol and bile salts and prevent their reabsorption, leading the liver to synthesize new bile salts from cholesterol. This is therefore a mechanism for depleting the body of cholesterol and may be useful in the treatment of high cholesterol levels.

The digestion products encounter two main barriers to their absorption. At the surface of the microvillus membrane is a region known as the unstirred water layer. The mixed micelles are small enough to diffuse readily into this layer. It is a few hundred micrometres thick and is retained by mucopolysaccharides secreted by the epithelial cells (mucosa). Its pH is relatively acidic. This will promote the protonation of non-esterified fatty acids (i.e. they become uncharged) so that they can more easily leave the micelles and move into the epithelial cell membrane. This membrane is highly convoluted (hence the description brush-border) to increase its absorptive capacity. There is a suggestion that pancreatic lipase may be loosely bound to the brush-border membrane, which would bring the products of its action even more closely into contact with the absorptive surface.

The second barrier is the brush-border membrane itself. It has been speculated for many years that fatty acids in their protonated form might diffuse across this membrane (and other cell membranes). However, it now looks increasingly likely that there is a specific transport protein to facilitate their movement into the cell. Two candidate proteins have been identified in enterocytes: plasma membrane fatty acid binding protein (FABPpm) and fatty acid translocase (FAT). The former, despite its name, is not related structurally to the family of intracellular fatty acid binding proteins (Section 2.2.1). FAT has been identified with a cell surface receptor CD36, which is a member of the family of scavenger receptors (Section 5.2.4.4). The relative importance of these two proteins in fatty acid uptake by enterocytes is unknown, if indeed

they play any role. This is an area of intense interest at present.

5.1.3 The intracellular phase of fat absorption involves recombination of absorbed products in the enterocytes and packaging for export into the circulation

For efficient absorption into the enterocytes to occur, it is essential that an inward diffusion gradient of lipolysis products is maintained. Two cellular events ensure that this occurs. First, the long-chain fatty acids entering the cells bind to a cytosolic fatty acid binding protein (FABPc). There are two FABPc expressed in enterocytes. One is found only in the intestine and is called I-FABPc (also known as FABP2); the other is known as liver FABP (L-FABPc) and is also expressed in liver and kidney. They are small proteins (molecular mass 14–15 kDa) and bind one or two fatty acids, respectively. The FABPc are believed to play a role in targeting fatty acids within the cell, and also in protecting the cell from the potentially cytotoxic effects of high fatty acid concentrations. They also provide the concentration gradient that ensures efficient fatty acid uptake from the intestinal lumen. It is not clear whether the two FABPc isoforms have different roles, although it has been suggested that I-FABPc is involved in the uptake of dietary fatty acids for triacylglycerol synthesis, whilst L-FABPc in enterocytes is involved in uptake of fatty acids delivered in the blood, which may be substrates for oxidation and for phospholipid synthesis. Both these FABPc bind long-chain unsaturated fatty acids with higher affinity than saturated fatty acids, and this may explain the more rapid absorption of oleic than stearic acid.

Up to this stage, the absorption process has not been dependent on a source of energy. The next phase, which removes fatty acids, thereby maintaining a gradient, is the energy-dependent re-esterification of the absorbed fatty acids into triacylglycerols and phospholipids. The first step in re-esterification is the ATP-dependent 'activation' of fatty acids to their acyl-CoA thioesters (Section 2.2.1). Again, the preferred substrates are the long-chain fatty acids. There are several isoforms of acyl-CoA synthetase (ACS), one of which, ACS5, is prominent in the intestine. The ACSs are all membrane-associated and are situated on the endoplasmic reticulum as well as on mitochondrial and peroxisomal membranes. The acyl-CoA thioesters are themselves bound by a specific, 10 kDa, cytosolic acyl-CoA binding protein which, like FABPc, may direct the acyl-CoA within the cell.

In humans and other simple-stomached animals, the major acceptors for esterification of acyl-CoA are the 2-monoacylglycerols that together with the non-esterified fatty acids are the major forms of absorbed lipids. Resynthesis of triacylglycerols, therefore, occurs mainly via the monoacylglycerol pathway (Section 3.4.2). In ruminant animals, the major absorbed products of lipid digestion are glycerol and non-esterified fatty acids and resynthesis occurs via the glycerol phosphate pathway (Section 3.4.1.1) after phosphorylation of glycerol catalysed by glycerol kinase. It should be noted, however, that lipids usually form a minor part of ruminant diets, so that glucose is probably the major precursor of glycerol phosphate in the ruminant enterocyte.

The main absorbed product of phospholipid digestion is monoacylphosphatidylcholine (lysophosphatidylcholine). A fatty acid is re-esterified to position 1 to form phosphatidylcholine by an acyl transferase located in the villus tips of the intestinal brush-border. The function of this phospholipid will be to stabilize the triacylglycerol-rich particles (chylomicrons) exported from the cell as described later. It is thought that the phosphatidylcholine used for the synthesis and repair of membranes in the enterocytes (cells with a rapid turnover) is synthesized by the classical CDP-choline pathway (Section 7.1.5) in cells at the villus crypts.

The absorption of cholesterol is slower and less complete than that of the other lipids, about half of the absorbed sterol being lost during desquamation of cells. Most of the absorbed portion is esterified either by reversal of cholesteryl esterase or via acyl-CoA:cholesterol acyltransferase (Section 7.5.6). The latter enzyme is induced by high concentrations of dietary cholesterol.

During fat absorption, the biosynthetic activity of the enterocyte is geared to packaging the resynthesized absorbed lipids in a form that is stabilized for transport in the aqueous environment of the blood. Within minutes of absorption products entering the enterocyte, fat droplets can be seen within the cysternae of the smooth endoplasmic reticulum, where the enzymes of the monoacylglycerol pathway are located. The rough endoplasmic reticulum is the site of the synthesis of phospholipids (Section 7.1) and apolipoproteins (Section 5.2.2), which provide the coat that stabilizes the lipid droplets. One apolipoprotein in particular, apolipoprotein B48 (apoB48), provides a 'skeleton' which associates with lipid during its synthesis, forming the immature chylomicron particle. These immature particles gain further triacylglycerol in a process that involves the microsomal triacylglycerol transfer protein (MTP). They migrate through the Golgi apparatus where carbohydrate moieties are added to the apolipoproteins, and the fully formed chylomicrons are exported in secretory vesicles that move to the basolateral surface of the enterocyte. The final phase of transport from the cells involves fusion with the membrane and secretion into the intercellular space by exocytosis (Fig. 5.1). Chylomicron assembly in the enterocyte is similar to the assembly of very low density lipoprotein (VLDL) particles in hepatocytes (Section 5.2.3). The process is illustrated in Fig. 5.4.

The chylomicrons do not enter the plasma directly. Instead they are secreted into the tiny lymph vessels that are found inside each of the intestinal villi, called lacteals because of their milky appearance after the ingestion of fat. From here the chylomicrons pass via the thoracic duct (a main branch of the lymphatic system) and enter the circulation in the subclavian vein, from where they reach the heart for distribution around the body. Thus, dietary lipids, contained in the chylomicrons, are unique amongst the products of intestinal digestion and absorption in that they do not enter the hepatic portal vein and traverse the liver before entering the systemic circulation.

Chylomicrons are the main route for the transport of dietary long-chain fatty acids. Those with chain lengths of less than twelve carbon atoms are absorbed in the non-esterified form, passing directly into the portal blood and are metabolized directly by β-oxidation in the liver. There are several reasons for this partition. Firstly, short- and medium-chain fatty acids are more readily hydrolysed from triacylglycerols and since they occupy mainly position 3, are not retained in the 2-monoacylglycerols. Secondly, they are more likely to diffuse into the aqueous phase rather than the mixed micelles and for this reason are more rapidly absorbed. Around $4\,g\,day^{-1}$ short- and medium-chain fatty acids enter the diet from dairy products or foods that incorporate coconut or palm kernel oils. Short-chain fatty acids derived by microbial fermentation of non-starch polysaccharides (dietary fibre) in the colon are also absorbed and contribute to lipid metabolism.

5.1.4 Malassimilation of lipids, through failure to digest or absorb lipids properly, can arise from defects in the gut or other tissues but may also be induced deliberately

Failure to assimilate lipids of dietary origin into the body may arise from defects in digestion (maldigestion) or absorption (malabsorption).

Maldigestion can occur because of incomplete lipolysis. Thus, pancreatic insufficiency, which may result from pancreatitis, pancreatic tumour, diseases of malnutrition such as kwashiorkor, or rarely from an inherited mutation in pancreatic lipase, can lead to a failure to secrete enough lipase or the production of an enzyme with reduced or no activity. Alternatively, the lipase may be fully functional but a failure to produce bile (generally arising from hepatic insufficiency) may result in an inability to effect the micellar solubilization of lipolysis products. This, in turn, feeds back to cause an inhibition of lipolysis. Gastric disturbances that result in abnormal acid secretion also inhibit pancreatic lipase and, furthermore, gastric problems may cause poor initial emulsification of the lipid in the stomach, further reducing the efficiency of digestion. Thus, maldigestion can arise from defects in a variety of organs con-

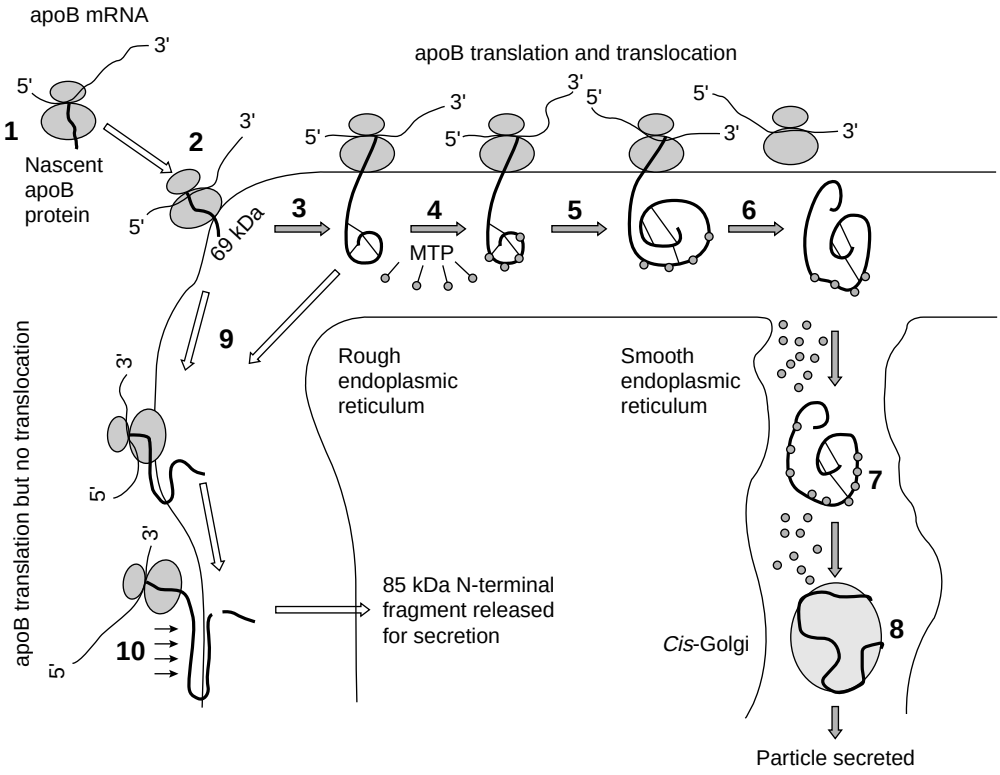


Fig. 5.4 Assembly of triacylglycerol-rich lipoprotein particles, chylomicrons in the enterocyte, and VLDL in the hepatocyte. Apolipoprotein B48 (apoB100 in hepatocytes) is synthesized on the rough endoplasmic reticulum (ER) (upper left). At step 4, the N-terminal region acquires a small amount of 'core lipid' (triacylglycerols and cholesteryl esters) in a process mediated by the microsomal triacylglycerol transfer protein (MTP). After further protein chain synthesis (step 5), a lipid-poor 'primordial' particle is produced. There is debate about whether this is released into the ER lumen as shown at step 6, or whether the next stage, of bulk lipid addition (step 7), occurs whilst the primordial particle is still attached to the ER membrane. There is also debate about whether MTP is involved in this stage also (consensus is that it probably is). The left side of the diagram indicates that if lipid availability is low and lipid addition fails to occur, then the nascent apoB is directed into a degradative pathway (steps 9 and 10). Reproduced, with permission of the Nutrition Society, from White, D.A., Bennett, A.J., Billett, M.A. & Salter, A.M. (1998) The assembly of triacylglycerol-rich lipoproteins: an essential role for the microsomal triacylglycerol transfer protein. *British Journal of Nutrition*, **80**, 219–229.

tributing to different aspects of the digestive process.

Malabsorption may occur, even when digestion is functioning normally, due to defects in the small intestine affecting the absorptive surfaces. There may be a variety of causes, some common ones being bacterial invasion of the gut or sensitization of the gut to dietary components such as gluten, as in coeliac disease. Malabsorption syndromes (often called sprue) are characterized by dramatic changes in the morphology of the intestinal mucosa. The epithelium is flattened and irregular and atrophy of the villi reduces the absorbing surface. Tropical sprue is a prevalent disease in many countries of Africa and Asia. Malabsorption of fat can also occur in certain inherited disorders in which the formation of chylomicrons is impaired, so that fat cannot be transported out of the cell. Triacylglycerols then accumulate in the enterocyte. One such condition is known as chylomicron retention disease or Anderson's disease. The molecular defect is not known.

Digestion and absorption may also be interfered with deliberately to reduce fat absorption in an attempt to control body weight. Synthetic fat substitutes, in particular sucrose-polyesters (sucrose with long-chain fatty acids esterified to its hydroxyl groups; Section 4.1.3.6), can be used in cooking, and are completely resistant to the action of pancreatic lipase. They are used at present in some snack foods that might otherwise contribute significantly to energy intake. Similarly, a bacterial metabolite known as tetrahydrolipstatin (or orlistat) is licensed for prescription as a drug to treat obesity. Tetrahydrolipstatin is a potent irreversible inhibitor of pancreatic lipase and so reduces the absorption of dietary fat. Certain plant sterols (phytosterols) can interfere with the absorption of cholesterol of both dietary and biliary origin, and are being marketed as components of spreads that can help to lower cholesterol levels in the blood.

A common feature of all fat malassimilation syndromes is increased excretion of fat in the faeces (steatorrhoea), which arises not only from unabsorbed dietary material but also from the bacterial population that usually proliferates in the gut and the breakdown of cells. The bacteria undoubtedly affect the composition of the excreted fat. For example, a major component (absent from the diet) of faecal fat, 10-hydroxystearic acid, was shown by tracer studies to be formed by bacteria from stearic acid. This is a normal component but found in particularly high concentration in patients with steatorrhoea.

Patients with poor fat absorption are very much at risk from deficiencies of energy, fat-soluble vitamins and of essential fatty acids (Section 4.2). The clinical management of fat malassimilation is facilitated, according to the nature of the defect, by giving bile salts in tablet form or a pancreatic extract containing pancreatic lipase and other enzymes, or by replacing normal dietary fats by medium-chain triacylglycerols (MCT). This product is composed largely of triacylglycerols with C8 and C10 saturated fatty acids refined from coconut oil, and can be purchased as a cooking oil or as a fat spread. The medium-chain fatty acids are rapidly hydrolysed and efficiently absorbed into the portal blood, thereby bypassing the normal absorptive route of long-chain fatty acids and chylomicron formation.

Some people are unable to take any nutrition by mouth, usually because of inflammatory conditions of the bowel or infection following surgical intervention. In early attempts at complete nutrition of such people using the intravenous route, their energy requirements were supplied in the form of glucose and amino acids. Several such patients developed symptoms of essential fatty acid deficiency (Section 4.2.3.1). In addition, the large carbohydrate loads caused metabolic problems, not least because such patients often have a reduced ability to handle glucose, associated with their inflammatory condition. In the early 1960s Arvid Wretlind in Sweden developed the first synthetic lipid emulsion that could be infused intravenously. It was based on an emulsion of soybean oil stabilized with lecithin, and was soon available commercially. Now there is a number of such intravenous lipid preparations with different combinations of fatty acids, including some enriched with the very long-chain polyenoic fatty acids 20:5*n*-3 and 22:6*n*-3.

5.2 TRANSPORT OF LIPIDS IN THE BLOOD: PLASMA LIPOPROTEINS

Lipids, being insoluble in water, need to be combined with proteins for transport in the blood. These plasma lipoproteins represent a continuum of lipid–protein complexes in which the ratio of lipid to protein, and hence their density, varies.

5.2.1 Lipoproteins can be conveniently divided into groups according to density

The biological problem of how to transport water-immiscible lipids in the predominantly aqueous environment of the blood has been solved by emulsification. Aggregates of hydrophobic molecules (particularly triacylglycerol and cholesteryl esters) are stabilized with a coat of amphipathic compounds: phospholipids, unesterified cholesterol and proteins. The resulting particles are the lipoproteins. The protein moieties are known as

apolipoproteins and, as will be discussed later, have more than a stabilizing role. They also confer specificity on the particles, allowing them to be recognized by specific receptors on cell surfaces, and they regulate the activity of some enzymes involved in lipoprotein metabolism.

The lipoprotein system developed quite early in evolution. Birds, fishes, amphibians and even roundworms have a system for delivery of lipids from lipogenic organs to the developing egg that is closely related to the mammalian lipid transport system, and some of the proteins involved are homologous. For instance, the protein involved in lipid transport in these various groups is known as vitellogenin and is related to mammalian apolipoprotein B. The vitellogenin receptor of the chicken oocyte will, in fact, recognize mammalian apolipoprotein B, suggesting a common origin with mammalian lipoprotein receptors (Section 5.2.4). The mammalian lipase gene family (pancreatic, lipoprotein, hepatic and endothelial lipases; Section 3.5.2) is homologous to proteins in egg yolk of *Drosophila* that are presumably involved in related functions.

There are several types of lipoproteins with differing chemical compositions, physical properties and metabolic functions (Table 5.1), but their common role is to transport lipids from one tissue to another, to supply the lipid needs of different cells. The different lipoproteins may be classified in a number of ways depending on their origins, their major functions, their composition, physical properties or method of isolation. They differ according to the ratio of lipid to protein within the particle as well as in the proportions of lipids (triacylglycerols, esterified and non-esterified cholesterol and phospholipids). These compositional differences influence the density of the particles: in general, the higher the lipid to protein ratio, the larger the particle and the lower its density. There is a strong relationship between biological function and the density class into which a particle falls. It is convenient, therefore, to make use of density to separate and isolate lipoproteins by ultracentrifugation, and it is now usual to classify plasma lipoproteins into different density classes. It is almost by good fortune for the lipid biochemist that this classification is also, at least approximately, one of function. From lowest to highest density, these are: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). (An additional class of intermediate density lipoproteins, IDL, is sometimes included between VLDL and LDL.) As density increases, so the ratio of triacylglycerols to phospholipids and cholesterol decreases (Table 5.1): chylomicrons and VLDL particles are often grouped as the triacylglycerol-rich lipoproteins, whereas LDL and HDL are more important as carriers of cholesterol.

It is important to emphasize that the classes are

Table 5.1 Composition and characteristics of the human plasma lipoproteins

	Chylomicrons	VLDL	LDL	HDL
Protein (% particle mass)	2	7	20	50
Triacylglycerols (% particle mass)	83	50	10	8
Cholesterol (% particle mass; free + esterified)	8	22	48	20
Phospholipids (% particle mass)	7	20	22	22
Particle mass (daltons)	$0.4–30 \times 10^6$	$10–100 \times 10^6$	$2–3.5 \times 10^6$	$1.75–3.6 \times 10^5$
Density range (g ml^{-1})	<0.95	0.95–1.006	1.019–1.063	1.063–1.210
Diameter (nm)	80–1000	30–90	18–22	5–12
Apolipoproteins	AI, AII, AIV, B-48, CI, CII, CIII, E	B-100, CI, CII, CIII, E	B-100	AI, AII, AIV, CI, CII, CIII, D, E

not homogeneous: there is a wide variety of particle sizes and chemical compositions within each class, and there is overlap between them. It is also important to realize that lipoprotein particles are not molecules – they are aggregates of lipid and protein molecules with a degree of structural organization. The term particle mass, rather than molecular mass, is used to describe their mass.

5.2.2 The apolipoproteins are the protein moieties that help to stabilize the lipid; they also provide specificity and direct the metabolism of the lipoproteins

The proteins associated with the plasma lipoproteins are known as apolipoproteins. At first a series of letters, A–E, was used to identify apolipoproteins as they were separated by electrophoresis, but it soon became apparent that most of these could be divided further into several individual proteins (Table 5.2). These are usually referred to in abbreviated form: apoAI, apoCIII, etc. The complete amino acid sequences of the nine major human apolipoproteins are now known [AI, AII, AIV, B (-48 and -100), CI, CII, CIII, D and E]. The apolipoproteins other than apoB are often referred to as soluble apolipoproteins. They may exist in lipid-free form in the plasma and they may exchange between lipoprotein particles. Apolipoproteins of the groups A, C and E have similar gene structures and some homologous stretches of sequence, and are believed to have evolved from a common ancestral gene, whereas the genes for apoB and apoD have distinct structures. The genes for apoAI, CIII and AIV are arranged in one cluster spanning about 15 kb on human chromosome 11, and the genes for apoCI, CII and E in a cluster on chromosome 19 (Table 5.2).

The apolipoproteins fulfil two main functions. First, they stabilize the lipid particles in the aqueous environment of the blood, and maintain their structural integrity. Secondly, they are important in 'identifying' the lipoprotein and directing its metabolism in specific ways. The known metabolic functions of the apolipoproteins are shown in Table 5.2.

Much interest has centred on the properties of apoB since it is common to the triacylglycerol-rich lipoprotein particles (chylomicrons, secreted from the small intestine, and VLDL secreted from the liver) and cholesterol-rich LDL. It is now realized that apoB synthesis is intimately linked to the assembly of the triacylglycerol-rich lipoprotein particles. In fact the particle forms around one molecule of the protein (Fig. 5.4), which never leaves the particle until it is ultimately catabolized. There are two isoforms of apoB in mammals. They are coded for by the same gene. A full-length transcript produces a very large protein, of 4536 amino acids, known as apoB100 (since it is 100% of the possible length). A specific enzyme system present in some cells edits the mRNA coding for apoB to introduce a stop codon about half-way along. This transcript codes for a shorter protein, apoB48 (2152 amino acids), so-called because it is 48% of the length of apoB100. In humans the intestine secretes apoB48 and the liver apoB100, and these proteins are therefore specific markers of chylomicrons and VLDL, respectively. In the rat, the liver secretes both apoB100- and apoB48-containing VLDL particles, and the intestine, as in humans, only apoB48.

5.2.3 The different classes of lipoprotein particles transport mainly triacylglycerols or cholesterol through the plasma

Chylomicrons are the largest and least dense of the lipoproteins and their function is to transport lipids of exogenous (or dietary) origin. Because the principal dietary fat is triacylglycerol, this is true also of the chylomicrons. The triacylglycerol core is stabilized with a surface monolayer of amphipathic molecules: phospholipids, unesterified cholesterol and proteins (Table 5.1). The core lipid also contains some cholesteryl esters and minor fat-soluble substances absorbed with the dietary fats: fat-soluble vitamins, carotenoids and traces of environmental contaminants such as pesticides. It seems that the enterocyte continually secretes particles. Between meals these are small and poor in lipid, which is derived from biliary phospholipids or the lipids of cells shed from the gut mucosa, and from plasma

Table 5.2 Characteristics of the human apolipoproteins

Shorthand name	Molecular mass (daltons)	Amino acid residues	Function	Major sites of synthesis	Chromosomal location
AI	28 000	243	Cholesterol efflux from cells; activates LCAT	Liver; intestine	11q13-qter
AII	17 000	154	May inhibit HL activity; inhibits AI/ LCAT	Liver; intestine	1p21-qter
AIV	44 500	376	Activates LCAT; some evidence for AIV as a satiety factor	Intestine	11q13-qter
B48	241 000	2152	Structural component of chylomicrons	Intestine	2p23-p24
B100	513 000	4536	Structural component of VLDL; binds to LDL-R	Liver	2p23-p24
CI	6 600	57	Activates LCAT	Liver	19q13.2
CII	8 800	79	Activates LPL (essential cofactor)	Liver	19q13.2
CIII	8 800	79	Inhibits LPL?; may be involved in hepatic uptake of apoE-containing particles	Liver; intestine	11q13-qter
D	22 000	169	Involved in cholesteryl ester transfer?	Brain, adipose tissue (role in brain may not be connected with lipid metabolism)	3p14.2-qter
E	34 000	279	Ligand for LDL-R	Liver (60–80%); other tissues including adipose tissue (remainder)	19q13.2

The functions of many of the apolipoproteins have not been fully clarified. HL: hepatic lipase; LCAT: lecithin-cholesterol acyltransferase; LDL-R: LDL-receptor (B/E receptor); LPL: lipoprotein lipase.

non-esterified fatty acids. When fat is being absorbed the particles increase in size. Thus, increased lipid transport into the circulation after a fat-rich meal is achieved mainly by an increase in the size of the chylomicrons secreted, a mechanism that allows rapid changes in flux through this pathway.

VLDL particles, like chylomicrons, contain predominantly triacylglycerols (Table 5.1) and their function is to transport triacylglycerols of endogenous origin, synthesized in the liver. VLDL are spherical particles with a core consisting mainly of triacylglycerols and cholesteryl esters, with unesterified cholesterol, phospholipids and protein mainly on the surface (Fig. 5.5). As with the chylomicrons, each VLDL particle is assembled around one molecule of apoB (in humans, apoB100), which remains with the particle throughout its lifetime. The particles also contain apoCI, CII, CIII and apoE in variable amounts, decreasing relative to apoB as particle density increases (i.e. as the particles

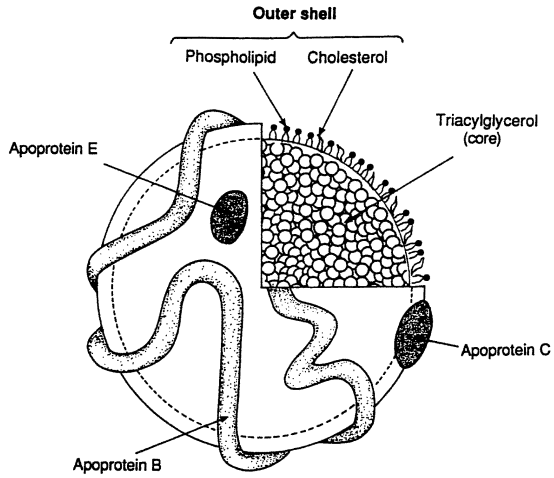


Fig. 5.5 Structure of a VLDL particle. (Other lipoprotein particles are similar in structure.) A monolayer of amphipathic molecules (phospholipids, unesterified cholesterol) stabilizes a droplet of hydrophobic lipids (triacylglycerols and cholesteryl esters) in the core. Apolipoprotein B wraps around the surface of the molecule, and other (soluble) apolipoproteins are present on the surface. Reproduced, with permission, from Gibbons, G.F. & Wiggins, D. (1995) Intracellular triacylglycerol lipase: its role in the assembly of hepatic very-low-density lipoprotein (VLDL). *Advances in Enzyme Regulation*, **35**, 179–198.

become smaller). The triacylglycerol-fatty acids secreted in VLDL are mobilized from stores within the hepatocyte by an unidentified lipase (Section 3.5.2). They originate from plasma non-esterified fatty acids and triacylglycerol-fatty acids taken up by the liver, and to a variable extent from *de novo* synthesis in the liver. The glycerol 3-phosphate used for their esterification may come either from glucose metabolism or from the phosphorylation of glycerol, taken up by the liver (released in turn mainly from adipose tissue), by the enzyme glycerol kinase.

As in chylomicron biosynthesis in the intestinal mucosa, nascent VLDL particles originate in the lumen of the endoplasmic reticulum where they form around an emerging molecule of newly synthesized apoB100. They acquire phospholipids and further triacylglycerol, the latter by a process involving the microsomal triacylglycerol transfer protein (MTP). A few individuals have been identified who have mutations rendering MTP inactive; such persons cannot secrete VLDL particles. The resulting syndrome is known as abetalipoproteinaemia (lack of apoB100 in plasma). The mature particles move to the Golgi apparatus where some of the apolipoproteins are glycosylated. The Golgi vesicles then migrate to the cell surface where VLDL are exported by exocytosis into the subendothelial space, the space of Disse. The regulation of VLDL assembly and secretion is complex. A proportion of all the apoB100 synthesized fails to associate with triacylglycerol and enters a degradative pathway. Some nascent particles fail to acquire sufficient triacylglycerol to become 'competent' for further loading with triacylglycerol, and are also degraded. Thus, increased availability of hepatic triacylglycerol may lead to a greater proportion of nascent VLDL particles reaching sufficient size for secretion.

LDL particles are the major carriers of plasma cholesterol in humans although this is not the case for all mammalian species (Section 5.2.6). They are derived from VLDL in the plasma by a series of degradative steps that remove triacylglycerols (described in more detail in Section 5.2.5), resulting in a series of particles that contain a progressively lower proportion of triacylglycerols and are correspondingly richer in cholesterol, phospholipids and protein. The intermediate particles (IDL) are usually present at relatively low concentrations. During the transformations, the apoB component remains with the LDL particles and the apoC and E components are progressively lost. Once only apoB remains, the particle is a mature LDL particle. ApoB has an important role in the recognition of LDL by cells since it must interact with specific cell-surface receptors before the LDL particle can be taken up and metabolized by the cell. Other receptors (for example on macrophages) recognize modified LDL and are responsible for the degradation of LDL particles that cannot be recognized by normal cell-surface LDL-receptors (Section 5.2.4).

There is a variety of types of particle classified as HDL. They are very numerous in plasma: it has been estimated that there are 10–20 times as many HDL particles in the circulation as all other lipoprotein particles combined. HDL precursor parti-

cles have not been found within cells. Liver cells in culture secrete apoAI, and in vivo it seems that apoAI becomes associated with some phospholipid molecules as it leaves the cell. ApoAI has a very high affinity for phospholipid, a property that is essential to understanding HDL metabolism. The precursor particles in the circulation are described as pre-β HDL (from their migration pattern on electrophoresis). The smallest particles (pre-β1 HDL) are essentially apoAI with some phospholipid molecules. Further addition of phospholipid molecules leads to discoidal particles also called pre-β1 HDL. They acquire cholesterol from cells and from other lipoprotein particles (Section 5.2.5) and 'mature' into spherical, cholesterol-rich particles. These particles can give up their cholesterol (again by routes described later) and hence can recycle via lipid-poor forms (Fig. 5.6). Most HDL cholesterol is carried by the larger, spherical particles, which are divided by centrifugation into two subclasses, HDL_2 (larger particles) and HDL_3 (smaller).

A further class of lipoprotein particles is found to a variable extent, and is known as lipoprotein(a) (always read as 'Lipoprotein-little a'). Lipoprotein(a), abbreviated Lp(a), is found at a somewhat higher density than LDL in the ultracentrifuge. Its structure is of an LDL particle, to the apoB100 of which is covalently attached a large additional protein, apolipoprotein(a). Apolipoprotein(a) is related to the protein plasminogen, precursor of the enzyme plasmin in the fibrinolytic system. Its structure is characterized by multiple repeats of a 114-amino acid cysteine-rich domain known as a kringle (because its tertiary structure resembles the twisted baked product known as a kringle). The number of kringles varies from person to person and is genetically determined. Variations in the number of kringles give the Lp(a) particles a range of densities. The plasma concentration of Lp(a) particles varies considerably, but is of interest because of a relationship between Lp(a) concentration and risk of atherosclerosis.

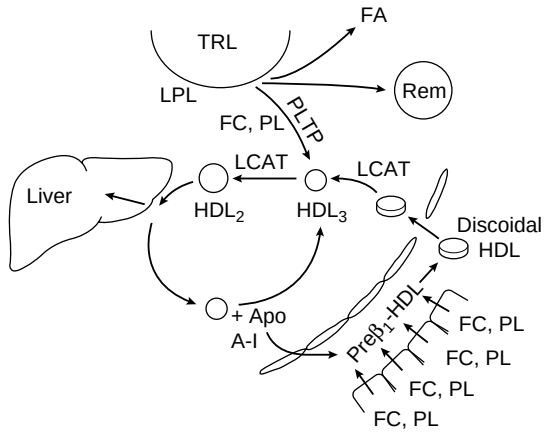


Fig. 5.6 The HDL pathway. Lipid-poor apoAI or pre-β1 HDL acquires phospholipids (PL) and free cholesterol (FC) by interaction with cell membranes, forming discoidal HDL in the circulation. Through the action of lecithin-cholesterol acyltransferase (LCAT), and the acquisition of further lipids arising during the action of lipoprotein lipase (LPL) on triacylglycerol-rich particles (TRL) [phospholipid transfer protein, PLTP, is involved in this transfer], these particles swell and become spherical 'mature HDL'; smaller spherical particles are known as HDL3; more lipid-rich particles as HDL2. (Fatty acids, FA, are also produced by the action of LPL, and the smaller particles that remain are known as remnants (rem).) These mature HDL particles can deliver their lipid content to the liver, possibly via the receptor SR-BI (see Section 5.2.4.4), to recycle as lipid-poor apoAI. An alternative route for delivery of lipid to the liver is through transfer of TRL remnants via the cholesteryl ester transfer protein (see Fig. 5.11). Based on Fielding, P.E. & Fielding, C.J. (1996) in *Biochemistry of Lipids, Lipoproteins and Membranes* (eds. D.E. Vance & J. Vance), pp. 495–516. Elsevier, Amsterdam.

5.2.4 Specific lipoprotein receptors mediate the cellular removal of lipoproteins and of lipids from the circulation

One function of the apolipoproteins has already been described as targeting of lipoproteins to specific destinations. This is achieved by the interaction of the apolipoproteins with specific cell-surface receptors. The triacylglycerol-rich lipoprotein particles, chylomicrons and VLDL, are largely confined to the vascular compartment by their size until they have undergone extensive lipolysis, allowing them to pass through the fenestrations in

the endothelial lining of the capillaries. Some of the receptors involved are therefore expressed on endothelial cells, but most are expressed on parenchymal cells, and part of their selectivity may depend upon the exclusion, by virtue of size, of the larger particles. Before describing the major receptors involved in lipoprotein metabolism in further detail, it is useful to make some general points about membrane-bound receptors.

5.2.4.1 Membrane receptors

Membrane receptors are proteins designed to recognize specific molecules and bind them in preparation for the initiation of a biological process that takes place in the membrane. The process may be the transport of a molecule (e.g. glucose, LDL) across the membrane or the triggering of a chemical message by a hormone or a growth factor. The recognition step demands that the receptor has specificity, like an enzyme for its substrate. Binding is followed frequently, but not always, by internalization in which the membrane in the vicinity of the receptor forms a vesicle that encapsulates the receptor–ligand complex and enters the cell. Once inside, the receptor complex can be degraded by lysosomal enzymes and the receptor proteins may be recycled to be reinserted into the plasma membrane again. The number of membrane receptors is usually responsive to the availability of the specific ligand. This requires a mechanism for the control of the biosynthesis of the receptor. Receptors are usually glycoproteins, as described for the LDL-receptor below. The functional properties of receptors in membranes appear to be intimately related to the microenvironment provided by membrane lipids. Membrane proteins are on average associated with 30–40 molecules of phospholipid per molecule of peptide. These annular phospholipids are required for functional activity or for the stabilization of protein conformation. The structural and compositional changes of the lipid bilayer, which provides a fluid matrix for proteins, can induce alterations in functional properties of proteins in the intact membrane, for example by allowing changes in protein conformation or the diffusion or position of proteins in the membrane. This may be an important means by which diet – affecting the nature of the membrane phospholipid fatty acids – can affect hormone action, for instance.

5.2.4.2 The LDL-receptor

The best-characterized lipoprotein receptor is the LDL-receptor, also known as the B/E receptor because it recognizes (i.e. binds to) homologous regions on apolipoprotein E and on apolipoprotein B100 (but not apoB48). Mutations in this receptor causing loss of function result in marked elevation in the plasma LDL-cholesterol concentration, the condition known as familial hypercholesterolaemia (Section 5.4.2). It is the receptor responsible for the removal of LDL particles from the circulation. The structure of this receptor is shown in Fig. 5.7.

The receptor is synthesized on the rough endoplasmic reticulum as a precursor of molecular mass 120 kDa. About 30 min after its synthesis, the protein is modified to a mature receptor with an apparent mass of 160 kDa. The increase in molecular mass coincides with extensive modifications of the carbohydrate chains. The precursor molecule contains up to 18 *N*-acetylglucosamine molecules attached in *O*-linkage to serine and threonine residues (Fig. 5.7) as well as two high mannose-containing *N*-linked chains. The latter are modified extensively and the *O*-linked chains are elongated by addition of one galactose and two sialic acid residues to each *N*-acetylglucosamine during post-translational processing in the Golgi network.

The receptor is exported to specialized regions of the cell membrane known as coated pits, where the cytoplasmic leaflet is coated with the protein clathrin. After binding of an LDL particle through interaction of its apolipoprotein B100 molecule with the ligand-binding domain of the LDL-receptor, the receptor–LDL complex is internalized by endocytosis of the coated pit (Fig. 5.8). The coated vesicle so formed loses its clathrin coating, and its interior becomes acidified (an endosome). The LDL particle and receptor dissociate in the low pH environment of the endosome, and the receptor is recycled back to the cell surface. The remainder of the particle is transferred to lysosomes, where the cholesteryl esters are hydrolysed by lysosomal acid hydrolases,

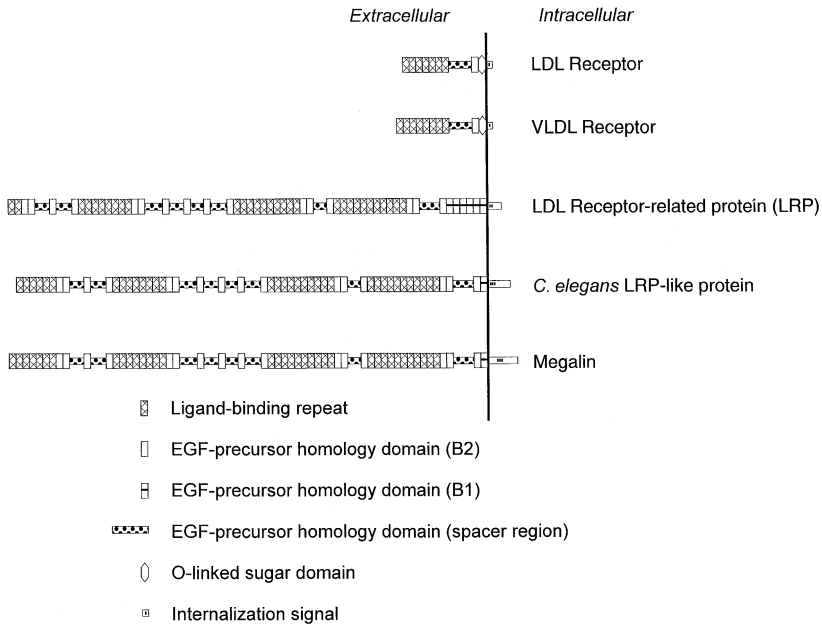


Fig. 5.7 The structure of the LDL-receptor and related receptors. (The apoE receptor 2 is not shown but it is similar to the LDL and VLDL-receptors.) Each of the receptors in this family is built up from repeats of simpler units, some named according to their homology with the epidermal growth factor (EGF) receptor. The ligand binding domains are responsible for binding to the apolipoproteins of the target lipoprotein particles. The internalization signal domain is responsible for internalization of the receptor after the ligand is bound. Based upon Fig. 4 in Schneider, W.J. (1996) in *Biochemistry of Lipids, Lipoproteins and Membranes* (eds. D.E. Vance & J. Vance), pp. 517–541. Elsevier, Amsterdam.

and the cholesterol adds to the cellular cholesterol pool. Cholesteryl esters may also be resynthesized by the action of acyl-CoA: cholesterol acyltransferase. The size of this cellular cholesterol pool in turn regulates expression of the LDL-receptor so that cellular cholesterol content is strongly regulated, as described in Section 5.3.1

LDL-receptors are expressed by most cell types. There is some debate, however, about their biological function. They represent a means by which cells can acquire cholesterol exported from the liver. Most cells expressing LDL-receptors, however, also have the enzymic capability of synthesizing cholesterol *de novo*, from acetyl-CoA. The alternative view is that LDL-receptors are there to remove cholesterol from the plasma. The fact that they are highly expressed in steroidogenic tissues such as the adrenal cortex argues for the former. However, in whole-body terms, the main site of LDL-receptor-mediated LDL removal is the liver. Cholesterol delivered to the liver can be removed from the body either by excretion directly in the bile, or after conversion to bile salts. Increasing LDL-receptor activity with the 'statin' drugs (see Section 5.3.1) can lead to marked reduction in plasma cholesterol concentration.

5.2.4.3 The LDL-receptor-related protein and other members of the LDL-receptor family

Because of its ability also to bind apoE, the LDL-receptor may play a role in removal of chylomicron remnants from the circulation. But it cannot be the major receptor involved in this process, because humans or animals lacking functional LDL-receptors (as in familial hypercholesterolaemia) do not show accumulation of chylomicron remnants. Recently a related receptor has been identified, known as the LDL-receptor-related protein or LRP. This is the best candidate at present for the

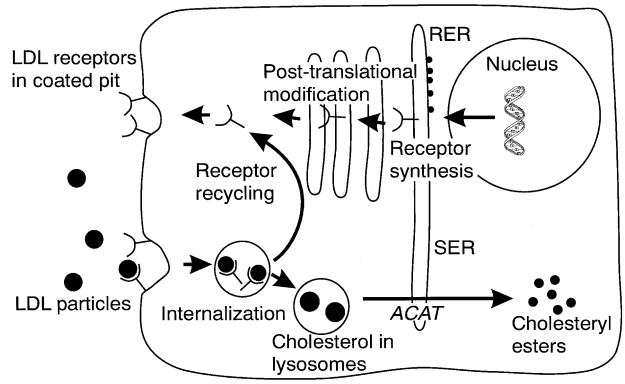


Fig. 5.8 Cell biology of the LDL-receptor. Acyl-CoA cholesterol acyltransferase (ACAT) synthesizes cholesteryl esters that may be stored in the cell. The synthesis of the LDL-receptor is closely regulated at the level of gene expression by the cellular cholesterol content (see Fig. 5.13). Redrawn from Frayn, K.N. (1996) *Metabolic Regulation: a Human Perspective* (Portland Press), and Krieger, M. (1999) *Annual Review of Biochemistry*, **68**, 523–558.

chylomicron remnant receptor. The LDL-receptor and LRP, together with the other receptors (described below), form a family of receptors with structural homologies (Fig. 5.7).

LRP binds to apoE, in which chylomicron remnants are relatively enriched. It is expressed in most tissues, with the liver, brain and placenta as major sites of expression on a whole-body basis. Its expression is not regulated by cellular cholesterol content as is that of the LDL-receptor. However, in adipocytes, where it is also expressed, its concentration on the cell surface is rapidly increased by insulin. This increase in receptor appearance on the cell membrane is too rapid to be accounted for by an effect of insulin on protein synthesis, leading to the suggestion that the LRP, like the glucose transporter GLUT4, is present within the cells and translocates to the cell membrane when stimulated by insulin. This would represent a mechanism for directing some chylomicron remnants to adipose tissue in the period following a meal, although the major site of chylomicron remnant uptake is still considered to be the liver.

Both hepatocytes and adipocytes also produce apolipoprotein E, which may be expressed on the cell surface. In the liver it has been suggested that chylomicron remnants, once they become small enough via the activity of lipoprotein lipase in the capillary beds of other tissues, may enter the subendothelial space (the space of Disse). Here they bind to hepatic lipase, which is also expressed on the hepatocyte membrane. Further triacylglycerol hydrolysis by hepatic lipase alters the particle composition so that cell membrane-attached apoE binds to the particle. As the remnant particle becomes further enriched with apoE by this means, it binds to adjacent LRP and is then internalized by a process very similar to that described for the LDL-receptor (Fig. 5.8). Lipoprotein lipase also binds to the LRP. Since lipoprotein lipase is not synthesized in the adult liver, it is presumed that remnant particles carry this enzyme from peripheral tissues to the liver, where it assists in binding of the particles to LRP.

Unlike the case for LDL-receptor, no human LRP deficiency state has yet been found. Attempts to create mice lacking the LRP have not been successful – the phenotype is lethal at an early embryonic stage. It may therefore be an essential protein for life.

A related receptor protein was discovered in 1992 by searching for genes with homology to the LDL-receptor gene. This receptor binds to, and internalizes, VLDL particles particularly avidly. It is expressed mainly in tissues outside the liver that might be expected to have a requirement for fatty acids: heart, skeletal muscle and adipose tissue. Within those tissues the so-called VLDL-receptor is

expressed mainly by the endothelial cells, which suits its supposed role as a receptor for large, triacylglycerol-rich particles that cannot cross the endothelial barrier. The precise physiological role of the VLDL-receptor is unknown, however. Its structure is shown in Fig. 5.7.

A further member of the family in mammals is known as megalin or lipoprotein receptor-related protein-2 (LRP-2; Fig. 5.7). It is expressed in absorptive epithelial cells of the proximal tubules of the kidney. It has been suggested that its function is the re-uptake of fat-soluble vitamins that would otherwise be lost by urinary excretion. In 1996 yet another member of the family was described, the apoE receptor 2. This is, again, closely homologous to the LDL and VLDL-receptors. It is highly expressed in the brain and may play a role in lipid transport into the central nervous system.

This family of receptors is related also to the vitellogenin receptor responsible for delivery of lipid to the developing avian egg yolk, and there are closely related proteins in the fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* (Fig. 5.7).

5.2.4.4 Scavenger receptors

Unrelated structurally to the LDL-receptor family are several receptors with the generic name of 'scavenger receptors'. These were first identified as receptors in macrophages that mediate the uptake of LDL particles that have been chemically modified so that their affinity for the LDL-receptor is reduced. One such modification is oxidation, the significance of which will be discussed in Section 5.4.1. There are at least three families of scavenger receptors, classes A, B and C. They are characterized by a broad substrate specificity, and they may well have roles in macrophage function in host defence, removal of foreign substances, etc., beyond their role in lipid metabolism. There are two related receptors in class A, the type I and type II scavenger receptors. They are expressed on the macrophage cell surface in clathrin-coated pits, similar to the LDL-receptor (Fig. 5.8). They bind and internalize modified LDL particles, which are then degraded by similar processes to those shown in Fig. 5.8. However, a crucial difference from the LDL-receptor system is that the cellular cholesterol content does not regulate expression of the scavenger receptors. Thus, their activity can lead to unregulated accumulation of cholesterol in macrophages, an event that may initiate the series of changes that lead to atherosclerosis (Section 5.4.1).

Class B scavenger receptors include the receptor known as Scavenger Receptor Class B type 1 or SR-BI, and a protein that had been previously studied as a widely expressed cell-surface antigen given the name CD36. SR-BI was found to bind HDL particles with high affinity, and is now recognized as a long-sought HDL receptor. Its function in this role is covered in more detail in the following section. CD36 appears to have multiple roles. Amongst other physiological roles, it has been suggested as a long-chain fatty acid transporter and also given the name fatty acid translocase (FAT).

5.2.5 The lipoprotein particles transport lipids between tissues but they interact and are extensively remodelled in the plasma compartment

It is convenient to describe lipoprotein metabolism as consisting of three pathways, but it will become clear that these are interrelated and interact with one another.

The pathway for transport of dietary fat is known as the exogenous pathway (Fig. 5.9). As the triacylglycerol-rich chylomicron particles, secreted from enterocytes, enter the plasma, they interact with other particles (presumably by simple physical contact) and acquire other apolipoproteins, especially CII, CIII and apoE. ApoCII is essential for their further metabolism. The chylomicron particles come into contact with the enzyme lipoprotein lipase, which is expressed in a number of extrahepatic tissues that can use fatty acids, including adipose tissue, skeletal and cardiac muscle and mammary gland. Lipoprotein lipase was described in Section 3.5.2. It is anchored to the luminal aspect of the endothelial cells lining the capillaries in these tissues by binding to heparan sulphate proteoglycans. As chylomicrons pass through the capillaries

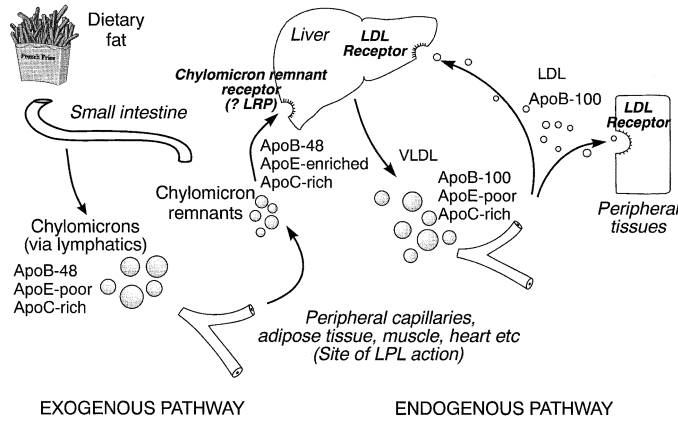


Fig. 5.9 Overview of the exogenous and endogenous pathways of lipoprotein metabolism. Lipolysis of particles by lipoprotein lipase (LPL) in capillaries of extra-hepatic tissues is simplified: VLDL particles, in particular, may go through several cycles of lipolysis, and there is an intermediate class of particle known as Intermediate Density Lipoprotein (IDL) formed before final conversion to LDL particles. VLDL particles themselves may also be removed by VLDL receptors expressed in peripheral tissues. Interactions with HDL are shown in part in Fig. 5.11, but HDL is also an important donor of apolipoproteins (e.g. apoCII to nascent chylomicrons). Ideas for this figure came from Herz, J. (1998) in *Lipoproteins in Health and Disease* (eds. D.J. Betteridge *et al.*), pp. 333–359. Arnold, London.

they bind to lipoprotein lipase and to the proteoglycan chains. LPL, with apoCII as an essential activator (cofactor), hydrolyses the core triacylglycerol in the particle. The rate of lipolysis is rapid, and it has been estimated that somewhere between 10 and 40 lipoprotein lipase molecules must act simultaneously on a chylomicron. The non-esterified fatty acids that are generated can diffuse into the adjacent tissues, either by simple diffusion across the cell membranes or by facilitated diffusion, using one of the recently described fatty acid transport proteins (see Section 5.1.3 for a description of this in the enterocyte). The triacylglycerol-depleted chylomicron particle shrinks, and as a consequence some of the amphipathic surface monolayer becomes redundant. Surface components dissociate and are acquired by other lipoproteins, particularly HDL. A phospholipid-transfer protein in the plasma seems to mediate this transfer. The particle dissociates from lipoprotein lipase (the process of lipolysis having taken perhaps a matter of minutes). It is known as a chylomicron remnant. It may undergo a further round of lipolysis in another tissue bed, but after loss of about 80% of its original content of triacylglycerol it will be removed rapidly from plasma. Shrinkage of the particle allows it to cross the endothelial barrier and interact with cell-surface receptors. In addition, conformational changes to the apolipoproteins caused by the shrinkage of the particle expose different regions, which bind to specific cell-surface receptors, probably mainly LRP (see Section 5.2.4 above). The half-life of chylomicron-triacylglycerol in the circulation is about 5 min. The half-life of a chylomicron particle has been estimated at 13–14 min.

The exogenous pathway is regulated mainly by the rate of entry of dietary fat, but the tissue disposition of the dietary fatty acids is regulated by tissue-specific regulation of lipoprotein lipase activity. Adipose tissue lipoprotein lipase activity is up-regulated by insulin and therefore plays an important role in removal of dietary fatty acids in the period following a mixed meal (i.e. a meal that contains both fat and carbohydrate, since the latter will stimulate insulin secretion). The mechanism for activation of adipose tissue lipoprotein lipase by insulin is complex. Whilst insulin increases transcription of the lipoprotein lipase gene in adipose tissue, the major point of regulation in the short

term (the hours following a meal) appears to be a diversion within the adipocyte of lipoprotein lipase between a degradative pathway, and secretion in an active form for export to the endothelium. Since the enzymes of triacylglycerol synthesis in adipocytes are also activated in the fed state, the net effect is that dietary fatty acids tend to be readily deposited as adipocyte triacylglycerol. In contrast, lipoprotein lipase in skeletal, and particularly cardiac, muscle is down-regulated by insulin, and increases in activity during fasting, and in skeletal muscle with exercise training. In these conditions, therefore, fatty acids tend to be directed to the tissues that need them for oxidation (Fig. 5.10).

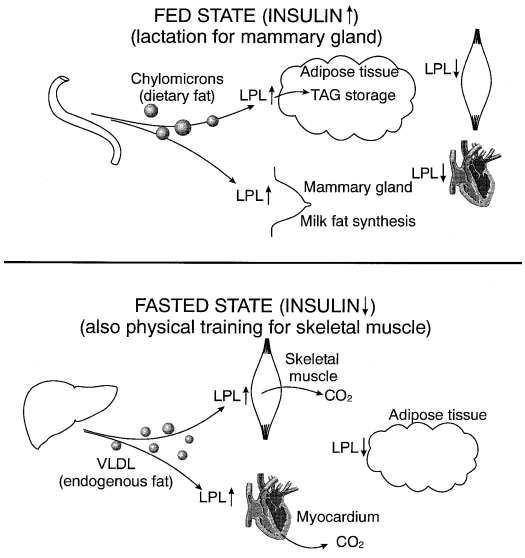


Fig. 5.10 Tissue-specific regulation of lipoprotein lipase (LPL) in relation to nutritional state directs the flow of triacylglycerol-fatty acids to different tissues according to needs.

The secretion of VLDL from the liver, and its further metabolism, is described as the endogenous pathway (Fig. 5.9). It is very similar initially to the exogenous pathway. VLDL particles also interact with lipoprotein lipase in capillaries and their core triacylglycerol is hydrolysed. The half-life of VLDL particles in the circulation is typically 12 h, but their fates are heterogeneous. As the particles lose their triacylglycerol through successive interactions with lipoprotein lipase, they also lose their surface apolipoproteins as described above, until they become LDL particles. However, along the way some of the particles are removed intact by endothelial VLDL-receptors. LDL particles have an even longer half-life in the circulation, typically 2–2.5 days, and they are removed by LDL-receptors on cell surfaces (Section 5.2.4 above) in the sub-endothelial space. The major site for removal of LDL particles is normally the liver although the LDL-receptor is a mechanism for delivering cholesterol to most cells. The expression of LDL-receptors is closely regulated to maintain cellular cholesterol homeostasis (Section 5.3.1). Thus, the endogenous pathway is a means of delivering fatty acids to tissues via lipoprotein lipase and perhaps the VLDL-receptor, and cholesterol via the LDL-receptor.

The third pathway is that of HDL metabolism (Fig. 5.11). As described above, HDL particles may not be secreted as such: rather, the basic components of apoAI and phospholipid are secreted and the particles cycle through various stages in the plasma. If the LDL pathway of cholesterol delivery to tissues is regarded as 'forward cholesterol transport', the HDL pathway represents 'reverse cholesterol transport' or the movement of cholesterol out of tissues and its transport to the liver for ultimate excretion in the bile. (The liver is the only mammalian organ that can excrete cholesterol from the body.)

As described earlier (Section 5.2.3 and Fig. 5.6), immature lipid-poor HDL particles (pre-β1 HDL) interact with cell membranes to acquire unesterified cholesterol. There has been much debate about whether this involves a specific receptor. Recent advances in the molecular biology of cellular cholesterol efflux have come about through the identification of the gene defect in Tangier disease, a rare inherited disease in which cholesterol accumulates in tissues.

The gene responsible for Tangier disease encodes a protein that is a member of a family of cell-membrane-associated transporter proteins that have the property of binding ATP on their cytoplasmic side (through the so-called ATP-binding cassette, ABC: this particular protein has now become known as ABC1). Hydrolysis of ATP can

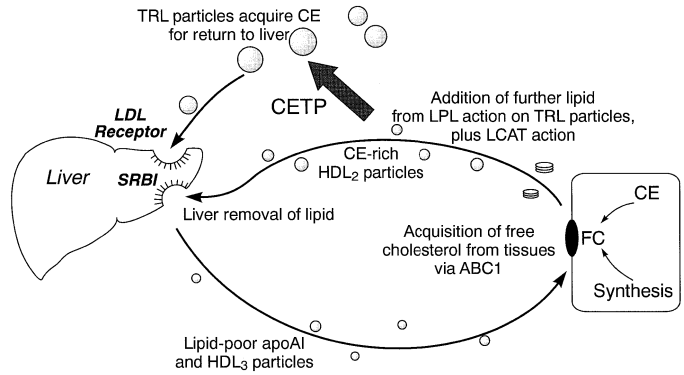


Fig. 5.11 The pathway of reverse cholesterol transport. For more details of HDL metabolism see Fig. 5.5. Lipid-poor apoAI acquires free cholesterol (FC) and phospholipid from peripheral tissues via the ATP-binding cassette protein-1 (ABC1). The lipid content of the HDL particles is increased by transfer of additional cholesterol from the lipolysis of triacylglycerol-rich lipoprotein (TRL) particles. Esterification of this cholesterol by lecithin-cholesterol acyltransferase (LCAT) produces mature, lipid-rich HDL_2 particles. These can return their cholesterol to the liver in two ways. They may deliver it directly via a hepatic receptor (SR-BI) or they may exchange it for triacylglycerol from the TRL via the action of cholesteryl ester transfer protein (CETP). The TRL particles then carry this cholesterol to the liver for receptor-mediated uptake. (CE denotes cholesteryl esters.)

then power the transfer of substances across membranes. In the case of cholesterol efflux, ABC1 seems to mediate the transfer of cholesterol from 'cholesterol rafts' in the cell membrane to the lipid-poor HDL particles (where it binds to apoAI). The unesterified cholesterol acquired by the particles becomes esterified with a long-chain fatty acid through the action of the enzyme lecithin-cholesterol acyltransferase (LCAT), which is associated with HDL particles. This enzyme catalyses the transfer of a fatty acid from phosphatidylcholine to cholesterol to form a cholesteryl ester (Fig. 5.12). The phospholipid substrate for the reaction is present in the HDL particle. The hydrophobic cholesteryl ester moves to the core of the particle, which swells and becomes spherical as more cholesterol is acquired and esterified. The other product, lysophosphatidylcholine, is transferred to plasma albumin from which it is rapidly removed from blood and reacylated. This is probably the origin of the bulk of HDL-cholesterol in plasma, although some is also acquired from the surface components of chylomicrons and VLDL particles when their triacylglycerol is hydrolysed by lipoprotein lipase, as described above.

To complete the pathway of reverse cholesterol

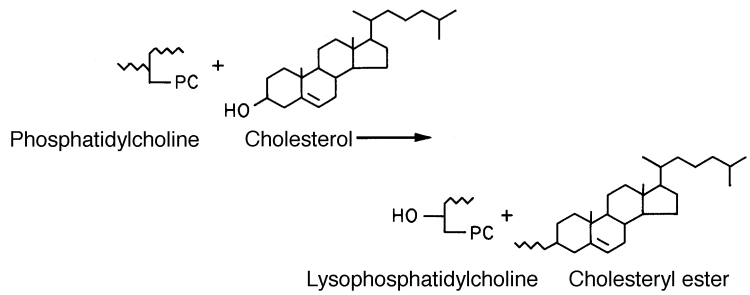


Fig. 5.12 The lecithin-cholesterol acyltransferase (LCAT) reaction.

transport, HDL-cholesterol must reach the liver. There are at least two routes by which this can occur. First, the scavenger receptor SR-BI (Section 5.2.4.4) is expressed in the liver and also in steroidogenic tissues (e.g. adrenal gland and ovary). 'Docking' of HDL particles with SR-BI is followed by off-loading of their cholesteryl ester content. The cholesteryl esters enter the cellular pool and may be hydrolysed by lysosomal acid hydrolases as shown for LDL-receptor-mediated uptake (Fig. 5.8). This process is fundamentally different from the uptake of LDL particles by the LDL-receptor, however, and has been called 'selective lipid uptake'. The difference is that the particle itself is not internalized and the cholesterol-depleted particle leaves the receptor to re-enter the cycle of the HDL pathway.

The second mechanism for delivery of HDL-cholesterol to the liver brings us to an important way in which the lipoprotein pathways interact. Human plasma contains a protein known as cholesteryl ester transfer protein (CETP), which is secreted from the liver and from adipose tissue. CETP mediates the exchange of 'neutral lipids' (triacylglycerol and cholesteryl esters) between particles, according to concentration gradients. When HDL particles become enriched in cholesteryl esters, they may exchange these esters for triacylglycerol carried in the triacylglycerol-rich lipoproteins, i.e. chylomicrons and VLDL. Thus, chylomicrons and VLDL acquire cholesterol that stays with the particle since it is not removed by lipoprotein lipase, and it is eventually removed when the remnant particle (or LDL particle) is taken up by receptors, which, as we have seen, occurs mainly in the liver. Thus cellular cholesterol in extra-hepatic tissues, setting out for the liver in an HDL particle, may 'change vehicles' part way and end up being carried in a remnant particle. The HDL particles acquire triacylglycerol in this process and this is hydrolysed by the enzyme hepatic lipase attached to the endothelial cells lining the hepatic sinusoids. What remains is a lipid-poor HDL particle, ready to recycle via acquisition of cellular cholesterol.

