VITAMINS AND HORMONES

Volume XIV

Robert S. Harris

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VOLUME XIV

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VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

Edited by

ROBERT S. HARRIS
Professor of Biochemistry of Nutrition
Massachusetts Institute of Technology
Cambridge, Massachusetts

G. F. MARRIAN
Professor of Medical Chemistry
University of Edinburgh
Edinburgh, Scotland

KENNETH V. THIMANN
Professor of Plant Physiology
Harvard University
Cambridge, Massachusetts

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CONTRIBUTORS TO VOLUME XIV

JAMES ASHMORE, *Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts*

S. H. EPPSTEIN, *Research Laboratories, The Upjohn Company, Kalamazoo, Michigan*

A. BAIRD HASTINGS, *Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts*

PETER KARLSON, *Max Planck-Institut für Biochemie, Tübingen, Germany*

G. A. LEVVY, *Rowett Research Institute, Bucksburn, Aberdeenshire, Scotland*

JOHN A. LORAINE, *Clinical Endocrinology Research Unit (Medical Research Council), University of Edinburgh, Scotland*

J. S. LOWE, *Department of Biochemistry, The University of Liverpool, England*

JEAN MAYER, *Department of Nutrition, Harvard University School of Public Health, Boston, Massachusetts*

P. D. MEISTER, *Research Laboratories, The Upjohn Company, Kalamazoo, Michigan*

OLAF MICKELSEN, *Laboratory of Biochemistry and Nutrition, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland*

R. A. MORTON, *Department of Biochemistry, The University of Liverpool, England*

H. C. MURRAY, *Research Laboratories, The Upjohn Company, Kalamazoo, Michigan*

D. H. PETERSON, *Research Laboratories, The Upjohn Company, Kalamazoo, Michigan*

ALBERT E. RENOLD, *Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts*

KENNETH H. SHULL, *Department of Nutrition, Harvard University School of Public Health, Boston, Massachusetts*

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EDITORS' PREFACE

The Editors are pleased to present this fourteenth volume of *Vitamins and Hormones.*

The eight chapters were written by authors located in three countries (Great Britain, Germany, and the United States). Of the two chapters on vitamins, one relates to the intestinal synthesis of vitamins in non-ruminants, and its importance in the nutrition of the host. This supplements the treatment of ruminants in Volume XII, and the two together bring out the widespread nutritional implications of these syntheses. The conversion of carotene to vitamin A, discussed in the second chapter, offers quite a different example of how vitamins may be formed internally.

The remaining chapters refer to hormones: two deal with the hormonal control of carbohydrate metabolism, and the others with insect hormones, the bioassay of gonadotropins and the microbiological transformations of steroids. This last covers a field of great and growing importance in the manufacture of steroids for clinical use. The article on insect hormones is probably the first published summary of the very recent work on the biochemistry of these substances. The trend of the contributions as a whole is to stress the increasing degree to which the actions of vitamins and hormones are interrelated with the whole field of metabolism in all its aspects.

The Editors are indebted to the authors whose devotion to science and to other scientists has activated them to accept our invitation to contribute to this volume, even though it may have seriously interfered with the progress of their own scientific programs. Their reward is in the satisfaction that comes from serving well.

Robert S. Harris
Guy F. Marrian
Kenneth V. Thimann

August, 1956

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Intestinal Synthesis of Vitamins in the Nonruminant

OLAF MICKELSEN

Laboratory of Biochemistry and Nutrition, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland

I. Introduction

1. Early Work on the Relation of the Intestinal Flora to Nutrition

The realization that viable bacteria are present in the gastrointestinal tract of normal individuals raised the question in Pasteur's mind whether they were necessary for the well-being of the host (Johansson and Sarles, 1949; Rettger and Cheplin, 1921). It was demonstrated as early as 1874

that the meconium of the newborn infant is sterile (Billroth, 1874). The characteristic flora that is established in the infant shortly after birth depends to a large degree upon the nature of its food (Escherich, 1885; Tissier, 1900). The work of Tissier (1900) showed that the flora in the breast-fed infant is predominantly *Lactobacillus bifidus*, whereas that in the bottle-fed baby is *Lactobacillus acidophilus*. The flora becomes more complex as supplementary foods are added to the child's diet. The varieties of bacteria in the gastrointestinal tract increase to a certain extent with age (Rettger and Cheplin, 1921).

More than fifty different species of microorganisms have been isolated from the feces of man. A number of these are present only occasionally and may be associated with specific diseases. Others, however, are present so frequently that they have been classed as normal inhabitants. About a third of the dry matter in the stool of a healthy adult represents bacteria. Most of these bacteria, even in the fresh stool, are dead (Todd *et al.*, 1953).

In the early days of bacteriology the bacteria in the gastrointestinal tract were viewed entirely from the standpoint of harmful and deleterious agents. Bouchard (1884) is credited with the theory of intestinal intoxication. According to this hypothesis, the "toxins" resulting from intestinal "putrefaction" of proteins are absorbed and then produce their debilitating effects. The urinary excretion of indole, phenol, ethereal sulfates, and other substances was used in evaluating the extent of intestinal putrefaction in many of the early studies on the effect of diet in "improving" the intestinal flora (Rettger and Cheplin, 1921).

Metchnikoff's (1907) hypothesis that the longevity of certain nomadic tribes and eastern Europeans who used fermented milk was due to the predominant *Lactobacillus bulgaricus* flora in their intestines initiated a wave of enthusiastic food faddism. The wave still flows on, as is evidenced by the popularity of and interest in yoghurt (a curdled milk containing primarily *L. bulgaricus*). The primary argument put forth by the early advocates of the *L. bulgaricus* and *acidophilus* flora was the necessity of suppressing the proteolytic organisms. By this means autointoxication of the body by the bacterial "toxins" produced in the gastrointestinal tract was supposedly reduced. There is a possibility that the drastic changes in the intestinal flora brought about by the dietary procedures advised by the adherents of the "autointoxication" theory may have had significant repercussions on the vitamin balances of the subjects.

2. Refection

While the preceding work on intestinal flora was being carried out, Eijkman (quoted by Kon, 1953) noticed that when he fed his chicks

potato starch instead of rice starch, they did not develop polyneuritis. There is a possibility that the failure to develop polyneuritis was due to a condition similar to the refection[1] described by Fridericia *et al.* (1927), who observed that one of three rats on a diet of rice starch, water-extracted casein, butterfat, and salt mixture started to gain weight. All the previous rats on the same vitamin B deficient diet and the other two in the same group continued to lose weight and finally died after showing typical symptoms of polyneuritis. The animal that behaved differently continued to gain for the remaining 20 weeks of the study. This animal had white, bulky feces which, when incorporated into a vitamin B-deficient diet, produced a refected state in the recipient. Although it is not known yet how refection is spontaneously established in an animal, there has been ample confirmation of its existence (Johansson and Sarles, 1949; Kon, 1953).

Fridericia *et al.* (1927) and Roscoe (1927) showed that a poorly digested carbohydrate, such as raw starch, had to be present before refection occurred. If the starch were boiled so that no starch grains were left or if sucrose were used in its place, refection did not develop. These observations have been confirmed and extended by Guerrant *et al.* (1935, 1937). On the basis of these studies it is believed that the relatively nondigestible carbohydrates reach the lower part of the intestinal tract where they provide a medium for the synthesis of the B vitamins by the microflora.

The B vitamins thus synthesized were absorbed either directly or after the rat had consumed its feces. The consumption of feces was believed by Roscoe (1927) to be an integral part of refection. When Roscoe transferred a refected rat to a cage with large mesh screen bottom, the refected state disappeared and the rat started to lose weight. The refected state was restored when the rat was returned to its original cage. "The inference drawn from this work was that to continue in the refected state it was necessary for the rats to eat some of their feces. Whatever construction of cage is used, there are some rats that cannot be prevented from eating their own feces, for they secure them and consume them as they leave the body." Guerrant *et al.* (1937), however, state that refection can be maintained in some rats that show no evidence of coprophagy. A similar conclusion appears in the studies of Ford *et al.* (1953). These

[1] Refection has been defined by Guerrant (1955) as " . . . the condition . . . in which the test animals were able to synthesize within their digestive tract and to absorb therefrom enough of some of the B-vitamins to meet their requirement. The phenomenon invariably appears in the highest incidence in young rats subsisting on purified diets containing a high percentage of uncooked starch, either potato or rice starch, but very seldom on commercial corn starch."

latter workers emphasized that the slightly acid cecal contents (pH 5–6.6) in the refected rats compared with the controls (pH 7–7.5) favored an increased absorption of the synthesized vitamins (see also Kon, 1953).

Earlier than the above, Cooper (1914) showed that an alcohol extract of normal chicken or rabbit feces would cure polyneuritis in pigeons. Although Cooper recognized that the B vitamins present in the feces might represent unabsorbed vitamins, he did suggest that the bacteria growing in the large intestine might be their source. Although he was unable to find any activity in an alcoholic extract of 2 g. of *B. coli*, he concluded that the amount of B vitamins in that particular organism was much less than that in yeast.

a. Effect of Sulfonamides.[2] Coates *et al.* (1946) found that sulfonamides have a depressing effect, especially on thiamine synthesis, in the intestine of the refected rat. When they added either sulfapyrazine, sulfathiazole, sulfasuxidine, sulfaguanidine, or sulfathalidine to their purified diet containing potato starch, with no added vitamins other than those in cod liver oil, the refected rats very shortly started to lose weight. The weight loss was accompanied by a marked reduction in the thiamine content of the feces. Symptoms of polyneuritis were seen in a number of the rats that received the sulfonamides. This observation, together with the fact that the urine of refected rats (both the sulfonamide-supplemented and the controls) contained no thiamine, suggested to them that thiamine was formed in limiting amounts in the intestine of the refected animal. Although the sulfonamides produced a marked reduction in both urine and fecal riboflavin levels, these rats still excreted approximately 5–10 μg. of the vitamin per day in their urine.[3] Feeding cooked or finely milled starch (both processes increase digestibility) in the absence of sulfonamides produced a weight loss in refected rats (Ford *et al.*, 1953), with a reduction in the fecal thiamine and riboflavin excretions. The urinary riboflavin increased, however, which might have resulted from the accompanying loss of body weight (Mickelsen *et al.*, 1945).

Further studies by Ford *et al.* (1953) showed that the refected rat on a diet containing only traces of riboflavin, niacin, pantothenic acid, biotin, folic acid, and vitamin B_6 excreted substantial but variable amounts of these substances in its urine. In many cases the urinary

[2] This section is devoted to a consideration of the influence of sulfonamides on refection. The effect of sulfonamides on specific vitamins is presented in Section IV; a survey of the earlier work is presented in Section I, 4.

[3] There is no specific indication in the reports of Coates *et al.* (1946) and Ford *et al.* (1953) that coprophagy was prevented. There is, however, presumptive evidence that coprophagy was prevented since the changes in the urinary excretion of certain B vitamins (especially thiamine) did not parallel the fecal concentrations.

excretion was as great as that seen on the stock diet. The addition of succinylsulfathiazole to the refection-producing diet decreased the urinary excretion of all these vitamins (except pyridoxine) to half or less that of their controls. The urinary excretion of pyridoxine was not changed, even though the fecal excretion was reduced to about 30% or less that of the presulfonamide period. A comparable reduction of the vitamins in the cecal content was observed following sulfonamide supplementation. These findings, especially those on the urinary vitamin excretion, indicate that the microflora in the rat can synthesize considerable amounts of the B vitamins when the animal is in the refected state and that these vitamins can be absorbed, presumably directly from the large intestine.

b. Influence of Carbohydrates. The role played by carbohydrates in the development of refection has been reviewed by Johansson and Sarles (1949), Elvehjem (1946, 1948), Elvehjem and Krehl (1947), and Kon (1953). For this reason carbohydrates will be mentioned only in the reports on the individual vitamins. Suffice it to say that refection occurs in the rat only when there is a poorly digested carbohydrate in the diet. The mechanism whereby the presence of these carbohydrates in the lower part of the gastrointestinal tract brings about a change in the flora is still unknown.

c. Influence of the Cecum. Although Griffith (1935) and Taylor *et al.* (1942) presented evidence which indicated that the cecum contributed few, if any, vitamins to rats in a nonrefected state, there has been relatively little of this work done with refected rats. Guerrant *et al.* (1935) observed that the one cecectomized rat that survived had a reduced amount of the B vitamins in its feces while being fed a diet which produced refection in normal rats. Results at variance with the preceding were reported by Kon *et al.* (1938) who found that their four cecectomized rats grew as well as the sham-operated controls when fed the refection-producing diet. A more recent publication from the latter group indicates that they were unable to produce refection "in a group of more than fifty rats after removal of the caecum" (Coates *et al.*, 1946). In view of the conflicting results, it is impossible to determine whether the cecum is necessary for the development of refection in the rat. It is unfortunate that observations were not made in the cecectomized rats which did not become refected to see if the lower part of the large intestine showed an outpouching (for a discussion of the role of the cecum in the synthesis of vitamins see Section III).

d. In Animals Other than the Rat. Very little work has been done to determine whether refection can be established in animals other than the rat. In 1897 Eijkman was unable to produce polyneuritis in hens fed corn starch instead of polished rice (quoted by Kon, 1953). Whether

this was due to a refected state in the hens was never determined. The type of dietary carbohydrate influences the synthesis of folic acid (Luckey *et al.*, 1946b) and biotin in the chick (Couch *et al.*, 1948). Although these situations may be related to refection, they may not be the same, since the original work of Fridericia *et al.* (1927) showed that if the diet of refected rats was supplemented with 5% brewer's yeast, they lost their refected state within 24 hours as shown by the change in the color, size, and consistency of their feces. Refection could be produced only on a vitamin B-deficient diet. These workers also reported that when mice fed a vitamin B-deficient diet were given feces from refected rats, one mouse out of 19 appeared to have become refected as shown both by the appearance of characteristic stools and a gain in body weight. A number of other mice in the above group showed the characteristic stools but died before any weight gains were evident. Three pigeons fed polished rice supplemented with feces from refected rats failed to become refected.

3. Coprophagy[4]

The ingestion of feces by animals has been recognized for many years. It was incorporated in certain agricultural practices where swine were permitted to follow cattle in pasture.

The necessity for controlling coprophagy in nutritional studies was pointed out by Steenbock *et al.* (1923). When they kept their rats on wire screens, the requirements for the B vitamins increased markedly in comparison to the requirements of similar animals maintained on shavings. It was shown by Osborne and Mendel (1911) that rats on a vitamin-low diet will consume feces and have a preference for the feces dropped by well-fed rats.

Coprophagy is a common phenomenon in the rabbit. Through some unexplained mechanism, the rabbit produces a soft feces high in water and vitamin content in addition to a hard feces (Thacker and Brandt, 1955). The soft feces are presumably voided during the night and con-

[4] The classic definition for coprophagy as given in Webster's unabridged dictionary is "the act or habit of eating dung or excrement." The illustrative phrases under this and related words indicate that the word includes not only the consumption of an animal's own feces but also the feces from any animal. Furthermore, the *Oxford English Dictionary* indicates that coprophagy was used to describe insects that lived in dung (presumably cows' dung) in 1826, long before its application to mammals. There has been a tendency in the field of nutrition to limit coprophagy to the consumption of an animal's own feces. This narrow concept probably arose from the practice of keeping only one animal in a cage as a means of preventing coprophagy. Considerable work has been done on coprophagy by such investigators as Osborne and Mendel (1911), Steenbock *et al.* (1923), Roscoe (1931), Schwartzer (1937), Geyer *et al.* (1947), Barki *et al.* (1949), and Kulwich *et al.* (1954).

sumed by the rabbit as they are dropped (Southern, 1940). The hard feces are excreted during the day. Under normal circumstances a rabbit may consume from 54 to 82% of the feces voided (Eden, 1940).

Early in the studies on the nutritional needs of the rabbit it became obvious that this animal did not require a dietary source of thiamine. Further studies showed that the rabbit did not require any pantothenic acid, riboflavin, biotin, or folic acid in its diet. These findings (reviewed by Olcese *et al.*, 1948) were unexpected, since it was assumed that only ruminants were able to satisfy their requirements for all of the B vitamins by means of bacterial synthesis.

The consumption of soft or night feces by the rabbit may explain that animal's independence of a dietary supply of many B vitamins. The soft feces contain large amounts of the water-soluble vitamins. Kulwich *et al.* (1953) collected the soft feces for a three-day period by fitting the rabbits with collars which prevented coprophagy, and they found that the feces made a significant contribution to the rabbit's vitamin economy. The vitamin contents of the soft feces, calculated as a percentage of the rabbit's vitamin intake on a stock diet, were: niacin 83, riboflavin 100, pantothenic acid 165, and vitamin B_{12} 42. The work of Kulwich *et al.* (1953) showed that there was no essential difference in the urinary excretion of the above water-soluble vitamins whether the rabbits were collared or not. It is difficult to understand why a doubling of the riboflavin intake and an increase of two and one-half fold in the pantothenic acid intake should not be associated with some increase in the urinary excretion of these factors.

The consumption of feces by poultry has been encouraged commercially on a limited scale in the so-called "built-up litter" plans. According to this procedure, ground up corn cob, bark, straw, or other similar product is used as litter for growing chickens. This may be one means whereby both the known and as yet unrecognized growth essentials are added to a ration (Jacobs *et al.*, 1954). The vitamin content of the litter may be fairly high, since the fecal bacteria continue to synthesize vitamins for some time after the feces are voided (Lamoreux and Schumacher, 1940).

4. Survey of Work on Sulfonamides

The advent of the relatively insoluble sulfonamides permitted intensive investigations on the effect of the intestinal microflora on the vitamin requirements of animals. One of the first reports on the use of sulfonamides in nutrition work was that by Black *et al.* (1941). They used a purified diet on which they secured good growth in rats. The addition of 0.5% sulfaguanidine to that diet produced a marked inhibition in growth. The addition of liver extract or *p*-aminobenzoic acid (PABA) to the diet

overcame the effect of the sulfonamide. The observed effects were explained on the assumption that sulfaguanidine had inhibited, in the gastrointestinal tract of the rats, the bacterial synthesis of certain nutrients not known at that time. This, and the work that followed, led to the recognition that vitamin K (Black *et al.*, 1942), folic acid (Nielsen and Elvehjem, 1942; Spicer *et al.*, 1942), and biotin (Daft *et al.*, 1942) were required by the rat. There was a suggestion from the work of Light *et al.* (1942) that the feces of rats fed sulfonamides had a reduced content of B vitamins. There was, however, no correlation between the reduction in vitamin content of the feces and the growth-depressing effect of the different sulfonamides studied.

Sulfonamides vary somewhat in producing vitamin deficiencies. This phase of the subject and a review of the earlier work in this field have been presented by Daft and Sebrell (1945). The early work can be summarized by saying that when the more insoluble sulfonamides, such as sulfaguanidine, sulfapyridine, sulfadiazine, and sulfathiazole, were incorporated into purified diets, they produced in the rat deficiencies of vitamin K, folic acid, and biotin. It is assumed that these vitamin deficiencies resulted from the action of the sulfonamides on the microbial flora of the gastrointestinal tract. The organisms synthesizing the vitamins were presumably inhibited to such an extent that the animal's requirement was no longer met or the metabolism of the microorganisms was changed in such a way that they were no longer able to synthesize an adequate amount of the vitamin for the host. In addition, some of these drugs produced disturbances in the thyroid gland and kidneys which were probably due to a specific sulfonamide toxicity (Daft and Sebrell, 1945).

While the primary nutritional effect of the sulfonamides appeared to be in decreasing the weight gains of animals, there were a few indications that these compounds stimulated growth under certain conditions. In 1944 Briggs *et al.* secured a slight growth response in chicks when 0.5% sulfasuxidine was added to a purified ration containing 2% solubilized liver, which supplied such factors as folic acid and probably vitamin B_{12}. This was in contrast to the action of the same sulfonamide when added to an incomplete purified ration. In the absence of the liver, the chicks on the same purified diet containing the sulfonamide gained only one-fourth as much as the controls. This observation was confirmed in the same laboratory by Moore *et al.* (1946). They found that 1% sulfasuxidine added to the above purified diet containing folic acid in place of the liver produced a marked improvement in the growth of the chicks. These workers recognized the possibility that the growth-stimulating effect of the sulfonamides might result from their action in inhibiting the

growth of organisms that produced harmful "toxins" or by decreasing the growth of organisms that competed with the host for certain vitamins.

There are still some problems involving sulfonamides that have not been clarified. One of these is the differential effect of some sulfonamides on vitamin-deficient and on adequate diets, which was shown in the work of Briggs *et al.* mentioned above (1944). Another involves the discrepancies between the reduction of vitamins in the feces and the growth-depressing effects of the different sulfonamides as brought out by the work of Light *et al.* (1942). It can be argued that if the vitamins in the feces reflect the level of microbial activity in the intestine, then there should be a parallelism between the effects of the sulfonamides on growth and the excretion of vitamins. The solution of these problems awaits future research.

The recent work on sulfonamides as they affect the vitamin economy will be considered subsequently when the individual vitamins are reviewed.

5. Survey of Earlier Work on Nutritional Effects of Antibiotics

It has often been pointed out that the environment must be receptive before any discovery will be recognized and exploited. The growth-stimulating effect of small amounts of antibiotics added to the diets of animals is a case in point. Although one of the earliest papers on this subject was that of Moore *et al.* (1946), little was done in this field until the report by Stokstad and Jukes in 1950.

After Briggs *et al.* (1944) found that chicks raised on a purified diet containing all the known nutrients plus 0.5% sulfasuxidine grew better than the unsupplemented birds, Moore *et al.* (1946) in the same laboratory observed that 10 mg. % of streptomycin added to a complete purified diet produced a considerable increase in the growth of their chicks. The primary purpose of these studies was to sterilize the gastrointestinal tract in order to secure a method of evaluating the role of its flora in providing the chick with essential nutrients. Since sulfonamides and antibiotics did not eliminate the bacteria, the primary attention of the investigators was focused on other means of accomplishing that purpose. For this reason the incidental observations of the growth-stimulating effect of streptomycin and the earlier one on sulfasuxidine were not followed up.

During the next four years the animal protein factor (which was eventually shown to be primarily vitamin B_{12}) monopolized the attention of most workers in this field. It was only after the isolation of vitamin B_{12} that the growth-stimulating effect of antibiotics was fully recognized.

The search for potent, yet inexpensive, sources of the animal protein

factor led to a variety of fermentation broths. The spent mash from the production of antibiotics turned out to be an excellent source. Furthermore, this material was well suited for large-scale use in commercial animal feeds. These broths increased the rates of weight gain for chicks, turkeys, and pigs (Stokstad, 1954a), but the increases could not be accounted for entirely on the basis of the vitamin B_{12} content. Even though only a very small amount of antibiotic remained in the broth, it occurred to Stokstad and Jukes (1950) that the antibiotic might be responsible for the growth effects. They showed that the addition of 10 mg. of crystalline Aureomycin to 100 g. of an all-plant ration supplemented with adequate amounts of vitamin B_{12} increased body weights of chicks to almost the same extent as the fermentation residue. This paper by Stokstad and Jukes initiated a tremendous amount of research on the growth-promoting action of antibiotics and related compounds. From this observation there developed a large-scale business which provides feed manufacturers with antibiotics. In 1951 the feed industry in the United States used 17.5 million dollars worth of antibiotics (*Chem. Eng. News*, 1952). This sum went up to 19.4 million in 1953 (*Chem. Eng. News*, 1955) and to 25.8 million in 1954 (unofficial estimate by Tariff Commission). With a continuing reduction in the unit cost of the antibiotics, the increases are actually greater than the preceding sums indicate.

A large fraction of the research studies and practical evaluation of antibiotic addition to animal feeds has been done with poultry. Such substances as procaine penicillin, Aureomycin, terramycin, bacitracin (Braude *et al.*, 1953) and carbomycin (Reynolds *et al.*, 1954) are fully effective as growth promoters when added to the feed at levels from 1 to 2 g. per ton. These levels are as low as the recommended allowances of thiamine for chicks (National Academy of Sciences, 1954), which indicates that the potency of the antibiotics added to the diet is as great as that of some vitamins.

The potency of low levels of antibiotics in stimulating the growth of animals receiving what had been considered an adequate ration has focused attention on antibiotics from a nutritional standpoint. The earlier work on the nutritional aspects of antibiotics has been covered by a number of reviews (Mickelsen, 1953; Stokstad, 1953; Braude *et al.*, 1953; Jukes and Williams, 1953; Stokstad, 1954a; Jukes, 1955). With the above background, an attempt will be made in a later section to evaluate the influence of the antibiotics on the synthesis of vitamins in the gastrointestinal tract of nonruminants. The literature dealing with ruminants has been reviewed by Kon and Porter (1954).

In addition to antibiotics, arsenicals, surface-active agents (sur-

factants) and, in a few cases, vitamin C are effective in stimulating the growth of animals maintained on vitamin-deficient diets. The activity of these substances has been reviewed by Frost (1953), Mickelsen (1953), Stokstad (1954a), and Frost *et al.* (1955).

II. Mechanisms whereby the Intestinal Microflora Influences Vitamin Economy

1. Microbial Synthesis with Subsequent Absorption of Vitamins

There was a suggestion in 1915 by Theiler (quoted by Kon and Porter, 1954) that ruminants secure their water-soluble vitamins as a result of the synthesis by bacteria in the animal's digestive tract. Confirmation of this has been provided by a great deal of research (Kon and Porter, 1954). From a purely anatomical viewpoint it is readily conceivable that the vitamins synthesized by the microflora in the rumen can be made available to the host. A large proportion of the bacteria are likely to be disintegrated with the release of their nutrient content during the digestive process which occurs after the food leaves the rumen. The vitamins and other nutrients can then be absorbed in that part of the intestinal tract where such processes normally occur.

The nonruminant presents a markedly different picture. Although there are some bacteria normally present in the stomach and upper part of the intestine (Twiss and Hanssen, 1936; Weinstein, 1936), it is doubtful whether much synthetic activity goes on in those places. By far the largest number of bacteria and greatest bacterial activity occurs in the lower part of the intestinal tract and in such specialized organs as the cecum. It has been known since the studies of Cooper (1914) that the feces contain vitamins, but there is still considerable debate about whether vitamins synthesized by bacteria in the lower part of the intestinal tract are available to the host.

That the presence of an abnormal bacterial flora may augment the dietary vitamin supply was considered as early as 1918 by Pacini and Russell (1918). They were led to this hypothesis by their observation that clinicians "have long recognized that in certain infectious diseases growth is induced, apparently, by the infection. In typhoid fever, for example, the patient may grow from 1 to even several inches in height. Yeasts and bacteria are so low in the zoological scale of life as to be virtually on the same plane; and there is no evident error of logic in inferring that the growth incident to bacillary infections may be due to substances, elaborated by the bacteria, such as the substances known to produce growth, which are elaborated by yeasts."

Pacini and Russell (1918) showed that an acid-alcohol extract of

typhoid bacteria contained some vitamins. They fed this extract to rats that had consumed a diet devoid of vitamins and had reached a plateau in weight. The addition of the extract produced an immediate growth response. From this observation Pacini and Russell concluded that the typhoid bacilli synthesized vitamins that were required by the rat for growth. More recently it was shown that *Corynebacterium diphtheriae* can synthesize and secrete into the surrounding media pantothenic acid, pyridoxine, biotin, folic acid, and vitamin B_{12} (Jannes, 1953). There is no evidence other than the preceding which would permit a comparison of the nutritional benefits that might ensue from a bacterial invasion with the pathological alterations resulting therefrom.

There is evidence for the microbial synthesis and direct absorption in the rat of folic acid, biotin, and vitamin K. Deficiencies of these vitamins can be produced in the rat most readily by adding certain sulfonamides to the diet. When the other essential nutrients are present, rats grow normally in the absence of these vitamins. Under such conditions coprophagy presumably does not occur. It would seem possible to secure an unequivocal answer to the question of fecal consumption by rats fed diets deficient in folic acid, biotin, and vitamin K. Analyses of stomach contents for coproporphyrin should show whether any feces were present since this porphyrin is found in feces but not in foods (Schwartz *et al.*, 1951).

In relation to the other vitamins it is a question whether they are absorbed from the intestine after being synthesized by the bacteria therein. There is ample evidence that bacteria normally present in the intestine can synthesize a variety of vitamins (Najjar and Barrett, 1945; Peterson and Peterson, 1945), but the evidence for the direct absorption of these vitamins is still debatable.

In an effort to answer the above problem, Mitchell and Isbell (1942) studied the vitamins elaborated by bacteria in the cecum, and they found that most of the thiamine, riboflavin, and niacin in the ceca of rats is within the bacterial cells, but large amounts of pantothenic acid and folic acid diffuse into the surrounding medium. Biotin and pyridoxine move freely from cells to medium. On the basis of content of the various vitamins in the cecum and the intake-excretion ratio, these workers calculated that rats on a ground beef diet might have secured from the intestinal flora the following percentages of their requirements: thiamine 8–18, riboflavin 5–19, niacin 1.8–3.5, pantothenic acid 11–58, pyridoxine 130–230, biotin 230–420, and folic acid 67–71. Experiments such as these should be repeated using purified diets where the intake of the specific vitamin can be controlled and the fate of labeled vitamins may be studied. It is only when such studies are made that more rational values

will be secured for the contribution of the intestinal flora to vitamin economy. Mitchell and Isbell's values may be criticized, since their calculation includes all the pyridoxine that can come from intestinal synthesis. A pyridoxine deficiency can be produced in a rat fed a sucrose diet deficient in that vitamin.

Additional support for the idea that vitamins synthesized in the cecum are absorbed comes from the work of Luckey *et al.* (1955). They found that the concentration of thiamine in the livers of their normal (or conventional) birds was greater than that in their germ-free birds. The chicks in both groups were raised on a thiamine-free diet. The higher liver levels of thiamine in the normal birds were associated with higher cecal levels.

Presumptive evidence for the absorption of nutrients from the cecum and colon of guinea pigs was provided by Hagen and Robinson (1953). When they injected the sodium salts of acetic, propionic, butyric, or valeric acids into various parts of the isolated gastrointestinal tract, practically all the acid disappeared from the intestinal contents two hours after injection.

The application of results secured by *in vitro* studies to the problem of intestinal synthesis is fraught with a number of difficulties. *In vitro* studies have shown that the synthesis of vitamins by bacteria is influenced by the composition of the medium and especially by the metallic ion content (Koser, 1948). These studies may be further complicated by the difference in results secured under aerobic and anaerobic conditions as shown by the work of Benesch (1945). He found that when the cecal contents from a patient were incubated in a medium containing niacin the vitamin was destroyed under anaerobic conditions and synthesized under aerobic conditions. Finally, results obtained with a single bacterium may not be comparable to those obtained in studies of the normal intestinal tract. Suggestive proof for the latter comes from the observation of Phillips *et al.* (1955), who were unable to implant *Endamoeba hystolytica* in the intestinal tract of germ-free guinea pigs.

2. Microbial Synthesis without Subsequent Absorption of the Vitamin

The evidence indicating that vitamins synthesized in the gastrointestinal tract are not absorbed is not as good as that indicating that folic acid, biotin, and vitamin K are absorbed by the rat. The evidence for the nonavailability of such vitamins as thiamine, niacin, and pantothenic acid rests largely on the observation that these substances are excreted by animals which show signs of vitamin deficiency. Some evidence has come from the studies of changes in the urinary excretion of vitamins when the fecal concentration was changed. Another source

of evidence has been the absorption of vitamins following a retention enema.