5.2.6 Species differ quantitatively in their lipoprotein profiles

In some species, like man, guinea-pig and pig, lipoproteins of the LDL type (in which apolipoprotein B predominates) account for more than 50% of the total substances of density < 1.21 g ml^{-1}. They are the LDL mammals. In the majority of mammals, however, HDL are the predominant class and may account for up to 80% of plasma substances of density < 1.21 g ml^{-1}. Herbivorous species, with the exception of guinea-pigs, camels and rhinos, and carnivores are HDL mammals. It is worth noting that although rats are often used for the study of lipid biochemistry, their lipoprotein pattern is of the HDL type and very different from that of man. Another distinct difference is that the rat liver secretes VLDL particles that contain either apoB48 or apoB100 (whereas in humans the liver secretes only apoB100-containing particles). This almost certainly relates to the fact that their LDL-cholesterol levels are so relatively low, since apoB48-containing particles tend to be cleared from the circulation rapidly (as with chylomicrons in man). Caution needs to be exercised in extrapolating results on experimental animals to the human situation.

Whichever species one is studying, it is often necessary to quantify various aspects of the lipoprotein system. Analytical methods are summarized in Box 5.1, p. 192–3.

5.3 THE CO-ORDINATION OF LIPID METABOLISM IN THE BODY

Several genes involved in lipid metabolism are themselves regulated by lipid or lipid-related molecules, and adipose tissue itself secretes proteins that regulate food intake and fat storage.

The flux of fatty acids through the pathways described in this chapter can vary enormously. The fat content of the diet varies in different parts of the world, between different people within one country, and even from day to day in one individual. The rate at which we transport fatty acids through the plasma varies considerably during a normal day. For non-esterified fatty acids it is high at night

during fasting, suppressed after meals, and stimulated to very high levels during aerobic exercise; for triacylglycerol-fatty acids it is roughly the converse. This requires co-ordination between metabolic pathways in different organs, in relation both to the influx of dietary lipids and the body's requirement for lipids. In Chapters 4 and 5 we have seen how this co-ordination can be brought about by hormones, and in particular by insulin whose secretion from the pancreas is stimulated in the 'fed' state. Insulin acts both by affecting the activity of enzymes on a short-term basis (e.g. by reversible phosphorylation/dephosphorylation) and on a longer term basis by regulation of gene expression. However, the main stimulus for insulin secretion is a rise in the plasma glucose concentration. It would seem sensible that the body should also have means for homeostasis of lipid metabolism that do not depend upon the simultaneous ingestion of carbohydrate in appropriate amounts.

Over the past few years there have been great advances in our understanding of how this is achieved. There are several, possibly interrelated, systems that regulate gene expression to control the flux through various pathways of lipid metabolism, and whose regulators are themselves lipids or products derived from lipids.

5.3.1 The sterol regulatory element binding protein (SREBP) system controls pathways of cholesterol accumulation in cells and may also control fatty acid synthesis

The American biochemists and 1985 Nobel Prize winners, Mike Brown and Joseph Goldstein, showed that cellular cholesterol content is regulated by two parallel mechanisms. When the content of unesterified cholesterol in cells increases, the expression of the LDL-receptor protein decreases. In addition, the key enzymes of cholesterol biosynthesis (hydroxymethylglutaryl(HMG)-CoA synthase, HMG-CoA reductase, squalene synthase, farnesyl diphosphate synthase; Section 7.5.1) are repressed. Thus, any further increase in cellular cholesterol is minimized. Conversely, if the cellular unesterified cholesterol content falls, these pathways are activated (Fig. 5.13). The molecular basis for this regulation is now clear. The genes for all these proteins contain an upstream sequence known as the sterol regulatory element-1 (SRE-1). The protein that binds to SRE-1, and activates expression of the gene, is part of a larger protein called sterol regulatory element binding protein (SREBP). SREBP is normally localized in the endoplasmic reticulum. In the absence of cholesterol a specific protease releases a peptide from SREBP, which migrates to the nucleus and binds to SRE-1,

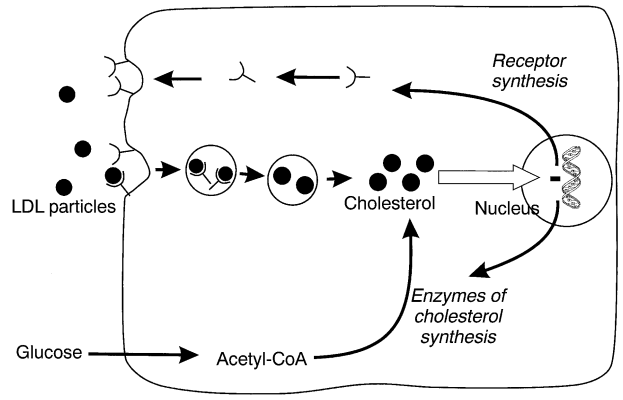


Fig. 5.13 Regulation of the cellular cholesterol content. When the content of unesterified cholesterol in cells increases, the expression of the LDL-receptor protein and the enzymes of cholesterol biosynthesis is repressed.

Box 5.1 Methods for lipoprotein analysis

It may be useful to analyse various aspects of the lipoprotein system. This might include separation of the various classes of lipoprotein particle, analysis of the apolipoproteins present in plasma, or analysis of the chemical species; or, of course, a combination of any of these.

Lipoprotein separation

There are various methods for separation of lipoproteins. They are mostly based on physical properties. It must be remembered that physical properties and biological function are closely but not absolutely related. Thus, separation of a so-called chylomicron fraction on the basis of flotation of large, triacylglycerol-rich particles will produce a population of particles that contain some large particles secreted by the liver (which, on biological function, should be called VLDL). Similarly, the next population to be harvested, which will be mainly VLDL, will also contain small chylomicrons and chylomicron remnants. The main physical methods are flotation in the ultracentrifuge, electrophoresis, gel filtration and precipitation. They are compared in the table below.

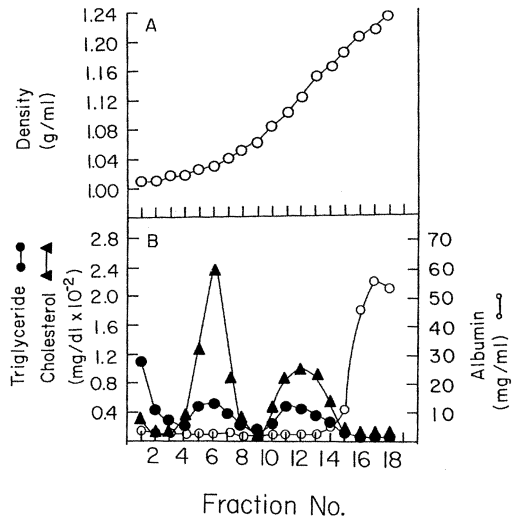
The principles of sequential flotation were developed by the Californian pioneer in lipid research, Richard Havel, in the 1950s (he is still active in the field today). Essentially, the plasma is laid in a tube under a salt solution of known density, which, on ultracentrifugation, allows one species of particle to be 'floated'. The particles are harvested and the density re-adjusted to prepare the next fraction. An alternative is to prepare a density gradient in which all types of particle will be separated in one centrifugation (usually faster but giving less clear separation). An example is shown in the figure right.

The figure shows the distribution of components after a single density-gradient centrifugation for 90 min at 65 000 rpm. The components were pumped from the bottom, out of the top of the tube. The density initially was from 1.006 (top) to 1.30 (bottom) g ml^{-1}. The major peaks eluting are (left to right) VLDL (major component triacylglycerol, ●), LDL (major component cholesterol, ▲), HDL (both triacylglycerol and cholesterol) and finally albumin from the bottom of the tube. Reproduced from Chung, B.H., Segrest, J.P., Ray, M.J., Brunzell, J.D., Hokanson, J.E., Krauss, R.M., Beaudrie, K. & Cone, J.T. (1986) Single vertical

Method	Advantages	Disadvantages
Ultracentrifugal flotation	Regarded as the 'reference method'.	Relatively low capacity (usually performed in a swinging-bucket rotor with only 4 or 6 buckets).
	Can be used to prepare large amounts of material for further analytical work.	Alterations in particle composition are known to occur during isolation.
Electrophoresis (usually on agarose gel)	Quick to perform on large numbers of samples.	Semi-quantitative. Not preparative.
Gel filtration	Appears to cause least alteration to the particles.	Very limited capacity (usually performed in FPLC mode, running one sample at a time).
Precipitation	Can be applied to large numbers of samples.	Separation is often based upon empirical findings and the basis is not always understood: possibility for artefacts therefore exists.

Continued

Box 5.1 ***Continued***



spin density gradient ultracentrifugation. In: *Methods in Enzymology*, Vol. 128 *Plasma Lipoproteins Part A. Preparation, Structure, and Molecular Biology* (eds. J.P. Segrest & J.J. Albers), pp. 181–209. Academic Press, Orlando, FL.

Because of the problem of lack of absolute separation of particles according to biological function, other techniques have been developed. For instance, chylomicrons and VLDL differ in that the chylomicrons carry the shorter apolipoprotein apoB48, whereas VLDL carry apoB100. Specific antibodies have been prepared that bind to the C-terminal region of apoB100, and not to apoB48. These may be attached to a support such as Sepharose gel. A fraction containing the triacylglycerol-rich lipoproteins is prepared. This is passed through the gel, and VLDL particles are then bound and retained whilst chylomicron particles pass through and may be collected. Such methods are as yet only used in specialized research applications.

Lipoprotein classes may also be separated by various precipitation techniques. The precise physico-chemical basis for these is not always known. For instance, a combination of heparin and $MnCl_2$ will precipitate particles containing apoB (chylomicrons, VLDL, LDL); after centrifugation to remove these, the supernatant contains HDL whose cholesterol content may be measured. When fasting plasma (i.e. with no chylomicrons present) is incubated with the detergent sodium dodecyl sulphate (SDS), VLDL particles separate (they float to the top). Analysis of the infranatant gives the concentration of (for instance) cholesterol in LDL+HDL. If the HDL-cholesterol concentration is measured by heparin–$MnCl_2$ precipitation as above, subtraction will give cholesterol concentrations in VLDL and LDL. The more subtractions that are performed, the less reliable is the result.

Analysis of apolipoproteins

It is often useful to measure the concentrations of specific apolipoproteins in plasma. The usual methods are electrophoretic, or immunological. Electrophoresis is usually carried out on denaturing SDS-polyacrylamide gels and separates the proteins according to their size. ApoB100, because of its very large size, remains near the origin. The separated apolipoproteins may be quantified by staining and measurement of absorbance. There is a great variety of immunological techniques, each based on the availability of an antibody or antiserum specifically directed against one apolipoprotein. For instance, the technique of immunoturbidimetry relies on the solubilization of the apolipoproteins in detergents. They are then allowed to bind to a specific antibody (e.g. anti-apoAI), forming complexes that make the solution turbid in proportion to the amount of the specific protein present. The turbidity is measured in a spectrophotometer or similar instrument. Such techniques are easily automated and hence can have high throughput.

Analysis of lipid components

The analysis of concentrations of triacylglycerol, cholesterol, etc. in plasma or in isolated lipoprotein fractions is usually based upon enzymic methods and is readily automated for high throughput. These methods are described in textbooks of clinical chemistry and will not be given in more detail here.

activating transcription. When cellular cholesterol levels are high, this protease is inhibited and gene expression is repressed. The natural regulator of this system appears not to be cholesterol itself but a hydroxylated derivative of cholesterol, an oxysterol. The working of the system is illustrated in Fig. 5.14.

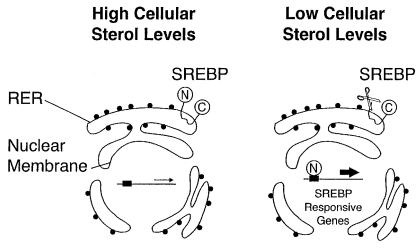


Fig. 5.14 The Sterol Regulatory Element Binding Protein (SREBP) system. The precursor protein (125 kDa) is associated with the rough endoplasmic reticulum (RER) (left). When cell cholesterol content is low, a protease (scissors) cleaves this to release the transcriptionally active 68 kDa N-terminal portion, which binds to specific promoter sequences in target genes (e.g. LDL-receptor, enzymes of cholesterol synthesis; see Fig. 5.13). When cell cholesterol content is high, this pathway does not operate and transcription of the target genes is low. Reproduced from Edwards, P.A. & Davis, R. (1996) in *Biochemistry of Lipids, Lipoproteins and Membranes* (eds. D.E. Vance & J. Vance), pp. 341–362. Elsevier, Amsterdam.

There are three known isoforms of SREBP: SREBP-1a, SREBP-1c and SREBP-2. The first two arise from differential splicing of the transcript from one gene, whereas SREBP-2 is the product of a separate gene. It seems that SREBP-1a and 1c are concerned more with regulation of genes involved in fatty acid metabolism including fatty acid synthase, acetyl-CoA carboxylase and stearoyl-CoA ($\Delta 9$) desaturase (Section 2.2.8), whereas SREBP-2 regulates transcription of genes involved in cholesterol metabolism (Section 7.5.7). SREBP-1c is also expressed in adipose tissue (where it is also known as adipocyte determination and differentiation factor-1, ADD-1), and has been shown to regulate fatty acid synthesis by increasing the expression of fatty acid synthase in adipose tissue as well as liver. Leptin production and secretion (Section 5.3.4) are also increased. Expression of SREBP-1c is itself under regulation by insulin. Thus, when insulin levels are high, more SREBP-1c is produced and fatty acid synthesis is up-regulated. Since this also requires the involvement of cholesterol (or a derivative), it might be that the regulation really works more in the negative way: in times of fasting (low insulin), SREBP-1c expression is severely reduced, and fatty acid synthesis cannot be activated by the SREBP pathway. Some of the genes whose expression is regulated by the SREBP system are shown in Table 5.3.

The SREBP system has been manipulated to alter cholesterol metabolism. Certain fungal metabolites, compactin from *Penicillium* spp. and mevinolin from *Aspergillus terreus*, were found in the 1970s to inhibit HMG-CoA reductase. Synthetic derivatives of these molecules are now widely used as drugs (the 'statins') to lower plasma cholesterol concentrations. The main mode of action is that inhibition of hepatic cholesterol synthesis reduces hepatocyte unesterified cholesterol concentrations, and this results in up-regulation of hepatic LDL-receptor expression: hence more LDL particles are removed from the circulation.

5.3.2 The peroxisome proliferator activated receptor (PPAR) system regulates fatty acid metabolism in liver and adipose tissue

A number of apparently unrelated chemicals can induce the proliferation of the subcellular oxidative organelles called peroxisomes, especially in the livers of rodents. These agents were suggested to work through a common receptor called the peroxisome proliferator activated receptor (PPAR). It is now realized that there is a family of PPARs and that a major role is to regulate fatty acid metabolism. Like the SREBP system, the PPARs are activated by lipids – in this case derivatives of fatty acids – and act upon specific response elements, peroxisome proliferator response elements (PPRE), in the regulatory region of genes concerned with lipid metabolism (Table 5.4).

PPAR-α is expressed mainly in the liver. It is activated by long-chain fatty acids, amongst other factors. The actual ligand is thought to be a meta-

Table 5.3 Genes whose expression is increased by the sterol regulatory element binding protein (SREBP) system

Gene	Function in cell
LDL-receptor	Import of LDL particles
HMG-CoA synthase	*de novo* cholesterol synthesis
Squalene synthase	*de novo* cholesterol synthesis
Farnesyl diphosphate synthase	*de novo* cholesterol synthesis
Lanosterol synthase	*de novo* cholesterol synthesis
Acetyl-CoA carboxylase	*de novo* fatty acid synthesis
Fatty acid synthase	*de novo* fatty acid synthesis
Stearoyl-CoA desaturase	Synthesis of oleate
Glycerol 3-phosphate acyltransferase	Triacylglycerol and phospholipid synthesis
Lipoprotein lipase	Import of lipoprotein-triacylglycerol fatty acids
PPAR-γ	Induction of adipocyte differentiation
Leptin	Secretion from adipocyte to signal fat store size

Stimulation occurs in response to low cellular cholesterol content. These functions have been shown in hepatocytes and adipocytes. Based on Sul, H.S. & Wang, D. (1998) Nutritional and hormonal regulation of enzymes in fat synthesis: studies of fatty acid synthase and glycerol 3-phosphate acyltransferase gene transcription. *Annual Review of Nutrition*, **18**, 331–351; Worgall, T.S. & Deckelbaum, R.J. (1999) Fatty acids: links between genes involved in fatty acid and cholesterol metabolism. *Current Opinion in Clinical Nutrition and Metabolic Care*, **2**, 127–133.

bolic derivative of a fatty acid such as a member of the prostaglandin family. One candidate is 15-deoxy-$(\Delta)^{12,14}$-prostaglandin J_2, which has been shown to be particularly active in cellular systems. The activated PPAR-α then dimerizes with another receptor, the retinoid-X receptor, which must itself be activated by binding 9-*cis*-retinoic acid (Section 4.2.4.1). The heterodimer migrates to the nucleus where it binds to the PPRE and activates gene expression. Some genes whose regulation is altered by PPAR-α are listed in Table 5.4. In general PPAR-α activation can be seen to up-regulate the secretion of the apolipoproteins forming HDL, and to increase hepatic fatty acid oxidation.

Another isoform, PPAR-γ, is expressed mainly in adipose tissue and its major role in that tissue is the regulation of fat storage. Activation of PPAR-γ occurs in a similar way through the binding of a fatty acid or a fatty acid derivative, and after dimerization with the activated retinoid-X receptor, PPAR-γ binds to PPREs in the adipocyte nucleus to activate expression of specific genes. In this case the genes include those of adipocyte differentiation from precursor cells (immature adipocytes), so activation of PPAR-γ causes the recruitment of new adipocytes, as well as increased fatty acid deposition by up-regulation of the expression of genes regulating fat storage. These include components of the pathway of glucose uptake and synthesis, glucose transporter-4 (GLUT4) and phosphoenolpyruvate carboxykinase (an enzyme of gluconeogenesis). Presumably their function is to increase availability of glycerol 3-phosphate for triacylglycerol synthesis. Therefore, in times of excess fatty acid availability, this system ensures the co-ordinated storage of the excess fatty acids in adipose tissue. The co-ordinated working of the PPAR-α and PPAR-γ systems is illustrated in Fig. 5.15, p. 197.

There is cross-talk between the SREBP and PPAR pathways. One of the SREBP isoforms, SREBP-1c (ADD-1), is expressed in adipocytes. As described above, SREBP-1c activation in adipose tissue increases expression of fatty acid synthase, and thus potentially the generation of ligands for PPAR-γ. But there is a more direct link in that SREBP-1 activation directly increases PPAR-γ expression.

Just as the SREBP system has been manipulated pharmacologically to alter cholesterol metabolism, so the PPAR system is the target for drug inter-

Table 5.4 Peroxisome proliferator activated receptors (PPARs): tissue distribution and effects of activation

Receptor	Other names	Main tissue distribution	Genes whose expression is increased by PPAR activation	Genes whose expression is suppressed by PPAR activation
PPAR-α		Liver (main site); also kidney, heart, muscle, brown adipose tissue	Apolipoprotein AI; apolipoprotein AII; enzymes of peroxisomal fatty acid oxidation; liver FABP; CPT-1; enzymes of mitochondrial fatty acid oxidation	Apolipoprotein CIII
PPAR-δ	PPAR-β, NUC-1, FAAR (fatty acid activated receptor)	Widespread	Not known although HDL concentrations increase with activation	Not known
PPAR-γ1		Widespread at low levels		
PPAR-γ2		Adipose tissue	Factors involved in adipocyte differentiation; adipose tissue FABP (also known as aP2); lipoprotein lipase; fatty acid transport protein; acyl-CoA synthase; GLUT4; phosphoenolpyruvate carboxykinase	Leptin

Based on information in Schoonjans, K., Staels, B. & Auwerx, J. (1996) Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. *Journal of Lipid Research*, **37**, 907–925; Jump, D.B. & Clarke, S.D. (1999) Regulation of gene expression by dietary fat. *Annual Review of Nutrition*, **19**, 63–90; Auwerx, J. (1999) PPARγ, the ultimate thrifty gene. *Diabetologia*, **42**, 1033–1049; Clarke, S.D. (2000) Polyunsaturated fatty acid regulation of gene transcription: a mechanism to improve energy balance and insulin resistance. *British Journal of Nutrition*, **83** (Supplement 1), S59–S66.

vention. A group of agonists of the PPAR-α receptor, known as fibric acid derivatives or fibrates, are useful drugs in the treatment of patients with elevated plasma triacylglycerol concentrations and low HDL-cholesterol concentrations. Their effects are apparently brought about by up-regulation of lipoprotein lipase and hepatic fatty acid oxidation, thus allowing the body to dispose of excess fatty acids, and also secretion of apolipoproteins AI and AII, leading to increased HDL-cholesterol concentrations. More recently a new group of drugs, the thiazolidinediones or 'glitazones', have been developed, which appear to improve the sensitivity of the body to insulin. These are finding a place in the treatment of type 2 (maturity-onset) diabetes, which is usually associated with obesity and in which the ability of tissues to respond to insulin is reduced (Section 5.4.4). It has been discovered that the thiazolidinediones are potent agonists of PPAR-γ in adipose tissue. How activation of PPAR-γ in adipose tissue should lead to a widespread improvement in response to insulin is not clear, although it is interesting that an early effect of thiazolidinedione treatment is a reduction in the plasma concentration of non-esterified fatty acids.

5.3.3 Other nuclear receptors are activated by fatty acids and affect gene expression

It seems likely that new mechanisms for the regulation of gene expression by lipid-related

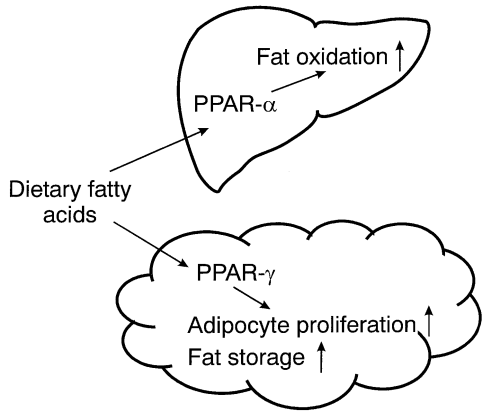


Fig. 5.15 The peroxisome proliferator activated receptor (PPAR) system and regulation of fatty acid disposition in liver and adipose tissue.

molecules will continue to be uncovered. One example is the nuclear receptor/transcription factor known as Hepatic Nuclear Factor-4α (HNF-4α). Fatty acyl-CoA esters appear to be an important natural ligand for HNF-4α, which in turn activates expression of a number of genes including apolipoprotein CIII, and pyruvate kinase. It has been suggested that the down-regulation of apoCIII expression by PPAR-α is mediated by competition with HNF-4α.

5.3.4 Adipose tissue secretes hormones and other factors that may themselves play a role in regulation of fat storage

The role of white adipose tissue as an organ of triacylglycerol storage was described in Section 3.3.1. In recent years it has become clear that adipocytes are also active in secretion of a number of peptides and other compounds that may in turn help to regulate both lipid metabolism and other functions. Some of these are listed in Table 5.5. The hormone leptin is a protein of 167 amino acids. It was discovered in 1994 by positional cloning of the *ob* gene responsible for gross, spontaneous obesity in a mutant strain of laboratory mice. Only mice homozygous for the mutation (*ob*/*ob* mice) show the obese phenotype. Painstaking work from the laboratory of Jeffrey Friedman at Rockefeller University in New York led to the identification of the gene and the realization that it coded for a novel hormone, expressed almost exclusively in white adipose tissue. The hormone was called leptin (Greek leptos, thin). When purified recombinant leptin was injected into *ob*/*ob* mice, they lost weight and became normal. The weight loss was the result of both decreased food intake, and increased energy expenditure. In normal animals, and humans, leptin secretion increases with increasing fat stores. Leptin receptors are widespread, and there are several isoforms produced by alternative splicing of transcripts from a single gene. A short, membrane-spanning form, believed to be responsible for leptin transport, is found in the choroid plexus, the brain region that governs transport of peptides between the bloodstream and the hypothalamus. In the hypothalamus itself, a brain region that controls many bodily functions including appetite and the activity of the autonomic nervous system, the long-form, signalling isoform of the leptin receptor is expressed. Leptin carries a signal from adipose tissue to the brain that the fat stores are growing and that it is appropriate to reduce food intake and increase energy expenditure. It also signals to other organs and tissues, and the widespread role of leptin in regulation of energy metabolism is now being discovered. One important role is that of a signal to the reproductive system that the fat stores are sufficient to support pregnancy and birth. Female and male *ob*/*ob* mice are sterile, and their fertility returns on treatment with recombinant leptin. This is probably the explanation also for the common observation that extreme thinness (arising, for instance, either from eating disorders or from regular physical training) is associated with amenorrhoea (absence of menstruation).

Although the main factor regulating leptin secretion from the adipocyte is cell size (i.e. triacylglycerol content), there is additional modulation by the short-term feeding state. This appears to be mediated largely by insulin, which acts through the adipocyte determination and differentiation factor-1, ADD-1 (or SREBP-1c), to increase leptin expression. Thus, leptin secretion increases on feeding (acting to reduce further food intake) and is

Table 5.5 Proteins and other factors secreted by adipocytes

Secreted product	Potential role
Proteins	
Lipoprotein lipase	Transport to endothelium; uptake of circulating triacylglycerol fatty acids.
Complement components D (adipsin), B, C3	Formation of C3a-desarg or acylation stimulating protein; stimulation of fatty acid storage.
Cholesteryl ester transfer protein	Systemic participation in reverse cholesterol transport; adverse role in generation of atherogenic lipoprotein phenotype in obesity/ insulin resistance.
Apolipoprotein-E	Lipoprotein metabolism.
Angiotensinogen	Regulation of blood pressure (after conversion to angiotensin-II).
Leptin	Hormone signalling size of fat stores, playing widespread role in metabolic regulation.
Plasminogen activator inhibitor-1 (PAI-1)	Increases coagulating ability of blood.
Tissue factor	Coagulation pathway.
Tumour necrosis factor-α	Cytokine, perhaps reducing fat storage; systemically, may reduce sensitivity to insulin (although there is some doubt about adipose tissue's contribution to systemic concentration).
Interleukin-6	Cytokine with role in inflammatory processes.
Other factors	
Prostaglandins PGE_2, PGI_2 (prostacyclin)	Probably local role in regulation of blood flow and lipolysis.
Non-esterified fatty acids, glycerol	Products of lipolysis; distribution to other tissues.

This tabulation is not exhaustive and is expanding all the time.

suppressed during fasting (so acting to increase food intake).

The leptin system also operates in humans, although it became clear soon after the discovery of leptin that the vast majority of obese humans have high, not low leptin concentrations in their circulation, just as appropriate for their enlarged fat stores. The message is that the majority of human obesity does not result from a failure of adipose tissue to secrete leptin, but from a failure of the brain to respond to it. The first clinical trials of recombinant leptin as a treatment for human obesity have recently been reported. Obese patients treated with high doses of leptin, by subcutaneous injection once a day, showed some weight loss, although it was not marked – perhaps not surprisingly in view of their 'leptin resistance'. However, we can be certain that the leptin system plays an important role in humans. A small number of families has been identified in which there are one or two individuals who have shown remarkable obesity from a very early age. Sequencing of the leptin gene has shown that these families carry a mutation rendering it ineffective, and that some of the rare cases of extreme precocious obesity are homozygous for this mutation or for a mutation in the leptin receptor. One pair of grossly obese cousins studied by Stephen O'Rahilly and his group at the Department of Medicine in Cambridge, UK, has displayed an almost incredible craving for food and failed ever to lose weight despite periods in hospital to try to restrain their eating. They are both homozygous for a particular frameshift mutation in the leptin gene. They have now been treated with

recombinant human leptin, which has normalized their eating behaviour and led for the first time to weight loss. There appears to be little effect on energy expenditure, so in humans the leptin system probably regulates food intake more than energy expenditure. These may be rare accidents of nature, but they show that the leptin system is crucial for normal regulation of feeding in humans.

Other proteins secreted from adipocytes that have a role in lipid metabolism include lipoprotein lipase (secreted for transport to the capillary endothelium), apolipoprotein E (see Table 5.5) and cholesteryl ester transfer protein. Adipocytes also secrete a number of peptide components of the complement system, which is important in host defence against infection. It has recently been realized that these include the factors known as D, B and C3, all components of the alternative (as opposed to the classical) complement pathway. Factors D (also known as adipsin, to reflect its origin), B and C3 interact to produce a peptide of 76 amino acids that is known to immunologists as C3a-desarg (formed by removal of a C-terminal arginine from the fragment C3a). C3a turns out to be identical to a protein that had been identified by Allan Sniderman and Katherine Cianflone, of McGill University in Montreal, as a potent stimulator of fatty acid esterification in adipocytes and termed by them acylation stimulating protein (ASP, Section 3.6.2). Production of ASP by adipocytes is stimulated by the presence of chylomicrons. This suggests a co-ordinated system of regulation, whereby the arrival in adipose tissue capillaries of dietary fat in the form of chylomicron-triacylglycerol triggers ASP secretion, which in turn stimulates the storage of dietary fatty acids as adipocyte-triacylglycerol after their release from chylomicrons by lipoprotein lipase (Fig. 5.16).

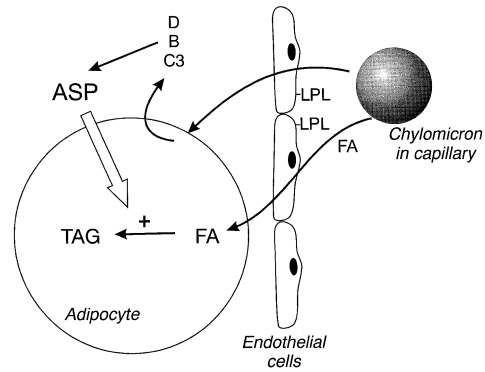


Fig. 5.16 The acylation stimulating protein (ASP) system in adipose tissue. Arrival of a chylomicron particle in the capillary sends a signal to the adipocyte (probably by transfer of a protein carried by the chylomicron to a specific receptor on the adipocyte surface), which increases secretion of the three proteins D, B and C3, components of the alternative complement pathway. These interact to form the protein C3a-desarg or ASP. ASP acts via a receptor (not yet characterized) to stimulate triacylglycerol (TAG) synthesis from fatty acids (FA) released by the action of lipoprotein lipase attached to the capillary endothelial cells on the chylomicron-TAG.

5.4 DISEASES INVOLVING CHANGES OR DEFECTS IN LIPID METABOLISM

Defects in a variety of lipid metabolic pathways can give rise to inappropriately high (or sometimes low) concentrations of lipoproteins in the blood. This in turn may be associated with a range of chronic conditions including atherosclerosis, obesity and diabetes.

In Chapter 4 we described deficiency diseases that arise because the diet contains insufficient of several types of lipids that the body is unable to synthesize for itself. Here, the discussion is devoted to diseases that involve a defect, or defects, in a metabolic pathway, usually because an enzyme or some other protein is deficient or not functioning correctly. This may be because of a mutation in the protein, or a mutation in another protein that in some way regulates the protein we are interested in, or (more commonly) a collection of mutations (or polymorphisms), each of which may be insignificant on its own, but which act together with lifestyle factors (such as excessive energy or fat intake, or lack of exercise) to produce the observed phenotype.

The common outcome of many of these conditions is an increased risk of atherosclerosis, a process that involves accumulation of fatty deposits in arteries. This condition will therefore be described first, followed by a consideration of the disorders of

lipid metabolism and how they lead to generation of atherosclerosis. Finally, we will consider briefly some diseases of lipid storage that may have different clinical outcomes.

5.4.1 Atherosclerosis

Atherosclerosis involves the build-up of deposits in arterial walls, characterized by high concentrations of lipids that derive from plasma lipoproteins; lipids are also involved in the formation of thrombi that may lead to the blockage of blood vessels narrowed by atherosclerosis.

Cardiovascular disease is a broad term for a number of conditions involving pathological changes in blood vessels associated mainly with the heart and brain. The major causes of death are ischaemic heart disease (IHD), often called coronary heart disease (CHD), and ischaemic stroke (failure of blood flow to a region of the brain). The common, chronic condition underlying these diseases is atherosclerosis. In IHD, the coronary arteries that supply oxygen and nutrients to the heart muscle (myocardium) become narrowed by deposits in the arterial wall to such a degree as to prevent the coronary circulation meeting the metabolic demands of the heart. This may result in chest pain, especially on exertion, because the myocardium is deficient in oxygen and lactic acid accumulates locally. This is the condition known as angina. Local and systemic factors may also increase the likelihood of platelet aggregation and blood clot (thrombus) formation at the site of narrowing. This can result in complete blockage of an artery, a heart attack (myocardial infarction) or stroke if it is in the brain. The immediate effect is failure to supply a region of the myocardium, or brain, with oxygen. If the blockage is of a major vessel, this may be fatal. Peripheral vascular disease involves blood vessels in, for example, the limbs and although it is not a major cause of mortality, it is associated with considerable morbidity and distress for the patient.

Atherosclerosis is an irregular thickening of the inner wall of the artery that reduces the size of the arterial lumen (Fig. 5.17), particularly near junctions in the arterial tree. The thickening is caused by the accumulation of atherosclerotic plaques, consisting of a proliferation of smooth muscle cells, connective tissue, mucopolysaccharides, fat-filled foam cells, in which the predominant lipid is cholesteryl ester, and deposits of calcium. The artery wall is locally thickened and loses elasticity. The term atherosclerosis was coined in 1904, from the Greek athere, meaning porridge or gruel and referring to the soft consistency of the core of the plaque, and sclerosis, hardening.

The first stage in atherosclerosis is the fatty streak, a yellowish, minimally raised spot (the spots later merging into streaks) in the arterial wall. Forty-five per cent of infants coming to post mortem examination during the first year of life have fatty streaks in their aorta, so this is an early development in life, and the development of the mature atheromatous plaque must be a long-term process. The origin of the fatty streak has mostly been studied in animal models of hyperlipidaemia, usually rabbits with a genetic predisposition to high blood cholesterol levels, fed on a high-fat diet containing predominantly saturated fatty acids and cholesterol. It is preceded by the adhesion of monocytes and T-lymphocytes to an area of endothelium. The monocytes migrate into the subendothelial space where they differentiate into macrophages, which then begin to engulf large amounts of lipid via the scavenger receptor pathway. Current understanding is that the main source of this lipid is LDL particles that have become chemically modified, probably through the process of lipid peroxidation, which leads in turn to peroxidation of the apoB100 (see Box 4.3). This reduces the affinity of the LDL particles for the LDL-receptor, and instead makes them ligands for the family of scavenger receptors. As we saw in Section 5.2.4.4, lipid uptake by the scavenger receptor pathway is not subject to feedback regulation by cellular cholesterol content; therefore, the macrophages may engulf large amounts of lipid, giving them a foamy appearance under the microscope. These are so-called foam cells, and are characteristic of the atherosclerotic plaque.

Further development of the atherosclerotic plaque involves proliferation of smooth muscle cells of the arterial wall and the elaboration of a connective

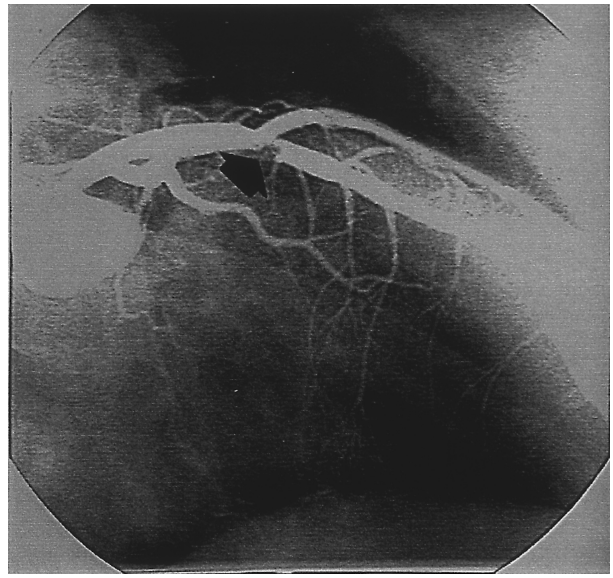


Fig. 5.17 An angiogram (an X-ray picture obtained after injection of dye into the coronary arteries) showing a constriction (arrow) of the left anterior descending coronary artery. The lesion has all the hallmarks of a complex atherosclerotic plaque. The patient had experienced one myocardial infarction and was experiencing recurrent chest pain. Reproduced from Ambrose, J.A. (1996) Coronary pathophysiology and angiographic correlations in acute myocardial infarction. In: *Atherosclerosis and Coronary Artery Disease* (eds. V. Fuster, R. Ross & E.J. Topol), pp. 797–805. Lipincott-Raven, Philadelphia.

tissue matrix, forming a fibromuscular cap to the lesion. Within the lesion there may be breakdown of dead macrophages and release of their contents, with formation of a semi-liquid pool of extracellular lipid. The lid of the lesion may remain firm, in which case the lesion may protrude into the arterial lumen, obstructing flow, but not causing acute damage. However, for reasons that are not understood, some plaque caps become unstable and are damaged, and the contents of the plaque are exposed, resulting in the normal response to damage to a vessel wall – thrombus formation. The processes involved in the development of ischaemic heart disease and myocardial infarction, and the possible involvement of lipids, are illustrated in Fig. 5.18.

Examination of the lipid contents of atherosclerotic plaques shows cholesterol and fatty acids that appear to have been derived from the circulation. This has been shown in animal experiments using radioactive labelling, and more recently in humans by analysis of the fatty acid content of atherosclerotic plaques and showing a relationship to dietary fatty acids. In addition, plaques can be shown to contain apoB100. This all points to circulating lipoprotein particles as the major source of plaque lipid.

Several aspects of lipid metabolism relate to the risk of atherosclerosis and CHD. The first is the LDL-cholesterol concentration. Over many years, epidemiological observations of the differences in CHD rates between different countries have shown a strong relationship with average (across the population) serum LDL-cholesterol levels. In addition, people with a defect in LDL removal, usually through a mutation in the LDL-receptor (the condition of familial hypercholesterolaemia; Sections 5.2.4.2 and 5.4.2) have a very high risk of developing CHD at an early age. Only 25 years ago the 'lipid hypothesis' of CHD – that is, the hypothesis that elevated serum LDL-cholesterol concentrations play a direct role in CHD – was the

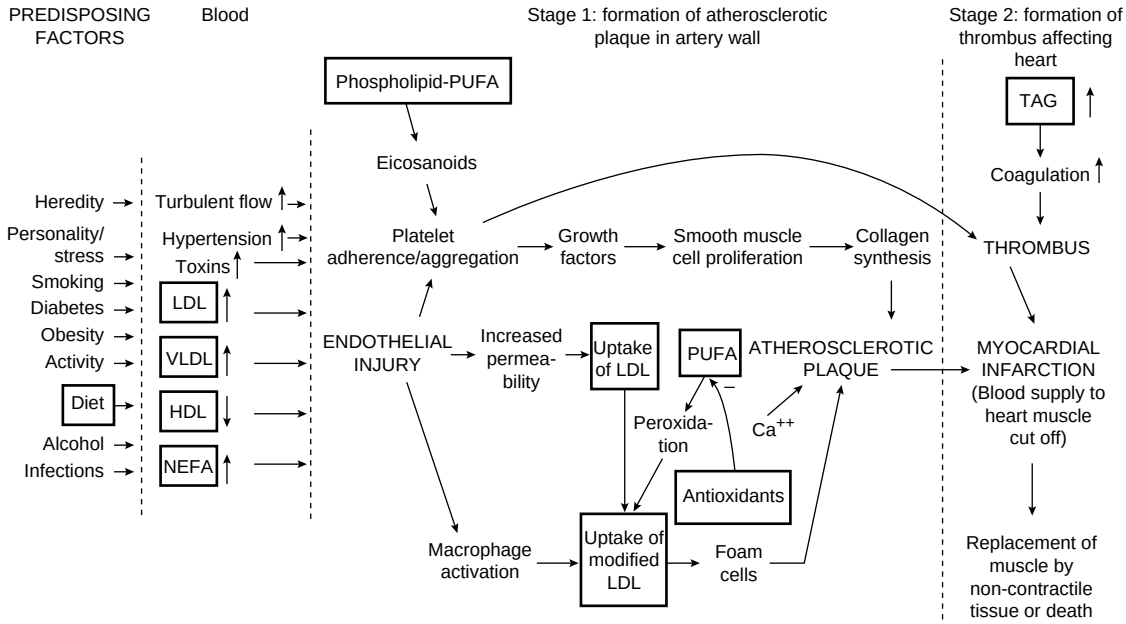


Fig. 5.18 Possible processes involved in the development of ischaemic heart disease and myocardial infarction. Boxes indicate points at which lipids may be involved.

subject of considerable controversy. Now it is more widely accepted. A major reason for this acceptance has been that several major prospective clinical trials have shown that lowering LDL-cholesterol concentrations with the 'statin' drugs, inhibitors of HMG-CoA reductase (Section 5.3.1), leads to marked reductions in mortality from CHD, in proportion to the reduction in LDL-cholesterol achieved.

Despite widespread acceptance of the role of LDL-cholesterol, it remains true that many patients suffering an early myocardial infarction have relatively normal levels of LDL-cholesterol (Fig. 5.19). Increasingly, other abnormalities of lipid metabolism are being seen as associated with development of atherosclerosis. The clearest relationship is with the serum HDL-cholesterol concentration, which, in epidemiological studies, is strongly negatively related to CHD risk. This is understandable if we consider HDL-cholesterol concentrations to reflect activity of the reverse cholesterol transport pathway, removing excess cholesterol from peripheral tissues, such as macrophages, to the liver for ultimate disposal. Again, epidemiological studies have now been reinforced by clinical trials. Fibrate drugs that raise HDL-cholesterol concentrations by acting as PPAR-α agonists (Section 5.3.2) reduce mortality from CHD.

Beyond the positive relationship of CHD with elevated LDL-cholesterol, and its negative relationship with HDL-cholesterol, there are other more subtle effects. Increasingly there is recognition of a phenotype that predisposes to CHD, known by a variety of names, including the atherogenic lipoprotein phenotype, the metabolic syndrome, syndrome X, or hyperapobetalipoproteinaemia (a high concentration of apoB in the circulation). The characteristics usually associated with the atherogenic lipoprotein phenotype are listed in Table 5.6. A brief description of its origin in terms of lipid metabolism may be given as follows. It appears to stem from a disturbance of the metabolism of the triacylglycerol-rich lipoproteins, with over-production of VLDL from the liver. That, in turn, may result from increased delivery of non-esterified fatty acids to the liver. An increased circulating con-

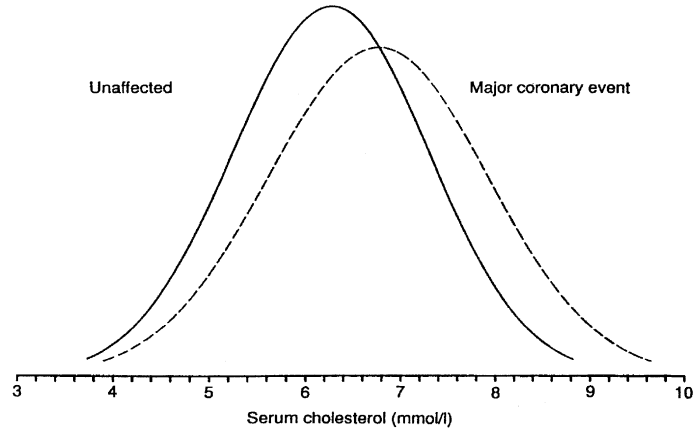


Fig. 5.19 Distribution of serum cholesterol concentrations in 438 men who had a major coronary event and 7252 unaffected men. Note that there is considerable overlap between the distributions. An elevated serum cholesterol concentration is certainly a major risk factor for development of coronary heart disease, but nevertheless many people experiencing the disease have relatively normal cholesterol concentrations. Data from the British Regional Heart Study: redrawn by Wald, N.J. (1992) in *Coronary Heart Disease Epidemiology* (eds. M. Marmot & P. Elliott). Oxford University Press, Oxford. Reproduced with permission.

centration of VLDL particles will compete for clearance by lipoprotein lipase when chylomicrons become enriched with triacylglycerol in the postprandial period. This will lead to a prolonged residence of partially lipolysed chylomicron and VLDL remnants in the circulation, something that has often been noted to be associated with increased CHD risk. If the circulating pool of triacylglycerol-rich lipoproteins is increased, there will be increased opportunity for the action of the cholesteryl ester transfer protein, a normal part of the reverse cholesterol transport pathway (Fig. 5.11), to exchange triacylglycerol from VLDL and chylomicron remnants for cholesteryl esters from HDL and LDL particles. VLDL and chylomicron remnant particles therefore become enriched in cholesteryl esters. Some people believe that these become atherogenic particles – that is, they may themselves penetrate the arterial wall and become engulfed by macrophages. (There is some evidence for the presence of apoB48 in arterial lesions, although this is difficult to demonstrate as apoB48 is enormously outnumbered in the circulation by apoB100.) But in addition, LDL and HDL particles thereby lose cholesteryl esters.

Table 5.6 Features of the atherogenic lipoprotein phenotype

Feature	Comment
Hypertriglyceridaemia	May be relatively mild, but usually is more marked after meals.
Low HDL-cholesterol concentration	Reflects impairment of triacylglycerol-rich lipoprotein metabolism.
Small, dense LDL particles	Total LDL-cholesterol concentration may be normal, hence number of particles must be increased.
Hyperapobetalipoproteinaemia (high concentration of apoB100 in plasma)	Reflects increased number of LDL particles, as above.
Insulin resistance	These features are often associated with impaired action of insulin on metabolic processes.

The triacylglycerol that they have gained in exchange can be hydrolysed by hepatic lipase, which acts preferentially on these smaller particles. Therefore the particles have lost both triacylglycerol and cholesterol. The result is a low HDL-cholesterol concentration (associated with increased risk of CHD as discussed above) and a predominance of small, relatively lipid-poor LDL particles. In the past few years these small, dense LDL particles have attracted much attention as the probable atherogenic particles of the atherogenic lipoprotein phenotype. There is always a population of LDL particles of different sizes in the circulation, but some people tend to have a predominance of large, buoyant cholesterol-rich LDL particles (known as pattern A) and others a predominance of smaller, denser relatively lipid-poor LDL particles (pattern B). There is some evidence for a genetic component to this LDL phenotype, but it is clearly modifiable by diet and by other influences on the lipoprotein phenotype. Pattern B is associated with much higher risk of CHD than is pattern A, even though the total LDL-cholesterol concentration may be the same. The increased number of LDL particles that must be present in pattern B (to give the same total LDL-cholesterol concentration) accounts for the term hyperapobetalipoproteinaemia, which covers the same, or a closely related, phenotype. It is postulated that smaller LDL particles may more readily cross the endothelial wall and hence become substrates for macrophage uptake. In addition, there is considerable experimental evidence that small, dense LDL particles are more susceptible to peroxidation than are larger, more buoyant particles, and hence again more likely to be substrates for macrophage uptake.

Once the atherosclerotic plaques have developed, further events must follow to lead to myocardial infarction or ischaemic stroke. These are aggregation of platelets, probably at the site of rupture of the atherosclerotic lesion, and then the formation of a thrombus. These processes may be related to aspects of lipid metabolism. Platelet aggregation, an early step, is propagated by the formation of the eicosanoid, thromboxane A_2, from arachidonic acid in platelets. Release of thromboxane A_2 by activated platelets leads to a cascade effect on aggregation. Formation of thromboxane A_2 is inhibited by aspirin (an inhibitor of the cyclo-oxygenase pathway), and this is the reason that low doses of aspirin can markedly reduce the risk of re-infarction in people who have already suffered one myocardial infarction. Platelet aggregation is also inhibited by *n*-3 polyunsaturated fatty acids. This is probably mediated via the generation of eicosanoids of the 3-series rather than the 2-series. The blood coagulation pathways are also related to lipid metabolism. High circulating concentrations of the activated form of coagulation factor VII, often known as VIIa (or, if measured by assay of its coagulant activity, as VIIc), are associated with increased risk of CHD. Generation of factor VIIa is now known to relate to triacylglycerol metabolism. There is a relationship between circulating concentrations of VIIa and of triacylglycerol, and the elevation of plasma triacylglycerol concentrations that occurs after a meal is associated with activation of factor VII.

Myocardial infarction may lead to death. If it does, this is not usually because of complete obstruction of blood flow to the myocardium, but because the electrical rhythm of the heart becomes grossly disturbed, leading to the critical conditions of ventricular fibrillation or cardiac arrest. Then blood supply to the rest of the body, including the brain, ceases. The stress reaction that sets in at the onset of myocardial infarction can lead to very high plasma concentrations of non-esterified fatty acids, and there is considerable evidence that these may themselves be a potent cause of ventricular dysrhythmias. It has been postulated that the stresses associated with modern living lead to consistent elevations of the plasma non-esterified fatty acid concentration that are now inappropriate, and may lead to – or potentiate – ventricular dysrhythmias. The UK biochemist Eric Newsholme has put forward this view, and suggests that in earlier times we would have oxidized the excess fatty acids in muscular activity; hence the hazards of stressful, sedentary living. There is an interesting recent development in this story. The American nutritionist Alexander Leaf has shown that, amongst fatty acids, the *n*-3 polyunsaturated fatty acids appear to have a unique ability to stabilize the heart

rhythm. In several studies in experimental animals he has shown that acute administration of *n*-3 polyunsaturated fatty acids can prevent death from coronary artery obstruction. There is convincing support now from human dietary trials. In the Diet and Reinfarction (DART) study, conducted in the UK, survivors of myocardial infarction were randomized into two groups, one of which was given advice to eat oily fish (and if this advice was not followed, they received supplementation with fish-oil capsules). Over the next two years, the fish-eating group suffered the same rate of new myocardial infarctions as did the control group; but their death rate was significantly reduced, by 29%. It has been proposed that this reflects protection against the fatal dysrhythmias that often accompany myocardial infarction. More recently, the Lyon Diet-Heart Study also investigated the effects of dietary modification in people who had suffered from a myocardial infarction. In this case the 'test' group was asked to follow a Mediterranean-style diet, which was particularly rich in α-linolenic acid (18:3*n*-3). The control group was given conventional dietary advice. The trial was stopped prematurely, after an average of 2.5 years on the diet, because the results were so clearly significant, with lower death rates in the Mediterranean-diet group. A four-year follow-up of the participants showed that even after the formal trial had finished, the subjects had largely kept to their diets, and the difference in all-cause mortality between the groups was marked (about half in the test group compared with the control group). For 'cardiac deaths' the ratio was 3:1.

5.4.2 Risk factors for CHD and the effects of diet

From the preceding description, it will be clear that the major lipid risk factors for CHD are: an elevated circulating LDL-cholesterol concentration; a low HDL-cholesterol concentration; a predominance of small, dense LDL particles; and elevated circulating triacylglycerol concentrations (particularly in the postprandial period). The total cholesterol concentration in serum is often listed as a risk factor, but this reflects the fact that LDL-cholesterol is the major component of total serum cholesterol. These risk factors are modified to a great extent by genetic and environmental factors, as described in more detail in Section 5.4.3, but they are also modulated to a considerable extent by dietary factors, which will be reviewed briefly here.

The realization of a connection between serum cholesterol concentrations and CHD risk began in the 1950s with the work of the celebrated American nutritionist, Ancel Keys. Keys had been travelling in the Mediterranean countries, investigating the apparently very low incidence of chronic diseases including CHD. He questioned whether this might be related to aspects of the diet in those areas. This led to the foundation of the Seven Countries Study, an international study of CHD and associated factors in a number of countries (it soon expanded beyond seven) with widely differing incidences of CHD. There were two major, early findings. Comparing average values in one country with another, there was a strong positive relationship between serum cholesterol concentration and incidence of CHD, and a strong positive relationship between consumption of saturated fat and serum cholesterol. This led to the recognition that the nature of the fatty acids in the diet is an important influence on the serum cholesterol concentration: saturated fatty acids tend to raise it, polyunsaturated fatty acids to lower it. For some years it was felt that monounsaturated fatty acids were neutral in this respect. However, the answer to any experimental trial is dependent upon how the experiment is conducted, and it is now realized that if monounsaturated fatty acids replace saturated fatty acids in the diet, they reduce serum cholesterol concentration. Amongst these fatty acid classes, not all fatty acids have the same effect. Of the saturated fatty acids, for instance, those with chain length below 12C seem to have no effect; 12C, 14C and 16C lengths raise cholesterol, whereas stearic acid, 18:0, may slightly reduce cholesterol (perhaps because of rapid desaturation to oleic acid). In contrast to the effects of dietary fatty acids, the effect of dietary cholesterol on serum cholesterol concentration is relatively weak over any reasonable range of intake. This can be understood if we remember the powerful systems that regulate cholesterol accumula-

tion in cells. The relative effects of fatty acids and cholesterol have been combined by many investigators to produce equations that predict the change in serum cholesterol in response to any dietary manipulation (Box 5.2). The mechanism for the effects of different dietary fatty acids on serum cholesterol concentration is not clearly understood. It appears to reflect a shift in the hepatic cholesterol pool between esterified and unesterified forms, with up-regulation of hepatic LDL-receptors (and a consequent reduction in serum LDL-cholesterol concentration) when polyunsaturated fatty acids predominate.

The *n*-3 polyunsaturated fatty acids have a special role. They are relatively neutral in terms of serum cholesterol, but they have a potent effect in reducing serum triacylglycerol concentrations. They also reduce platelet aggregation, and may stabilize the myocardial rhythm, as described above.

The double bonds in dietary unsaturated fatty acids are mostly of the *cis* geometrical configuration. However, some foods contain significant quantities of isomeric fatty acids in which the double bonds are in the *trans* configuration (Figs 1.1 and 4.1; Table 4.2 and Section 4.1.3.1). These are often produced as a result of hydrogenation of unsaturated fatty acids. This occurs in the reducing environment of the bovine rumen, so that dairy products and beef fat contain small proportions of the *trans* fatty acids. It also occurs during the hydrogenation of vegetable oils to produce 'harder' fats, e.g. in the production of margarine. A number of epidemiological studies have shown a relationship between *trans* fatty acid intake and cardiovascular disease, and controlled feeding studies suggest that dietary *trans* fatty acids raise serum cholesterol concentrations to a very similar extent to saturated fatty acids. Given the similarity of their molecular configurations, this is perhaps not

Box 5.2 Effects of dietary fatty acids on serum cholesterol concentrations

In many experiments in healthy subjects, the fatty acid composition of the diet has been manipulated to assess the effect on the serum cholesterol concentration. These studies have been summarized by a number of investigators to produce 'predictive equations'. Two examples are given here.

Hegsted *et al*. (1965) produced the equation

$$\Delta\text{serum cholesterol} = 0.026\times (2.16\Delta\text{SFA} - 1.65\Delta\text{PUFA} + 6.66\Delta\text{Chol} - 0.53)$$

where Δserum cholesterol represents the change in serum cholesterol concentration (mmol l^{-1}), ΔSFA and ΔPUFA are changes in the percentage of dietary energy derived from saturated and polyunsaturated fatty acids, respectively, and ΔChol is the change in dietary cholesterol in 100 mg day^{-1}. Note that the term for ΔSFA is positive (an increase in saturated fat intake raises serum cholesterol) whereas that for ΔPUFA is negative (an increase in polyunsaturated fat intake reduces serum cholesterol).

Yu *et al*. (1995) collated data from 18 studies in the literature that gave information on individual fatty acids in the diet. Their predictive equation was:

$$\Delta\text{serum cholesterol} = 0.0522\Delta(12{:}0 \text{ to } 16{:}0) - 0.0008\Delta 18{:}0 - 0.0124\Delta\text{MUFA} - 0.0248\Delta\text{PUFA}$$

where ΔMUFA is the change in the percentage of dietary energy derived from monounsaturated fatty acids (other terminology as above). Note that most saturated fatty acids are shown as raising serum cholesterol, but stearic acid (18:0) as slightly lowering it.

References

Hegsted, D.M., McGandy R.B., Myers M.L. & Stare F.J. (1965) Quantitative effects of dietary fat on serum cholesterol in man. *American Journal of Clinical Nutrition*, **17**, 281–295.

Yu, S., Derr, J., Etherton, T.D., Kris-Etherton, P.M. (1995) Plasma cholesterol-predictive equations demonstrate that stearic acid is neutral and monounsaturated fatty acids are hypocholesterolemic. *American Journal of Clinical Nutrition*, **61**, 1129–1139.

surprising. The content of *trans* fatty acids is being reduced by many food manufacturers as a result of this finding.

In most naturally occurring *cis*-polyunsaturated fatty acids, the double bonds are separated by a methylene bridge; e.g. the most common form of linoleic acid in nature is (*cis*-9,*cis*-12) 18:2. However, a large number of isomers of linoleic acid is found. Some of these have the double bonds between consecutive pairs of carbon atoms [the most common is (*cis*-9,*trans*-11) 18:2, also known as rumenic acid]. This arrangement of double bonds is similar to that seen in benzene, and is known as conjugated. The electrons become delocalized over conjugated double bonds, and this can confer unusual chemical properties. The group of isomers of linoleic acid with this configuration has become known (collectively) as conjugated linoleic acid (CLA). CLA is formed in the rumen of ruminant animals, and is found in milk fat, cheese and beef (Section 4.1.3). It has come to prominence because of claims from animal studies that CLA can protect against some forms of cancer. Dietary CLA has also been shown to alter body composition in mice, with a loss of body fat, and in cultured adipocytes to reduce the activity of lipoprotein lipase. More recently, it has been claimed that dietary CLA may protect against atherosclerosis. However, the evidence in this respect is not clear-cut: there have also been demonstrations that high levels of CLA fed to rodents can predispose to the formation of fatty streaks in the aorta (presumed to be the precursors of atherosclerotic lesions). Various mechanisms for the potential beneficial effects have been proposed, and may differ for the different isomers. For instance, some isomers are potent agonists of PPAR-α (Section 5.3.2).

The total quantity of dietary fat has an important influence on serum lipoprotein concentrations, beyond the nature of the fatty acids it contains. It is a common observation that a change to a diet containing a low proportion of energy from fat, and a correspondingly higher proportion from carbohydrate, is associated with a reduction in serum HDL-cholesterol concentration and an elevation of serum triacylglycerol concentration. The reduction in serum HDL-cholesterol concentration may reflect reduced flux of fat through the exogenous lipoprotein pathway, which involves the transfer of surface components from the chylomicrons, as they are lipolysed by lipoprotein lipase, to HDL particles. The elevation of serum triacylglycerol concentration is not clearly understood. It is thought to represent increased secretion of VLDL-triacylglycerol from the liver, perhaps because of a change in the metabolic partitioning of fatty acids in the liver between oxidation (favoured when fat levels in the diet are high) and esterification (favoured when carbohydrate is plentiful, and insulin levels are high). A recent study using isotopic methods to assess hepatic fatty acid metabolism has suggested, however, that the elevation of triacylglycerol concentration on a high-carbohydrate diet may reflect impaired removal of triacylglycerol from the circulation in peripheral tissues. These observations have led to questions about the safety of low-fat diets, since both these changes (depression of HDL-cholesterol, elevation of triacylglycerol concentrations) appear to be deleterious in terms of CHD risk. Such questioning is important, but many nutritionists feel that a shift towards lower fat diets may play an important role in reducing the incidence of obesity. The answers are not yet clear. We need to know: (1) Are the changes in lipoprotein particles that lead to elevation of triacylglycerol concentrations the same as those seen in subjects at increased risk of CHD? (2) Whether the changes in lipid concentrations are maintained long term (there is some evidence that they are not, but there are few long-term studies) (3) Whether the changes in lipid concentrations depend upon the nature of the dietary carbohydrate: in many short-term experimental studies, much of the dietary carbohydrate is in the form of simple sugars, which may have a particularly marked triacylglycerol-raising effect.

5.4.3 Hyperlipoproteinaemias (elevated circulating lipoprotein concentrations) are often associated with increased incidence of cardiovascular disease

Diseases that involve elevation of circulating lipid concentrations (hyperlipoproteinaemias or hyperlipidaemias) are often associated with increased incidence of atherosclerosis and myocardial infarction. They are often sub-divided into primary and secondary hyperlipoproteinaemias. Primary hyperlipoproteinaemias are considered to arise directly from a genetic cause, while the secondary condition arises from some other medical or environmental cause such as diabetes or obesity. If the diabetes or obesity is controlled or cured, the hyperlipoproteinaemia will disappear or lessen. However, the simplicity of this classification is now under question as we recognize increasingly that most diseases reflect interactions between genome and environment. For instance, even in familial hypercholesterolaemia, caused by a defect in the LDL-receptor, the degree of elevation of plasma cholesterol concentration is dependent upon other lifestyle and medical factors such as diet and thyroid hormone status.

A different classification is based upon the observed lipid phenotype. The classification normally used is that proposed in 1967 by the American clinician and biochemist, Donald Fredrickson (Table 5.7). Fredrickson believed this was a classification of familial (i.e. primary) hyperlipidaemias, but it is now recognized to include many conditions that we would regard as secondary.

There are a few well-described single-gene mutations that lead to 'classic' primary hyperlipoproteinaemias, but for many conditions the background is polygenic, and the phenotype may be correspondingly more variable according to environmental factors. A given phenotype may result from different causes. For instance, amongst single-gene mutations, those that abolish activity of the LDL-receptor lead to familial hypercholesterolaemia, but an identical phenotype (Fredrickson Type IIa) is produced by rarer mutations in apoB100 that affect its ability to bind to the LDL-receptor (the condition is known as familial defective apolipoprotein B100 or FDB, but in most clinical situations would not be distinguished from familial hypercholesterolaemia). Complete lack of activity of lipoprotein lipase leads to familial chylomicronaemia syndrome or Fredrickson Type I hyperlipoproteinaemia, but again an identical phenotype is caused by mutations in apoCII that abolish its ability to activate lipoprotein lipase. For clinical purposes it may not be important to distinguish these causes, although genetic analysis is increasingly being applied to these conditions.

Secondary hyperlipoproteinaemias may be associated with many conditions, but amongst the most common are diabetes and obesity (see the following section), thyroid disease (low thyroid hormone concentrations lead to hypercholesterolaemia and hypertriglyceridaemia), over-consumption of alcohol (hypertriglyceridaemia), and liver and kidney diseases. Some drug treatments for other medical conditions may also lead to hyperlipoproteinaemias. A prominent example that is causing concern at present is a marked hypertriglyceridaemia that is commonly observed in patients with human immunodeficiency virus (HIV) infection treated with viral protease inhibitors.

5.4.4 Obesity and diabetes are associated with increased risk of cardiovascular diseases

Mortality from cardiovascular disease (mainly coronary heart disease and ischaemic stroke) is considerably increased in obese compared to lean individuals. Almost all this increase can be accounted for by alterations in plasma lipid and lipoprotein concentrations. Statistically, it is sometimes claimed that obesity *per se* is not a significant risk factor for cardiovascular disease, but this merely implies that it has no residual effect beyond what would be expected from the alterations in lipid concentrations. The typical alterations in lipid concentrations in obesity are those described earlier as the atherogenic lipoprotein phenotype (Table 5.6). Because concentrations of some lipoprotein

Table 5.7 The classification of hyperlipidaemias according to phenotype

Type	Plasma cholesterol	Plasma triacylglycerol	Particles accumulating	Usual underlying defect	Treatment
I	+	+++	Chylomicrons	Lipoprotein lipase deficiency; apolipoprotein CII deficiency.	Low-fat diet; medium-chain triacylglycerols to replace long-chain fatty acids.
IIa	++	N	LDL	LDL-receptor defect or LDL overproduction.	Reduce dietary saturated fat and cholesterol; resins; 'statin' drugs (HMGCoA reductase inhibitors).
IIb	++	++	VLDL, LDL	VLDL or LDL overproduction or impaired clearance.	Reduce dietary saturated fat and cholesterol; reduce weight; fibrate drugs (PPAR-α activators); statins.
III	+	++	Chylomicron remnants and VLDL remnants	Impaired remnant removal; may be due to particular isoform of apolipoprotein E, or apo-E deficiency.	Reduce dietary saturated fat and cholesterol; fibrates; statins.
IV	N or +	++	VLDL	VLDL overproduction or clearance defect.	Reduce weight if obese, reduce alcohol intake; *n*-3 PUFA supplementation; fibrates.
V	+	+++	Chylomicrons, VLDL and remnants	Lipoprotein lipase defect (not complete absence) or apolipoprotein CII deficiency.	Reduce weight if obese, reduce alcohol intake; *n*-3 PUFA supplementation; fibrates.

This is known as the Fredrickson classification. N: normal; +: mildly raised; ++: moderately raised; +++: severely raised. Resins are non-absorbed compounds that bind bile salts and cholesterol in the gastrointestinal tract so that they are excreted (see Section 5.1.2)

species (particularly HDL) may be decreased, this is often described as a dyslipidaemia rather than a hyperlipidaemia.

It is a long-standing observation that the health risks of obesity vary according to the distribution of fat over the body. The observation of different patterns of fat distribution is an old one; a 25 000-year-old carved figurine shows a female, perhaps thought to be especially fertile, with large fat deposits around her hips and breasts; Shakespeare in *Comedy of Errors* has Dromio referring to Nell, who clearly carried her fat around her midriff, with the words: 'No longer from head to foot than from hip to hip: she is spherical, like a globe'. This subject was first investigated systematically by the French physician Jean Vague in Marseilles in the 1940s. (Jean Vague is still active and publishing today.) In a classic paper published in 1947 Vague drew attention to two broad patterns of fat distribution, one typically female with fat mainly on the lower body (often thought of as pear-shaped) and one typically male with abdominal and upper-body fat accumulation (often envisaged as apple-shaped; Fig. 5.20). He pointed out that the male pattern can

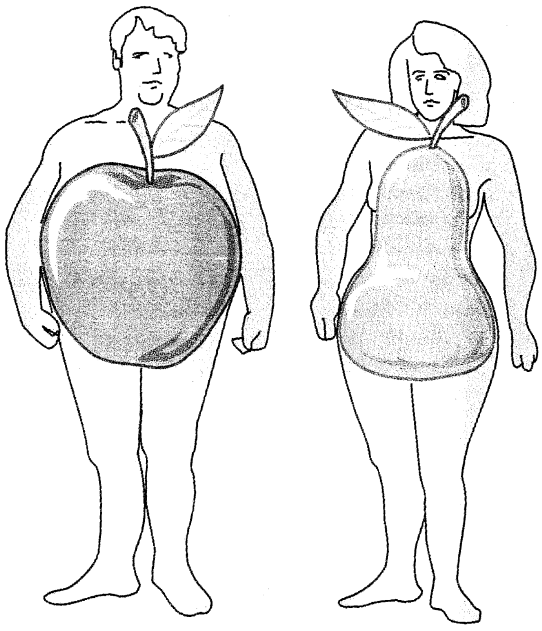


Fig. 5.20 Characterization of the two extremes of body fat distribution as apple- and pear-shaped. Reproduced with permission from Frayn, K.N. (1997) *Biological Sciences Review*, **10**, 17–20.

also occur in women, and vice versa, and that irrespective of sex, the upper-body (apple) type of fat distribution was associated with a much higher incidence of several diseases including coronary heart disease and risk of developing diabetes.

One widely accepted hypothesis for the link between obesity, especially of the upper-body variety, and dyslipidaemia predisposing to coronary heart disease, suggests that it is mediated by the abdominal fat releasing some factor or factors that affect both lipid metabolism, and sensitivity of tissues to insulin. This factor might be a cytokine or other hormone, but it might equally be non-esterified fatty acids. Abdominal adipose tissue is more metabolically active than is lower-body (buttock and hip) fat, and releases non-esterified fatty acids more readily upon stimulation by adrenaline and noradrenaline. Similarly, insulin is less effective at suppressing non-esterified fatty acid release from the upper-body than the lower-body depots. The reasons for this have been investigated thoroughly in adipocytes removed from different depots, and they reflect mainly differential expression of hormone receptors and other 'downstream' components of hormone-signalling chains.

Increased delivery of non-esterified fatty acids into the circulation, particularly if not restrained normally by insulin, can be seen to lead to many changes characteristic of the atherogenic lipoprotein phenotype. Increased delivery of non-esterified fatty acids to the liver will tend to stimulate VLDL-triacylglycerol secretion. Increased VLDL-triacylglycerol secretion will lead to competition with the influx of chylomicron-triacylglycerol for clearance by lipoprotein lipase in the period following meals. Thus, hypertriglyceridaemia and particularly increased postprandial hypertriglyceridaemia may be explained. It was described earlier (Section 5.4.1) how this can lead to depression of HDL-cholesterol concentrations and the production of small, dense LDL particles through the action of cholesteryl ester transfer protein (CETP). It is interesting also that the activity of CETP is itself increased by high concentrations of non-esterified fatty acids.

With the advent, in the last few decades, of imaging techniques such as computer-assisted tomography and magnetic resonance imaging, it has been appreciated that upper-body fat accumulation is associated with increased deposition of adipose tissue within the abdominal cavity, surrounding the visceral organs – so-called visceral fat. Two of the larger intra-abdominal depots, those associated with the greater omentum and the mesentery, have the property that the blood passing through them is conducted to the hepatic portal vein. It has also been found in many studies that adipocytes isolated from these depots (taken at abdominal surgery) is even more metabolically active than is abdominal fat from the subcutaneous depot. Therefore some people now consider that liberation of non-esterified fatty acids from these depots, reaching the liver directly (where they may influence VLDL-triacylglycerol secretion), plays a particular role in the dyslipidaemia of upper-body obesity. This remains to be confirmed because it is very difficult to study the metabolic pattern of these adipose tissue depots within the body.

Increased circulating concentrations of non-esterified fatty acids have effects in addition to those on VLDL secretion from the liver. The supply of fatty acids to the liver is a major determinant of the rate of release of glucose from the liver. This may arise because fatty acids are readily oxidized in the liver, and this provides a source of energy for the process of gluconeogenesis (glucose synthesis from other precursors). Therefore increased non-esterified fatty acid concentrations may lead to hepatic over-production of glucose. Fatty acids are a preferred metabolic fuel for many tissues including skeletal muscle, where they displace glucose as a fuel for oxidation. This can lead to inefficiency of glucose disposal, particularly in the postprandial period when skeletal muscle glucose uptake is normally stimulated by insulin. These changes in glucose metabolism together tend to raise concentrations of glucose in the plasma. The rise may be counteracted by an increased secretion of insulin from the pancreas. This is considered by many to be a plausible scenario for the development of insulin resistance, a situation in which insulin concentrations are high and do not regulate glucose metabolism normally. It was described earlier (Table 5.6) as a feature of the atherogenic lipoprotein phenotype, and is a very typical finding in upper-body obesity.

In some people, increasing insulin resistance can be matched by increasing insulin secretion from the pancreas. In this case it is accompanied by the dyslipidaemia described above, but glucose metabolism remains relatively normally controlled. In others, however, presumably those with a genetic predisposition, the pancreatic β cells fail to match the demand and become 'exhausted', so that insulin concentrations in plasma no longer match the glucose concentrations, which may then rise to high levels. This is the condition of diabetes mellitus, diagnosed by measurement of increased plasma glucose concentrations, fasting or after an oral glucose dose.

There are two major types of diabetes mellitus. The type described above, very often associated with insulin resistance, usually coming on in later life (after the age of 40), and associated with marked insulin resistance, is called Type II diabetes mellitus. In older literature it is called non-insulin-dependent diabetes mellitus (because insulin is not usually necessary for treatment) or maturity-onset diabetes. The other major type is Type I, or insulin-dependent or juvenile-onset diabetes. This almost always develops in childhood and requires insulin for treatment. People with Type I diabetes are often thin, although insulin resistance is also present (less marked than in Type II). Type I diabetes mellitus arises because of an autoimmune process that destroys the insulin-secreting β-cells of the pancreas and thus its aetiology is not directly concerned with lipid metabolism.

Both types of diabetes are associated with considerably increased risk of cardiovascular disease, including coronary artery disease, peripheral vascular disease and ischaemic stroke. About 75% of people with diabetes will eventually die from coronary heart disease, an incidence that is about twofold to sixfold greater than that for non-diabetic people matched for age, sex and for major risk factors including LDL-cholesterol concentration. The reasons for this are not entirely clear. In Type II diabetes there is clear association with the atherogenic lipoprotein phenotype (Table 5.6). In Type I diabetes the alterations in lipid metabolism may be more subtle and involve changes in the composition of lipoprotein particles that have not been fully delineated. Increased oxidative stress is one unifying hypothesis that has attracted some attention, since peroxidation of LDL particles may precede their uptake by macrophages in the arterial wall.

The treatment of diabetes is not strictly within the scope of this book except in so far as it relates to lipid metabolism. Type I diabetes requires injections of insulin. Type II diabetes is usually managed with drugs that boost insulin secretion (sulphonylureas) or apparently increase sensitivity to insulin by an unknown mechanism (metformin, a biguanide drug). Recently, however, a new class of agents has been discovered and is being introduced for clinical use in Type II diabetes. These are the thiazolidinediones or 'glitazones', agonists of PPAR-γ, as described in Section 5.3.2.

5.4.5 Hypolipoproteinaemias are rare conditions of abnormally low plasma lipoprotein concentrations

These conditions in which lipoprotein concentrations are below normal or there is a complete absence of one lipoprotein class are much rarer than those characterized by raised lipoprotein concentrations (Table 5.7). There are some 'classic' single-gene mutations in this group.

Chylomicron retention disease (Anderson's disease) is usually diagnosed in childhood as a result of poor growth and intestinal problems. The enterocytes are unable to secrete chylomicrons, and although other lipoprotein species are present in plasma their concentrations are low and their composition abnormal. It is a familial disease which is recessively expressed, and occurs mainly in males. The precise defect is not known and may differ between different families. It is not a defect in microsomal triacylglycerol transfer protein (MTP) although people with that defect (see below) may exhibit some impairment of fat absorption.

Abetalipoproteinaemia is a complete absence of apoB100 in plasma, i.e. there are no VLDL or LDL particles. It is an autosomal recessive disease. There may be signs of fat malabsorption, and neurological and visual impairment. A characteristic finding is abnormally shaped red blood cells (often star-shaped) which reflects abnormal membrane phospholipid composition, secondary to the changes in lipoprotein concentration and composition. The molecular defect is in MTP, which plays a critical role in VLDL assembly (Fig. 5.4). There are also conditions in which levels of apoB100 are low but not absent (hypobetalipoproteinaemia). This may represent the heterozygous form of abetalipoproteinaemia or it may be a separate condition, associated with a mutation causing a truncation of apoB100.

There are several diseases associated with low concentrations of HDL, but the classic single-gene condition associated with absence of HDL is Tangier disease, named after a small island off the coast of Virginia, USA, where it was first described by Donald Fredrickson in 1961. Lack of HDL is associated with cholesterol accumulation in tissues, especially macrophages that accumulate in lymphoid tissue such as the tonsils. Since these macrophages have accumulated their lipid initially by taking up LDL particles, and since LDL particles also carry fat-soluble pigments such as β-carotene, the tonsils in Tangier disease are typically orange in colour, a hallmark of the disease. The gene responsible for Tangier disease was described in Section 5.2.5. It encodes the cholesterol efflux regulatory protein, responsible for efflux of cholesterol from tissues. Paradoxically, sufferers are not at greatly increased risk of cardiovascular disease.

A further defect leading to low, but not absent concentrations of HDL-cholesterol is a defect in lecithin-cholesterol acyltransferase (LCAT). LCAT is responsible for the esterification of cholesterol acquired by HDL particles either by interaction with cell membranes or during the shedding of excess surface components as triacylglycerol-rich lipoproteins are hydrolysed by lipoprotein lipase. A striking feature of this disease is corneal opacity, caused by lipid infiltration of the cornea. This led Swedish investigators who described one variant, to give it the name Fish Eye Disease (because the opacified corneas were thought to resemble the eyes of boiled fish). Plasma lipoproteins are abnormal in composition in LCAT deficiency, with an excess of unesterified cholesterol as expected. As in Tangier disease, the patients are unexpectedly not at greatly increased risk of atherosclerosis.

5.5 SUMMARY

In this chapter we looked at how dietary lipids are taken up into the body, how lipids are transported around the body, and how these systems are regulated according to supply and demand. In addition, diseases relating to lipid transport have been considered. Emphasis has been given to processes in the human body, but differences between humans and other mammals have been touched upon.

Humans and some other mammals take in a large part of their dietary energy in the form of fat. This dietary fat is a source of energy, of fuel for storage, and a precursor for many important biological

molecules. In order for the body to use it, it has first to be taken up from the intestinal tract. This involves emulsification, to produce a large surface area upon which various lipolytic enzymes can act to cause hydrolysis of ester bonds. Simpler molecules (e.g. monoacylglycerols, fatty acids) are released and taken up into absorptive cells of the small intestine. Here, larger molecules (e.g. triacylglycerols, cholesteryl esters) are resynthesized and packaged for export to the circulation. The distribution of water-insoluble lipid molecules around the body in the aqueous environment of the blood plasma has been achieved early on in evolution by the production of emulsion-like particles in which hydrophobic molecules are surrounded by an amphipathic shell, mainly of phospholipids. These are the lipoprotein particles. There are various classes of lipoproteins, distinguished by size, composition and metabolic function. The largest and most lipid-rich are the chylomicrons, synthesized in the small intestine and carrying dietary fat (mainly triacylglycerol) into the circulation. The triacylglycerol in these particles is delivered to tissues in the form of fatty acids after hydrolysis by the enzyme lipoprotein lipase, attached to the capillary endothelium. The activity of lipoprotein lipase is regulated in different tissues according to the need for fatty acids. Thus, dietary fatty acids are distributed according to the metabolic needs of the various tissues.

During fasting, no dietary triacylglycerol enters the system but the liver secretes triacylglycerol-rich particles, the very low density lipoproteins. They also deliver fatty acids to tissues via lipoprotein lipase. The smaller particles that remain after loss of most of the triacylglycerol are known as low-density lipoproteins. They are the main transporters of cholesterol in the circulation. They may be taken up by tissues via the cell-surface low-density lipoprotein receptor, thus delivering cholesterol from liver to other tissues. Tissues can also export excess cholesterol through the pathway of reverse cholesterol transport. This involves the high-density lipoprotein particles, which can pick up cholesterol from cell surfaces and transport it to the liver where it can be excreted in the bile, either intact or after conversion to bile acids.

Lipoprotein and lipid metabolism are regulated in a number of ways. Lipoproteins are targeted by the proteins (apolipoproteins) that they carry on their surface, which interact with specific receptors on cell surfaces. The expression of these receptors, especially the low-density lipoprotein receptor, is regulated according to the cell's lipid status. Fatty acids and cholesterol, and derivatives of them, can directly regulate gene expression to control the synthesis of lipids and of receptors.

Excessive concentrations of lipids in the circulation are associated with a number of diseases, especially cardiovascular disease in which the blood supply to various organs is impaired. This can lead to partial or complete loss of function, e.g. of the brain (stroke), heart (coronary heart disease and myocardial infarction) or limbs (peripheral vascular disease). A common underlying process is that of atherosclerosis, the build-up of fatty deposits in the arterial wall. Circulating lipid concentrations are responsive to the diet. In particular, the nature of the predominant dietary fatty acids regulates the serum cholesterol concentration (saturated fatty acids tend to raise it, polyunsaturated to lower it).

FURTHER READING

Auwerx, J. (1999) PPARγ, the ultimate thrifty gene. *Diabetologia*, **42**, 1033–1049.

Besnard, P., Niot, I., Bernard, A. & Carlier, H. (1996) Cellular and molecular aspects of fat metabolism in the small intestine. *Proceedings of the Nutrition Society*, **55**, 19–37.

Betteridge, D.J., Illingworth, D.R. & Shepherd, J. (1999) *Lipoproteins in Health and Disease*. Arnold, London.

Caspary, W.F. (1992) Physiology and pathophysiology of intestinal absorption. *American Journal of Clinical Nutrition*, **55** (Supplement), 299S–308S.

Clarke, S.D. (2000) Polyunsaturated fatty acid regulation of gene transcription: a mechanism to improve energy balance and insulin resistance. *British Journal of Nutrition*, **83** (Supplement 1), S59–S66.

Cooper, A.D. (1997) Hepatic uptake of chylomicron remnants. *Journal of Lipid Research*, **38**, 2173–2192.

Fielding, G.J. & Fielding, P.E. (1995) Molecular physiology of reverse cholesterol transport. *Journal of Lipid Research*, **36**, 211–228.

Gurr, M.I. (1996) Dietary fatty acids with *trans* unsaturation. *Nutrition Research Reviews*, **9**, 259–279.

Hussain, M.M., Strickland, D.K. & Bakillah, A. (1999) The mammalian low-density lipoprotein receptor family. *Annual Review of Nutrition*, **19**, 141–172.

Jump, D.B. & Clarke, S.D. (1999) Regulation of gene expression by dietary fat. *Annual Review of Nutrition*, **19**, 63–90.

Krieger, M. (1999) Charting the fate of the 'good cholesterol': identification and characterization of the high-density lipoprotein receptor SR-BI. *Annual Review of Biochemistry*, **68**, 523–558.

Pariza, M.W., Park, Y. & Cook, M.E. (2000) Mechanisms of action of conjugated linoleic acid: evidence and speculation. *Proceedings of the Society of Experimental Biology and Medicine*, **223**, 8–13.

Salter, A.M. & White, D.A. (1996) Effects of dietary fat on cholesterol metabolism: regulation of plasma LDL concentrations. *Nutrition Research Reviews*, **9**, 241–257.

Sethi, S., Gibney, M.J. & Williams, C.M. (1993) Postprandial lipoprotein metabolism. *Nutrition Research Reviews*, **6**, 161–183.

Sul, H.S. & Wang, D. (1998) Nutritional and hormonal regulation of enzymes in fat synthesis: studies of fatty acid synthase and glycerol-3-phosphate acyltransferase gene transcription. *Annual Review of Nutrition*, **18**, 331–351.

Vance, D.E. & Vance, J.E., eds. (1996) *Biochemistry of Lipids, Lipoproteins and Membranes*. Elsevier, Amsterdam.

White, D.A., Bennett, A.J., Billett, M.A. & Salter, A.M. (1998) The assembly of triacylglycerol-rich lipoproteins: an essential role for the microsomal triacylglycerol transfer protein. *British Journal of Nutrition*, **80**, 219–229.

Worgall, T.S. & Deckelbaum, R.J. (1999) Fatty acids: links between genes involved in fatty acid and cholesterol metabolism. *Current Opinion in Clinical Nutrition and Metabolic Care*, **2**, 127–133.

Zammit, V.A. (1999) The malonyl-CoA-long-chain acyl-CoA axis in the maintenance of mammalian cell function. *Biochemical Journal*, **343**, 505–515.

6 Lipids in cellular structures

One of the most important general functions for lipids is their role as constituents of cellular membranes. These membranes not only separate cells from the external environment, but also compartmentalize cells and provide a special milieu for many important biochemical processes. Here we shall describe the different types of lipids found and their distribution in various cellular membranes, as well as aspects of their function there.

6.1 CELL ORGANELLES

Lipids contribute to the structures of a variety of membranes in different parts of cells and in different organisms. The composition of these lipids varies considerably depending on the type and function of the membrane.

Eukaryotic cells characteristically contain a range of different cell organelles. All of these are separated from the cytosol (and usually from each other) by membranes. Some organelles such as mitochondria or chloroplasts are surrounded by two distinct membranes whereas others, such as microbodies, have a single membrane only. The relative numbers and detailed structure of most of these organelles vary from cell to cell. Moreover, in some cases, for example chloroplasts, the morphology will change depending on the developmental stage of the cell and will even be affected by the environment and metabolic state of the organelle.

Even in prokaryotes, many species contain a complex internal membrane structure. This is especially true in photosynthetic organisms such as purple or green bacteria and cyanobacteria (Fig. 6.1). Moreover, in some organisms membrane (or

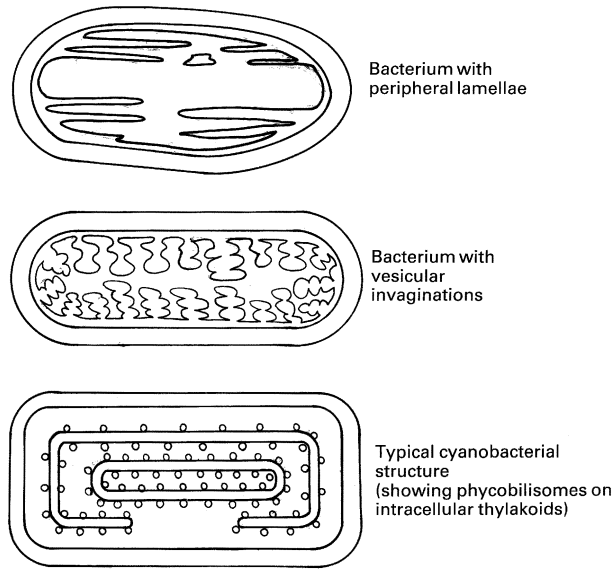


Fig. 6.1 Diagrammatic representation of internal membrane systems of bacteria and cyanobacteria.

organelle) formation can be induced (Table 6.1) – thus providing a convenient experimental system from which to isolate membranes and in which to study membrane biogenesis.

Since organelles and their membranes carry out such a wide range of different functions, it is scarcely surprising that their lipid compositions vary. Moreover, the percentage of lipid and protein in different membranes can range from 80% lipid in myelin to only about 25% in mitochondria (Table 6.2). Almost all membrane lipids are amphipathic molecules (Section 6.5) such as phospholipids or glycolipids; neutral lipids such as triacylglycerols have little part in membrane structure. In organ-

Table 6.1 Experimental systems for studying membrane induction

Organism	Stimulus	Morphological change
Photosynthetic bacteria	Starvation, light	Photosynthetic internal membranes formed
Euglena	Starvation, light	Chloroplasts produced
Yeast	Glucose in medium reduced	Mitochondria produced
Etiolated plants	Light	Etioplasts converted to chloroplasts
Animals	Fed barbiturates	Liver and endoplasmic reticulum proliferates
Animals	Fed clofibrate	Liver peroxisomes proliferate

Table 6.2 Lipid composition of different membranes

	Membrane (% total lipid)				
	Chloroplast (spinach)	Protoplast (*B. megaterium*)	Mitochondrion (rat)	Erythrocyte (rat)	Myelin (rat)
Lipid:protein (wt/wt)	1:1	1:3	1:3	1:3	3:1
Phospholipid	12	48	90	61	41
PC	tr	0	40	34	12
PE+PI+PS	tr	19	41	11	26
PG	12	26	–	–	–
DPG	–	3	7	–	–
SPH	–	–	2	16	3
Glycolipid	80	52	–	11	42
MGDG	41	–			
DGDG	23	–			
SQDG	16	–			
Sterol, sterol ester	tr	0	tr	28	17
Acylglycerols	–	–	10	–	–
Pigments	8	–	–	–	–

PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; PG = phosphatidylglycerol; SPH = sphingomyelin; MGDG = monogalactosyldiacylglycerol; DGDG = digalactosyldiacylglycerol; SQDG = sulpholipid; DPG = diphosphatidylglycerol; tr = trace. The glycolipids in rat membranes are sphingolipids.

isms that make or utilize sterols, these tend to be concentrated in the external surrounding membrane (compare chloroplast or mitochondria with erythrocyte or myelin membranes in Table 6.2).

Before describing the composition, structure and function of membranes, it is necessary to detail the molecules that make up the lipid components.

6.2 GLYCEROLIPIDS

Glycerolipids are the most widespread of membrane lipids. Phosphoglycerides dominate in higher animals whereas glycosylglycerides are important in plants.

Three important classes of membrane lipid are widely distributed – glycerolipids, sphingolipids and steroids. Of these, glycerolipids are quantitatively by far the most important group. They can be conveniently divided into two main groups – those containing phosphorus (phosphoglycerides) and those without phosphorus but containing a sugar constituent (glycosylglycerides). Confusingly, some compounds (e.g. sphingomyelin) can be classified in more than one group.

6.2.1 Phosphoglycerides are the major lipid components of most biological membranes

The stereochemistry of phosphoglycerides was discussed in Chapter 1. The phosphoglycerides comprise a very widespread and diverse group of structures. In most membranes they are the main lipid components and, indeed, the only general exceptions to this statement are the photosynthetic membranes of plants, algae and cyanobacteria and the archaebacterial membranes.

Usually, phosphoglycerides contain fatty acids esterified at positions 1 and 2 of glycerol. They are, thus, diacylphosphoglycerides. These lipids are named after the moiety which is attached to the phosphate esterified at position 3 of glycerol. Thus, the compounds can be thought of as derivatives of diacylglycerols in which the hydroxyl on carbon atom 3 is esterified with phosphoric acid, which in turn is esterified with a range of molecules – organic bases, amino acids, alcohols.

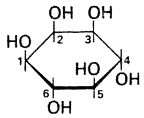
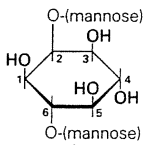
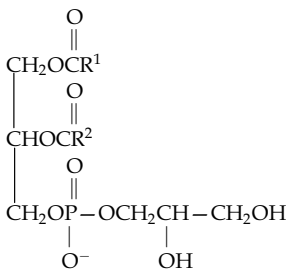
The simplest phosphoglyceride contains only phosphoric acid attached to diacylglycerol and is called phosphatidic acid (Table 6.3). Where addi-

Table 6.3 Structural variety of different diacylglycerophospholipids

X	Name of phospholipid	Source	Remarks
H	Phosphatidic acid	Animals, higher plants, micro-organisms.	Only small amounts normally found. Main importance as a biosynthetic intermediate.
$OH{\cdot}CH_2CH_2N^+(CH_3)_3$ Choline	Phosphatidyl choline (lecithin)	Animals; first isolated from egg yolks; higher plants; rare in micro-organisms.	Most abundant animal phospholipid.
$OH \cdot CH_2CH_2NH_3^+$ Ethanolamine	Phosphatidyl ethanolamine	Animals; higher plants; micro-organisms.	Widely distributed but in small amounts. Minor component of old 'cephaline' fraction. *N*-acetyl derivatives in brain; fatty amides in wheat flour, peas.
$OH \cdot CH_2 \cdot CH(COO^-) \cdot NH_3^+$ Serine	Phosphatidylserine	Animals; higher plants; micro-organisms.	Widely distributed but in small amounts. Minor component of old 'cephaline' fraction. Serine as L isomer. Lipid usually in salt form with K^+, Na^+, Ca^{2+}.

Continued on p. 218

Table 6.3 Continued

X	Name of Phospholipid	Source	Remarks
 *Myo*-inositol	Phosphatidylinositol	Animals, higher plants, micro-organisms.	The natural lipid is found as a derivative of *myo*-inositol-1-phosphate only. The major inositol-containing phosphoglyceride (about 80% of total in animals). 3-Phosphate derivatives of inositol phosphoglycerides present in very small amounts.
Inositol-4-phosphate	Phosphatidylinositol phosphate	Animals; small amounts in yeast; plants.	Mainly nervous tissue, but also plasma membranes of other cells.
Inositol-4, 5-*bis*phosphate	Phosphatidylinositol *bis*phosphate	Animals; small amounts in yeast; plants.	Distribution as above. Both compounds have very high rates of turnover.
 Inositol mannoside	Phosphatidylinositol mannoside X = 0, monomannoside X = 1, dimannoside etc	Micro-organisms (*M. phlei, M. tuberculosis*).	
$CH_2OH \cdot CHOH \cdot CH_2OH$ Glycerol	Phosphatidylglycerol	Mainly higher plants and micro-organisms.	'Free' glycerol has opposite stereochemical configuration to the acylated glycerol, i.e. 1,2-diacyl-1-*sn*-glycerol. Probably most abundant phospholipid because of its prevalence in photosynthetic membranes.
 Phosphatidylglycerol	Diphosphatidylglycerol (*bis*phosphatidylglycerol, cardiolipin)	Animal; higher plants; micro-organisms.	Major component of many bacteria, localized in inner mitochondrial membrane of eukaryotes.

tional 'X' groups are esterified to the phosphate moiety the lipids are called phosphatidyl-X. Major types of such diacylphosphoglycerides are shown in Table 6.3 where relevant comments about their distribution and properties are made also.

6.2.2 Phosphonolipids constitute a rare class of lipids found in few organisms

In 1953, Rouser and his associates first identified a phosphonolipid in biological extracts – from a sea

anemone. Subsequent work has shown that such lipids, which contain a C–P bond, are significant constituents of lower animals such as molluscs, coelenterates and protozoa. Phosphonolipids can be detected in bacteria and mammals, but only in very low quantities. Two types of glycerophosphonolipids have been found (Fig. 6.2) and there are also other structures that contain a sphingosine (and, sometimes, a galactosyl) residue instead of glycerol. Usually, organisms tend to accumulate phosphonolipids based on either glycerol or on sphingosine rather than both types. In protozoa such as *Tetrahymena*, the glycerophosphonolipids are concentrated in the ciliary membranes. This is perhaps because the phosphonolipids are particularly resistant to chemical as well as enzymic attack.

6.2.3 Glycosylglycerides are particularly important components of photosynthetic membranes

Glycolipids that are based on glycerol have been found in a wide variety of organisms. However, whereas in animals they are only found in very small quantities, they are major constituents of some micro-organisms and are the main lipid components of the photosynthetic membranes of algae (including the blue-greens or cyanobacteria) and plants (Table 6.4). Their structure is analogous to that of glycerophospholipids with the sugar(s) attached glycosidically to position 3 of glycerol and fatty acids esterified at the other two positions.

Since the membranes of higher plant chloroplasts are the most prevalent on land, and the photosynthetic membranes of marine algae are the most common in the seas and ocean, it follows that these glycosylglycerides are the most abundant membrane lipids in Nature, in spite of the sparse attention paid to them in most standard biochemistry textbooks! The two galactose-containing lipids – monogalactosyldiacylglycerol and digalactosyldiacylglycerol – represent about 40% of the dry weight of photosynthetic membranes of higher plants. Position 1 of the galactose ring has a β-link to glycerol whereas, in digalactosyldiacylglycerol there is an α,1-6 bond between the sugars. Whereas galactose is almost the only sugar found in the glycosylglycerides of higher plants, other sugars such as glucose may be found in algae, particularly marine species. In bacteria several combinations of residues may be found in diglycosyldiacylglycerols (Table 6.5). The most common combinations are two glucose, two galactose or two mannose residues linked α,1-2 or β,1-6. Such glycosylglycerides do not form a large proportion of the total lipids in bacteria, but are found more frequently in the Gram-positives or photosynthetic Gram-negatives. In addition, bacteria may contain higher homologues with up to seven sugar residues.

Apart from the galactose-containing lipids, a third glycosylglyceride is found in chloroplasts (Table 6.4). This is the plant sulpholipid. It is more correctly called sulphoquinovosyldiacylglycerol and contains a sulphonate constituent on carbon 6 of a deoxyglucose residue. This sulphonic acid group is very stable and also highly acidic so that the plant sulpholipid is a negatively charged molecule in Nature. Although this sulpholipid occurs in small amounts in photosynthetic bacteria and some fungi, it is really characteristic of the photosynthetic membranes of chloroplasts and cyanobacteria.

All three chloroplast glycolipids usually contain large amounts of α-linolenic acid (Table 6.6). In fact, monogalactosyldiacylglycerol may have up to 97%

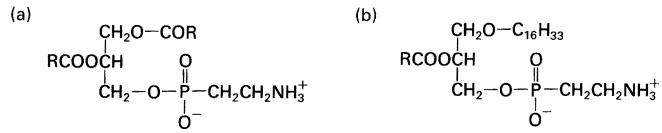
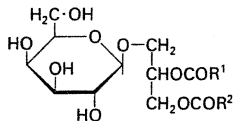
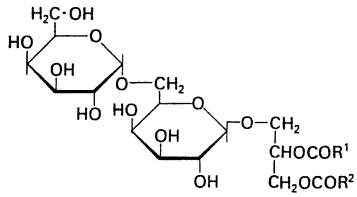
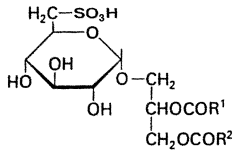


Fig. 6.2 Glycerophosphonolipids: (a) phosphatidylethylamine; (b) an analogue, which is a derivative of chimyl alcohol rather than a diacylphosphonolipid. It is named 1-hexadecyl-2-acyl-*sn*-glycero(3)-2-phosphonoethylamine.

Table 6.4 Major glycosylacylglycerols of plant and algal photosynthetic membranes

Common name	Structure and chemical name	Source and fatty acid composition
Monogalactosyl-diacylglycerol (MGDG)	 1,2-diacyl-[β-D-galactopyranosyl-(1′→3)-*sn*-glycerol	Especially abundant in plant leaves and algae; mainly in chloroplast. Contains a high proportion of polyunsaturated fatty acids. *Chlorella vulgaris* MGDG has mainly 18:1, 18:2 when dark grown but 20% 18:3 when grown in the light. *Euglena gracilis* MGDG has 16:4. Spinach chloroplast MGDG has 25% 16:3, 72% 18:3. Also found in the central nervous systems of several animals in small quantity.
Digalactosyl-diacylglycerol (DGDG)	 1,2-diacyl-[α-D-galactopyranosyl-(1′→6′)-β-D-galactopyranosyl-(1′→3)]-*sn*-glycerol	Usually found together with MDGD in chloroplasts of higher plants and algae. Not quite so abundant as MGDG. Also has high proportion of polyunsaturated fatty acids, especially 18:3. In both lipids the glycerol has the same configuration as in the phospholipids.
Plant sulpholipid (sulphoquinovosyl-diacylglycerol; SQDG)	 1,2-diacyl-[6-sulpho-α-D-quinovopyranosyl-(1′→3)-*sn*-glycerol. [D-quinovose is 6-deoxy-D-glucose. Note the carbon–sulphur bond]	Usually referred to as a 'sulpholipid' as distinct from a 'sulphatide', which is reserved for cerebroside sulphates. Typical lipid of chloroplast membranes but present elsewhere in some marine algae. Also found in cyanobacterial photosynthetic membranes and, to a lesser extent, in purple photosynthetic bacteria. Contains more saturated fatty acids (mainly palmitic) than the galactolipids, e.g. spinach leaf sulpholipid has 27% 16:0, 39% 18:2, 28% 18:3.

of its total acyl groups as this one component in some plants! The reason for this exceptional enrichment is not known although speculations have been made (Section 6.5.11). Moreover, a unifying theory connected with photosynthesis is not possible since many cyanobacteria do not make α-linolenate and marine algae contain little of the acid. In the plant sulpholipid the most usual combination of acyl groups is palmitate/α-linolenate. Interestingly, unlike animal lipids (Section 6.2.1) most palmitate is esterified at position 2 and most of the α-linolenate at position 1. This distribution of fatty acids stems from the special features of plant fatty acid metabolism (Section 2.2.5) and because the sulpholipid is assembled via the 'prokaryotic pathway' of metabolism. Since the position at which saturated and unsaturated fatty acids are attached to the glycerol has a marked effect on their melting properties (Section 6.5.9), fatty acid distribution may have functional significance. It has also been speculated that the different distribution of acyl moieties between plants and animals may be related to specific interactions with membrane proteins (Section 6.5.11).

Bacteria contain a number of phosphatidylglycolipids. These compounds are confined to certain types of Gram-positive organisms such as the streptococci or mycoplasmas. In addition, different species of algae and bacteria (including archaebacteria) contain various sulphoglycolipids –

Table 6.5 Some glycosylglycerides found in bacteria

Glyceride	Structure of glycoside moiety	Occurrence
Monoglucosyldiacylglycerol	α-D-Glucopyranoside	*Pneumonoccus, Mycoplasma*
Diglucosyldiacylglycerol	β-D-Glucopyranosyl-(1→6)-*O*-β-D-glucopyranoside	*Staphylococcus*
Diglucosyldiacylglycerol	α-D-Glucopyranosyl-(1→2)-*O*-α-D-glucopyranoside	*Mycoplasma, Streptococcus*
Dimannosyldiacylglycerol	α-D-Mannopyranosyl-(1→3)-*O*-α-D-mannopyranoside	*Micrococcus lysodeikticus*
Galactofuranosyldiacylglycerol	β-D-Galactofuranoside	*Mycoplasma, Bacteroides*
Galactosylglucosyldiacylglycerol	α-D-Galactopyranosyl-(1→2)-*O*-α-D-glucopyranoside	*Lactobacillus*
Glucosylgalactosylglucosyldiacylglycerol	α-D-Glucopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→2)-*O*-α-D-glucopyranoside	*Lactobacillus*

usually with the sulphur present in a sulphate ester attached to the carbohydrate moiety. Lipids containing taurine seem to be significant components of some protozoa.

6.2.4 Betaine lipids are important in some organisms

Betaine ether-linked glycerolipids are naturally occurring lipids which are important in many lower plants (e.g. bryophytes), algae, fungi and protozoa. Because of their structure (Fig. 6.3) and properties, they are often envisaged as substituting for phosphatidylcholine in membranes. A number of structures has been identified but diacylglyceryltrimethylhomoserine (DGTS) is the most common. Typically, it represents 10–20% of all membrane lipids but, in some species of mosses, it may be around 40% of the total.

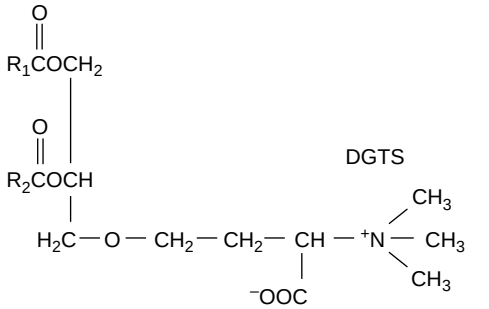
6.2.5 Ether-linked lipids and their bioactive species

Apart from the betaine ether-linked glycerolipids (Section 6.2.4), there are many lipids that have ether links to carbon position 1 (and sometimes position

Table 6.6 Fatty acid compositions of glycosylglycerides in two plants

		(% total fatty acids)				
Plant leaf		16:0	16:3	18:1	18:2	18:3
Spinach	MGDG	trace	25	1	2	72
('16:3-plant')	DGDG	3	5	2	2	87
	SQDG	39	–	1	7	53
Pea	MGDG	4	–	1	3	90
('18:3-plant')	DGDG	9	–	3	7	78
	SQDG	32	–	2	5	58

'16:3-plants' contain hexadecatrienoate in their monogalactosyldiacylglycerol while '18:3-plants' contain α-linolenate instead. The reason for this is provided by the differences in fatty acid metabolism between these two types of plants (see Section 2.2.5).



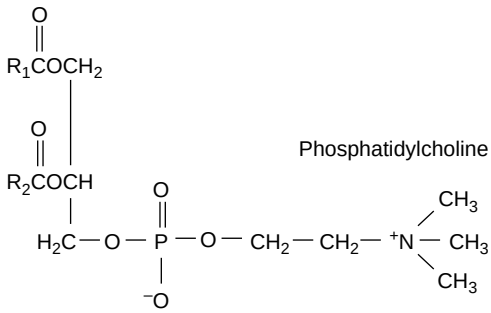
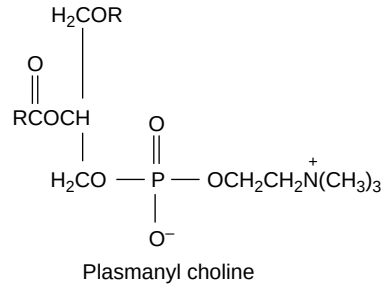
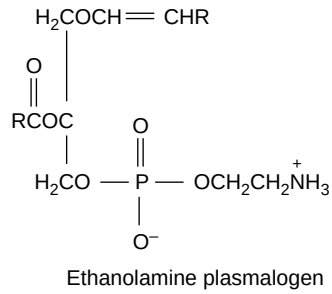


Fig. 6.3 Comparison of the structure of the betaine lipid, DGTS with phosphatidylcholine. DGTS = 1,2-diacylglyceryl-3-*O*-4′*(N,N,N*-trimethyl)-homoserine.

2) of the glycerol backbone. In mammals, there are almost exclusively ether linkages in the choline or ethanolamine glycerolipid class. If the ether link is unsaturated (Fig. 6.4) then the lipid is termed a plasmalogen. Indeed, of the ether-linked phosphoglycerides, ethanolamine plasmalogen is the most widespread. It is especially abundant in nervous tissues and in white blood cells and platelets. The ether lipids are not confined to mammals but are also found in lower animals, some plants and many bacteria. Particular interest has recently been paid to the biologically active platelet activating factor (PAF) (Fig. 6.4) and its derivatives (see Section 7.1.10). PAF has a similar structure to the ether derivative of phosphatidylcholine (plasmanylcholine) except that the fatty acid at the *sn*-2 position is replaced by an acetyl group.





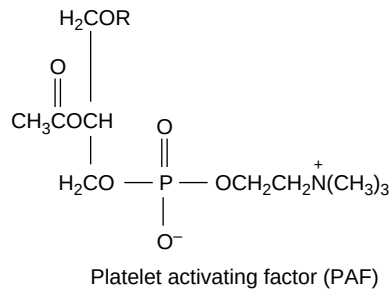


Fig. 6.4 Some important ether lipids.

6.3 SPHINGOLIPIDS

Lipids based on a sphingosine rather than a glycerol backbone are also widespread in membranes but are particularly abundant in nervous tissue. Generally, they tend to be concentrated in the outer leaflet of the plasma membrane.

The lipids described in Section 6.2 were based on a glycerol backbone. However, another important group of acyl lipids have sphingosine-based structures. Both glycolipids and phospholipids are found, with some compounds capable of dual

classification, i.e. phospholipids containing sugar residues. More than 300 different types of complex sphingolipids have been reported and this does not take account of variations in the ceramide backbone!

Sphingosine (D-erythro-2-amino-*trans*-4-octadecane-1,3-diol or 4D-sphingenine) is a long-chain amino alcohol (Figs 6.5 and 6.6). Several other sphingosylalcohols are also found in naturally occurring sphingolipids and these differ mainly in the number and position of double bonds and whether they are hydroxylated or not. (Some examples are given in Fig. 6.6.) Both the amino and the alcohol moieties of sphingosine can be substituted to produce the various sphingolipids (Fig. 6.5).

Attachment of an acyl moiety to the amino group yields a ceramide. This acyl link is resistant to alkaline hydrolysis and, therefore, can be easily distinguished from the *O*-esters found in glycerol-based acyl lipids. The simplest glycosphingolipids are the monoglycosyl ceramides (or cerebrosides). In animals galactosylcerebroside is the most common. Further attachment of hexosides to gluco-

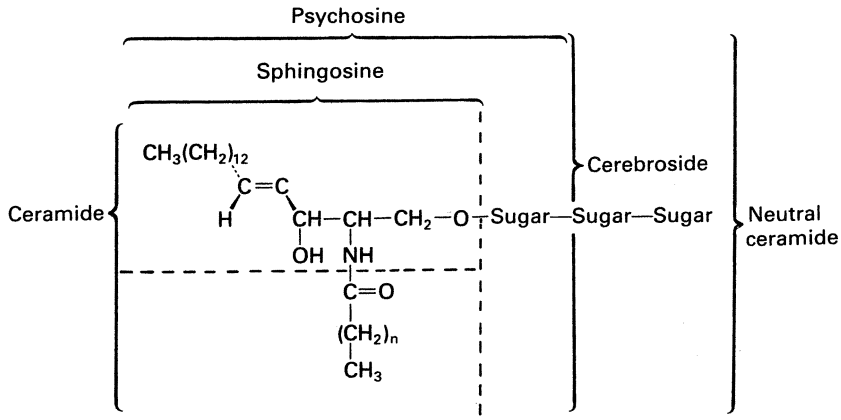


Fig. 6.5 Basic structure of sphingolipids.

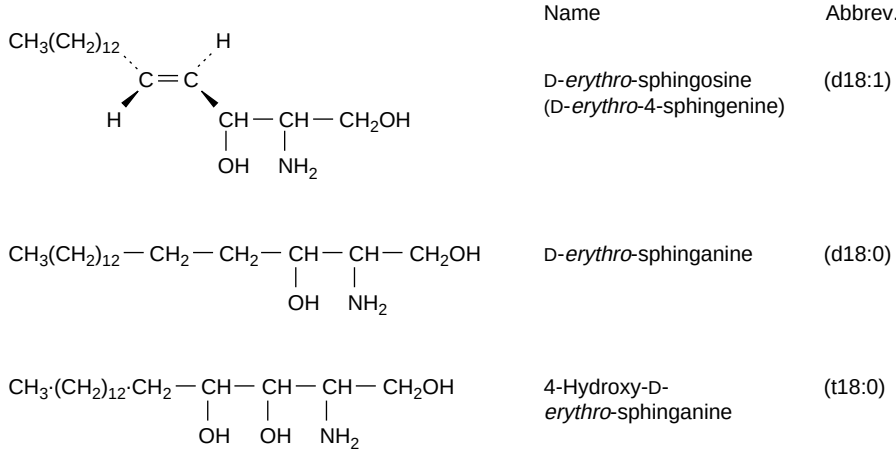


Fig. 6.6 Some variations of the sphingosine base.

sylcerebroside yields the neutral glycosylsphingolipids (or neutral ceramides) (Table 6.7). These are usually written with a shorthand nomenclature, e.g. Glc-Gal-Glc-Cer would be glucosyl-(1-4)galactosyl-(1-4)glucosyl-ceramide. The deacylated product of galactosylcerebroside, *O*-sphingosyl-galactoside is called psychosine.

Some glycosphingolipids contain one or more molecules of sialic acid linked to one or more of the sugar residues of a ceramide oligosaccharide. These lipids are called gangliosides (Table 6.8). Sialic acid is *N*-acetyl neuraminic acid (NANA or NeuAc; Fig. 6.7). Glycolipids with one molecule of sialic acid are called monosialogangliosides; those with two sialic residues are disialogangliosides and so on. Disialogangliosides may have each sialic moiety linked to a separate sugar residue or both sialic acids may be linked to each other and one of them linked to a central sugar residue. Because of their complex structure and, hence, cumbersome chemical names, many shorthand notations have been employed. One of the most commonly used is that introduced by the Swedish scientist, Svennerholm. The parent molecule is denoted as G_{M1} and other derived structures are as shown in Table 6.8. The subscripts M, D, T and Q refer to monosialo-, disialo-, trisialo- and quatra (tetra)sialo-gangliosides, respectively. A more recent nomenclature system, due to the German, Wiegandt, is also included in Table 6.8. The latter method has the advantage that, once the symbols have been learnt, the structure can be worked out from the shorthand notation and it can be applied to non-sialic acid-containing glycolipids as well. However, it has yet to supersede the Svennerholm notation, which will be used in this book.

Instead of glycosylation of the alcohol moiety of the sphingosine base, esterification with phosphocholine can take place. The phospholipid produced thus is called sphingomyelin.

Three structures constitute the main bases in animals with sphinganine and sphingosine being present in the sphingomyelin of most tissues, while a trihydroxylated derivative of sphinganine, called phytosphingosine (t18:0 in Fig. 6.6) is found in kidney. This base is also prominent in phytoglycolipid, which is a sphingolipid exclusive to plants that contains inositol as well as several other monosaccharide residues.

Physical techniques have revolutionized the analysis and identification of sphingolipids. In many cases a combination of gas–liquid chromatography, mass spectrometry and nuclear magnetic resonance spectroscopy provides sufficient information to elucidate a complete structure. The position of attachment of the sugar residues can be worked out from the use of glycosidase enzymes or by permethylation analysis. On the other hand the molecular weight and sequence of some quite complex glycosphingolipids can often be established in one step by the use of the gentler techniques of mass spectrometry such as fast-atom bombardment.

As a generalization, sphingolipids have a rather specific distribution in cells where they are concentrated in the outer (external) leaflet of the plasma membrane.

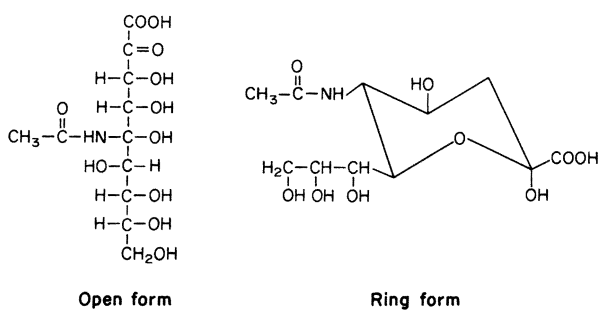


Fig. 6.7 *N*-acetylneuraminic acid (NeuAc) or sialic acid.

Table 6.7 The structure of some glycosyl ceramides [Cer = ceramide; glc = glucose; gal = galactose; GalNAc = *N*-acetyl galactosamine]

Accepted nomenclature	Old trivial name	Structure	Description	General remarks
MONOGLYCOSYL CERAMIDES	Cerebroside	Cer-gal Cer glc In all glycosyl ceramides and gangliosides there is an O-glycosidic linkage between the primary hydroxyl of sphingosine and C-1 of the sugar	'Cerebroside' originally used for galactosyl ceramide of brain but now widely used for monoglycosyl ceramides. Sugar composition depends largely on tissue. Brain cerebroside mainly galactoside while serum has mainly glucose. In animals the highest concentration is in brain. Monogalactosyl ceramide is largest single component of myelin sheath of nerve. Intermediate concentrations in lung and kidney. Also found in liver, spleen, serum with trace amounts in almost all tissues examined.	*Fatty acids:* cerebrosides containing galactose are characterized by having large concentrations of (a) hydroxy acids, (b) long-chain odd and even fatty acids, in comparison with other lipids. The hydroxy acids include α-hydroxy acids formed by α-oxidation. This oxidation mechanism is probably also responsible for odd-chain acids. Typical acids are: 22:0 (behenic); 24:0 (lignoceric); 24:1 (nervonic); α-OH-24:0 (cerebronic)
Sulphatide	Cerebroside sulphate	Cer-gal-3-sulphate	Very generally distributed like cerebrosides. Fatty acid and base composition similar.	
DIGLYCOSYL CERAMIDES	Cytosides			Base: d18:1 (sphingosine) is dominant. Smaller amounts of d18:0 (sphinganine) (especially leaves, wheat flour). Hydroxylated bases (e.g. t18:0) also occur (see Fig. 6.6).
(a) Lactosyl ceramide	Cytolipin H	Cer-glc-(4←1)-gal	Major diglycosyl ceramide. Widely distributed. Key substance in glycosyl ceramide metabolism. It may accumulate, but as it is a precursor for both ceramide oligosaccharides or gangliosides it may be present in trace quantities only.	
(b) Digalactosyl ceramide		Cer-gal-(4←1)-gal	Minor diglycosyl ceramide. Found especially in kidney (human and mouse).	

Continued on p. 226

Table 6.7 Contd

Accepted nomenclature	Old trivial name	Structure	Description	General remarks
TRIGLYCOSYL CERAMIDES Digalactosyl glucosyl ceramide		Cer-glc-(4←1)-gal-(4←1)-gal	Source: kidney, lung, spleen, liver. Most analyses have been on human tissue.	In general, each *organ* has a dominant type of glycolipid but its nature may depend also on the *species*, e.g. monoglycosyl ceramides are dominant in brain; trihexosides and aminoglycolipids in red cells. A ceramide with a 30C ω-hydroxy fatty acid is a major skin component, where it is largely responsible for water impermeability. Modern analytical techniques are revealing that most types are widespread among tissues. Cerebrosides are widespread, but minor components of higher plants, the best characterized being those of bean leaves and wheat flour where glucose is probably the only sugar. Sphingolipids are rare in micro-organisms.
TETRAGLYCOSYL CERAMIDES				
(a)	Aminoglycolipid globoside	Cer-glc-(4←1)-gal-(4←1)-gal-(3←1)-β-*N*-acetyl galactosamine	Most abundant lipid in human erythrocyte stroma.	
(b)		Cer-glc-(4←1)-gal-(4←1)-gal-(3←1)-α-*N*-acetyl galactosamine	So-called 'Forssman Antigen'.	
(c)	Asialoganglioside	Cer-glc-(4←1)-gal-(4←1)-galNAc-(3←1)-gal	Basic ganglioside structure without *N*-acetyl neuramic acid (sialic acid) residues. Intermediate in ganglioside biosynthesis (rat, frog, brain).	

Table 6.8 Structure of some gangliosides

Class	Structure	Wiegandt shorthand	Svennerholm shorthand	Composition and occurrence
MONOSIALOGANGLIOSIDE	(a) Cer-glc-(4β←1)-gal-(4β←1)-galNAc-(3β←1)-gal 3 (↑) 2 NANA	$G_{GNT}1$	G_{M1}	*Major bases:* C_{18} and C_{20} sphingosines. Minor amounts of dihydroanalogues. *Fatty acids:* large amounts of 18:0 (86–95% in brain). *Occurrence:* mainly in grey matter of brain but also in spleen, erythrocytes, liver kidney. Modern analytical techniques have shown them to be present in a much wider range of tissues than previously realized. Main gangliosides of human brain are $G_{GNT}1$, 2a, 2b, 3a. Gangliosides appear to be confined to the animal kingdom. In man, cattle, horse, main ganglioside outside brain is $G_{LACT}1$. *N*-glycolyl-neuraminic acid is chief sialic acid in erythrocyte and spleen gangliosides of horse and cattle. *Physical properties:* insoluble in non-polar solvents; form micelles in aqueous solution.
Tay–Sachs Ganglioside	(b) Cer-glc-(4β←1)-gal-(4β←1)-galNAc 3 (↑) 2 NANA	$G_{GNTrII}1$	G_{M2}	
Haematoside	(c) Cer-glc-(4^{β}←1)-gal-(3^{β}←2)-NANA	$G_{LACT}1$	G_{M3}	
DISIALOGANGLIOSIDE	(a) Cer-glc-(4β←1)-gal-(4β←1)-galNAc-(3β←1)-gal 3 3 (↑) (↑) 2 2 NANA NANA	$G_{GNT}2a$	G_{D1a}	
	(b) Cer-glc-(4β←1)-gal-(4β←1)-galNAc-(3β←1)-gal 3 (↑) 2 NANA-(8←2)-NANA	$G_{GNT}2b$	G_{D1b}	
TRISIALOGANGLIOSIDE	Cer-glc-(4β←1)-gal-(4β←1)-galNAc-(3β←1)-gal 3 3 (↑) (↑) 2 2 NANA-(8←2)-NANA NANA	$G_{GNT}3a$	G_{T1}	

Abbreviations in column 2 are the same as in Table 6.7. NANA = *N*-acetylneuraminic acid (sialic acid). Wiegandt abbreviations: G = ganglioside. Subscript denotes sialic-free oligosaccharide. Arabic numeral gives number of sialic residues. T = basic tetraose; Tr = triose; Lact = lactose. Trisaccharides and disaccharides that can be derived from the basic tetraose are distinguished by I, II, etc., I describing the sugar that originated from the non-reducing end of the tetrasaccharide; a, b refer to positional isomers with respect to sialic residues.

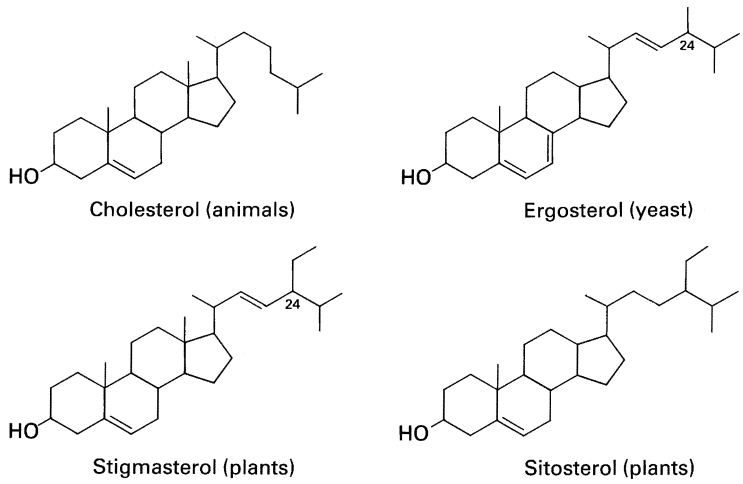


Fig. 6.8 Major types of membrane sterols.

6.4 STEROLS

Sterols are common in the membranes of eukaryotic organisms, where they are important in providing stability, but are rare in prokaryotes. In the membranes of higher animals cholesterol is the almost exclusive sterol constituent whereas in plants, other sterols, such as β-sitosterol, predominate.

6.4.1 Major sterols

Cholesterol and the functionally related sterols of fungi and plants are significant components of many organisms, especially of their external cellular membranes. However, they are not needed by all types of organisms nor, indeed, are they components (or, therefore, necessary) for all classes of membranes even in organisms with an appreciable sterol content. Sterols are common in eukaryotes, but rare in prokaryotes. Whereas vertebrates synthesize cholesterol, invertebrates rely on an external sterol supply.

Yeast and fungi, in the main, have side-chain-alkylated compounds, while plants and algae contain sitosterol and stigmasterol as their most abundant sterols (Fig. 6.8). However, it should be emphasized that, as with most situations in biology, too many generalizations should not be made. For example, cholesterol, which in many animal tissues comprises over 95% of the sterol fraction, cannot be regarded as exclusively the animal sterol. Thus, marine invertebrates, whether or not they are sterol auxotrophs, discriminate much less than the mammalian intestine in the sterols that they absorb and, therefore, have compositions dependent on the general availability of sterols. Furthermore, whereas plants typically have C24-ethyl sterols such as stigmasterol, some species of red algae contain only cholesterol.