The report of Najjar and Holt (1943) that thiamine and riboflavin were synthesized by the bacteria in the gastrointestinal tract of human subjects after they were absorbed, elicited a number of studies on the fecal excretion of the B vitamins (for review see Najjar and Barrett, 1945). Hathaway and Strom (1946) repeated Najjar and Holt's work with older adults. They fed the synthetic diet used by Najjar and Holt to three normal women for seven weeks. This was followed by a month on a diet of natural foods. The daily thiamine content of the synthetic diet was 1.0 mg. and that of the natural foods, 0.84 mg. In spite of the lower thiamine intake on the natural diet, the fecal excretion was much higher than on the synthetic diet. However, practically all of the increase in fecal thiamine on the natural diet occurred in the combined thiamine fraction (primarily cocarboxylase) which, according to Najjar and Barrett (1945), could not be absorbed from the lower part of the gastrointestinal tract. The urinary thiamine excretion in the women studied by Hathaway and Strom reflected the intake changes. For this reason they concluded that "fecal synthesis of thiamine was not an important factor in the thiamine economy of these three subjects."

A criticism of Najjar and Holt's work came from Alexander and Landwehr (1946), who found that when thiamine was given to one subject as a retention enema, there was no increase in the urinary excretion during the next 24 hours. They were able to recover all of the administered thiamine in the stool sample. On this basis they doubted whether the thiamine produced in the lower intestinal tract was available to the host.[5] That there was synthesis of thiamine in the intestine is apparent from their finding that over a period of seven days a normal young man excreted in his urine and stools almost as much of this vitamin as there was in his food. When allowance is made for the breakdown products, one of which (the pyrimidine fraction) they measured, the over-all balance required a considerable contribution of thiamine by the bacteria in the intestinal tract.

Additional studies, such as those by Denko and co-workers (1946a,b), indicate that synthesis of many of the vitamins probably occurs in the intestinal tract of man even when the diet is restricted therein, but they do not offer a means of determining whether these vitamins are absorbed.

[5] Prior to this Najjar and Holt (1943), and Najjar and Barrett (1945) reported that when 50 mg. of thiamine were given to two subjects by means of a high enema, all the vitamin was recovered in the urine. Such was not the case, however, when the same dose of cocarboxylase was given. It would seem possible to answer the question of absorption from the lower intestine by means of the tagged vitamin.

Perhaps an answer to the above problem will come from studies with germ-free animals. One report by Luckey *et al.* (1955) indicated that a more severe deficiency developed in germ-free birds fed diets deficient in niacin and riboflavin than in conventional chicks. Actually, the conventional birds on the low-niacin diet grew at a fairly good rate for three weeks (when the experiment ended), whereas the germ-free birds showed a weight plateau after 10 days. Both the germ-free and conventional birds died at the same time when placed on a thiamine-deficient diet. On the basis of these findings one might conclude that the conventional birds secured some niacin and riboflavin from the synthetic activity of the intestinal microflora. Such an explanation is open to question since, on the deficient diet, the cecal concentration of vitamins was almost as great in the germ-free as in the conventional birds.

From the standpoint of intestinal synthesis of vitamins, the high vitamin levels in the cecal contents of the germ-free birds raises the question: Do the vitamins in the gastrointestinal tract of the germ-free birds represent the unabsorbed dietary fraction? Luckey *et al.* (1955) suggest that "fecal vitamin excretion may represent a relatively less efficient absorption of dietary vitamins, . . . or an excretion of the vitamin from the tissues into the intestinal lumen . . . ." If this is so, it is unfortunate that they did not establish a balance sheet for the vitamins studied.

The work with germ-free animals (Reyniers *et al.*, 1950; Luckey *et al.*, 1955) indicates the need for caution in arriving at any conclusions about intestinal synthesis of vitamins. An additional complication was introduced into this area by the work of Emerson and Obermeyer (1945). They maintained their animals on a thiamine-deficient ration until there was a cessation of weight gain. The feces from half of the rats were fed by stomach tube to the other half of the group. After fecal analysis, the quantity fed was adjusted to provide 5 μg. of thiamine per rat per day. The feces fed did not affect the course of the deficiency. However, 5 μg. of crystalline thiamine given each day produced "spectacular weight gains." It is difficult to determine whether the nonavailability of the fecal thiamine is a consequence of the deficiency present in the recipient animals or the nature of the feces from the donors. Since the feces from refected animals were able to cure a thiamine deficiency (Guerrant *et al.*, 1937) and since Hayashi (1951) reported that rats on a thiamine-deficient diet increased their urinary excretion of this vitamin when they were fed *L. bifidus*, the work of Emerson and Obermeyer should be extended in order to evaluate the factors that may influence the nature and availability of the fecal vitamins.

In view of the above, we may appear to be throwing caution to the

winds by saying that if thiamine, niacin, riboflavin, pyridoxine, pantothenic acid, vitamin B_{12}, and vitamin A are absorbed after intestinal synthesis in the rat fed a purified-type diet, the extent to which this occurs is limited. The primary basis for the above statement is that deficiency of any one of these vitamins can be produced by feeding a diet low in that vitamin.

3. Destruction of Vitamins by the Microflora

Although it had been assumed that intestinal bacteria destroyed certain vitamins, it was not until the work of Abdel-Salaam and Leong (1938) that evidence therefor was secured. They found that when the cecal contents of normal rats were incubated, there was an increase in the thiamine content during the first 24 hours. However, on longer incubation the thiamine content decreased. The latter effect they attributed to bacterial action.

There have been a number of reports from Japan on the isolation of thiamine-splitting bacteria from human patients. According to Fujita *et al.* (1952a), Chang in 1948 observed that the feces from a patient with signs of thiamine deficiency contained a thiaminase.[6] In the following year Matsukawa and Misawa (Fujita *et al.*, 1952a) isolated from the feces of a patient with diarrhea an organism that had thiaminase activity. The organism appeared to be a new species and was named *Bacillus thiaminolyticus*. Kimura *et al.* (1952) have described the cultural and immunological characteristics of this organism. The enzyme present has been studied by Fujita *et al.* (1952b,c) and Fujita and Tashiero (1952).

Since the above work two other microorganisms have been isolated from human feces by Japanese investigators. In 1953 Aoyama described a *Bacillus aneurinolyticus* which contains a thiaminase that apparently is different from that present in *B. thiaminolyticus*, especially in its response to activators. *B. aneurinolyticus* still contains 3 to 4 μg. of thiamine per gram of wet cells even when the thiaminase activity is at its peak. Thiaminase produced by this organism is present both within and without the cells (Fujita *et al.*, 1953). Another organism with a similar action on thiamine is *Clostridium thiaminolyticum* described by Liao (1953). This organism differs from the two previous ones in that it is anaerobic, produces gas, and does not contain any catalase.

Preliminary evidence indicates that the bacteria which contain enzymes that split thiamine may be of public health importance in

[6] The thiaminase present in fish is not necessarily of bacterial origin even though the visceral contents are a potent source. The enzyme is widely distributed in fish organs (for review see Harris, 1951).

Japan. Partial confirmation of this comes from work with experimental animals, and the rest from clinical reports.

Matsukawa *et al.* (1955) reported that when they fed a culture of *B. thiaminolyticus* to hens, symptoms of polyneuritis developed within 40 days. As soon as the bacterium could be detected in the feces, the thiamine content thereof started to go down. On autopsy they found the thiamine content of the organs of the birds fed the bacterial suspension much lower than in the controls receiving the same diet without the bacteria. They were able to detect the enzyme from the midpart of the ileum on down, with by far the highest concentration in the cecum. Since the diet provided the hens with 150 μg. of thiamine per day (an adequate intake) and since the thiamine-splitting bacteria were present only in the lower part of the intestine, these findings suggest that the intestinal flora may have a more dramatic effect, at least under special conditions, on vitamin metabolism than has heretofore been considered possible.

The above observations become more important in view of the report by Hamada (1953) that about 3% of the people in a large city such as Kobe have *B. thiaminolyticus* in their intestinal tracts. A high proportion of these people exhibit signs of thiamine deficiency. The presence of these bacteria was associated with a proteolytic type of flora. When a lactic acid bacterial culture was fed *B. thiaminolyticus* was eliminated from the feces and the stool remained that way for at least a month after feeding of the lactic culture was stopped. The author said nothing about the effect of this procedure on the symptoms of polyneuritis.

It has been recognized for many years that certain bacteria isolated from the intestinal tract can destroy ascorbic acid. In a review of this subject, Eddy and Ingram (1953) refer to the work of Stepp and Schröder (1935) which showed that when ascorbic acid was incubated with suspensions of bacteria commonly found in the intestinal tract, one out of eight strains of *B. coli* and one strain of *B. paratyphosus* produced a rapid disappearance of the vitamin.

Folic acid and its derivatives may also be destroyed by bacteria commonly found in the intestinal tract. This was originally observed by Stokes and Larsen (1945), who found a 60 to 80% destruction of folic acid by *Streptococcus lactis* R cells. Results similar to these have been reported by Woods (1954) for *Str. fecalis* bacteria. The folic acid is cleaved between C_9 and N_{10} with the formation of *p*-aminobenzoylglutamic acid and a mixture of free pteridines (Webb, 1955).

The work of Kendall and Chinn (1938) showed that by suitable techniques a variety of bacteria which decompose ascorbic acid could be isolated from stomach contents or feces of patients with achlorhydria.

The most active organisms in this respect were members of the mucosus capsulatus and enterococcus groups. These bacteria apparently carry the destruction of vitamin C beyond the dehydroascorbic acid stage since treatment with hydrogen sulfide did not restore the vitamin. They also showed that other strains of *B. mucosus capsulatus*, *B. alcaligenes*, and the Flexner bacillus protected the vitamin from destruction. The "protective" effect did not overcome or reduce the rate of destruction by the *B. mucosus* and enterococcus groups.

Young and James (1942) and Young and Rettger (1943) have reviewed the clinical reports which suggest that a deficiency of ascorbic acid might be associated with the destruction of the vitamin by the microbial flora. The work of Young and James (1942) confirmed that of Kendall and Chinn (1938) and showed that the decomposition of ascorbic acid occurred under both anaerobic and aerobic conditions. The decomposition was dependent upon intact cells, with the rate of decomposition being greatest when the cells were most viable. The organisms are of only minor importance in producing a vitamin deficiency, since *in vitro* studies showed that they attacked the vitamin only after the glucose had disappeared from the medium (Young and James, 1942).

On the basis of the available evidence it appears that the destruction of vitamins by bacteria in the gastrointestinal tract may be of more than just theoretical interest. It is hoped that the reports of the Japanese workers with the bacteria isolated from the feces of patients with signs of polyneuritis will soon be extended to include a clinical evaluation of the influence of the bacteria on the course of a dietary thiamine deficiency.

4. Absorption of Nutrients by the Microflora

It has been recognized since 1933 that thiamine in fresh yeast is not so available to rats as that in dried yeast (Walker and Nelson, 1933). A partial explanation for the nonavailability of the vitamin in viable yeast was the observation that from 55 to 95% of the yeast cells passed through the stomach of rats in an intact condition (Montgomery *et al.*, 1931). The thiamine in the yeast could be made available to normal adults by boiling the yeast just prior to feeding; the boiling destroyed the yeast cells (Parsons and Collord, 1942). The thiamine which was not absorbed during the period of yeast feeding could be recovered in the stools. These workers reported that live yeast could be recovered in the stools three days after the fresh yeast was added to the diet.

A continuation of the yeast work (Ness *et al.*, 1946) showed that when as little as 15 g. of certain types of live yeast were added to a diet to provide the normal adult with a constant thiamine intake, the yeast actually caused a depletion of the body's stores of thiamine as reflected by the

urinary excretion levels. Even when the yeast was added to the diet for only one day, the low urinary thiamine levels persisted for several days thereafter (Parsons *et al.*, 1947). Additional evidence for the above has been presented by Kingsley and Parsons (1947) and Parsons *et al.* (1945b). Comparable results were secured when these studies were extended to rats (Parsons *et al.*, 1945a).

The poor availability of thiamine in live yeast was confirmed by the work of Kline and Nelson (1955), who fed three human subjects in one day five yeast cakes which contained, according to the label, 2.5 mg. of thiamine. The bioassay technique of Oser *et al.* (1945), as modified by Kline (1947), showed that only 19% of the thiamine in the yeast was available. In another experiment in which 10 yeast cakes were fed to six subjects, the availability of the thiamine was 15.6%.

Not only does fresh yeast interfere with the absorption of thiamine, but it also withholds its riboflavin (Price *et al.*, 1947). Again there was a reciprocal relation between the urinary and fecal excretions indicating that the riboflavin that was not absorbed could be recovered in the feces.

It is surprising that the above observations were not considered when the refected state in the rat was studied. Guerrant *et al.* (1935) suggested that the poorly digested carbohydrates which produced refection in rats did so as a result of stimulating the growth of yeast in the cecum. If, as is likely, this yeast remained viable through most of the remaining intestinal tract, it is difficult to understand how the rat could absorb any of the B vitamins contained therein. The work of Parsons *et al.* (1945a) indicates that even when the rat consumed its feces, the vitamins in the yeast should not have been available. Further work should be done to identify the organisms in the feces of refected rats which are contributing the B vitamins. The work of Parsons *et al.* (1947) might well be extended to rats fed yeast over a long period of time in an effort to see whether the body stores of the animal are actually reduced.

Another type of thiamine absorption by the microflora is that studied by Citron and Knox (1954). They isolated a strain of *Staphylococcus aureus* from the gastric contents of a man who had severe polyneuritis. Studies with the organism *in vitro* showed that in the presence of glucose it removed 75% of the thiamine in the medium within one and one-half hours. The rate of removal of thiamine was very much reduced when there was no glucose. The thiamine was not destroyed by the organism, since practically all the thiamine could be recovered when the bacterial cells were destroyed. At first glance it appears difficult to see how this organism could be of etiological importance in polyneuritis. The work of Cregan *et al.* (1953) indicates that even in cases of achlorhydria the upper intestinal tract contains practically no viable organisms. They

postulated an antibacterial mechanism in the small intestine which was independent of the gastric germicidal barrier. The staphylococcus was isolated by Citron and Knox (1954) from the stomach and presumably would have been destroyed by the time it had reached the small intestine. Under these circumstances the thiamine should be available for absorption. Cregan *et al.* (1953) criticized the claim of other clinicians (Welbourn *et al.*, 1951) that the signs of vitamin B deficiency seen in cases of gastrectomy or perhaps also in sprue, pernicious anemia, and nutritional megaloblastic anemia (Frazer, 1949) were due to absorption or destruction of vitamins by bacteria in the small intestine. The conclusion of Cregan *et al.* cannot be accepted unreservedly, since de la Huerga and Popper (1952) claim that in patients with liver disease there are large numbers of bacteria in the intestinal juice removed from the duodenum by means of a Miller-Abbott tube. It appears impossible to arrive at any decision on the relative density of bacteria in the upper part of the intestinal tract partly because the small number of papers on this subject report conflicting findings.

Certain bacteria, such as *E. coli* (Davis and Mingioli, 1950) and *L. casei* (Hoff-Jorgensen, 1952), are known to absorb large amounts of vitamin B_{12}. For a time it was thought that some of these organisms were of importance in reducing the amount of vitamin B_{12} available to pernicious anemia patients (Levanto, 1955; Bethell, 1954a). Work with antibiotics (Schilling, 1953; Ungley, 1950), which presumably reduced the number of bacteria in the upper part of the intestinal tract, led Schilling to state that "no evidence was found to suggest that intestinal bacteria were preventing vitamin B_{12} absorption."

There appears to be one situation where the absorption of vitamin B_{12} by the intestinal flora has been observed. The megaloblastic anemia associated with fish tapeworm has been reported to be the result of true vitamin B_{12} deficiency. Von Bonsdorff (1948) reviewed the earlier work in this field and indicated that from $\frac{1}{8}$ to $\frac{1}{4}$ of the people in Finland were carriers of the tapeworm. The frequency of anemia among them, however, is relatively small. In patients who had anemia the tapeworm was located high in the intestinal tract. Von Bonsdorff and Gordin (1951) showed that feeding vitamin B_{12} to some of the tapeworm patients who had anemia improved their blood picture. In these cases the tapeworm did not interfere with the intrinsic factor elaborated by the gastric mucosa.

The tapeworm is able to absorb large amounts of vitamin B_{12} from the intestinal tract (von Bonsdorff and Gordin, 1953). When dried and fed to addisonian pernicious anemia patients, it serves as a good source of vitamin B_{12}. These patients were given normal human gastric juice

as a source of the intrinsic factor. The tapeworm alone had no hematopoietic effect (von Bonsdorff and Gordin, 1952). On the basis of the studies with pernicious anemia patients these workers concluded that 5 to 10 g. of dried tapeworm contained 5 μg. of vitamin B_{12}. They also found that the tapeworm is not able to absorb vitamin B_{12} to any appreciable extent in *in vitro* studies.

There is a suggestion that *Endamoeba histolytica*, when present in the intestinal tract, may absorb riboflavin to produce deficiency signs. A patient whose stools showed *E. histolytica* had ulcerations at the angles of the mouth and depigmentation of lips (Mukherjee and Chakraborty, 1955). These signs did not respond to oral administration of riboflavin and vitamin B complex, but did respond to intramuscular injections of liver extract containing large amounts of B vitamins. This claim must be accepted with reservations, since the oral lesions are nonspecific (Cayer *et al.*, 1945; Finnerud, 1944) and if the lesions were actually due to a vitamin deficiency, then there would be only a small chance that they were due to the *E. histolytica*, since these organisms are found primarily in the large intestine (Faust, 1954) where presumably only small amounts of dietary riboflavin should be present.

III. Site of Intestinal Synthesis

The work on the nutritional effects of sulfonamides focused attention on the lower part of the intestinal tract as the site of bacterial synthesis of vitamins. The more insoluble sulfonamides were most effective in producing vitamin deficiencies. These insoluble compounds, it was reasoned, were the only ones which reached the lower part of the intestinal tract where bacterial synthesis was most prominent. Earlier, in conjunction with the work on refection, a number of attempts had been made to determine the site of vitamin synthesis. Since the ceca of the refected rats were markedly increased in size, a number of studies were made of cecectomized animals.

Perhaps the first ones to use cecectomized rats for this purpose were Guerrant *et al.* (1935). They observed that the ceca of rats fed dextrinized cornstarch rations were much larger than those of animals fed sucrose rations. Since it was only the cornstarch-fed rats that became refected, they reasoned that the enlarged cecum was associated with the condition. To test their hypothesis, they removed the ceca of two rats; one animal survived. After it had recovered from the operation, it was fed the vitamin B-free, cornstarch ration. Even though feces from refected rats were added to the ration, the rat lost weight. The only time the rat gained weight was when the ration was supplemented with yeast. Furthermore,

the feces from the cecectomized rats contained much less vitamin B than the feces of normal rats maintained on the same cornstarch diet. The pictures of the gastrointestinal tracts of their animals indicated that a compensatory increase had occurred in the diameter of the large intestine and colon of the cecectomized rat. If this was a mechanism to compensate for the removal of the cecum, then in that rat it did not permit the synthesis of the B vitamins normally occurring in refected rats.

Griffith (1935) cecectomized over 200 rats but was primarily interested in determining "whether the rat regularly received significant amounts of vitamin G from the intact cecum." On a stock diet the cecectomized rats grew at the same rate as unoperated controls and showed no outward signs of impairment. Griffith's rats did not become refected when put on a cornstarch diet that was deficient primarily in thiamine. On this diet both the cecectomized and normal rats developed polyneuritis at about the same time. That the feces of the cecectomized rats did contain some vitamin G, which was presumably synthesized in the gastrointestinal tract, was shown by the improvement in growth when rats on the vitamin G-low ration were permitted access to their feces. Griffith called attention to the fact that "following cecectomy there was a marked dilatation of the first portion of the colon with a thinning and outpouching of the wall. The fecal contents were fluid and of the consistency of the usual cecal material. The semi-solid fecal masses were formed farther down in the distal portion of the colon than is commonly the case."

Work along the above line was done by Taylor *et al.* (1942), who found that cecectomy had no influence on the rate of growth of rats on a pyridoxine-free diet. They also noted that cecectomized rats grew and developed normally when fed a stock ration (Purina). However, there was a difference between the cecectomized and normal rats when the stock diet was diluted with a mixture of sucrose, casein, and salts. When fed the latter diet the cecectomized rats did not gain weight, but the normal rats did. The feces of the cecectomized rats had a lower concentration of folic acid, pyridoxine, and pantothenic acid and a higher concentration of thiamine than the normals. These observations confirm the previous suggestions that the cecum is probably the primary site of bacterial vitamin synthesis.

Schweigert *et al.* (1945a) found evidence that on high sucrose diets normal rats secured no thiamine, riboflavin, pantothenic acid, or pyridoxine from bacterial synthesis in the intestinal tract. Their conclusions were based on the fact that cecectomized and normal rats showed the same response to the deficient diets. These studies confirmed the observa-

tions of Taylor *et al.* (1942) on the nonabsorption of pyridoxine synthesized in the intestine when rats are fed a sucrose diet. Schweigert and co-workers (1945a) fed some of their rats diets containing lactose as the only carbohydrate. When this was done the riboflavin excretion in the urine and feces was twice the intake. Cecectomy reduced the riboflavin excretion to the same extent as did the addition of sulfasuxidine to the lactose diet of normal rats (a 30% reduction). The addition of sulfasuxidine to the lactose diet of cecectomized rats did not influence the riboflavin excretion. Essentially similar results were secured with the dextrin diet. It is difficult to determine whether any of these changes are significant, since only averages are given. Assuming the changes to be significant the results suggest that riboflavin synthesis occurred in the cecum.

The studies of Schweigert *et al.* are in line with the earlier work of Morgan *et al.* (1938), who found that rats on a lactose diet did not require any dietary source of riboflavin or pyridoxine. Here again was presumptive evidence for the intestinal synthesis of riboflavin and pyridoxine.

Cecectomized rats were used by Day *et al.* (1943) for the study of vitamin K synthesis. When these rats were fed a vitamin K-deficient diet only 20% of the animals showed elevated prothrombin times. It was only when 1% sulfasuxidine was added to the ration that there was a high incidence of hypoprothrombinemia. Normal animals fed the sulfasuxidine-supplemented ration showed only a low incidence of hypoprothrombinemia. These findings indicated that the cecum is of some importance in the synthesis of vitamin K in the rat but, since only a small percentage of the cecectomized rats on the basal vitamin K-deficient diet showed signs of vitamin deficiency, the vitamin must have been formed in other parts of the intestinal tract and then absorbed. The synthetic activity may have been transferred to the outpouching of the colon described by Griffith (1935) as a compensatory mechanism occurring in cecectomized rats.

Another point brought out by the above work is the variability in the response of rats to a vitamin K deficiency. A similar variability was observed by Greaves (1939) among normal rats maintained on a vitamin K-deficient ration. In the latter work 12 of 77 rats showed elevated blood clotting time.

The work of Greaves added confirmation to the suggestion of vitamin K synthesis in the intestinal tract. An ether extract of feces, collected from rats with bile fistulas into the colon, or normal rats maintained for as long as 180 days on vitamin K-free diets, completely protected chicks fed a vitamin K-free diet. The bile fistula animals actually excreted significant amounts of vitamin K when they were exhibiting a bleeding

tendency which could be cured with a vitamin K concentrate. These rats developed hemorrhagic tendencies within a week after the operation. On this basis Greaves concluded that the vitamin K synthesized in the intestinal tract is probably absorbed through the walls of the cecum.[7]

Kornberg *et al.* (1944) showed that rats made hypoprothrombinemic by a diet supplemented with sulfadiazine had very little if any vitamin K in their cecal contents or feces. The action of subcutaneously administered PABA in overcoming the effect of the sulfonamide was shown by them to be explainable on the basis of its presence in the cecum.

The above studies indicate that the cecum is an important site for the synthesis of a number of vitamins (especially folic acid, biotin, and vitamin K) in normal rats. In the refected rat the cecum is probably the primary site for the synthesis of most of the B vitamins. The increased synthesis of various vitamins produced by a change in carbohydrates may also be mediated through the cecum. This conclusion is based primarily on the enlargement and changes in flora of the cecum associated with the different carbohydrates (Johannson and Sarles, 1949).

It is difficult to evaluate the role played by other parts of the lower intestinal tract in vitamin synthesis, since cecectomy results in a compensatory enlargement of the areas distal and proximal to the site of the excised cecum. Under such circumstances, these areas may take over some of the functions normally occurring in the cecum. Similar limitations would no doubt hold true for any attempt to use individuals with ileostomies or resections of the large intestine.

Much less work has been done with cecectomized poultry. Sunde *et al.* (1950), like others (Mayhew, 1934), found that cecectomized birds could develop and lay eggs normally when fed a commercial ration. As a matter of fact, the cecectomized birds produced eggs that had a higher biotin content than the eggs from normal birds, as determined by hatchability studies and biotin assays. The same results were secured on a purified diet containing sucrose or dextrin and on a commercial ration. The explanation proposed for this unexpected finding was that cecectomy removed a large number of yeasts from the intestinal tract and thus reduced the amount of biotin that was absorbed by these organisms. Unfortunately no autopsy observations were made to determine whether the yeast population was actually reduced.

The above work was confirmed by Couch *et al.* (1950), who also

[7] Mitchell and Isbell (1942), on the basis of the amounts of vitamin extractable from the cecal walls compared with those in an aqueous extract of cecal contents, claimed that B vitamins could be readily absorbed by that organ. They did not study vitamin K.

showed that the feces from cecectomized birds contained the same concentration of riboflavin, niacin, pantothenic acid, biotin, and folic acid as the feces from normal birds. These results suggest that some part of the intestinal tract in the cecectomized birds may have taken over the functions of the cecum. Since the ceca in fowl are proportionately much longer than in rats, it would be highly desirable to study cecectomized birds to determine the extent of the adaptation that has occurred in the lower intestinal tract.

Additional evidence for the importance of the cecum as the primary site of intestinal synthesis comes from comparing the vitamin contents of intestinal juice secured from different parts of the digestive tract. McGregor *et al.* (1947) found that the biotin concentration in the cecum and large intestine of the rat was greater than in the stomach or small intestine. On a stock diet, the increase was twofold; on a cooked egg-white ration it was somewhat higher; the concentration factor was still greater (3 to 10 times) on the raw egg-white ration.

Couch *et al.* (1950) found that the concentration of riboflavin, niacin, pantothenic acid, folic acid, and biotin was greater in the cecum of chicks than in the remainder of the intestinal tract. The cecum of the chicken also has a higher concentration of vitamin B_{12} than the intestinal contents (Simonnet *et al.*, 1953).

In the pig, Barber *et al.* (1953) found a higher concentration of vitamin B_{12} in the cecal contents than in the rest of the intestine.

The work of Huang *et al.* (1954) showed that in the rabbit absorption of nutrients in the cecum and large intestine represents a considerable portion of the total absorptive mechanism. They used chromic oxide as a reference and on this basis found that the cecum "utilizes" most of the fat and crude fiber while the large intestine absorbs most of the protein. Their data also showed that the vitamin B_{12} increased, on a dry weight basis, from 11 mμg. per g. of material in the small intestine to 4,944 mμg. per g. of cecal contents. The concentration of this vitamin decreased in the large intestine.

From the present evidence it would appear as though a variety of vitamins can be synthesized in the cecum and that some of these are subsequently absorbed from the large intestine. Alterations in the microbial flora of the intestinal tract may favor an increase of those organisms which produce the water-soluble vitamins in diffusible form, thus permitting them to be absorbed by the host. Other circumstances may favor the development of organisms which require the water-soluble vitamins, thus producing a competitive situation in which the host comes out second best.

IV. Influence of Intestinal Microflora on Vitamins

1. Thiamine

It is commonly conceded that animal cells cannot synthesize thiamine. All of the thiamine an animal uses for its metabolic activity must come, under normal circumstances, by absorption from its intestinal tract. This holds true for most of the other vitamins to be considered in this review. The evidence will be reviewed which bears on the intestinal synthesis of the vitamin. The primary problem is to evaluate the extent to which the vitamins synthesized in the digestive tract are absorbed by the host.

a. Excretion Studies—Animals. The evidence that first comes to mind for the synthesis and absorption of thiamine from the gastrointestinal tract is that of refected rats (see Section I, part 2). The diets fed these rats contained rice starch or potato starch. Although no thiamine values for these starches have been published, it is likely that they are comparable to cornstarch which has no detectable thiamine (Anderson *et al.*, 1946). In spite of the absence of thiamine from the diet, the refected rats were able to grow. There is still some question whether the rats secured the thiamine after ingesting their feces. There are enough reports, however, to support the idea that some rats absorb some of the thiamine synthesized in the intestine.

b. Excretion Studies—Man. When thiamine balance studies are carried out with human subjects there is some variation in the results observed. The sum of the urinary and fecal thiamine frequently has been less than the dietary intake where the intake of the vitamin was above the minimal requirement. This was true in the experiment of Hathaway and Strom (1946), where the thiamine balance was negative for all three women maintained on a purified diet (thiamine intake = 1.0 mg. per day, which is close to the requirement) for seven weeks and then for a month on a natural diet (thiamine intake = 0.84 mg.). The work of Denko *et al.* (1946a) indicated that for seven men who were maintained on an intake of 1.44 mg. per day, the sum of the urinary and fecal excretion ranged from 29 to 126% of the intake with an average of 78%. When these men were put on a diet containing 0.53 mg. of thiamine, the sum of the urinary and fecal thiamine was practically the same as the intake. Results similar to these were obtained with women by Oldham *et al.* (1946). When one makes allowance for the pyrimidine component of thiamine, the total excretion in the above experiments would be considerably greater than the intake (Mickelsen *et al.*, 1946, 1947).

The work that is most frequently thought of in this connection is that of Najjar and Holt (for review see Najjar and Barrett, 1945). These

investigators observed that the sum of the urinary and fecal excretions was greater than the intake, especially when the intake was very low. They maintained one young adult on a purified diet providing 187 μg. of thiamine per day. At the end of three months when the experiment was terminated the subject was still "vigorous and healthy in every respect." This subject maintained a high level of free thiamine excretion in the feces. The other eight subjects in the study developed deficiency signs when the free thiamine in their feces fell below 30 μg. per day. For this reason Najjar and Holt maintained that adults can supplement their dietary thiamine with that which is synthesized in the intestine. However, only the free thiamine, according to these workers, is available (see Section III for discussion of thiamine absorption following retention enemas).

The fecal excretion of thiamine appeared to be increased in normal young women when the fiber content of the diet was raised (Williamson and Parsons, 1945). There is considerable individual variation in the fecal thiamine excretion (Mickelsen *et al.*, 1946) and some indication that on a constant thiamine intake the variation may be an individual characteristic.