It is generally believed that the main function for membrane sterol is in the modulation of fluidity (Section 6.5.9). The latter is mediated by the interaction of sterol with the glycerolipid components. For that purpose, cholesterol seems to have been optimally designed. Any changes in the structure appear to reduce its effect on membranes (Fig. 6.9). Points to note are:

(1) All *trans*-fusion of the rings gives a planar molecule capable of interacting on both faces.
(2) The 3-hydroxy function permits orientation of cholesterol in the bilayer.
(3) In naturally occurring sterols, the side chain is in the (17β, 20*R*) configuration, which is thermodynamically preferred and permits maximal interaction with phosphoglycerolipids.
(4) Methyl groups at C10 and C18 are retained and provide bridgeheads.

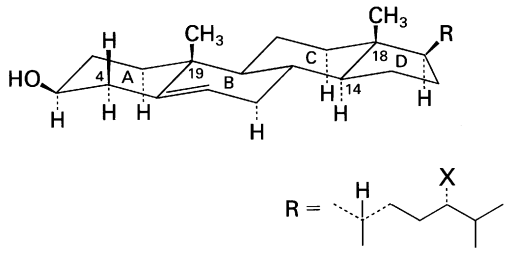


Fig. 6.9 The fused-ring structure of sterols showing important features. Reproduced with kind permission of K. Bloch from (1983) *Critical Reviews in Biochemistry*, **17**, 47–92.

(5) An unmodified isooctyl side chain renders the core of the bilayer relatively fluid (cf. substitutions at C24 in ergosterol or stigmasterol).
(6) The tetracyclic ring is uniquely compact and rigid. Other molecules of comparable hydrophobicity are much less restrained conformationally. Because of these properties such sterol molecules are able to separate or laterally displace both the acyl chains and polar head groups of membrane phospholipids.

As mentioned above, cholesterol is, by far, the most important sterol in mammalian tissues. So far as plants and algae are concerned, the major structures are sitosterol (~70%), stigmasterol (~20%), campesterol (~5%) and cholesterol (~5%). Yeasts can accumulate large amounts of sterols (up to 10% of the dry weight) and phycomycetes contain almost exclusively ergosterol. This compound is the major sterol of other yeasts and mushrooms except the rust fungi in which it is absent and replaced by various C29 sterols. With very few exceptions, sterols are absent from bacteria.

The 3-hydroxyl group on ring A can be esterified with a fatty acid. Sterol esters are found in plants as well as animals. Although sterols are present in most mammalian body tissues, the proportion of sterol ester to free sterol varies markedly. For example, blood plasma, especially that of humans, is rich in sterols and like most plasma lipids they are almost entirely found as components of the lipoproteins (Section 5.2). About 60–80% of this sterol is esterified. In the adrenals, too, where cholesterol is an important precursor of the steroid hormones, over 80% of the sterol is esterified. By contrast, in brain and other nervous tissues, where cholesterol is an important component of myelin, virtually no cholesterol esters are present.

The 3-hydroxyl group can also form a glycosidic link with the 1-position of a hexose sugar (usually glucose). Sterol glycosides are widespread in plants and algae and the 6-position of the hexose can be esterified with a fatty acid to produce an acylated sterol glycoside.

6.4.2 Other steroids

A full description of the various types of steroids, their metabolism and function, is beyond the scope of this book. Cholesterol is the essential precursor for bile acids, corticoids, sex hormones and vitamin D-derived hormones, as has been well established for all vertebrates. Their synthesis is outlined briefly in Section 7.5.6.

6.5 MEMBRANE STRUCTURE

The detailed structure of membranes differs considerably according to their location. However a general model for most membranes is consistent with an extended bilayer of amphiphilic lipids with hydrophobic moieties directed to the centre and hydrophilic head groups at the two surfaces. Ionic or hydrophobic interactions occur between lipids and membrane proteins. The latter are located at the surface or embedded within the membrane.

6.5.1 Early models already envisaged a bilayer of lipids but were uncertain about the location of the proteins

It has been known for a long time that membranes were composed basically of lipid and protein. The question was how were these constituents arranged in the membrane and how could their special properties be reconciled with their possible physiological functions?

In 1925, the Dutch workers Gorter and Grendel

extracted the lipids from erythrocytes and calculated the area occupied by the lipid from a known number of cells when it was spread as a monolayer on a *Langmuir Trough*. There was sufficient, they claimed, to surround a red cell in a layer two molecules thick. (Actually these workers made two mistakes which happened to exactly cancel each other out. First, they didn't know how hard to compress the monolayer and, hence, estimate the area accurately. Secondly, they made no allowance for the contribution of proteins.) Nevertheless, the estimates made by Gorter and Grendel turned out to be quite accurate and were accepted at the time to indicate that a bimolecular lipid leaflet surrounded cells. This organization, which provides a permeability barrier between various cellular compartments, was further refined by Danielli and Davson. They were unaware of Gorter and Grendel's paper and proposed a similar model in 1935, but realized that the measured surface tension of the membrane was too low to be accounted for by lipid and proposed that a layer of protein was present at each surface.

With the advent of electron microscopy came pictures of membranes that seemed to support the Danielli model. For the purposes of electron microscopy, the specimen has to be dehydrated, stained (usually with osmium tetroxide or potassium permanganate – so-called positive staining) and embedded in a plastic material such as an epoxy resin. The sample sections are then cut and examined under the microscope. Much of the interpretation of the structures observed depends on how much shrinkage occurs during dehydration of the sample, and what regions of the membrane take up the stain; it is now generally accepted that osmium tetroxide accumulates at the polar regions of lipid and protein. In just about every membrane examined in this way a triple-layered structure [two dark lines on either side of a light band; see Fig. 6.10(b)], was seen on the micrographs and on this basis Robertson put forward the unit membrane hypothesis, which held that every membrane had a basic structure consisting of a bimolecular lipid leaflet sandwiched between a layer of protein on one side and glycoprotein on the other. Further support for the hypothesis came from X-ray studies on myelin by Finean. Myelin is an ideal material for this purpose; it can be easily isolated in a pure state, has a simple composition and regular repeating features providing good diffraction patterns.

In Table 6.2 we have tried to indicate that from the point of view of composition at least, there is no such thing as a typical membrane. With such widely differing compositions it is unreasonable to expect a universal structure. Myelin, the structure of which lent most support to the unit membrane theory, seems to be the most atypical.

6.5.2 The lipid–globular protein mosaic model now represents the best overall picture of membrane structure

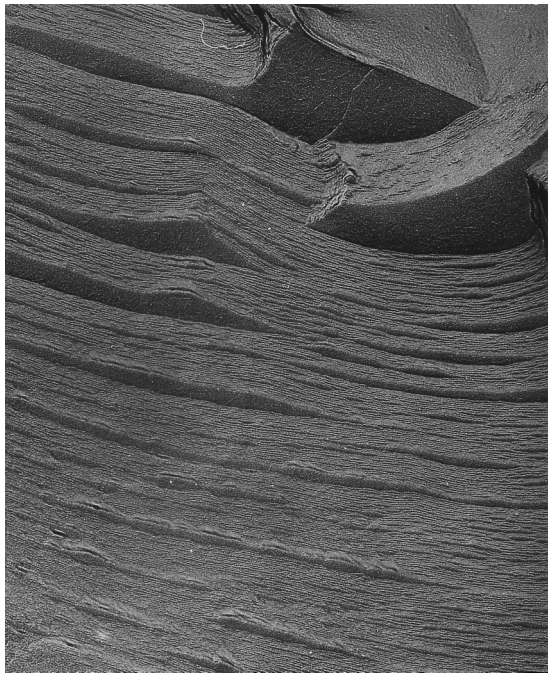
Implicit in the bimolecular leaflet model is the idea that the protein is spread as an extended sheet (β-conformation) over the ionic heads of the phospholipids and that the binding between phospholipid and protein is essentially electrostatic. Several observations about membrane lipids and proteins were not consistent with this model.

First, the protein component of many membranes represents well over 50% of the bulk of the membrane material. The average amino acid composition of membrane proteins shows no marked preponderance of ionic or hydrophobic residues. Thus, if the protein were extended as a sheet, a significant proportion of the hydrophobic groups would have to be in contact with the water.

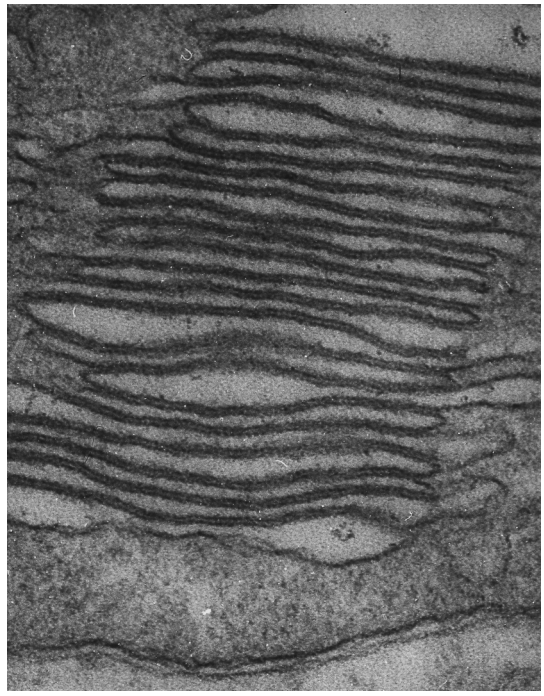
Secondly, increasing the ionic strength of the medium surrounding the membrane does not dissociate a large fraction of membrane proteins from lipids as would be expected if electrostatic interactions were predominant.

Thirdly, when the lipid moiety is removed, the proteins are not very soluble in water; in fact they tend to interact hydrophobically with one another.

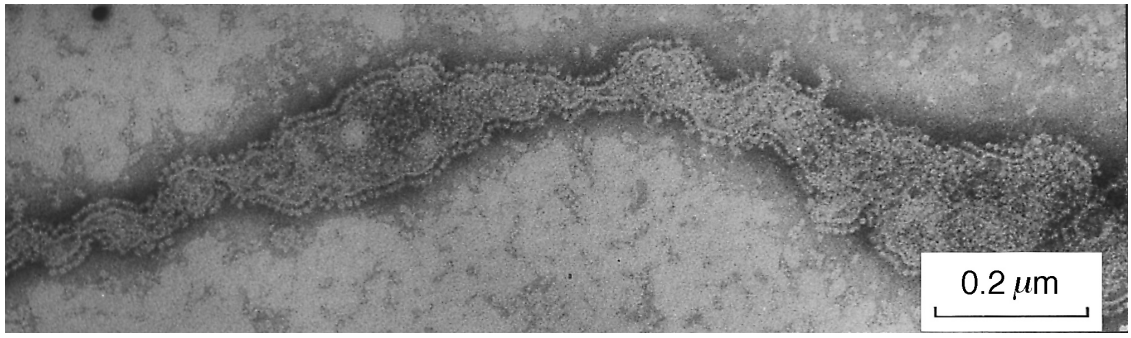
Lastly, most membrane lipids are zwitterionic rather than having a net charge and would have no strong electrostatic interactions. Those that are charged tend to be acidic and preferably interact with predominantly basic proteins, which are not common in membranes.



(a)



(b)



(c)

Fig. 6.10 Examples of some different electron microscopic techniques used for examining membrane preparations. (a) An electron micrograph of a 2% dispersion of dioleoyl phosphatidylcholine in water. This is an example of the 'freeze-etching' technique and illustrates the way in which phosphatidylcholine molecules take up a lamellar configuration when dispersed in water. Magnification is ×98 000. (b) An electron micrograph of the chloroplast lamellae of a green narcissus petal. The specimen was fixed in glutaraldehyde osmium, embedded in epon and post-stained with lead citrate. This is an example of the 'positive staining' technique and clearly illustrates the typical 'unit membrane' feature of two dark lines separated by a light band. Magnification is ×142 500. (c) An electron micrograph of beef heart mitochondria membranes. This is an example of the 'negative staining' technique and illustrates the regular array of globular lipid/protein particles (known as 'elementary particles') attached to the membrane. Some detached particles can be seen. The particles correspond to the F1 component of ATP synthase. Magnification ×93 000.

Subsequent observations such as the known rapid lateral diffusion of lipid and protein in the plane of the membrane and knowledge that proteins are often inserted into and through the lipid matrix have been added to the points made above. These considerations have been allowed for in the proposal by Singer and Nicholson in 1972 of their fluid mosaic model for membrane structure (Fig. 6.11). In the diagram an asymmetric lipid bilayer forms the basis of the membrane structure with proteins spanning the membrane or embedded into the hydrophobic core region.

Two techniques in the electron microscopist's armoury have helped to encourage a reappraisal of membrane structure. One is negative staining, in which the sample is not fixed and embedded but dispersed in an aqueous solution of the negative stain (phosphotungstate) and dried down on a support film [Fig. 6.10(c)]. Stain accumulates in the hydrophilic regions. The other method, drastically different from the staining methods and therefore useful to give independent corroboration, is the freeze-etching technique [Fig. 6.10(a)].

The freeze-etching technique allows a three-dimensional view of membrane surfaces to be made. Furthermore, the analogous freeze-fracture method permits visualization of the interior of the membrane bilayer. With both techniques intrinsic membrane proteins can be seen that penetrate into the bilayer or pass through it, just as proposed in the Singer/Nicholson model (Fig. 6.11).

6.5.3 Membrane structure is not static but shows rapid movement of both lipid and protein components

The ability of many membrane lipids to form the basic bilayer structure is caused by a number of properties, the most important of which is their amphipathic character. Amphipathicity is caused by the lipids having a polar or hydrophilic head

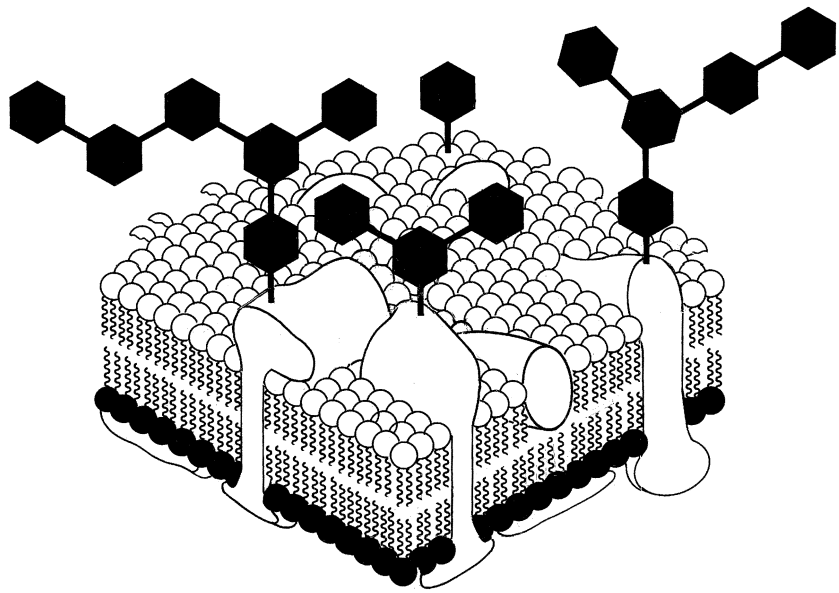


Fig. 6.11 The Singer and Nicholson fluid-mosaic model of membrane structure. The figure shows the topography of membrane protein, lipid and carbohydrate in the fluid-mosaic model of a typical eukaryotic plasma membrane. Phospholipid asymmetry results in the preferential location of phosphatidylethanolamine and phosphatidylserine in the cytosolic monolayer. Carbohydrate moieties on lipids and proteins face the extracellular space. Reproduced with kind permission of Dr. P.R. Cullis and the Benjamin/Cummings Company, from *Biochemistry of Lipids and Membranes*, Benjamin/Cummings, Menlo Park, CA.

group region and a non-polar or hydrophobic part. Thus, such molecules will naturally orientate themselves to ensure that the polar groups associate with water molecules while the hydrophobic tails interact with each other. Provided the molecule in question is roughly cylindrical in dimension and has no net charge then bimolecular planar leaflets will be the most stable configuration in an aqueous system. However, it is also true that many major lipid components of membranes do not form bilayers when isolated and placed into aqueous systems in vitro. Some of the packing characteristics of different lipids and the structures they form are shown in Fig. 6.12.

Israelachvili, in a masterly exposition in 1978, has discussed the theoretical shapes of these structures and the consequences for cells (see Further Reading). In some naturally occurring membranes, a very high proportion of the lipid is capable of adopting the hexagonal II phase (inverted micelles) rather than bilayers. In this arrangement cylinders of lipids are formed with an aqueous interior in contact with the head groups of the lipids (Fig. 6.12). The cylinders interact with each other by hydrophobic forces and this can be clearly seen in freeze-fracture micrographs as a regular corrugated pattern.

In general, biological membranes contain an appreciable fraction (up to 40 mol%) of lipid species that, individually, prefer the hexagonal II arrangement. But it has been discovered that, depending on the acyl chain composition, temperature and head group size and charge, complete bilayer stabilization can be achieved by the addition of 10–50 mol% of bilayer-forming lipid species. Moreover, these properties are displayed without any stabilizing

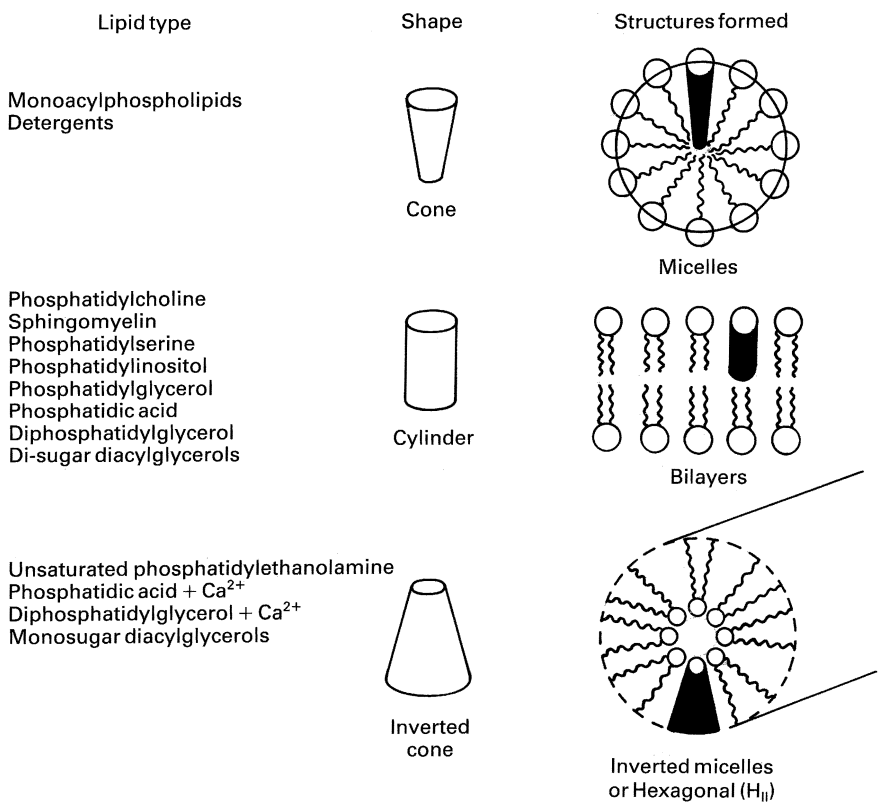


Fig. 6.12 Lipid shapes and their packing characteristics.

effects from membrane proteins. Thus, many bacteria contain unsaturated phosphatidylethanolamine and diphosphatidylglycerol in large amounts while chloroplast thylakoids contain up to 50% of their lipid as monogalactosyldiacylglycerol. Such lipids form hexagonal II phases on their own but, clearly, the bacterial and chloroplast membranes provide good permeability barriers with the vast bulk of the lipid arranged in a bilayer in vivo. Therefore, other factors such as the ionic environment, interaction with other lipids and the contributions of membrane proteins are obviously important.

For certain purposes, it seems important for non-bilayer phases to be present in membranes. A good example is in membrane fusion or in the budding of vesicles. These aspects are discussed in Section 6.5.10.

The fluidity of membranes is dependent on the nature of the hydrophobic moieties of lipids. When lipids are isolated the acyl chains of each type undergo transitions from a viscous gel to a fluid state at a certain temperature. Above the transition temperature, the molecules exist in a liquid-crystalline state where the acyl chains, but not the head group, are fluid. This is the normal state of membrane lipids and ensures proper functioning of the membrane proteins. When the temperature of a cell is lowered, three phenomena can contribute to impairment of membrane function. First, ice crystals formed in the aqueous compartments can cause physical damage. Second, transition of the acyl chains into the gel phase leads to changed enzyme activity and altered transport. Third, temperature lowering can lead to the membrane lipids becoming phase-separated. Concentration of particular lipid types by this phase separation can cause the formation of non-bilayer structures. Lipid adaptations to environmental temperatures are discussed in Section 6.5.9.

6.5.4 Further remarks on the lipid composition of membranes

In Section 6.1, we emphasized that different membranes contain quite different lipid compositions. Because such compositions are maintained generally within quite strict limits, one must presume that a membrane's complement of lipids is particularly adapted to its needs. However, the overall lipid composition of a membrane is itself a simplification which needs some qualification. First, individual lipid classes (e.g. phosphatidylcholine) consist of a large number of molecular species where the combinations and positional distribution of acyl chains differ. The nature of these individual molecular species is important for the physical properties, membrane function and further metabolism of the lipids. Second, lipids are not distributed evenly in membranes. For example, all naturally occurring membranes, which have been examined, show sidedness, i.e. two halves of the bilayer are dissimilar (Section 6.5.5). In addition, lateral segregation has been shown in a few instances or implied from the specific association of certain lipids with particular membrane proteins (Section 6.5.6).

6.5.5 Transbilayer lipid asymmetry is an essential feature of all known biological membranes

The first natural membrane to be examined for asymmetry was that from erythrocytes. Such cells are very convenient for membrane studies since erythrocytes can be easily purified in large quantities and contain only a single membrane. In order to study the two leaflets of such membranes, intact cells were probed (or labelled) in order to establish those components that were in the outer leaflet (i.e. that were accessible). In a second experiment, the erythrocytes were broken by a suitable method (e.g. osmotic shock, sonication) to allow access of the probe to the inner surface as well (Fig. 6.13). Control experiments are also necessary to evaluate the total membrane proteins and lipids, since not all of these may be accessible to the probes used.

Various methods have been used to evaluate the lipid composition of inner and outer membrane leaflets. These are listed in Table 6.9. Enzymic digestion is probably the most common method since it is easily carried out and allows analysis of almost all membrane lipids. A drawback with the method, however, is that removal of lipids from the

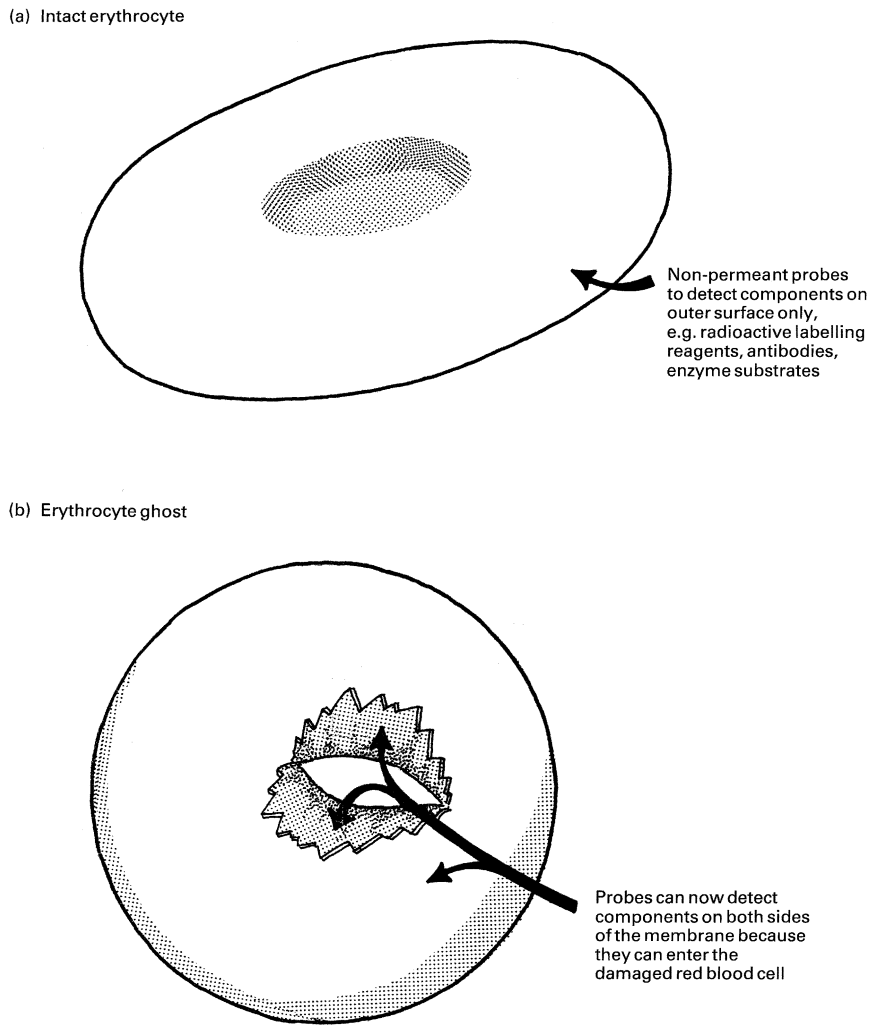


Fig. 6.13 Design of an experiment to establish transbilayer composition in erythrocytes (red blood cells).

outer leaflet enhances transfer of replacement lipids from the inner leaflet (transbilayer movement). In addition, the products of lipid digestion may be detergents, which could themselves alter membrane structure (e.g. lysophospholipids). In some instances, similar methods (i.e. radiolabelling, enzyme digestion, antibody labelling) may be used to analyse membrane proteins and, in addition, if the latter are enzymes, then they can be measured with their substrates.

An important caveat for all experiments to probe membrane sidedness is that the membrane itself is not permeable to the probe, otherwise the latter will, of course, label both sides of the bilayer.

All membranes that have been examined so far show very different lipid (and protein) compositions for their two leaflets. A common feature of mammalian cell membranes is that the aminophospholipids and phosphatidylinositol are concentrated in the cytosolic leaflet (Fig. 6.14). The sphingolipids are highly concentrated in the external leaflet of the plasma membrane. By contrast, in

Table 6.9 Methods used to determine membrane sidedness

Method	Notes
Antibody labelling	Antibodies can be raised to specific proteins or certain lipids. Distribution of antibodies is measured with a radiolabel (^{125}I) tag, a linked enzyme assay or by immunogold visualization.
Enzymic digestion	Specific enzymes are the best to use since large-scale phospholipid or protein digestion can destroy the membrane's structure. Measurement of the decrease in given constituents allows an estimation of their accessibility and, hence, distribution.
Chemical (or radio) labelling	Various compounds that react with primary amino groups (phosphatidylethanolamine, phosphatidylserine, proteins) most used.
Lipid exchange proteins	These allow exchange of specific lipids between the outer leaflet and liposomes in the suspension medium. Thus, the natural membrane composition is retained. By initially (radio) labelling the lipid in liposomes, the amount of the same substance in the outer leaflet can be estimated. Unfortunately, such exchange proteins are few and not widely available.
Assay of enzymic function	Only useful for a limited number of proteins.
NMR	Signals measured in the absence and presence of paramagnetic ions – these act as chemical shift or broadening agents. Results are difficult to interpret and not very specific.

the plant membrane example shown in Fig. 6.14 (castor bean glyoxysome) while phosphatidylethanolamine was mainly in the cytosolic (i.e. outside) leaflet, phosphatidylinositol and phosphatidylserine were mainly in the opposite side. This latter example emphasizes that generalization should not be made from system to system without firm evidence. It should also be noted that the distribution calculated may be biased if significant amounts of lipids are inaccessible to probes and, therefore, cannot be located.

Nevertheless, lipid asymmetry in membranes raises an important question as to how it arises and also has obvious implications for membrane function. The preservation of asymmetry clearly depends on a very low frequency of exchange from one surface to another or a controlled reciprocal exchange.

An interesting example of the importance of lipid asymmetry has emerged recently. All body cavities in mammalian organisms are lined with epithelia. In most cases (e.g. the gastrointestinal and urinary tracts) a single layer of cells is involved and these cells have their plasma membrane divided into two parts. An apical portion, which borders the lumen, is connected to adjacent cells through tight junctions. The remainder of the plasma membrane faces the underlying tissue and is called the basolateral portion. Several studies have shown that the apical portion is significantly less fluid (and, hence, less passively permeable and more mechanically stable) than the basolateral membranes. It turns out that much of the rigidity is due to a very high proportion of glycosphingolipids in the outer leaflet. Such lipids are much more highly ordered than phospholipids owing to their extensive hydrogen-

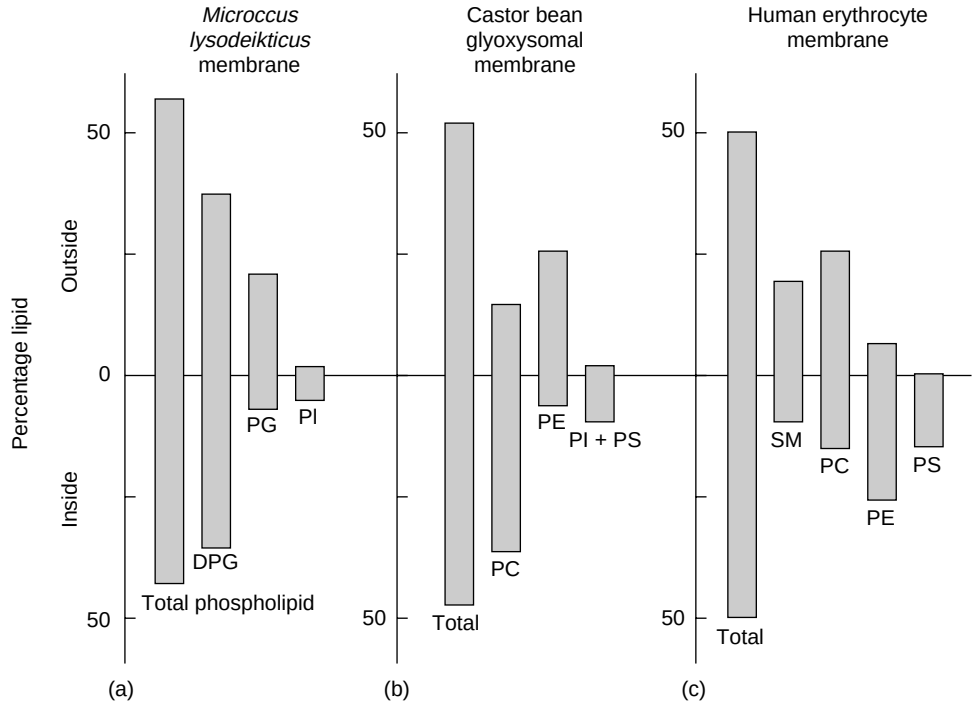


Fig. 6.14 Asymmetry of lipid distribution in a bacterial, plant and an animal membrane. PG = phosphatidylglycerol; PI = phosphatidylinositol; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PS = phosphatidylserine; SM = sphingomyelin; DPG = diphosphatidylglycerol.

bonding capacity. Division of the plasma membrane into apical and basolateral parts is also an example of lateral heterogeneity (below).

6.5.6 Lateral heterogeneity is probably important in some membranes at least

So far, techniques have not been developed that allow us to examine the distribution of lipids within the plane of most membranes. However, in a few cases the obvious morphological differences in certain regions of membranes can be exploited to allow sub-fractionation and afterwards lipid analysis. Thus, chloroplast thylakoids can be divided into the appressed and non-appressed membranes. (More detail can be found in the review by Gounaris and colleagues in Further Reading.) 'Appressed' membranes are sometimes referred to as 'stacked' membranes and have their outer surfaces close together, rather than facing the stroma as in 'non-appressed' or 'non-stacked' membranes. These membranes are known to have very different protein compositions and this introduces different charges on to the membrane surfaces. The membrane vesicles deriving from appressed or non-appressed regions can then be separated using two-phase partition systems. Analysis of such membranes showed that, in contrast to the very different protein contents, the lipids are rather similar. The ratio of lipid to protein is, however, very different with non-appressed regions being enriched 3–4-fold in lipid compared to appressed membranes.

In photosynthetic bacteria, where the cytoplasmic membrane becomes infolded to produce photosynthetic membranes (Fig. 6.1) the two regions can be separated. Here, there is a clear difference in lipid composition with the photo-

synthetic membrane being enriched in phosphatidylglycerol compared to the cytoplasmic membrane. In addition, the carotenoid and bacteriochlorophyll pigments are exclusive to the photosynthetic membrane though this probably reflects their binding to specific proteins rather than a special property of the lipids themselves.

Micro-lateral heterogeneity is present in membranes where particular lipids are found tightly bound to individual proteins. The clearest examples of this are the pigment–protein complexes of photosynthetic organisms. However, certain purified proteins appear to be highly enriched in specific lipids which are very tightly bound and survive solubilization and purification steps in the presence of detergents. Thus, chloroplast ATPase from spinach and the green alga *Dunaliella salina* have been reported to contain highly saturated molecular species of sulphoquinovosyldiacylglycerol. In addition, cytochrome c from mitochondria is a very basic protein that in vivo has been suggested to interact with diphosphatidylglycerol. However, in many other cases, what were thought to be specific interactions with proteins turn out to be general properties of lipids, which can be replaced by artificial compounds or even detergents while still preserving functional activity. The functions of lipids in providing permeability barriers (Section 6.5.8) interacting with proteins (Section 6.5.11), aiding membrane fusion (Section 6.5.10) and in drug delivery (Section 6.5.13) are discussed later.

6.5.7 Physical examination of membranes reveals their fluid properties

Fluidity in membranes is an easily visualized phenomenon and is a term that is widely used. However, it can be misleading. For example, it is commonly assumed (and stated) that making membrane lipids more saturated or adding cholesterol makes a membrane less fluid. The assumption is that such alteration will reduce the speed of movement of lipids. However, introduction of cholesterol into phosphatidylcholine model membranes has no effect on lateral movement and may actually increase rotational diffusion rates. What cholesterol and increased saturation do is to increase the order in the hydrocarbon matrix and this is what can be measured easily by NMR or ESR order parameters.

Spectroscopic studies have demonstrated that lipid molecules in membranes normally show considerable freedom of movement. Lipids can rotate, bob up and down in the plane of the bilayer, show fluid movement down the hydrocarbon chain and, to a small extent, undergo flip-flop (i.e. movement from one leaflet to the other). Flexing of the hydrocarbon chains occurs fastest with the methyl ends of the chains undergoing the most movement. Rotation of the molecule is also fast (say about every 10^{-7} s in artificial systems) but flip-flop is much slower (Fig. 6.15) and preserves the bilayer asymmetry of natural membranes.

Although it is accepted generally that membrane lipids show considerable movement, their rate of flip-flop, in particular, can be influenced by proteins. For example, when the cytoskeleton proteins of erythrocytes are modified their transverse asymmetry is changed. Moreover, the sided distribution of lipids in artificial membranes can be changed by posing a transmembrane pH gradient and this redistribution may also occur in vivo. Furthermore, specific proteins, such as an aminophospholipid translocase, have been identified that assist the movement of specific lipids across bilayers.

6.5.8 General functions of membrane lipids

Detailed discussion of membrane properties and functions is beyond the scope of this book but numerous detailed reviews are available. Nevertheless, a few remarks centred on the role of lipids can be made. In the previous sections, we discussed current ideas for membrane structure and the concept that, although lipids are capable of adopting non-bilayer structures when isolated, by and large they participate in bilayers in vivo.

Under normal conditions the membrane bilayer is in a 'fluid' state. Membrane proteins can migrate within the plane of the membrane with diffusion

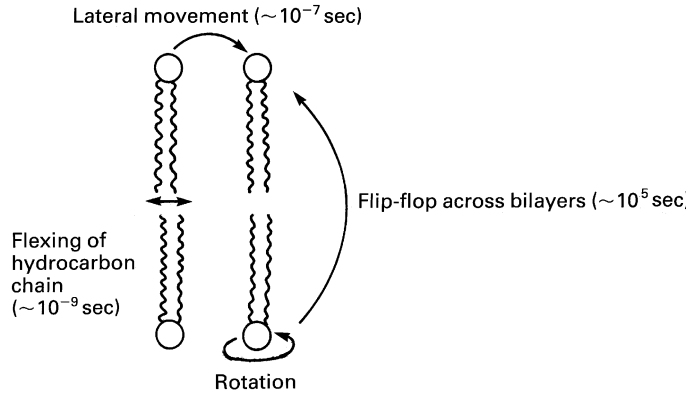


Fig. 6.15 Lipid movement in an artificial membrane.

coefficients of about $10^{-10}\,cm^2\,s^{-1}$ while lipids diffuse with coefficients of about $10^{-8}\,cm^2\,s^{-1}$. Overall behaviour might be considered, therefore, in thermodynamic terms. But generalized deductions relating the fluidity of the membrane to enzyme activity are difficult to make for several reasons. For example, motion in a given lipid molecule may include rapid rotations, but slow lateral movement. Also, increased disorder in a bilayer may not correlate with increased translational motion. Moreover, all membranes so far examined have shown transbilayer asymmetry while there is evidence in several cases for at least small areas of concentration of certain lipids, i.e. micro-lateral heterogeneity (Section 6.5.6). These sorts of consideration complicate the interpretation of experiments designed to show how the bilayer lipids affect membrane proteins at a molecular level.

Enzymologists frequently study how reaction rates, substrate binding, etc. change under a variety of experimental conditions. Parameters that may be changed include pH, ionic concentration and the addition of water-soluble inhibitors. However, the membrane protein is only probed in regions where it is exposed to bulk water or at the interfacial region where such water meets the bilayer. This often only represents a small part of the whole membrane protein.

Lipids can interact with proteins in several ways. First, there are interactions with the hydrophobic proteins that penetrate into or through the bilayer. These are interactions with the membrane's integral or intrinsic proteins. Secondly, the water-soluble extrinsic proteins can interact electrostatically with the charged head groups of the membrane (phospho)lipids. Thirdly, covalent lipid modifications (such as palmitoylation; see Section 6.5.14) may take place and these will help proteins to associate with and/or become anchored to the membrane. Some basic types of arrangement of membrane proteins are indicated in Fig. 6.16.

Interactions of lipids with a variety of extrinsic proteins have been studied. The proteins that have been studied (e.g. cytochrome c, spectrin) are usually basic molecules that interact with negatively charged lipids. Even if no negatively charged lipid is tightly bound to these proteins, they induce a time-averaged enrichment of negatively charged lipids in their vicinity. However, this has not been shown to lead to the formation of crystalline domains via local fluidity changes. Indeed, there may be an increase in fluidity due to a disturbance of the hydrophobic interactions of the acyl chains in the bilayer. In addition, divalent cations compete with basic proteins for the negatively charged head groups. In fact, both calcium and basic proteins are able to induce the formation of hexagonal II phases in micelles of diphosphatidylglycerol. This property may explain the ability of cytochrome c to induce non-bilayer structures in diphosphatidylglycerol-containing systems and also to translocate rapidly across bilayers such as the inner

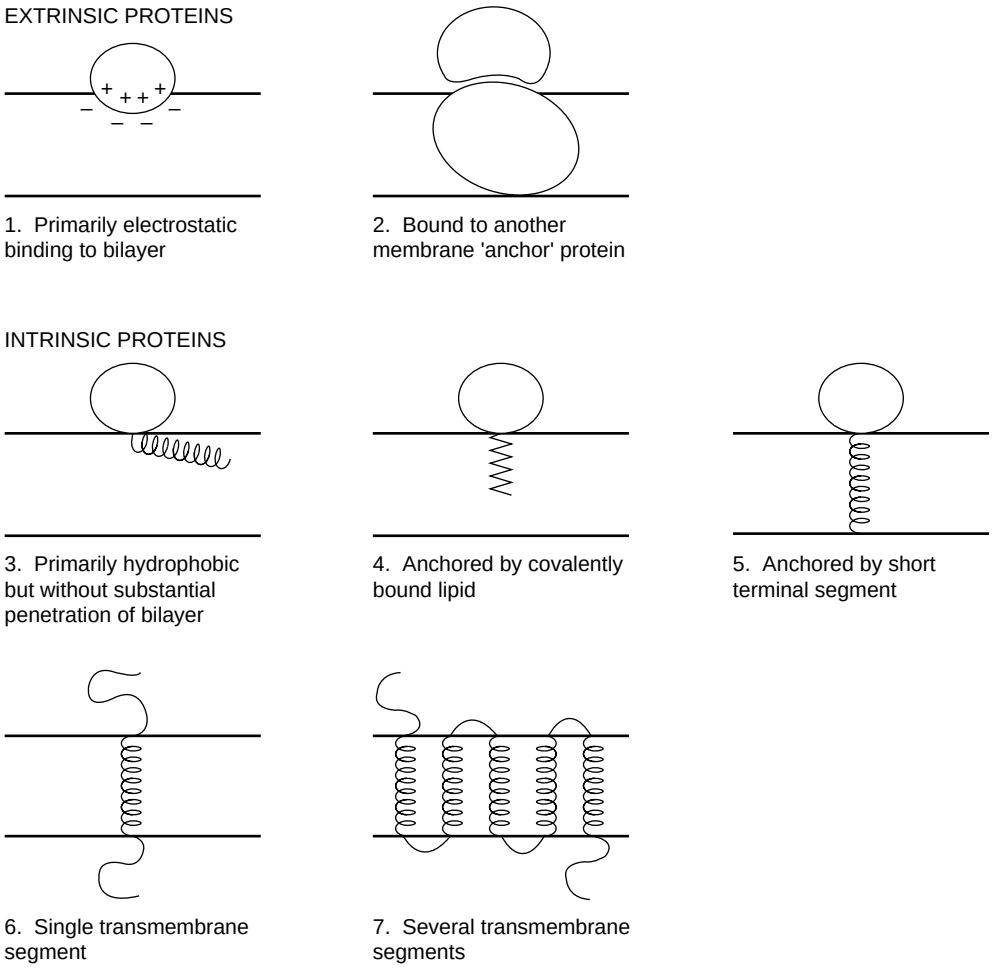


Fig. 6.16 Arrangement of proteins in membranes.

mitochondrial membrane (which contains high amounts of diphosphatidylglycerol).

To remove intrinsic proteins from a membrane it is necessary to solubilize the molecules using detergents that can break the hydrophobic interactions with lipids. The lipids adjacent to the proteins experience a different environment from the bulk lipid of the membrane and this has led to the concept of boundary lipids. ESR (electron-spin resonance) studies showed that lipids residing at the lipid–protein interface exhibited increased-order parameters (restricted motion) in the acyl chain region. Recent experiments, however, paint a different picture. First, the lipid–protein interactions in general appear to be relatively non-specific with most isolated integral proteins capable of being reactivated by a whole host of membrane lipids. Secondly, the evidence for a long-lived boundary of lipid is less convincing when further experiments are considered. When NMR (nuclear magnetic resonance) data are used, boundary–bulk lipid exchange appears rapid. This reflects the time-scale of NMR ($\sim 10^{-6}$ s) versus ESR ($\sim 10^{-10}$ s) measurements.

Although the general concept of long-lived boundary lipids surrounding intrinsic proteins

does not appear to be viable at present, there are well-documented cases where particular lipids are associated with individual proteins. For example, the purple membranes of *Halobacterium halobium*, apart from having a rigidly defined protein structure, also contain a unique lipid composition. The lipids appear to be interdigitated between the protein rods and have a composition quite distinct from the rest of the membrane.

Membrane proteins that clearly have an important part spanning the bilayer are those concerned with transport. These molecules must have at least two portions: (1) a domain, such as a channel, that allows the movement of the substrate vectorally across the membrane; (2) a binding site for the substrate that confers selectivity and also, when occupied, initiates transport. There may also be regulatory domains allowing the opening of the channel gate. Each of these three parts could be modulated by lipids in bilayer.

An example of the type of information obtained for lipid–protein interactions with a transporter is that derived from the experiments conducted by Melchior and Carruthers on the erythrocyte sugar transporter. The protein is particularly useful because it can be purified and reconstituted into lipid vesicles of different composition and the exact number of reconstituted transporters determined through the binding of radiolabelled cytochalasin B, a specific inhibitor. Furthermore, the kinetic properties, complete amino acid sequence and arrangement of the protein in erythrocyte membranes are known. Comparison of transport rates with the number of reconstituted transporters allowed estimation of the turnover number (T_n), and allowed comparison between different lipids used for the reconstitution (Table 6.10). It was found that several factors influenced transport rates. In order of importance these were: lipid head group > lipid acyl chain length and saturation index > lipid backbone >> bilayer fluidity. Negatively charged phosphoglycerides such as phosphatidylserine were the most active for reconstruction and suggested that the normal tertiary structure of the translocating domain might need a high surface potential. In fact, for the best lipids, such as phosphatidylserine, the fluidity of the bilayer had little effect on transport rate.

In contrast, for a lipid showing slower turnover numbers, such as phosphatidylcholine, transporter T_n increased markedly when the bilayer changed from the crystalline to the gel state. Thus, the nature of the lipid head group(s) influenced the degree to which bilayer fluidity altered transport activity.

The length of the acyl chains (and hence mem-

Table 6.10 Factors influencing the activity of the erythrocyte sugar transporter in reconstituted lipid vesicles

Factor and effect	Experimental result
Nature of the phospholipid head group (alters surface charge/potential)	Vesicles composed of single phospholipids had the following order of activity: PS>PA>PG>>PC.
Acyl chain length (important to be correct for size of transmembrane protein portions)	For saturated chain acyl chains: C18>C20>C16>C14. For C18 chains: stearoyl>elaidoyl>oleoyl.
Bilayer fluidity (affects immediately environment of hydrophobic domains)	Relatively little change in activity on going from gel to crystalline phases except for micelles containing PC.
Cholesterol inclusion (affects lipid lateral mobility)	Lipid packing alterations correlate with transporter activity changes.

brane thickness) was also important – not surprisingly because the sugar transporter is known to contain 12 membrane-spanning hydrophobic domains. In effect the thickness of the membrane has to be right for the correct arrangement of these hydrophobic domains.

Of course, natural membranes contain not just a single lipid species but a whole host of different molecular types. Thus, experiments to determine the effect of lipids on membrane proteins in vivo are extremely difficult to interpret. In animal membranes, cholesterol is frequently present in addition to phosphoglycerides. Addition of cholesterol to a lipid bilayer induces complex alterations in lipid packing. McConnell and colleagues at Stanford showed that these alterations in lipid packing were associated with altered lateral mobility within the bilayer. In experiments with the sugar transporter referred to above, changes in transporter activity correlated closely with cholesterol-induced alterations in lipid packing. They did not, however, apparently result from cholesterol-induced changes in bilayer lipid order. Experiments with other systems (such as the Ca^{2+}-Mg^{2+}-ATPase from endoplasmic reticulum) have also shown no consistent correlation in bilayer fluidity and enzyme activity. Once more, the length of the acyl chains seemed a more important parameter.

6.5.9 Membrane lipids are modified to maintain fluidity at low temperatures

We have already seen that membrane lipids can exist in the gel – or liquid – crystalline phases, depending on the prevailing temperature. Whereas isolated lipid samples show a sharp gel–liquid phase transition, naturally occurring lipids show broad non-cooperative transitions due to the heterogeneity of the acyl chains. For a series of molecular species of, say, phosphatidylcholine, the temperature (T_c) for the gel–liquid phase transition depends on the fatty acid composition (with *cis*-unsaturated fatty acids lowering T_c) (see Table 6.11 and Fig. 6.17) and even on the position of acyl esterification.

It was thought that the membranes of natural organisms could not function when their lipids were in the gel–crystalline phase and that changes in growth or other physiological parameters seen in prokaryotes on lowering temperature could be explained simply by proposing that phase transitions had taken place. However, it is now clear that while a correlation can sometimes be seen between, for example, enzyme activity changes and the temperature for membrane lipid phase changes, this relationship is by no means simple nor the correlation universal. Nevertheless, as a general rule it can be stated that once significant areas of a

Table 6.11 Effect of fatty acyl groups on the temperature (T_c) of the gel to liquid-crystalline phase transition of phosphatidylcholines

Lipid species	T_c (°C)
(a) Saturated	
Dimyristoyl-PC (14:0, 14:0-PC)	24
Dipalmitoyl-PC (16:0, 16:0-PC)	41
Distearoyl-PC (18:0, 18:0-PC)	55
(b) Unsaturated	
Palmitoyl, oleoyl-PC (16:0, 18:1-PC)	−1
Dioleoyl-PC (18:1, 18:1-PC)	−19
Stearoyl, oleoyl-PC (18:0, 18:1-PC)	6
Stearoyl, linoleoyl-PC (18:0, 18:2-PC)	−16
Stearoyl, linolenoyl-PC (18:0, 18:3-PC)	−13
Stearoyl, arachidonyl-PC (18:0, 20:4-PC)	−13

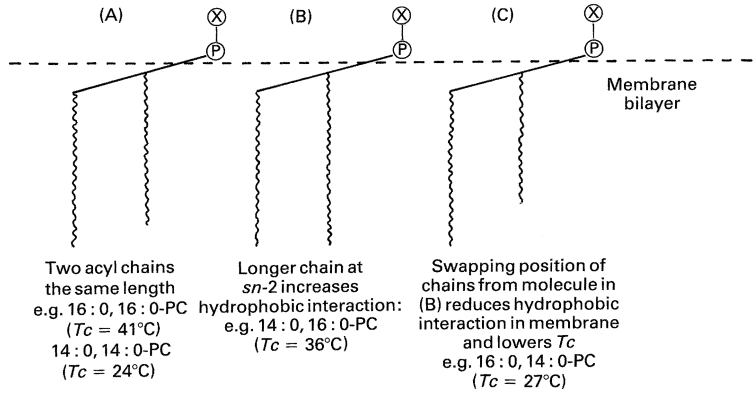


Fig. 6.17 The basis for re-tailoring of lipids during temperature adaptation.

natural membrane are in the gel state then function is significantly impaired. It is known, for example, that as temperatures are lowered phase separation of certain lipid species takes place and membrane proteins are squeezed out of such areas leading to the formation of protein-free areas, which can be seen by electron microscopy. Studies with various fatty acid-requiring mutants of *Escherichia coli* have shown that if less than half of the lipids of the cytoplasmic membrane are fluid then it becomes non-functional and the bacterium dies.

Thus, poikilotherms (organisms unable to regulate their own temperatures), which grow at low temperatures, have a pattern of membrane lipids possessing lower phase transition temperatures than those of the same organisms existing under hotter conditions. Furthermore, following changes in environmental temperature, an alteration in the composition of membrane lipids is seen. Although an increase in fatty acyl unsaturation at lower temperatures is best known, it is not, by any means, the only change that can take place.

The process of temperature adaptation has been studied in some detail in cyanobacteria by Murata's group and in the protozoon *Tetrahymena* and the alga *Dunaliella* by Thompson in Texas. A number of lipid changes has been observed by these workers and others (Table 6.12), but they occur on a different time-scale. A sudden change in growth temperature could immediately alter membrane fluidity with harmful consequences for the organism concerned. Murata has proposed that an emergency response involves re-tailoring (altering the position of fatty acid acylation) of molecular species. Such re-tailoring has been shown in vitro to lead to significant differences in the T_c of the lipid concerned. This is because the glycerol backbone of a membrane lipid is not exactly parallel to the surface of the bilayer and, hence, two acyl chains of equal length penetrate the hydrophobic membrane region to a different extent (Fig. 6.17). After the emergency response has occurred, other changes in lipid composition can take place on a longer time-scale. For organisms operating the aerobic pathway for desaturation an increase in *cis*-unsaturation is a relatively quick and easy modification to make. In contrast, bacteria such as *E. coli* could only increase their unsaturation by *de novo* synthesis of more palmitoleate and vaccenate (Fig. 2.8). Such micro-organisms have been known, however, in some cases to lower the T_c of any given lipid by shortening the acyl chain. Some bacilli can even reduce the degree of unsaturation of membrane lipids by hydrogenating double bonds in response to increased environmental temperatures (Section 2.26). The slowest changes to take place are those involving membrane lipid proportions or the ratio of lipid to protein in the membrane. These alterations might include, for example, an increase in lipid to protein ratio in the plasma membranes of cold-stressed plant roots (Table 6.12).

The process of low-temperature adaptation has

Table 6.12 Changes in membrane composition caused by lowered growth temperatures in different poikilotherns

1. Re-tailoring of molecular species	Quick change involving swapping acyl chains on the glycerol backbone.
2. Increase of fatty acyl unsaturation	Relatively quick for organisms using the aerobic pathway of desaturation; involves *in situ* modification of lipids.
3. Chain shortening	Relies on the lower melting temperatures of shorter acyl chains; relatively uncommon to see shortening of existing acyl chains; same net effect more commonly seen when *de novo* synthesis produces shorter chain products
4. Alteration of fatty acid type	Some micro-organism may alter amounts of branched-chain acids – this will also modify fluidity.
5. Lipid classes altered	Changes in membrane lipid classes often seen; where phospholipid interconversion is easy (e.g. PE→PC) then this could be adaptive because PC has a lower T_c than PE for the same molecular species.
6. Lipid-to-protein ratio changed	Usually the ratio is increased significantly at lower temperatures – due to increased net lipid synthesis.

been studied in some detail in the soil protozoon, *Acanthamoeba*. This unicellular animal has an important role in grazing soil bacteria and, hence, keeping their numbers roughly constant. It is also unusual (for an animal) in being able to synthesise linoleic acid (Section 2.2.5). When *Acanthamoeba* is cooled, a succession of events occurs (Table 6.13) involving increased gene expression of the oleate desaturase that forms linoleate. In turn this leads to increased enzyme activity, membrane fluidity changes and a renewed ability to phagocytose.

An interesting application of membrane lipid biochemistry to a natural phenomenon has been made by Murata in Okasaki. He has examined the process of frost sensitivity in plants. It is well known that, whereas some plants can survive low or, indeed, freezing temperatures, many others drop their leaves and/or are killed by the first cool nights of autumn. It seemed logical to look for differences in the membrane composition of these two groups of plants to explain the difference. A superficial examination showed that both types of leaves contained the same classes of lipids with the usual exceptionally high content of linolenic acid. Murata noticed, however, that the phosphatidylglycerol fractions from frost-sensitive as opposed to frost-resistant plants were markedly different. Thus, frost-sensitive species had a much higher proportion (40–80%) of the 'disaturated' species, which had T_c values of about 40°C. (For this purpose the unusual *trans*-3-hexadecenoic acid, which is present in phosphatidylglycerol and which has a similar melting point to palmitic acid, is classed as saturated.) In contrast, frost-resistant leaves only had about 15% of the disaturated species. Murata, therefore, proposed that the basis for frost sensitivity lay in the phase separation of a significant quantity of phosphatidylglycerol during exposure to low temperatures – this leading to membrane damage, leakage of intra-organellar contents and cell death. The experiment also emphasized how important it is to examine lipid composition in detail rather than merely taking average values.

Table 6.13 Sequence of events to restore membrane function on cooling *Acanthamoeba* (a poikilotherm)

1. Drop in environmental temperature causes loss of membrane fluidity and this is sensed by the animal (protozoon).
2. Induction of transcription of Δ12-fatty acid desaturase gene caused.
3. Activity of Δ12-desaturase causes oleate in membrane lipids to be converted to linoleate.
4. Membrane lipid fluidity is restored by increased unsaturation of acyl chains.
5. Phagocytosis recommences and *Acanthamoeba* can feed, grow and reproduce again.

More recently, Murata has engineered plants to become low-temperature tolerant. This has been done in two ways. Either the ability to form unsaturated fatty acids has been increased by cloning desaturase genes into the plants or, alternatively, the pathway of phosphatidylglycerol synthesis has been modified in order to increase the production of molecular species containing unsaturated fatty acids. In both cases, the transgenic plants are low-temperature tolerant – thus showing that genetic modification of membrane lipids can be used to create useful new crop varieties.

In further experiments with Hungarian coworkers Horvath and Vigh, Murata suggested that the 'trigger' for increased gene expression when organisms are cooled can be the physical state of the membrane itself. Thus, reduction in the membrane 'fluidity' either by cooling or by chemical modification caused increased expression of various desaturase genes. Therefore, organisms have the ability to self-regulate their membrane fluidity.

6.5.10 Lipids and membrane fusion

Membrane fusion is a very common process in cells. In eukaryotic organisms, many different fusion events can occur at any given time, participating in such diverse functions as secretion, endocytosis, intracellular digestion in lysosomes, cell division and the adjustment of mitochondrial numbers to a cell's energy requirements. Just a short consideration of the overall events of fusion will make it obvious that, during the process, the normal lipid bilayer structure must disappear.

First of all, biochemists studied the process in model systems, employing lipid vesicles. It has been recognized for some time that such vesicles can be induced to fuse when incubated in the region of their gel to liquid-crystalline transition temperature. Furthermore, it was also noted that Ca^{2+} addition would induce fusion in phosphatidylserine-containing systems, possibly by inducing phase separation of the lipid and, hence, a local crystalline point for fusion. However, it was also noted that in more complex mixtures, Ca^{2+} was not always able to induce fusion.

An important clue as to the mechanism of lipid membrane fusion has come from the work of de Kruijff and Cullis. They proposed that fusion proceeded because of the ability of lipids to undergo polymorphism (i.e. to adopt different structures). Three types of observation support the hypothesis. First, fusogens (such as monoacylglycerols) induce H_{II} (inverted micellar structures; Fig. 6.12) phase structures, consistent with a role for non-bilayer structures in fusion. Second, promotion of fusion of, for example, phosphatidylserine-containing systems by Ca^{2+} is accompanied by H_{II} structures. Third, a number of factors, such as pH changes or elevated temperatures that cause H_{II} formation, also promotes fusion of lipid vesicles.

Siegel and coworkers have examined fusion in more detail particularly in relation to two possible mechanisms – via inverted micelle intermediates or by stalk structures (Fig. 6.18). They produced evidence for either process being important although more recent experiments favour the stalk hypothesis. In either mechanism, however, non-lamellar lipid phases are initially involved. Furthermore, artificial membranes with inverted cone-shaped lipids (Fig. 6.12, supporting positive curvature) added to the *cis*-sided leaflets block stalk formation whereas their addition to *trans*-leaflets promotes pore opening. Cone-shaped lipids (Fig. 6.12) have the opposite effects. These results illustrate the potential for different-shaped lipids to influence membrane stability in various structures (Section 6.5.11).

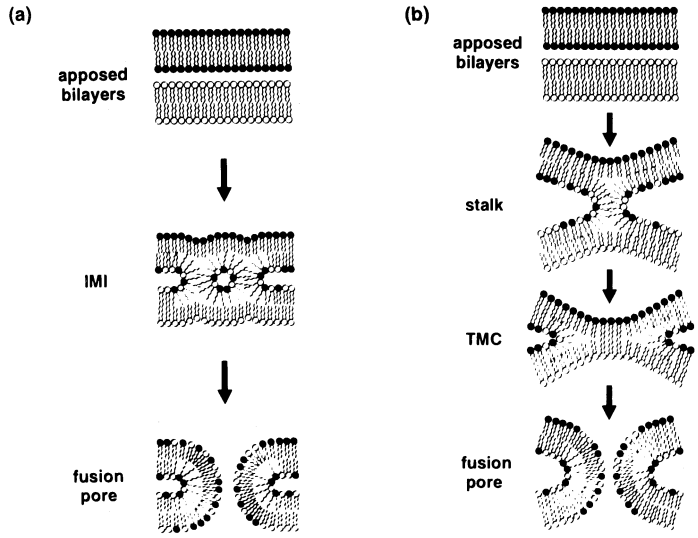


Fig. 6.18 Two possible mechanisms for membrane fusion. In method (a) inverted micellar intermediates (IMI) are involved and, in (b), the formation of a stalk. Reproduced from Cullis *et al.* (1996) with kind permission of the authors and Elsevier Science.

Extension of the Cullis–de Kruijff hypothesis to natural membrane events has been difficult to prove. Nevertheless, confirmatory evidence has been obtained with a number of systems such as the release of chromatin granule contents during stimulation of the adrenal medulla. The exocytosis accompanying this event seems to depend on the ability of Ca^{2+} to promote non-bilayer structures.

Evidence from freeze-fracture microscopy has also implicated hexagonal II phases in two natural membrane systems where a kind of 'arrested fusion' exists. These are in the tight junctions between epithelial and endothelial cells and in the contact sites between the inner and outer membranes of mitochondria or of Gram-negative bacteria like *E. coli*.

Fusion of biological membranes is now thought to require the action of specific fusion proteins. Of these, the viral fusion proteins that help the virus fuse with the host cell membrane during infection are the best understood. These proteins undergo dramatic and spontaneous conformational changes upon activation. For the influenza and the human immunodeficiency (HIV) viruses, the fusion peptide inserts itself into the membrane and then reorients itself thus forcing the fusion membranes together and allowing lipid mixing.

Fusion of intracellular eukaryotic membranes involves several protein families. These are termed SNAREs [soluble NSF (*N*-ethylmaleimide *S*ensitive *F*actor) receptors], SM proteins (Sec 1/Munc 18 homologues where, for example, Sec derives from *sec* mutants of yeast that have temperature-sensitive blocks in the secretory pathway) and Rab proteins (small GTPases, which in yeasts genetically interact with SNAREs and SM proteins). The latter probably function in the initial membrane contact connecting the fusion proteins, but are not involved in fusion itself. Although there is much genetic and structural information about these three components, which are essential for fusion (evidence from gene deletions or mutations), exactly what they do in the process is still unclear. Furthermore, although the mechanism of membrane fusion seems broadly similar in different situations, there must clearly be specificity to proteins such as in intracellular membrane traffic.

In the foregoing section we have suggested ways in which cells could initiate membrane fusion through allowing areas of hexagonal II phase.

Conversely, there may be situations where the synthesis of large amounts of hexagonal II-forming lipids could destabilize the normal bilayer structure. Thus, when the anaerobic bacterium *Clostridium butyricum* is grown in the absence of biotin (when it is reliant on exogenous fatty acids) its membrane lipid composition can be made more unsaturated by supplying *cis*-monounsaturated acids rather than saturated ones. Since phosphatidylethanolamine and its plasmalogen are major membrane lipids in the bacterium, and their unsaturated species form the hexagonal II phase (Fig. 6.12) an increase in unsaturation might destabilize the membrane. *C. butyricum* reacts by reducing the proportion of phosphatidylethanolamine and increasing that of its glycerol acetal. Since the latter forms bilayers then this change restabilizes the membrane structure. Similarly, in *Acholeplasma laidlawii* where monoglucosyldiacylglycerol and diglucosyldiacylglycerol are major membrane lipids, the former is hexagonal II-forming while the latter gives bilayers (Fig. 6.12). Under conditions such as increased temperature or unsaturation, which would tend to favour the formation of non-bilayer structures, the monoglucosyl-lipid is converted to diglucosyldiacylglycerol. This change in lipid proportions would again be expected to preserve membrane bilayer stability.

6.5.11 Lipids and proteins interact in order to determine membrane structure and shape

As discussed in Section 6.5.3 (Fig. 6.12) the shape of complex lipid molecules can give rise to polymorphism. In addition, they may also contribute to curved regions of bilayers. Israelachvili suggested that, for regions of high curvature (such as at the ends of thylakoid stacks in chloroplasts) cone-shaped lipids would be needed in one leaflet and inverted cone-shaped lipids in the other leaflet (Fig. 6.19). This is an attractive idea but examination of the transbilayer distribution of chloroplast lipids by various techniques has shown that the enrichment of monogalactosyldiacylglycerol (inverted cone) is the opposite of that required, i.e. it is more abundant in the outer leaflet. Nevertheless, because it is entirely possible that a small proportion of certain lipids may be concentrated laterally in certain membrane regions, it is still plausible that lipids alone may play a significant role in determining natural membrane shape. Experiments with whole membrane fractions would not pick up such a specific distribution, of course, but would merely provide an averaging of transbilayer composition. Indeed, some of the results with differently shaped lipids during membrane fusion experiments (Section 6.5.10) would support a role for different lipids in helping to stabilize regions of high membrane curvature.

Much more likely is the co-operation of membrane lipids and proteins in controlling membrane shape and properties. For example, membranes containing very large integral protein complexes often contain high amounts of lipid able to form H_{II} phases when purified. Thus, chloroplast thylakoids contain around 50% of the non-bilayer-forming monogalactosyldiacylglycerol while the photosynthetic membrane of purple non-sulphur bacteria is enriched in unsaturated phosphatidylethanolamine (also H_{II}-forming). It has been suggested that these inverted cone-shaped lipids may help to

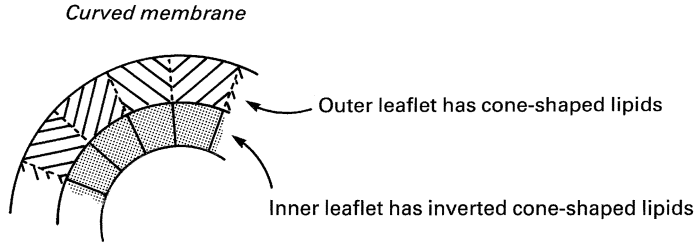


Fig. 6.19 How lipids could cause bilayer curves.

package the integral proteins and ensure a good bilayer structure, which must of course be impermeable to ions and other molecules.

A situation where proteins and lipids appear to interact in determining membrane properties is in the erythrocyte. This simple cell can have its membrane composition altered in a non-destructive fashion by the use of phospholipid exchange proteins. The Dutch biochemists op den Kamp and van Deenen and their colleagues introduced specific phosphatidylcholine molecules into the outer leaflet by such means and showed that, depending on the molecular species substituted, the cells became more or less fragile and displayed susceptibility to osmotic shock. They could lose their flexibility and show an altered ion permeability. A surprising finding was that substitution with certain species resulted in a changed cell shape (Fig. 6.20). These results were explained by assuming that replacement of the natural slightly cone-shaped phosphatidylcholine molecules by the cylindrical dipalmitoyl species would cause the membrane to become convex while enrichment with the cone-shaped dilinoleoyl species would alter the shape to concave (Fig. 6.20).

Sickle cell anaemia is characterized by an amino-acid substitution of the β-chain of haemoglobin. The erythrocytes have a sickle shape and have changes in their membrane characteristics. Studies with such cells (including experiments on repeated sickling/unsickling) suggest that sickling results from a local uncoupling of the lipid bilayer and the cytoskeleton. This leads amongst other things to a very much increased transbilayer movement (flip-flop) of lipids. In agreement with this suggestion it has been noted that membranes that do not contain a strictly organized cytoskeleton, such as the endoplasmic reticulum of rat liver or the plasma membrane of Gram-positive bacteria, also show relatively fast bilayer lipid movement.

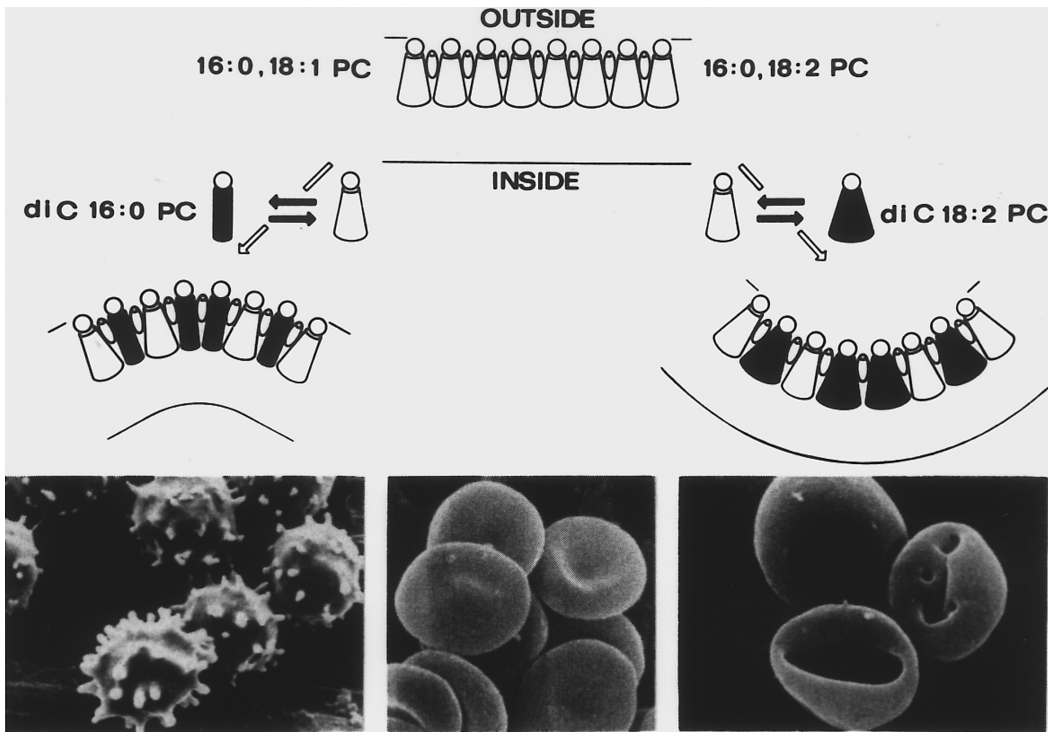


Fig. 6.20 The relationship between lipid composition and erythrocyte shape. Reproduced with kind permission of Professor L.L.M. van Deenen and Elsevier Trends Journals, from *Trends in Biochemical Sciences* (1985), p. 322, Fig. 3.

Other experiments with erythrocytes also confirm the interaction of protein and lipids. In patients with hereditary pyropoikilocytosis the spectrin dimers cannot associate into the normal tetramers and this gives rise to enhanced thermosensitivity of the cytoskeleton. The cells also have an abnormal cell shape, increased osmotic fragility and an instability of the lipid bilayer manifesting itself in the temperature-sensitive nature of flip-flop movement. Also, in mutants lacking one or more of the membrane-spanning proteins that anchor the cytoskeleton in the bilayer, transbilayer mobility of lipids was enhanced. These observations emphasize that proteins play a role in determining lipid properties in membranes as well as vice versa.

6.5.12 Why are there so many membrane lipids?

In biological membranes from different organisms, there is a bewildering array of different lipid molecules. Moreover, each lipid class also contains a variety of molecular species that differ in such aspects as their acyl moieties or sphingosine backbones. The fact that the lipid composition of a given membrane is generally maintained within strict limits points to a functional advantage. Perhaps this is most easily seen in the photosynthetic membranes of the oxygen-evolving organisms. Thus, in the millions of years since cyanobacteria entered into a symbiotic relationship as chloroplasts in simple algae and these developed to higher plants, the unique glycosylglyceride/phosphatidylglycerol composition has been kept.

A clue as to why there are so many lipids may be developed from a realization that membrane lipids have many, diverse, functions:

(1) they act as permeability barriers for cells and organelles;
(2) they provide the matrix for assembly and function of a wide variety of catalytic processes;
(3) they directly participate in metabolism, e.g. as donors for the synthesis of molecules or as substrates for desaturation;
(4) they participate in a multitude of membrane fusion events;
(5) they actively influence the functional properties of membrane-associated processes.

Most experiments aimed at showing particular functions for membrane lipids use in vitro reconstituted systems. Unfortunately, although this approach yields important clues as to function, it is fraught with artefacts and may not reflect the in vivo situation at all. To look at the in vivo situation, genetics has been used. However, mutations cannot be made directly on the lipids themselves, but only on enzymes metabolizing them. Moreover, mutations in such genes may compromise many interacting functions or even the viability of the organism itself! The more complex the organism, the more difficult the experimental interpretations become. However, by careful manipulation of genes in *E. coli* it has recently been possible to develop secure arguments for the function of particular lipids in that bacterium.

In *E. coli* it has been demonstrated that the acidic phospholipids, phosphatidylglycerol and diphosphatidylglycerol (cardiolipin), which together make up less than 30% of the total phospholipid, had important functions for DNA-binding proteins (such as DNA topo-isomerase or polymerase). Phosphatidylglycerol was absolutely essential and null mutants were non-viable. By changing the expression of the phosphatidylglycerol synthase gene, the phospholipid could be varied from 3% to 20% and functions such as the initiation of replication and in the membrane translocation of proteins were revealed. Cardiolipin was found to function with the ADP/ATP carrier while phosphatidylethanolamine was needed for active transport events and the proper folding of proteins like lactose permease.

These experiments with mutants of *E. coli*, together with data from the glycosylglyceride-enriched *Acholeplasma laidlawii*, also showed clearly that organisms maintain the proportions of non-bilayer-forming versus bilayer-forming lipids rather carefully (Section 6.5.3). Thus, for example, phosphatidylethanolamine mutants of *E. coli* regulate their diphosphatidylglycerol content according to the

divalent cations present in the medium to ensure that normal membrane structures are maintained. In wild-type *E. coli* fatty acid composition rather than lipid class is usually changed while *Acholeplasma* will adjust its monoglycosyl-(non-bilayer-forming) to diglycosyldiacylglycerol (bilayer-forming) ratio in response to the addition of sterol, hydrocarbons or detergents to its growth medium.

6.5.13 Liposomes and drug delivery systems

In Section 6.5.1 we described how many membrane lipids, when isolated, could spontaneously form bilayers in aqueous systems. To prevent any contact of the hydrophobic acyl chains with the aqueous medium, such bilayers close to form vesicles. Usually, under the experimental conditions used to rehydrate lipid samples and with the commonly used membrane lipids (such as phosphatidylcholine) stable multilayered structures are formed. These structures, which have been compared to the multilayered appearance of onions, were termed 'liposomes' by Bangham. He realized when studying such systems that, because of their non-toxic nature and impermeability to many solutes, they would make very good agents for drug delivery and other clinical applications. Because liposomes contain alternating layers of bilayer and aqueous space, hydrophobic solutes can be contained within the bilayer and water-soluble materials within the aqueous compartments. Both types of molecules are prevented from leaking by the hydrophilic or hydrophobic layers, respectively.

There are several fundamental problems to the use of liposomes for drug delivery (Table 6.14). First, the liposomes must be able to carry a sufficient quantity of trapped drug. Their capacity can be increased by making liposomes of very large volumes using special procedures such as the reversed-phase evaporation method. A recent technique uses transmembrane pH gradients across large unilamellar vesicles to encapsulate lipophilic, cationic drugs (e.g. the anticancer drugs doxorubicin or vincristine) with high (nearly 100%) efficiency and at high concentrations. Second, once in the blood circulation, serum factors such as lipoproteins tend to interact with the liposomes and cause leakage. This leakage can be reduced by using more saturated phospholipids and increased cholesterol proportions in the liposomes. Third, because of the size of liposomes they tend to be taken up by the macrophages of the reticulo-endothelial system. This means that the liposomal contents are almost always discharged in the liver or spleen, regardless of which organ site is really the target. Covalent linking of hydrophilic polymers (e.g. polyethyleneglycol) to the head group of certain lipids will reduce this. In addition, liposomes will not normally cross the endothelial barrier and, hence, gain access to extravascular tissue. Access, is a particular problem for the brain with its 'blood–brain barrier'. Finally, attempts are being made to encourage specific interaction with and uptake by certain cells or tissues through the covalent attachment of antibodies.

Table 6.14 Problems with the design of liposomes for drug delivery

1. Producing liposomes with a sufficient volume and concentration of trapped drug.
2. Preventing leakage of contents induced by serum constituents such as lipoproteins.
3. Reducing the preferential uptake of liposomes by macrophages of the reticulo-endothelial system.
4. Allowing access to extravascular tissue.
5. Specific targeting of liposomes to the required cells/tissues.

Despite these problems, the enormous potential for the use of liposomes in medicine ensures a very active research field. Some successes have been recorded already. Thus Fabry's disease (a lipidosis; Section 7.8), which mainly affects the liver, has been treated by enzyme-replacement therapy while parasites such as *Lieshmania*, which reside in macrophages, have been destroyed by drugs delivered in liposomes. Macrophages can also be activated by certain activating factors that can be liposome-delivered. Such treatment renders the macrophages remarkably active in recognizing and destroying diseased tissues including transformed cells.

A particularly exciting development of the use of liposomes has been the attempt to utilize the

changed patterns of the surface glycoproteins and glycolipids in malignant cells (Section 4.4.3). The aim is to incorporate a specific recognition molecule onto the surface of liposomes that carry highly toxic molecules such as the protein ricin. Treatment of patients should result in the specific uptake of liposomes only by cancer cells, which would then be destroyed. In theory these techniques would enable very small numbers of cancer cells to be destroyed before they had a chance to form a sizeable malignancy and might be particularly useful for the treatment of secondaries or in following up surgical intervention. Certainly some success has been achieved by encapsulation of anticancer drugs (e.g. vincristine) when the toxic side-effects of the therapy seem to be reduced.

Liposomes have more recently been used as a vehicle for DNA transfection. Ultimately this technique can be used for gene therapy. The method has been termed 'lipofection' and is very dependent on the lipid mixture and dioleoyl-phosphatidylethanolamine seems to be rather effective. As discussed in Section 6.5.3 (see Fig. 6.12) unsaturated phosphatidylethanolamine readily forms non-bilayer structures so it is thought that, after uptake of the DNA-containing liposome by endocytosis, the phosphatidylethanolamine disrupts the endosome sufficiently for some DNA to escape before its destruction in the lysosome. The escaped DNA can then enter the nucleus where transcription can take place. Significant rates of transfection have been achieved and uses for human gene therapy are being investigated.

An interesting commercial application of liposomes has been the development of skin preparations. These types of moisturizing creams are lotions that are claimed to remove wrinkle-lines and are anti-ageing. The main constituents of these preparations are liposomes and the use of such vehicles might be expected to aid in the skin penetration of other components. Although the scientific basis for some of the claims made by the manufacturers are difficult to understand, the lotions do represent a novel and unexpected use of liposomes!

6.5.14 Lipid anchors for proteins

As discussed in Section 6.5.8 (Fig. 6.16) proteins can interact or become embedded in the membrane in various ways. One type of interaction is via lipid anchors. There are four major classes of covalently bound lipids for membrane proteins (Table 6.15).

Table 6.15 Lipid anchors for proteins

Type	Attachment	Examples
1. Myristoylation	Co-translational; glycine at N-terminus.	cAMP protein kinase; G-proteins (Go, Gi); various viral proteins.
2. Palmitoylation	Post-translational; cysteine in body of protein.	Rhodopsin; fibronectin; Ca^{2+} – ATPase.
3. Prenylation	Post-translational; cysteine near C-terminus. Separate enzymes for farnesyl or geranylgeranyl groups. Often carboxymethylated also.	*Ras* proteins; G-protein (γ subunits); lamin B.
4. GPI (glycosylphosphatidylinositol)	Post-translational; at C-terminus; sensitive to phospholipase C.	Alkaline phosphatase; 5′-nucleotidase; acetylcholinesterase.

6.5.14.1 Acylation

Acylation of proteins can use myristoyl or palmitoyl groups. Myristoylated proteins (Fig. 6.21) come in many shapes, sizes and functions. The myristate is attached co-translationally to a glycine at the N-terminus. A 7–10 amino-acid consensus sequence is usually present: $G^1N^2X^3X^4X^5X^6R^7R^8$. Position 2 is usually a small, uncharged residue such as Asn, Gln or Ser. Positions 3 and 4 are permissive and X^5 is usually Ser or Thr. Basic residues are preferred at positions 7 and 8.

N-Myristoylation confers upon a protein a significant tendency to associate with membranes but the interaction is not hydrophobic enough to maintain a long-term association. However, these properties are ideal for the function of many of the myristoylated proteins that have been discovered – such as protein tyrosine kinases or the ADP ribosylation factor.

Unlike myristoylation, the attachment of palmitate is a post-translational and reversible modification. A specific palmitoyl-CoA:protein S-palmitoyltransferase is involved, which in rat liver is located in the plasma membrane. At least two thioesterases are known to remove the fatty acid. Palmitoylation can occur on more than one cysteine residue for a given protein as in G-protein α subunits or *Ras* proteins.

Because of its reversible nature, protein palmitoylation can be regarded as a method for regulating protein targeting in membranes. In some cases, palmitoylation enhances the loose association of previously myristoylated (or farnesylated; see later) proteins with membranes. A good example is with the G-protein α subunit. This is myristoylated and randomly and reversibly associates with different membranes until it encounters the plasma membrane where it binds to $G_{\beta\gamma}$. In this complex palmitoylation of G_α is facilitated and the doubly lipidated protein remains in stable association with the plasma membrane.

In animals and yeast cells acylated and particularly doubly acylated proteins are enriched in plasma membrane lipid 'rafts' and in caveolae-vesicular invaginations commonly found in animal cells.

6.5.14.2 Prenylation

A third type of modification involves prenylation. A covalent attachment is formed with either a C15 farnesyl or a C20 geranylgeranyl isoprenoid chain. These hydrophobic chains are bound via a stable thioether bound to a cysteine residue near the carboxyl terminus. Specific transferase enzymes are involved and the prenyl chains remain for the life of the protein.

As mentioned above, attachment of a farnesyl residue only makes a protein loosely associated with membranes. This association is considerably increased (10–20×) by modification of the carboxyl terminus. Thus, the remaining amino acids are removed by a specific carboxy peptidase and the prenylated cysteine is carboxylmethylated.

The longer geranylgeranyl chain gives the protein a much higher membrane affinity (up to 45×) than a farnesylated protein. Thus, geranylgeranylated proteins are usually membrane located whereas farnesylated ones are cytosolic and only loosely associate with membranes. Reversible methylation/demethylation can be used to influence the strength of the latter association (see above).

Prenylated proteins have been implicated in a wide variety of functions including signalling, cell division and stress responses.

6.5.14.3 GPI-anchors

A fourth type of lipid anchor is via glycosylphosphatidylinositol (GPI). This was actually the first type of lipid anchor to be discovered when it was noticed that exposure of plasma membranes to a phosphatidylinositol-specific phospholipase C released a number of hydrolytic enzymes. Further examination revealed that all were attached via a GPI-anchor. Well over 150 distinct proteins are now known to be linked to the outer surface of the plasma membrane via a highly conserved core structure (Fig. 6.21). Examples of proteins believed to be attached thus to membranes include alkaline phosphatase, acetylcholinesterase, 5′-nucleotidase and the variant surface glycoprotein (VSG) from *Trypanosoma brucei*. (The latter is responsible for the

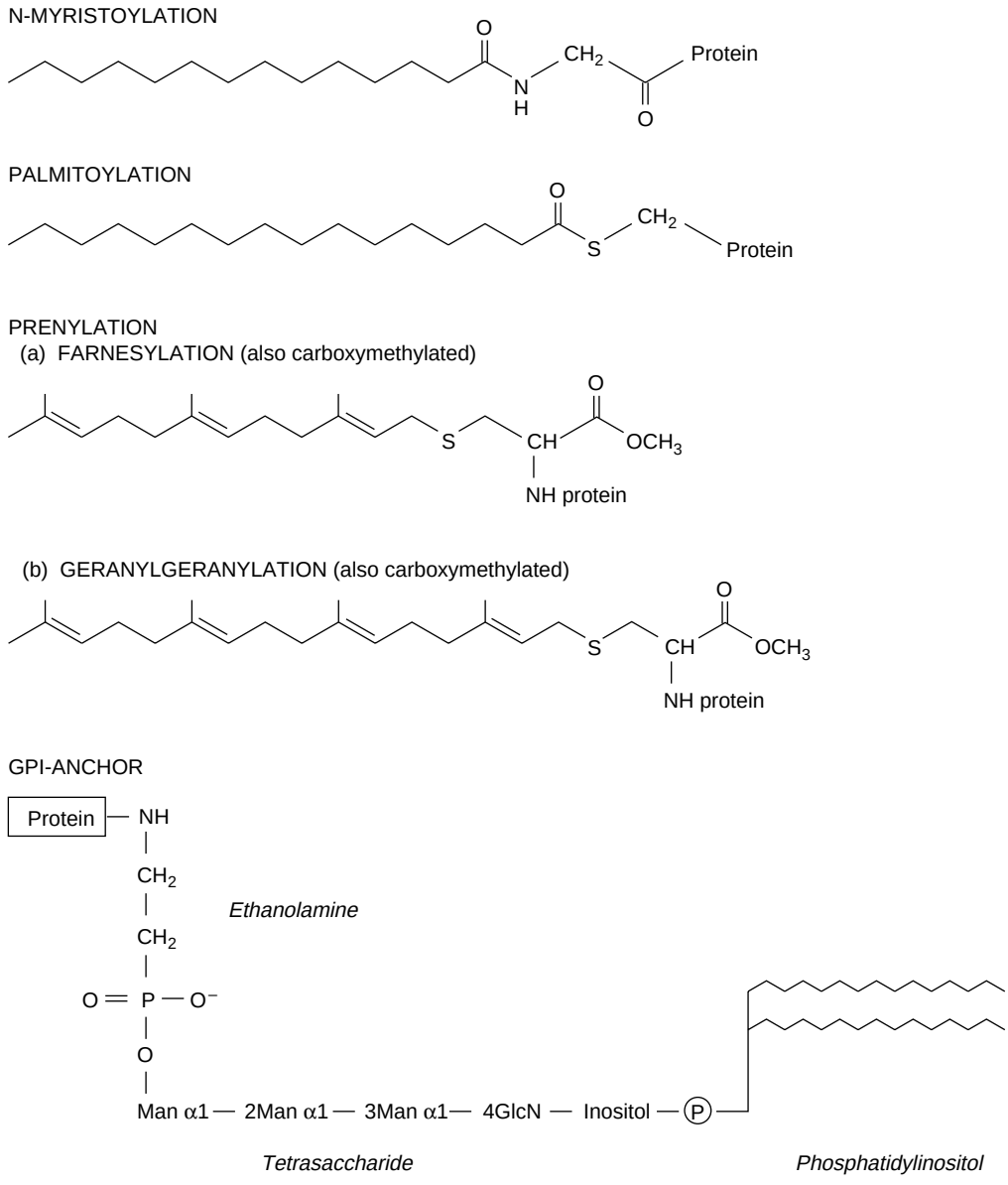


Fig. 6.21 Structures of lipid anchors on proteins.

ability of the parasitic protozoon to evade the victim's immune defence system and so cause sleeping sickness.)

The core structure, first determined for the VSG protein, has been found in a wide variety of GPI-anchors from protozoa and plants through to mammalian brain tissue, although there may be specific additions (which can include saccharides, fatty acids or another phosphorylethanolamine) for individual GPI-proteins.

It will be noted that it is only the diacylglycerol domain of phosphatidylinositol that holds the

membrane protein in place. However, such an anchor is at least as strong as a hydrophobic sequence of amino acids spanning the entire membrane.

At present we do not have a definite reason as to why some membrane proteins are attached through phosphatidylinositol. One possibility is that it may provide a means of selectively mobilizing these proteins. Indeed, there is some evidence that endogenous phospholipases may cause the release of VSG and alkaline phosphatase in soluble forms and other proteins such as acetylcholinesterase are also known to be released under certain physiological conditions.

Another advantage is that the anchor itself targets the attached protein from the endoplasmic reticulum, where it is made, to the cell surface. This is achieved by association with plasma membrane-destined lipid 'rafts'.

Once on the surface, GPI-anchored proteins (e.g. alkaline phosphatase, 5′-nucleotidase) can recruit nutrients for the cell by metabolizing non-utilizable substrates. Other GPI-anchored proteins may act as ligands to bind nutrients such as folate and iron and participate in their internalization. Further GPI-anchored proteins can participate in cell signalling pathways.

6.6 LIPIDS AS COMPONENTS OF THE SURFACE LAYERS OF DIFFERENT ORGANISMS

In addition to their roles in biological membranes, lipids of all kinds are located in various cell-surface structures that are not strictly 'membranes'. Thus, they are found in the waxy outer coatings of plant stems, roots and leaves; in bacterial cell-wall structures; in insect waxes and on the mammalian skin surface.

6.6.1 Cutin, suberin and waxes – the surface coverings of plants

Stems, leaves and roots of plants are covered with a layer of fatty material. The structural component is a hydroxy fatty acid polymer – cutin. Underground parts and wound surfaces are covered in another type of lipid-derived polymeric material – suberin. Both these polymers are associated with, or embedded in, a complex mixture of lipids imprecisely called waxes. (Strictly speaking a wax is an ester between a long-chain fatty acid and a fatty alcohol; see Section 3.7.)

The main components of plant waxes are listed in Table 6.16. In general, most of the major constituents are non-polar molecules with long hydrocarbon chains. The pathway of synthesis determines whether the final products have odd- or even-numbered

Table 6.16 The major components of plant waxes

Compound	Structure	Occurrence
n-Alkanes	$CH_3(CH_2)_nCH_3$	Most plants; major components usually 29C or 31C.
iso-Alkanes	$CH_3CH(CH_3)R$	Not as widespread as *n*-alkanes; usually 27C, 29C, 31C and 33C.
Ketones	$R^1 \cdot CO \cdot R^2$	Not as common as alkanes; usually 29C and 31C.
Secondary alcohols	$R^1 \cdot CH(OH)R^2$	As common as ketones; usually 29C and 31C alcohols.
	$CH_3CH(OH)R$	Uncommon in cuticular waxes; more common in suberin waxes; odd-chain length, 9–15C.
β-Diketones	$R^1 \cdot COCH_2CO \cdot R^2$	Usually minor, but in some species (e.g. barley) may be major components; mainly 29C, 31C and 33C.
Primary alcohols	$R \cdot CH_2OH$	Most plants; even chains predominate, usually 26C and 28C.
Acids	$R \cdot COOH$	Very common; even chains predominate, usually 24–28C.

Abbreviations: R, R^1 and R^2 are alkyl chains.

carbon chains. While chain lengths of 20–35 carbon atoms are usually encountered, fatty acids and hydrocarbons with fewer than 20C and esters with more than 60C are known. The composition and quantity of epicuticular waxes can vary widely not only from species to species but also between organs or even cell types. Moreover, environmental influences can change the composition.

Whereas cutin contains dihydroxy fatty acids as major components, suberin has ω-hydroxy and dicarboxylic fatty acids. Phenolic constituents are present in both (Table 6.17). They are especially prevalent in suberin where they are believed to have an anti-microbial function. The exact composition of these polymers varies with the age and development of a given tissue.

Cell walls of fungi have significant amounts of aliphatic hydrocarbons, which are presumed to act as a desiccation barrier – similar to higher plants. In contrast, bacteria do not usually contain large quantities of waxy materials in their surface layers. However, as will be seen in subsequent sections, different bacterial classes contain other characteristic lipids.

6.6.2 Mycobacteria contain specialized cell-wall lipids

The mycobacterial cell wall contains three components. First, there is a skeleton consisting of arabinogalactan mycolate covalently linked through a phosphodiester bond to peptidoglycan. In addition, peptides that can be removed by proteolysis and free lipids are important components.

The skeleton is a branched polymer of D-arabinose and D-galactose in a 5:2 ratio. Every tenth arabinose has a mycolic acid esterified to the 5′-hydroxyl. These mycolic acids are 60C–90C fatty acids that are 2-branched or 3-hydroxylated (Fig. 6.22). They may also contain other substituted

Table 6.17 The main compositional differences between cutin and suberin

Monomer	Cutin	Suberin
Dicarboxylic acids	Minor	Major
In-chain-substituted acids	Major	Minor (sometimes substantial)
Phenolics	Low	High
Very long chain (20–26C) acids	Rare and minor	Common and substantial
Very long chain alcohols	Rare and minor	Common and substantial

Source: Kolattukudy, P.E. (1980) *Biochemistry of Plants*, Vol. 4 (eds P.K. Stumpf & E.E. Conn), p. 591. Academic Press, New York.

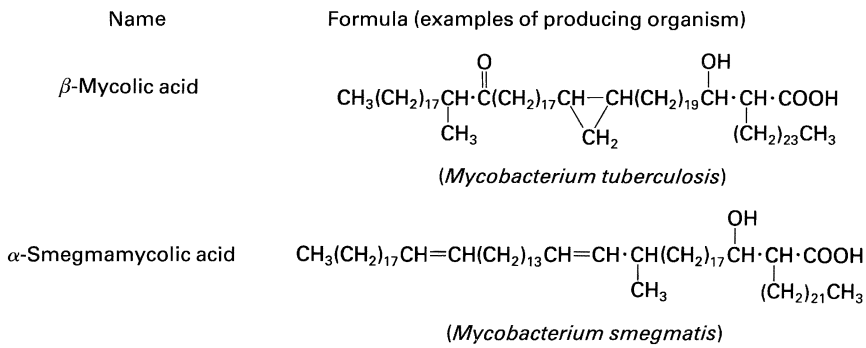


Fig. 6.22 Structure of two mycolic acids.

groups such as cyclopropane rings or methoxy groups. The acids are often named after the type of bacteria in which they are found (e.g. nocardomycolic acid from *Nocardia* spp.).

The extractable free lipids comprise a mixture of cord factors, mycosides, sulpholipids and wax D. Together they represent about 25–30% of the mycobacterial cell wall. Cord factors are esters of the disaccharide, trehalose, with two mycolic acids (Fig. 6.23). Cord factor is so called because it is found in the waxy capsular material of virulent strains of tubercle and related bacteria. The factor causes the bacteria to string together in a long chain or cord. The compound is highly toxic and is somehow intimately associated with the virulence of the organism. It interacts strongly with host cell membranes thus impairing their function. Cord factor probably acts as a hapten and binds to albumin in plasma, thus forming an antigen. Like extracts of mycobacterial cell walls, cord factor acts as an immunostimulant. Killed mycobacteria (or their cell walls) are suspended in Freund's adjuvant, which is commonly injected with antigens to increase the titre of antibody.

The sulpholipids (Fig. 6.24) consist of trehalose, which is sulphated at the 2-position and acylated at several positions on both sugar residues. Instead of mycolic acids, the sulpholipids have a mixture of palmitic acid and very long chain (31C–46C) fatty acids with up to 10 methyl branches – known as phthioceranic acids.

Mycosides (Fig. 6.24) are again characteristic of mycobacteria. The basic structure for mycosides A and B is a long-chain, highly branched, hydroxylated hydrocarbon terminated in a phenol group. Acyl groups are esterified to hydroxy groups of the hydrocarbon chain. In contrast, mycoside C is a glycolipid peptide.

6.6.3 Lipopolysaccharide forms a major part of the cell envelope of Gram-negative bacteria

In Gram-negative bacteria, the wall is far more complex than for Gram positives and contains glycopeptide, lipopolysaccharide, phospholipid and protein. Also, there is not such a clear distinction between the wall and the membrane as in Gram positives. Up to 20% of the wall contents may be lipids, but only a proportion of these are readily extractable by conventional solvent methods. This is because of the covalent nature of the lipopolysaccharide linkages.

The cytosol of Gram-negative bacteria is surrounded by a complex cell envelope consisting of at least three layers (Fig. 6.25). The cytoplasmic membrane is composed of the familiar phospholipid bilayer with integral and peripheral proteins.

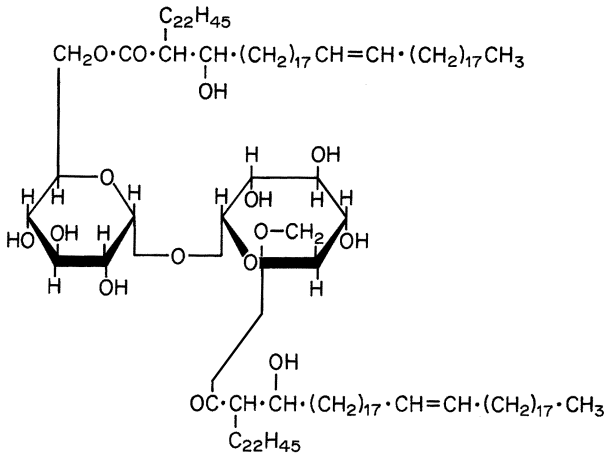


Fig. 6.23 Cord factor.

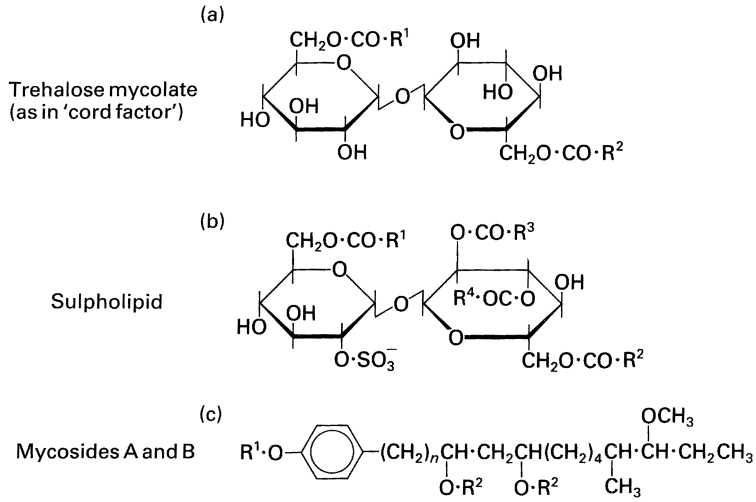


Fig. 6.24 Some mycobacterial lipids. Structures of mycobacterial lipids: (a) trehalose mycolate, where R^1 and R^2 are mycolic acids; (b) sulpholipid, where R^1–R^4 are palmitic acid or very long chain branched fatty acids; (c) mycosides A and B, where R^1 is a mono- or trisaccharide and R^2 is a 12C–18C saturated fatty acid or a mycocerosic acid [e.g. $CH_3(CH_2)_{21}(CH(CH_3)CH_2)_2CH(CH_3)COOH$].

Then comes a peptidoglycan layer, which is separated from the outer membrane by a periplasmic space. The outer membrane is extremely asymmetric, but contains lipopolysaccharide and enterobacterial-common antigen in its outer leaflet. Phospholipid molecules are missing from the outer membrane in many Gram-negative bacteria but are present in some species. In those bacteria containing phospholipids in the outer membrane, phosphatidylethanolamine is, by far, the most common constituent (~85%).

The lipopolysaccharide is involved in several aspects of pathogenicity. It is a complex polymer in four parts (Fig. 6.26). Outermost is a carbohydrate chain of variable length (called the *O*-antigen), which is attached to a core polysaccharide. The core polysaccharide is divided into the outer core and the backbone. These two structures vary between bacteria. Finally the backbone is attached to a glycolipid called lipid A. The link between lipid A and the rest of the molecule is usually via a number of 3-deoxy-D-manno-octulosonic acid (KDO) molecules. The presence of KDO is often used as a marker for lipopolysaccharide (or outer membrane) even though it is not present in all bacterial lipopolysaccharides.

Lipid A is composed of a disaccharide of glycosamines (Fig. 6.27). The amino groups are substituted with 3-hydroxymyristate while the hydroxyl groups contain saturated (12C–16C) acids and 3-myristoxymyristate. Phosphate and KDO groups are also substituted. Unsaturated and cyclopropane fatty acids, which are common in other lipid types, are absent from lipopolysaccharide.

Studies on the synthesis of lipopolysaccharide have made extensive use of bacteria mutants that are deficient in one or more of the necessary reactions. Rothfield, Horecker and their colleagues in the USA initiated such experiments. It was discovered that formation took place in several stages.

Lipid A, which anchors lipopolysaccharide in the membrane, is made first. Hydroxy acids are added first to the disaccharide, followed by KDO and then saturated fatty acids. The hydroxy fatty acids come from acyl-CoA substrates whereas CMP-KDO is the source of the second addition units. After the addition of saturated fatty acids, sugars are added

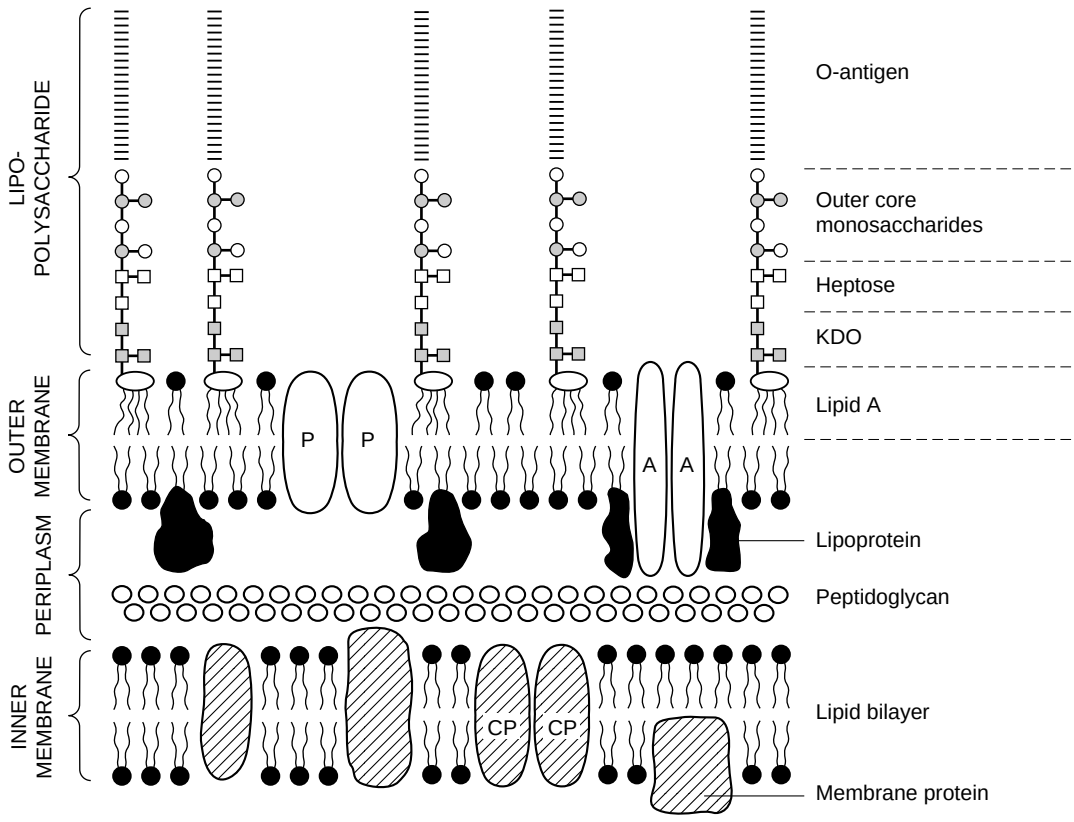


Fig. 6.25 Organization of the membrane system of a Gram-negative bacterium. P = porin; A = transmembrane peptidoglycan-associated protein; CP = carrier protein. Based on information contained in Wilkinson (1996).

from nucleotide diphosphate derivatives. Various deficient mutants, which lack either glucosyl- or galactosyl-transferase, have been isolated. These reactions build one half of the molecule. Another lipid, phosphatidylethanolamine, has been suggested to be intimately involved in the binding of the transferase enzymes to the lipopolysaccharide acceptor.

The *O*-antigen is made in three stages. The oligosaccharide units are transferred from nucleotide diphosphate carriers to a galactose attached to another lipid carrier; a 55C polyisoprenoid molecule. The oligosaccharide units are then polymerized and lipid carriers are released in the process. Finally, the complete *O*-antigen is transferred to the R-core, with the release of the final isoprenoid carrier.

Lipopolysaccharide synthesis occurs on the inner membrane from which the molecule must be transferred to the outer membrane. This occurs at specific membrane sites generally where the two membranes adhere to each other.

6.6.4 Gram-positive bacteria have a completely different surface structure

The cell walls and membranes of most Gram-positive bacteria contain a series of highly anionic polymers. Most important among these is teichoic acid, which is a polymer of glucose 1-phosphate or ribitol phosphate. Membrane teichoic acids are based on glucose 1-phosphate and a proportion may be linked to glycolipids to give lipoteichoic

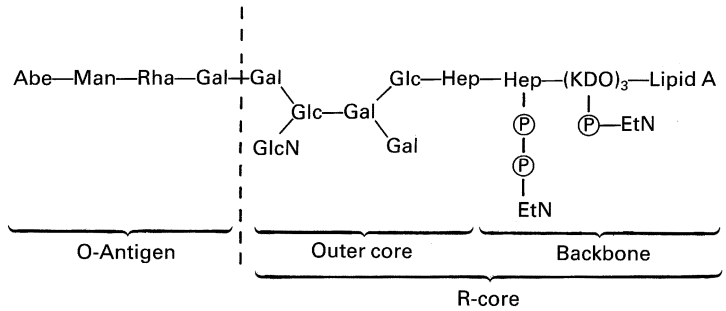


Fig. 6.26 Generalized structure of lipopolysaccharide from a Gram-negative bacterium.

acids. The details of these structures and the amount of substitution of the teichoic acids vary with bacterial species.

Apart from the glycolipid linked to teichoic acid, the only significant lipid in most Gram-positive bacteria is that in the cytoplasmic (protoplast) membrane. Notable exceptions are the mycobacteria the special lipids of which have been dealt with above.

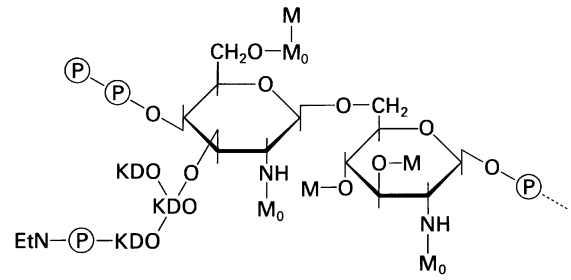
6.6.5 Insect waxes

A majority of studies on animal waxes have been done with arthropods, particularly insects. These waxes consist of a complex mixture that includes straight-chain saturated and unsaturated hydrocarbons, wax esters, sterol esters, ketones, alcohols, aldehydes and acids. The composition may be influenced by the plants on which the insect feeds. Futhermore, the relative proportions of wax components can vary widely between species. For example, the amount of hydrocarbon can vary from 100% in the surface lipids of the field cricket (*Nemobius fasciatus*) to less than 1% in tobacco budworm pupae.

Not only do insect waxes prevent desiccation they may also have other diverse functions such as acting as pheromones or kairomones, helping in recognition and in thermoregulation.

6.6.6 Lipids of skin – the mammalian surface layer and skin diseases

The main function of the skin – one of the largest organs of the human body – is that of a protective barrier. The epidermis, which is separated from a predominantly acellular dermis by a basement membrane, is composed of four layers. The outer-



KDO = deoxy-D-mannooctulosonic acid, Ⓟ = phosphate, EtN = ethanolamine, M = myristrate and M_0 = β-hydroxymyristrate.

Fig. 6.27 Lipid A.

most of these four layers, the stratum corneum, consists of impermeable cellular layers composed of flattened, densely packed, anucleate cells embedded in a waxy lamellar matrix of complex intercellular lipids. The latter are derived from the lipid-filled membrane-coating granules of the cells of the next layer, the stratum granulosum. In addition, the lipid mixture sebum is secreted by the sebaceous glands. Typical compositions of sebum and epidermal lipids are shown in Table 6.18.

Table 6.18 The main lipid classes of sebum and the skin's epidermis

Sebum lipids	Epidermal (*Stratum corneum*) lipids
40% Triacylglycerol	50% Ceramides
25% Wax esters	25% Fatty acids
15% Fatty acids	19% Cholesterol
12% Squalene	4% Cholesterol sulphate
8% Others	2% Others

The composition of human skin surface lipids varies over the body, but in sebaceous gland-enriched areas (such as the face) the secretion, sebum, may represent 95% of the total. When sebum is freshly formed it contains mainly triacylglycerols, wax esters and squalene. However, due to bacterial action, unesterified fatty acids are rapidly released. These acids are both an irritant and comedogenic (give rise to 'black-heads') and have been implicated in acne vulgaris – an extremely common complaint of puberty. It is often stated that the severity of acne correlates with the amount of seborrhoea and many treatments are designed primarily to remove such lipids from the skin surface.

More serious than acne, but less prevalent, are the skin diseases atopic eczema and psoriasis. Lipids have been suggested as being involved in both of these complaints although it must be stated that the evidence at present is equivocal. It has been noted in patients with atopic eczema that there is a disturbance in the normal tissue complement of polyunsaturated fatty acids. For convenience, blood samples have usually been analysed and these usually exhibit reduced levels of γ-linolenic (all *cis*-6,9,12-octadecatrienoic) acid. γ-Linolenate is an intermediate in the normal conversion of the essential fatty acid, linoleate, to the eicosanoid precursor arachidonate:

$$c,c\text{-}9,12\text{-}18{:}2 \xrightarrow{\Delta 6\text{-desaturase}} c,c,c\text{-}6,9,12\text{-}18{:}3 \xrightarrow{\text{elongase}} c,c,c\text{-}8,11,14\text{-}20{:}3 \rightarrow c,c,c,c\text{-}5,8,11,14\text{-}20{:}4$$

Moreover, symptoms of atopic eczema have been reported to be alleviated by γ-linolenate-rich lipids, such as the oil from evening primrose seeds. These results are interpreted to indicate a deficiency of the Δ6-desaturase responsible for linoleate metabolism, but they do not explain the beneficial effect of some other treatments.

Psoriasis is a severely debilitating skin disease affecting 2% of the population world-wide. It is responsible for more days spent in hospital than any other skin complaint and present treatments are rudimentary and rather unsatisfactory. Characteristically there is an impairment of normal cell differentiation and development, excessively high levels of some arachidonate metabolites and a potentiation of the symptoms in patients with lithium therapy. The symptoms have been drawn together recently with the suggestion that defective phosphoinositide metabolism (Sections 7.9 and 7.10) is involved.

Finally a few remarks should be made about essential fatty acid (EFA) deficiency (Sections 2.4 and 4.2.3). Linoleic acid is the precursor of the eicosanoids, leukotrienes, prostaglandins, thromboxanes and related compounds (Section 2.4). Until recently it was thought that the classic skin symptoms resulting from a deficiency were due to a lack of eicosanoids. However, the specific role of *n*-6 acids (such as linoleate) as opposed to *n*-3 acids (such as α-linolenate) in membrane fluidity and membrane function cannot explain their essentiality. For example, aspirin ingestion, which effectively prevents prostanoid formation, does not result in the appearance of EFA-deficiency symptoms. It is also generally accepted that at least some cell lines in culture do not have an EFA requirement – thus suggesting that EFAs are not essential for the

formation and function of cellular membranes in general.

Recently, several linoleic acid-rich lipids such as acylglucosylceramide, acylceramide and a specific wax ester have been identified in human, pig and rat epidermis (Fig. 6.28). These lipids form part of the intercellular lipid-rich matrix serving as a water permeability barrier. In EFA deficiency the linoleate of these lipids is replaced by oleate, a change that is associated with a loss of the barrier function (see Section 4.2.3.4).

6.7 SUMMARY

A major characteristic of eukaryotic cells is the presence of cellular organelles. These organelles are bound by one or more membranes that serve as a barrier to the free passage of molecules – thus maintaining the unique composition and environment of individual organelles. Not only do individual organelles have a characteristic complement of enzymes (and, therefore, metabolic pathways) they also have membranes made up of a special blend of lipid molecules. In a few cases, such as the diphosphatidylglycerol of the inner mitochondrial membrane, these lipid distributions are so specific that they can be used to aid identification of subcellular fractions. In some instances, organelles can be induced or caused to undergo functional development or morphological change. By the use of such systems, the role of lipids in membrane biogenesis and function can be studied.

Most cellular membranes are composed predominantly of proteins and phosphoglycerides. Therefore, phosphoglycerides are significant components of prokaryotes and eukaryotes. In animals and non-photosynthetic cells of higher plants, phosphatidylcholine is the main phosphoglyceride. This molecule is not, however, a major component in bacteria. Phosphatidylethanolamine is a major component in most organisms while phosphatidylglycerol is common in prokaryotes and is the dominant (in many cases exclusive) phosphoglyceride of the photosynthetic membranes of oxygen-evolving species. As well as these molecules, phosphatidylinositol is quantitatively important forming a smaller but significant percentage of the membrane lipids in many cases. Phosphorylated

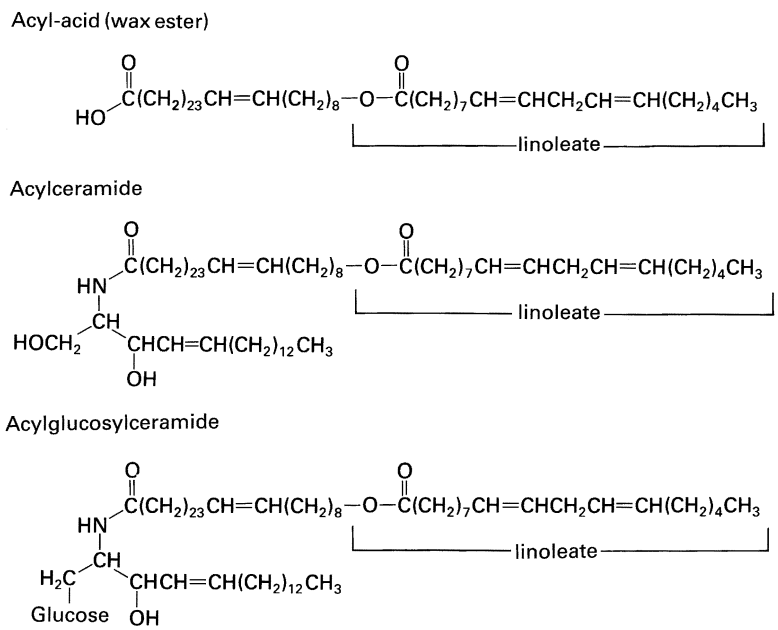


Fig. 6.28 Structures of linoleic acid-rich skin epidermal lipids.

derivatives of phosphatidylinositol are found in the plasma membranes of animals and plants, while diphosphatidylglycerol is exclusively located in the inner mitochondrial membrane of eukaryotes. Together with phosphatidylethanolamine and phosphatidylglycerol, it is a major component of many bacteria.

In contrast to the quantitative importance of the phosphoglycerides, phosphonolipids (which contain a stable carbon–phosphorus linkage) have been found in significant quantities in only a few organisms such as sea urchins and protozoa. Similarly, ether-linked lipids also are less prevalent than the acyl phosphoglycerides, although phospholipids such as choline and ethanolamine plasmalogens are widespread but usually minor components in animals. The betaine ether-linked glycerolipids are major lipids in many lower plants.

Glycosylglycerides are the major lipids of the photosynthetic membranes of higher plants, algae and cyanobacteria. Because of the preponderance of such membranes the main glycosylglyceride, monogalactosyldiacylglycerol, is actually the most abundant lipid in the world. The other glycosylglycerides found in plants and algae are digalactosyldiacylglycerol and the plant sulpholipid, sulphoquinovosyldiacylglycerol. Different lipids containing various glycosidic residues are found in micro-organisms such as *Pneumococcus* or *Mycoplasma* while the galactosylglycerides are minor constituents of mammalian brain.

The glycerolipids are distributed in individual membranes at characteristic ratios. There is also good evidence that biological membranes are asymmetric with regard to their lipid as well as their protein distribution. There may also be some lateral heterogeneity with particular regions of the membrane being enriched with certain lipids.

In contrast to the glycerolipids, sphingolipids are based on a sphingosine backbone. Sphingolipids are only found in any quantity in animals where they tend to be concentrated at the cell surface – in keeping with their function there. Sphingolipids have an acyl residue attached through the amino group of sphingosine while carbohydrates (or phosphorylcholine in the case of sphingomyelin) are linked via the alcohol residue. Depending on the nature of the sugar residues, ceramides, sulphatides and gangliosides are formed. Each cell contains a characteristic pattern of such molecules.

Sterols are based on the cyclopentanoperhydrophenathrene structure, which consists of a fused four-ring system. Three of the rings are six-membered while ring D has five carbons. A side-chain is attached to ring D and sterols contain an hydroxyl function at C3 of ring A. Cholesterol is, by far, the most common sterol in animals while different sterols occur in other organisms. Thus, ergosterol is important in yeasts while stigmasterol and β-sitosterol are common in plants.

Important steroids include the (pro-)vitamins D, bile acids and various steroid hormones such as testosterone, oestrogens, progesterones and glucocorticoids. Sterols may also be derivatized to give sterol esters, sterol glycosides and acylated sterol glycosides.

The basic structure of membranes is usually envisaged as the fluid-mosaic model of Singer and Nicholson. In this, amphiphilic lipids are arranged in a bimolecular layer with the hydrophobic moieties in the centre of the membrane and the hydrophilic head groups at the two surfaces. Stability is achieved by various ionic or hydrophobic interactions between the lipids and the membrane proteins. The latter can be embedded within the membrane (intrinsic proteins) or attached to the surface (extrinsic proteins). Many membrane proteins are large enough to actually span the membrane.

By a combination of enzyme digestion, chemical or antibody labelling or physical techniques such as NMR, it has been shown that all membranes thus examined are clearly asymmetric with regard to their lipids as well as their proteins. This asymmetry is maintained because, although lipids show rapid rotation and translational movement, they only move from one leaflet to the other at very slow speeds.

For a membrane to function effectively, the acyl chains of its lipids must be above their gel–liquid transition temperature. Organisms take care to maintain membrane fluidity by a variety of methods. Membrane fluidity contributes to important properties such as the operation of transport

proteins and to the rapid diffusion of hydrophobic electron carriers like quinones.

Apart from a general function in providing the essential bimolecular layer, which is the foundation of biological membranes, lipids have more specific functions. One essential role is in membrane fusion – a process essential for secretion, endocytosis, intracellular digestion by lysosomes and cell division. The ability of membrane lipids to adopt non-bilayer phases is important for fusion. Moreover, lipids and proteins interact to determine membrane shape and properties and recent experiments with bacterial mutants have demonstrated the essential role of particular lipid components in specific membranes.

Artificial membrane systems have been used for many years to help scientists understand how membranes work. More recently, lipid vesicles (usually multilayered and called liposomes) have been employed as drug carriers for medical treatments. They have high capacities for both hydrophobic and hydrophilic drugs and often show better release and toxicity characteristics than other forms of treatment.

Lipids can also be used to modify proteins covalently. Four types of lipid anchor are used – myristoylation, palmitoylation, prenylation and the glycosyl phosphatidylinositol anchor. Depending on the structure, they will cause proteins to be tightly or loosely associated with membranes.

The surface layers of different organisms often have lipids as significant components. The surface coverings of plants consist of waxes and cutin on the aerial parts while suberin covers the underground tissues or wound surfaces. Very long chain lipids are characteristic of these surface coverings. Dependent on whether the surface layer is wax, cutin or suberin and also, to some extent, the plant tissue in question then the individual lipid composition will vary. Hydrocarbons, very long chain acids and alcohols, their esters and long-chain aldehydes and ketones are all found with hydroxylated fatty acids prominent in suberin.

In contrast to plants, micro-organisms have rather specialized and, in some cases, unique surface coverings. Mycobacteria contain large amounts of cell-wall lipids such as mycosides while Gram-negative bacteria contain lipopolysaccharide. The latter is composed of four parts. On the outside of the cell is a polysaccharide of variable structure often called the *O*-antigen. This is attached to a core polysaccharide in two parts – the backbone of which is linked to lipid A. In contrast, Gram-positive bacteria have a completely different surface structure. Lipids are less prevalent although a proportion of the membrane teichoic acids are linked to a glycolipid, such as diacyldiglycosylglycerol, to give lipoteichoic acid.

As in plants, waxes are important on the outer surface of insects. The composition of the waxy layer varies widely and is often influenced by the plant food that the insect eats.

The skin of mammals contains large amounts of lipids that act as a protective barrier. The epidermal lipids themselves contain a high proportion of ceramides, many of which contain linoleic acid as a major component. Cholesterol and its sulphate ester are also important. The lipid content is supplemented by the secretion of sebum from the sebaceous glands. Sebum is rich in triacylglycerols, wax esters and the terpenoid squalene and its secretion helps to maintain the skin in a waterproof and flexible condition. Several skin diseases, including acne, eczema and psoriasis, have lipids as major factors in their progenesis.

FURTHER READING

General – Lipids

Gunstone, F.D., Harwood, J.L. & Padley, F.B., eds. (1994) *The Lipid Handbook*, 2nd edn. Chapman & Hall, London.

Harwood, J.L. & Russell, N.J. (1984) *Lipids in Plants and Microbes*. George Allen and Unwin, Hemel Hempstead.

Larsson, K., ed. (1994) *Lipids: Molecular Organisation, Physical Functions and Technical Applications*. The Oily Press, Dundee.

Ratledge, C. & Wilkinson, S.G., eds. (1988) *Microbial Lipids*, Vol. 1. Academic Press, London.

Stumpf, P.K. & Conn, E.E., eds. (1980, 1987) *The Biochemistry of Plants*, Vol. 4 and Vol. 9. Academic Press, New York. [Contains chapters specific to topics in the following sections on Glycerolipids, Sterols and Surface Layers.]

Vance, D.E. & Vance, J.E., eds. (1996) *Biochemistry of Lipids, Lipoproteins and Membranes*. Elsevier, Amsterdam.

[Contains several chapters specific to topics in the following sections.]

Glycerolipids

Ansell, G.B. & Hawthorne, J.N., eds. (1982) *Phospholipids*. Elsevier, Amsterdam.

Cevc, G., ed. (1993) *Phospholipids Handbook*. Marcel Dekker, Basel.

Dembitsky, V.M. (1996) Betaine ether-linked glycerolipids: chemistry and biology. *Progress in Lipid Research*, **35**, 1–52.

Kates, M., ed. (1990) *Glycolipids, Phosphoglycolipids and Sulphoglycolipids*. Plenum Press, New York.

Kaya, K. (1992) Chemistry and biochemistry of taurolipids. *Progress in Lipid Research*, **31**, 87–108.

See also specific chapters in multiauthor volumes in the *General* section above.

Sphingolipids

Hori, T. & Sugita, M. (1993) Sphingolipids of lower animals. *Progress in Lipid Research*, **32**, 25–46.

Kanfer, J.N. & Hakomori, D.-I. (1983) *Sphingolipid Biochemistry*. Plenum Press, New York.

Leeden, R.W. & Yu, R.K. (1982) Gangliosides: structure, isolation and analysis. In: *Methods of Enzymology* (ed. V. Ginsberg), Vol. 83, pp. 139–219. Academic Press, New York.

Makita, A., Handa, S., Taketonii & Nagai, Y. (1982) *New Vistas in Glycolipid Research*, pp. 55–69. Plenum Press, New York.

See also chapter by Merril and Sweeley in the multiauthor volume edited by Vance and Vance in the *General* section above.