A determination of the significance of fecal thiamine excretion is complicated by the report that the feces of germ-free chicks contain fairly large amounts of thiamine (Luckey *et al.*, 1955) and that feces of newborn infants contain 44 μg. of thiamine per 100 g. (Horikawa, 1951). The preceding work suggests that the thiamine in the feces comes not from bacterial synthesis but from one of the following sources: (1) Incomplete absorption of dietary sources [support for this comes from the work of Kirk and Chieffi (1951), who found that when either young or old adults were given 5-mg. supplements of thiamine the fecal excretion increased enough to account for one-half to three-fourths of the dose and, when the supplements were discontinued, the fecal excretions returned to their original values] and (2) body stores of the vitamin excreted into the intestinal tract, which is not too probable since work with radioisotopically labeled thiamine suggested that a negligible amount appeared in the feces when the injected dose was small (McCarthy *et al.*, 1954); the fecal excretion was, however, of some importance when large doses of radioisotopically labeled thiamine were given (Borsook *et al.*, 1940). Unfortunately, the work on the fecal excretion of the isotopically labeled thiamine in both of the above studies was limited to a measurement of radioactivity.

c. Influence of Sulfonamides on Thiamine Synthesis. The work of Slater (1946) and Kratzing and Slater (1950) indicated that sulfadiazine and sulfamerazine spare the dietary requirement of both man and rats

for thiamine. The original work reported an increase in urinary thiamine in a young man from 136 μg. per day to 344 μg. on the fifth day after a five-day period of sulfadiazine supplementation (2 to 5 g. per day). During this time the subject was maintained on a constant diet providing 0.57 mg. of thiamine per day.[8] They extended their work to rats maintained on a thiamine-low diet (6.0 μg. rat/day as a separate supplement) containing all of the other vitamins in adequate amounts (Kratzing and Slater, 1950). The rats fed this diet plus 0.55% sulfadiazine showed an increase in the thiamine content of the carcass and liver of about one and one-half times that of the controls. The increase in thiamine content of the sulfadiazine rats occurred even though the growth rates of both groups had been kept the same through controlled feeding. Sulfamerazine, another fairly soluble drug, produced similar results, while sulfanilamide, sulfapyridine, sulfathiazole, succinylsulfathiazole, and 2-amino-4 methylpyrimidine (a part of the thiamine molecule) were inactive. Since the two sulfonamides that increased thiamine levels in the rat also produced a tripling in the weight of the thyroid gland, Kratzing and Slater suggested that the metabolic rate was reduced by the sulfonamides and with that went a decreased requirement for thiamine.

The above work, which appears to have been overlooked, should be repeated and extended. There is a possibility that sulfadiazine and sulfamerazine reduce the requirement of the rat for thiamine but it is not too likely that the mechanism involves a reduction of the basal metabolic rate. Sulfonamides have been reported to have no effect on the basal metabolism of rats or rabbits (Long and Bliss, 1939). The only work along this line was done with rats on a pyridoxine-deficient diet. The results indicated that thiouracil not only depressed the metabolic rate but also decreased the growth rate below that of the controls (Beaton *et al.*, 1953).

A thiamine-sparing effect for sulfasuxidine was reported by Lih and Baumann (1951) on the basis of increased growth of rats when the sulfonamide was added to a thiamine-low diet.

Even though Kratzing and Slater (1950) found the injected sulfonamide to be as active as that fed, this observation cannot be used as an argument for excluding their action on the intestinal tract. These compounds are relatively soluble and would very likely appear in the intestines after subcutaneous injection. Kornberg *et al.* (1944) showed that the cecal concentration of sulfadiazine was as high when the sulfonamide

[8] It should be noted that Sarett (1952) observed no change in urinary excretion of thiamine following oral supplementation of 6 subjects, each with 1 g. of streptomycin per day for 8 to 22 days; this in spite of the fact that Lih and Baumann (1951) showed streptomycin to be almost as potent as penicillin in sparing the rat's requirement for thiamine.

was injected subcutaneously as when it was fed in the diet. The injected rats received a dose which was equal to that of the rats on the dietary supplement. Additional evidence for the suggestion that the sulfonamide affected the bacteria in the intestinal tract comes from the work of Tittler and Bovell (1954), who found that of a large number of sulfonamides, sulfadiazine was the only one that reduced the requirement of *Tetrahymena feleii* for thiamine when added to the medium.

That sulfasuxidine may influence the rat's requirement for thiamine as indicated by urinary excretion values was shown by Schweigert *et al.* (1945a). When they added 0.75% sulfasuxidine to the diets of rats maintained on a constant thiamine intake, the sulfonamide-supplemented rats excreted as much thiamine as the unsupplemented controls. This was true whether the dietary carbohydrate was lactose, sucrose, or dextrin. They also observed that rats on the lactose diet excreted large amounts of thiamine; with sucrose or dextrin as the dietary carbohydrate, the excretion values were equal but about one-half those on the lactose diet. Removal of the cecum reduced the thiamine excretion only in the lactose-fed rats, and there the excretion level was brought down to that of the sucrose- and dextrin-fed rats.

There is one report (Grundy *et al.*, 1947) which indicates that feeding 4 g. phthalylsulfathiazole per day to five normal young men for periods of 8 to 17 days had no influence on urinary thiamine excretion. These subjects were maintained on a constant vitamin intake prior to and during the study. Certain sulfonamides may reduce the rat's requirement for thiamine. The work of Kratzing and Slater is highly suggestive that sulfadiazine and sulfamerazine have such actions.

d. Influence of Antibiotics on Thiamine Synthesis. It has been shown that increased growth results from the addition of small amounts of antibiotics to a diet deficient in thiamine. Both rats and chicks show this response. Lih and Baumann (1951) showed that when 5 mg. % of penicillin was added to a purified diet containing 25 μg. % thiamine, the rats weighed one and one-half times as much as their controls at five weeks. The weight gain produced by penicillin supplementation was the same as that secured on the diet containing 100 μg% thiamine. The growth response with penicillin was far greater than that with streptomycin, sulfasuxidine (1%), or Aureomycin (in decreasing order of activity); terramycin and chloromycetin were inactive. No growth stimulation was observed when antibiotics were added to diets containing adequate thiamine.

Stokstad (1954a) pointed out "that a simple growth response to an antibiotic on a diet marginal in a certain vitamin does not establish a vitamin sparing effect. The only true criterion is a reduction in the vitamin

requirement as measured by a dosage response curve, or a greater response to an antibiotic on a suboptimum than on an optimum level of the vitamin." Using the second of these criteria, Lih and Baumann (1951) have established a thiamine-sparing effect for certain antibiotics.

Results similar to the above were secured by Sauberlich (1952), who also noted that the effect of the antibiotic was greater on a dextrin-containing diet than on sucrose. Guggenheim *et al.* (1953) went on to show that when the antibiotic supplement increased the weight gain of rats on the thiamine deficient diet, there was no change in the fecal excretion of the vitamin. The total amount of thiamine in the liver was related to a certain extent to the growth-promoting action of the antibiotic. Results similar to these were secured by Balakrishnan *et al.* (1954).

Schendel and Johnson (1954) found that the growth-stimulating effect of Aureomycin was apparent in rats fed a thiamine-low diet, whether the supplements were given orally or subcutaneously. The above observations were extended by Jones and Baumann (1955), who attempted to produce the same growth as with the oral supplement by regulating the injected dose of thiamine. When the diets of half of each group were supplemented with an antibiotic, a greater growth response occurred in the group receiving the vitamin orally. Under these circumstances penicillin was more active than Aureomycin, with streptomycin relatively inactive. Jones and Baumann stated that "although the antibiotics stimulated growth even when there was no thiamine in the small intestine to be diverted to the microorganisms, the antibiotics were less effective than when some thiamine was present in the digestive tract from the diet."

The work of Waibel *et al.* (1953) showed that when penicillin was added to the diet of the chick it exerted a thiamine-sparing effect similar to that in the rat. Penicillin was more active in this respect than Aureomycin. The chick differs from the rat, however, in that the thiamine-sparing effect was not altered by the type of carbohydrate in the diet.

Coates *et al.* (1951) reported that chicks on a thiamine-free diet showed no improvement in growth or survival when penicillin was added to the diet. This in itself is not necessarily proof that the antibiotic had no sparing effect, since a number of reports indicate that the antibiotic may have no effect in the absence of a particular vitamin, but shows a sparing effect in the presence of suboptimal amounts (Lih and Baumann, 1951).

e. Influence of Diet on Thiamine Synthesis. Considerable work has been done by Balakrishnan and Rajagopalan (1952) to show that when a thiamine-deficient diet, be it purified or composed of foods commonly eaten in southern India, was supplemented with milk curds, there ap-

peared to be an increase in intestinal synthesis of thiamine as shown by increased weight gain and increased urinary and fecal thiamine excretions. Further studies showed that sulfaguanidine added to the diet reduced thiamine excretion in both feces and urine, but the effect was less when curds were present in the diet (Balakrishnan and Rajagopalan, 1953). The effect of the sulfonamide could be overcome by PABA (Balakrishnan and Rajagopalan, 1954).

It has been reported that the addition of large amounts of ascorbic acid to a purified diet reduces the rat's requirement for certain B vitamins (McDaniel and Daft, 1954). Actually, 5% ascorbic acid added to a thiamine-free diet prevented or delayed the signs of deficiency (Daft and Schwarz, 1952). It is not known whether the ascorbic acid produces its effect through a change in the intestinal contents. This is a possibility since Katznelson (1947) showed that ascorbic acid reduced the requirement of *B. paraalevi* for thiamine. Furthermore, Fang and Butts (1953) found that ascorbic acid not only spared the requirement of *Lactobacillus fermenti-36* for thiamine, but it also permitted the organism to synthesize the vitamin.

In a series of papers Satoh (1952) has claimed that the addition of as little as 5 mg. of onion oil or garlic oil to the diet of a healthy adult provided with 1.7 to 1.9 mg. of thiamine per day, increased both urinary and fecal thiamine. The oral administration of 10 mg. of the oil prevented or cured beriberi. Results similar to the above were seen in dogs and mice. The material responsible for the activity was thought to be a volatile organic sulfide, the activity of which was destroyed on heating. Should this work be confirmed, it would be highly important to determine the nature of the substance responsible for the effect. The abstract implies that a few slices of onion are able to spare the thiamine requirement. Under these circumstances it is not likely that the thiamine in the onions is producing the effect since 100 g. of onions (a large serving) contain only 30 μg. of thiamine (U.S. Dept. Agr. Handbook No. 8, 1950).

f. General. The present evidence indicates that a variety of substances such as antibiotics, sulfonamides, ascorbic acid, and certain carbohydrates may reduce the animal's requirement for thiamine. Evidence for this sparing effect in some cases is available, not only from body weight gains but also from increased storage of the vitamin both in the liver and the entire carcass. There are still many problems that remain to be answered before a final picture is available of how the thiamine-sparing effect is mediated. The most attractive hypothesis still revolves around some change in the intestine which permits an increased absorption of thiamine.

There is ample evidence indicating that the intestinal synthesis of thiamine occurs in both man and animals. It is likely that a portion of the

thiamine so synthesized is absorbed. However, except under rather unusual conditions [such as refection in rats or the studies of Najjar and Holt (1943) with children fed purified diets] the amount thus secured is insufficient to supply the requirement of nonruminants other than rabbits (for which see Section I, part 5).

The bacterial destruction of thiamine in the gastrointestinal tract has been considered in Section II, part 3. It would appear from the available literature that about 3% of the people in some Japanese cities harbor one or more types of bacteria which destroy thiamine (Hamada, 1953). There are some reports which indicate that these organisms may be the cause of certain cases of polyneuritis.

2. Niacin

The observation by Krehl *et al.* (1945) that tryptophan can supply part of the niacin requirement of rats fed a corn diet led them to suggest that the transformation of tryptophan to the vitamin was mediated through the intestinal flora. Later it was demonstrated that the conversion occurred in the tissues (Henderson and Hankes, 1949; Hundley, 1949b). These workers removed the intestines of rats and found that when tryptophan was injected, the animals excreted N^1-methylnicotinamide in amounts equivalent to the amino acid injected.

a. Excretion Studies—Animals. There was still the possibility that the intestinal tract might influence the niacin available to the animal. Shourie and Swaminathan (1940) found that rats on a low intake of niacin excreted more than they received in their diet, while on a high intake they excreted much less than the intake. Since there was no difference in the niacin contents of the livers, muscle, or blood of the rats on the high and low niacin intakes nine weeks after starting the diets, it was obvious that both synthesis and destruction of niacin occurred. Certain bacteria that are normal inhabitants of the intestinal tract have been shown by *in vitro* studies to be able to synthesize niacin (Burkholder and McVeigh, 1942; Ellinger *et al.*, 1947; Thompson, 1942), whereas other bacteria consume it (Koser and Baird, 1944; Benesch, 1945).

The rabbit secures all its B vitamins except niacin through the activity of the intestinal microflora (Wooley and Sebrell, 1945). The rabbit probably secures some niacin from this source (like the other nutrients synthesized in the intestine, the niacin becomes available through coprophagy). The exact amount of niacin secured from the intestinal tract is difficult to determine, since balance studies are complicated by the contribution that trytophan makes to the body's niacin pool. This

reservation makes it difficult to interpret niacin balances. The results of Olcese *et al.* (1949) showed that rabbits on a niacin intake of 11.2 μg. per day excreted 38.8 μg. in their feces and 139.9 μg. in their urine. As the niacin intake was increased, the percentage recovered decreased.

b. Excretion Studies—Man. The difference in the balance picture secured on an adequate and an inadequate intake of niacin was also observed by Denko *et al.* (1946a,b) in seven young men. These investigators studied the fecal niacin and the urinary excretion of niacin, and N^1-methylnicotinamide, which is the primary excretion product of niacin in man (Hundley, 1954). On a low intake of niacin (5.9 to 6.8 mg./day) the excretion of N^1-methylnicotinamide in the urine increased to the point where the total excretion, expressed as niacin, exceeded the intake (Denko *et al.*, 1946b). On a higher intake (15.6 mg./day) the excretion of niacin and N^1-methylnicotinamide accounted for 31% of the intake (Denko *et al.*, 1946a). Results similar to the latter were secured by Oldham and co-workers (1946a). Young women on niacin intakes of 10 to 20 mg. per day excreted about 20% of the intake as niacin, with about twice as much in the feces as in the urine.

c. Influence of Sulfonamides on Niacin Synthesis. The influence of the sulfonamides on the intestinal contribution of niacin is still controversial, especially as it pertains to man. The work of Teply *et al.* (1947) suggested that the rat secured some of its niacin through the microbial activity in the intestinal tract. When phthalylsulfathiazole was added at a level of 2% to a purified diet containing sucrose, it reduced the rate of weight gain. The rate was restored to normal by the addition of both folic acid and niacin; neither one alone was effective. In contrast to this is the observation that the addition of 2% sulfasuxidine to a niacin-free diet increased the growth rate of chicks when either glucose or starch was the carbohydrate (Anderson *et al.*, 1950).

The work of Ellinger and co-workers suggested that human beings obtain fairly large amounts of niacin as a result of intestinal microbe activity. This conclusion was based on their interpretation of urinary excretion studies. Ellinger and Coulson (1944) found fairly large amounts of "nicotinamide methochloride" (N^1-methylnicotinamide) excreted in the urine of their subjects. Since they assumed that only 15% of the niacin is excreted in that form, the dietary intake based on the preceding assumption was "inconceivably high if one is to accept the values for the content of nicotinamide and similar compounds in the foods." Urinary excretion values higher than those expected indicated to Ellinger and Coulson (1944) that the subjects were receiving some niacin from intestinally synthesized sources.

When succinylsulfathiazole was given to three normal subjects and to

two patients with symptoms of pellagra, there was a reduction of about 60% in the urinary excretion of N^1-methylnicotinamide. Sulfathiazole had no influence on the urinary excretion in either group (Ellinger *et al.*, 1945). These observations confirmed the faith of Ellinger and co-workers in the availability to man of the niacin synthesized by the bacteria in the intestinal tract. The results of feeding another sulfonamide (*p*-amino-methylbenzene sulfonamide) to two human subjects led them to conclude that this sulfonamide increased the intestinal synthesis of niacin (Ellinger and Emmanuelowa, 1946). Their conclusion was based on the urinary excretion of N^1-methylnicotinamide. When the latter data are plotted, it is apparent that there was a peak in the excretion three or four days after the sulfonamide was started. From then on it declined, and whether the cessation of sulfonamide feeding on the seventh day had any influence on the trend is problematical. Similar experiments were carried out on rats with essentially the same results.

The fact that Najjar *et al.* (1946) found the feeding of succinylsulfathiazole to have no influence on the excretion of N^1-methylnicotinamide prompted Ellinger (1950) to defend his observations by suggesting that the difference in results was due to differences in diets. Grundy *et al.* (1947) stated, without presenting any data, that feeding 4 g. phthalylsulfathiazole per day to normal young men for 8 to 17 days had no influence on their niacin excretion. It is possible that sulfonamides are selective as far as their influence on the excretion of vitamins is concerned, and that their effects may depend upon the diet.

In rats, the addition of 2% succinylsulfathiazole to a purified diet containing 18% casein had no effect on the liver stores of niacin (Skeggs and Wright, 1946). This was true whether the carbohydrate in the diet was sucrose, dextrin, or cornstarch. Regardless of the kind of dietary carbohydrate, the sulfonamide had no influence on the fecal excretion of niacin.

In order to evaluate properly the influence of the sulfonamides on the contribution of the intestinal flora to the nutritional requirements of the host, it will be necessary to extend observations such as those of Ellinger. The work with sulfonamides showed that changes occurred in the intestinal flora shortly after the sulfonamide supplementation was started but that the flora returned to the original type within six weeks (Gant *et al.*, 1943). For this reason the long-term influence of the flora on vitamin requirements may be different from that secured in the short-term studies mentioned above.

It would appear that sulfonamides produce little, if any, change in the niacin that may normally be available to the host as a result of microbial synthesis in the intestinal tract. An exception to this is the chick, in which

sulfasuxadine increased the growth rate when a niacin-free diet was fed (Anderson *et al.*, 1950).

d. Influence of Antibiotics on Niacin Synthesis. A number of studies have been made of the effect of antibiotics on the niacin requirement of animals. There is conflicting evidence on this score for the chick. A number of workers have reported that neither Aureomycin nor procaine penicillin had any influence on the growth of chicks fed various levels of niacin (Stokstad *et al.*, 1951; Jukes and Williams, 1953). This was true whether the diet contained sucrose or dextrin (Nelson and Scott, 1953). There is one report that chicks fed a niacin-free diet supplemented with procaine penicillin gained less weight than the unsupplemented controls (Coates *et al.*, 1951). Another report showed that the addition of Aureomycin to a diet free of niacin produced a slightly heavier weight in chicks at 3 weeks when compared with the controls (Biely and March, 1951). The significance of these reports will have to await clarification and a more complete presentation of data than that in the Biely and March report.

In the rat, neither growth nor urinary excretion of N^1-methylnicotinamide was influenced by the addition of streptomycin, Aureomycin, terramycin, or penicillin to a 9% casein-cornstarch diet. Aureomycin and, to a lesser extent, terramycin did, however, increase liver niacin (Halevy *et al.*, 1955).

In man, 4 or 6 g. streptomycin per day as a supplement to a normal diet had no effect on the urinary excretion of N^1-methylnicotinamide, even though the antibiotic was fed for as long as 20 days (Sarett, 1952). The antibiotic also had no influence on the conversion of tryptophan to niacin as shown by increased N^1-methylnicotinamide excretion following oral supplements of tryptophan (Sarett, 1952). This observation confirmed the suggestions of Henderson and Hankes (1949) and Hundley (1949b) that tryptophan is converted to niacin in the tissues.

The present evidence indicates that antibiotics have little or no effect on the niacin that may be available to the host as a result of intestinal synthesis.

e. Influence of Diet on Niacin Synthesis. Najjar *et al.* (1946) stated that niacin synthesized in the intestinal tract is available to man. This conclusion was based on the fact that adolescents could be maintained on a purified diet of vitamin-free casein, Crisco, Dextrimaltose, minerals, and adequate amounts of the necessary vitamins except niacin. Over a period of 3 months, the niacin intake was 1.5 to 2.0 mg. per day. In spite of the low intake there was no change in the urinary excretion of N^1-methylnicotinamide, nor were there any signs of deficiency. These facts suggested to them the "synthesis of the vitamin by intestinal bacteria."

Another explanation that was not apparent at the time the work was done is that the casein in the diet supplied enough tryptophan to cover not only the requirement for the amino acid but also the need for niacin. The latter is more than a possibility, since a number of workers have shown that tryptophan can cure human pellagra (Goldsmith *et al.*, 1952; Bean *et al.*, 1951) and that the diet must be low in both niacin and tryptophan in order to secure signs of a deficiency.

The influence of dietary carbohydrates upon the growth of animals maintained on low-niacin diets has been reviewed by Krehl (1949), Hundley (1954), and Johansson and Sarles (1949). Dextrin, glucose, cornstarch, and sucrose are effective in decreasing order in overcoming a niacin deficiency in the rat produced by adding corn to a purified ration (Krehl, 1949).

The apparent niacin-sparing effect of the above carbohydrates is based on the differential growth seen in animals when the individual carbohydrates are incorporated into a niacin-low diet. The explanation proffered is that dextrin favors a microbial flora in the intestine which synthesizes niacin or makes it available to the host.

That other mechanisms may be operative has been suggested by Hundley (1949a), who found that on niacin-free diets rats grew better when carbohydrates containing no fructose were used. Carbohydrates containing either glucose or galactose produced good growth, which was improved to only a slight extent by the addition of niacin. On the niacin-free diets the concentration of niacin in the liver and muscles of the rats was the same regardless of the carbohydrate in the diet. The niacin levels were within the range indicative of a deficiency, yet the rats on the glucose and starch diets grew at a good rate (13 to 14 g. per week). Hundley concluded that "more niacin is actually needed in the metabolism of fructose, since rats receiving fructose grew only when the concentration of tissue niacin approached normal, while rats receiving glucose grew well in the presence of reduced niacin levels."

Another complicating factor is the report of Anderson *et al.* (1950) that chicks grow better on a niacin-free diet when glucose is present compared with starch (88 vs. 44 g. in 4 weeks).

A critical evaluation of the present data leads to the conclusion that it is doubtful whether any niacin is provided to man by the intestinal bacteria. The situation in animals is still debatable.

3. Riboflavin

a. Excretion Studies—Animals. Griffith's work (1935) with cecectomized rats led him to the conclusion that if coprophagy were prevented, the animal was unable to utilize the riboflavin synthesized in its intestinal

tract. This work was done before there was a clear distinction between the different members of the vitamin B complex. That riboflavin was synthesized in the gastrointestinal tract of the rat was definitely shown by Tange (1939), who isolated the compound from the feces of rats fed a lactose or dextrinized cornstarch diet deficient in the B vitamins. The identity of the substance in the feces with pure riboflavin was shown by absorption spectra studies.

With the development of methods for the determination of riboflavin, it was soon apparent that rats excreted in urine and feces almost as much of the vitamin as there was in their diet (Sure and Ford, 1943; Laszt and Torre, 1943). The fecal riboflavin was not influenced by changing the intake from 10 μg. to 40 μg. per rat per day (Obermeyer *et al.*, 1945).

That the riboflavin content of the feces is related to the bulk thereof was shown by the work of Yasuda (1953). He found that the addition of either filter paper or absorbent cotton increased riboflavin synthesis by the intestinal flora. These increases were associated with increases in urinary riboflavin, suggesting that some of the vitamin synthesized in the intestine was absorbed.

The rabbit dramatically demonstrates the utilization of riboflavin synthesized by the intestinal microflora—the vitamin is very likely absorbed primarily after the consumption of the soft feces which are a potent source of vitamins. Olcese *et al.* (1948) found that on an average daily intake of 11 μg. of riboflavin the young rabbit excreted 113 μg. of the vitamin in the urine and 14 μg. in the feces. When the riboflavin intake was increased to about 400 μg. per day, the urinary and fecal excretions increased approximately threefold but at the higher level the intake exceeded the total excretion.

The problem of riboflavin synthesis by bacteria in the intestinal tract of the chicken has not been clarified. The work of Lamoreux and Schumacher (1940) showed that when adult cockerels were fed a riboflavin-low diet, the feces contained about 2.5 times as much riboflavin as was present in the diet consumed by the birds. Cecectomized birds produced feces with the same riboflavin content as normals when put on the riboflavin-low diet. The fecal riboflavin increased very markedly when the droppings were left at room temperature for 24 hours.[9] No such increase in riboflavin concentration occurred when the feces were incubated at 107°F. (body temperature of chickens). On the basis of these observations Lamoreux and Schumacher concluded that "most of the riboflavin consumed is excreted in some measurable form." It would not

[9] One of the organisms involved in the synthesis of riboflavin was isolated from droppings and shown to be a bacillus (Schumacher and Heuser, 1941).

seem possible for an animal that requires riboflavin to continue excreting more of the vitamin than it ingested unless there were synthesis of the substance in the intestinal tract. A more rational explanation for the findings of Lamoreux and Schumacher would be that the cecectomized birds showed a compensatory hypertrophy of the lower part of the intestine which permitted the microbiological reaction to occur. There has been no evidence that the chick can absorb any of the riboflavin produced in its intestinal tract.

b. Excretion Studies—Man. Prior to the paper by Najjar *et al.* (1944) no work appears to have been done on the fecal excretion of riboflavin by human subjects. The above investigators found a high fecal riboflavin among adolescent subjects maintained on a purified diet (casein, Crisco, Dextrimaltose, minerals, and vitamins) providing 70 to 90 μg. of riboflavin per subject per day. The fecal excretion on the deficient diet was the same as on the control diet (riboflavin intake not stated). The low riboflavin diet with and without succinylsulfathiazole was fed for three months. During this time no signs of deficiency were seen. For this reason these workers stated that "under these conditions the intestinal bacteria can synthesize enough of this factor to supply the requirements of the individual" for three months.

Denko and co-workers (1946b) also observed no change in the fecal riboflavin excretion when their subjects were changed from an intake of 1.8 mg. per day to 0.36 mg.

Hathaway and Lobb (1946) fed the purified diet of Najjar *et al.* to three women who received 1.09 mg. of riboflavin per day. The fecal excretions under these circumstances were essentially the same as those observed in the study of Najjar *et al.* (200 to 500 μg. per day), even though the intake was ten times as high. When the same subjects received a diet of natural foods providing 1.33 mg. of riboflavin per day, the fecal excretion was about 2.8 times greater. These results indicate that fecal riboflavin levels are influenced primarily by the type of diet, and when the diet is maintained constant, within limits, the riboflavin intake has little influence on the amount in the feces. The data of Hathaway and Lobb, unfortunately, permit no decision as to whether any of the riboflavin synthesized by intestinal bacteria was absorbed.[10]

An influence of the diet on fecal riboflavin excretion was also noted by Iinuma (1955). When a normal subject consumed a vegetable diet, the fecal riboflavin was five times that on a meat diet. The vegetable diet resulted in a marked increase in the weight of the stools which appeared to be associated with the increased riboflavin therein. In spite

[10] On both the purified and natural diets more than half the riboflavin in the feces was free, i.e., extractable with water.

of the lower riboflavin intake when the meat diet was fed (0.63 mg./day vs. 0.85 mg. from the vegetable diet) and the lower fecal riboflavin, the daily urinary riboflavin increased from about 180 μg. on the vegetable diet to 400 μg. on meat.[11]

Suggestive evidence that riboflavin is not absorbed from the lower part of the intestinal tract came from the work of Everson *et al.* (1948). When they gave 2 mg. riboflavin by retention enema to seven young women, there was no change in urinary excretion during the subsequent two days. The enema was retained for 10.5 to 39 hours.

The evidence at hand indicates that riboflavin is synthesized in the intestinal tract of humans but the extent to which it is absorbed is questionable and, probably, negligible, since a riboflavin deficiency can be produced in some people by feeding a diet which provides a deficient amount of the vitamin (Sebrell and Butler, 1938; 1939).

The amount of riboflavin in the feces of both man and rats appears to be related to the weight of the stool. Long-term studies should be made to determine whether substances such as cellulose influence the rat's requirement for riboflavin.

c. Influence of Sulfonamides on Riboflavin Synthesis. In the rat the addition of sulfonamides to the diet appears to have no influence on the riboflavin requirement. In some cases the addition of sulfasuxidine reduced the combined urinary and fecal riboflavin excretion (Schweigert *et al.*, 1945a), but in other cases succinylsulfathiazole had no effect on the amount of the vitamin in the feces (Skeggs and Wright, 1946). The type of carbohydrate in the diet had relatively little influence on the responses seen in the two preceding reports.

That the changes in fecal riboflavin brought about by the sulfonamide have no effect on the dietary requirement was shown by Schweigert *et al.* (1945b). They found that the addition of succinylsulfathiazole to a purified diet containing dextrin produced no change in the riboflavin concentration in the liver or muscles of rats. Results similar to these were reported by Skeggs and Wright (1946). In both cases the rats receiving the sulfonamide grew more slowly than the controls. The poorer growth was probably attributable to the absence of folic acid in the diet.

There was no change in the urinary or fecal excretion of riboflavin when 1% sulfasuxidine was added to a purified diet which provided rabbits with 11 μg. of riboflavin per day (Olcese *et al.*, 1948).

Sure and Easterling (1947) found that when succinylsulfathiazole was added to a variety of diets, the riboflavin in the feces of rats decreased.

[11] Yasuda (1953) found higher urinary riboflavin excretions in rats fed meat diets than diets containing fish. He also noted a marked increase in fecal riboflavin synthesis when cellulose was added to the diet.

There was no change, however, in the urinary excretion of the vitamin, suggesting that the fecal riboflavin was not being absorbed.

By feeding succinylsulfathiazole to 12 subjects on a purified casein-Dextrimaltose diet, Najjar *et al.* (1944) produced a slight increase in fecal riboflavin, but no effect on the urinary excretion was noted except for a reduction in one subject who had previously excreted more than any of the other 11. When the deficient diet was started, there was a reduction in the riboflavin excretion as measured by the one-hour sample collected before breakfast. The decreased urinary excretion was associated with the reduced intake. Thereafter there was no change (except as noted above) in the fasting one-hour excretion throughout the three-month experimental period. Confirmation for the above appears in the paper by Grundy *et al.* (1947), who stated (but presented no data) that 4 g. of phthalylsulfathiazole fed daily to five normal men for 8 to 17 days had no effect on the urinary excretion of riboflavin.

Although sulfonamides may influence the fecal excretion of riboflavin, they appear to have no influence on the animal's dietary requirement for it.

d. Influence of Antibiotics on Riboflavin Synthesis. The evidence for a sparing effect of antibiotics in rats fed a riboflavin-free diet is controversial. Daft and Schwarz (1952) claimed that rats on a diet "devoid" of riboflavin reached weights over 500 g. when the diet was supplemented with 20 mg. % Aureomycin. There are, however, two reports that neither penicillin nor Aureomycin added to a riboflavin-free diet had any effect on the growth of rats (Sauberlich, 1952; Lih and Baumann, 1951). In the presence of suboptimal amounts of riboflavin, Aureomycin and penicillin supplements were equally effective in stimulating growth while streptomycin was less so (Lih and Baumann, 1951). There is an indication that the growth-stimulating effect of the antibiotic is seen whether the limiting amount of riboflavin is present in the diet or is given by injection (Jones and Baumann, 1955), which is contrary to the thiamine situation. The increased growth produced by the antibiotic added to the riboflavin-low rations is accompanied by an increased content of riboflavin in the liver (Lih and Baumann, 1951). However, the latter was only a reflection of the larger liver sizes, since there was no difference in the amount of riboflavin per gram of liver or plasma in the rats on the basal and antibiotic-supplemented rations.

For chicks the evidence of any sparing effect is equivocal. Biely and March (1951) found that the addition of Aureomycin to a corn-casein-gelatin-mineral-vitamin ration increased growth when riboflavin was omitted from the vitamin mixture. Unfortunately, they did not report the growth secured with added riboflavin and omitted all statistical

data needed for a critical evaluation of the effect. Jukes and Williams (1953) found suggestions of a riboflavin-sparing effect in chicks fed a sucrose-casein type diet. Coates *et al.* (1951) found that penicillin added to a purified diet free of riboflavin had no effect on growth of day-old chicks. The addition of Aureomycin to a natural grain ration was reported to have no influence on the serum riboflavin levels in chicks (Common *et al.*, 1950). This observation is not unexpected, since work with other species indicates that the riboflavin level in the blood is not influenced by dietary intake (Pearson *et al.*, 1944; Axelrod *et al.*, 1941).