Sterols

Bloch, K. (1983) Sterol structure and membrane function. *Critical Reviews of Biochemistry*, **17**, 47–92.

Yeagle, P.L. (1985) Cholesterol and the cell membrane. *Biochimica et Biophysica Acta*, **822**, 267–287.

See also specific chapters in the multiauthor volumes in the *General* section above.

General – Membranes

Finian, J.B. & Michell, R.H., eds. (1981) *Membrane Structure*. Elsevier, Amsterdam.

Gounaris, K., Barber, J. & Harwood, J.L. (1986) The thylakoid membranes of higher plants. *Biochemical Journal*, **237**, 313–326.

Hoffman, J.F. & Giesbisch, G., eds. (1970) *Current Topics in Membrane and Transport*, Annual Series. Academic Press, New York.

Houslay, M.D. & Stanley, K.K. (1982) *Dynamics of Biological Membranes*. Wiley, Chichester.

Israelachvili, J.N. (1978) The packing of lipids and proteins in membranes. In: *Light Transducing Membranes: Structure, Function and Evolution* (ed. D.W. Deamer), pp. 91–107. Academic Press, New York.

Manson, L.A. (1971) *Biomembranes*, Series. Plenum Press, New York.

Prasad, R., ed. (1996) *Manual on Membrane Lipids*. Springer, New York.

Quinn, P.J. & Cherry, R.J., eds. (1992) *Structural and Dynamic Properties of Lipids and Membranes*. Portland Press, London.

Robertson, R.N. (1983) *The Lively Membranes*. Cambridge University Press, Cambridge.

See also chapter by Cullis *et al.* in the multiauthor volume edited by Vance and Vance in the *General* section above.

Lipids and Membrane Structure

Carruthers, A. & Melchior, D.L. (1986) How bilayer lipids affect membrane protein activity. *Trends in Biochemical Sciences*, **11**, 331–335.

Dai, J. & Sheetz, M.P. (1998) Cell membrane mechanics. *Methods in Cell Biology*, **55**, 157–171.

de Kruiff, B., *et al.* (1984) Lipid polymorphism and membrane function. In: *Enzymes of Biological Membranes* (ed. A. Martinosi), pp. 131–204. Plenum Press, New York.

Edidin, M. (1997) Lipid microdomains in cell surface membranes. *Current Opinion in Structural Biology*, **7**, 528–532.

Fenske, D.B., Monch, M.A., Hope, M.J. & Cullis, P.R. (1995) The functional roles of lipids in biological membranes. In: *Biomembranes* (ed. A.G. Lee), Vol. 1, pp. 1–28. JAI Press, London.

Jacobson, K., Sheets, E.D. & Simson, R. (1995) Revisiting the fluid mosaic model of membranes. *Science*, **268**, 1441–1442.

op den Kamp, J.A.F. (1979) Lipid asymmetry in membranes. *Annual Review of Biochemistry*, **48**, 47–71.

Petty, H.R. (1993) *Molecular Biology of Membranes. Structure and Function*. Plenum Press, New York.

Pugano, R.E. & Sleight, R.G. (1985) Emerging problems in the cell biology of lipids. *Trends in Biochemical Sciences*, **10**, 421–425.

Siegenthaler, P.-A. (1998) Molecular organisation of acyl lipids in photosynthetic membranes of higher plants. In: *Lipids in Photosynthesis: Structure, Function and Genetics* (ed. P.-A. Siegenthaler & N. Murata), pp. 119–144. Kluwer, Dordrecht.

Singer, S.J. & Nicholson, G.L. (1972) The fluid mosaic

model of the structure of cell membranes. *Science*, **175**, 720–731.

Tocanne, J.-F., Dupou-Cezanne, L. & Lopez, A. (1994) Lateral diffusion of lipids in model and natural membranes. *Progress in Lipid Research*, **33**, 203–238.

Van Meer, G. (1988) How epithelia grease their microvilli. *Trends in Biochemical Sciences*, **13**, 242–243.

Zhang, F., Lee, G.M. & Jacobson, K. (1993) Protein lateral mobility as a reflection of membrane microstructure. *BioEssays*, **15**, 579–588.

Effects on membranes during temperature adaptation

Avery, S.V., Lloyd, D. & Harwood, J.L. (1995) Temperature dependent changes in plasma membrane lipid order and the phagocytotic activity of the amoeba *Acanthamoeba castellanii* are closely related. *Biochemical Journal*, **312**, 811–816.

Cossins, A.J. (1994) *The Temperature Adaptation of Biological Membranes*. Portland Press, London.

de Mendoza, D. & Cronan, J.E. (1983) Thermal regulation of membrane fluidity in bacteria. *Trends in Biochemical Sciences*, **8**, 49–52.

Lee, A.G. (1991) Lipids and their effects on membrane proteins: evidence against a role for fluidity. *Progress in Lipid Research*, **30**, 323–348.

Lynch, D.V. & Thompson, G.A. (1984) Retailored lipid molecular species: a tactical mechanism for modulating membrane properties. *Trends in Biochemical Sciences*, **9**, 442–445.

Murata, N., Ishizaki-Nishizawa, O., Higashi, S., Hayashi, H., Tasaka, Y. & Nishida, I. (1992) Genetically-engineered alteration in the chilling sensitivity of plants. *Nature*, **356**, 710–713.

Murata, N. & Wada, H. (1995) Acyl lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria. *Biochemical Journal*, **308**, 1–8.

Vigh, L., Los D.A., Horvath, I. & Murata, N. (1993) The primary signal in the biological perception of temperature: Pd-catalysed hydrogenation of membrane lipids stimulated the expression of the desA gene in *Synechocystis* PCC 6803. *Proceedings of the National Academy of Science, U.S.A.*, **90**, 9090–9094.

Lipids and membrane fusion

Fisher, D. & Geisow, M.J. (1986) Molecular mechanisms of membrane fusion. *Biochemical Society Transactions*, **14**, 241–257.

Hay, J.C. & Scheller, R.H. (1997) SNAREs and NSF in targeted membrane fusion. *Current Opinion in Cell Biology*, **9**, 505–512.

Jahn, R. & Sudhof, T.C. (1999) Membrane fusion and exocytosis. *Annual Review of Biochemistry*, **68**, 863–911.

Siegel, D.P. (1993) Energetics of intermediates in membrane fusion: comparison of stalk and inverted micellar intermediate mechanisms. *Biophysical Journal*, **65**, 2124–2140.

Wilschut, J. & Hockstra, D. (1984) Membrane fusion: from liposomes to biological membranes. *Trends in Biochemical Sciences*, **9**, 479–483.

Lipids and proteins interact to determine membrane structure and shape

Dowhan, W. (1997) Molecular basis for membrane phospholipid diversity; why are there so many lipids? *Annual Review of Biochemistry*, **66**, 199–232.

Eisenberg, D. (1984) Three-dimensional structure of membrane and surface proteins. *Annual Review of Biochemistry*, **53**, 595–623.

Israelachvili, J.N. (1978) The packing of lipids and proteins in membranes. In: *Light Transducing Membranes: Structure, Function and Evolution* (ed. D.W. Deamer), pp. 91–107. Academic Press, New York.

op den Kamp, J.A.F., Roelofsen, B. & van Deenen, L.L.M. (1985) Structural and dynamic aspects of phosphatidylcholine in the human erythrocyte membrane. *Trends in Biochemical Sciences*, **10**, 320–323.

Liposomes and drug delivery systems

Gregoriadis, G., ed. (1993) *Liposome Technology*. CRC Press, London.

Lasic, D.D. (1993) *Liposomes: from Physics to Applications*. Elsevier, Amsterdam.

New, R.R.C., ed. (1990) *Liposomes: a Practical Approach*. IRL Press, Oxford.

Sato, T. & Sunamoto, J. (1992) Recent aspects in the use of liposomes in biotechnology and medicine. *Progress in Lipid Research*, **31**, 345–372.

Lipids that play specific roles in membrane protein anchoring

Dunphy, J.T. & Linder, M.E. (1998) Signalling functions of protein palmitoylation. *Biochimica et Biophysica Acta*, **1436**, 245–261.

Englund, P.T. (1993) The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. *Annual Review of Biochemistry*, **62**, 121–138.

Thompson, G.A. & Okuyama, H. (2000) Lipid-linked proteins of plants. *Progress in Lipid Research*, **39**, 19–40.

Zhang, F.L. & Carey, P.J. (1996) Protein prenylation: molecular mechanism and functional consequences. *Annual Reviews of Biochemistry*, **65**, 241–269.

Surface layers

Goldfine, H. (1982) Lipids of prokaryotes – structure and distribution. *Current Topics in Membrane Transport*, **17**, 1–43.

Hamilton, R.J., ed. (1995) *Waxes: Chemistry, Molecular Biology and Functions*. The Oily Press, Dundee. [See chapters by: Bianchi; Nelson & Blomqvist; Rawlings; and Reiderer & Schreiber.]

Perisho, K., *et al.* (1988) Fatty acids of acylceramide from comedones and from the skin surface of acne patients and control subjects. *Journal of Investigative Dermatology*, **90**, 350–353.

Wilkinson, S.G. (1996) Bacterial lipopolysaccharides: themes and variations. *Progress in Lipid Research*, **25**, 283–344.

Yardley, H.J. & Summerly, R. (1981) Lipid composition and metabolism in normal and diseased epidermis. *Pharmaceutical Therapy*, **13**, 357–383.

See also specific chapters in the multiauthor volumes edited by Stumpf and Conn in the *General* section above.

7 Metabolism of structural lipids

7.1 PHOSPHOGLYCERIDE BIOSYNTHESIS

Phosphoglycerides are all formed from a basic parent compound – phosphatidic acid – which is also a key intermediate in triacylglycerol biosynthesis. Phosphatidic acid itself arises from acylation of glycerol 3-phosphate. There is considerable variation in the way in which the polar head groups are attached to the phosphate of phosphatidic acid.

The first phospholipid to be discovered was phosphatidylcholine, which was demonstrated by Gobley (1847) in egg yolk. The lipid was named lecithin after the Greek *lekithos* (egg yolk). Interest in the chemistry of phospholipids began with the extensive investigations of Thudicum, who isolated and analysed lipids from many animal tissues, particularly the brain, and published his results in *A Treatise on the Chemical Constitution of the Brain* (1884). It began to be appreciated later that the difficulties involved in handling these substances and obtaining a pure product were enormous. Another factor tending to discourage research into phospholipids was the very prevalent but erroneous idea that they were metabolically inert and that once laid down during the initial growth of the tissue their turnover was very slight and, hence, a purely structural significance was ascribed to them.

7.1.1 Tracer studies revolutionized concepts about phospholipids

The myth that phospholipids were slowly turning over structural molecules was exploded by the Danish chemist Hevesy, who in 1935 demonstrated that a radioactive isotope of phosphorus (^{32}P) could be rapidly incorporated as inorganic orthophosphate into tissue phospholipids. By this time it was also known that the stable isotopes ^{2}H and ^{15}N were incorporated into proteins and fats. These studies gave rise to two important concepts. First, molecules (including lipids) in living cells were subject to turnover and were continuously replaced (or parts of them were replaced) by a combination of synthesis and breakdown. Secondly, tracer methods showed the presence of metabolic pools. These are circulating mixtures of chemical substances, in partial or total equilibrium with similar substances derived by release from tissues or absorbed from the diet, which the organism can use for the synthesis of new cell constituents.

That tissues are able to synthesize their own phospholipids could also be inferred from the fact that most of their constituents (choline is one exception) are not essential dietary requirements. However, in spite of the impetus given to biosynthetic studies by the tracer work in the 1930s and 1940s, the major treatise on phospholipids in the early 1950s (Wittcoff's *Phosphatides*) could give virtually no information about their biosynthesis.

7.1.2 Formation of the parent compound, phosphatidate, is demonstrated

The different parts of phosphoglycerides – fatty acids, phosphate and base – are capable of turning over independently. Thus, to study phospholipid synthesis we must first learn the origin of each constituent and then how they are welded together. Eighteen years after Hevesy's demonstration of the rapid rate of phospholipid turnover, came the first real understanding as to how complete phospholipids are built up.

Two American biochemists, Kornberg and Pricer, found that a cell-free enzyme preparation from liver would activate fatty acids by forming their

coenzyme A esters. They then went on to demonstrate that these activated fatty acids could be used by an acyl transferase to esterify 3-*sn*-glycerol phosphate forming 1,2-diacyl-3-*sn*-glycerol phosphate (phosphatidate). We now know that there are two distinct acyl-CoA:glycerol phosphate-*O*-acyltransferases, specific for positions 1 and 2 (Section 3.4.1). Furthermore, organisms containing the Type II fatty acid synthases (Section 2.2.3.2), which form acyl-ACP products, use these as substrates for the acyltransferases. Such organisms are bacteria like *Escherichia coli*, cyanobacteria, algae and higher plants. In fact, plant cells produce phosphatidate within their plastids using acyl-ACPs but also contain acyl-CoA:glycerol phosphate acyltransferases on the endoplasmic reticulum for the synthesis of extra-chloroplast lipids such as phosphatidylcholines or triacylglycerols.

Phosphatidate is the parent molecule for all glycerophospholipids and was at first thought not to be a normal constituent of tissue lipids. Later studies have shown it to be widely distributed, but in small amounts. In fact, it is very important for the concentration of phosphatidate to be carefully regulated because of its function as a signalling molecule (Section 7.11).

Thus glycerol phosphate is one of the building units for phospholipid biosynthesis. It is mainly derived from the glycolytic pathway by reduction of dihydroxyacetone phosphate, though other methods are used to various extents by different organisms or tissues. Likewise, phosphatidate can also be produced by direct phosphorylation of diacylglycerol using diacylglycerol kinase.

7.1.3 A novel cofactor for phospholipid synthesis was found by accident

After the problem of phosphatidic acid biosynthesis had been solved, interest then grew in the pathways to more complex lipids. What was the origin of the group 'X' attached to the phosphate? The first significant finding in connection with phosphatidylcholine biosynthesis was made when Kornberg and Pricer demonstrated that the molecule phosphorylcholine was incorporated intact into the lipid. This they did by incubating phosphorylcholine, labelled both with ^{32}P and ^{14}C in a known proportion, with an active liver preparation and finding that the ratio of ^{32}P to ^{14}C radioactivities remained the same in phosphatidylcholine. Choline may come via a number of pathways having their origins in protein metabolism. For instance, the amino acid serine may be decarboxylated to ethanolamine, which may then be methylated to form choline. Phosphorylcholine arises by phosphorylation of choline with ATP in the presence of the enzyme choline kinase. The story of how the base cytidine, familiar to nucleic acid chemists, was found to be involved in complex lipid formation is a good example of how some major advances in science are stumbled upon by accident, although the subsequent exploitation of this finding by the American biochemist, Kennedy, typifies careful scientific investigation at its best. Kennedy proved that cytidine triphosphate (CTP), which was present as a small contaminant in a sample of ATP, was the essential cofactor involved in the incorporation of phosphorylcholine into lipid and later isolated the active form of phosphorylcholine, namely cytidine diphosphocholine (CDP-choline). The adenine analogue had no reactivity.

7.1.4 The core reactions of glycerolipid biosynthesis are those of the Kennedy pathway

Readers will already have come across the basic Kennedy pathway in the biosynthesis of triacylglycerols (Section 3.4.1.1). A simplified version is shown in Fig. 7.1. Four reaction steps (involving three acyltransferases and phosphatidate phosphohydrolase) are used to produce storage triacylglycerols. Two intermediates, phosphatidic acid and diacylglycerol are used for the formation of anionic phosphoglycerides (phosphatidylglycerol, phosphatidylinositol, diphosphatidylglycerol) and zwitterionic phosphoglycerides (phosphatidylcholine, phosphatidylethanolamine), respectively. Diacylglycerol is also used to produce the glycosylglycerides (Section 7.3.1).

For phosphoglycerides, as well as other glycerolipids, the first two acyltransferases (which esterify positions 1 and 2 of glycerol) have typical fatty

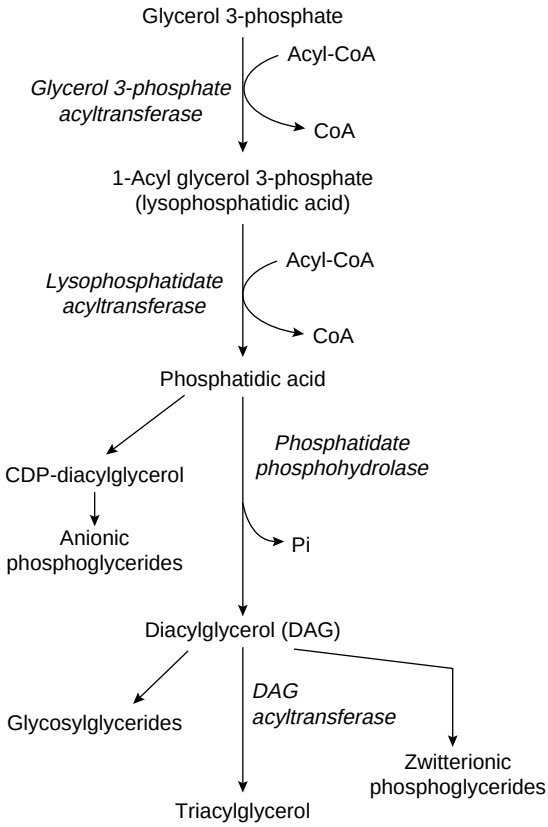


Fig. 7.1 The basic Kennedy pathway for glycerolipid biosynthesis in animals and plants.

acyl specificities. Thus, in animals and for the extrachloroplastic (endoplasmic reticulum) acyltransferases in plants, saturated fatty acids (e.g. palmitate) are preferred for esterification at position 1, whereas unsaturated fatty acids (e.g. oleate) are attached to the 2-hydroxyl. In chloroplasts, by contrast, the acyltransferases that utilize acyl-ACP typically place a saturated or unsaturated acid at position 1, while palmitate is preferred at position 2. Therefore, in plants, the subcellular origin of the backbone of glycerolipids can be deduced from the fatty acid distribution (Section 3.4.1.3).

Glycerol 3-phosphate acyltransferase (GPAT) catalyses the first, committed, step in glycerolipid assembly. In mammals, two isoforms (present in mitochondria and the endoplasmic reticulum) have been reported and can be distinguished by their relative sensitivity to *N*-ethylmaleimide. In most tissues the mitochondrial activity is relatively minor (about 10% total) but, in liver, it is as active as the endoplasmic reticulum isoform. Furthermore, the liver mitochondrial enzyme is under both nutritional and hormonal control. This property allowed the first isolation of a cDNA for a mammalian enzyme involved in glycerophospholipid synthesis when the cDNA for mouse GPAT was isolated by Sul and coworkers using differential screening. The cDNA was shown to code for the mitochondrial isoform by transfecting cell cultures and measuring an increase in acyltransferase activity in mitochondria, but not in the endoplasmic reticulum. When the deduced amino acid sequence of the mouse GPAT was compared to other acyltransferases a conserved arginine residue was identified that might be useful for binding the negatively charged substrates glycerol 3-phosphate or acyl-CoA. The identification of a conserved arginine agreed with the finding that arginine-modifying agents such as phenylglyoxal inhibited the enzyme.

Lysophosphatidate acyltransferase (LPAT) in most eukaryotic organisms has a high specificity for unsaturated fatty acids. Indeed, in plant seeds the high specificity of this enzyme has proved to be an obstacle for the engineering of 'designer' oils in transgenic plants where the accumulation of unusual fatty acids at position 2 is severely restricted. However, as noted above in plants, a second isoform of the enzyme is present in the chloroplasts that uses acyl-ACP substrates and prefers palmitoyl-ACP rather than oleoyl-ACP as substrate.

LPAT cDNAs have been cloned from yeast, plants and mammals. In humans, isoforms are present with different tissue distributions. Intriguingly, the human α-isoform is present at very high levels in testes where it has been suggested that it acts to generate phosphatidic acid for signalling purposes (Section 7.11). The acyltransferase is increased substantially (59-fold) when pre-adipocytes differentiate into adipocytes, perhaps by phosphorylation under hormonal influences.

Phosphatidate phosphohydrolase catalyses the key dephosphorylation reaction, which yields

diacylglycerol (Fig. 7.1) and thus directs carbon away from acidic (anionic) phosphoglyceride synthesis. Two forms of the enzyme have been identified in animals. One is present in the cytosol/endoplasmic reticulum and its activity is altered by translocation. The cytosolic form is inactive and it is translocated to the endoplasmic reticulum under the influence of fatty acids, fatty acyl-CoAs and phosphatidic acid itself. This makes good sense because a build-up in its substrate (phosphatidate) will activate the enzyme. Moreover, when the enzyme is used for triacylglycerol production (Sections 3.4.1.1 and 3.6.2), the supply of acyl-CoAs (or fatty acids) will stimulate the Kennedy pathway itself.

A second isoform of animal phosphatidate phosphohydrolase is present in the plasma membrane where it is thought to have a role in signal transduction (Section 7.11). Multiple isoforms have also been detected in yeast and in plants, where they again have different subcellular locations.

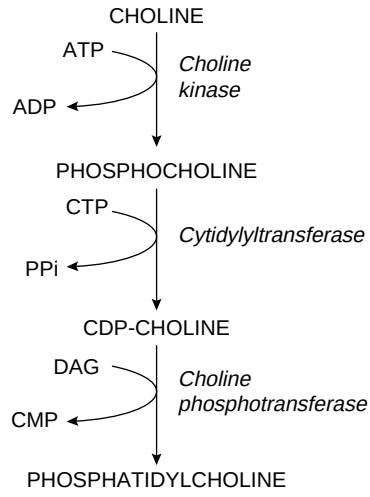


Fig. 7.2 The CDP-base pathway for phosphatidylcholine synthesis in eukaryotes. Equivalent enzymic reactions are used for the conversion of ethanolamine to phosphatidylethanolamine. DAG = diacylglycerol.

7.1.5 The zwitterionic phosphoglycerides can be made using CDP-bases

As can be seen in Fig. 7.1, diacylglycerol generated by phosphatidate phosphohydrolase can have several fates, one of which is to be used for generation of the zwitterionic phosphoglycerides, phosphatidylcholine and phosphatidylethanolamine. In animals and plants the CDP-base pathway is the main pathway for production of phosphatidylcholine, which represents 40–50% of the total lipids in most of their membranes.

Three enzymic steps are used (Fig. 7.2). First, the base (either choline or ethanolamine) is phosphorylated by a kinase that uses ATP. In most tissues, including those from animals, plants and the yeast *Saccharomyces cerevisiae*, separate choline and ethanolamine kinases are found. In fact, in yeast the genes for these enzymes have been mapped to separate chromosomes. However, some choline kinases do have limited activity with the alternative ethanolamine substrate.

The second enzyme in the pathway is a cytidylyltransferase, which links the phosphorylated base to CMP to create CDP-choline or CDP-ethanolamine. In those tissues where phosphatidylcholine synthesis has been studied in detail, the cytidylyltransferase seems to be important in regulating the overall rate of phospholipid formation. This includes the initiation of lung surfactant synthesis (Section 7.7), indole 3-acetic acid-induced plant growth and the formation of Golgi-derived secretory vesicles in yeast. In animals, the cytidylyltransferase contains lipid binding and phosphorylation domains. Near the N-terminus is a nuclear localization signal. Although the enzyme tends to be mainly soluble (cytoplasmic) it will associate with membranes, including the nuclear envelope, when it is activated. Binding of phospholipids and phosphorylation at a number of sites activate the cytidylyltransferase.

In plants choline phosphate cytidylyltransferase activity was increased long term by gene expression, as well as short term by activation. Of the factors identified as being important, CTP and AMP concentrations were significant. This was interesting because, in yeast, the supply of CTP (by CTP synthetase) was also found to stimulate the formation of phosphatidylcholine.

The CDP-choline or CDP-ethanolamine produced by the cytidylyltransferases are rapidly utilized by phosphotransferase enzymes, which release CMP and transfer the phosphorylated base to diacylglycerol. The phosphotransferases are integral membrane proteins and the ethanolaminephosphotransferase has never been purified from any source. However, from selectivity experiments with different diacylglycerols or by examining the molecular species of phosphatidylethanolamine or phosphatidylcholine formed, separate phosphotransferase enzymes seem to be present in both animals and plants. In yeast, the genes for these two individual phosphotransferases have been isolated.

As well as being involved in the production of phosphatidylcholine, CDP-choline can be used for the formation of the sphingosine-containing phospholipid, sphingomyelin (Section 6.3). However, the major pathway for the formation of sphingomyelin transfers phosphorylcholine from phosphatidylcholine to a ceramide (Section 7.4.6).

7.1.6 CDP-diacylglycerol is an important intermediate for phosphoglyceride formation in all organisms

In Fig. 7.1 it was seen that phosphatidic acid can be converted into CDP-diacylglycerol (CDP-DAG) using another cytidylyltransferase (CDP-diacylglycerol synthase, CDP-DAG synthase). The CDP-DAG is an important intermediate for the acidic phosphoglycerides, phosphatidylinositol, phosphatidylglycerol and diphosphatidylglycerol in animals and plants. In yeast it also has a role in phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine production, whereas in *E. coli*, all phosphoglycerides are formed via CDP-diacylglycerol.

We will begin by considering the situation in animals and plants for which the main reactions are shown in Fig. 7.3. The CDP-diacylglycerol intermediate can react with either *myo*-inositol to give rise to the various inositol-containing phospholipids or with glycerol 3-phosphate. Phosphatidylinositol is the main inositol-containing phospholipid in all eukaryotes. It can be further phosphorylated and the 4-kinase and 5-kinase enzymes are the most important quantitatively (Fig. 7.3). Another important phosphorylation is that catalysed by a 3-kinase (Section 7.10) where a series of inositol-lipids, important for signalling, is produced.

The reaction of glycerol 3-phosphate with CDP-diacylglycerol forms an intermediate that does not accumulate because it rapidly loses a phosphate to yield phosphatidylglycerol (Fig. 7.3). In animals, almost all of the phosphatidylglycerol is converted into diphosphatidylglycerol (cardiolipin), which accumulates as an important membrane constituent of the inner mitochondrial membrane. An exception is the epithelial cell of the lung that produces pulmonary surfactant (Section 7.7) containing significant amounts of phosphatidylglycerol.

The formation of phosphatidylglycerol in plants and algae is most active in the chloroplast where, of course, phosphatidylglycerol is the only significant phosphoglyceride (Section 6.2.1).

In yeast, the CDP-diacylglycerol pathway is not only important for acidic phosphoglyceride formation but is also the main way in which the zwitterionic phospholipids are made. A key reaction in the latter connection is the reaction of CDP-diacylglycerol with serine to produce phosphatidylserine. The overall scheme for phosphoglyceride synthesis in yeasts is shown in Fig. 7.4.

After CDP-diacylglycerol has been converted into phosphatidylserine, a decarboxylation yields phosphatidylethanolamine, which can then be methylated three times to produce phosphatidylcholine. These reactions are described in the following section in more detail and are typical of bacterial systems. Indeed, phospholipid synthesis in yeast can be regarded as a more complex version of *E. coli* metabolism, but where the modifications have not gone quite as far as in animals or plants.

7.1.7 Phospholipid formation in *E. coli* is entirely via CDP-diacylglycerol

E. coli makes use of reactions that are found in yeast for the production of phosphoglycerides via CDP-diacylglycerol. The reactions and the enzymes (and genes) catalysing these steps are shown in Fig. 7.5.

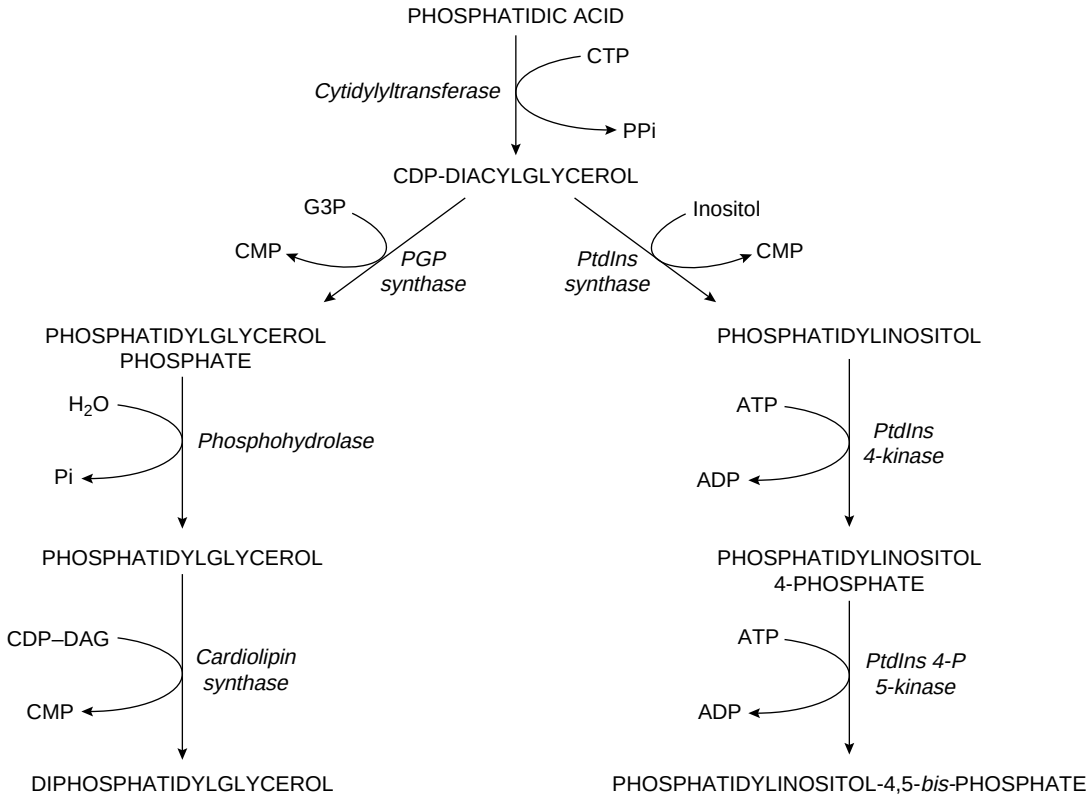


Fig. 7.3 Pathways for the synthesis of acidic phosphoglycerides in animals and plants. PtdIns = phosphatidylinositol; PGP = phosphatidylglycerol phosphate; G3P = glycerol 3-phosphate; CDP-DAG = CDP-diacylglycerol.

Because of the availability of mutants with deleted or severely reduced gene expression, we have been able to learn much about the function of specific phospholipids recently from experiments with *E. coli* (Section 6.5.12). This bacterium has one of the simplest phospholipid compositions (75% phosphatidylethanolamine, 15% phosphatidylglycerol, 10% diphosphatidylglycerol) and so is relatively straightforward to study. It does not methylate phosphatidylethanolamine to produce phosphatidylcholine. In bacteria containing phosphatidylcholine, such as the photosynthetic bacteria (*Rhodobacter* spp., etc.), this lipid is made entirely by three successive methylations, using S-adenosylmethionine, of the head group of phosphatidylethanolamine (Fig. 7.4).

7.1.8 Differences between phosphoglyceride synthesis in different organisms

A major difference between phosphoglyceride formation in yeasts and bacteria compared to animals is in the production of phosphatidylserine. In the simpler organisms, phosphatidylserine plays a major role as an intermediate in the production of phosphatidylethanolamine (and phosphatidylcholine) and is made from CDP-diacylglycerol (Figs 7.4 and 7.5). However, in animals it is made via an exchange reaction in which the head group of an existing phospholipid (usually phosphatidylethanolamine or phosphatidylcholine) is exchanged for serine. The reactions have been studied in most detail using Chinese hamster ovary cell mutants

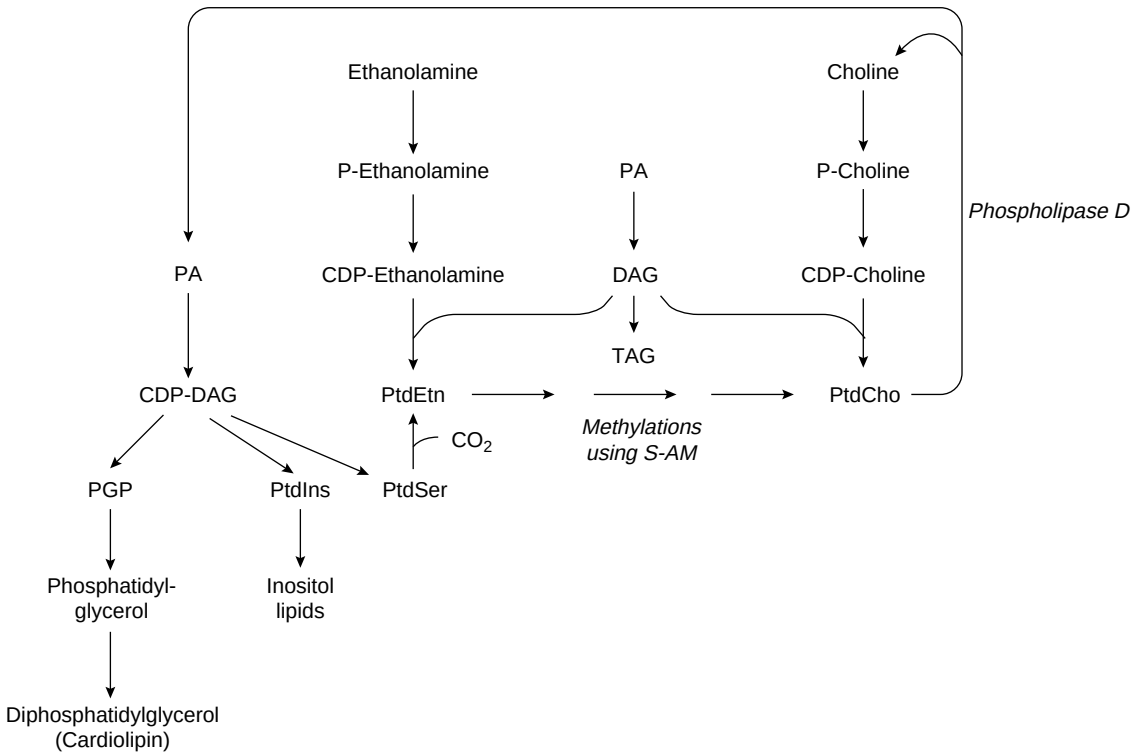


Fig. 7.4 Phosphoglyceride synthesis in yeast (*Saccharomyces cerevisiae*). PtdIns = phosphatidylinositol; PtdCho = phosphatidylcholine; PtdEtn = phosphatidylethanolamine; PtdSer = phosphatidylserine; PGP = phosphatidylglycerol phosphate; G3P = glycerol 3-phosphate; CDP-DAG = CDP-diacylglycerol; S-AM = S-adenosylmethiomine.

and separate enzymes are involved in exchanging serine on the two phospholipids. Further fine-tuning of the proportions of different phosphoglycerides can be made by the presence of phosphatidylserine decarboxylase (Fig. 7.6).

For the other phospholipids, the importance of different pathways in different organisms is listed in Table 7.1. With the exceptions of cardiolipin production, the same pathways are used by different organisms, but it is the number of enzymes present or their relative activity that is different.

7.1.9 Plasmalogen biosynthesis

Ether lipids are widespread in animal tissues and, of these, the plasmalogens are the most important. In mammals, ether links are virtually exclusively confined to the choline and ethanolamine glycerolipid classes.

Although the pathway for the formation of ethanolamine (and choline) plasmalogens uses CDP intermediates, there are several points of difference with those for the synthesis of diacylphospholipids. First, the ethanolamine (choline) phosphotransferase enzyme uses a plasmalogenic diacylglycerol (1-alkenyl-2-acyl-*sn*-glycerol). Hajra and his group showed that the starting point for its synthesis was dihydroxyacetone phosphate (see also Section 3.4.1.2). This compound was acylated, the acyl group at position 1 being substituted by a long-chain alcohol and then the keto group at position 2 reduced. The newly created hydroxyl at *sn*-2 could then be acylated and the phosphate group removed to produce a plasmalogenic diacylglycerol (Fig. 7.7).

It is interesting to note that the acyldihydroxyacetone phosphate at the start of the pathway can also be reduced to form lysophosphatidate thus

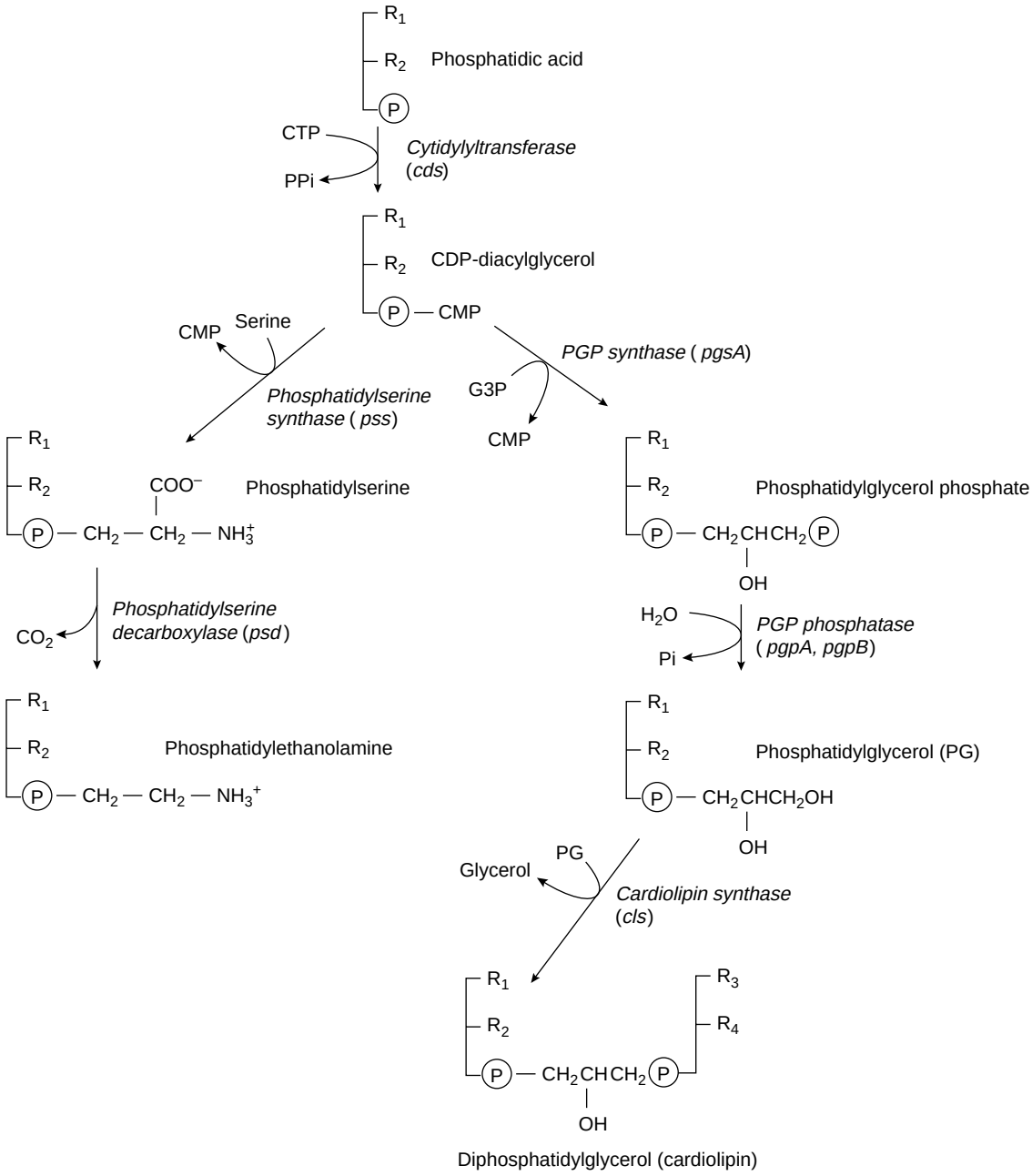


Fig. 7.5 Phosphoglyceride synthesis in *E. coli* (gene nomenclature for the enzymes concerned is shown in parentheses).

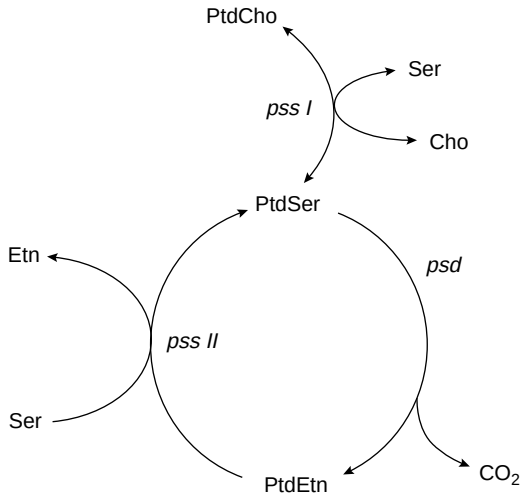


Fig. 7.6 Phosphatidylserine formation in mammals: *pssI* codes for phosphatidylserine synthase I; *pssII* codes for phosphatidylserine synthase II; *psd* codes for phosphatidylserine decarboxylase.

providing another potential source of phosphatidate and a link between acyl- and alkyl-glycerolipid formation.

Further work on the pathway, which particularly involved the laboratories of Snyder in the USA and Paltauf in Austria, demonstrated that the 1-alkyl, 2-acyl-*sn*-glycerol was used by phosphotransferase enzymes to give saturated ether products.

The desaturation of positions 1 and 2 of the alkyl chain to form an alkenyl chain is catalysed by cell-free extracts of intestinal epithelial cells, tumour cells and brain. The enzyme is present in the microsomal fraction but the reaction is stimulated by a high molecular weight, heat-labile factor in the soluble cytosol. The fact that a reduced pyridine nucleotide and molecular oxygen are absolute requirements and that the reaction is inhibited by cyanide but not by CO strongly suggest that this enzyme is very similar to the fatty acyl-CoA desaturase described in Section 2.2.5. This provides another interesting example of an enzyme catalysing a modification of a hydrocarbon chain in the intact lipid molecule.

For choline plasmalogen the situation appears even more complicated because the desaturation of the alkyl chain to form the unsaturated alkenyl chain does not take place on a choline alkyl phospholipid. Instead, the ethanolamine plasmalogen appears to be made first and then the head group exchanged for choline or the ethanolamine plasmalogen is converted via a series of reactions into the unsaturated ether equivalent of diacylglycerol (alk-1-enylacylglycerol). A choline phosphotransferase enzyme can then produce choline plasmalogen.

One of the most exciting discoveries in the ether lipid field has been that of certain acetylated forms of alkylglycerolipids (originally described as platelet activating factor) with potent biological activities. Most important of these is 1-alkyl-2-acetyl-*sn*-glycerol 3-phosphocholine the metabolism and function of which are detailed next.

Table 7.1 Major differences in phosphoglyceride synthesis in different organisms

	Main pathway used		
Lipid	Animals	Yeast	*E. coli*
Phosphatidylcholine	CDP-choline	CDP-DAG	Not present
Phosphatidylethanolamine	CDP-Etn	CDP-DAG	CDP-DAG
Phosphatidylinositol	CDP-DAG	CDP-DAG	Not present
Phosphatidylglycerol	CDP-DAG	CDP-DAG	CDP-DAG
Cardiolipin	CDP-DAG[a]	CDP-DAG[a]	CDP-DAG[a]
Phosphatidylserine	Exchange	CDP-DAG	CDP-DAG

[a] Cardiolipin synthase uses CDP-DAG substrate in animals and yeast but phosphatidylglycerol in *E. coli*.
CDP-Etn: CDP-ethanolamine.

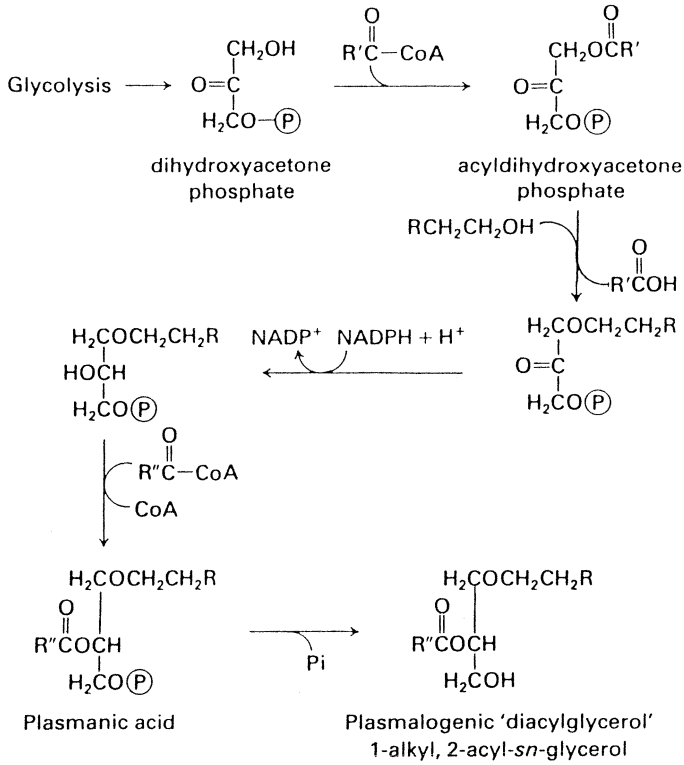
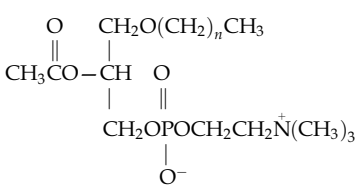


Fig. 7.7 Generation of substrate for plasmalogen synthesis.

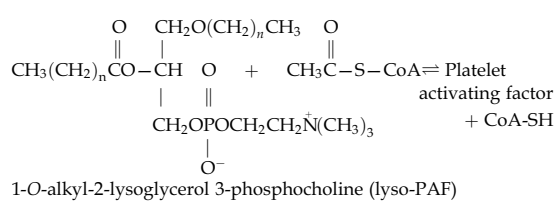
7.1.10 Platelet activating factor (PAF): a biologically active phosphoglyceride

The action of platelet activating factor (PAF) was first observed when a 'fluid phase mediator' was released from leukocytes to cause platelets to release vasoactive amines. It was identified as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine by Hanahan's group in Texas in 1980:



The compound has several unique features: (1) it was the first well-documented example of a biologically active phosphoglyceride; (2) it has an ether link at position 1 (fatty acyl derivatives have 1/300th the activity); and (3) an acetyl moiety is present at the 2-position (butyryl substitution lowers activity some 1000-fold).

In hypersensitivity reactions, PAF appears to be made mainly via a remodelling pathway from alkylacylglycerophosphocholines. Phospholipase A_2 removes the acyl group from position 2, where it is replaced using acetyl-CoA to yield PAF:



1-*O*-alkyl-2-lysoglycerol 3-phosphocholine (lyso-PAF)

In contrast, synthesis *de novo* beginning by the acetylation of 1-alkyl-glycero-3-phosphate and finishing by using a cholinephosphotransferase is the

primary source of endogenous levels of PAF in cells and blood. PAF is inactivated by removal of its acetyl group by an acetylhydrolase to yield the biologically inactive lyso-PAF. There are two different acetylhydrolases – one in cells and the other in the extracellular (plasma) compartment.

PAF is bound to platelets through a single class of specific receptors (about 250 per cell). Specific receptors have also been documented on many other cell types and it seems likely that most biological responses to PAF are receptor-dependent.

Platelet activating factor is a very potent molecule having biological activity at 5×10^{-11} M concentrations. Some of its effects are listed in Table 7.2 where it will be seen that this molecule has wide-ranging effects in many tissues.

In terms of its biochemistry, PAF has general effects on intracellular regulation by increasing Ca^{2+} uptake and increasing protein phosphorylation via protein tyrosine kinases, protein kinase C and G-protein receptor kinase. It alters inflammatory responses by altering expression of cytokines like tumour necrosis factor-α. Recently, it has been shown to activate immediate-early genes (e.g. *c-fos*, *c-jun*) that produce proteins to change other gene expression. PAF also interacts with other biologically active lipids by increasing arachidonate turnover (Section 2.4) and activating the phosphatidylinositol-specific phospholipase C (Section 7.9).

A chemically synthesized derivative of PAF (with a methoxy group at position 2) shows highly selective antitumour properties. Apparently this derivative prevents membrane formation by inhibiting the cytidylyltransferase of the CDP-choline pathway (Section 7.1.5).

7.2 DEGRADATION OF PHOSPHOLIPIDS

A variety of hydrolytic enzymes, the phospholipases, exists to remove selectively the different constituents of the phospholipid molecule: the acyl groups at positions 1 and 2 of phosphoglycerides, the phospho-base moiety or the base alone.

Phospholipases are classified according to the positions of their attack on the substrate molecule as illustrated in Fig. 7.8. Phospholipases of type A yield a monoacyl (lyso) phospholipid while phospholipases C and D yield a lipid (diacylglycerol and phosphatidic acid, respectively) and a water-soluble product. Phospholipase D can use an hydroxyl in an organic molecule instead of water and, thus, catalyse transphosphatidylation rather than hydrolysis. (This activity can result in the production of the artefact phosphatidylmethanol during

Table 7.2 Some effects of platelet activating factor (PAF) in different cells/tissues

Cell/tissue	Effect	Implications
Platelet	Degranulation, aggregation	Amine release, coronary thrombosis
Neutrophil	Chemotaxis, aggregation, superoxide generation	Antibacterial activity
Alveolar macrophage	Respiratory bursts, superoxide generation	Antibacterial activity
Liver	Inositide turnover, glycogenolysis stimulation	Overall control of activity
Exocrine secretory glands	Similar effects to acetylcholine	Overall control of activity
Leukaemic cells	Specific cytotoxicity towards the cells	Treatment?
Vascular permeability	Mimics acute and chronic inflammation	Pathogenesis of psoriasis?
Lungs	Increases airway and pulmonary oedema; decreases compliance	Asthma and other respiratory conditions

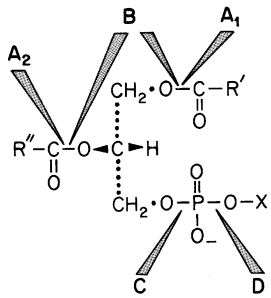


Fig. 7.8 Sites of action of phospholipases A, B, C and D.

methanol extraction of tissue extracts if the enzyme is not inactivated!) Phospholipases of type B differ from phospholipases A_1 or A_2 in that they can hydrolyse acyl groups at both positions, but they are relatively rare. There are also lysophospholipases and acyl hydrolases (which can act against acyl lipids in general) in some tissues.

All true phospholipases share the same general property of having relatively low activity against monomeric soluble phospholipids but become fully active against aggregated structures, such as phospholipid solutions above their critical micellar concentration or membrane phospholipids in bilayer or hexagonal structures. They play an obvious role as digestive enzymes whether it be in the digestive secretions of mammals (Section 5.1.1) or in bacterial secretions. In addition, some enzymes, such as phospholipase A_2, play important roles in re-modelling the acyl composition of membrane lipids. Several types of phospholipase are needed for the production of lipid-derived signalling molecules (Sections 2.4, 7.9 and 7.11) and which are associated with cellular regulation.

7.2.1 General features of phospholipase reactions

As mentioned above, phospholipases are distinguished from general esterases by the fact that they interact with interfaces in order to function. The difference in reaction velocity with substrate concentration for these two types of enzymes is illustrated in Fig. 7.9. Whereas esterases show classical Michaelis–Menten kinetics, the phospholipases show a sudden increase in activity as the substrate (phospholipid) concentration reaches the critical micellar concentration (CMC) and the molecules tend to form aggregates or micelles with polar ends in the aqueous environment (Fig. 7.10). Because of the physical nature of the substrate, enzyme activity is dependent both on the (hydro-

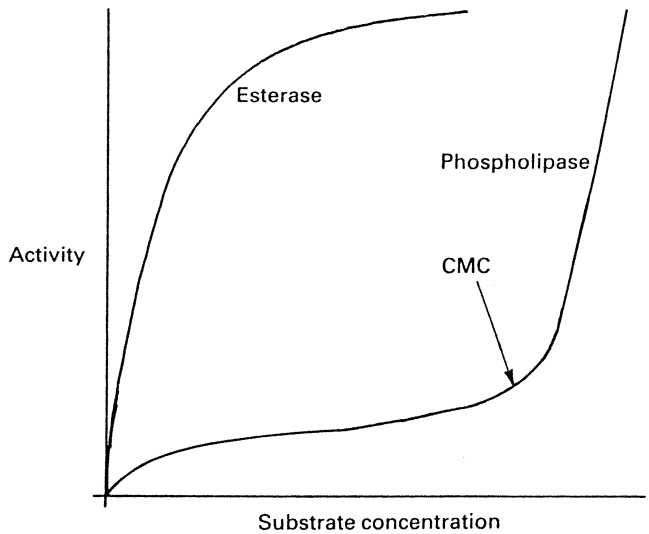


Fig. 7.9 Enzyme kinetics for esterases and phospholipases. CMC = critical micellar concentration.

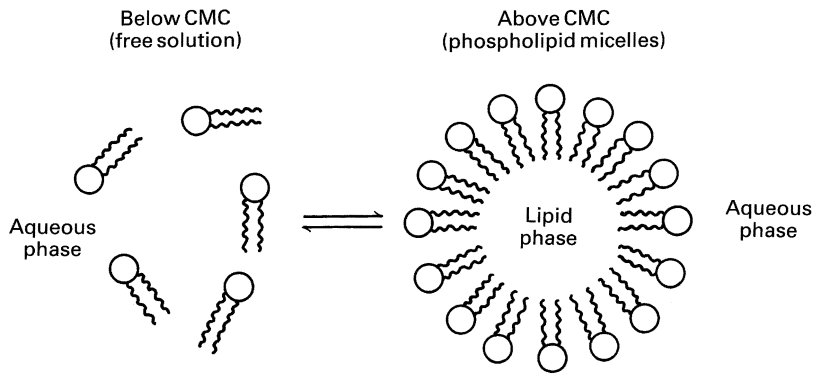


Fig. 7.10 Aggregation of a phospholipid at its critical micellar concentration (CMC).

phobic) interaction with the aggregate and on the formation of a catalytic Michaelis complex. Several factors have been suggested as being responsible for the increased rate of hydrolysis at interfaces:

(1) the very high local substrate concentration;
(2) substrate orientation and physical state at the interface;
(3) increased rates of diffusion of products from the enzyme;
(4) increased enzyme activity due to conformational changes on binding to the interface.

For a typical phospholipid in solution, formation of a micelle increases its effective concentration by at least three orders of magnitude – depending on the CMC (factor 1 above). The British biochemist Dawson and also Dutch workers including van Deenen, de Haas and Slotboom have studied how the nature of aggregated lipid (factor 2) influences markedly the activity of phospholipases. Such activity seems to depend on four parameters – surface charge, the molecular packing within the aggregate, the polymorphism of the aggregate and the fluidity of the phospholipid's acyl chains. The surface charge can be quite different from the bulk pH and is influenced by ionic amphipaths as well as ions in the aqueous environment. This explains why, for example, the phospholipase B from *Penicillium notatum* will not attack pure phosphatidylcholine but is active in the presence of activators, such as phosphatidylinositol, which give phosphatidylcholine micelles a net negative charge. Molecular packing is particularly important for the acylhydrolases, but is also relevant to the phosphodiesterases (phospholipase C). Changes in activity with this parameter have been referred to in the example of diethylether activation above. Polymorphic states can include micelles, bilayer structures and hexagonal arrays (Section 6.5.3). There is some evidence that certain phospholipases attack preferentially hexagonal structures and since these are believed to be formed transiently at sites of membrane fusion (Section 6.5.10), the phospholipase may help to remove fusative lipid and help re-establish the normal membrane bilayer. In addition, it has been well-established that gel-phase lipids are attacked preferentially by phospholipases from several sources.

Factor 3 is particularly important for phospholipases A and B where both products from a typical membrane (natural) phospholipid are hydrophobic. In the case of phospholipases C and D, where one product is water-soluble, action at an interface is still useful because the water-soluble product can move into the aqueous environment while the other product can diffuse into the hydrophobic phase. Similar physico-chemical arguments explain why many phospholipases show increased activity in the presence of organic solvents. Thus, when diethylether is used with phospholipase A assays it is thought that accumulating fatty acids are more readily removed. In addition, the solvent may allow more ready access

of the enzyme to the hydrocarbon chains of the phospholipid and, by reducing micellar size, increases the effective surface area for reaction. Finally, conformational changes (factor 4) have been shown to take place when some digestive phospholipases interact with substrate and/or Ca^{2+}. Kinetic studies indicate that such a conformation change is needed for maximal activity.

7.2.2 Phospholipase A activity is used to remove a single fatty acid from intact phospholipids

Phospholipase A enzymes are divided into two basic groups, depending on which acyl moiety is hydrolysed. Phospholipases A_1 comprise a large group of enzymes some of which may also degrade neutral lipids and, so, act as lipases. Usually the enzymes have a wide specificity and act well on lysophospholipids. Their function in most cases is obscure – except for the role of lipases with phospholipase A_1 activity in lipoprotein metabolism (Section 3.5.2 and below).

The first example of a phospholipase A_1 to be purified was from *E. coli*, which actually has two separate enzymes – a detergent-resistant enzyme in the outer membrane and a detergent-sensitive enzyme in the cytoplasmic membrane and soluble fractions.

In animals, phospholipase A_1 is present in lysosomes and has an appropriately low pH optimum (about 4.0). It does not need Ca^{2+} for activity but Ca^{2+} and charged amphipaths influence hydrolysis by altering the surface charge on the substrate micelle or membrane.

Also present in animals are two enzymes that, while preferentially hydrolysing triacylglycerols, will also hydrolyse the bond at position 1 of phospholipids. These are the extra-hepatic lipoprotein lipase (Section 3.5.2) and the hepatic lipase. The latter enzyme is activated by apolipoprotein E to hydrolyse phospholipids and by phospholipids to act as a lipase.

Phospholipases A_2 are extremely widespread in Nature and their activity was noted first by Bokay over a hundred years ago. He studied the degradation of phosphatidylcholine by pancreatic secretions and at the turn of the twentieth century cobra venom phospholipase activity was discovered. These two sources of phospholipase have proved very useful for purifying and studying the enzyme.

Phospholipase A_2 enzymes can be divided into four main groups – based both on their properties and on sequence similarities (Table 7.3). Sufficient quantities of the phospholipases from pancreas and cobra venom (Group I) have been purified for detailed X-ray analyses to be made. Detailed reaction mechanisms have also been proposed (see Waite, 1996, in Further Reading).

Pancreatic phospholipases are synthesized as zymogens that are activated by the cleavage of a heptapeptide by trypsin. Cleavage of this peptide exposes a hydrophobic sequence, which then allows interaction of the enzyme with phospholipid substrates. The enzyme is very stable and its seven disulphide bonds no doubt play a key role here. Chemical modification and NMR studies have shown clearly that the catalytic and binding sites are distinct in both the pancreatic and snake venom phospholipases A_2. In confirmation, it is known that the zymogen form of the pancreatic enzyme is also active even though its binding site is masked. Ca^{2+} is absolutely required for activity and seems to interact with both the phosphate and carbamyl groups of the ester undergoing hydrolysis.

The sequences of pancreatic phospholipase A_2 and the enzymes from the venoms of Elapids (cobras, kraits and mambas) are closely related in contrast to the enzymes from vipers [e.g. *Crotalus* (rattlesnakes)], which are Group II enzymes (Table 7.3). Subtle modifications in the sequence of the Group I–II enzymes causes significant changes in their crystal structure and substrate interaction. For example, pancreatic enzymes (Group IB) crystallize as monomers, rattlesnake (Group IIA) as dimers and cobra phospholipases A_2 (Group IA) as trimers.

Phospholipase A_2 enzymes also have other important metabolic functions in addition to the overall destruction of phospholipids as catalysed by digestive pancreatic or venom enzymes. An enzyme in mitochondrial membranes seems to be intimately connected with the energy state of this organelle. Thus, the phospholipase is inactive in fully coupled mitochondria and only becomes

Table 7.3 Phospholipase A_2 enzymes

Source	Location	Mass (kDa)	Ca^{2+} needed?
Group I			
A Cobras, kraits	Secreted	13–15	mM
B Pancreas			
Group II			
A Rattlesnakes, vipers	Secreted	13–15	mM
B Gaboon viper			
Group III			
Bee, lizard	Secreted	16–18	mM
Group IV			
Kidney, macrophages, platelets	Cytosolic	85	μM
Others			
Myocardium	Cytosolic	40	None
Liver, macrophages	Lysosomal	—	None

Based on Table II in M. Waite (1996) Phospholipases in *Biochemistry of Lipids, Lipoproteins and Membranes* (eds. D.E. Vance and J.E. Vance), pp. 211–236 with kind permission of the author and Elsevier Science.

active when ATP and respiratory control drop to low levels. Also, the widespread distribution of phospholipases A_2 allows many tissues to perform re-tailoring of the molecular species of membrane lipids by the Lands mechanism. In this process, named after Lands, the American biochemist who first described it, cleavage of the acyl group from the *sn*-2 position yields a lysophospholipid that can be re-acylated with a new fatty acid from acyl-CoA (Fig. 7.11).