For pigs, Terrill and co-workers (1955) found no indication that Aureomycin added to a cornstarch-casein diet spared the dietary riboflavin requirement.

For normal adults, what little evidence there is indicates that the oral administration of a variety of antibiotics has little or no influence on urinary riboflavin excretion (Sarett, 1952; Montenero and Frongia, 1952). However, there may be a decrease in the riboflavin excretion of patients under similar circumstances (Montenero and Frongia, 1952).

There is one report of a clinically diagnosed riboflavin deficiency appearing in a patient treated with chloramphenicol (Montenero and Frongia, 1952). That the lesions seen in this case represent a true deficiency is open to question, since some of the more widely-used antibiotics, such as penicillin, produce oral lesions in a small percentage of individuals (Welch, 1954).

There is an indication from the work of Foster and Pittillo (1953) that riboflavin competitively overcame the growth inhibition of Aureomycin and that, for certain mutants of *B. subtilis*, Aureomycin competitively inhibited the utilization of riboflavin.

It would appear as though antibiotics spare the rat's dietary requirement for riboflavin when small amounts of the vitamin are present in the diet. However, the antibiotics do not have any prominent influence on the riboflavin requirement of pigs, chicks, or man.

e. Influence of Diet on Riboflavin Synthesis. The work of Guerrant and Dutcher (1934) suggested that the increased growth of rats fed cornstarch diets compared with sucrose diets was associated with a greater fecal excretion of vitamin G (the obsolete name for riboflavin). The addition of 10 or 20% cottonseed oil to the sucrose diet appeared to have no effect on either growth or riboflavin excretion in the feces. It was subsequently shown that dietary fat increased the rat's requirement for riboflavin and aggravated the deficiency symptoms (Mannering *et al.*, 1941). Whether the fat acts on the intestinal tract in a manner that is the reverse of lactose, starch, and dextrin is hard to say. The

presence of fat in the sucrose ration had no influence on fecal riboflavin but did accentuate the vitamin deficiency (Mannering *et al.*, 1944).

The work of Mannering *et al.* (1944) indicated that rats receiving suboptimal amounts of riboflavin grew better when fed a dextrin or cornstarch ration than a sucrose ration. Under these circumstances, the fecal riboflavin excretion was related to the growth-promoting action of the dietary carbohydrate. The exception to this was lactose, which resulted in the highest fecal excretion of riboflavin but poor growth.[12] Schweigert *et al.* (1945a) found results similar thereto in their study of the effect of sucrose and lactose on the riboflavin requirement of the rat. Both of these studies were extensions of the earlier work of Morgan *et al.* (1938), who showed that lactose spared the rat's requirement for riboflavin.

That dietary factors other than fats and carbohydrates may have a marked influence on fecal riboflavin excretion, which presumably resulted from a change in intestinal activity, was shown by Sure (1945). When he fed dried whole liver, the urinary and fecal excretions of riboflavin were twice that when an equivalent amount of riboflavin was fed. The increased excretion occurred in the presence of a greater body weight gain.

It should be pointed out that Coates *et al.* (1946) found almost as much riboflavin in the urine and feces of their refected rats as in the animals receiving a stock diet. This occurred in spite of the fact that the potato starch diet was free of the vitamin.

The present evidence indicates that the intestinal flora of rats can synthesize fairly large amounts of riboflavin and that a portion of this vitamin is available to the host, as shown by the high urinary excretion of refected rats and by the sparing effects of dextrin and cornstarch on the riboflavin requirement. The evidence from both types of studies is open to the question, "Did the rats secure the riboflavin as a result of ingesting their feces?"

4. Pantothenic Acid

a. Excretion Studies—Animals. There are apparently only a few reports on the fecal excretion of pantothenic acid by animals. Silber (1945) found that the fecal excretion of pantothenic acid by dogs showed considerable variation, some of which was attributed to the effect of the food in decreasing absorption of the vitamin. The 40 mg. daily supplement of pantothenic acid used by Silber was far larger than the dog's requirement (Schaefer *et al.*, 1942) and might have been responsible for some of the variation observed in fecal excretion. In no case did the fecal excretion amount to much more than 50% of the intake. It is

[12] The poor growth of the rats fed the lactose ration might have been due to the toxicity of lactose (Mitchell, 1927; Schantz *et al.*, 1938).

impossible to say whether the 50% of the oral dose not accounted for by the urinary and fecal excretions represented destruction by intestinal bacteria.

Skeggs and Wright (1946) found that the fecal excretion of pantothenic acid by rats receiving adequate amounts of the vitamin was much greater than the intake. The excretion varied very markedly with the type of dietary carbohydrate.

On an inadequate intake of pantothenic acid, the fecal excretion of rats was much higher than the intake (Nelson *et al.*, 1947). When the pantothenic acid content of the diets was adjusted to the same level (0.7–0.9 μg./g.), the urinary excretion was much higher on a 64% casein diet than on the 24% level (4.5 vs. 1.6 μg./day). Although there was a similar relation for the fecal pantothenic acid excretion, the results were not significant because of the great variability. The increased urinary excretions were associated with greater weight gains. It is possible, as the authors suggest, that dietary protein exerts a sparing effect on the pantothenic acid requirement, but it must be recognized that an effect of the diet upon the intestinal flora has not been ruled out.

Rabbits maintained on a low dietary intake of pantothenic acid (8 or 17 μg. per day) showed a urinary excretion of the vitamin which was four to five times the intake. A marked increase in the pantothenic acid intake increased the urinary excretion but had no effect on the fecal excretion. At the higher level the intake was twice the total excretion (Olcese *et al.*, 1948).

b. Excretion Studies—Man. The fecal excretion of pantothenic acid in young women consuming nothing but milk was less than 10% of the intake (Gardner *et al.*, 1943). In these three subjects the sum of the urinary and fecal excretions (6.0 and 0.41 mg./day respectively) was almost the same as the intake (6.9 mg.). Results similar to these were secured by Oldham *et al.* (1946) with 12 young women maintained on a varied diet. Seven normal young men, while on an adequate intake of pantothenic acid (about 5 mg./day), showed a higher and more variable fecal pantothenic acid excretion (Denko *et al.*, 1946a) than the women in the preceding study. Again, the total excretion was, on an average, greater than the intake. Even when the pantothenic acid intake was reduced to 1.3 mg., the fecal excretion went up while the urinary excretion decreased. Under these circumstances the combined excretion was 3.5 times as great as the intake.

The latter study of the excretion of pantothenic acid on a reduced intake provides good evidence that the vitamin is synthesized in the intestinal tract, but none of these studies offer evidence that the vitamin so synthesized is absorbed.

c. Influence of Sulfonamides on Pantothenic Acid Synthesis. Practically all the work on the influence of sulfonamides on the requirement for pantothenic acid was done in the early 1940's. These papers were reviewed by Daft and Sebrell (1945). It was shown that when rats were fed diets containing adequate amounts of vitamins supplemented with sulfonamides, they developed signs suggestive of a deficiency of pantothenic acid. West *et al.* (1943) observed deficiency symptoms in rats receiving sulfapyridine-supplemented diets. In these cases large doses of pantothenic acid produced cures. Other workers (Martin, 1942; Wright and Welch, 1944), adding sulfaguanidine or sulfasuxidine to diets containing adequate amounts of pantothenic acid, observed some of the deficiency signs seen by West *et al.* The problem became more complicated when it was shown that some of the symptoms seen in the above experiments could be cured by supplementing the diet with biotin or folic acid. Wright and Welch (1944) were unable to cure the pantothenic acid deficiency symptoms either with large oral doses of the vitamin or with smaller doses given subcutaneously. When the pantothenic acid was given in the absence of folic acid or biotin, the liver levels of pantothenic acid remained low. On the other hand, all deficiency signs disappeared and growth was restored as soon as biotin and folic acid were added to the ration. Pure biotin added to the diet had only a very limited effect on both growth and pantothenic acid levels in the liver; folic acid alone was not tested. These observations led Wright and Welch to suggest that "pantothenic acid metabolism is impaired when inadequate amounts of folic acid and biotin are available."

Confirmatory evidence for the essential role of folic acid in pantothenic acid deposition in the liver of the rat was provided by Giroud *et al.* (1954). They added succinylsulfathiazole to a folic acid-deficient diet fed to adult female rats six days before mating. The livers removed from both the mothers and fetuses at parturition showed a slight reduction in pantothenic acid for the group receiving the sulfonamide. Folic acid supplements brought the level of pantothenic acid in the liver up to normal even in the presence of the sulfonamide.

Suggestive evidence for an interrelationship among bacteria between pantothenic acid and folic acid or one of its derivatives comes from the work of Purko *et al.* (1953, 1954). They found that *B. linens* requires either pantothenic acid or PABA for growth on a synthetic medium. Neither folic nor folinic acids will replace PABA on a molar basis. They suggested that PABA functions in the synthesis of pantothenic acid and vice versa.

It is difficult to understand why the suggested interrelation between pantothenic acid and folic acid has not been studied more completely. The chick would seem a good subject for such experiments since it must

have a dietary source of folic acid even in the absence of sulfonamides (Hogan and Parrott, 1940).

That sulfonamides can reduce the fecal excretion of pantothenic acid by rats was shown by Miller (1945) and Skeggs and Wright (1946). The latter group showed that this was true whether the diet contained dextrin, sucrose, Cerelose, or cornstarch. The reduction in fecal excretion was associated with even more marked reductions in the concentration of pantothenic acid in the liver. Since there was no evidence to indicate that the rats had not consumed their feces, there is a possibility that the liver stores reflected the pantothenic acid secured by fecal consumption.

Normal young men were reported to show no change in pantothenic acid excretion when their diets were supplemented for 8 to 17 days with 4 g. phthalylsulfathiazole (Grundy *et al.*, 1947).

Aside from increasing the requirement for pantothenic acid in the rat, sulfonamides appear to have no influence on the pantothenic acid requirement in other animals or in man.

d. Influence of Antibiotics on Pantothenic Acid Synthesis. For rats there appears to be a definite sparing effect of certain antibiotics on the dietary pantothenate requirement. Lih and Baumann (1951) found that the addition of Aureomycin, penicillin, or streptomycin to a sucrose ration containing all of the known nutrients except pantothenic acid stimulated the growth of their animals. This affect was even more pronounced when suboptimal amounts of pantothenic acid (4 mg./kg.) were present in the ration. Under these circumstances the growth response was essentially the same as that secured by doubling the level of vitamin in the ration. These findings were confirmed by Sauberlich (1952), Giunchi *et al.* (1954),[13] and Daft and Schwarz (1952).[14]

Guggenheim and co-workers (1953) reported that neither penicillin nor Aureomycin had a sparing effect for pantothenic acid when the ration was devoid of the vitamin. Under these circumstances there was also no change in the pantothenic acid content of the liver, but there was a doubling of the urinary excretion of the vitamins with no change in the fecal excretion. However, in the presence of small amounts of dietary pantothenic acid an increase in weight occurred with terramycin, streptomycin, penicillin, and Aureomycin (in decreasing order of potency). The weight gains were associated with increased urinary excretion and increased amount of the vitamin in the liver; however, the concentration

[13] Giunchi *et al.* (1953) found that chloromycetin and Aureomycin restored to normal the ability of their pantothenic acid-deficient rats to produce antibodies while penicillin and streptomycin were less effective. The activity of the antibiotics in restoring antibody response was related to their pantothenic acid-sparing activities.

[14] Daft and Schwarz reported that rats on a pantothenic acid-deficient diet attained weights over 500 g. when 20 mg. % Aureomycin was added to the diet.

(i.e., μg./g.) of the vitamin in the liver was not changed to any significant extent. There was a variable effect of the different antibiotics on the fecal excretion of pantothenic acid with penicillin having no effect and the other three increasing the excretion.

The growth-stimulating effect of the antibiotics for rats on diets low in pantothenic acid was apparent even when the vitamin was given subcutaneously (Schendel and Johnson, 1954). There was a higher concentration of pantothenic acid in the small intestines of the rats receiving the antibiotic than in the control rats. The above studies were extended by Jones and Baumann (1955) who found that, as in their work with thiamine and riboflavin, the antibiotic was more effective when the vitamin was present in the diet than when it was given subcutaneously.

For chicks the evidence of a sparing effect of the antibiotic is not so clear. Coates and co-workers (1951) found no improvement in the growth of chicks fed a purified diet free of pantothenic acid when procaine penicillin was added thereto. Jukes and Williams (1953) found that Aureomycin added to a purified diet deficient in pantothenic acid produced increases in the growth of young chicks. When the preceding was repeated, the growth increases were not significant. Although Slinger and Pepper (1954) stated that procaine penicillin reduced the requirement of turkey poults for pantothenic acid, their conclusion may be questioned. The practical type of ration used by them must have been deficient in a factor(s) other than pantothenic acid, since the addition of penicillin produced a much greater growth response than the addition of adequate amounts of pantothenic acid.

For swine, Catron *et al.* (1953a) reported that Aureomycin spares the requirement for pantothenic acid, but Luecke *et al.* (1952, 1953) denied it. The pig apparently requires less pantothenic acid in the diet as the protein level is increased above that needed for normal growth (Luecke *et al.*, 1952; Wahlstrom, 1954).[15] Until the relationship between these two nutrients is worked out, it may be difficult to determine the effect of an antibiotic on the pig's dietary requirement for pantothenic acid.

Concerning man there is one report that indicates no change in the urinary excretion of pantothenic acid when either penicillin, Aureomycin, or chloramphenicol (Scuro, 1953) was added to diets providing constant intakes of the vitamin. Each antibiotic was given for a period of 15 days.

Antibiotics have a marked sparing effect for dietary pantothenic acid in the rat, but not in the chick or in man, and probably not in swine. It could be interesting to know what is responsible for the differential effect of the antibiotics in these species.

[15] A similar suggestion was made for rats by Nelson and Evans (1945, 1947) and Nelson *et al.* (1947).

e. Influence of Diet on Pantothenic Acid Synthesis. Before very many members of the vitamin B complex had been distinguished, Morgan *et al.* (1938) showed that in rats a cornstarch diet deficient in pantothenic acid (at that time called filtrate factor) produced only slightly less weight gain than did their yeast-supplemented diet. Krehl and Carvalho (reported by Elvehjen and Krehl, 1947) found that rats on a pantothenic acid-deficient diet grew much better when dextrin was the carbohydrate rather than sucrose. The increased growth was associated with higher levels of the vitamin in the liver, muscles, and cecal contents.

Skeggs and Wright (1946) found that rats fed purified rations containing adequate amounts of pantothenic acid showed a higher fecal excretion when dextrin was present in the ration than when sucrose, Cerelose (a commercial glucose), or cornstarch were used. The effect of the carbohydrate on fecal pantothenic acid excretion may partly explain the higher excretion of rats fed a stock ration than those fed a purified diet (428 vs. 35 μg. per day) even though the latter diet contained more of the vitamin than the former (44 vs. 14 μg. per g.; Miller, 1945). Although there was a twofold increase in the pantothenic acid concentration in the feces of the rats fed the stock diet, the fivefold increase in quantity of feces was primarily responsible for the increased vitamin excretion.

On a constant vitamin intake, there was a tendency for the pantothenic acid in the feces to increase with increases in the dietary protein levels (Wright and Skeggs, 1946).

The addition of 2% ascorbic acid to a pantothenic acid-deficient ration permitted rats to grow (Daft, 1951), with some rats attaining weights over 500 g. (Daft and Schwarz, 1952). The animals that grew well on the ascorbic acid-supplemented diets showed no histological indications of a pantothenic acid deficiency. A number of 6-carbon compounds such as glucuronolactone, glucuronic acid, and *d*-isoascorbic acid produced results similar to ascorbic acid (Hundley and Ing, 1953). The best evidence that increased amounts of pantothenic acid are available to the animal receiving ascorbic acid came from the study of Everson and associates (1954). When adult female rats were fed a pantothenic acid-deficient ration containing 2% ascorbic acid just prior to mating, the concentration of pantothenic acid in the carcass of the mother rat, as well as in the young, was as great as when the diet was supplemented with adequate pantothenic acid.

It is impossible to say whether the effect of vitamin B_{12} in sparing the chick's dietary requirement for pantothenic acid (Yacowitz *et al.*, 1951) involves the gastrointestinal tract.

The presence of dextrin, as the sole carbohydrate in the diet, or large

amounts of vitamin C reduce the rat's dietary requirement for pantothenic acid. From the available evidence it would appear as though both of these actions involved the flora of the intestinal tract. Again, no statement can be made about whether the vitamins are absorbed directly after synthesis or following ingestion of feces.

5. Pyridoxine

a. Excretion Studies—Animals. No papers could be found which reported fecal excretion of pyridoxine in animals.

b. Excretion Studies—Man. Suggestive evidence for the synthesis of pyridoxine in the gastrointestinal tract of man was provided by the work of Linkswiler and Reynolds (1950). They fed diets providing a constant daily intake of pyridoxine for each subject over a 10-day period. The daily intake for the 10 subjects ranged from 0.53 to 1.21 mg.[16] On this intake the fecal excretion of the vitamin equalled the intake in 4 subjects. When the urinary pyridoxine was added to the fecal excretion, 6 of the 10 subjects showed an excess of excretion over intake. The primary urinary metabolite of pyridoxine is 4-pyridoxic acid (Rabinowitz and Snell, 1949). The urinary excretion of 4-pyridoxic acid in the study of Linkswiler and Reynolds (1950) was greater than the pyridoxine intake. This situation was true for all subjects maintained on these levels of pyridoxine. When the 4-pyridoxic acid was added to the pyridoxine excreted in the urine and feces, the excretion was three to seven times greater than the intake. Supplementing the basal diet each day with 15 mg. of pyridoxine reduced the urinary recovery of pyridoxine and 4-pyridoxic acid to about one half the intake. If it can be accepted that the only source of 4-pyridoxic acid is pyridoxine and that there was no downward trend in the urinary excretion values throughout the 10-day experimental period, then the data of Linkswiler and Reynolds indicate that a fair share of pyridoxine may be synthesized in the intestinal tract and subsequently absorbed by man.

The preceding work is exceedingly important in suggesting that another vitamin besides biotin, folic acid, and vitamin K may be available to man following synthesis in the intestinal tract. Since the study of Linkswiler and Reynolds (1950) shows higher urinary excretions of pyridoxine and its recognized degradation product than can be accounted for on the basis of dietary intake, data should be made available on the excretion throughout the experimental period. Other tests of vitamin B_6 adequacy should be used such as the tryptophan load test used by Greenberg *et al.* (1949) with their human subjects. Finally, it should be estab-

[16] The intake of pyridoxine provided by the basal diet approaches the suggested minimal requirement of adults (National Academy of Sciences, 1953, p. 25).

lished that the assay procedures used for the vitamin B_6 compounds quantitatively measure all the substances that are biologically active.

The work of Denko *et al.* (1946a) indicates that normal young men excrete an average of 57% of their pyridoxine intake in the urine and feces, with some values as high as 87%. Had the 4-pyridoxic acid excretion been determined, it is likely that the excretion would have exceeded the intake. Fecal excretion of pyridoxine appears to be unaffected by changes in dietary intake (Denko *et al.*, 1946b).

The evidence suggests that under normal circumstances man secures a certain amount of pyridoxine as a result of bacterial synthesis in the intestine. Before this suggestion becomes more than that, additional studies should be made. In infants the microflora is apparently not able to provide very much of the vitamin since a deficiency of pyridoxine has been produced in them by diets very low in the vitamin (Molony and Parmelee, 1954; Coursin, 1954; May, 1954). Support for the preceding comes from the observation that the excretion of 4-pyridoxic acid rapidly falls to zero and urinary excretion of pyridoxine reaches that value shortly after a diet very low in pyridoxine is fed to infants (Snyderman *et al.*, 1953).

c. Influence of Sulfonamides on Pyridoxine Synthesis. The addition of 0.5% sulfathalidine to a pyridoxine-deficient ration containing dextrin reduced the weekly gain of rats from 23 to 10 g. (Sarma *et al.*, 1946). When pyridoxine was added to the sulfonamide-containing diet, the higher growth rate was restored. Sulfasuxidine added to a sucrose diet containing no added pyridoxine produced a rapid loss of weight in rats and death, whereas the animals on the sulfonamide-free diet continued to grow but at a slower-than-normal rate. Pyridoxine supplementation prevented the deleterious effects of the sulfonamide (Carpenter *et al.*, 1948). Sulfamerazine added to either a sucrose or dextrin ration free of pyridoxine reduced the growth rate of rats below that of the unsupplemented groups (Sauberlich, 1952).

Although sulfonamides accentuate and hasten a pyridoxine deficiency in the rat, presumably by interfering with microbial synthesis, the evidence from short term experiments indicates that a similar situation does not exist in man. When normal young men on a diet containing a small amount of pyridoxine were given 4 g. phthalylsulfathiazole per day for 8 to 17 days, there was no change in the fecal or urinary excretions of pyridoxine (Grundy *et al.*, 1947). Sulfasuxidine at a level of 12 g. per subject per day for six days not only produced no change in pyridoxine excretion but, in addition, had no effect on urinary excretion of 4-pyridoxic acid (Linkswiler and Reynolds, 1950).

Certain sulfonamides increase the rat's requirement for pyridoxine

both on sucrose and dextrin diets. In man, sulfonamides do not affect the urinary excretion of either pyridoxine or 4-pyridoxic acid.

d. Influence of Antibiotics on Pyridoxine Synthesis. Aureomycin was reported by Linkswiler *et al.* (1951) to spare the dietary requirement of rats for pyridoxine. They found that the antibiotic produced growth stimulation in rats receiving minimal levels of the vitamin; these growth responses were equivalent to doubling the vitamin intake. In addition, the antibiotic equalized the potency of pyridoxal, pyridoxamine, and pyridoxine when added to a purified ration. In the absence of the antibiotic, pyridoxal and pyridoxamine were about three-fourths as active as pyridoxine (Sarma *et al.*, 1946). The antibiotic was presumably acting here the same as the dextrin diet in the case of chicks by inhibiting the bacteria which selectively absorb pyridoxal or pyridoxamine from the upper part of the intestinal tract.

That there may be a difference between Aureomycin and penicillin in their effect on a pyridoxine-low ration was suggested by Sauberlich (1952). Penicillin G added to the pyridoxine-deficient sucrose or dextrin ration produced a considerably greater gain in weight than that secured on the unsupplemented diets. Aureomycin under the same circumstances had no effect. Results of a similar nature were reported by McDaniel and Daft (1955). They found that penicillin overcame all deficiency symptoms and improved growth, whereas Aureomycin accentuated the pyridoxine deficiency.

Most of the reports indicate that Aureomycin (Stokstad, 1954a; Jukes and Williams, 1953) or penicillin (Coates *et al.*, 1951) have no pyridoxine-sparing effect for chicks. There is, however, one report of increased growth associated with the addition of Aureomycin to purified diets fed chicks (Waibel *et al.*, 1952a). The growth-promoting effect was apparent regardless of the type of carbohydrate in the diet, or whether the pyridoxine level was inadequate or adequate.

The evidence for any pyridoxine-spring effect of antibiotics is still open to question. The differential effect of penicillin and Aureomycin in rats should be investigated and when it is, statistical data should be provided which will permit an evaluation of the results secured.

e. Influence of Diet on Pyridoxine Synthesis. The report of György and co-workers (1937) indicated that it was impossible for them to secure signs of pyridoxine deficiency in rats fed a cornstarch diet whereas they were successful when a sucrose diet was used. A slight vitamin B_6-sparing effect of cornstarch was observed by Morgan *et al.* (1938), and of cooked rice starch by Chick *et al.* (1940).

Conclusive evidence for the stimulation of bacterial synthesis of pyridoxine in rats with subsequent absorption from the intestine was

provided by Sarma *et al.* (1946). When they used dextrin in their pyridoxine-deficient ration, their rats grew much better than on sucrose. The improvement in growth was associated with an increased urinary excretion of 4-pyridoxic acid. Confirmation of the pyridoxine-sparing effect of dextrin was reported by Sauberlich (1952).

Another dietary factor that spares the requirement of pyridoxine is ascorbic acid. When a pyridoxine-deficient diet contained 5% ascorbic acid, rats grew about half as well as those on the complete diet (McDaniel and Daft, 1954).

In chicks the addition of 20% lactose to a purified diet containing suboptimal amounts of pyridoxine produced a considerable growth stimulation compared with the results with sucrose or glucose diets (Waibel *et al.*, 1952a). Dextrin diets resulted in an equalization of the growth-promoting effects of pyridoxal, pyridoxamine, and pyridoxine. Pyridoxine is more active than the other two when incorporated into a sucrose diet. Waibel *et al.* suggested that their observations could be explained by assuming a change in the duodenal flora "so that bacteria, which either selectively destroy or utilize pyridoxal and pyridoxamine in preference to pyridoxine" are decreased.

The type of carbohydrate markedly influences the dietary requirement of both the rat and the chick for pyridoxine. Presumably this effect is brought about through a change in the intestinal flora. Whether the pyridoxine synthesized in the intestinal tract as a result of dietary alterations is absorbed directly or after coprophagy is unknown. However, chicks raised on wire screens have not been reported to be afflicted with the above experimental limitation (i.e., coprophagy) and for this reason the suggestion can be made that the vitamin is absorbed directly by them. Dextrin in the diet equalizes the relative activity of the different forms of pyridoxine for the chick.

6. Biotin

a. Excretion Studies—Animals. The synthesis of biotin in the intestinal tract of the rat was shown by Nielsen *et al.* (1942), who found that rats fed a purified diet excreted from 6 to 12 times the biotin intake. Even when fed a stock diet, which contained 300 times as much biotin as the purified diet, the rats still excreted almost three times as much biotin as their intake. Rats fed a purified diet containing no biotin excreted large amounts of the vitamin in their feces (Skeggs and Wright, 1946).

Since the rat can grow normally on a ration containing no detectable amount of biotin (Skeggs and Wright, 1946) and since its requirement for the vitamin is evident only when raw egg white or sulfonamides are added to a biotin-free diet (Daft *et al.*, 1942), it is apparent that the rat

secures the biotin it requires from that synthesized in its gastrointestinal tract. In this respect the mouse and chick differ either qualitatively or quantitatively from the rat. A biotin deficiency can be produced in the chick (Hegsted *et al.*, 1940, 1942) and in certain strains of mice[17] (Nielsen and Black, 1944) within a few weeks when a diet deficient in the vitamin is used. The very young pig is like the chick in requiring a dietary source of biotin in order to prevent specific skin lesions (Lehrer *et al.*, 1952). Evidence for the establishment of a flora that synthesizes biotin was seen in the increasing urinary excretion of biotin as the pigs became older (Lehrer *et al.*, 1952). Shortly after weaning (3–5 weeks) a biotin deficiency can be produced in the pig only by incorporating raw egg white into the diet (Cunha *et al.*, 1946) or by feeding sulfathalidine (Lindley and Cunha, 1946).

b. Excretion Studies—Man. Since the urinary excretion of biotin in many cases exceeded the intake, with the fecal excretion being two to five times the intake, Oppel stated in 1942 that the biotin provided by intestinal synthesis was adequate for the needs of man and "there is thus reason to doubt that human subjects require any biotin in their food. . . . " A number of reports confirm the excess urinary biotin over intake (Gardner *et al.*, 1943) and indicate that the greatest excess of output over intake occurs on low biotin intakes; as the intake was increased, the ratio decreased (Gardner *et al.*, 1945, 1946).

c. Influence of Sulfonamides on Biotin Synthesis. It was only a short while after the introduction of sulfonamides to nutritional research that the rat's requirement for biotin became evident (Daft *et al.*, 1942). The addition of 1% sulfaguanidine or sulfasuxidine to a purified ration on which rats would normally grow very well produced a cessation of growth and a variety of symptoms, some of which could be cured with crystalline biotin.

The absence of pantothenic acid from the purified ration containing succinylsulfathiazole aggravated the biotin deficiency in rats (Emerson and Wurtz, 1944). Whereas the rats receiving pantothenic acid in the succinylsulfathiazole rations showed only minimal signs of biotin deficiency ("spectacled eyes and sparse mole-like fur"), those receiving the same ration without pantothenic acid showed signs of a severe biotin deficiency ("sore mouth, ventral alopecia, and encrustations"). The supplementation of the diet with biotin overcame the deficiency in both cases.

Folic acid and vitamin B_{12} appear to hasten the development of a

[17] Fenton *et al.* (1948) and Wilson *et al.* (1949) reported that their strains of mice did not require a dietary source of biotin and that a deficiency could be produced only by feeding egg white or a sulfonamide.

biotin deficiency in swine fed sulfonamides (Cunha *et al.*, 1948), presumably by influencing intestinal synthesis. (These pigs were on raised floors and coprophagy was reported to have been absent.) When a purified ration containing 2% sulfasuxidine was supplemented with either folic acid or vitamin B_{12}, signs of biotin deficiency appeared two weeks earlier than in the group receiving the unsupplemented ration containing the sulfonamide.

Evidence for a reduction in the intestinal synthesis of biotin in the rat was presented by Nielsen *et al.* (1942), who found that when they added 0.5% sulfaguanidine to a purified ration, both the fecal and urinary excretions of biotin were reduced. The reduction was only relative, however, for all the rats receiving the sulfaguanidine still excreted 5 to 40 times as much biotin as they consumed. Similar reductions in the urinary biotin excretion have been observed when succinylsulfathiazole was used (Ham and Scott, 1953; Halevy *et al.*, 1955). Not only was the reduction in biotin synthesis evident from excretion studies, but it was also reflected in hepatic stores of the vitamin (Wright and Welch, 1944; Halevy *et al.*, 1955). The liver biotin levels in the rats receiving the succinylsulfathiazole were from one-third to three-fourths those in the rats not receiving the sulfonamide. In addition to a reduction in liver levels of biotin, succinylsulfathiazole reduced the concentration of biotin in the cecum (Halevy *et al.*, 1955).

Even though the avidin present in raw egg white has a high affinity for biotin, which makes the use of this substance an effective means of producing a biotin deficiency, the addition of raw egg white to a ration containing succinylsulfathiazole did not aggravate the biotin deficiency in rats (Emerson and Wurtz, 1945). In conjunction with these experiments Emerson and Wurtz used a purified diet containing 2% dried beef liver. Adding 0.75% succinylsulfathiazole to the latter ration increased the growth rate over that of the nonsupplemented controls. These workers stated that "this growth stimulating effect of succinylsulfathiazole has been observed routinely in this laboratory with rats receiving purified diets containing liver." Again, we can only say that "hindsight is easier to come by than foresight."

Although certain strains of mice may require a source of biotin in their diets in order to grow at a normal rate and to prevent the development of a mild biotin deficiency, they do secure some of the vitamin from bacterial synthesis. This was shown by Nielsen and Black (1944), who found that when 0.6% sulfasuxidine was added to the biotin-free ration, mice grew at a slower rate than those on the basal diet and showed an alopecia which could be cured by feeding biotin.

Feeding normal young men 4 g. phthalylsulfathiazole per day for 8

to 17 days was reported to reduce the urinary and fecal excretion of biotin (Grundy *et al.*, 1947). The statistical significance (which was omitted from the paper) of these observations is open to question because of the variability in the excretion values before, during, and after the period when the sulfonamide was fed.

Sulfonamides when added to a purified diet bring out the rat's requirement for biotin presumably by inhibiting bacterial synthesis. Evidence therefor comes from the reductions in urinary excretion and liver stores resulting from sulfonamide feeding. Sulfonamides accentuate a biotin deficiency in mice but appear to have no influence on the urinary excretion of human subjects.

d. Influence of Antibiotics on Biotin Synthesis. Most antibiotics appear to have no or only a minor influence on the bacterial synthesis of biotin in the rat but may have a sparing effect on the dietary requirement of poultry for this vitamin. The addition of either streptomycin, Terramycin, Aureomycin, or penicillin to a purified cornstarch diet containing no added biotin had no effect on the growth of rats over a period of five weeks (Halevy *et al.*, 1955). Aureomycin and penicillin had no effect on the concentration of biotin in the urine, cecum, or liver of the rat. Although streptomycin produced a reduction in the cecal level and urinary excretion of biotin, the significance of that observation is open to question. Streptomycin had no influence on the level of biotin in the liver. In all cases the liver levels were considerably above the 0.35 μg./g. of fresh liver which was proposed by Wright and Welch (1944) as being the level below which deficiency signs appear. Procaine penicillin or bacitracin added to a purified diet containing sucrose increased the fecal excretion of "free biotin" by rats (Chow *et al.*, 1953). Terramycin decreased the "extra-dietary" biotin excreted by rats fed a cornstarch ration (Ham and Scott, 1953). Here again there is no evidence that the reduction in intestinal microbial biotin synthesis has any implication as far as growth or other physiological functions are concerned.

A very high level of streptomycin in the diet is the exception to the above. When Emerson and Smith (1945) added 160–375 mg.[18] of the antibiotic to a kilogram of a purified diet (in most of the other reports, the levels used have been 10–25 mg.) their rats grew fairly well and showed no deficiency signs. It was only when the level of streptomycin was increased to 600–875 mg./kg. of diet that poor growth and alopecia were seen. The two conditions were curable by adding biotin to the diet.

Turkey poults are similar to chicks in requiring a dietary source of

[18] The units used in the original paper were converted to a mg. basis using the conversion factor of 1 unit = 1 μg. streptomycin suggested in the Dispensatory of the U. S. of America (1950).

biotin. When either terramycin or penicillin was added to a practical ration, the dietary requirement of poults for biotin was reduced (Slinger and Pepper, 1954). A suggestion of a similar effect appears in the slightly increased biotin levels in eggs from hens receiving a practical ration supplemented with penicillin (Waibel *et al.*, 1952b).

Some antibiotics decrease while others increase the intestinal synthesis of biotin in the rat, but apparently have no other effect so far as vitamin economy is concerned. There are indications that the biotin requirement of poultry is spared by antibiotics. This area requires further work before definite statements can be made.

e. Influence of Diet on Biotin Synthesis. As in the case of most of the vitamins, dextrin is superior to sucrose in reducing the dietary requirement for biotin. In the laying hen, where a portion of the biotin comes from bacterial synthesis, dextrin-fed hens produced eggs that had lower embryonic mortality and less abnormalities indicative of a biotin deficiency than the sucrose-fed hens (Couch *et al.*, 1948). The yolks of eggs from hens fed the dextrin ration contained 0.15 μg./g. biotin, while sucrose-fed hens had 0.02 μg./g. Additional evidence for the biotin-sparing effect of dextrin comes from the work of Ham and Scott (1953). They found that in rats fed a raw potato starch diet the combined urinary and fecal biotin was about ten times as great as in rats fed a sucrose diet.

The intestinal synthesis of biotin appears to be influenced by a number of dietary components. Individuals (patients with chronic illnesses) who had eaten no food for days according to Oppel (1942) still excreted biotin in their urine at a rate which fell within the lower normal range. It is remarkable that the biotin excretion was maintained since complete abstinence from food results in a decreased intestinal activity, as shown by the absence of stools during such a period (Keys *et al.*, 1951). There is, of course, the possibility that the biotin excreted during starvation represents that which is liberated from the tissue being destroyed; if this were so, one might expect it to behave in a manner analogous to riboflavin (Mickelsen *et al.*, 1945).

Dietary components other than carbohydrates influence the intestinal synthesis of biotin. Large amounts of fat (28% corn oil or butterfat) in a purified diet containing sucrose appeared to decrease the synthesis of biotin by intestinal bacteria in the rat since significant growth responses were secured when biotin was added to such diets (Barki *et al.*, 1949). Although Couch *et al.* (1949) claimed that dietary fat increased the intestinal synthesis of biotin in the hen as shown by egg hatchability studies, their evidence is not conclusive. There was practically no difference in the biotin content of the egg yolks from the hens receiving the

high-fat (20%) and low-fat (5%) rations. The observed differences in hatchability of the eggs may well have been associated with some influence of the diet other than its effect on the biotin requirement.

The protein level of the diet was reported to influence the urinary biotin and for this reason was claimed to have an effect on intestinal synthesis by bacteria (Chow *et al.*, 1953). Unfortunately, these workers compared protein-free diets with 22% casein and 25% soy protein diets. There is no question about the significance of the urinary excretions but it may well be that the protein-free ration had an effect on over-all metabolic reactions which in turn might have depressed biotin synthesis.

Considering the large volume of work that has been done on the nutritional aspects of biotin, it is surprising that more unequivocal evidence is not available on the dietary factors that influence the intestinal bacterial synthesis of the vitamin. At present one can only state that dextrin favors bacterial synthesis in the hen and there are suggestions that a number of other factors such as protein and fat might affect it also.

7. Choline

Perhaps the first suggestion that choline might be synthesized by intestinal flora came from du Vigneaud *et al.* (1939). In their studies on the utilization of homocystine for the growth of rats, a few animals grew slowly on the basal ration. These workers suggested that the phenomenon might be analogous to refection. Results comparable to these were observed by Bennett and Toennies (1946) and extended by the finding that the addition of 2% succinylsulfathiazole could overcome the condition (Bennett, 1946). Another suggestion for choline synthesis came from work with "heavy water." When du Vigneaud *et al.* (1945) injected rats with deuterium oxide, about 8% of the choline methyl groups were labeled after three weeks. Since they were convinced that animal tissue does not have the ability to synthesize choline, they thought the labeled choline came from synthesis by bacteria in the intestine. As vitamin B_{12} may have been involved in these earlier studies, the validity of the interpretations must be established by future research.

When sulfasuxidine (1%) was added to a low-protein, high-fat, starch diet, free of choline, the weights of rats were increased and their survival was markedly increased (Handler and Follis, 1948). The mechanism whereby the sulfasuxidine exerted its choline-sparing action is indeed a puzzle, since PABA at a level of 2% had essentially the same effect as the sulfonamide on growth response, survival and condition of the liver.

Ignoring any complication in interpretation that may result from an

interaction between choline and vitamin B_{12},[19] the work of Baxter (1947) has shown that the rat's requirement for choline can be reduced by substituting cornstarch for sucrose in the ration regularly used or by adding Aureomycin to a sucrose diet (Baxter and Campbell, 1952). Since no choline was found in the feces of any rats (Baxter, 1947), it would appear as though intestinal synthesis of choline under any circumstances was very limited, or that excess choline was destroyed by bacterial action. That there was probably an actual synthesis with consequent absorption is indicated by the slight increase in choline content of the liver of the rats on the Aureomycin-supplemented, choline-free diet (Baxter and Campbell, 1952).

The work of Baxter and Campbell (1952) is important in showing that very high levels of Aureomycin (500 mg. %) were required to produce the observed choline-sparing effects on the kidney lesions. This level is about 100 times that required for the production of a sparing effect with other vitamins. Even at that high level there was practically no effect when a compound as closely related chemically as terramycin was used; chloromycetin, streptomycetin, and penicillin were without any influence. The possibility that the observed effects were due to contamination of the Aureomycin with vitamin B_{12} was ruled out by purifying the antibiotic and also by feeding large amounts of the vitamin.

That the intestinal bacteria may destroy choline is suggested by the work of de la Huerga and Popper (1951). They found that about two-thirds of a dose of 2 to 8 g. choline appeared in the urine of patients with liver disease, presumably as trimethylamine. A variety of bacteria commonly found in feces were able to carry out the same reaction, and since the conversion could be reduced by antibiotics[20] and sulfonamide feeding, they assumed that the destruction was of bacterial origin. Samples of intestinal contents removed by Miller-Abbott tube showed large amounts of the trimethylamine compound in the small intestine just below the duodenum (de la Huerga *et al.*, 1953). Bacteriological studies run in conjunction with these tests showed a slight relation between the bacterial flora and destruction of choline (de la Huerga and Popper, 1952). The presence of 4000 or more bacteria per milliliter of intestinal contents removed from an area within one foot of the pylorus of patients with liver disease gives some indication of the potential magnitude of microbiological action on nutrients, even in the upper part of the intestinal

[19] For a discussion of the various factors that might complicate the interpretation of any findings implying choline synthesis or degradation see Jukes (1955).

[20] Kaplan *et al.* (1954) found that a dog receiving 0.75 g. Aureomycin for six weeks showed no reduction in intestinal choline destruction as reflected by urinary trimethylamine excretion.

tract where absorptive potentialities are at their maximum. Since the above work was done with patients, there is a possibility that the high bacterial count in the duodenal contents is not a normal situation.

8. Folic Acid

a. Species Differences in Dietary Requirement for Folic Acid. There is good evidence that the following animals require a dietary source of folic acid: monkey,[21] chick[22] (Hogan and Parrott, 1940), duck (Miller *et al.*, 1953), turkey (Richardson *et al.*, 1945), guinea pig (Woolley and Sprince, 1945), gosling (Briggs *et al.*, 1953a), mink (Schaefer *et al.*, 1946), and fox (Schaefer *et al.*, 1947). Mice apparently secure some folic acid from bacterial synthesis in the intestinal tract, since some mice have been maintained on a deficient diet for weeks, but folic acid supplementation of the diet improves growth (Nielsen and Black, 1944) and reproduction (Cerecedo and Mirone, 1947).[23] The concept that man is dependent upon a dietary source of folic acid is based on the fact that a variety of anemias, both in adults and infants, respond to vitamin therapy, but, as yet, the evidence for an absolute dietary requirement is only presumptive (Bethell, 1954b).

The following animals can apparently secure their requirement for folic acid by means of intestinal bacterial synthesis: rats, swine, dogs (Stokstad, 1954b), and rabbits (Simpson *et al.*, 1949).

b. Excretion Studies—Animals. Perhaps the best evidence for the synthesis of folic acid by intestinal bacteria and for its subsequent utilization comes from the work of Ford *et al.* (1953) with refected rats. These animals excreted substantial amounts of folic acid in their urine even though the diet was free of the vitamin. The feces and cecal contents also contained appreciable amounts of folic acid. The folic acid in the cecal material, feces, and urine was reduced when sulfasuxidine was added to the diet. The rats showed a marked reduction in the cecal and fecal folic acid, but only a slight lowering in the urinary excretion, when cooked starch (a more digestible starch than raw starch) was used in the diet. Again, it must be recognized that folic acid synthesized by the intestinal microflora may have been utilized only after the rats consumed their feces.

[21] In the early work on the anemia in monkeys the curative substance was called vitamin M. This was shown to be the same as folic acid. For a review of the early work see Day (1944) and Stokstad (1954b).

[22] The anti-anemia factor for chicks was originally called vitamin B_c.

[23] Weir *et al.* (1948) reported that mice on a purified diet required no dietary folic acid. Their conclusion may be open to question since they started with mice that were considerably heavier than the ordinary weanling mouse.

Rats fed a purified diet free of folic acid excreted in the feces 5.3 μg. folic acid per day; on a stock diet providing about 7 μg. folic acid per day, the daily fecal excretion was 43 μg. (Miller, 1945).

Rats fed a diet containing succinylsulfathiazole had large amounts of folic acid in their feces, even when they showed deficiency symptoms (Wright *et al.*, 1945; Skeggs and Wright, 1946). Guinea pigs have been reported to have more folic acid in their feces than they ingested with a normal diet, with only a slight reduction when a deficient diet was fed (Wichmann *et al.*, 1954).

Rabbits maintained on a folic acid-free diet showed an increase in their urinary excretion up to 10 μg. per day by the tenth week (Simpson *et al.*, 1949). These levels undoubtedly reflect the rabbit's ingestion of its soft feces.

c. Excretion Studies—Man. Studies of normal adults showed that the urinary and fecal excretion of folic acid exceeded the intake (Denko *et al.*, 1946a). When the subjects were fed a vitamin-low diet, there was a slight increase in fecal folic acid compared with the normal diet, with the result that on the restricted vitamin intake the folic acid excretion was four to five times the intake (Denko *et al.*, 1946b). The addition of sulfathalidine to the normal diet produced a sharp reduction of folic acid in the feces of the above subjects, with a prompt increase when the drug was withdrawn (Grundy *et al.*, 1947). Patients with pernicious anemia or rheumatoid arthritis excreted 0.5 mg. folic acid daily in their stools (Girdwood, 1950). Patients who received no food or water for about 36 hours showed only traces of folic acid in their stomachs and intestinal contents.

Folic acid is synthesized by the microflora in the intestine. For the rat, swine, and dog, the folic acid so synthesized is all that is required for growth and normal blood formation. Other species are either partially or completely dependent upon a dietary source.

d. Effect of Sulfonamides on Folic Acid Synthesis. The addition of insoluble sulfonamides such as sulfaguanidine or succinylsulfathiazole to purified diets[24] resulted in poor growth, leucopenia, granulocytopenia, and a number of other symptoms in the rat (Daft and Sebrell, 1945; Robinson, 1951; Stokstad, 1954b). Earlier work showed that these lesions could be cured with liver extracts. As soon as pure folic acid became available, Daft and Sebrell (1943) showed that it would cure the blood dyscrasia produced by the sulfonamides. When sulfaguanidine was added to a purified diet, achromotrichia appeared in black rats; it was cured by a folic acid concentrate (Martin, 1942). The addition of certain

[24] In the absence of sulfonamide, the diets produced good growth and no abnormalities in the rats.

sulfonamides to purified diets resulted in a multiple deficiency in rats, which was cured by folic acid, biotin, and vitamin K (Daft and Sebrell, 1945; Stokstad, 1954b).

The deficiencies produced by the sulfonamides resulted in a decrease in the liver levels of folic acid (Ransone and Elvehjem, 1943). Supplementing the diet of the sulfonamide-fed rats with a liver extract increased folic acid levels in the livers of these rats above those of the controls (Schweigert *et al.*, 1945b). Sulfonamides also reduced the folic acid in the feces of rats; on a purified diet, the excretion was 5.3 μg. per day, which was reduced to 0.54 μg. by 0.5% phthalylsulfathiazole. On Purina Chow the excretion was 42.7 μg., which was reduced to 4.2 μg. by 2% succinylsulfathiazole (Miller, 1945). The fact that sulfonamide supplementation of the Purina ration lowered the fecal folic acid to only the same level as that on the unsupplemented purified diet may explain why a deficiency has not been produced under these circumstances on the stock diet.

In rabbits, the addition of succinylsulfathiazole to a purified diet free of folic acid produced a reduction in the urinary excretion of the vitamin by the fourth week (Olcese *et al.*, 1948; Simpson *et al.*, 1949). The sulfonamide also produced a reduction in rate of weight gain, but had no effect on the hemoglobin level (Simpson *et al.*, 1949).

Baby pigs (started at 4 days of age) fed a synthetic milk containing 2% sulfathalidine (on basis of solids) showed no signs of folic acid deficiency (Johnson *et al.*, 1948). Older pigs fed a purified diet containing 2% sulfasuxidine stopped growing after about 120 days and developed a normocytic anemia which could be cured with pure folic acid (Cartwright and Wintrobe, 1949). The anemia resulting from the sulfasuxidine was not apparent during the first 13 weeks of the experiment, nor were weight gains disturbed until after the sixth week (Cunha *et al.*, 1948). It was suggested that the baby pigs did not develop a folic acid deficiency when fed the sulfonamide because of the high protein level (30% casein) in their diets (Stokstad, 1954b), but the older pigs on a 26% casein diet did eventually develop a deficiency on a similar diet (Cunha *et al.*, 1948). These differences between baby pigs and the length of time required to produce a folic acid deficiency should be supplemented with studies of the urinary excretion and liver stores of the vitamin.

The chick's requirement for folic acid is not influenced by the addition of carboxylsulfathiazole, sulfamerazine (Oleson *et al.*, 1946), sulfadiazine, or sulfaguanidine (Hutchings *et al.*, 1946) to purified diets. Neither 1 nor 2% sulfathiazole added to a purified diet had any influence on growth or hemoglobin levels of chicks fed marginal amounts of folic acid over a period of six weeks (Robertson *et al.*, 1946). There is no apparent explana-

tion for the earlier reports of the sulfonamides increasing a folic acid deficiency in the chick (Briggs *et al.*, 1943; Luckey *et al.*, 1946a).

The folic acid requirement of the monkey was not influenced by the addition of succinylsulfathiazole (Elvehjem, 1948; Tappan and Elvehjem, 1953).

A change in the intestinal flora resulting from the feeding of sulfonamides was observed by a number of groups. It had been shown that *E. coli* and a number of other organisms commonly found in the intestinal tract of rats were able to synthesize folic acid when grown in a medium free thereof (Thompson, 1942). The addition of 0.5% sulfaguanidine or succinylsulfathiazole to purified diets produced a decrease in the *E. coli* counts in both the feces and cecal contents of rats within three days (Gant *et al.*, 1943; Miller, 1945). Ten days later, the counts began to increase and gradually returned to normal. It was suggested that the so-called "sulfa-resistant" organisms which increased in spite of continued sulfonamide feeding had lost the ability to synthesize certain nutrients required by the rat. That this may not be the case was shown by the work of Miller who found that the production of folic acid by *E. coli* was reduced in cultures containing increasing concentrations of sulfanilamide (Miller, 1944) or sulfathiazole (Miller, 1945). However, both the normal and sulfa-resistant strains of *E. coli* produced essentially the same amounts of folic acid.

The addition of certain sulfonamides to purified diets brings out the animal's requirement for folic acid, the presumed mechanism being through an interference with intestinal bacterial synthesis. However, it is apparent that more than an interference occurs, since rats showing prominent signs of folic acid deficiency still show high levels of the vitamin in the feces. Sulfonamides do not affect the requirement of the chick or monkey for folic acid.

e. Influence of Antibiotics on Folic Acid Synthesis. There is some indication that antibiotics have a slight sparing effect on the chick's dietary requirement for folic acid. This is based on the increased growth rate of chicks produced by penicillin or Aureomycin supplementation of a low folic acid ration containing large amounts of corn (Biely and March, 1951; March and Biely, 1953) and a slight growth stimulation produced by penicillin (Coates *et al.*, 1951; Coates, 1953), Aureomycin, or a mixture of penicillin and bacitracin (Rhodes *et al.*, 1954) added to a purified ration free of folic acid. The importance of nondietary factors in influencing the action of antibiotics in the folic acid requirement was stressed by Briggs *et al.* (1953b). They found that the first groups of chicks raised on a folic acid-free diet in new quarters showed only mild

signs of a deficiency. At that time the addition of Aureomycin to the diet accentuated the deficiency. Over a period of two years the deficiency in the chicks fed the basal ration increased and the effect of Aureomycin gradually disappeared.

Chicks that responded to antibiotics with an increased growth rate when fed a purified diet free of folic acid showed an increase in coliform bacteria and a reduction in lactobacilli in the intestinal tract (Rhodes *et al.*, 1954). Since the coliform bacteria have a higher synthetic activity as far as vitamins are concerned and the lactobacilli have a relatively higher requirement for folic acid, the above observations on the changes in intestinal flora of the antibiotic-supplemented chicks fit in with the hypothesis that the vitamin-sparing action of antibiotics is brought about through an effect on the intestinal flora.

In rats made deficient in folic acid by means of a diet containing sulfonamide, terramycin produced an increase in weight and concentration of folic acid in the liver (Halevy *et al.*, 1955). Although no data were presented, it was stated that the sulfonamide-fed rats had ceca which were enlarged to such an extent that their total folic acid content was greater in deficient animals than in the controls. When rats were fed the same diet without sulfonamide, streptomycin supplementation reduced the urinary excretion and liver concentration of folic acid but penicillin was without effect.

Five patients and a normal adult receiving 1 g. of streptomycin four times daily exhibited an increased urinary folic acid excretion which was accompanied by a marked reduction in fecal coliforms and an almost complete absence of other aerobes (Sarett, 1952).

There is currently some confusion about the action of Aureomycin in counteracting the toxicity produced in rats by feeding the folic acid antagonist aminopterin. The first reports indicated that Aureomycin did overcome the deficiency even though the antibiotic had no effect in overcoming the folic acid deficiency produced by sulfonamides. Since the citrovorum factor (a folic acid derivative) did cure both deficiencies, it was suggested that Aureomycin spared the rat's requirement for the citrovorum factor (Waisman *et al.*, 1951). Streptomycin, terramycin, penicillin, and chloromycetin were reported to be inactive in curing either type of folic acid deficiency (Waisman and Cravioto, 1952). However, Sauberlich (1953) found that neither Aureomycin nor penicillin was able to cure the deficiency produced by aminopterin. There is no explanation for these differences other than the variability in response that has been observed by different investigators who fed the same antibiotic under essentially the same conditions.

Antibiotics have a slight folic acid-sparing effect in chicks but apparently not in rats.

f. Influence of Diet on Folic Acid Synthesis. A variety of dietary constituents influence the intestinal synthesis of folic acid. Powdered milk (which was reported to have very little folic acid),[25] supplemented with minerals and vitamins other than folic acid, increased the folic acid content of the liver and feces of rats compared with the animals raised on a purified diet free of folic acid (Wright *et al.*, 1945). In order to produce a folic acid deficiency with the milk powder, 20% succinylsulfathiazole had to be used. Increasing concentrations of the sulfonamides progressively lowered the concentration of liver folic acid. It was only on the 20% level that the concentration reached the low levels seen on the purified diet containing 2% of the sulfonamide. The very low liver levels of folic acid were related to the development of a deficiency in the rats. No such relationship was seen in the fecal folic acid excretion. In another laboratory (Day *et al.*, 1946) the addition of 8% succinylsulfathiazole to either fresh or powdered whole milk (containing minerals and vitamins other than folic acid) resulted in a decreased rate of growth in rats which could be overcome by the addition of folic acid.

In low-fat diets, the type of carbohydrate exerted a marked influence on the folic acid requirement of chicks (Luckey *et al.*, 1946b). At all levels of folic acid, better growth was secured on dextrin than on sucrose diets. The glucose and corn starch diets produced growth which was in between that of the dextrin and sucrose diets. That dextrin has an influence on the intestinal microflora was shown by the finding that coliform bacteria isolated from dextrin-fed birds "produced significantly more folic acid than did those from the sucrose controls" (Monson *et al.*, 1954).

High-fat diets containing low levels of folic acid and dextrin as the carbohydrate produced lower weight gains and higher mortality in chicks than the low-fat diet (Luckey *et al.*, 1946b). It was only with relatively high levels of folic acid that good growth was secured. Additional evidence that fats, especially those that are rancid, increase the chick's requirement for folic acid was provided by March and Biely (1955). When they added 6% herring or cottonseed oil to a corn and herring meal ration, the oil

[25] The interpretation of folic acid balance studies such as these is open to a certain amount of question. There is a possibility that the analytical values for folic acid in foods may not be a true index of what is actually present. The work of Chang (1953) emphasized this point. He showed that when special precautions were taken, as much as ten times the usually accepted values could be secured for the folic acid content of liver.

depressed growth. Addition of folic acid restored the growth rate of chicks to that on the ration containing no extra oil. A highly rancid oil depressed growth more than the regular herring oil. The addition of folic acid to the ration overcame the depression. There is a possibility that folic acid may be destroyed by the rancid oils and that the above effects are not due to any direct action of the oils on the intestinal flora. Since only weight data were given in the above reports, no conclusion can be made about the mechanism involved.

Purified rations containing high levels (50%) of casein did not produce as good growth in chicks when graded levels of folic acid were added to the ration as did a ration containing lower levels (18% casein plus 10% gelatin) (Luckey *et al.*, 1946b).

A number of vitamins have been reported to affect the requirement for folic acid. The extent to which these substances produce the observed effects by acting on the microflora is unknown. For this reason only a brief reference will be made to them. In the early work on folic acid, O'Dell and Hogan (1943) reported that the incidence of anemia in chicks fed a folic acid-deficient ration was reduced when additional pyridoxine was added thereto. They believed that pyridoxine "aids in the bacterial synthesis of the anti-anemia vitamin in the intestine."

Ascorbic acid has been related to folic acid activity for a long time. The early observation of Briggs *et al.* (1944) of a growth-stimulating action of ascorbic acid for chicks may have been associated with its sparing effect on the crude folic acid concentrate used (Dietrich *et al.*, 1949). When crystalline folic acid was used, the addition of ascorbic acid (100 mg. %) to a purified diet increased both weight gains (from 19 to 33 g. in two weeks) and liver levels of folic acid (from 2.82 to 4.65 μg./liver) (Dietrich *et al.*, 1949). Increases comparable to these were also seen when the diets contained vitamin B_{12} or suboptimal amounts of folic acid. Since ascorbic acid was shown to stimulate certain organisms (Rohn and Hagerty, 1938), it seemed logical to suggest that it was acting in a similar manner on the bacteria which synthesized folic acid in the intestinal tract of the chick.

Additional evidence for a sparing effect of ascorbic acid on the folic acid requirement comes from the work of May *et al.* (1952). When monkeys were raised on dried cow's milk supplemented with minerals and vitamins other than ascorbic acid, folic acid, and vitamin B_{12}, they developed scurvy and a megaloblastic anemia; the latter was cured by folic acid. When ascorbic acid was added to the diet, the improvement in the monkeys was accompanied by an increase in liver folic acid. This increase occurred only with *l*-ascorbic acid (nutritionally active form)

but not with the *d*-form. If the ascorbic acid were acting on the intestinal bacteria to stimulate folic acid synthesis primarily through an oxidation-reduction potential, then the *d*-isomer should have been as active as the *l*-isomer. As the King of Siam said, "It is a puzzlement."

Folic acid synthesis in the intestinal tract of the guinea pig was increased by the addition of agar, potassium acetate, and magnesium oxide to a purified diet (Wichmann *et al.*, 1954). Since an increase in the bulk of the stools was associated with an increased fecal content of riboflavin (see Section IV, 3a, 3b), it is likely that the agar in the preceding work was the agent primarily responsible for the increased folic acid excretion in the feces of the guinea pigs.

There are a number of dietary factors that influence the synthesis of folic acid by intestinal bacteria. As measured by growth, folic acid is more available to both the rat and chick when dextrin rather than sucrose is the carbohydrate in the diet. Ascorbic acid increases the folic acid available to both monkeys and chicks but its mode of action is unknown.

9. Vitamin B_{12}

Although vitamin B_{12} has existed in crystalline form for only about eight years, a tremendous number of studies related to it have been made. After the isolation of the vitamin a number of related compounds were secured. These compounds were closely related to vitamin B_{12} in structure and like it were effective in stimulating the growth of some microorganisms used in its assay. They were, however, inactive for the rat (Lewis *et al.*, 1952) and for chicks (Coates *et al.*, 1953). Unfortunately, many investigators have not distinguished the different compounds and have reported their results only in terms of vitamin B_{12} when, in reality, they were probably measuring a certain amount of pseudo vitamin B_{12} together with the compound active for higher animals (true vitamin B_{12}).

a. Excretion Studies—Animals. In order to study the intestinal synthesis of vitamin B_{12} in rats, Davis and Chow (1951) maintained rats on a vitamin B_{12}-deficient diet for their first year of life. When radioactive cobalt was added to the diet, radioactivity appeared in the butanol extract of the feces (in which vitamin B_{12} is soluble and inorganic cobalt practically insoluble). The addition of 40 mg. % Aureomycin to the diet stimulated vitamin B_{12} synthesis in the gastrointestinal tract of the rat as shown by an increase of two- to threefold in the radioactivity of the butanol extract of the feces. They attempted to show that the substance soluble in butanol was vitamin B_{12} by the close correlation between its growth-stimulating activity in the microbiological assay and its radio-

activity, as well as its similarity to vitamin B_{12} on chromatographic analysis. In view of more recent work which indicates that it is very difficult in some cases to separate vitamin B_{12} from its related substances (Briggs and Daft, 1955), there is a good possibility that the results of Davis and Chow (1951) were complicated by the presence of pseudo vitamin B_{12}.

Additional evidence for the synthesis of vitamin B_{12} by the intestinal microflora of rats was presented by Johansson *et al.* (1953), who found a high concentration of the vitamin in the colons of rats maintained on a diet free of vitamin B_{12}. Here again, part of the activity was probably due to pseudo vitamin B_{12}.

Rabbits fed a corn-soybean diet deficient in vitamin B_{12} show a high microbial intestinal synthesis of the vitamin. In the presence of minimal amounts of vitamin B_{12} in the feed, the fecal excretion was 221 times greater than the intake (Kulwich *et al.*, 1953). When a commercial stock ration was fed, the rabbits that were fitted with collars which prevented coprophagy excreted soft feces that contained 2 μg. of vitamin B_{12} per gram (Kulwich *et al.*, 1953). Actually, the intestinal flora of all animals synthesize so much vitamin B_{12} that the intestinal contents of slaughtered animals have been proposed as a commercial source of the vitamin (van Oss, 1954).

There is still some question about the amount of vitamin B_{12} synthesized in the intestinal tract of the nonruminant. Reports have appeared which indicate that freshly voided feces from chickens (McGinnis *et al.*, 1947), swine (Groschke *et al.*, 1950), and horses (Miller and Groschke, 1950) contained no vitamin B_{12}, but on standing at room temperature for several hours, the feces showed high levels of the vitamin. Since no special precautions were described in the studies with rats and rabbits, some of the reported vitamin content may have been formed after the feces were voided.

b. Excretion Studies—Man. Shortly after the isolation of vitamin B_{12}, it was shown that the feces of normal individuals contained large amounts of vitamin B_{12} (Bethell *et al.*, 1948; Callender *et al.*, 1949). Later work indicated that there was no difference in the vitamin B_{12} excretion between normal people and those with pernicious anemia (Girdwood, 1950; Callender and Spray, 1951). Both groups excreted in their feces from 4 to 22 μg. of vitamin B_{12} per day. According to Heinrich and Lahann (1954), the average human diet provides 0.2 to 3.5 μg. of vitamin B_{12} per day. Since a normal individual excretes considerably more vitamin B_{12} than the reported intake, a large share of the fecal excretion must represent intestinal synthesis.

A note on a balance study showed that normal young women receiving

1.1 μg. vitamin B_{12} per day excreted in their feces from 8 to 65 μg. of the vitamin (Broberg *et al.*, 1953). In this case precautions were taken to minimize bacterial action after the feces were voided. For this reason the large amounts of vitamin B_{12} in the feces must have been synthesized in the intestinal tract.

In a review on the importance of the intestinal bacteria in vitamin B_{12} nutrition, Hausmann (1955) mentioned that a patient with pancreatitis who was fed intravenously for 23 days and given 8 g. phthalylsulfathiazole daily by mouth still excreted in the feces 1 μg. of vitamin B_{12} per day toward the end of the study. Another patient who had approximately half his large intestine removed showed a normal excretion of vitamin B_{12} in his stools. The difficulty in interpreting excretion studies involving vitamin B_{12} is shown by the report (Ludwig, 1951) that even after 30 days of starvation, a fecal sample secured from a patient still contained some vitamin B_{12} as shown by microbiological assay. However, the red pigment isolated from the feces was inactive when injected into a patient with pernicious anemia.