Some phospholipases A_2 play key roles in signal transduction. The Group IV cytosolic phospholipases A_2 translocate to membranes when the intracellular Ca^{2+} concentration rises. They have a very high specificity for arachidonate at the *sn*-2 position of phospholipids and a distinct catalytic mechanism compared to the Group I–III enzymes. Ca^{2+} is not involved in catalysis itself but, by promoting enzyme–membrane interaction, increases activity several-fold. Within its catalytic domain a highly conserved sequence around the active-site serine (ser 228) is identical with that of phospholipase B from *Penicillium* (below) and another serine (ser 505) can be phosphorylated by mitogen activated protein (MAP) kinase. Phosphorylation increases the activity significantly and this can account for typical agonist stimulation of phospholipase A_2 activity. In addition, a number of cellular agonists (such as the inflammatory cytokines interleukin-1 and tumour necrosis factor) increase the cellular content of cytosolic phospholipase A_2 by gene expression.

Several phospholipases A_2 are Ca^{2+}-independent. One of these, which is found in blood, has particularly good activity against short-chain acyl groups. It is believed to be important for inactivating PAF (Section 7.1.10).

Finally, it is well-recognised that many phospholipases A have high activity against oxidized or oxygen-containing acyl groups. They can, therefore, play a key role in removing such moieties from oxidized lipids in order to preserve normal lipid function.

7.2.3 Phospholipase B and lysophospholipases

Only one phospholipase B has been highly purified – that from *Penicillium notatum*. This enzyme will, of course, also hydrolyse lysophospholipids and, indeed, under most conditions has much greater activity towards the latter. In the presence of

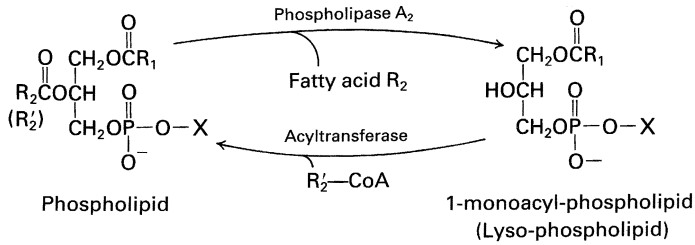


Fig. 7.11 The 'Lands Mechanism' for substitution of the acyl species at the *sn*-2 position of phospholipids.

detergents (Triton X-100 is usually used) it shows roughly equal activity towards diacyl- and monoacylphospholipids. The enzyme appears to have two distinct binding sites and the intermediate lysophospholipid must move from one to the other during hydrolysis.

In contrast to phospholipase B, lysophospholipases are widely distributed and are found in micro-organisms, bee venoms and mammalian tissues. Many of them have limited activity towards intact phospholipids.

In both heart and liver there are two isoforms – a large size (63 kDa) that also catalyses transacylations and a small form (22 kDa) that only catalyses hydrolysis.

An obvious function of lysophospholipases is to prevent a significant build-up of lytic monoacylphospholipids, which would clearly damage membrane function. In addition, the transacylation property of some lysophospholipases suggests that they could play a significant role in molecular species re-modelling by a process that would be independent of the Lands mechanism (Fig. 7.11).

7.2.4 Phospholipases C and D remove water-soluble moieties

In accordance with the observations showing the independent turnover of phosphate and base moieties, other enzymes named phospholipases C and D are known to hydrolyse each of the two phosphate links (Fig. 7.8). Phospholipases C have been traditionally associated with secretions from certain pathological bacteria. Thus, much of the damage caused by *Clostridium welchii* when giving rise to gas gangrene, is caused by phospholipase C in its toxins. The enzymes from *Bacillus cereus* are the best studied – three different proteins having been isolated, one having a broad specificity, one being a sphingomyelinase and one attacking phosphatidylinositol specifically.

In mammalian tissues, phospholipases C are found in the cytosol and in lysosomes. In the latter, the enzymes have a broad substrate selectivity, so do not need Ca^{2+} and, as expected, have an acidic pH optimum (about 4.5).

The cytosolic phospholipases C have a neutral pH optimum, require Ca^{2+} and can be divided into three groups. The best studied are those acting on inositol phospholipids, particularly phosphatidylinositol-4,5-*bis*phosphate. These are exceptionally important in signal transduction (Section 7.9). Another enzyme hydrolyses phosphatidylcholine while there are also specific sphingomyelinases, which are phospholipase C enzymes that release ceramide from sphingomyelin (Section 7.4.7). Both of these types of phospholipase C are also important for signal transduction (Sections 7.9 and 7.12).

Traditionally, phospholipase D has been thought of as a plant enzyme and it has been purified from several tissues including cabbage and carrots.

In addition to catalysing hydrolysis of phospholipids the enzyme will cause phosphatidate exchange. This allows the formation of new phospholipids in the presence of an appropriate alcohol. For example:

$$\text{Phosphatidylcholine} + \text{glycerol} \rightleftharpoons \text{phosphatidylglycerol} + \text{choline}$$

Such transphosphatidylation reactions were first noticed because phosphatidylmethanol was formed when plant tissues were extracted with

methanol. However, transphosphatidylation does not seem to be a physiological function for plant phospholipase D because the phosphatidylglycerol produced in the above reaction is a racemic mixture rather than having the two glycerols in the opposite configuration as occurs naturally. Nevertheless, the activity can be used in the laboratory for the preparation of phospholipids.

Mammalian phospholipases D have been found in many tissues and it is clear that, like the plant enzymes, they play a role in lipid signalling reactions and the control of cellular activity (Section 7.11). Interestingly, the product of phospholipase D activity, phosphatidic acid, binds to some of the phosphatidylinositol-4,5-*bis*phosphate-hydrolysing phospholipase C enzymes, causing their activation. This is an example of 'cross-talk' between signalling pathways where products or intermediates in one pathway affect the activity of a second pathway.

7.2.5 Phospholipids may also be catabolized by non-specific enzymes

Not all phospholipid hydrolysis is catalysed by phospholipases. The action of two triacylglycerol lipases in this regard has already been mentioned. In addition, acyl hydrolases are present, particularly in plant leaves. These enzymes have activity towards a variety of lipids, including partial glycerides, glycosylglycerides and phosphoglycerides. The leaf acyl hydrolases have very high activities and they are rather resistant to denaturation. Failure to inactivate the enzymes leads to spoilage of vegetables during freezing.

7.3 METABOLISM OF GLYCOSYLGLYCERIDES

The addition of sugar molecules to diacylglycerols to form glycosylglycerides involves the UDP-sugars familiar in carbohydrate biochemistry. By analogy with phospholipases, a battery of enzymes exists to remove selectively the acyl groups and the sugar moieties of glycosylglycerides.

7.3.1 Biosynthesis of galactosylglycerides takes place in chloroplast envelopes

Since the galactosylglycerides are confined (almost exclusively) to chloroplasts, it would seem natural to seek an active enzyme preparation from these organelles. First, it was shown that carefully prepared isolated chloroplasts could incorporate ^{14}C-galactose into the lipids. The nature of the substrates involved was not clear until Ongun and Mudd in California showed that an acetone powder of spinach chloroplasts would catalyse the incorporation of galactose from UDP-galactose into monogalactosyldiacylglycerol if the acetone-extracted lipids were added back. The acceptor proved to be diacylglycerol or, for the synthesis of digalactosyldiacylglycerol, a monogalactosyldiacylglycerol acceptor was needed. Further work on the enzymes involved was carried out by Joyard and Douce in Grenoble who showed that the reactions were confined to the chloroplasts envelope – hence the need for carefully prepared chloroplasts.

The reactions are, therefore:

$$\text{1,2-diacylglycerol} + \text{UDP-galactose} \rightleftharpoons \text{monogalactosyldiacylglycerol} + \text{UDP}$$

$$\text{monogalactosylglycerol} + \text{UDP-galactose} \rightleftharpoons \text{digalactosyldiacylglycerol} + \text{UDP}$$

The UDP-galactose is produced in the cytosol. In contrast, the diacylglycerol is synthesized by a phosphatidate phosphohydrolase localized in the envelope. The two galactosyltransferases have slight differences in their enzymic characteristics and, of course, result in the formation of β- and α-glycosidic bonds.

More recently, in Wintermanns' laboratory in The Netherlands, it was noticed that isolated chloroplast envelopes were capable of forming digalactosyldiacylglycerol from labelled monogalactosyldiacylglycerol by a reaction that did not require UDP-galactose. Further examination revealed that inter-lipid galactosyltransfer was involved thus:

$$2\ \text{monogalactosyldiacylglycerol} \rightleftharpoons \text{digalactosyldiacylglycerol} + \text{diacylglycerol}$$

This enzyme is also capable of generating higher homologues by further transfer of galactose from monogalactosyldiacylglycerol. The trigalactosyl- and tetragalactosyldiacylglycerols thus formed are detected in small quantities in many isolated chloroplasts. It is, however, unclear at present whether such higher homologues are actually present in vivo or are artefacts of the isolation and analytical processes. The relative rate of formation of digalactosyldiacylglycerol by the two pathways is also an area of controversy.

One difference in the fatty acyl compositions of galactosylglycerides, which has been noticed repeatedly, in the analysis of plants is the presence of hexadecatrienoate (16:3) in monogalactosyldiacylglycerol (but little in digalactosyldiacylglycerol) from certain plants. The presence of 16:3 in some plants seems to be related to the provision of palmitate at the *sn*-2 position of monogalactosyldiacylglycerol where it acts as a substrate for fatty acid desaturases. In such plants the diacylglycerol for galactolipid biosynthesis is generated by phosphatidate phosphohydrolase within the chloroplast. In contrast, the source of diacylglycerol for galactolipid biosynthesis in other plants comes from outside the chloroplast and it does not contain 16-carbon acids at the *sn*-2 position. A glance at Table 7.4 will also reveal that even in plants, like spinach, which do contain hexadecatrienoate in their monogalactosyldiacylglycerol, little is present in the digalactosyl derivative. It is presumed that this is due to substrate specificity of the second galactosyltransferase, as well as to the desaturation of palmitate to hexadecatrienoate on monogalactosyldiacylglycerol referred to above.

7.3.2 Catabolism of glycosylglycerides

Enzymes are present in higher plant tissues that rapidly degrade glycosylglycerides. The initial attack is by an acyl hydrolase (Section 7.2.5), which removes acyl groups from both positions and has very high activity in some tissues such as runner bean leaves or potato tubers. Indeed, homogenization of the latter at a suitable pH in aqueous media results in the complete breakdown of all membrane lipids within a minute! The activity of acyl hydrolases (and other lipid degradative enzymes) in many vegetables even at low temperatures makes it necessary to blanch (boil) such products before storage in a deep-freeze.

Several acyl hydrolases, with slightly different specificities, have been purified from various plant tissues. The enzymes from runner bean leaves are remarkably stable to heating (only 10% activity is lost after 30 min at 70°C) and solvents and can be conveniently purified using hydrophobic chromatography. Further breakdown of the galactosylglycerides occurs by the action of α- and β-galactosidases.

7.3.3 Relatively little is known of the metabolism of the plant sulpholipid

The plant sulpholipid, sulphoquinovosyldiacylglycerol, is also rapidly broken down by the acyl

Table 7.4 Fatty acid composition of galactosyldiacylglycerols from *Euglena gracilis* cells or spinach chloroplasts

Lipid		Fatty acid (% total)						
E. gracilis		16:0	16:3	16:4	18:1	18:2	18:3	Other
	MGDG	6	—	32	9	6	41	6
	DGDG	17	—	7	19	12	26	19
Spinach								
	MGDG	trace	25	—	1	2	72	trace
	DGDG	3	5	—	2	2	87	1

MGDG = monogalactosyldiacylglycerol; DGDG = digalactosyldiacylglycerol.

hydrolases mentioned above. It is not known if any plants can metabolize the sulphoquinovose moiety itself although it is known to be catabolized by soil micro-organisms.

So far as the biosynthesis of sulpholipid is concerned it has been remarked that there are more ideas about the possible synthetic pathway(s) than there are experimental facts! What we do know for sure is that the final step involves the transfer of the sulphoquinovose from UDP-sulphoquinovose to diacylglycerol in a manner analogous to monogalactosyldiacylglycerol formation:

$$\text{UDP-sulphoquinovose} + \text{diacylglycerol} \rightleftharpoons \text{sulpholipid} + \text{UDP}$$

This reaction is also located in chloroplasts.

Evidence has been recently produced that the sulphoquinovose moiety is synthesized from a pathway beginning with UDP-glucose.

7.4 METABOLISM OF SPHINGOLIPIDS

The sphingosine backbone of sphingolipids arises from a condensation of palmitoyl-CoA with serine. Acyl-CoAs donate acyl groups to the NH_2 moiety of sphingosine. In cerebroside biosynthesis, the sugar moieties are supplied from UDP derivatives whereas in ganglioside biosynthesis, cytidine phosphates are used. A range of hydrolytic enzymes is available to degrade sphingolipids.

7.4.1 Biosynthesis of the sphingosine base and ceramide

The pathway by which sphingosine is formed was elucidated first by the German biochemist Stoffel in Cologne. He demonstrated the pathway by isolating intermediates (Fig. 7.12) and, thus, disproving a previously accepted alternative scheme. The first intermediate is 3-ketodihydrosphingosine (3-ketosphinganine), which is formed by the condensation

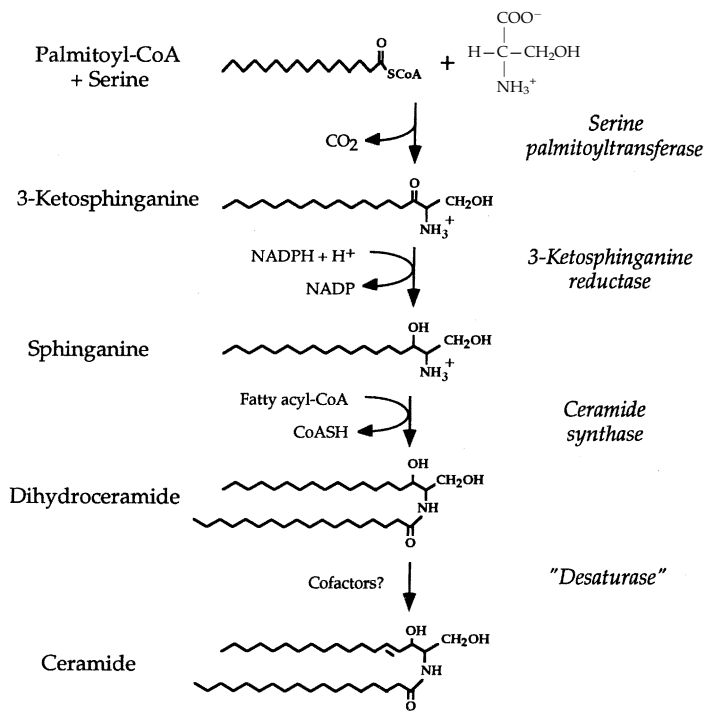


Fig. 7.12 Biosynthesis of ceramide.

of palmitoyl-CoA with serine using a pyridoxal phosphate-requiring enzyme. The selectivity of the serine palmitoyltransferase accounts for the prevalence of 18C bases in most sphingolipids. Some very potent inhibitors of the reaction have been recently developed including a myriocin that is at least ten times better as an immunosuppressant than cyclosporin A.

3-Ketosphinganine is then reduced to sphinganine using NADPH. The desaturation of the hydrocarbon chain of sphinganine to yield sphingosine uses a flavoprotein enzyme – possibly analogous to the acyl-CoA dehydrogenase of β-oxidation (Section 2.3.1). However, it appears to take place after an *N*-acyl group has been attached to the base by ceramide synthase. The latter uses acyl-CoAs as donors (Fig. 7.12).

Potent inhibitors of ceramide synthase (fumonisins) are produced by some strains of *Fusarium moniliforsue* a very common contaminant of corn. Fumonisins cause important veterinary diseases (e.g. horse leukoencephalomalacia, pig pulmonary oedema) and have been implicated in human cancer. Similar mycotoxins are produced by fungi that grow on other plants.

7.4.2 Cerebroside biosynthesis

Two pathways for the synthesis of galactosylceramide from sphingosine have been proposed:

1. sphingosine + UDP-gal ⇌ psychosine + UDP
   psychosine + fatty acyl-CoA ⇌ galactosylceramide + CoA
2. ceramide + UDP-gal ⇌ galactosylceramide + UDP

In the experiments leading to the proposal of pathway (1), the acylation of psychosine occurred by a non-enzymic reaction and, therefore, the second pathway (i.e. galactosylation of ceramide, pathway (2), is thought to be the major pathway. The reaction was first demonstrated by Morell and Radin and the UDP-galactose:ceramide galactosyltransferase has now been purified from rat brain. It is a membrane-bound enzyme that has to be solubilized with detergent and contains tightly bound phospholipid, even in its purified form. Interestingly, in view of the concentration of α-hydroxy fatty acids in galactosylceramides (Table 6.7) the enzyme was inactive with ceramides containing non-hydroxy fatty acids.

Glucosylceramide, which is a precursor for neutral glycosphingolipids and for gangliosides, is formed in an analogous reaction by a ceramide containing non-hydroxy fatty acids.

7.4.3 Formation of neutral glycosphingolipids

A whole host of higher homologues of ceramide are present in different tissues (Section 6.3). These are produced by direct transfer of a single sugar usually from its UDP derivative (Table 7.5). The enzymes are specific for this individual glycosphingolipid substrate and, therefore, sugar addition is in a defined order. These reactions give rise to the different core sphingolipid structures illustrated in Fig. 7.13.

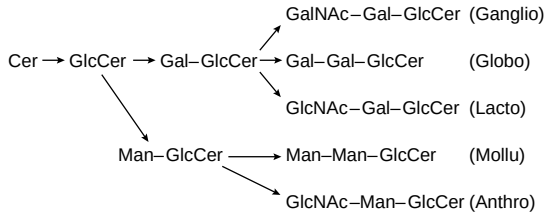


Fig. 7.13 Formation of different core glycosphingolipids from ceramide. Glc: glucose; Gal: galactose; Man: mannose; GalNAc: *N*-acetylgalactosamine; GlcNAc: *N*-acetylglucosamine; Cer: ceramide.

Whereas formation of the cerebrosides (Glu-Cer, Gal-Cer) occurs on the cytoplasmic face of the endoplasmic reticulum, the more complex neutral glycosphingolipids are made in the lumen of the Golgi apparatus.

7.4.4 Ganglioside biosynthesis

Gangliosides contain sialic acid residues that are added using a cytidine derivative. This compound, CMP-neuraminic acid (Fig. 7.14) is formed by an enzymic reaction from CTP and is analogous to the

Table 7.5 Substrates used for the formation of sphingolipids

Group to be added	Substrate used
Glucose	UDP-glucose
Galactose	UDP-galactose
N-Acetylgalactosamine	UDP-*N*-acetylgalactosamine
Fucose	GDP-fucose
Sialic acid (*N*-acetyl neuraminic acid)	CMP-NeuAc
Sulphate	PAPS

CMP-NeuAc = CMP-*N*-acetyl neuraminic acid; PAPS = 3′-phosphoadenosine-5′-phosphosulphate.

cytidine derivatives used in phospholipid biosynthesis.

The gangliosides are made by transfer of the neuraminic acid residue to the growing sugar chain at various stages and four series of gangliosides result from these reactions (Fig. 7.15).

The picture of ganglioside synthesis was built up largely by the work of Roseman, Brady and their colleagues in the USA. The way in which they worked out the sequence of sugar additions was by testing the specificity of each individual transferase for a specific acceptor. Each enzyme requires a specific substrate as a donor and the end-product of the previous step has much the greatest activity as acceptor.

In recent years considerable information has accumulated to show that gangliosides, like other glycosphingolipids, have important properties for cells (Section 6.3). The relative amounts of gangliosides for a given cell are not always constant. Changes occur with the developmental stage of the cell, its environment and whether it is subject to a number of pathological conditions. With neuronal development a general increase in the quantity of total gangliosides and in the proportion of the more highly sialylated compounds is seen. Small shifts in composition also occur after prolonged nerve stimulation or during temperature adaptation in poikilothermic animals or during hibernation in mammals. A number of drug-induced changes (e.g. with opiates) has also been noted.

The changes in ganglioside patterns, which occur during cell development and growth, imply that these lipids may also play a role in cell-contact inhibition. This suggestion is reinforced by several lines of evidence. First, exogenous gangliosides (or antibodies to them) have been shown to control growth and differentiation in a number of systems. Second, oncogenic transformation, which results in a loss of growth regulation mechanisms (such as the cell-to-cell contact-dependent inhibition of growth), is usually paralleled by an irreversible reduction in the levels of the more complex gangliosides (and neutral glycosphingolipids). Third,

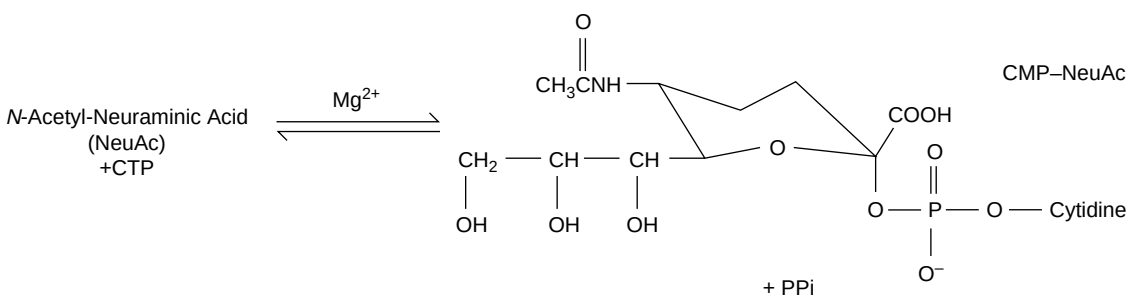


Fig. 7.14 Formation of CMP-NeuAc.

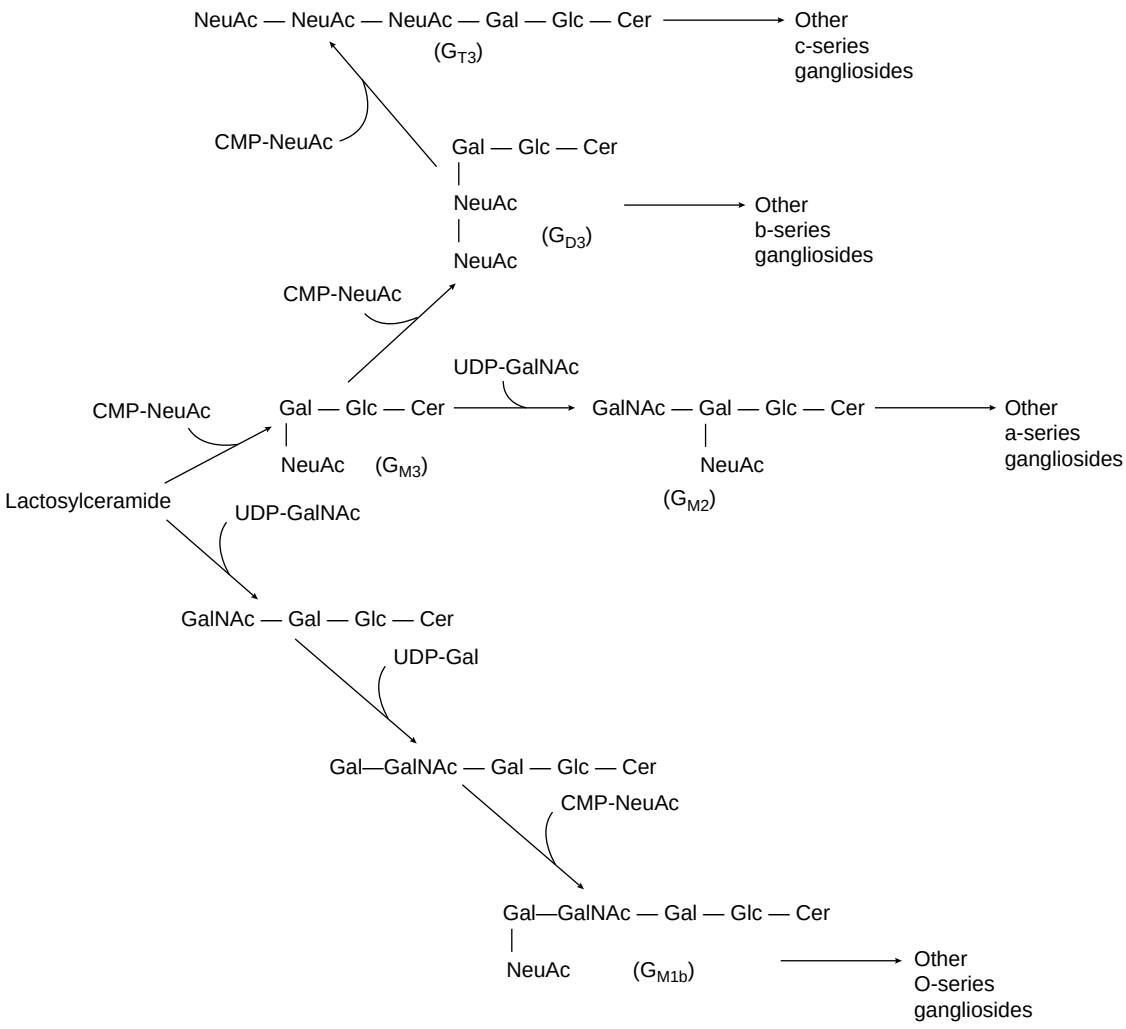


Fig. 7.15 Key steps in the initial formation of gangliosides.

gangliosides make an important contribution to the characteristic immunoexpression of individual cell types. Fourth, they act as binding sites for a number of important compounds. Originally, several toxins, such as tetanus toxin, botulinum toxin and cholera toxin, were shown to interact tightly with gangliosides, but there is now some indication that they may function as receptors for interferon and for certain cell-growth and differentiation factors. Brief reference to the role of glycosphingolipids in the immune system, especially in relation to cancer, can be found in Section 4.4.2.

7.4.5 Sulphated sphingolipids

The major sulphated glycolipid in mammalian tissues is galactosylceramide with a sulphate attached to the 3-position of the sugar (Table 6.7). It is commonly known as sulphatide. However, various other sulphated glycolipids (e.g. lactosylceramide

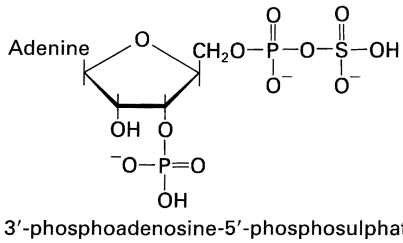


Fig. 7.16 Phosphoadenosine phosphosulphate: the donor of sulphate groups.

sulphate) have also been detected in small amounts in different animal tissues.

Sulphatide is an important constituent of the myelin sheath of nervous tissue and the incorporation of radioactive sulphate into the sulphatide molecule has been used to study myelination. The donor of the sulphate group is the complex nucleotide 3′-phosphoadenosine 5′-phosphosulphate, usually abbreviated in scientific papers to PAPS (Fig. 7.16). PAPS itself is produced from ATP in two steps via an adenosine 5′-phosphosulphate (APS) intermediate. The synthesis of sulphatide is catalysed by a sulphotransferase, which has been detected in the microsomal fraction from a number of tissues:

$$\text{galactosylceramide} + \text{PAPS} \rightleftharpoons \text{3-sulpho-galactosylceramide} + \text{PAP}$$

7.4.6 Sphingomyelin is both a sphingolipid and a phospholipid

The biosynthesis of this sphingolipid is closely tied to that of phosphatidylcholine, since the phosphocholine moiety of sphingomyelin is donated directly from phosphatidylcholine to ceramide using sphingomyelin synthase:

$$\text{phosphatidylcholine} + \text{ceramide} \rightleftharpoons \text{sphingomyelin} + \text{diacylglycerol}$$

Sphingomyelin synthase appears to be present in both the plasma membrane and the Golgi apparatus perhaps as isoforms. Other potential pathways of synthesis appear to be of minor importance.

7.4.7 Catabolism of the sphingolipids

Sphingolipids are widely distributed in animal tissues and constitute significant components of the human diet. It is not surprising, therefore, that the small intestinal cells contain enzymes for breaking down such lipids into their constituent parts. In addition, turnover of the various endogenous and secreted sphingolipids takes place. In general, sphingolipids are internalized with endocytic vesicles, sorted in early endosomes and recycled back to the plasma membrane or transported to lysosomes where they are degraded by specific acid hydrolases. Phagocytic cells, particularly the histiocytes or macrophages of the reticuloendothelial system (located primarily in bone marrow, liver and spleen) play a prominent role here.

The predominant sphingolipid type varies between tissues and, therefore, the important catabolic enzymes will be different. For white or red blood cells, lactosylceramide (cer-glc-gal) and haematoside (cer-glc-gal-NANA) are major components. In contrast, the brain composition is dominated by complex gangliosides. During the neonatal period, the turnover of gangliosides is particularly rapid as sphingolipids are broken down and then resynthesized.

Usually each of the catabolic enzymes is specific for a particular chemical bond. Thus, a combination of a number of enzymes is needed to ensure the complete breakdown of a given sphingolipid. Catabolism begins by attack on the terminal hydrophilic portions of the molecules. The enzymes responsible are glucosidases, galactosidases, hexosaminidases, neuraminidases and a sulphatase. As an example of the specificity of such enzymes it has been noted that β-galactosidases have been found which are specific for Cer-glc-gal and for Cer-glc-gal-gal. Another enzyme (from spleen) cleaves the glucose from Cer-glc, but is inactive on Cer-gal, whereas an intestinal enzyme is active with both substrates. Brain contains an enzyme that cleaves ceramide (but not cerebroside) to yield free fatty acid and sphingosine. The sulphatases are responsible for the cleavage of the sulphate ester from sulphatides while *N*-acetyl neuraminic acid is hydrolysed from gangliosides by neuraminidases.

The breakdown of the various sphingolipids usually proceeds smoothly and it has been suggested that the various catabolic enzymes are aligned in an ordered fashion on the lysosomal membrane – thus ensuring more efficient hydrolysis than would be the case for various substrates allowed random access to enzymes freely admixed within the organelle. Sometimes one of the breakdown enzymes is missing or has very low activity. When this happens there is a build-up of one of the intermediate lipids. Such accumulation can impair tissue function and gives rise to sphingolipid storage diseases called 'lipidoses'. These diseases are discussed in Section 7.8 and general pathways for sphingolipid breakdown are shown in Fig. 7.17 together with the enzyme deficiencies in the different storage diseases.

Because of their role in generating lipid-derived second messengers (Section 7.12), the catabolism of sphingomyelin and ceramide is especially important. Several sphingomyelinases with neutral to alkaline pH optima have been described. They are found in several cellular compartments including the plasma membrane, cytosol and nuclear membrane. In fact, five distinctly different sphingomyelinases have been identified in mammalian cells – based on their pH optima, cellular distribution and cation requirement.

When ceramide is generated, it can be acted upon by acidic or neutral ceramidases. The neutral

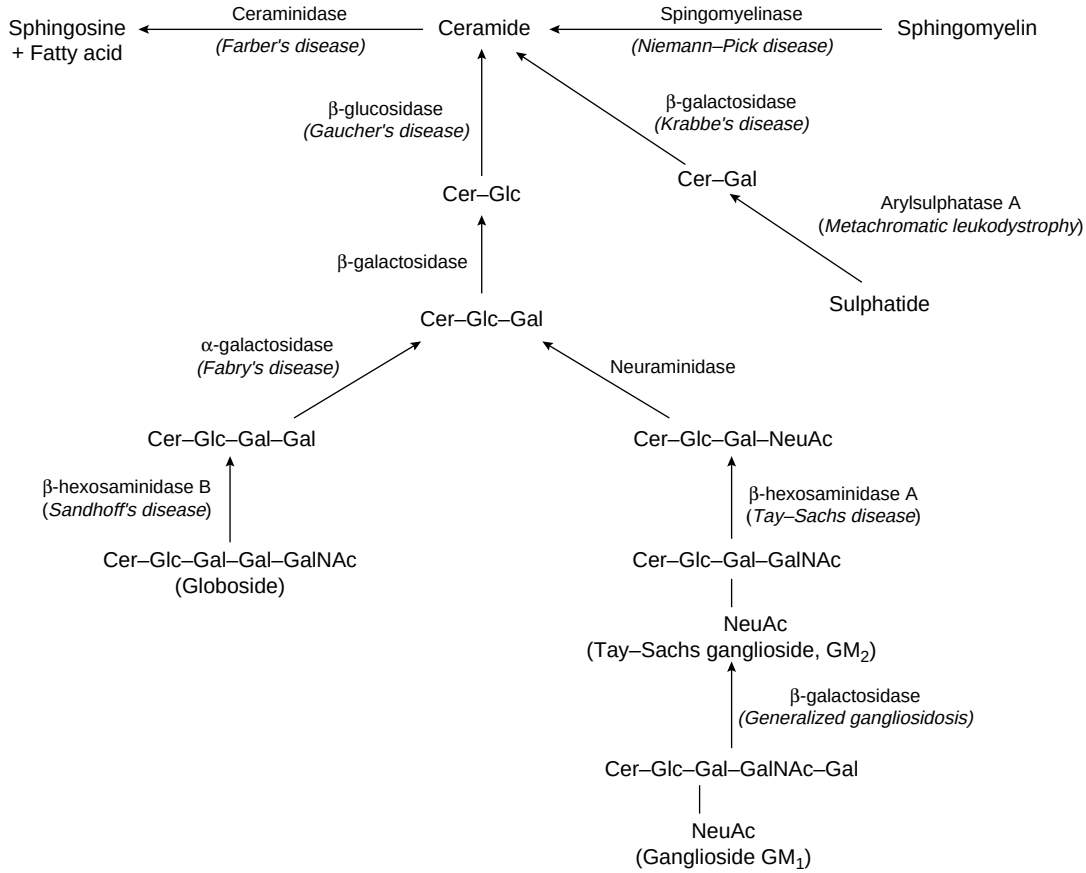


Fig. 7.17 Pathways for sphingolipid catabolism showing enzyme deficiencies in lipid storage diseases (Section 7.8).

ceramidases moderate release of sphingosine from ceramides produced in the plasma membrane for participation in signalling.

Both ceramide and sphingosine can be phosphorylated by kinases. While sphingosine-1-phosphate is known to be important for cellular regulation (Section 7.12) there is less evidence for a significant role for ceramide phosphate.

7.5 CHOLESTEROL BIOSYNTHESIS

Cholesterol biosynthesis, in common with fatty acid synthesis, starts from the 2-carbon compound acetyl-CoA. However, the condensation follows a quite different pathway. Hydroxymethylglutaryl-CoA is formed and is a key intermediate in the biosynthesis of cholesterol and a host of other polyisoprenoids. After the stage at which squalene (a straight-chain polyisoprenoid) is formed, a cyclization reaction gives rise to sterols and eventually, cholesterol.

As mentioned in Section 6.4, cholesterol is a major and important constituent of animal membranes. Different sterols may be important in other organisms. Sterols are, however, a relatively small group of a very large class of biogenetically related substances – the polyisoprenoids (or terpenoids). These compounds are all derived from a common precursor isopentenyl diphosphate. Polyisoprenoids can be open-chain, partly cyclized, or fully cyclized substances. They contain a basic structure of a branched-chain C_5 (isoprenoid) unit.

For a membrane constituent, cholesterol has a long scientific story. In 1816, Chevreul coined the term cholesterine (from the Greek 'chole' meaning bile and 'stereos' meaning solid) for an alcohol-soluble substance that could be isolated from bile stones and in 1843 Vogel was one who identified it in several normal animal tissues as well as atheromatous lesions. After various advances in knowledge of its chemistry, the structure of cholesterol was finally solved, principally by Wieland and Windaus around 1932.

7.5.1 Acetyl-CoA is the starting material for terpenoid as well as fatty acid synthesis

The biosynthesis of cholesterol is well covered in standard biochemistry textbooks and only the more important features will be outlined here. However, it should be added that a great deal of work has gone into solving the pathway of cholesterol formation with those concerned receiving no less than 13 Nobel prizes between them!

The precursor pool in mammalian cells is the cytosolic acetyl-CoA. This acetyl-CoA may be derived, for example, from β-oxidation of fatty acids by mitochondria or microbodies (Section 2.3.1). The acetyl-CoA pool is in rapid equilibrium with intracellular and extra-cellular acetate, which allows radiolabelled acetate to be used conveniently to measure cholesterol synthesis in tissues.

The first two steps involve condensation reactions catalysed by a thiolase and hydroxymethylglutaryl-CoA (HMG-CoA) synthetase. Both enzymes are soluble and the first reaction is driven to completion by rapid removal of acetoacetyl-CoA by the second step (Fig. 7.18).

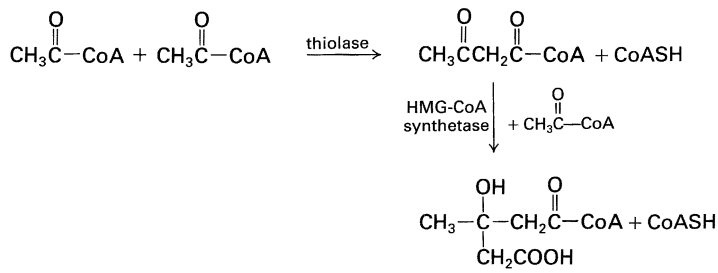


Fig. 7.18 Formation of hydroxymethylglutaryl-CoA (HMG-CoA).

HMG-CoA synthetase has been studied in considerable detail and the reaction mechanism defined. It shows a very high degree of specificity with regard to the stereochemistry of the acetoacetyl-CoA substrate and the condensation proceeds by inversion of the configuration of the hydrogen atoms of acetyl-CoA. In addition to cytosolic HMG-CoA synthase, a second synthase is found in mitochondria. Not only has this been shown to be a different protein, the HMG it forms has a different function. HMG in the cytosol is destined for mevalonate formation, whereas in mitochondria it is broken down by HMG-CoA lyase to yield acetyl-CoA and acetoacetate (Section 2.3.1.6). The cytosolic HMG-CoA synthase seems to be one of the pathway enzymes important for controlling cholesterol synthesis and its activity is changed by transcriptional modulation.

The next reaction, which results in mevalonate production, is catalysed by the membrane-localized HMG-CoA reductase. It is a highly regulated enzyme with a short half-life ($T_{½}$ about 3 h) and it is usually considered to catalyse the reaction with the most control over the rate of sterol synthesis. Even in a normal diurnal cycle, its activity will vary about tenfold.

HMG-CoA reductase activity is regulated at the transcriptional level and by post-transcriptional methods. When sterols are added to animal diets there is a decline in the mRNA levels for HMG-CoA reductase (together with HMG-CoA synthase, farnesyl diphosphate synthase and the LDL-receptor (Section 5.3.1). These four mRNAs increase when cells are deprived of sterols. Brown and Goldstein showed that the 3′-flanking region of the gene for HMG-CoA reductase (and the LDL-receptor) contained one to three copies of a nucleotide sequence called the sterol regulatory element I (SRE-I). A transcription factor, sterol regulatory element binding protein I (SREBP-I) specifically binds to SRE-I, which is within the promoter for HMG-CoA reductase (and the LDL-receptor). The transcription factor, SREBP-I, is synthesized as a 125 kDa precursor bound to the endoplasmic reticulum. In cells deprived of cholesterol, the factor is cleaved and a 68 kDa N-terminal fragment is released, which is targeted to the nucleus, where it binds to SRE-I and promotes expression of HMG-CoA reductase.

HMG-CoA reductase expression is also regulated by changes in mRNA translation and stability and by protein turnover. The degradation of HMG-CoA reductase protein in the endoplasmic reticulum is regulated through the eight trans-membrane domains, perhaps by farnesol or its diphosphate derivative or via oxysterols.

HMG-CoA reductase is also regulated by a reversible phosphorylation/dephosphorylation cycle (Fig. 7.19). The phosphorylated reductase is inactive and the amounts of the phosphorylated enzyme can be shown to be increased when its activity is decreased by mevalonate or glucagon.

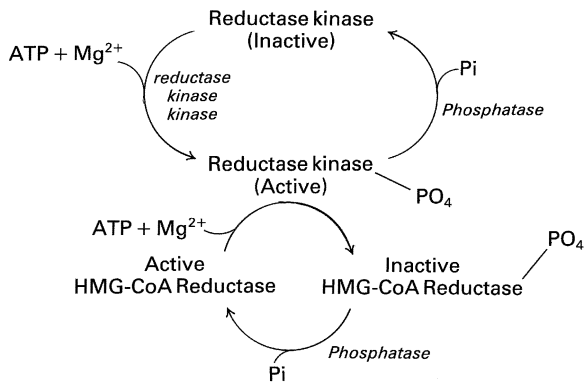


Fig. 7.19 Regulation of HMG-CoA reductase activity by phosphorylation–dephosphorylation.

The protein kinase, which inactivates HMG-CoA reductase, is itself subject to phosphorylation. Interestingly, both ATP and ADP are needed. Apparently ADP binds to a different site on the reductase kinase and acts as an allosteric effector. The phosphatases, which activate HMG-CoA reductase, are highly sensitive to NaF. The kinase and phosphatases are each present in the cytosol as well as endoplasmic reticulum. Recently the protein kinase, which phosphorylates HMG-CoA reductase, has been identified as an AMP-activated protein kinase. Interestingly, this kinase also phosphorylates (and thus regulates) acetyl-CoA carboxylase (Section 2.2.8.1).

Because of the involvement of cholesterol in the aetiology of arterio-vascular disease (Section 5.4) considerable efforts have been made to develop suitable pharmaceutical agents able to reduce its formation. Two interesting compounds that inhibit HMG-CoA reductase are natural antibiotics isolated from the moulds *Penicillium* spp. and *Aspergillus terreus* and named compactin and mevinolin, respectively. These compounds are competitive inhibitors of HMG-CoA reductase with a K_i for the enzyme of about 10^{-9} M compared to 10^{-6} M for HMG-CoA. Surprisingly, they increase mRNA and protein levels for the enzyme, but this is consistent with a downstream metabolite of mevalonate inhibiting transcriptions and HMG-CoA reductase turnover (as discussed above). Nevertheless, these fungal metabolites rapidly enter cells and inhibit cholesterol biosynthesis (Section 5.3.1).

7.5.2 Further metabolism generates the isoprene unit

Once mevalonate has been formed it is sequentially phosphorylated by two separate kinases yielding mevalonate 5-diphosphate. A third ATP-consuming reaction involving a decarboxylase then generates the universal isoprene unit, isopentenyl diphosphate (Fig. 7.20). The function of the ATP in this reaction appears to be to act as an acceptor for the leaving OH group in the dehydration part of the reaction.

7.5.3 Higher terpenoids are formed by a series of condensations

Isopentenyl diphosphate is potentially a bifunctional molecule. Its terminal vinyl group gives it a nucleophilic character whereas when it isomerizes to 3,3-dimethylallyl diphosphate, the latter is electrophilic. Thus, longer chain polyprenyls are formed by a favourable condensation of isopentenyl diphosphate first with dimethylallyl diphosphate and later with other allylic diphosphates. The initial interconversion of isopentenyl diphosphate and dimethylallyl diphosphate is promoted by an isomerase. The successive condensations yield the 10C compound geranyl diphosphate and then the 15C farnesyl diphosphate. The two molecules of farnesyl diphosphate condense to form presqualene diphosphate, which is reduced by NADPH to give the 30C open-chain terpenoid squalene. Each condensation reaction with IPP represents a novel method of C–C bond formation since, in the formation of other types of natural products (peptides, sugars, fatty acids, etc.), the reactions involve Claisen- or aldol-type condensations.

7.5.4 A separate way of forming the isoprene unit occurs in plants

In plant chloroplasts a mevalonate-independent pathway for isopentenyl diphosphate formation is present. This pathway was discovered first by Rohmer when he was studying the biosynthesis of hopanoids (pentacyclic steroid-like molecules) in bacteria, but is used generally by algae and higher plants for plastid isoprenoid production. The pathway has not been fully defined yet but uses 1-deoxy-D-xylulose-5-phosphate rather than mevalonate as a precursor of the isoprenoid unit. Because isopentenyl diphosphate can be transported from the plastid, it may contribute to sterol biosynthesis in the cytosolic compartment. However, the portion of sterol precursor carbon originating from this route is not yet known.

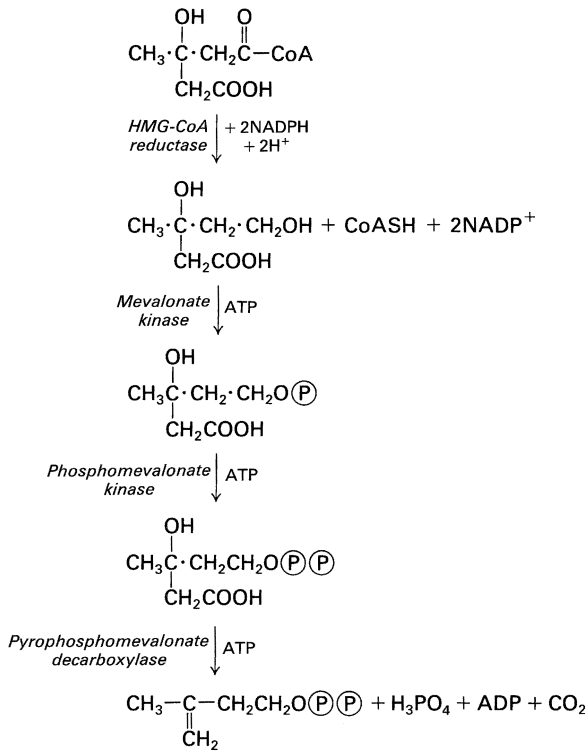


Fig. 7.20 Formation of isopentenyl diphosphate from HMG-CoA.

7.5.5 Sterol synthesis requires cyclization

Formation of sterols from squalene involves cyclization. First, a microsomal mixed-function oxidase (squalene epoxidase) forms squalene-2,3-oxide in the presence of NADPH, FAD and O_2 (there is no requirement for cytochrome P450 in this reaction). The cyclization of the oxide to lanosterol then takes place by a concerted reaction without the formation of any stable intermediates. This conversion, which has been described as the most complex known enzyme-catalysed reaction, depends on a cyclase with a molecular mass of only 90 kDa. In plants and algae squalene-2,3-epoxide is cyclized to cycloartenol, which is the precursor of stigmasterol whereas lanosterol is the precursor of cholesterol and ergosterol (Fig. 7.21).

The conversion of lanosterol to cholesterol involves a 19-step reaction sequence catalysed by microsomal enzymes. The exact order of the reaction has not been delineated and, indeed, there may be more than one pathway. The main features of the transformation are the removal of three methyl groups, reduction of the 24(25)-double bond and isomerization of the 8(9)-double bond to position 5 in cholesterol.

7.5.6 Cholesterol is an important metabolic intermediate

Cholesterol is not only an important membrane constituent in animals but also plays a vital role as a metabolic intermediate (Fig. 7.22). It acts as a precursor for the steroid hormones (glucocorticoids, aldosterone, oestrogens, progesterones, androgens), for the bile acids (and their salts) and can also be esterified. Even if cholesterol was not required continuously because of membrane turnover, a

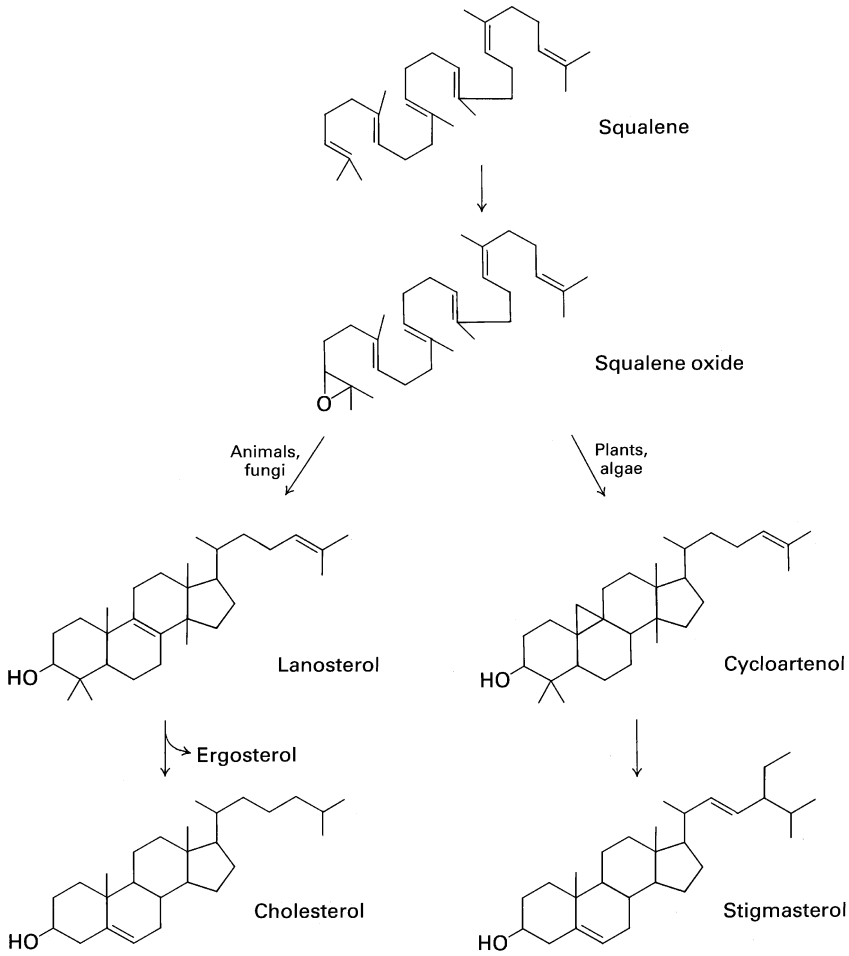


Fig. 7.21 Conversion of squalene into sterols.

considerable amount would be needed for bile production – even though greater than 80% of the bile salts are absorbed from the large intestine and re-utilized. Thus, under physiological conditions cholesterol is needed mainly in cell division (growth or replacement of desquamated cells), in the replacement of cholesterol metabolized to steroid hormones in the adrenals and other endocrine glands or catabolized to bile acids by the liver.

Because high levels of unesterified cholesterol are thought to be deleterious to cells, excess of this sterol is converted into cholesteryl ester:

$$\text{acyl-CoA} + \text{cholesterol} \rightleftharpoons \text{cholesteryl ester} + \text{CoA}$$

This reaction (catalysed by the enzyme acyl-CoA cholesterol acyltransferase) occurs on the endoplasmic reticulum and the cholesteryl ester can accumulate as lipid droplets in the cytosol. Such lipid droplets are often relatively abundant in steroidogenic tissues where the cholesteryl ester can act as a readily available precursor for steroid hormone production. Cellular cholesterol homeostasis regulation by the acyl transferase is also dependent on the production of free cholesterol in the lysosome and its transport to the endoplasmic reticulum. Hereditary diseases (such as cholesterol ester storage disease) exist where either of these processes is impaired.

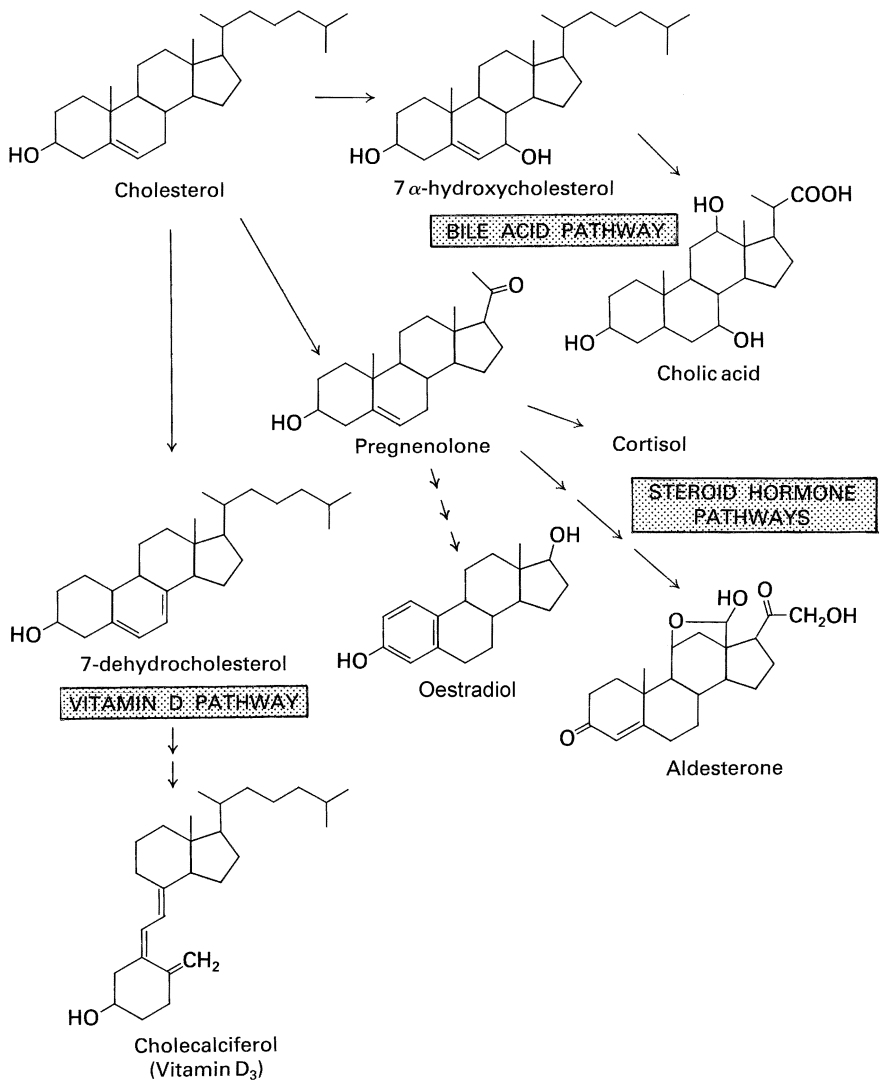


Fig. 7.22 Metabolism of cholesterol to bile acids, calciferols and steroid hormones.

7.5.7 It is important that cholesterol concentrations in plasma and tissues are regulated within certain limits and complex regulatory mechanisms have evolved

Cholesterol balance in cells is maintained by a number of factors (Table 7.6). In fact, it has been found that uptake of lipoproteins may influence cholesterol synthesis itself via the LDL-receptor mechanism described in Sections 5.2.4 and 5.3.

Early experiments showed that the synthesis of cholesterol was reduced when animals were fed cholesterol and led eventually to the identification of the sterol regulatory element binding protein (SREBP) system. The molecular basis for the regulation of the activities of the enzymes of

Table 7.6 Factors influencing cellular cholesterol concentrations

1. Uptake of intact lipoproteins via receptors.
2. Uptake of free cholesterol from lipoproteins by lipid transfer.
3. Cholesterol synthesis.
4. Cholesterol metabolism (e.g. to hormones).
5. Efflux of cholesterol.
6. Esterification of cholesterol by acyl-CoA:cholesterol acyltransferase.
7. Breakdown of cholesterol esters by neutral cholesterol esterase.

cholesterol biosynthesis, involving the SREBP system, was described in detail in Section 5.3.1 and Figs 5.13 and 5.14. Table 7.7 further summarizes the main mechanisms for the regulation of the key enzyme in the pathway, HMG-CoA reductase (Section 7.5.1).

Table 7.7 Methods to regulate activity of HMG-CoA reductase

Transcription
mRNA stability
Translation
Protein stability
Protein phosphorylation/dephosphorylation

Although sterols are present in most mammalian body tissues, the proportion of sterol ester to free sterol varies markedly. For example, blood plasma, especially that of humans, is rich in sterols and like most plasma lipids they are almost entirely found as components of the lipoproteins; about 60–80% of this sterol is esterified. In the adrenals, too, where cholesterol is an important precursor of the steroid hormones, over 80% of the sterol is esterified. However, in brain and other nervous tissues, where cholesterol is a major component of myelin, virtually no cholesterol esters are present. Cholesterol esters are formed by the action of a microsomal acyl-CoA:cholesterol acyltransferase (ACAT), which is present in most cells. Under normal conditions the enzyme is considered rate-limiting for cholesterol esterification. It is regulated by progesterone and may be modulated by phosphorylation/dephosphorylation like HMG-CoA reductase. Under conditions where cells take up a large amount of cholesterol, such as via LDL-receptors, ACAT is induced. The enzyme is particularly important in intestine and is relatively low in liver where the lipoproteins made for secretion into the serum contain little if any cholesterol ester.

In contrast, cholesterol esters are formed in blood by another acyltransferase, the lecithin:cholesterol acyltransferase. Most of the cholesterol that accumulates in arterial plaques during the development of arteriosclerosis is in the esterified form. An understanding of cholesterol transport and cholesteryl ester metabolism is crucial for the understanding of this disease, as discussed in Section 5.4.1.

7.6 SPECIFIC ROLES

Membrane lipids can have specific roles in addition to their normal function in membrane structure. Clearly the role of amphipathic lipids in forming the bilayer that is the foundation of normal membrane structure is one of the most important functions for any molecule in Nature. However, there are more specialized functions that we should not forget. In the following sections, we will deal firstly with the role of phospholipids as components of lung surfactant (where they, literally, help the breath of life) and then in cell signalling.

Lipid signalling is one of the most rapidly growing areas of lipid biochemistry. We have already come across examples of this in the section on eicosanoids (Section 2.4). In addition, there are many examples of intact lipids or their derivatives (Table 7.8) which are important in controlling such aspects of cellular activity as hormone action, cell differentiation and apoptosis.

Of course, whenever a biochemical plays an important role in biology then things can go wrong. Examples of diseases where lipids are involved are also included in the following sections. In addition, some previous sections of the book also cover aspects of disease (Sections 4.2.3, 4.2.4, 4.3, 4.4 and 5.4).

Table 7.8 Examples of lipids or their derivatives that have roles in signalling

Precursor	Signalling molecule	Example of role
20C Polyunsaturated fatty acid	Eicosanoid (animals)	Inflammation, pain
α-Linolenic acid	Jasmonic acid (plants)	Stress responses
Platelet activating factor (PAF)	PAF	Inflammation
Phosphatidylcholine	Phosphatidic acid	Phagocytic respiratory burst
Phosphatidic acid	Diacylglycerol	Protein kinase C regulation
Phosphatidyl inositol-4,5-*bis*phosphate	Diacylglycerol; inositol-1,4,5-*tris*phosphate	Protein kinase C regulation; calmodulin kinase control
Sphingomyelin	Ceramide	Apoptosis
Ceramide	Sphingosine; sphingosine-1-phosphate	Protein phosphorylation; release of calcium

7.7 PULMONARY SURFACTANT

A unique lipoprotein, comprising almost entirely dipalmitoylphosphatidylcholine as its lipid component, acts as a lung surfactant. Its function is to lower alveolar surface tension and prevent lung collapse.

Every time we breathe out, our lungs are prevented from collapsing by a unique lipoprotein mixture termed the 'pulmonary surfactant'. Pulmonary surfactant is adsorbed at the alveolar air–liquid interface where it lowers the surface tension and, therefore, reduces the contractile force at the surface and the work of lung expansion. Collapse is prevented by the formation of a solid film on the alveolar surface during expiration. A dramatic example of its importance is provided by the disease *acute respiratory distress of the new-born*. In this complaint, premature infants who have not yet begun to synthesize pulmonary surfactant are unable to expand their lungs properly and suffer from various complications, which, even with the best modern treatments, results in death for over 25% of affected individuals.

Surfactant has a unique composition. Pulmonary surfactant can be isolated by carefully washing out lungs repeatedly with isotonic saline. Such material, of course, only represents a proportion of the total lung surfactant – that which has already been secreted into the alveolar spaces. The important compositional features of pulmonary surfactant are emphasized in Table 7.9. First, surfactant is a lipid-rich lipoprotein mixture. Moreover, in contrast to other lipoproteins, the lipids are dominated by a single class and, indeed, a single molecular species – dipalmitoylphosphatidylcholine. Thus, the lipids of lung surfactant have a composition particularly suited to the rapid formation of stable lamellar structures. The small amounts of unsaturated phosphatidylcholine are believed to help in the rapid spreading of surfactant at body temperatures and phosphatidylglycerol may assist in the morphological dissolution of lamellar bodies in the aqueous sub-phase to provide a constant source of renewal of the surface monolayer.

Four characteristic proteins are present in human surfactant [surfactant protein-A (SP-A), SP-B, SP-C and SP-D] and these help with properties such as assisting the spreading of surfactant over the air–liquid interface in the lung or in the re-absorption of used surfactant into the alveolar type II epithelial cells, which synthesize and secrete surfactant. SP-B and SP-C are small and remarkably hydrophobic. In fact, they co-purify with lipid in normal extraction systems!

The type II cells which are present at the alveolar surface are the exclusive sites of surfactant synthesis.

Table 7.9 Composition of alveolar surfactant

	(% w/w)		
	Human	Rabbit	Rat
Total protein	15	8	10
Total lipid	85	91	89
	(% w/w of total lipid)		
Phosphatidylcholine	74	83	73
(dipalmitoyl-phosphatidylcholine)	(68)[a]	(63)[a]	(82)[a]
Phosphatidylglycerol	6	3	5
Sphingomyelin	4	2	2
Other phospholipids	7	3	9
Cholesterol (free and esterified)	4	3	3
Other lipids	5	6	8

[a] Percentage of phosphatidylcholine.