There is ample evidence that large amounts of vitamin B_{12} are synthesized in the intestinal tracts of both animals and man. As yet, only minor attention has been given to the characterization of the material in feces which gives a positive response in the microbiological assays. It is doubtful whether very much, if any, of the vitamin B_{12} synthesized by the intestinal microflora is available to most nonruminants unless feces are consumed.

c. Influence of Sulfonamides on Vitamin B_{12} Synthesis. There is one report which indicates that phthalylsulfathiazole may reduce the rat's requirement for vitamin B_{12}. Schultze (1950) reported that the addition of the sulfonamide to a soy-protein ration reduced the incidence of uremic poisoning in young rats from 35 to 6%. Since the same effect was seen following vitamin B_{12} injection into the young rats, Schultze suggested (but provided no proof) that the sulfonamide stimulated the synthesis of vitamin B_{12} by the intestinal microflora.

The addition of sulfamethazine was reported to have no effect on the growth of chicks fed a vitamin B_{12}-deficient diet supplemented with various levels of the vitamin (Stokstad and Jukes, 1951).

d. Influence of Antibiotics on Vitamin B_{12} Synthesis. Much of the work on the relationship between antibiotics and vitamin B_{12} has been limited to growth studies. The assumption has frequently been made that where the antibiotics produced stimulation in growth, they did so as a result of influencing the intestinal flora in such a way that more of the vitamin became available to the host. This assumption may not be valid, since a number of substances, especially methionine, can reduce the animal's

requirement for the vitamin. It has not, in all cases, been possible to determine which substance was being spared by the antibiotic. The interpretation of most growth studies is limited by that complication.

Growth of rats fed a vitamin B_{12}-free diet was stimulated by the addition of antibiotics (Johansson *et al.*, 1953; Peterson and Johansson, 1953–4). Although Johansson *et al.* (1953) originally reported that with the microbiological assay they could detect "no conclusive effect of Aureomycin on the concentration of vitamin B_{12} in the intestinal tract" of rats, a subsequent paper from the same laboratory reported a highly significant effect. In the later work they reported that Aureomycin increased the "free" (noncellular) vitamin B_{12} in the colon and cecum (Peterson *et al.*, 1953). The increase was greatest when small amounts of the vitamin were present in the diet. In practically all cases the increase of "free" vitamin B_{12} was at the expense of the "bound" (cellular) vitamin with no change in the total vitamin activity. Furthermore, they reported a highly significant ($p = <0.005$) correlation between weight gain of the rats and the free vitamin B_{12} in the colon. A possible explanation for the high correlation is the consumption of feces which may have occurred according to the report. Coprophagy seems like the most logical explanation, since one would hardly expect any large amount of nutrients to be absorbed in the colon.

For swine, the evidence for a vitamin B_{12}-sparing effect of antibiotics is contradictory. Some laboratories have reported a growth stimulatory effect from the antibiotics while others secured no effect. This is true for baby pigs fed synthetic soybean milk deficient in vitamin B_{12} (Wahlstrom and Johnson, 1951; Sheffy *et al.*, 1952) and for older pigs (Catron *et al.*, 1951a, 1953b; Kline *et al.*, 1954). The variability that can be expected in this area was brought out by a collaborative study in Great Britain, where the antibiotics stimulated the growth of pigs on all-vegetable rations at some laboratories but not at others (Braude *et al.*, 1955).

Growth responses in pigs resulting from the addition of procaine penicillin, Aureomycin hydrochloride, and Aureomycin or streptomycin fermentation mashes to an all-vegetable ration deficient in vitamin B_{12} were reported by Barber *et al.* (1953). In all experiments the antibiotics produced a significant increase in body weight gain. Since the diets were deficient in vitamin B_{12}, this might be considered presumptive evidence for a vitamin-sparing effect. However, there was no effect of the antibiotics on the vitamin B_{12} concentration in the contents of the stomach, duodenum, cecum, or colon. Unfortunately, it is impossible to determine whether they measured total or "free" vitamin B_{12} activity.

Contradictory reports on the vitamin B_{12}-sparing effect of antibiotics for chicks have appeared from the same laboratory (Oleson *et al.*, 1950;

Stokstad and Jukes, 1951). Another report of a growth-stimulating effect following the addition of antibiotics to a vitamin B_{12}-free diet can be criticized for the poor growth of the chicks in even the best groups (Jenkins *et al.*, 1954). The diet must have been deficient in a number of factors, since the New Hampshire X Barred Plymouth Rock birds gained only 146 g. in four weeks on the best diet, whereas these birds should have gained more than 250 g. in that time (Stokstad and Jukes, 1951).

Feeding four patients with pernicious anemia 2 or 3 g. of Aureomycin per day was reported by Lichtman *et al.* (1950) to produce a hematological response which was slow in all cases as far as red cell count and reticulocytosis were concerned. Since pernicious anemia is presumably due to a deficient production of the intrinsic factor, it is hard to see how this study has any bearing on the "vitamin B_{12}-sparing effect" of antibiotics. It should be mentioned that Ungley (1950) gave 3 g. of Aureomycin per day for five days to one patient with pernicious anemia and found no change in the hematological picture.

The evidence at best is only suggestive that antibiotics may spare the requirement of certain species for vitamin B_{12}; this is true for poultry and swine, whereas most of the work indicates that rats do not show the effect. There is, however, evidence that antibiotics increase the intestinal synthesis of vitamin B_{12} in the rat.

e. Influence of Diet on Vitamin B_{12} Synthesis. Cobalt is the first dietary factor that comes to mind in considering the dietary components that may influence vitamin B_{12} synthesis. There is sufficient evidence that ruminants maintained on a low-cobalt diet show an increase in intestinal synthesis of vitamin B_{12} and a restoration of normal growth and development when the diet is supplemented with cobalt (for review see Kon and Porter, 1954, and Heinrich and Lahann, 1954).

The evidence for an increase in vitamin B_{12} synthesis in the gastrointestinal tract of nonruminants following cobalt supplementation is not so dramatic as is the case with sheep, but it does appear to occur. Although rats on a vitamin B_{12}-deficient diet do not respond to added cobalt with an increased rate of growth (Tappan *et al.*, 1950; Lewis *et al.*, 1952), they do show an increased concentration of vitamin activity (probably some of it as pseudo vitamin B_{12}) in their intestinal contents (Lewis *et al.*, 1952) and in their feces (Davis and Chow, 1951). The type of diet used may influence the growth responses to added cobalt, since a note by Burns and Salmon (1954) indicated that growth stimulation attributable to cobalt was seen only when the ration contained less than 0.2% choline.

For the pig, cobalt supplementation of a ration low in vitamin B_{12} has been reported by two groups to stimulate growth (Dinusson *et al.*,

1950; Robinson, 1950), while another laboratory (Kline *et al.*, 1954) found that the mineral produced no effect when added to a purified ration containing 0.1 p.p.m. of cobalt and no vitamin B_{12}. No increase in the intestinal synthesis of vitamin B_{12} was observed when radioactive cobalt was fed to pigs for a six-week period (Braude *et al.*, 1949).

For chicks, the reports indicate that the addition of cobalt to a ration low in vitamin B_{12} may increase weight gains (Burns and Salmon, 1954; Davis *et al.*, 1953; Jenkins *et al.*, 1954).

The amount and kind of protein in the diet may have some influence on the intestinal synthesis of vitamin B_{12}. On a protein-free diet rats excreted considerably less vitamin B_{12} (as measured by the radioactivity of the butanol extract of feces following an oral dose of radioactive cobalt) then they did when soybean meal or casein was in the diet (Chow *et al.*, 1953). A patient with pernicious anemia who was fed a protein-rich normal diet for five days and then a diet free of animal protein showed the same fecal vitamin B_{12} excretion regardless of the diet fed (Ludwig, 1951). Another patient with pernicious anemia who was transferred from a normal diet to one providing only 1 g. of protein per day also showed no change in fecal vitamin B_{12} excretion.

On the basis of the growth rate of rats maintained on a vitamin B_{12}-free diet, Peterson *et al.* (1953) showed that dextrin produced better growth than sucrose. They secured presumptive evidence that the difference between the carbohydrates was due to their effect on intestinal synthesis of vitamin B_{12}; supplementation of the sucrose ration with vitamin B_{12} produced slightly better growth than on the unsupplemented dextrin diet. Lewis (1952) found that the intestinal synthesis of vitamin B_{12} in the rat is higher when the diet contains dextrose than when it contains sucrose.

Mink are unusual in that they showed a higher intestinal vitamin B_{12} synthesis when sucrose was present in the diet than when dextrin diets were fed (Leoschke, 1953; Leoschke *et al.*, 1953). Evidence therefor was provided both by the increased growth on diets free of sucrose and vitamin B_{12} and by the higher concentration of the vitamin in the feces (0.98 μg./24 hr. on sucrose vs. 0.16 μg. on dextrin).

The superiority of sucrose over dextrin should be checked, since the results may be important in evaluating the explanation offered for the development of the refected state. The accepted explanation therefore involves the rats' relatively poor digestibility of starch, which permitted large amounts of the carbohydrate to reach the lower part of the intestinal tract, where its presence stimulated microbial activity (see Section I, 2b). Do the observations of Leoschke *et al.* (1953) mean that sucrose is not readily absorbed by mink? Further work should be done in an effort to

determine why sucrose has a vitamin B_{12}-sparing action in mink. Since the requirements of mink for unidentified factors are similar to those of foxes (Schaefer *et al.*, 1948), one might wonder whether foxes show the same response to sucrose.

There are a number of suggestions that a variety of vitamins added to a vitamin B_{12}-free diet spare the requirement for that substance. Whether the action of these vitamins is mediated through an increased intestinal synthesis is hard to determine. Thiamine (Bhagwat and Sohonie, 1954), pantothenic acid (Yacowitz *et al.*, 1951; Evans *et al.*, 1951; Catron *et al.*, 1953a), folic acid (Dietrich *et al.*, 1949), and riboflavin (Cooperman *et al.*, 1952) have been so implicated. A number of degradation products of vitamin B_{12} fall in this category (Emerson *et al.*, 1950, 1951; Lambooy and Haley, 1952; Cooperman *et al.*, 1952).

The large amount of work that has appeared on the factors that may influence the animal's requirement for vitamin B_{12} has tended to confuse rather than clarify the situation. It might be helpful if future workers in this area were to consider the influence that dietary alterations might have upon such factors as methionine and choline, which are known to spare the requirement of animals for vitamin B_{12}, and to supplement growth studies with analyses of tissues for vitamin B_{12} and intestinal contents for "free" vitamin B_{12}. Finally, an attempt should be made to determine whether the substances are "true" or "pseudo" vitamin B_{12}.

f. General. There is very little vitamin B_{12} in the intestine of fasting man or horse above the ileocecal valve (Dyke and Hind, 1952). The concentration of the vitamin in the ileum contents of the horse was 0.008 μg. per g., while the cecum contained 0.055 μg. per g. The vitamin B_{12} isolated from the intestinal contents of the horse was shown to be as active as the crystalline vitamin when injected into a patient with pernicious anemia (Dyke and Hind, 1952).

From a physiological standpoint, it is important to know whether the substances synthesized in the intestinal tract are available to the host. The studies on the absorption of vitamin B_{12} from the lower part of the intestinal tract indicate that under normal conditions only little vitamin B_{12} is absorbed in that part of the intestine. When tremendous doses of vitamin B_{12} are introduced into the lower part of the intestinal tract, a small amount can be absorbed. Ungley (1951) reported that "doses of free vitamin B_{12} as large as 1000 to 3000 μg. administered rectally sometimes gave responses no less than those one would expect from the same doses given orally." With smaller doses of vitamin B_{12} (25 μg. per day) plus normal gastric juice given by enema, no reticulocyte response was seen whereas the same dose by mouth was active (Girdwood, 1950). It is interesting to note that as early as 1931 Sharp *et al.* showed that a

reticulocyte response could be secured when desiccated hog stomach was given to a patient with pernicious anemia by rectum.

Contradictory conclusions have been secured on the absorption of vitamin B_{12} from the ceca of birds. Shrimpton (1954) found that chicks maintained from birth on a vitamin B_{12}-free diet showed an increase in carcass vitamin B_{12} content and that the substitution of potato starch for cornstarch in the vitamin B_{12}-free diet fed laying hens increased the vitamin B_{12} in the eggs. Although Shrimpton found no change in cecal pH or concentration of vitamin B_{12} as a result of substituting potato starch for cornstarch, he still felt that the vitamin in the cecum became more available to the birds. That the absorption of vitamin B_{12} from the cecum may be of only minor importance was reported by Jackson *et al.* (1955). They found that cecectomized birds did not develop a vitamin B_{12} deficiency any more rapidly than normal birds and that only minimal amounts of the vitamin were absorbed when radioactively labeled vitamin B_{12} was injected into the cecum.

A number of studies have shown that a variety of bacteria can synthesize vitamin B_{12} (Noer, 1950; Tanaka *et al.*, 1952; Darken, 1953; Witkeus *et al.*, 1954). The ability of some bacteria which are common inhabitants of the intestinal tract to synthesize vitamin B_{12} suggests that under proper conditions such synthesis could be stimulated.

There is as yet no conclusive experimental evidence to show that increased synthesis of vitamin B_{12} by microorganisms in the intestinal tract results in increased absorption by the host. Under certain conditions the feces contain large amounts of the vitamin even though dietary supplementation is necessary to prevent deficiency. It is generally believed that absorption of the vitamin takes place high in the small intestine. Von Bonsdorff (1953) found that the level at which the tapeworm adhered to the intestinal wall was important in the appearance of "tapeworm pernicious anemia." When a small amount of an anti-tapeworm drug (filicin) was given to dislodge the tapeworm adhering to the duodenal wall, the anemia in the patient cleared up spontaneously, even though the worm was present in the lower part of the intestine. On the basis of this and related observations it appears doubtful that significant amounts of vitamin B_{12} that may be formed in the lower part of the intestinal tract are absorbed directly.

10. Ascorbic Acid

The destruction of ascorbic acid by intestinal bacteria has been reviewed in Section II, part 3. It is doubtful whether microbial destruction of this vitamin ever plays an important role in nutrition. The primary basis for this statement is the observation of Young and James (1942) that when the medium contains 1% glucose, practically none of the

ascorbic acid is destroyed during the first 15 hours.[26] Under normal circumstances ascorbic acid is consumed in foods which contain at least 1% glucose, and so the vitamin should be protected.

The rat does not require a dietary source of ascorbic acid. However, when succinylsulfathiazole was added to a purified sucrose ration, the level of the vitamin in the liver was reduced from 300 to 200 μg./g. (Schwartz and Williams, 1952). Since the reduction in liver ascorbic acid was not associated with an increased urinary excretion or a transfer of the vitamin to other organs, and since the sulfonamide is poorly absorbed from the intestinal tract, Schwartz *et al.* (1954) concluded that the sulfonamide was interfering with the synthesis of some essential precursor of ascorbic acid by the intestinal flora.

It is worth noting that a group of rats that had been on the purified diet supplemented with succinylsulfathiazole for six weeks (at that time their liver ascorbic acid should have been down to 200 μg./g.) were then put on a diet of natural foods plus the sulfonamide. The liver level of the vitamin increased to 350 μg./g. within 12 days. This level was higher than that seen in rats on the basal purified diet. Could it be that the cellulose or other ingredients in the natural diet stimulated bacterial synthesis of an ascorbic acid precursor in the same way as riboflavin synthesis is increased in the intestinal tract by inert fillers (see Section IV, 3a)?

11. Vitamin A

Refection could not be produced in the rat, according to Fridericia *et al.* (1927), when a vitamin A-free diet was fed. This diet was made by oxidizing the lard used in their rice-starch ration.

It is not known whether the substances found by LePage and Pett (1941) in the feces of individuals receiving large doses of vitamin A resulted from bacterial action. These workers presented evidence indicating that the substances were probably degradation products of vitamin A.

There are a number of reports indicating that the growth-promoting effects of dietary antibiotics in poultry are associated with increased stores of vitamin A. Burgess *et al.* (1951) showed that when sodium penicillin G was added to a practical grain ration, the chicks grew considerably better than those fed the unsupplemented diet. The heavier chicks had 748 I.U. vitamin A in their livers compared with 423 I.U. for the lighter ones. The carotenoid level of the serum was also higher in the birds receiving penicillin (402 vs. 261 μg./100 ml.). The differences

[26] A possible explanation for this observation is the work which shows that glucose may be an important precursor in the synthesis of ascorbic acid (Horowitz *et al.*, 1952; Nath *et al.*, 1953).

in liver vitamin A were not associated with any change in percentage of protein, fat, or cholesterol fractions in the liver (Burgess *et al.*, 1953).

Coates *et al.* (1952) presented evidence supporting the above. Their data included a number of groups of chicks fed a practical diet with a fairly wide range of body weights. When the reviewer plotted body weights against liver vitamin A levels, it was apparent that the higher vitamin A levels were associated with the heavier birds (Fig. 1), and on this basis there was no difference between the penicillin-supplemented and control groups. Figure 1 does not include the data from one experiment in which the livers of the control and the unsupplemented birds

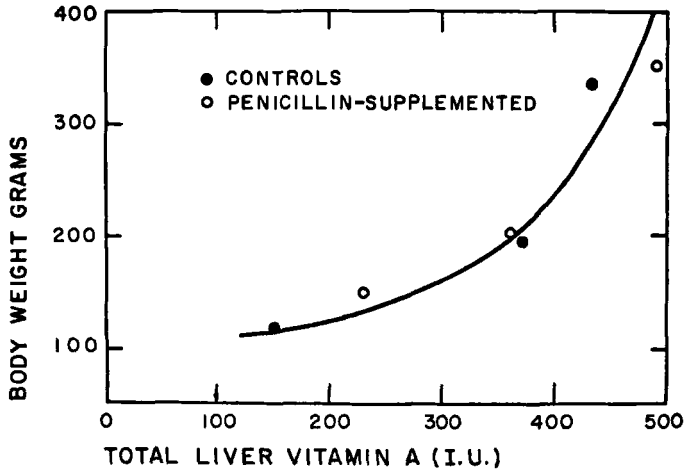

FIG. 1. Relation of liver vitamin A content to body weight of chicks (from Coates *et al.*, 1952).

represented 7.8 and 8.1% of the body weights. In the other six experiments the values ranged from 2.2 to 3.2%. The British workers were aware of the possible relationship presented in Fig. 1 even though they did not plot the data, for they state: "It is clear that the antibiotic raised the liver stores of vitamin A only when it also accelerated growth, hence in all probability the effect is not specific."

A number of factors made Coates and co-workers believe that the growth-promoting effects of antibiotics were associated with changes in the intestinal wall. In some of the preceding work they thought that the intestinal walls of the birds receiving penicillin were thinner than those of the control birds. When the length and weight of the intestinal tract were measured, substantiating evidence was secured (Coates *et al.*, 1955). On the basis of these findings they postulated that in the presence of a "high disease level" the intestinal wall is increased in thickness as

part of a defense mechanism. The thicker wall decreased the absorption of bacterial toxins and reduced the possibility of bacteria gaining entrance to the body. With the thickening of the intestinal wall went a decrease in the absorption of nutrients such as vitamin A. An analogous proposal was made by Almquist and Maurer (1955).

There are a number of factors that will have to be considered before the above hypothesis can be accepted. The most important is a re-evaluation of the data on liver and serum vitamin A levels. So far the only data which permit one to make any calculations show that there is no increased absorption of vitamin A by the penicillin-supplemented birds, at least not on the basis of liver vitamin A (Fig. 1). The data of Barber *et al.* (1953) indicate that in pigs there is no consistent relation between liver stores of vitamin A and growth-promoting effect of various antibiotics. A similar effect was reported for chicks in which the addition of Aureomycin or a mixture of Aureomycin and penicillin to a practical diet increased the concentration of both carotene and vitamin A in the livers of young birds without having any effect on the rate of weight gain (Almquist and Maurer, 1955). Furthermore, Coates *et al.* (1955) did not observe any differences when the intestinal tracts were examined histologically. Had there been a significant difference in the thickness of the wall, that should have been evident on microscopic examination of the fixed tissues. (See also Nutrition Reviews, 1956.)

The condition of the intestinal wall may be an important factor in explaining the growth-promoting action of antibiotics and should certainly be investigated from that standpoint. Even though the liver vitamin A does not appear to support the suggestion of increased absorptive activity, there are other observations which bear on this point. Catron *et al.* (1951b, 1953a) observed a faster rate of glucose absorption in pigs showing a growth response to antibiotics. Rats fed a diet containing streptomycin were reported to have an increased vascular bed around the intestinal tract (Sieburth *et al.*, 1954) which might be a mechanism for increasing intestinal absorption.

In rats, Aureomycin added to purified rations containing different levels of vitamin A does not stimulate growth (Hartsook *et al.*, 1953). Actually, Aureomycin accentuated the deficiency in the rats receiving the vitamin A-free diet. The antibiotic had no influence on either the concentration or the total amount of vitamin A in the livers of the rats.

In pigs fed all-vegetable rations the addition of either Aureomycin, penicillin, or streptomycin increased the growth rate to the same extent (Barber *et al.*, 1953). However, only Aureomycin increased both the concentration and total vitamin A in the livers, and penicillin and streptomycin had no effect on vitamin A stores.

Antibiotics in some cases have produced considerable weight gains in chicks raised on vitamin A-low diets. The livers of the birds showing the increased growth contained more vitamin A than the unsupplemented controls. However, the present evidence does not support the hypotheses that the antibiotics increased vitamin A absorption from the intestine.

12. Vitamin D

Only one paper was found in which the influence of sulfonamides on vitamin D metabolism was studied. Jukes and Stokstad (1946) added 0.5% sulfaguanidine to the AOAC[27] rachitic diet fortified with adequate B vitamins. Under these circumstances the sulfonamide had no influence on the utilization of vitamin D by chicks as shown by tibial bone ash at four weeks of age.

The situation regarding antibiotics is not so clear. There are a number of reports indicating that penicillin may have a sparing effect on the vitamin D requirement of rats and chicks. Migicovsky *et al.* (1951) used "The percentage of an oral dose of Ca^{45} which appears in the tibia 48 hours after dosing" as an index of the effectiveness of penicillin in sparing the rat's requirement for vitamin D. On the AOAC rachitogenic diet, 30 mg. of penicillin per kg. of diet increased the percentage absorption from 1.14 to 1.92. Whether this difference is significant will have to be determined by future work. Rekling *et al.* (1954) found a greater dose-response curve in penicillin-supplemented rats on the basis of the size of the calcified area of the tibia (planimetric measurement of the line test—see Bliss and György, 1951). A report similar to this has been published by Murray and Campbell (1955). In the latter case Aureomycin increased calcification only when the results for 10 assays were pooled, and even then the difference between the supplemented and unsupplemented rats was significant at only the 5% level.

There is a possibility that antibiotics may spare the chick's requirement for vitamin D. Ross and Yacowitz (1954) found that at all but the lower levels of vitamin D, penicillin-supplemented diets produced chicks with a higher bone ash than the unsupplemented diet. Lindblad *et al.* (1952) state that "previous work in our laboratories showed that dietary antibiotics reduced percentage of bone ash in chicks fed diets containing the levels of calcium and phosphorus recommended by the NRC."[28] In the same abstract they refer to their work with chicks which suggested that "aureomycin improves the utilization of calcium and phosphorus." Since the egg shell contains a large amount of mineral, the increase in density thereof resulting from penicillin supplementation of the hen's

[27] See Association of Official Agricultural Chemists, 1945, p. 625.

[28] National Research Council Publication No. 301.

diet (Gabuten and Shaffner, 1952) might indicate a vitamin D-sparing effect.

The evidence is at best only suggestive that antibiotics may spare the dietary vitamin D requirement of chicks. The data on which some of the above statements are based will have to become available before a final conclusion can be reached. Even if the evidence shows that antibiotics do spare the animal's dietary requirement for vitamin D, it may involve no action on the intestinal microflora, since the tests used in these studies are influenced by changes in the availability of minerals.

13. Vitamin E

Although some of the early work on vitamin deficiencies produced by feeding sulfonamides to rats showed that necrosis and calcification of skeletal muscle occurred in such cases (Ashburn *et al.*, 1942), it appears doubtful whether the observations can now be attributed to an interference with intestinal synthesis of vitamin E. The early work showed that the hyalinization, necrosis, and calcification of voluntary muscle in rats fed succinylsulfathiazole could be prevented by feeding α-tocopherol (Daft *et al.*, 1943). On the basis of such observations it was reasonable to suggest that sulfonamides interfered with the intestinal bacterial production of vitamin E (Daft and Sebrell, 1945; Sebrell, 1943–44). Very little work has been done on this problem since then. A recent report (Dessau *et al.*, 1954) on "necrosis of heart muscle fibers" in mice fed sulfaguanidine makes the earlier interpretation questionable. The heart lesions developed only in the complete absence of vitamins E and K. No lesions were seen when either of the vitamins was added to the purified diet containing thiamine, pyridoxine, riboflavin, calcium pantothenate, choline chloride, and vitamins A and D.

A note by Pindborg (1949) claims there is intestinal synthesis of vitamin E in rats fed a stock diet. The conclusion was based on the assumption that all of the dietary vitamin E was absorbed and on the observations that when feces of rats on the stock diet were added to a vitamin E-free diet at a level of 20%, rats did not show the incisor depigmentation seen in controls. That vitamin E in the feces may be of dietary origin, especially when the intake is as great as that from a stock diet, was shown by Klatskin and Molander (1952b). Actually, on the basis of incubating feces or jejunal fluid with α-tocopheryl acetate, it appears that in man at least there is no evidence of either synthesis or destruction of α-tocopherol in the gastrointestinal tract (Klatskin and Molander, 1952a).

The entire field of vitamin E metabolism is confusing at the present time, since there is one report that sulfaguanidine at a level of 1% in a purified diet containing all of the B vitamins required by the rat, plus

vitamin K, exerts a sparing action on the rat's requirement for vitamin E as shown by incisor depigmentation (Granados *et al.*, 1950). The preceding interpretation is open to question, since it has been shown that a number of deficiencies other than vitamin E will produce depigmentation of the incisors in rats (for discussion see Moore and Mitchell, 1955). Furthermore, there are indications that a number of compounds can replace the vitamin E requirement of animals (Dam *et al.*, 1951; Dam and Granados, 1952).

It is difficult to determine whether compounds such as the sulfonamides, which under some conditions increase the animal's requirement for vitamin E, do so as a result of producing "lipid peroxides in the tissues when insufficient vitamin E has been taken in" (Hove, 1955), or by some other mechanism.

14. Vitamin K

a. Excretion Studies—Animals. The dietary requirement of the young chick for vitamin K became apparent with the work of Dam (1935a,b) and Almquist and Stokstad (1935a,b). Since the vitamin was readily synthesized by bacteria in both water, when it became contaminated with feed, and feed, when it became wet (Ansbacher, 1939; Almquist, 1941), special precautions have to be taken to secure signs of the deficiency. A vitamin K deficiency may not appear if the diet of the breeding hens contains large amounts of alfalfa or other potent sources of the vitamin (Almquist and Stokstad, 1936).

The presence of vitamin K in the feces of birds showing deficiency signs was reported by Almquist and Stokstad (1936). An ether extract of these feces added to the deficient diet at a level equivalent to 5% of the original feces protected day-old chicks from hemorrhages. About half the vitamin K was synthesized after the feces were voided, since collecting feces directly in a phenol solution reduced the vitamin potency. These workers suggested that "the vitamin was evidently synthesized by bacterial action." Some birds were able to utilize vitamin K synthesized in the lower intestinal tract, since these workers mentioned "cases of spontaneous recovery which we have frequently observed."

The vitamin K requirement of older birds is questionable, according to the Committee on Poultry Nutrition of the National Research Council (National Academy of Science, 1954). This report states that "mature birds do not seem to be subject to the acute vitamin K deficiency, indicating that they may synthesize the vitamin to some extent." That older birds still require vitamin K is shown by the development of a hemorrhagic condition among laying hens given certain sulfonamides in their feed (see Section IV, 14c).

Until recently the intestinal flora would have been accepted as the source of the vitamin K for those birds that can presumably get along without any dietary supply. The recent report on the nutritional requirement of germ-free chicks at first glance makes the earlier postulate slightly suspect. Luckey *et al.* (1955) reported that germ-free chicks maintained on vitamin K-free diets did not show bleeding tendencies. The blood-clotting time increased in these chicks during the first week after hatching, but then promptly returned to normal and remained there for the duration of the 24-day experiment. From whence comes the vitamin K apparently required by the germ-free chick? One is tempted to say that instead of clarifying the situation as was originally anticipated (i.e., confirming preconceived ideas), the germ-free animal work has only compounded our confusion, at least in this particular area. It is obvious that there is still a great deal of work to be done before a final answer can be secured on the role of intestinal flora in supplying the vitamin requirement of man and animals.

The first interpretation that might be made of the observations of Luckey *et al.* is that germ-free chicks synthesize vitamin K in their tissues. Other possibilities are that germ-free birds do not require vitamin K, or that the birds were accidentally contaminated with bacteria. However one wishes to interpret the work of Luckey *et al.*, it is well to bear in mind the fact that a hemorrhagic condition develops in patients whose bile has been diverted from the intestinal tract (Quick *et al.*, 1935; Hawkins and Brinkhouse, 1936) and that this condition can be cured by the oral administration of vitamin K together with bile (Warner *et al.*, 1938; Butt *et al.*, 1938; Dam and Glavind, 1938).

b. Excretion Studies—Man. The contribution that synthesis by intestinal bacteria makes to the vitamin K needs of human beings is unknown. Part of the difficulty stems from the fact that as little as 1 μg. of the vitamin per day will correct hypoprothrombinemia in newborn infants (Sells *et al.*, 1941). Another difficulty arises from the inadequate understanding of the exact relation between body stores of vitamin K and prothrombin levels, especially in infants (Owen, 1954).

Although there are a few reports indicating that a deficiency of vitamin K occurred in adults primarily as a result of an inadequate diet (Kark and Lozner, 1939; Thompson and Hilferty, 1949), there is no unequivocal evidence on the quantitative requirement of adults for vitamin K. It is believed, primarily on the basis of animal experimentation, that man secures a substantial amount of his vitamin K from intestinal synthesis (Owen, 1954).

c. Influence of Sulfonamides on Vitamin K Synthesis. A deficiency of vitamin K as a result of feeding rats purified diets supplemented with

sulfonamides was recognized by Black *et al.* (1941). It was shown that the sulfonamide-fed rats had hypoprothrombinemia (Daft and Sebrell, 1945). When the sulfonamide was omitted from the ration, rats could maintain a normal level of prothrombin. These observations suggested that the rat's requirement for vitamin K was provided by intestinal synthesis.[29]

Interest in vitamin K has increased during the past few years, especially in the poultry field. There have been a number of cases where large commercial flocks of laying hens showed prolonged clotting times and high mortality, which was often associated with severe hemorrhages (Nutrition Reviews, 1954; Gray *et al.*, 1954). Attempts were made to reproduce the disturbance under laboratory conditions. There are a number of reports which show that chicks raised on wire screens and fed a practical-type ration develop bleeding tendencies (Griminger *et al.*, 1953; Anderson *et al.*, 1954; Sweet *et al.*, 1954). In these cases the difficulty was probably related to the use of a corn and soybean meal ration which was supplemented with a borderline amount of vitamin K and a drug used for the control of coccidiosis. Some of these drugs, such as sulfaquinoxaline and 3-nitro-4-hydroxyphenylarsonic acid, may inhibit the intestinal bacterial production of vitamin K (Yacowitz *et al.*, 1955).

It is doubtful that the sulfonamides act directly on the blood, even though a single oral dose of sulfaquinoxaline (400 mg./kg. body weight) produces a lowering of the prothrombin level of rats within 24 hours (Seeler *et al.*, 1944). Vitamin K prevented the depression of prothrombin level or promptly cured it once it developed. The mechanism whereby these large doses of sulfaquinoxaline exerted their action is unknown, but it did not appear to involve any changes in the liver, nor did the addition of the drug to blood *in vitro* have any affect (Mushett and Seeler, 1947).

When 0.1% sulfaquinoxaline was added to a practical ration containing 5% alfalfa (a good source of vitamin K) and 5 mg. of menadione per lb. (the chick's requirement is 0.18 mg./lb. according to the National Research Council Publication No. 301 (National Academy of Sciences, 1954)), 3-week old chicks showed external hemorrhages within one week (Yacowitz *et al.*, 1955). The interpretation of the above observation was complicated by the fact that the sulfonamide was less harmful when incorporated in the diets of day-old chicks. When lower levels of sulfaquinoxaline were used, the prolonged clotting time could be corrected by vitamin K (Sweet *et al.*, 1954).

[29] Additional evidence for the intestinal synthesis of vitamin K comes from the observations of Greaves (1939) that rats maintained on a vitamin K-free diet for as long as 180 days still had appreciable amounts of the vitamin in their feces.

d. Influence of Antibiotics on Vitamin K Synthesis. One report claims that either penicillin or terramycin added to a corn and soybean ration containing no other source of vitamin K, prolonged the blood clotting time in chicks (Griminger *et al.*, 1953); this effect could be overcome with vitamin K. However, other reports claim that 70 times the level of terramycin used above had no influence on blood clotting time of chicks even when fed for nine months (Reynolds *et al.*, 1953; Sweet *et al.*, 1954).

In human beings Aureomycin in a single dose of 250 mg. does not affect the prothrombin level but may produce a transitory reduction in the coagulation time (Saroff and Rosenak, 1954).

e. Influence of Diet on Vitamin K Synthesis. No papers could be found on this subject.

V. Conclusion

There is no question but that large amounts of many vitamins are synthesized by the bacteria in the intestinal tract. Ruminants are able to secure their requirements for many of the vitamins, especially the water-soluble ones, by this mechanism. Nonruminants, however, secure only a few vitamins by direct absorption after they are synthesized in the intestinal tract. The rat appears able to secure at least folic acid, biotin, and vitamin K thereby. The situation with human beings is not so definite. Under normal circumstances man is able to satisfy his requirement for biotin and vitamin K through the intestinally synthesized compounds. There is suggestive evidence that adults, but *not* infants, secure a part of their pyridoxine and vitamin B_{12} in the same way.

The advent of sulfonamides and antibiotics provided a valuable tool for the exploration of the interrelations between the intestinal flora and the nutritional requirements of the host. A few divergent reports appeared when the same sulfonamide was used in different laboratories but, by and large, fairly consistent results were obtained. However, the present reports from different laboratories using the same antibiotic frequently appear to be contradictory. The wider spectrum of bacteria inhibited by the antibiotics may partially explain the apparent confusion.

Whether an antibiotic or some other antimicrobial agent added to the diet will stimulate, depress, or have no effect on the growth of an animal depends to a large extent upon the microorganisms present in the intestinal tract of the animal or in its immediate environment. The possibilities are illustrated in guinea pigs, for which a number of laboratories have reported that the addition of Aureomycin produced death within a few days, whereas others have not seen that effect (Jukes, 1955). Recent work by Roine *et al.* (1955) indicates that where death did occur following antibiotic supplementation of the diet, it was due to the overgrowth of

and tissue invasion by *Listeria monocytogenes*. This observation suggests that those guinea pigs harboring a few *Listeria* organisms can apparently grow at a fairly good rate until something such as Aureomycin disturbs the microbial balance, with a consequent overgrowth of *Listeria monocytogenes*. In these cases where there are no *Listeria* (or other organisms that react to antibiotics in a similar manner) in the guinea pigs or in their immediate environment, the antibiotic does not produce death. Under such circumstances the action of the antibiotic will depend upon the microbial and dietary situations existing at the time of the experiment. That the environment may change and thus alter the over-all results of antibiotic supplementation of diets has been brought out by Libby and Schaibel (1955).

The synthesis or destruction of vitamins in the gastrointestinal tract will probably be influenced by any drastic changes in the intestinal flora. If the change in the synthetic activity is great enough, repercussions may be seen in the host. For the moment, a prediction of the nutritional effects that might result from the addition of an antimicrobial agent to the diet is rather hazardous. Although it is recognized that many antimicrobial agents when added to commercial diets will produce growth stimulation of farm animals, the exact mechanism whereby that effect is brought about is not known. It may well involve changes in the intestinal flora resulting in either synthesis of additional nutrients or making the nutrients more available to the host. Further work is needed to elucidate the mechanism involved.

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Some Aspects of Vitamin A Metabolism

J. S. LOWE AND R. A. MORTON

Department of Biochemistry, The University of Liverpool, England

I. General Introduction

The existence of a "fat-soluble Factor A" was postulated in 1913, and forty years later it could be claimed that the purely chemical problems of structure and synthesis in the field of vitamin A and its carotenoid precursors had, in the main, been solved. In contrast, the state of knowledge concerning the physiology and biochemistry of vitamins A is much less satisfactory. Progress has certainly been made in describing the deficiency syndrome and in partially elucidating many separate

aspects of it; the special subject of the mode of action of vitamin A in visual processes has been in large measure reduced to order (for recent reviews see Wald, 1953; Collins, 1954; Willmer, 1955). It must be admitted, however, that the mode of action of vitamin A in promoting growth and in maintaining "normal" states in epithelial tissues, in short its main systemic action, is hardly understood at all. There is as yet no master key to avitaminosis A, no biochemical common denominator to the changes which occur in bone and nerve, to hyperkeratosis (including xerophthalmia), and to retarded growth.

The present article is concerned with two divisions of the field, the conversion of carotene to vitamin A and the "systemic" mode of action.

II. Conversion of Carotene to Vitamin A

1. Intestine as Site of Conversion

Moore (1930, 1931) proved that carotene is utilized by the animal to form vitamin A, which is found in the liver and, in very much smaller amounts, in the lungs, kidneys, and intestines. As some carotene remained unchanged in the intestine many days after its administration, Moore (1931) suggested that the conversion was not associated with digestion but occurred instead in the liver. Olcott and McCann (1931) incubated liver from vitamin A-deficient rats with carotene and claimed the formation of vitamin A through the agency of a liver carotenase. The evidence did not, however, compel assent and numerous later investigators failed to obtain conversion *in vitro*. The existence of a liver carotenase remained in doubt.

Popper and Greenberg (1941), using fluorescence microscopy, showed that after carotene was fed to vitamin A-depleted rats, vitamin A appeared within 15 minutes in the intestinal wall and liver. Matterson (see Bieri and Pollard, 1953a) observed that carotene given orally elicited a more favorable response than did carotene introduced directly into the circulation. Although these observations implied that the intestine might be a site for the conversion of carotene to vitamin A, it was not until 1946 that Sexton *et al.* established that carotene given to rats by mouth caused an increase in the liver level of vitamin A rather than carotene, whereas parenteral injection of carotene resulted in a rise in liver carotene but not of vitamin A. They then suggested that carotene might be converted to vitamin A in the intestinal wall.

Verification of this idea came almost simultaneously from three groups of workers—Glover *et al.* (1947), Mattson *et al.* (1947) and Thompson *et al.* (1947). Following this work on rats, similar experiments on many other species have shown the intestine to be a major site for the

conversion of carotene to vitamin A. For a fuller review see Kon and Thompson (1951).

Accepting that the process occurs in the intestine, the next step is to see if it can be achieved *in vitro*. Glover *et al.* (1948a) did not obtain conversion when carotene was incubated with rat small intestine, and the perfusion technique as employed by Kon and Thompson (1951) also led to negative results. On the other hand, Wiese *et al.* (1947a) claimed a demonstrable formation of vitamin A from carotene, and Stallcup and Herman (1950), using calf tissues, observed considerable formation of vitamin A from carotene by both intestine and liver. Both groups relied upon the $SbCl_3$ color test for vitamin A without establishing spectroscopic specificity, so that the results are inconclusive (see Bieri and Pollard, 1953a). More recently, McGillivray (1951) used 2–3-ft. lengths of sheep intestine removed from the jejunum with control segments taken from either side of the test length. Colloidal carotene was introduced into the test section. All three sections were incubated anaerobically for 2 to 3 hours at 37°C. in Ringer-Locke solution. The sections were saponified and the vitamin A estimated spectrophotometrically on the unsaponifiable fraction. In some cases the extracts from the intestinal sections were pooled and chromatographed, leading to a fraction which, after a three-point correction procedure (McGillivray, 1950), exhibited a good vitamin A absorption curve. Further confirmation of the presence of vitamin A was obtained by measuring the absorption curve of the colored compound formed with glycerol dichlorohydrin. McGillivray reports that the incubated tissues "showed statistically significant increases in vitamin A content of up to 15%." When he employed segments removed from sheep on a low-carotene hay diet, statistical analysis showed "the increase in vitamin A content on incubation with colloidal carotene to be highly significant."

The problem was also investigated by Rosenberg and Sobel (1953a), who gave β-carotene (dispersed in Tween 80 in the presence of α-tocopherol) by mouth to vitamin A-depleted rats. These were at once killed and the stomach contents were squeezed into the intestine. This was tied off, removed, and incubated at 45°C. for 2 hours in normal Ringer solution. The unsaponifiable fraction of the intestine was then prepared and its absorption spectrum measured from 310 to 390 mμ (solvent, equal volumes of xylene and kerosene). The solution was then irradiated with ultraviolet light for 1 hour and its spectrum again determined. The difference in optical density at 328 mμ was the measure of vitamin A formed. Using this technique, Rosenberg and Sobel arrived at a mean figure of 4.24 μg. (range 3.54–6.65 μg.) vitamin A produced per rat intestine. Although both McGillivray and Rosenberg and Sobel have ob-

tained conversion of carotene to vitamin A outside the living animal, Bieri and Pollard (1953a), using rat, calf, and rabbit intestine under a wide variety of experimental conditions, have concluded that "the formation of vitamin A proceeds at a greatly reduced rate, if at all, in the isolated intestine." De and Sundarajan (1951) also tried without success to demonstrate the *in vitro* conversion of carotene by rat intestine, and other workers have failed using intestinal breis or homogenates.

2. Alternative Sites for Conversion

Evidence is accumulating that the intestinal wall is not the only site at which the conversion of carotene to vitamin A can occur. Tomarelli *et al.* (1946) showed intramuscular injections of carotene, dispersed (with α-tocopherol) in Tween 80, to be as effective in restoring growth of vitamin A-depleted rats as the same amounts of carotene given orally, whether as an aqueous dispersion or an oily solution. Bieri and Sandman (1951) have shown that although carotene administered intramuscularly is ineffective when dissolved in oils it becomes effective when dispersed in Tween 40. A similar result was noted when the water-solubilized carotene was injected intravenously into vitamin A-deficient rats (Bieri and Pollard, 1953b). Extending the work, Bieri and Pollard (1953c, 1954) have tried to locate the conversion. When the bile duct of rats was ligated and severed, vitamin A was still formed from injected carotene, even though it could not be carried by the bile to the gut and there converted. Moreover, conversion was not impaired when the small intestine was removed before administering carotene, and even removal of the kidneys and a partial (60–75%) hepatectomy still allowed formation of appreciable amounts of vitamin A. An extraintestinal site of conversion must therefore exist. Kon *et al.* (1955) have confirmed that injections of carotene dispersed in Tween 40 to rats led to a rise in blood and liver vitamin A.

Eaton *et al.* (1951) reported a very limited conversion of carotene to vitamin A, as judged by blood assay, in dairy calves given intravenous injections of aqueous suspensions of carotene. Warner and Maynard (1952) reported that, although intravenously injected carotene in coconut oil would not cure vitamin A-deficient calves, injection of aqueous colloidal suspensions of carotene significantly increased blood vitamin A. This experience was not reproduced by Church *et al.* (1953, 1954) using Hereford calves 4 months old with rather low liver reserves of vitamin A (mean 2.5 I.U./g. liver). These workers found that carotene injections caused no significant changes in plasma vitamin A values. The contradictory results may be due to variations between carotene dispersions or to differences between breeds, but it should be noted that Kon *et al.*

(1955), using both Ayrshire and Shorthorn calves, could find little evidence for the conversion to vitamin A of carotene dispersions when they were injected into the animals.

In the case of the sheep too the position is confused, although the balance is tilted in favor of the view that sheep can convert injected carotene to vitamin A. In 1949, Klosterman *et al.* briefly reported that when colloidal carotene (i.e. carotene dispersed with Tween 20) was injected into the blood of vitamin A-deficient lambs, it was removed rapidly with no concomitant increase in blood or liver vitamin A. On the other hand, Church *et al.* (1953, 1954) found that when yearling wethers were fed a low-carotene ration for 8 weeks and were then given by injection a solubilized, aqueous carotene preparation, there was a highly significant increase in plasma vitamin A, the mean values nine hours later being about twice the original value. After an initial rise, the blood carotene fell rapidly during the first six hours following injection and then declined slowly for 10 days.

Kirschman and MacVicar (1955) have recently reported briefly that intravenously administered carotene (solubilized in Tween 40) is converted to vitamin A by ilectomized sheep.

Using pigs, Hentges *et al.* (1952) found that water-miscible preparations of carotene administered intravenously or intramuscularly will cure the symptoms of vitamin A deficiency. Rabbits (Kon *et al.*, 1955; Bieri, 1955a) and chicks can also convert injected β-carotene (solubilized with Tween 40), although in the chick conversion occurs more rapidly when given by the oral route (Bieri, 1955a).

Reports from Bonfante (1954, 1955) claim that when oxen are allowed to absorb an aerosol (water containing carotene, 50 mg. per 100 ml.) for 45 minutes, the carotene content of the blood shows a slight decrease while the vitamin A content increases up to double within four hours. Bonfante takes this to mean that transformation of carotene to vitamin A can occur in the lungs.

Many observations now indicate that the enzymes effecting the conversion of carotene to vitamin A are to be found at sites additional to the intestinal wall. This is an intriguing swing of the pendulum, for it will be recalled that comparison of the effects of injected and orally administered carotene led to the experiments on the intestinal wall as the site of the conversion process. The nature of the carotene injection (dissolved in oil or dispersed by a detergent) may be a factor, but further work is necessary. It was natural in the earlier work to assume that since both vitamin A and the provitamins A were fat-soluble, they were best administered in oily solution. In the outcome, the dispersing action of a detergent seems to expose the provitamin more efficiently to enzyme action.

3. Mechanisms Involved

The mechanism of the conversion of β-carotene to vitamin A is still uncertain although, as will be seen, a new approach has recently been made (Glover and Redfearn, 1954). Karrer *et al.* (1931a,b) suggested that β-carotene is cleaved symmetrically by addition of two molecules of water at the 15, 15′ double bond to give two molecules of vitamin A alcohol:

$$C_{40}H_{56} + 2H_2O \rightarrow 2C_{20}H_{29}OH$$

This "hydrolytic central fission hypothesis" is by now unattractive biochemically, and the carotenase of Olcott and McCann (1931) would not today be expected to do more than catalyze an initial step in the conversion process. Hunter (1946) suggested that the first stage in the conversion was an oxidative attack on the central double bond to give vitamin A aldehyde (or a product subsequently converted to this); vitamin A alcohol presumably being formed at a later stage. This hypothesis gained support from the work of Glover *et al.* (1948b), who found that retinene was rapidly reduced to vitamin A alcohol on entering the gut wall, so that an enzyme system occurs in the intestinal wall for Hunter's second stage. Taking into account the dynamics of the process, Glover *et al.* (1948a) suggested the following sequence: (a) oxidation of β-carotene to vitamin A aldehyde, (b) reduction of vitamin A aldehyde to alcohol, (c) esterification and absorption of vitamin A alcohol. Since the amount of vitamin A found in the intestinal wall during conversion of β-carotene is small, it appears that reaction (a) is probably slow compared with (b) and (c). This implies that the over-all rate is controlled by the initial oxidative step, which agrees with the accepted finding that the rate of deposition of vitamin A in liver is greater when preformed vitamin A is fed than when β-carotene is used.

If it be assumed that only the central double bond is attacked, each β-carotene molecule could perhaps yield two molecules of vitamin A, so that under specially favorable conditions the potencies of β-carotene and vitamin A should be almost identical. If, however, other double bonds in β-carotene are open to attack, the maximum yield of vitamin A might be one molecule instead of two.

Most investigators have found the biological potency of β-carotene as measured by the growth response of rats at low dose levels to be half that of the same weight of vitamin A (see Hume, 1951). Several collaborative tests carried out in many different laboratories to establish vitamin A acetate as the new International Standard arrived at the 50% conversion figure; in other words, 0.6 μg. β-carotene was biologically equivalent to

0.3 μg. vitamin A alcohol (Hume, 1951). No laboratory concerned in the collaborative trials reported results approaching 100% conversion.

On the other hand, support for central fission has come from Koehn (1948), who has recorded 100% conversion in the presence of tocopherol. This might, as an antioxidant, prevent oxidative degradation. Koehn did not supply tocopherol to the control animals, and his diet was decidedly low in fat. Koehn's results were confirmed, however, by Burns *et al.* (1951), who found that tocopherol intake is critical in determining the growth response to β-carotene; the optimum dose was 1 mg. daily, but a 2-mg. dose diminished significantly the efficiency of utilization.

Despite these results, the weight of evidence favors a normal process of conversion of β-carotene such that 1 mole of β-carotene gives 1 mole of vitamin A.

Further doubts about fission occurring only at the 15–15′ double bond of β-carotene arise from physicochemical considerations. Zechmeister *et al.* (1943) have argued that the central double bond of an extended polyene chain behaves like a single bond and should be more stable than the terminal ethylenic linkages which have less single-bonded character.

A rather different picture of the process of conversion of β-carotene into vitamin A has recently been advanced by Glover and Redfearn (1954) and Redfearn (1954). These workers have prepared β-apo-8′-carotenal, β-apo-10′-carotenal and β-apo-12′-carotenal and fed them to vitamin A-deficient rats. All were transformed into vitamin A, thereby confirming and extending the findings of Euler *et al.* (1938). Evidence was also obtained that β-apo-10′-carotenal undergoes β-oxidation to give β-apo-12′-carotenal. Glover and Redfearn suggest that the initial attack on β-carotene occurs at one end of the molecule and then proceeds by progressive β-oxidation until retinene is formed, reduced to vitamin A alcohol and absorbed. The scheme proposed is shown in Fig. 1.

Support for this hypothesis comes from the fact that the chemical oxidation of "all *trans*" β-carotene has been shown to occur primarily at the end of the conjugated chain producing β-apo-8′, β-apo-10′-, and β-apo-12′-carotenal and retinene (Redfearn, 1954). That such a β-oxidation mechanism as Glover and Redfearn propose is possible *in vivo* is shown by the fact that fatty acids having branched methyl groups can be oxidized *in vivo* when the branching group is in the α-position but not in the β-position to the carboxyl group (Kuhn and Livada, 1933; Weitzel, 1951; Carter *et al.*, 1939). This is precisely the situation with the β-apocarotenals until retinene is reached, when the branched methyl appears in the β-position and hence further degradation in that direction is arrested.

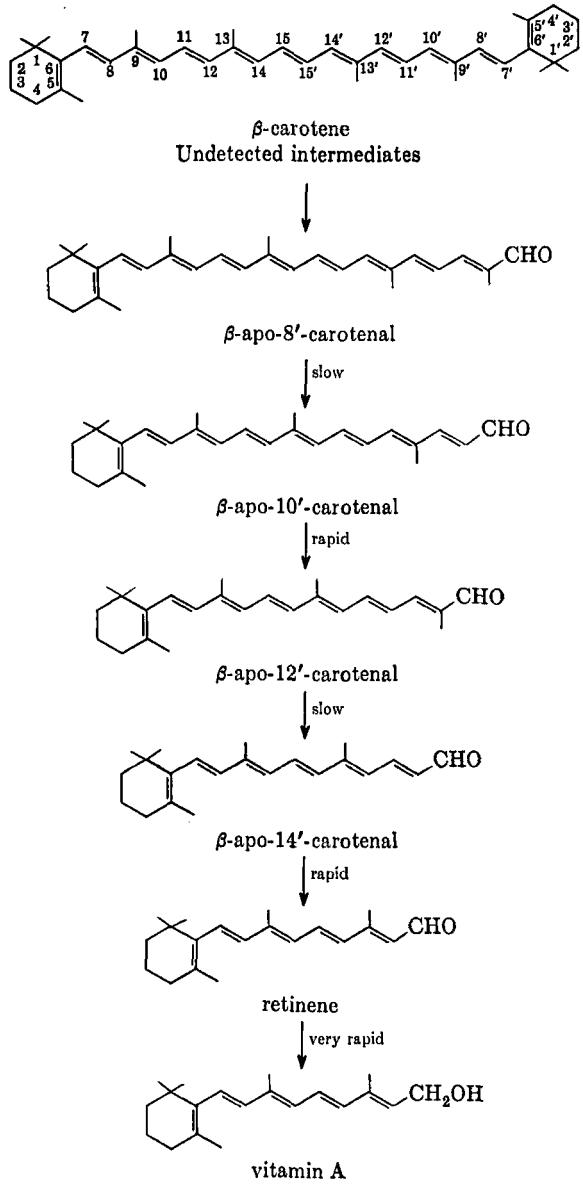


FIG. 1. Glover and Redfearn's (1954) scheme for transformation of β-carotene to vitamin A by terminal oxidations.

The chief merit of the above scheme is that it accounts for the experimental finding of 50% conversion. It is consistent with recent observations that β-carotene can be converted into vitamin A in extraintestinal tissues (page 100), since only normal β-oxidation processes need to be invoked and there is no need to postulate special enzymes, e.g., carotenase.

If the foregoing accurately describes the biochemical degradation of β-carotene the β-apo-carotenals ought perhaps to be normal constituents of the intestinal lipid. Festenstein (1951) has reported the presence of hitherto unidentified carotenoids in horse intestine. Glover and Redfearn (1954) point out that these substances have identical spectroscopic and chromatographic properties to the β-apo-10′- and -12′-carotenals.

The scheme proposed by Glover and Redfearn has many advantages and deserves to be tested fully.

4. Hormonal Effects on the Conversion of Carotene to Vitamin A

a. Thyroxine. Hormonal control of either the absorption of carotene or its conversion to vitamin A, or both, has been accepted for some time. The thyroid has been implicated in carotene-vitamin A metabolism since 1926 when Kunde noted the appearance of vitamin A-deficiency in thyroidectomized rabbits fed carotene. The view that hypothyroidism decreases the ability of the organism to convert carotene to vitamin A gradually gained ground largely on the basis of carotenemia and delayed dark adaptation in clinical hypothyroidism [for reviews see Drill (1943) and Goodwin (1952, 1954)].

Johnson and Baumann (1947) were the first to attack the problem on quantitative lines. They showed that, compared with control animals, rats receiving thiouracil or thiourea stored very little vitamin A in liver and kidney following carotene feeding. Hyperthyroid rats, however, accumulated more vitamin A than the normal animals, and the effects of thiouracil were neutralized by thyroxine, a clear indication that thiouracil was functioning through its antithyroid effect. Support came from Drill and Truant (1947), who found that carotene failed to prevent xerophthalmia in vitamin A-deficient thyroidectomized rats, although preformed vitamin A was effective. On the other hand, earlier work by Remington *et al.* (1942) had shown that xerophthalmia in hypovitaminotic thyroidectomized rats was alleviated equally rapidly by giving either vitamin A or carotene.

Cama and Goodwin (1949) argued that there were three possible explanations for the adverse action of thiouracil: (1) that the enzyme converting carotene into vitamin A in the intestinal wall is inhibited, (2) that the stability of carotene in the intestinal tract is decreased, and (3)

that the absorption of carotene from the lumen is interfered with. They showed by *in vitro* experiments that thiouracil had no effect on the stability of β-carotene in intestinal contents, but on the other hand they found that orally administered thiouracil decreased the absorption of β-carotene when fecal excretion was used as the criterion. Desiccated thyroid stimulated absorption, but a mixture of the two compounds had no net effect (see Table I).

TABLE I

EFFECT OF THIOURACIL AND DESICCATED THYROID ON EXCRETION OF CAROTENE BY RATS*

Diet†	β-Carotene excreted	Excretion above or below normal
	μg./3 rats/day	*% of normal*
Normal	10.14	—
Desiccated thyroid (100 mg./rat/day for 3 days)	8.72	−14.0
Thiouracil (100 mg./rat/day for 3 days)	15.90	+56.8
Desiccated thyroid and thiouracil (both 100 mg./day/rat for 3 days)	9.83	− 3.1

* From Goodwin (1954).

† Diet was ether-extracted food cubes + 30 μg. β-carotene/day.

Chanda *et al.* (1951a,b) confirmed this effect on the absorption of carotene by both sheep and goats. They also found that normal goats absorbed carotene more efficiently than normal cows, which is in harmony with the statement by Schultze and Turner (1945) that goat thyroid is more active than cow thyroid. All the foregoing experimental findings support the view that the relevant property of the thyroid hormone is promotion of the absorption of carotene.

Johnson and Baumann (1947) showed that the amount of vitamin A stored in the liver after a given dose of β-carotene increases if the rats are treated with thyroid hormone. This exposes another facet of the problem, namely, the choice between attributing the rise to improved absorption of carotene and consequent increase in substrate presented for conversion, or attributing it to thyroid stimulation of the enzymes concerned in the process (see Goodwin, 1954).

This tidy pattern of thyroid effects on carotene metabolism has received a severe jolt from recent work by Morgan and Arnrich (Arnrich and Morgan, 1954; Arnrich, 1955).

Using young male rats, Arnrich and Morgan (1954) found that carotene-fed (440 μg./day for seven weeks), thiouracil-supplemented

rats not only converted carotene to vitamin A but also stored about twice as much vitamin A in their livers as the control animals given carotene but not thiouracil. This result was confirmed with young male and female rats even when the carotene dosage levels were adjusted to body weight so that the more slowly growing, thyroid-deficient rats received less carotene than the actively growing controls. The difference in liver vitamin A levels disappeared when the carotene intake was increased from 86 μg. to about 1500 μg./day, but the hypothyroid effect was overcome by thyroxine. It is thus quite clear that thiouracil-treated rats can convert carotene to vitamin A.

When hypothyroid and normal rats were depleted of similar pre-existing stores of vitamin A, larger amounts of the vitamin were found in the thyroid-deficient animals than in the normals, indicating a decrease in the utilization rate of vitamin A. That this was due to retarded growth rather than a lowered metabolic rate is seen from the fact that, when the growth of normal rats was retarded through caloric restriction to match that of hypothyroid rats and the animals were again depleted of similar pre-existing stores of vitamin A, the resulting residual vitamin A stores were strictly comparable in the two groups but were much higher than in normals.

As already mentioned, Cama and Goodwin (1949) (using fecal excretion of carotene as a measure of absorption) found that thiouracil-treated rats had a reduced absorption of β-carotene. Arnrich and Morgan, however, studied the absorption of a single dose of 35 μg. carotene by measuring the residual amount of carotene in the walls and lumen contents of the intestinal tract two hours after dosing. They found that the removal of a given dose of carotene occurred at a similar rate in normal and hypothyroid rats, indicating little difference in absorption rate between the two groups for the dose given.

These results are not limited to the rat, as Arnrich (1955) has shown that comparable increases in liver vitamin A concentrations occur in normal and thiouracil-treated, young mature, pure-bred cocker spaniels following the ingestion of carotene. Compared with normal levels, thiouracil caused a rise in blood vitamin A, but this was accompanied by a rise in serum cholesterol and a significant increase in serum carotene. There may thus be a nonspecific effect on lipids.

From the work of Arnrich and Morgan it appears that neither absorption nor conversion of carotene to vitamin A is affected directly by thyroid activity. These findings are in disagreement with most of the earlier reports, but they support the findings of Remington *et al.* (1942) and Wiese *et al.* (1947b, 1948). Arnrich and Morgan suggest that the discrepancies may have arisen in part from differences in experimental

procedures, but in our view no neat and simple explanation can command assent if all the findings are given equal weight.

b. Insulin. There is a good deal of evidence that insulin is concerned in the metabolism of carotene and vitamin A. As early as 1904, von Noorden and Isaac commented on a yellow pigmentation occurring in the skin of some patients with diabetes. The condition, xanthosis diabetica, was also characterized by increased amounts of carotene in the blood. Ralli *et al.* (1935) and Ralli *et al.* (1936) gave diets with the same high carotene content to a group of patients with diabetes (some insulin-treated) and to a number of normal persons. The diabetic patients had higher blood carotene levels than the controls; the values rose more rapidly on the high carotene diet and fell more slowly when the carotene was withheld than in the normal persons. The liver carotene of diabetic patients who came to autopsy was higher than the normal. Brazer and Curtis (1940) found that juvenile diabetics had poor dark-adaptation; daily administration of "60,000 U.S.P. units of vitamin A," in the form of crystalline carotene dissolved in vegetable oil, for as long as 14 days did not affect the dark adaptation, but daily administration of 60,000 U.S.P. units of vitamin A from fish liver oil caused their dark adaptation to return to normal or nearly normal in periods ranging from 3 to 21 days. This work, with that of Heymann (1936), Stueck *et al.* (1937), Oliva and Pescamona (1939), and Galeone (1943) led to the belief that the efficiency of conversion of carotene into vitamin A was impaired in the diabetic state. This view was shaken by Kimble *et al.* (1946), who showed that, when a sufficiently large number of patients were examined, no statistically demonstrable increase in plasma carotene levels occurred in diabetes. However, Ralli and Claps (1949) reaffirmed an increased absorption of carotene in the diabetic and higher blood carotene levels.

This disconcerting and unsatisfactory situation persisted until Sobel *et al.* (1953) depleted rats of their vitamin A stores and induced diabetes in some of them by subcutaneous injections of alloxan. The animals were then given 0.2 ml. cottonseed oil containing 2 mg. carotene (90% β and 10% α) by stomach tube. The animals were killed 36 hours after dosing, and analyses for vitamin A were carried out on blood and liver. As can be seen from Table II, the livers of the nondiabetic rats contained about four times as much vitamin A as those of the diabetic animals. When preformed vitamin A was fed to alloxan-diabetic and to normal rats, the liver storage was about the same in both groups, so that the alloxan-diabetic rat had no failure to store vitamin A nor any unusual destruction or impaired absorption of the vitamin. Although the evidence points to impaired conversion of carotene to vitamin A in the alloxan-diabetic rat a cautious approach with the thyroid experiments in mind suggests that the absorption of carotene may be influenced.

Continuing their earlier work, Rosenberg and Sobel (1953b) have compared the *in vitro* conversion of carotene to vitamin A by isolated intestinal loops taken from alloxan-diabetic and nondiabetic vitamin A-depleted rats. As shown in Table III, it is clear that there is a very

TABLE II

MEAN VITAMIN A STORES OF ALLOXAN-DIABETIC AND NONDIABETIC VITAMIN A—DEFICIENT LITTERMATE RATS GIVEN 2000 μg. CAROTENE IN COTTONSEED OIL

No. of animals	Group	Blood sugar	Serum vit. A	Liver vit. A
		mg./100 ml.	*μg./100 ml.*	*μg./animal*
43	Diabetic	307 ± 125	41 ± 29	22 ± 20
10	Nondiabetic	87 ± 10	44 ± 30	88 ± 25
	P.	10^{-8}	0.2	10^{-3}

From Sobel *et al.*, 1953.