In these cells the necessary proteins and lipids are made on the endoplasmic reticulum from where they are assembled into lamellar bodies. Surfactant is then released from the latter by exocytosis.

Detailed studies of lipid metabolism in type II cells have been made either by the use of normal cells (isolated from whole lungs by collagenase treatment to separate them carefully from the more abundant type I cells or the underlying tissue) or with tumour cells that have the same morphological characteristics as type II cells. In particular, attention has been focused on the formation of dipalmitoylphosphatidylcholine. This major component of surfactant is made primarily by the CDP-base pathway (Section 7.1.5). However, the operation of this route results in phosphatidylcholine containing a number of molecular species without an especial abundance of the dipalmitoyl species. Two possibilities exist for enrichment of palmitate into phosphatidylcholine (or re-modelling as it has been termed). Either fatty acids can be removed from phosphatidylcholine by phospholipase A attack and then replaced by an acylation with palmitoyl-CoA (method A; Fig. 7.23) or two molecules of monopalmitoyl-phosphatidycholine could react together (method B; Fig. 7.23). Careful experiments by the Dutch workers Batenburg and van Golde have shown that method A (i.e. deacylation/reacylation) is the major method used.

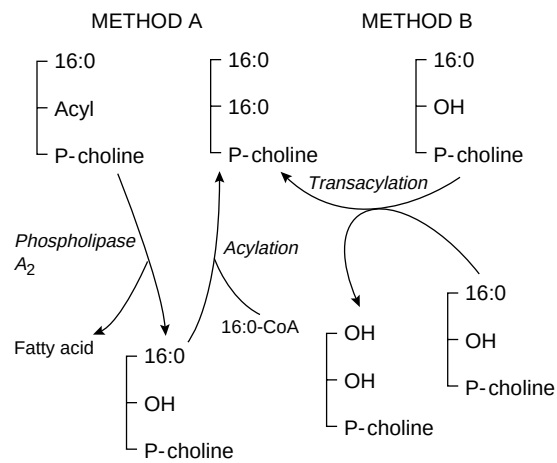


Fig. 7.23 Methods for the enrichment of surfactant phosphatidylcholine with palmitate.

The type II cell is important not only for surfactant synthesis but also seems to be involved in its recycling. In much the same way as it would be wasteful for an animal continually to produce bile salts without any reabsorption, so pulmonary surfactant is constantly taken up by alveolar cells. Little is known of this process (and still less of its control) but it appears that all major lipid components are recycled. The careful balance of secretion and re-uptake of surfactant ensures that optimal

amounts are always available to function in the alveolar spaces.

There would be little point in a young foetus producing surfactant until near the time for birth and active breathing. Accordingly, surfactant is produced only towards the end of term – in humans after about 8 months. After this time the typical surfactant components can be found, and tested for, in the amniotic fluid. Various tests have been used of which the phosphatidylcholine/sphingomyelin ratio is the most common. A ratio of at least 2 is taken to indicate significant surfactant production.

Respiratory distress syndrome of the new-born is a developmental disorder that is caused by immaturity of the baby's lungs. The lack of normal amounts of surfactant causes morphological alterations (hyaline membranes, atelactasis) and physiological changes (decreased lung compliance, hypoxaemia) and is a major cause of death in premature infants. About 25% of such babies die and some others are left with handicaps. There have been two approaches to the treatment of the disease. The first has been to hasten the development of the foetal lungs by the use of hormones, particularly corticosteroids. Such treatments obviously require considerable pre-warning of a possible caesarean delivery or premature natural birth and cannot be used in all cases owing to pregnancy complications. The second treatment is to instil lipid mixtures into the baby's lungs in order to reproduce the effects of natural surfactant until such time as the baby can synthesize its own. Various preparations are in current use and they include mixtures made of lipids alone or extracts from animal lungs that contain both phospholipids and the surfactant proteins. Use of these artificial surfactants has been very successful and has succeeded in reducing the incidence of respiratory distress significantly and also in allowing younger and younger babies to survive. The use of artificial surfactants is now routine in the UK for babies of less than 32 weeks' gestation.

Although respiratory distress of the new-born is a very tragic and dramatic disease, that of adults is far more prevalent and has a worse prognosis. In the USA alone it has been estimated that at least 150 000 persons die of the disease each year. Since the problem is not caused by lung immaturity, hormone therapy is of little use but, so far, little attention has been paid to its treatment by replacement therapy.

Several other diseases or conditions are known to affect surfactant metabolism and, hence, lung function. For example, dust-related industrial diseases such as silicosis lead to a massive accumulation of surfactant (up to 40 times normal amounts), which impairs gas exchange and breathing. In paraquat (a herbicide) poisoning, the opposite occurs and the type II cells no longer produce surfactant – leading to particularly painful efforts at breathing by the victim who dies within a few days.

7.8 LIPID STORAGE DISEASES (LIPIDOSES)

If tissues lack a key breakdown enzyme, lipids accumulate. The resulting pathologies are usually fatal. However, enzyme replacement therapies can be effective.

Several inborn errors of metabolism exist in which the missing enzyme is one that is involved in the breakdown of a specific lipid molecule. Since the biosynthesis of these lipids is not impaired, the result of the enzyme deficiency is the gradual accumulation of lipids in the tissues. Most of the important diseases of this type are ones that involve structural lipids, frequently glycosphingolipids, of the central nervous system and they are summarized in Table 7.10. The diseases are rare and frequently fatal, which serves to indicate how important it is that the amounts and types of lipids in membranes are strictly controlled to preserve biological function. Many of the lipids involved in these disorders are readily synthesized in the body, so that dietary treatment is ineffective. There is one lipid storage disease, Refsum's disease, however, that can be controlled by strict exclusion of a fatty acid from the diet. This disease is due to failure to break down by α-oxidation, the branched-chain fatty acid, phytanic acid (Section 2.3.2), which is formed from phytol, a universal constituent of green plants (Fig. 7.24). In patients, there is a characteristic build-up of phytanic acid in the blood

Table 7.10 Enzyme deficiencies and accumulating lipids in the main sphingolipidoses

Disease	Signs and symptoms	Major lipid accumulation	Enzyme defect
Ceramide lactoside; lipidosis	Slowly progressing brain damage.	Ceramide lactoside	Neutral β-galactosidase
Fabry's disease	Reddish-purple skin rash, kidney failure, pain in lower extremities.	Gal-Gal-Glu-ceramide	α-Galactosidase
Farber's disease	Hoarseness, dermatitis, skeletal deformation, mental retardation.	Ceramide	Ceramidase
Gaucher's disease	Spleen and liver enlargement, erosion of long bones and pelvis, mental retardation only in infantile form.	Glucocerebroside	Glucocerebrosidase
Generalized gangliosidosis (GM_1 gangliosidosis)	Mental retardation, liver enlargement, skeletal deformities, about 50% with red spot in retina.	Ganglioside GM_1	β-Galactosidase
Krabbe's disease (globoid leukodystrophy)	Mental retardation, almost total absence of myelin, globoid bodies in white matter of brain.	Galactocerebroside	Galactocerebroside β-galactosidase
Metachromatic leukodystrophy (two forms)	Mental retardation, psychological disturbance in adult form, nerves stain yellow-brown with cresyl violet dye.	Sulphatide	Aryl sulphatase A (sulphatide activator/ saposin inactivator-deficient form)
Niemann–Pick disease	Liver and spleen enlargement, mental retardation, about 30% with red spot in retina.	Sphingomyelin	Sphingomyelinase
Tay–Sachs disease	Mental retardation, red spot in retina, blindness, muscular weakness.	Ganglioside GM_2	Hexosaminidase A
Tay–Sachs variant	Same as Tay–Sachs disease, but progressing more rapidly.	Globoside (and ganglioside GM_2)	Hexosaminidase A and B

Reproduced with kind permission from *Annual Review of Biochemistry*, **47** (1978), 687 by Annual Reviews Inc.; modified with reference to Neufeld (1991).

where it may represent 30% of the total fatty acids. A condition of ataxic neuropathy develops and the disease is normally fatal. To survive, the patients must have a low phytol diet.

Since an important consequence of the accumulation of sphingolipids in the central nervous system is to produce mental retardation, it is important to be able to detect the defect as early as possible. It is now possible to detect the enzyme deficiency responsible for some of the lipid storage diseases *in*

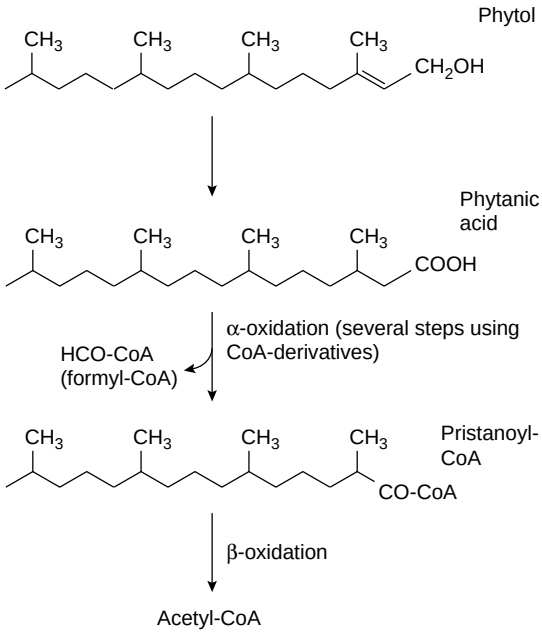


Fig. 7.24 The normal metabolism of phytol.

utero. Once the deficiency has been established, a possible treatment is enzyme replacement therapy. Thus, patients with Fabry's disease (Table 7.10) can be infused with normal plasma to provide active enzyme (β-galactosidase) for the hydrolysis of the substrate (Gal-Gal-Glu-ceramide) that accumulates in the plasma of these patients. Maximum enzyme activity occurs in six hours after infusion of the plasma and is detectable for seven days. The accumulated substrate decreases about 50% on the tenth day after infusion.

Another method involves the encapsulation of the appropriate enzymes in liposomes (Section 6.5.13) that can be targeted to the appropriate tissue. However, because of brain involvement in many sphingolipidoses, the use of liposomes is inappropriate owing to the 'blood–brain barrier' which prevents their uptake.

The sphingolipidoses are recessive diseases and heterozygous individuals have 50% of the normal enzyme levels in their tissues. This allows white blood cells to be tested and genetic counselling can then be given to carriers who wish to start families. In the future it may also be possible to use gene therapy.

Other examples of lipid storage diseases (involving fatty acid oxidation) were discussed before (Section 2.3) and include problems with β-oxidation in both mitochondria and peroxisomes.

7.9 THE 'PHOSPHATIDYLINOSITOL CYCLE' IN CELL SIGNALLING

The discovery of this cycle indicated that inositol phospholipids were important in hormone action.

As far back as 1953, the husband and wife team, the Hokins, observed that stimulation of pancreatic secretion with acetylcholine led to a markedly increased incorporation of ^{32}P into phospholipids. Further investigation showed that much of this incorporation was localized in phosphatidylinositol. This led to the concept that the head group of phosphatidylinositol could be released through phospholipase C action to yield diacylglycerol. The latter could then be converted back into phosphatidylinositol via phosphatidate (Section 7.1.6), thus completing the 'cycle'.

Since that time it has been realized that the more phosphorylated derivatives of phosphatidylinositol (especially phosphatidylinositol-4,5-*bis*phosphate) are more important in signal transduction and such molecules have extremely fast turnovers. The key enzymes here are the phosphoinositide-specific phospholipases C (PI-PLCs). PI-PLC isoenzymes have been found in a broad range of organisms and, although they have common catalytic properties, their regulation involves different signalling pathways. Originally three isoenzymes were isolated from brain cytosol and they were called PLC-β, PLC-γ and PLC-δ. All three could hydrolyse phosphatidylinositol, phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-*bis*phosphate, but preferred the latter. Surprisingly, the gene sequences of the three isoenzymes showed little homology except in two short regions, which were designated X and Y (Fig. 7.25). Since that time, other PI-PLCs have been cloned and the various cDNAs have been divided into the three groupings.

All three PI-PLC subgroups contain a pleckstrin homology (PH) domain (PH domains are named

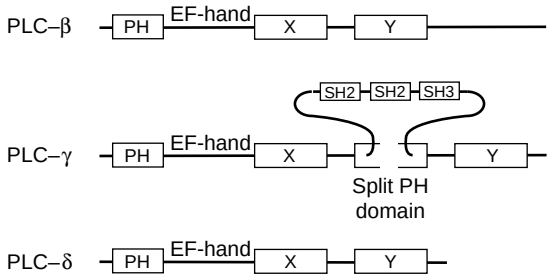


Fig. 7.25 Simplified structures of phosphoinositide-phospholipase C (PI-PLC) isoenzymes. PH = pleckstrin homology domain. SH2 and SH3 are Src-homology domains while EF-hand refers to domains in a helix-turn motif that bind calcium.

after their sequence similarity to the platelet protein, pleckstrin). These PH domains are binding sites for inositol lipids or inositol polyphosphates. This allows phospholipid–protein interactions that are downstream of agonist-stimulated receptor stimulation.

The different PI-PLC isoenzymes are stimulated in different ways:

- PLC-β types: G-protein-mediated activation
- PLC-γ types: tyrosine kinase-mediated regulation
- PLC-δ types: regulated by special class of GTP-binding protein, termed Gh

Some 20 years after the Hokin's first experiments, some detailed proposals as to how the inositol phospholipids could be involved in cell signalling and, in particular, in Ca^{2+}-mediated reactions were made by Michell and others. These were later refined by Berridge with the key concept that the PI-PLCs yielded two biologically active products – namely, diacylglycerol and inositol phosphates. An overall scheme for the activity of a PLC-β isoenzyme is shown in Fig. 7.26.

Basically the phospholipase C is activated in various ways (in Fig. 7.26 activation of a PLC-β

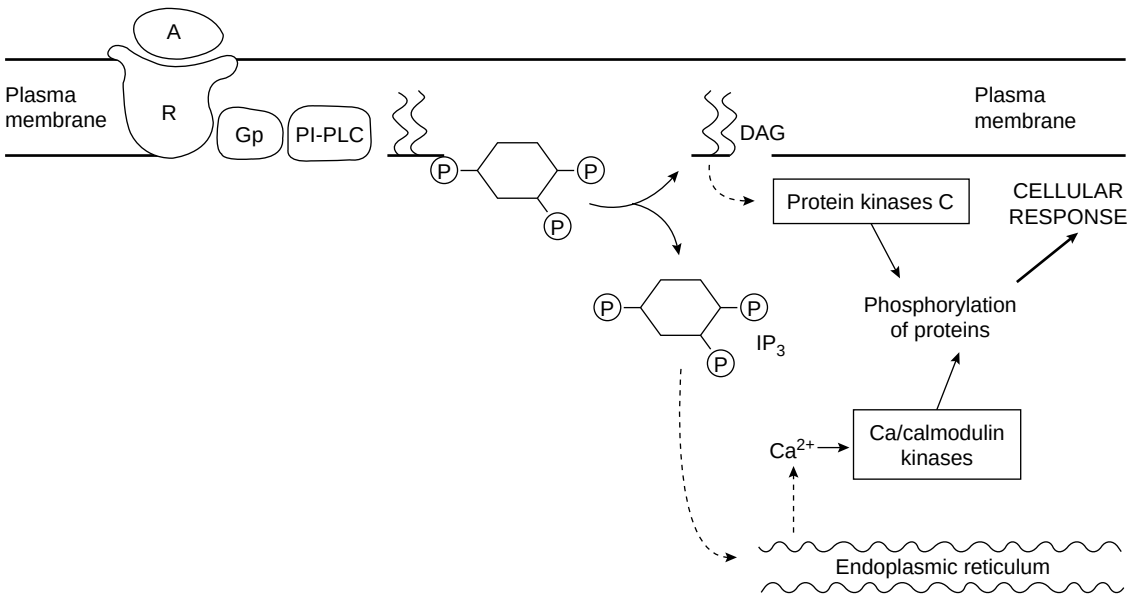


Fig. 7.26 Hydrolysis of phosphatidylinositol-4,5-*bis*phosphate by phospholipase C yields two second messengers
A: agonist that binds to receptor (R). The latter acts via G-protein (Gp) to activate a phosphoinositide-phospholipase C and causes hydrolysis of membrane-bound phosphatidylinositol-4,5-*bis*phosphate. The products, diacylglycerol (DAG) and inositol-1,4,5-*tris*phosphate (IP_3) activate protein kinases C and calmodulin kinases, respectively. This leads to a selective phosphorylation of proteins and a cellular response.

via a G-protein is shown) following (external) stimulation of the cell by, for example, a hormone. Activation of phospholipase C is mainly important in the hydrolysis of phosphatidylinositol-4,5-*bis*phosphate for two reasons. First, this is a preferred substrate for the enzyme and, second, the water-soluble inositol phosphates produced from most of the other inositol-phospholipids (e.g. inositol-1-phosphate) do not have a messenger function. Thus, phospholipase C activity leads to the production of diacylglycerol and inositol-1,4,5-*tris*phosphate, each of which leads to activation of protein kinases. Diacylglycerols activate protein kinases C. They bind to cysteine-rich regions of these kinases in co-operation with phosphatidylserine and Ca^{2+}. There are three classes of protein kinase C (conventional, novel and atypical) and only the first two are activated by diacylglycerols. (Diacylglycerols are also formed following phospholipase D hydrolysis of phosphatidylcholine and their production is mediated by sphingolipids; see Sections 7.11 and 7.12.)

On the other hand, the second product of phospholipase C hydrolysis of phosphatidylinositol-4,5-*bis*phosphate is the water-soluble inositol-1,4,5-*tris*-phosphate ($InsP_3$) (Fig. 7.26). There are various types of $InsP_3$ receptors expressed in mammalian cells and each is capable of forming intracellular Ca^{2+} channels and is regulated by both $InsP_3$ and cytosolic Ca^{2+}. Release of Ca^{2+} from internal stores then activates the Ca^{2+}/calmodulin protein kinases (Fig. 7.26) and can influence other cellular events (such as the interaction of diacylglycerol with protein kinases C).

In the diagram (Fig. 7.26) we have depicted the conventional view of plasma membrane-located metabolism of inositol phospholipid leading to release of Ca^{2+} from the endoplasmic reticulum. In addition, many features of the pathways are also present in nuclei. Thus, there is nuclear phospholipase C, which can generate diacylglycerol and a kinase that can convert the latter into phosphatidic acid (Section 7.1.6). $InsP_3$ receptors are also found. The metabolism of inositol phospholipids in the cell nucleus has been implicated in functions such as cell proliferation, differentiation and the control of transcription, DNA replication and chromatin structure.

7.10 A WIDER RANGE OF LIPID SIGNALLING MOLECULES

Phosphatidylinositol-3-kinase can generate other important signalling molecules. Over the last decade it has become apparent that, not only do many cells contain phosphatidylinositol-3-kinases but, on stimulation, such cells increase their concentration of 3-phosphorylated lipids markedly. (Normally the levels of such lipids are very low.) Two major isoforms of phosphatidylinositol-3-kinase have been found and activation connected to such events as stimulation of mitogenesis, triggering of differentiation and insulin responses (Table 7.11). Some forms of protein kinase C are stimulated by phosphatidylinositol-3,4,5-*tris*phosphate and this lipid can also activate serine/threonine kinases Akt (named after their similarity to the transforming oncogene of the retrovirus Akt 8).

Table 7.11 Cellular functions regulated by phosphatidylinositol-3-kinases

Apoptosis
Cellular proliferation
Vesicular trafficking
Cytoskeletal structure
Cellular morphology
Glucose utilisation
Protein biosynthesis
Lipid metabolism

The Akt/protein kinase B family of kinases contain a pleckstrin homology (PH) domain that can bind 3-phosphorylated inositol phospholipids. This leads to their activation via phosphorylation of the activation loop. In fact, the PH domain itself seems to inhibit the phosphorylation of Akt kinases and it is only after the 3-phosphorylated inositol lipids have bound that phosphorylation (at the cell membrane) occurs.

Phosphatidylinositol-3-kinase has other important roles. For example, it can activate MAP kinase (via its protein kinase activity). MAP kinase is part

of another important cell-signalling pathway and this activity of the 3-kinase provides a connection between two different signalling pathways. Such interactions are known as 'cross-talk'. The protein kinase activity of 3-kinases also has serine residues of the catalytic and regulatory subunits of itself as major substrates. Such autophosphorylation regulates 3-kinase activity.

Just as metabolism of phosphatidylinositol-4,5-*bis*phosphate was associated with nuclear membranes as well as the plasma membrane, 3-kinase activity also has a dual localization. Various agents (e.g. insulin, vitamin D) have been reported to increase translocation of the 3-kinase to the nucleus. Activity of the 3-kinase to produce phosphatidylinositol-3,4,5-*tris*phosphate in the nucleus then leads to activation of protein kinase C or phospholipase C-γ and, hence, to signalling actions.

7.11 PHOSPHOLIPASE D IN CELL SIGNALLING

After years of uncertainty about a role for phospholipase D, it was discovered that the enzyme can generate a wide range of compounds involved, inter alia, in vesicular trafficking, secretion, tumour promotion, membrane deterioration and senescence and as plant hormones.

Phospholipase D was first discovered in plants some 50 years ago. The enzyme is widespread and often very active in such organisms but is also present in mammals, fungi and bacteria. For a long time the presence of an active phospholipase D in plants was an enigma at best and, if its activity hindered lipid extraction or membrane preparation, then a nuisance. Phospholipase D hydrolyses phosphoglycerides at the terminal phosphodiester bond to produce phosphatidic acid. It can also transfer the phosphatidyl moiety to a variety of alcohols (by transphosphatidylation; Section 7.2.4) and this reaction is often used to assay the enzyme.

More recent studies have shown that activation of phospholipase D produces secondary messengers. Phospholipase D is involved in a wide variety of cellular and physiological processes including phytohormone action (in plants), vesicular trafficking, secretion, cytoskeletal arrangements, mycosis, tumour promotion, pathogenesis, membrane deterioration and senescence (Table 7.12).

Multiple phospholipase D activities have been found in several eukaryotes and, several phospholipase D genes have been cloned. For example, in the plant *Arabidopsis thaliana* seven genes, distributed between three separate chromosomes, have been found. In fact a phospholipase D gene superfamily, defined by a number of structural domains and sequence motifs, has been described that also includes phosphatidyltransferases and certain phosphodiesterases.

Many of the functions of phospholipase D (e.g. synthesis and release of matrix metalloproteinases in the metastasis of cancer cells) have been attributed to phosphatidic acid. However, the situation is complicated by the fact that this lipid can be rapidly converted to diacylglycerol by phosphatidate phosphohydrolase. Thus, in the long term, the rapid release of diacylglycerol from inositol lipids (Section 7.9) (Fig. 7.27) can be followed by a slower

Table 7.12 Some functions for phospholipase D in mammals[a] and plants

Mammals	Plants
Trafficking through the Golgi	Phytohormone action and signalling
Endosome/lysosome membrane fusions	Responses to wounding
Exocytosis	Plant–pathogen interactions
Mediation of agonist-induced cell responses	Membrane deterioration after stress
Neutrophil function; degranulation	Senescence, ageing
Synthesis and release of metalloproteinases	Membrane remodelling
	Membrane proliferation

[a] Most functions attributed to the phospholipase D1 isoform.

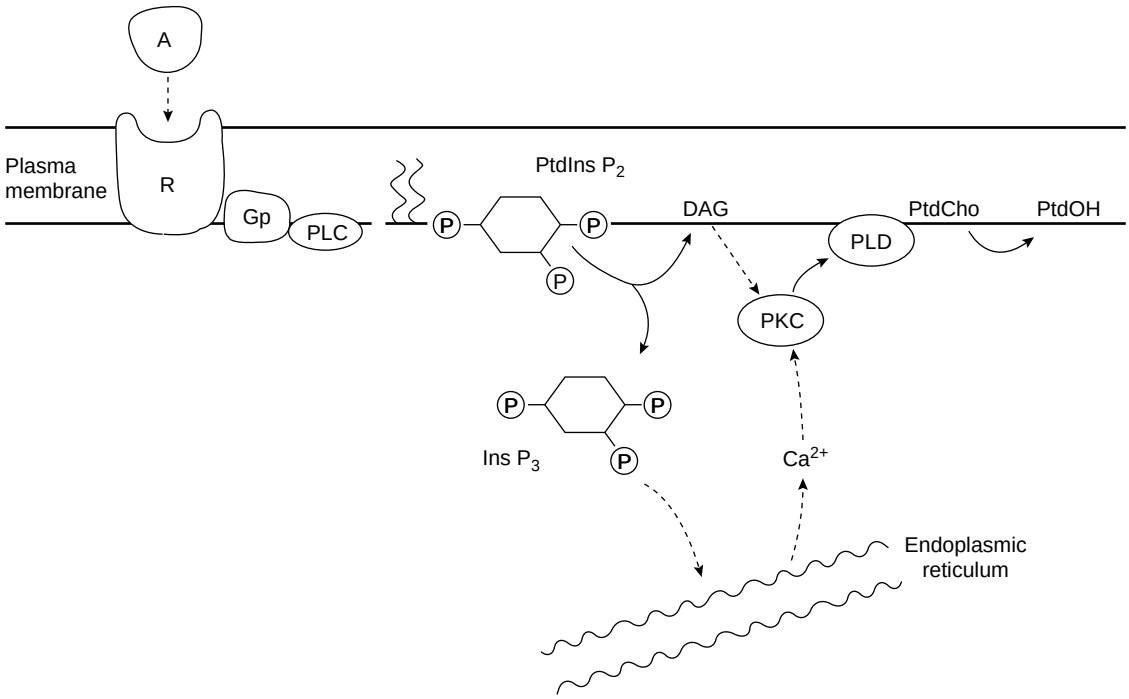


Fig. 7.27 How agonists acting through receptors linked to G-proteins can activate phospholipase D. Agonists (A) bind to receptors (R) and activate phospholipase C (PLC) via G-proteins (Gp). This releases inositol-1,4,5-*tris*phosphate (Ins P_3) by hydrolysis of phosphatidylinositol-4,5-*bis*phosphate. Ins P_3 causes Ca^{2+} mobilization from the endoplasmic reticulum and, together with the other PLC product diacylglycerol (DAG), Ca^{2+} causes translocation of protein kinase C (PKC) to the plasma membrane. PKC probably activates phospholipase D by phosphorylation of its regulatory domain. As a result phosphatidylcholine is hydrolysed to phosphatidic acid (PtdOH).

increase in diacylglycerol through phospholipase D action. Since the main substrate for phospholipase D is phosphatidylcholine (the main membrane phosphoglyceride; Section 6.2.1), this secondary production of diacylglycerol leads to an elevation in its levels, albeit at a slower rate of increase. This phenomenon is sometimes referred to as diacylglycerol amplification.

The mammalian phospholipase D has been well studied and is activated by phosphatidylinositol-4,5-*bis*phosphate and by ARF (ADP-ribosylation factor). Some plant phospholipase Ds also contain phosphatidylinositol-4,5-*bis*phosphate binding sites thus providing a connection (cross-talk) between inositol lipid signalling (Section 7.9 and Fig. 7.28) and the breakdown of phosphati-

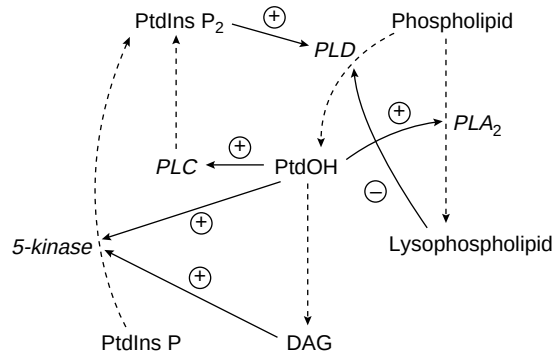


Fig. 7.28 Interactions of the phospholipase C and phospholipase D pathways for lipid-signalling molecules in plants. Enzymes are given in italics. PLA_2: phospholipase A_2; PLC: phospholipase C; PLD: phospholipase D. Continuous lines = effects of lipids on enzyme activity. Dotted lines = enzyme reactions.

dylcholine, which is the preferred substrate of many phospholipases D.

Not only will both phosphatidic acid and diacylglycerol have independent signalling properties but these lipids can be hydrolysed further to release polyunsaturated fatty acids. In plants this is especially important and can lead to jasmonic acid and other oxylipins being produced (Section 2.3.6).

Finally to add to the complexity of lipid signals and cross-talk between pathways, ceramides can activate phospholipase D to link sphingolipid metabolism with phosphoglycerides. The subject of sphingolipids in signalling is covered in the next section.

7.12 ROLE OF SPHINGOLIPIDS IN CELLULAR SIGNALLING

The most recent development in the saga of lipid signalling has been the discovery that sphingolipids, and in particular their breakdown products, have roles in modulating cell-surface receptor activity and recycling, in morphological changes during growth and differentiation, in apoptosis and in inflammatory responses.

At a number of places in this book we have described ways in which various sphingolipids can participate directly in different aspects of cell regulation. These include:

(1) Sphingolipids act as ligands for receptors (on neighbouring cells or in the extracellular matrix) and so regulate cell responses to the environment.
(2) They modulate receptor properties on the same cell.
(3) They are involved in membrane trafficking, receptor internalization and recycling and in secretory vesicle function.
(4) Sphingolipids have an important role in morphological changes during growth and differentiation.

In addition, hydrolysis of sphingolipids, like that of various phosphoglycerides (Sections 7.9–7.11), can give rise to second messengers. The main reactions involved in this generation of second messengers are those of the so-called sphingomyelin cycle (which is not necessarily a cycle; Fig. 7.29). Although in many cases there is a transient

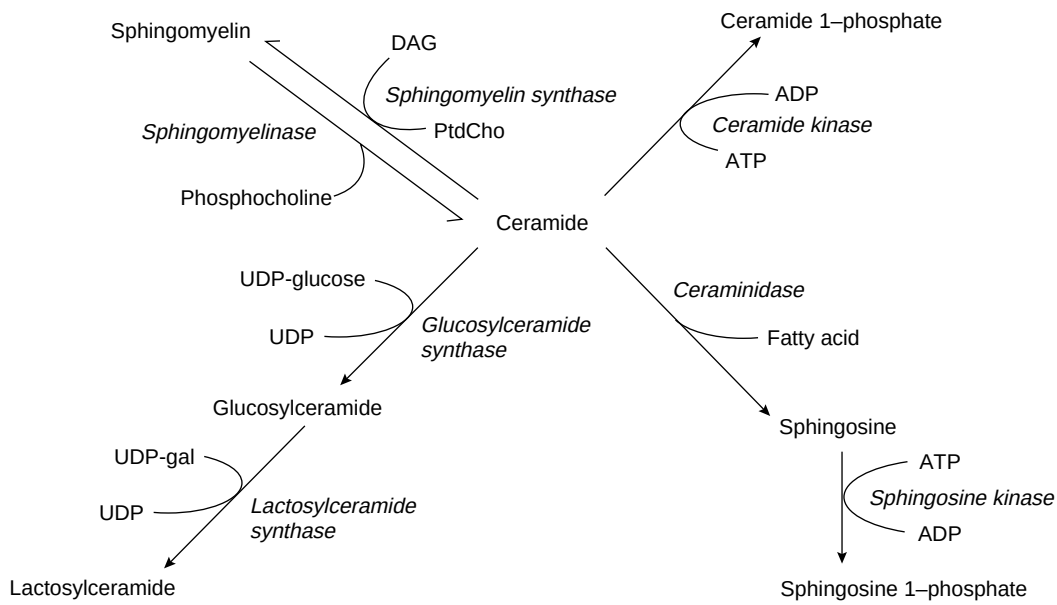


Fig. 7.29 The sphingomyelin cycle with modifications.

decrease in sphingomyelin levels as ceramide is formed followed by a return to normal levels, in some instances (particularly in apoptosis) there is a net loss of sphingomyelin and a net increase in ceramide. Several sphingolipid derivatives are formed which can have regulatory properties – lactosylceramide, glycosylceramide, ceramide, ceramide phosphate, sphingosine and sphingosine phosphate.

One of the first clues that sphingolipid catabolites could be important in cellular regulation was the observation by Hannun, Bell and colleagues that ceramide was released from sphingomyelin when HL-60 cells were stimulated by dihydroxyvitamin D_3, to make them differentiate. The sphingomyelinase enzyme responsible has now been found in eight different forms differing in pH optimum and subcellular localization. The best known is the acidic lysosomal sphingomyelinase, which is needed for turnover of membrane sphingomyelin and whose deficiency is responsible for Niemann–Pick disease (Section 7.8). There are also neutral (often Mg^{2+}-dependent) sphingomyelinases that are located in plasma membrane, endoplasmic reticulum or nuclear membranes. An alkaline sphingomyelinase also occurs in the gastrointestinal mucosa and in bile.

Ceramide is the best-studied of the sphingoid signalling molecules. Its production, by activation of sphingomyelinase, can be increased by a wide variety of different agents (Table 7.13). Ceramide will activate a number of important protein kinases, which in turn will affect important cellular activities (Fig. 7.30) such as growth arrest, apoptosis and inflammatory responses. It can also inhibit phospholipase D (Section 7.11) and affect phosphatidylinositol-3-kinase (Section 7.10), thus providing 'cross-talk' to these signalling pathways. Moreover, protein kinase C (the activity of which is altered by the diacylglycerol generated by both the phospholipase C and phospholipase D pathways; Sections 7.9 and 7.11) has been suggested, in at least some instances, to modify sphingomyelinase activity.

Ceramide is hydrolysed by ceramidase to yield sphingosine (or its equivalent base). Thus this enzyme has the intriguing property of converting one lipid messenger into another. Sphingosine bases will inhibit protein kinase C but activate several tyrosine protein kinases. They also interact with lipid metabolism by inhibiting phosphatidate phosphohydrolase, stimulating some isoforms of phospholipase C and D and enhancing prostaglandin production. Depending on the system studied they can either stimulate or inhibit growth and they may also play a role in apoptosis.

Sphingosine can be phosphorylated (Fig. 7.29) via a kinase. The product, sphingosine 1-phosphate

Table 7.13 Examples of stimuli known to increase ceramide production

Inducers of apoptosis	Tumour necrosis factor-α (TNF-α) Dexamethasone (a steroid) Nitric oxide
Inducers of differentiation	Vitamin D_3 TNF-α Nerve growth factor Retinoic acid
Damaging agent	UV light Heat shock Oxidative stress
Inflammatory cytokines	Interleukin-1α and IL-1β TNF-α Interferon-γ

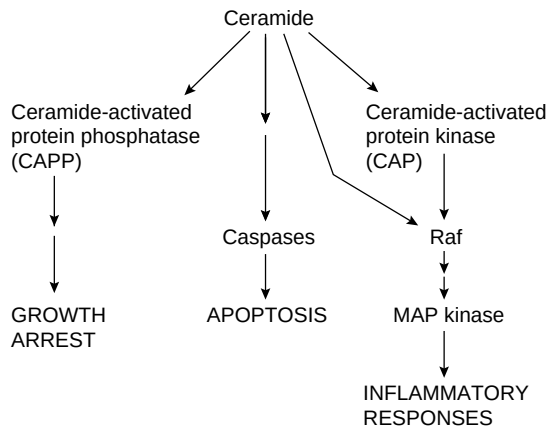


Fig. 7.30 Examples of the stimulation of protein modification by ceramide and subsequent cellular responses. Modified from Perry and Hannun (1998) with kind permission of the authors and Elsevier Science.

is a potent mitogen and can reverse the effects of ceramide on apoptosis. It can also cause release of calcium from internal stores. The mitogenic effects are probably through MAP kinases while cytoskeletal effects are quite common.

Other sphingosine derivatives such as ceramide 1-phosphate and lactosylceramide have also been reported to regulate cellular function, but they have not been nearly as well studied as ceramide, sphingosine and sphingosine 1-phosphate. However, two examples of the multiple effects of different sphingoid lipid messengers are shown in Fig. 7.31.

It is staggering how many cellular events seem to

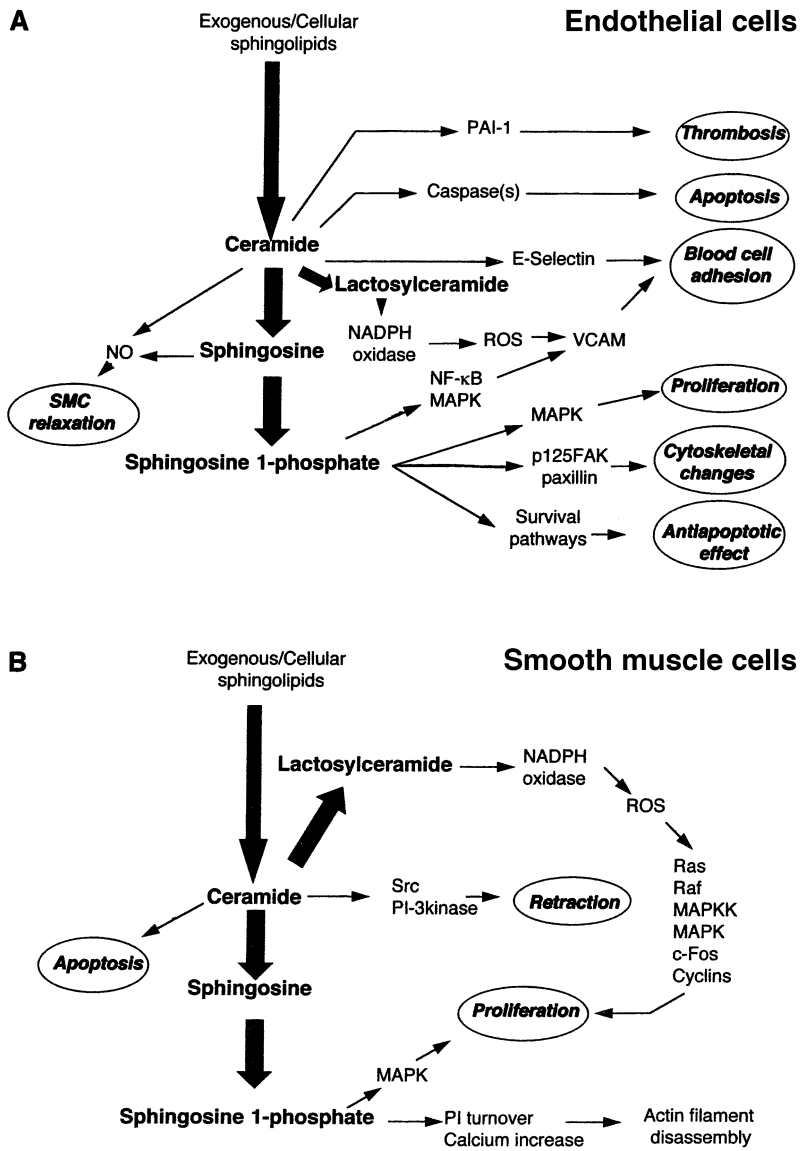


Fig. 7.31 Effects of sphingolipids in two different cell types – endothelial cells and smooth muscle cells. Based on Fig. 3 from Augé *et al.* (2000) with kind permission of the authors and Elsevier Science. ROS = reactive oxygen species.

be influenced by sphingolipid-derived messengers and, in only just over a decade, we have learnt much. But, at the same time, there is much still to elucidate and the next decade will undoubtedly be very busy in that regard!

7.13 SUMMARY

Glycerolipids are based on the trihydric alcohol glycerol. Phosphoglycerides are synthesized in one of three basic pathways. Either a CDP-base derivative reacts with diacylglycerol to produce the phospholipid or CDP-diacylglycerol can be used as an intermediate. The third type of pathway involves the conversion of one phospholipid into another. As a generalization, phosphatidylcholine and phosphatidylethanolamine are made by the CDP-base pathway in eukaryotes while the CDP-diacylglycerol pathway is used for acidic phospholipids such as phosphatidylglycerol and phosphatidylinositol. In both these pathways the hydrophobic part of the phospholipid ultimately derives from the acylation of glycerol 3-phosphate by the Kennedy pathway. The resultant phosphatidic acid can then be dephosphorylated to yield diacylglycerol or transferred to a cytidine nucleotide to produce CDP-diacylglycerol.

Different organisms make their phosphoglycerides in different ways. For example, *E. coli* makes all its phosphoglycerides from CDP-diacylglycerol. Moreover, whereas animals make phosphatidylserine by an exchange reaction, using another phosphoglyceride, micro-organisms form it from CDP-diacylglycerol and then use phosphatidylserine as a key intermediate to synthesize other phosphoglycerides.

The plasmalogen derivatives of phospholipids are made by the same basic pathway as the more usual diacyl analogues except that a 1-ether,2-acylglycerol substrate is used instead of diacylglycerol. The first documented example of a biologically active phosphoglyceride is platelet activating factor (PAF). Like plasmalogens, PAF contains an ether link at position 1, but with an acetyl moiety at position 2. PAF is bound to platelets via specific receptors. It has general effects on intracellular regulation, alters inflammatory responses and interacts with other biologically active lipids.

Phospholipases, responsible for the degradation of phospholipids, often have unique characteristics as enzymes. They usually operate best at the surface of immiscible solvents such as with lipid micelles. The micellar nature of their substrates makes the application of classical enzyme kinetics difficult. Many phospholipases (and lipases) are extremely stable proteins, which exhibit activity in organic solvents and at remarkably high temperatures.

Dependent on the position of attack, phospholipases are classified as A, B, C or D. Phospholipases A remove a fatty acid from phospholipids and are subdivided as A_1 or A_2 according to which acyl group is hydrolysed. They are important not only in lipid degradation but also in the turnover of acyl groups and in the release of fatty acids for particular purposes such as eicosanoid production. Phospholipase B removes the remaining fatty acid from a monoacyl (lyso) phospholipid while phospholipase C action gives rise to diacylglycerol and a phosphate-base moiety. A phosphatidylinositol-4,5-*bis*phosphate-specific phospholipase C is responsible for the generation of second messengers from this lipid. Phospholipase D removes the base moiety from a phospholipid to yield phosphatidate. Phospholipase D enzymes are very active in many plant tissues and can give rise to analytical artefacts if their activity is not carefully controlled. In addition, phospholipase D is recognized as being very important for lipid-signalling reactions in both plants and animals.

In photosynthetic membranes, the major lipid constituents are glycosylglycerides. Monogalactosyldiacylglycerol is formed by the transfer of galactose from UDP-galactose to diacylglycerol. Digalactosyldiacylglycerol can then be synthesized either by transfer of a second galactose from UDP-galactose to monogalactosyldiacylglycerol or by inter-lipid transfer between two molecules of monogalactosyldiacylglycerol. The formation of the third major glycosylglyceride, the plant sulpholipid (sulphoquinovosyldiacylglycerol) remains undefined although UDP-glucose appears to be a precursor of UDP-sulphoquinovose, which

is then used for transfer of sulphoquinovose to diacylglycerol.

Sphingolipids are based on sphingosine bases. Condensation of palmitoyl-CoA with serine yields 3-ketosphinganine, which can then be modified to produce other bases, after acylation of the amino group to yield a ceramide. Once ceramides have been synthesized, their alcohol moiety can be glycosylated to various degrees to form cerebrosides, neutral ceramides or gangliosides. Substrate-specific enzymes are used for the transfer of individual sugars during these syntheses. Usually UDP-sugars are the source of the sugar moiety although *N*-acetyl neuraminic acid is transferred from its CMP derivative. By contrast, sphingomyelin is produced by reaction of a ceramide with phosphatidylcholine.

Sphingolipids are broken down by substrate-specific enzymes. Sialic acid residues are removed by neuraminidases, galactose by galactosidases and glucose by glucosidases, etc. Almost all of these enzymes are found in lysosomes and their absence gives rise to the accumulation of the respective substrate sphingolipid in tissues. This causes various disease states known as lipidoses.

Cholesterol (and other sterols) is derived from acetyl-CoA. By a series of reactions the 5C-isoprene unit is formed and this can then self-condense to give a series of 10C, 15C, 30C, etc. isoprenoid molecules. Reduction of hydroxymethylglutaryl-CoA (HMG-CoA) is a key regulatory step in the overall process. The enzyme HMG-CoA reductase is regulated at the transcriptional level and by post-transcriptional methods. To form sterols from the open-chain isoprenes requires cyclization and various other modifications are also needed to form the final cholesterol molecule.

A new mevalonate-independent pathway for isopentenyl diphosphate formation has been found in algae and plants. This uses 1-deoxy-D-xylulose-5-phosphate rather than mevalonate as precursor of the isoprene unit.

Cholesterol itself is an important metabolic intermediate – being converted to cholesterol esters, to bile acids, to cholecalciferol (and vitamin D) or to various steroid hormones by different tissues. The synthesis of cholesterol and the regulation of its plasma circulating levels or conversion to other compounds is normally carefully controlled. Several enzymes of the cholesterol biosynthetic pathway are controlled through a specific transcription factor, the sterol regulatory element binding protein. Furthermore, the ratio of cholesterol to cholesterol ester is carefully regulated in different tissues by the activity of various acyltransferases.

Many membrane lipids can have specific roles in tissues in addition to their function in membrane structure. Many of these functions relate to their actions as lipid-signalling molecules where they can control diverse aspects of cellular activity such as hormone action, cell differentiation and apoptosis.

One specialized function is found in pulmonary surfactant where dipalmitoylphosphatidylcholine is crucial for the properties of the monolayer at the alveolar air–liquid interface. This monolayer lowers surface tension and prevents lung collapse when breathing out. There are also significant amounts of phosphatidylglycerol (which is unusual for animals) and this phosphoglyceride is believed to aid in the formation and maintenance of the surface layer. Deficiencies in pulmonary surfactant production can lead to respiratory distress. In premature babies this condition can be treated with exogenous surfactants, either artificial or extracted from animal lungs.

Several inborn errors of metabolism exist that involve deficiencies of specific enzymes of lipid metabolism. Prominent amongst these are a series of lipidoses in which sphingolipids build up in various tissues owing to reduced activity of a specific breakdown enzyme. The diseases are autosomal recessive and are particularly serious if the brain is involved. Carriers, who are heterozygous, can be diagnosed because they have 50% of the normal enzyme levels in their tissues. It has proved possible to treat some of the lipidoses by enzyme replacement therapy where the missing enzyme is supplied in liposomes and injected into patients.

Several phospholipids, apart from PAF, are important in cell signalling. The role of inositol phosphoglycerides was the first to be recognized here. In particular, the catabolism of phosphatidyl-

inositol-4,5-*bis*phosphate was found to be stimulated by a whole series of important agonists. The latter cause activation of a selective phospholipase C which catalyses the formation of diacylglycerol and inositol-1,4,5-*tris*phosphate – both of which have second messenger functions. Diacylglycerol is involved in the activation of protein kinases C. In addition, it can be hydrolysed to yield significant quantities of arachidonate, which is a precursor of eicosanoids. On the other hand, the water-soluble inositol-1,4,5-*tris*phosphate causes Ca^{2+} release from intercellular stores and activation of Ca/calmodulin protein kinases. The activity of the various protein kinases then regulates a host of cellular functions from metabolic regulation to differentiation. In addition to their role in the plasma membrane, inositol phosphoglycerides are also important in the cell nucleus where they are involved in the control of transcription, DNA replication and chromatin structure.

Phosphatidylinositol-3-kinase can generate other important signalling molecules. For example, some forms of protein kinase C are specifically stimulated by phosphatidylinositol-3,4,5-*tris*phosphate and this lipid can also activate some other types of serine/threonine kinases, of the Akt family. Phosphatidylinositol-3-kinase itself can act as a protein kinase and, hence, directly activate other cellular signalling pathways. It is also present in the nucleus.

In both plants and animals, phospholipase D activity has been shown to be involved in a wide variety of cellular and physiological processes including phytohormone action (in plants), vesicular trafficking, secretion, cytoskeletal arrangements, myosis, tumour promotion, pathogenesis and senescence. Some phospholipase Ds have binding sites for phosphatidylinositol-4,5-*bis*phosphate and this provides a connection (cross-talk) between two lipid-signalling pathways. The product of phospholipase D action (phosphatidic acid) is usually rapidly converted to diacylglycerol and both phosphatidic acid and diacylglycerol have independent signalling activities.

Not only do phosphoglycerides have signalling functions, but so do a number of sphingolipids or sphingolipid-derived compounds. Sphingolipids themselves play roles in cellular responses to the environment, modulating receptor responses, membrane trafficking and in controlling morphological changes during growth and differentiation. Catabolic products of sphingolipids such as the sphingolipid bases and ceramide or ceramide 1-phosphate are signalling molecules. For example, ceramide is important in growth arrest, apoptosis and inflammatory responses. It can also inhibit phospholipase D to provide another example of cross-talk between lipid-signalling pathways. Likewise sphingosine bases will interact with lipid metabolism by altering phospholipase C and D activity and eicosanoid production. They also have multiple effects on protein kinases. Although only recently discovered, sphingolipid-derived compounds have a huge array of effects on cells because of their activities in regulating protein kinases, calcium homeostasis and through interactions with other lipid-signalling pathways.

FURTHER READING

General

Cevc, G., ed. (1993) *Phospholipids Handbook*. Marcel Dekker, Basel.

Gunstone, F.D., Harwood, J.L. & Padley, F.B., eds. (1994) *The Lipid Handbook*, 2nd edn. Chapman & Hall, London.

Harwood, J.L., ed. (1998) *Plant Lipid Biosynthesis: Fundamentals and Agricultural Applications*. Cambridge University Press, Cambridge.

Moore, T.S., ed. (1993) *Lipid Metabolism in Plants*. CRC Press, Boca Raton. [Contains chapters specific to sections below on Phosphoglyceride Synthesis, Metabolism of Glycosylglycerides.]

Ratledge, C. & Wilkinson, S.G., eds. (1989) *Microbial Lipids*, 2 Vols. Academic Press, London.

Vance, D.E & Vance, J.E., eds. (1996) *Biochemistry of Lipids, Lipoproteins and Membranes*. Elsevier, Amsterdam. [Contains chapters specific to topics in the sections below.]

Phosphoglyceride synthesis

Carman, G.M. & Henry, S.A. (1999) Phospholipid biosynthesis in the yeast *Saccharomyces cerevisiae* and interrelationship with other metabolic processes. *Progress in Lipid Research*, **38**, 361–399.

Chao, W. & Olson, M.S. (1993) Platelet activating factor:

receptors and signal transduction. *Biochemical Journal*, **292**, 617–629.
Dircks, L. & Sul, H.S. (1999) Acyltransferases of de novo glycerophospholipid biosynthesis. *Progress in Lipid Research*, **38**, 461–479.
Hanahan, D.J. (1986) Platelet activating factor: a biologically active phosphoglyceride. *Annual Review of Biochemistry*, **55**, 483–509.
Harwood, J.L. (1989) Lipid metabolism in plants. *Critical Reviews in Plant Science*, **8**, 1–44.
Ishii, S. & Shimizu, T. (2000) Platelet-activating factor (PAF) receptor and genetically engineered PAF receptor in mutant mice. *Progress in Lipid Research*, **39**, 41–82.
Kent, C. (1995) Eukaryotic phospholipid biosynthesis. *Annual Review of Biochemistry*, **64**, 315–343.
Raetz, C.R.H. (1986) Molecular genetics of membrane phospholipid synthesis. *Annual Review of Genetics*, **20**, 253–295.
Shibuya, I. (1992) Metabolic regulations and biological functions of phospholipids in *E. coli*. *Progress in Lipid Research*, **31**, 245–300.
Smith, J.D. (1993) Phospholipid biosynthesis in protozoa. *Progress in Lipid Research*, **32**, 47–60.
Snyder, F. (1995) PAF and its analogs: metabolic pathways and related intracellular processes. *Biochimica et Biophysica Acta*, **1254**, 231–249.
Vance, J.E. (1998) Eukaryotic lipid biosynthetic enzymes: the same but not the same. *Trends in Biochemical Sciences*, **23**, 423–428.
Whatley, R.E., Zimmerman, G.A., Prescott, S.M. & McIntyre, T.M. (1996) Platelet-activating factor and PAF-like mimetics. In: *Lipid Second Messengers* (eds. R.M. Bell, J.H. Exton & S.M. Prescott), pp. 239–276. Plenum Press, New York.

See also the chapters by Rock *et al.* and Snyder in the multiauthor volume edited by Vance and Vance in the *General* section above.

Degradation of phospholipids

Dennis, E.A. (1994) Diversity of group types, regulation and function of phospholipase A_2. *Journal of Biological Chemistry*, **269**, 13057–13060.
Gelb, M.H., Jain, M.K., Hamel, A.M. & Berg, O.G. (1995) Interfacial enzymology of glycerolipid hydrolases: lessons from secreted phospholipases A_2. *Annual Review of Biochemistry*, **64**, 653–688.
Liscovitch, M., Czarny, M., Fiucci, G. & Tang, X. (2000) Phospholipase D: molecular and cell biology of a novel gene family. *Biochemical Journal*, **345**, 401–415.
Ohara, O., Ishizaki, J. & Arita, H. (1995) Structure and function of the phospholipase A_2 receptor. *Progress in Lipid Research*, **34**, 117–138.
Waite, M., ed. (1987) *The Phospholipases*. Plenum Press, New York.

Metabolism of glycosylglycerides

Browse, J. & Sommerville, C.R. (1991) Glycerolipid metabolism, biochemistry and regulation. *Annual Review of Plant Physiology & Plant Molecular Biology*, **42**, 467–506.
Douce, R., Joyard, J., Block, M.A. & Dorne, A.J. (1990) Glycolipid analysis and synthesis in plastids. In: *Methods in Plant Biochemistry* (eds. J.L. Harwood & J.R. Bowyer), Vol. 4, pp. 71–103. Academic Press, London.
Kates, M., ed. (1990) *Glycolipids, Phosphoglycolipids and Sulphoglycolipids*. Plenum Press, New York.
Pugh, C.E., Roy, A.R., Hawkes, T. & Harwood, J.L. (1995) A new pathway for the synthesis of the plant sulpholipid, sulphoquinovosyldiacylglycerol. *Biochemical Journal*, **309**, 513–519.

Metabolism of sphingolipids

Bell, R.M., Hannun, Y.A. & Merrill, A.H., eds. (1993) Sphingolipids, Part B: regulation and function of metabolism. *Advances in Lipid Research*, **26** (special issue).
Merrill, A.H. & Jones, D.D. (1990) An update on the enzymology and regulation of sphingolipid metabolism. *Biochimica et Biophysica Acta*, **1044**, 1–12.

See also the chapter by Merrill and Sweeley in the multiauthor volume edited by Vance and Vance in the *General* section above.

Cholesterol biosynthesis

Britton, G., Liaaen-Jensen, S. & Pfander, H., eds. (1998) *Carotenoids*, Vol. 3, *Biosynthesis and Metabolism*. Birkhauser, Basel. [Discusses the early steps of isoprene metabolism (including the non-mevalonate pathway), which are common to sterol formation.]
Brown, M.S. & Goldstein, J.L. (1986) A receptor-mediated pathway for cholesterol homeostasis. *Science*, **232**, 34–47.
Mercer, E.I. (1993) Inhibitors of sterol biosynthesis and their applications. *Progress in Lipid Research*, **32**, 357–416.
Ridgway, N.D., Byers, D.M., Cook, H.W. & Storey, M.K. (1999) Integration of phospholipid and sterol metabolism in mammalian cells. *Progress in Lipid Research*, **38**, 337–360.
Trzaskos, J.M. (1995) Oxylanosterols as modifiers of cholesterol biosynthesis. *Progress in Lipid Research*, **34**, 99–116.

Pulmonary surfactant

Batenburg, J.J. & Haagsman, H.P. (1998) The lipids of pulmonary surfactant: dynamics and interactions with proteins. *Progress in Lipid Research*, **37**, 235–276.
Bourbon, J.R., ed. (1991) *Pulmonary Surfactant: Biochemical,*

Functional, Regulatory and Chemical Concepts. CRC Press, Boca Raton.
Crenwels, L.A.J.M., van Golde, L.M.G. & Haagsman, H.P. (1997) The pulmonary surfactant system: biochemical and clinical aspects. *Lung*, **175**, 1–39.
Muller, B. & Von Wickers, P., eds. (1994) *Lung Surfactant: Basic Research in the Pathogenesis of Lung Disorders*. Karger Press, Basel.
Robertson, B., van Golde, L.M.G. & Batenburg, J.J., eds. (1984) *Pulmonary Surfactant*. Elsevier, Amsterdam.
van Golde, L.M.G., Batenburg, J.J. & Robertson, B. (1988) The pulmonary surfactant system: biochemical aspects and functional significance. *Physiological Reviews*, **68**, 374–455.

Lipid storage diseases

Brady, R.O. & Barton, N.W. (1994) Enzyme replacement therapy for Gaucher disease: critical investigations beyond demonstration of clinical efficacy. *Biochemical Medicine and Metabolic Biology*, **52**, 1–9.
Neufeld, E.F. (1991) Lysosomal storage diseases. *Annual Review of Biochemistry*, **60**, 257–280.
Scriver, C.R., Beaudet, A.L., Sly, W.S. & Valle, D., eds. (1995) *The Metabolic and Molecular Bases of Inherited Disease*, Vol. II, 7th edn. McGraw-Hill, New York.

See also the chapter by Merrill and Sweeley in the multi-author volume edited by Vance and Vance in the *General* section above.

Inositol lipids, phospholipase D and signalling

Bell, R.M., Exton, J.H. & Prescott, S.M., eds. (1996) *Lipid Second Messengers*. Plenum Press, New York.
Berridge, M.J. (1987) Inositol triphosphate and diacylglycerol: two interacting second messengers. *Annual Review of Biochemistry*, **56**, 159–193.
Berridge, M.J. (1993) Inositol triphosphate and calcium signalling. *Nature*, **361**, 315–325.
Bocckino, S.B. & Exton, J.H. (1996) Phosphatidic acid. In: *Lipid Second Messengers* (eds. R.M. Bell, J.H. Exton & S.M. Prescott), pp. 75–124. Plenum Press, New York.
Chan, T.O., Rittenhouse, S.E. & Tsichlis, P.N. (1999) AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annual Review of Biochemistry*, **68**, 965–1014.
Downes, C.P., ed. (1998) Lipids in signalling and recognition. [A special issue containing a dozen chapters on inositol lipids, phospholipase D and cell signalling.] *Biochimica et Biophysica Acta*, **1436**, 1–261.
Exton, J.H. (1998) Phospholipase D. *Biochimica et Biophysica Acta*, **1436**, 105–115.
Ferris, C.D. & Snyder, S.H. (1992) Inositol-1,4,5-trisphosphate-activated calcium channels. *Annual Review of Physiology*, **54**, 469–488.
Fruman, D.A., Meyers, R.E. & Cantley, L.C. (1998) Phosphoinositide kinases. *Annual Review of Biochemistry*, **67**, 481–507.
Liscovitch, M., Czarny, M., Fiucci, G. & Tang, X. (2000) Phospholipase D: molecular and cell biology of a novel gene family. *Biochemical Journal*, **345**, 401–415.
Martelli, A.M., Capitani, S. & Neri, L.M. (1999) The generation of lipid signalling molecules in the nucleus. *Progress in Lipid Research*, **38**, 273–308.
Martin, T.F.J. (1998) Inositol lipids as signalling molecules. *Annual Review of Cell Developmental Biology*, **14**, 231–264.
Singer, W.D., Brown, H.A. & Sternweis, P.C. (1997) Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. *Annual Review of Biochemistry*, **66**, 475–509.
Wang, X. (2000) Multiple forms of phospholipase D in plants: the gene family, catalytic and regulatory properties and cellular functions. *Progress in Lipid Research*, **39**, 109–150.

Sphingolipids and cell signalling

Allan, D. (2000) Lipid metabolic changes caused by short-chain ceramides and the connection with apoptosis. *Biochemical Journal*, **345**, 603–610.
Augé, N., Negre-Salvayre, A., Salvayre, R. & Levade, T. (2000) Sphingomyelin metabolites in vascular cell signalling and atherogenesis. *Progress in Lipid Research*, **39**, 207–229.
Dickson, R.C. (1998) Sphingolipid functions in *Saccharomyces cerevisiae*: comparison to mammals. *Annual Review of Biochemistry*, **67**, 27–48.
Hannun, Y.A., ed. (1998) *Sphingolipid-mediated Signal Transduction*. Springer, New York.
Martelli, A.M., Capitani, S. & Neri, L.M. (1999) The generation of lipid signalling molecules in the nucleus. *Progress in Lipid Research*, **38**, 273–308.
Merrill, A.H., Liotta, D.C. & Riley, K.E. (1996) Bioactive properties of sphingosine and structurally related compounds. In: *Handbook of Lipid Research* (ed. R.M. Bell), Vol. 8, pp. 205–237.
Perry, D.K. & Hannun, Y.A. (1998) The role of ceramide in cell signalling. *Biochimica et Biophysica Acta*, **1436**, 233–243.
Pfeilschifter, J. & Huwiler, A. (2000) Ceramides as key players in cellular stress response. *News in Physiological Sciences*, **15**, 11–15.

See also the chapter by Merrill and Sweeley in the multi-author volume edited by Vance and Vance in the *General* section above.

Index