TABLE III

AMOUNT OF VITAMIN A FORMED IN THE ISOLATED INTESTINE OF VITAMIN A—DEFICIENT, ALLOXAN-DIABETIC AND NONDIABETIC RATS

Litter	Animal	Alloxan diabetic	Control
		μg. Vitamin A per gut	
	1	0.70	4.21
1	2	0.65	3.12
	3	0.66	3.54
	1	0.42	2.08
2	2	0.38	4.66
	3	1.93	—
	1	1.08	5.35
3	2	1.16	4.66
	3	1.93	—
	1	0.38	2.08
4	2	0.24	6.00
	3	—	4.05
Mean ± S.D.		0.76 ± 0.50	4.25 ± 1.53

Animals from same litter, dose 150 μg. carotene, intestines incubated at 45° for 2 hours. From Rosenberg and Sobel, 1953b.

much decreased synthesis of vitamin A in the gut from the alloxan-treated rats.

The administration of alloxan to rats thus impairs the conversion of carotene to vitamin A at the same time as it induces the diabetic syndrome. Whether the two effects are parallel but independent or related and in sequence has not yet emerged. If the diabetes is primary, administration of insulin might reverse the inhibition of conversion; indeed, proof

of this impairment in the diabetic would require a reversal of the effect by insulin. The more recent work on rats shows a much closer parallel with the older literature on xanthosis diabetica than with the results of Kimble *et al.* (1946), which seem, at first sight, to rob the connection between diabetes and carotene of validity. In fact Kimble *et al.* (1946) studied 116 unselected diabetics, young and old, insulin-treated and untreated, some with infections common in diabetics, others with arteriosclerotic and cardiovascular conditions. It seems not unlikely that these complications may have masked any simple relationship analogous to that which can be discerned by controlled experiments on animals.

c. Adrenal Hormones. Two recent reports indicate some relationship between vitamin A metabolism and the adrenal. Clark and Colburn (1953, 1955) gave to each of 32 male rats the same amount (*about* 400 μg.) of β-carotene daily. The rats were divided into four groups: (1) *ad libitum*-fed controls, (2) rats receiving daily 3 mg. cortisone acetate subcutaneously, (3) pair-weighed to cortisone-treated rats, and (4) pair-fed to cortisone-treated rats. Half the rats were sacrificed at 10 days and the remainder at 20 days; the livers and kidneys were then analyzed for vitamin A and carotene. When sufficient carotene was given to the cortisone-treated rats, there was adequate conversion into vitamin A. However, there was less total vitamin A and more total carotene in the livers of the cortisone-treated rats than in their pair-fed or *ad libitum*-fed controls, especially after the 20-day treatment. In the kidneys, less total vitamin A was present in the cortisone-treated animals than in any of the controls except the 20-day, pair-weighed group. The experiments suggest a somewhat impaired conversion of carotene to vitamin A in the cortisone-treated rats.

Clark and Colburn (1955) also studied the effects of cortisone and inanition upon the liver vitamin A and carotene levels of rats on a vitamin A-free diet. These results showed that rats subjected to the double hazard of cortisone treatment and starvation lost their liver vitamin A at a much faster rate than rats subjected to starvation only. When the cortisone-treated, starved rats had no vitamin A remaining in their livers, they were given 60 μg. β-carotene daily for six days. Even this treatment failed to produce liver stores of vitamin A, but carotene tended to accumulate in the livers. This result supports the former one, since it indicates that on a low intake of β-carotene, adequate conversion does not occur.

The investigations of Clark and Colburn (1955) need to be extended and confirmed, but so many reports point to a connection between the adrenals and vitamin A metabolism (*vide infra*) that a cortisone effect is not altogether surprising.

5. Effects of Other Vitamins on the Conversion Process

a. Tocopherols. Sixteen years ago, Moore *et al.* (1939) and Moore (1940) reported the sparing effect *in vivo* of tocopherol on vitamin A and carotene. Since then much work on this topic has been interpreted in terms of a synergism between vitamin A and the tocopherols (see Moore, 1945), but the specific role of the tocopherols in the metabolism of carotene and vitamin A is not yet clearly understood.

The work of Hickman and his colleagues (Hickman *et al.*, 1944a,b; Harris *et al.*, 1944) was broadly consistent with synergism, but it was noted that doses exceeding 1.5 mg. α-tocopherol were less effective than smaller doses in increasing the growth of rats supplied with vitamin A. When small daily doses of carotene were given instead of vitamin A, tocopherol at doses up to 0.5 mg. increased the growth rate, but larger doses sometimes actually depressed it. On the other hand, when large doses of carotene were supplied, growth was little influenced by the tocopherol intake. These rather odd findings hinted that under certain conditions tocopherols could interfere with the conversion of carotene to vitamin A.

More recently similar results have been obtained not only with the different tocopherols (Swick and Baumann, 1952) but also with other antioxidants (see below).

While studying the effect of a fixed dose of α-tocopherol upon liver storage of vitamin A with rather widely varying daily intakes of vitamin A and carotene, Hebert and Morgan (1953) noted that the addition of 0.5 mg. α-tocopherol daily to the diet of rats partially depleted of vitamins A and E and receiving 35–129 μg. vitamin A per day (for 14–28 days) produced *no* change in liver vitamin A stores, except in the group fed the lowest level of vitamin A (35 μg./day). However, addition of 0.5 mg./day of tocopherol to the diet of similarly treated rats receiving 24–174 μg. carotene in oil per day for 14–28 days produced a significant increase in liver vitamin A when the *total* carotene intake was from 1200–2600 μg. Above and below these levels no effect was found.

Johnson and Baumann (1948) fed varying levels of tocopherol to vitamin A-depleted rats given daily doses of either vitamin A or β-carotene. When vitamin A was supplied there was no difference in the liver or kidney stores whether 0, 0.5, 2.5, 5.0 or 10 mg./day α-tocopherol was fed, but, when β-carotene was fed, the amounts of vitamin A deposited in the tissues varied inversely with the dose of tocopherol. In other words, less vitamin A was stored when large amounts of α-tocopherol were fed with carotene than when carotene was fed alone, although the tocopherol did not interfere with the storage of preformed vitamin A. When 5 mg.

tocopherol was fed eight hours after the ingestion of carotene, the liver vitamin A store was the same as that in rats receiving no tocopherol. Since the same amount of carotene was excreted whether the rats received 0, 0.5, 5, or 10 mg. tocopherol, it is clear that the tocopherol did not interfere with the absorption of carotene.

TABLE IV

EFFECT OF ANTIOXIDANTS ON CAROTENE AND VITAMIN A UTILIZATION

Daily supplement	No. of rats	Vitamin A deposited	
		Liver	Kidney
		($\mu g.$)	($\mu g.$)
33 μg. carotene	6 (1 M)	78 ± 12.0	16.0 ± 2.5
33 μg. carotene + 10 mg. DTBH	4 (1 M)	45.0 ± 5.3	14.5 ± 2.8
33 μg. carotene + 10 mg. TMBH	5 (1 M)	32.2 ± 2.0	19.2 ± 1.4
33 μg. carotene + 10 mg. TBA	6 (1 M)	39.1 ± 3.8	13.0 ± 3.5
13 μg. vitamin A	6 (2 M)	34.3	13.2
13 μg. vitamin A + 10 mg. α-tocopherol acetate	5 (2 M)	33.0	14.0
13 μg. Vitamin A + 10 mg. MTBH	5 (1 M)	34.1	12.0
13 μg. Vitamin A + 10 mg. TMBH	6 (2 M)	33.4	13.0

Carotene experiment lasted 25 days.
Vitamin A experiment lasted 20 days.
DTBH = 2,5-ditertiarybutylhydroquinone.
TMBH = 1,1,3,3-tetramethylbutylhydroquinone.
TBA = ditertiarybutyl-4-hydroxyanisole.
MTBH = monotertiarybutylhydroquinone.
Table modified from High *et al.* (1954).

High *et al.* (1954) carried out analogous experiments with other antioxidants. Table IV shows the results obtained when 10 mg. of the various antioxidants were fed daily either with 33 μg. carotene or 13 μg. vitamin A to vitamin A-deficient rats for 20–25 days.

It is clear that large amounts (10 mg.) of the various antioxidants decrease hepatic vitamin A deposition from β-carotene, while similar doses do not affect the liver vitamin A deposition when preformed vitamin A is given. In addition, the amount of carotene absorbed is unaffected by the antioxidants, although they markedly protect carotene against oxidative decomposition *in vitro*.

Large amounts of antioxidants and tocopherols thus decrease the deposition of vitamin A from β-carotene but do not affect the deposition

of preformed vitamin A. The facts that the antioxidants neither interfere with the absorption of carotene nor accelerate its oxidative destruction imply that large amounts of antioxidants, as well as vitamin E, act by suppressing directly or indirectly the oxidative processes involved in the enzymic conversion of carotene to vitamin A.

The matter does not, however, appear to be quite so simple, since Bieri (1955b) has found that when β-carotene, solubilized in aqueous Tween 40, was injected into rats dosed with large amounts of α-tocopherol, no inhibition of conversion occurred; in fact the amounts of vitamin A formed in rats with normal and elevated tissue tocopherol levels were found to be essentially the same. Unless it be assumed that the enzyme system which deals with injected carotene differs from that concerned with the conversion of orally administered carotene, it would appear that the inhibitory action of large amounts of tocopherols on orally administered carotene concerns stability and absorption, in disagreement with the experimental results of High *et al.* (1954).

A recent series of papers by Tappel (1953, 1954) has shown that α-tocopherol and other antioxidants with *in vivo* vitamin E-like activity effectively inhibit oxidation *in vitro* of unsaturated fatty acids and concurrent oxidative destruction of vitamin A catalyzed by hematin compounds. Tappel's findings might lead to an explanation of the sparing action of the tocopherols on vitamin A and carotene. In addition to this synergistic action, the tocopherols in large amounts can manifestly inhibit the conversion of carotene to vitamin A. How this happens is not known. More work is still needed on various aspects of the vitamin A-vitamin E interrelationships.

b. Vitamin B_{12}. High and Wilson (1953) suggest a relationship between vitamin B_{12} and the conversion of carotene to vitamin A. Young rats, deficient in vitamins A and B_{12}, were fed daily supplements of 33 μg. carotene (90% β, 10% α) dissolved in 0.2 ml. cottonseed oil for 21 days. On alternate days, the control animals were injected intramuscularly with 0.2 ml. saline solution and the test animals were likewise dosed with 0.2 ml. saline containing 0.28 μg. vitamin B_{12} in the form of either Cobione or Normocytin. To inhibit possible intestinal microbial synthesis of vitamin B_{12}, one series of animals was given sulfasuxidine in the diet (2 g./kg. diet—series 2, Table V). A third lot of animals (series 3, Table V) was fed an A.P.F. (animal protein factor) supplement (equivalent to about 55 μg. vitamin B_{12}/kg. diet). Results of this experiment are shown in Table V. From series 1, it is clear that vitamin B_{12} increases growth and deposition of vitamin A from carotene. When sulfasuxidine was added to the diet (series 2) the effect on growth was more marked

(an increase of 62% compared with 50% in series 1), but the storage of vitamin A in the livers and kidneys was about the same (around 37%). In similar experiments using preformed vitamin A (13 μg./day for 21 days), vitamin B_{12} was found to be comparatively ineffective with respect to storage of vitamin A, but the growth effect was still present.

It appears, therefore, that vitamin B_{12} influences the conversion of carotene to vitamin A. It is agreed that vitamin B_{12} is concerned in the metabolism of labile methyl groups including those of choline. As High

TABLE V

EFFECTS OF VITAMIN B_{12} AND A.P.F. SUPPLEMENT ON THE UTILIZATION OF CAROTENE

Series	Supplement	No. and sex of rats	Weight gain	Vitamin A deposited	
				Liver	Liver + kidney
			(g.)	(μg.)	(μg.)
1	33 μg. carotene + saline	8 F	36	46.4 ± 2.8	55.9 ± 2.4
	33 μg. carotene + liver extract	8 F	52	65.0 ± 5.1	74.9 ± 5.1
	33 μg. carotene + Cobione	5 F	60	61.1 ± 2.9	67.0 ± 2.9
	33 μg. carotene + Normocytin	8 F	49	64.6 ± 5.2	74.0 ± 5.4
2 (Sulfasuxidine)					
	33 μg. carotene + saline	6 (2 F)	37	36.6 ± 2.8	50.5 ± 3.2
	33 μg. carotene + Cobione	5 (2 F)	59	57.6 ± 3.3	70.8 ± 3.3
	33 μg. carotene + Normocytin	6 (1 F)	60	56.3 ± 2.3	70.2 ± 2.9
3	33 μg. carotene	8 (3 F)	47	39.7 ± 1.7	52.1 ± 1.4
	33 μg. carotene + A.P.F. supplement	8 (3 F)	53	49.9 ± 1.7	62.4 ± 1.4

After High and Wilson (1953).

and Wilson (1953) point out, Popper and Chinn (1942) claimed that choline deficiency impaired the utilization of both carotene and vitamin A for liver storage, while Clayton and Baumann (1944) found a normal distribution of vitamin A in the tissues of choline-deficient rats. If vitamin A storage from dietary carotene does depend on the choline intake, it is reasonable to expect some relationship between vitamin B_{12} and carotene metabolism. On this basis, and since vitamin B_{12} does not affect the storage of preformed vitamin A, it might be expected that choline would also be without effect on liver deposition of preformed vitamin A, which is actually the case (Guerrant and Thompson, 1952). Although it is clear that vitamin B_{12} status may influence the conversion

of carotene to vitamin A, the how and the where of the effect are still uncertain.

6. Phosphate and the Conversion Process

Interest has been shown in the influence of phosphate deficiency on the conversion of carotene to vitamin A. Ross and Gallup (1949) noted in Hereford cows an inverse relationship between plasma inorganic phosphate and carotene concentrations, and preliminary reports indicated that this held for Hereford steers (Thomas *et al.*, 1951). The observations were extended by Thomas *et al.* (1953), who kept four cows for about eight weeks before calving and up to 20 weeks thereafter on a low-phosphate diet, and five cows, similarly treated, but with a phosphate supplement. The nine cows all had the same daily intake of carotene in the form of prairie hay. The plasma content of carotene, vitamin A, and inorganic phosphate, and the liver content of vitamin A and carotene were measured at intervals spread over the experimental period. From the first to the fourth week after calving, the cows on the low phosphate intake had an average plasma carotene level of 158 μg./100 ml., while those on the high phosphate intake had 138 μg./100 ml. The difference, according to Thomas *et al.* (1953), "approaches statistical significance at 5% level." Apart from this no difference between the plasma carotene levels or the plasma vitamin A values in the two groups was shown. Liver vitamin A values for the low phosphate group were always higher than those of the phosphate-supplemented group—a fact which does not support the idea that phosphate deficiency affects the conversion of carotene to vitamin A, although as Thomas *et al.* (1953) suggest, it may indicate that phosphate is needed for mobilizing vitamin A from the liver and transferring it to milk during lactation. This suggestion is supported by a tendency for milk and colostrum from the cows on the low phosphate regimen to be higher in carotene and lower in vitamin A than milk from the cows receiving the phosphate supplement. Gallup *et al.* (1953), using Hereford steers again, found that the terminal plasma carotene levels of four steers on a low phosphate diet (119 μg.%) were considerably higher than the levels of five steers on the phosphate-supplemented diet (76 μg.%), but there was less difference in the plasma vitamin A values (17 μg.% and 22 μg.% respectively), and liver vitamin A values tended to be lower in the low-phosphate group. These experiments suggest that a relationship between phosphate intake and carotene conversion is possible. Gallup *et al.* also worked with lambs. There was no difference between the plasma carotene or vitamin A of the lambs on the low and adequate-phosphate rations, a result which agrees with that of Klosterman *et al.* (1952). In rats, it has been found that higher liver vitamin A stores result from a given amount of carotene in animals on a low phosphate diet than on a high phosphate

diet (Klosterman *et al.*, 1952) and, moreover, the amount of phosphate in the diet of rats does not appear to influence the absorption of carotene (Wilcox and Galloway, 1955).

These conflicting results do not encourage generalization. Different species may not respond in the same way to a low phosphate diet. It will be recalled that rat and sheep are efficient converters of carotene, there being practically no carotene in rat and sheep plasma, but cattle are poor converters, their plasma always containing carotene. Nevertheless, the suggestion that low dietary phosphate impairs the conversion of carotene to vitamin A seems incompatible with the finding that cattle on a low phosphate diet have higher liver vitamin A than cattle on the high phosphate diet.

7. Effect of Antibiotics on the Conversion Process

Addition of antibiotics to animal foodstuffs is now so common (for review see Stokstad, 1954) that it becomes important to note any effects it might have on vitamin A metabolism. Burgess *et al.* (1951) found that chicks responding to penicillin (30 mg./kg. diet), had higher liver reserves of vitamin A than untreated birds, and this finding was confirmed by Coates *et al.* (1952). Since the diet employed by Burgess *et al.* (1951) contained both carotenoid and vitamin A, the antibiotic might have affected the metabolism of the vitamin itself or that of the provitamins.

In rats vitamin B_{12} influences the conversion of carotene to vitamin A (*vide supra*), but recently, High (1955) has found that in the presence of penicillin it was ineffective. It is not easy to reconcile these various findings.

When aureomycin is the antibiotic used, the position is much clearer. High (1955) reported that, in rats, aureomycin feeding (50 mg./kg. diet) significantly increased vitamin A deposition from carotene but did not affect the amount of vitamin A deposited in the liver and kidneys when preformed vitamin A was administered. Similar results were obtained by Almquist and Maurer (1955) feeding chlortetracycline to chickens. The optimum level was about 55 mg./kg. diet. Additional support comes from Hartsook *et al.* (1953), who showed that aureomycin does not enhance absorption of vitamin A in the rat, and Murray and Campbell (1955), who, while agreeing, claimed that it consistently increased the apparent effectiveness of the vitamin A as judged by the vaginal smear technique.

Here, again, it is too soon to expect a unified picture, but this facet of the action of antibiotics deserves further study.

III. The Systemic Mode of Action of Vitamin A

1. Introduction

Vitamin A deficiency leads, as is well known, to changes in bone and nerve tissues, retarded growth, and hyperkeratosis. Perhaps the most

striking manifestation of vitamin A deficiency, as observed in experimental animals and man, is the failure to maintain the integrity of the various epithelial tissues, the organs affected including the eye and eyelids, respiratory tract, alimentary tract, urinogenital tract and skin. The different lesions in animals and man have been elegantly described by Wolbach (1954) and Mason (1954). The remainder of the present article will review work which may now or later shed light on the mode of action of vitamin A in epithelial tissues in general.

Vitamin A deficiency as seen in epithelia has been summarized by Wolbach and Howe (1925, 1926) as atrophy of many glands and replacement of many different single-layered epithelia by stratified keratinizing epithelium. The change from the cuboidal or columnar type of cell to the stratified type is known as keratinizing metaplasia. The sequence is as follows: atrophy of the epithelium concerned, reparative proliferation of the basal cells, and growth and differentiation of the new products into a stratified keratinizing tissue. Regardless of the original function and structure of the region, this replacement epithelium is identical in all locations and comparable in all its layers with epidermis.

When the vitamin A deficiency is brought to an end, the epithelium of each region returns to normal both in morphology and function. Wolbach and Howe (1933a,b) describe the process of repair as involving the removal of the layer of cells irreversibly differentiated toward keratinization by autolysis and leucocytic infiltration and direct differentiation of the stratum germinativum into the normal type. This means that the cells of the stratum germinativum of the replacement epithelium retain the identity of the original epithelium throughout the metaplasia.

Despite the unquestioned importance of vitamin A for the maintenance of normal epithelial tissue, there has never been a convincing demonstration of the presence of vitamin A in skin and other epithelial tissues known to undergo characteristic changes in depleted animals. Popper and his collaborators (for review see Popper, 1944) studied the distribution of the vitamin in tissues by fluorescence microscopy but were unable to detect any in the epidermis or epithelium of the mucous membranes, tissues which are considered to be the first to undergo change in vitamin A deficiency (Popper, 1941). Moreover, even after feeding high doses of vitamin A, only the fat cells of the cutis and subcutis were found to contain vitamin A and then only in small amounts. More recently, Edwards *et al.* (1951) claim to have demonstrated the presence of vitamin A in the intact human skin by means of a Harrison recording spectrophotometer, but they admit that it was not possible to distinguish between vitamin A present in the skin structure proper and vitamin A in the blood.

The fact that no vitamin A can be detected in epithelial tissue can

be looked at in three ways. First, epithelia may contain amounts of the vitamin so minute that the methods of detection are not sufficiently sensitive for the purpose. Second, it is possible that not vitamin A but some metabolite is essential for the maintenance of the integrity of the epithelia—in other words, search has been made for the wrong compound. Third, the derangement of epithelia in the absence of vitamin A might be due to the accumulation of some abnormal metabolite(s) in the epithelial tissue.

2. Alternative "Active Forms" of Vitamin A

In 1934 Randoin and Netter cured xerophthalmia in rats by feeding a diet apparently free from vitamin A or carotenoids. It was made up as follows: casein 15%, lard 20%, dried brewer's yeast 5%, dextrin 56%, and salt mixture 4%. The work opened up the possibility that the substance responsible for the well-being of epithelia was not vitamin A but a derivative with different chemical properties. This could mean that the systemic action of vitamin A is mediated not by the vitamin *per se*, but by a metabolite. The role of retinene in vision provides a precedent.

Randoin and Netter were followed by Le Gallic, who claimed that the vitamin A-replacing action of the above diet depended upon both lard and casein, which must be present in definite amounts, viz. 20–21.5% lard and 18% casein (Le Gallic, 1951a), although the casein can be replaced by either ovalbumin (Le Gallic, 1949) or fish muscle (Le Gallic, 1950a). If either the casein or the ovalbumin is subjected to vigorous peptonization (pH 2.8–3.0 at 50°C.), the vitamin A activity of the diet is lost, but the proteins can withstand milder treatment (pH 3.5 at 45° for 24 hours) without loss of vitamin A activity when incorporated in the diet (Le Gallic, 1949).

Le Gallic (1951b) extracted casein with various solvents (light petrol, chloroform, acetone, ether, and dichlorethane) and then incorporated the treated casein in the diet. The treatment with solvent did not result in a loss of vitamin A activity. It is now a well-established practice to use alcohol-extracted casein in vitamin A-free diets; refluxing with boiling alcohol removes traces of β-carotene and vitamin A (see Lowe *et al.*, 1953), but extraction of casein with ether or light petroleum does not. It is important that the casein to be used in Le Gallic's diet should be extracted with boiling ethanol.

Since the vitamin A-replacing activity of the lard-casein diet is not lost when 4% succinylsulfathiazole is incorporated into the diet, it seems reasonable to exclude a synthesis of provitamin A by the intestinal flora as an explanation (Le Gallic, 1950b).

Le Gallic focussed attention mainly on the protein part of the diet.

On the other hand, the vitamin A activity of diets containing lard has been noted by other workers; e.g. Mayer and Krehl (1948), who showed that for vitamin A-deficient rats, isocaloric replacement of sucrose by fats opposed development of the syndrome of vitamin A deficiency. This effect was particularly marked with lard. More recently, Kaunitz and Slanetz (1950a,b) claimed that an active material could be concentrated by molecular distillation of lard. The first 7% to be eliminated contained most of the vitamin A-replacing factor. Vitamin A itself was not present, the unsaponifiable fraction of the distillate in the Carr-Price color test showing evidence of less than 1 I.U. vitamin A/g. of unsaponifiable matter. Spectrophotometric analysis showed less than 10^{-7} g. of vitamin A/g. distillate, even assuming that the total absorption at 325 mμ was due to vitamin A. When the distillate was added at the level of 2% to a vitamin A-free diet, the test animals fared better than littermates on the same diet (without lard distillate) corrected by vitamin A palmitate (7.6 I.U./week, by injection).

In attempting to concentrate the "lard factor," Kaunitz and Slanetz (1951) found that mild saponification under N_2 reduced the activity of the unsaponifiable and fatty acid fractions, while hydrogenation destroyed the activity. Extraction with acetone, however, resulted in a fraction with two to three times the activity of the distillate.

This work has implications of such importance that its reproducibility must be tested. Lowe and Morton (1953), using both spectrophotometric and colorimetric tests, were unable to detect preformed vitamin A or carotenoids in lard or lard unsaponifiable matter before or after chromatography. Since the available lard distillate was required for bioassays, it was not saponified or chromatographed, but a 10% (w/v) solution of the distillate in $CHCl_3$ gave no color with the $SbCl_3$ reagent. More concentrated solutions (20% w/v) gave only an evanescent green. When the distillate was supplied at a level of 0.6 g./day, however, it cured xerophthalmia and restored normal growth in avitaminotic rats. Nieman (1954) and Le Gallic (1953) have also confirmed the vitamin A-like action of lard distillate.

Herb *et al.* (1953) have more recently reinvestigated the original distillates used by Kaunitz and Slanetz (1950a,b). Using as much as 3 kg. and subjecting this to further molecular distillation and chromatography, they obtained fractions which gave a positive Carr-Price test and exhibited a typical vitamin A absorption curve. A further batch of their original distillate gave fractions containing vitamin A after chromatography alone, and finally, they succeeded in showing the presence of vitamin A (color test and ultraviolet absorption) in a chromatographic fraction from undistilled lard unsaponifiable matter. It is not easy to

reconcile these later findings of Herb *et al.* (1953) with the earlier ones of Kaunitz and Slanetz (1950a,b), but the presence of vitamin A in molecular distillates of lard has also been demonstrated by Ames and Harris (1954), who converted the vitamin A to the anhydro derivative. They found that the amounts of vitamin A present were sufficient to account for all the biological activity.

At first sight these findings put an end to the "lard factor." In view of the failure of Lowe and Morton (1953) to detect vitamin A in the lard unsaponifiable matter (even after chromatography) it would appear that either the color test is powerfully inhibited or repeated molecular distillation produces vitamin A from some unknown precursor. The main difficulty in this problem would seem to be that the amounts of vitamin A (conventional or unconventional) are so small that the techniques employed to accumulate them cannot be guaranteed not to convert a hypothetical precursor of vitamin A to the conventional form. It is, of course, well known that kitol is converted to vitamin A in the process of molecular distillation.

Other work suggests that the systemic action of vitamin A may be mediated by a substance different from the known form of the vitamin A. Thus Le Gallic claimed that under certain circumstances the blood (1947) and the liver (1948) of albino rats possess vitamin A-like activity and yet contain no detectable amounts of vitamin A and less than 1 μg. carotenoid/100 ml. blood. Moore and Sharman (1951) have made numerous unsuccessful attempts, however, to substantiate Le Gallic's observations. Other investigators record unidentified substances with vitamin A activity in zoöplankton (Lane, 1950) and in shrimp oil (Grangaud and Massonet, 1948). It is interesting that shrimp oil exhibited intense antixerophthalmic activity but negligible action on growth. Grangaud *et al.* (1949) showed that the antixerophthalmic activity is associated with a substance with λ_{max} 484 mμ (astaxanthin) and, moreover, no vitamin A could be found in the oil either by the Carr-Price test or by spectrophotometric analysis (Grangaud *et al.*, 1950; Grangaud and Massonet, 1950). Recently, Grangaud *et al.* (1954) have shown that the method of chromatography employed enables them to free astaxanthin from added vitamin A and β-carotene.

The important issue in this work is whether some active derivative of vitamin A can be found to throw light on the systemic mode of action.

3. Protein and Amino Acid Metabolism

To account for the inability of vitamin A-deficient rats to produce normal epithelial cells, Mason and Ellison (1935) suggested that vita-

min A might have a role in protein metabolism within epithelial tissue. Since then several groups of workers have attempted to demonstrate such a relationship. Brown and Morgan (1948) studied both young and adult rats and used paired feeding. As the deficiency progressed, growth per gram of protein eaten declined in the deficient young rats to 65% of that of the pair-fed animals. The urinary nitrogen increased and the nitrogen balance was disturbed more in the deficient than in the pair-fed young animals. In the adult group, however, weight changes and nitrogen balance were alike in the pair-fed and deficient rats. In other words, the deficiency state decreased the utilization of nitrogen in the young rats, but the adults exhibited no difference in nitrogen metabolism. Brown and Morgan therefore concluded that vitamin A is essential for tissue protein growth but not for its maintenance.

Baumann *et al.* (1942) found that a diet low in protein reduced the storage of vitamin A in liver and increased the rate at which stores of vitamin A were depleted. On the other hand, Moore *et al.* (1952) found that a very liberal allowance of vitamin A (5000 I.U. vitamin A acetate for three days and 1000 I.U./week thereafter) enabled rats, at least partially, to resist the ill effects of a severe deficiency of protein.

The effects of "isocaloric, isonitrogenous and essentially isophosphoric" rations of casein, lactalbumin, gluten, and zein on the utilization of β-carotene were studied by James and El Gindi (1953), who found that the livers of the rats given casein were about 20% smaller in relation to body size than the livers of rats given the three other proteins. These smaller livers, however, stored from two to five times as much vitamin A as the livers of the rats receiving lactalbumin, gluten, or zein when the carotene intakes were the same for all four groups. These findings lend support to the idea that vitamin A is consumed in a liver process which transfers moieties from the ingested protein to other proteins and amino acids. This possibility has been investigated by Abdulnabi and Bieri (1953a), who studied the distribution pattern of amino acids in the testes and submaxillary glands (tissues severely affected by vitamin A deficiency) and the blood serum of both vitamin A-deficient and normal rats. Paper chromatography did not reveal any difference between the two groups, and the quantitative picture, as obtained by microbiological assay, in general supported the qualitative results. There were, however, three quantitative differences. In the testes of the deficient rats there was more tyrosine and phenylalanine, while in the normal livers there was more threonine than in the livers from the deficient rats. These results suggest that vitamin A might be involved in the metabolism of these three amino acids. If this were so, one might expect vitamin A to be implicated in some reaction characteristic of protein metabolism. This

idea has been tested by Abdulnabi and Bieri (1953b), who investigated the glutamic-aspartic transaminase system but found no difference in the reactivity of such systems in homogenates of liver, kidney, and heart from normal and vitamin A-deficient rats.

A few signposts thus point to a relationship between vitamin A and proteins, but there is a long road to be traveled.

4. Vitamin A and the Adrenals

The evidence for a relationship between vitamin A and the pituitary-adrenal function has been reviewed by Ershoff (1952), who emphasized the need for further study. The possible influence of cortisone on the conversion of carotene to vitamin A has been discussed (see p. 110).

Lowe *et al.* (1953) examined both histologically and histochemically the adrenals of 31 vitamin A-deficient rats (14 of which had severe xerophthalmia). Unlike Stoerk *et al.* (1952), they were unable to find any hyaline droplets in the adrenal cortical cells of the deficient rats, and the distribution of lipid and cholesterol appeared to be within the normal limits. However, the phospholipid content was not only reduced but confined to the zona glomerulosa. This picture has not been seen in the normal adrenal cortex (Harrison and Cain, 1947; Cain and Harrison, 1950), and it suggests disordered metabolism of adrenal cortical cells in vitamin A deficiency with consequent alteration of secretory activity. If the hypothesis of morphological and functional independence of the adrenal cortical zones be accepted (Deane and Greep, 1946; Harrison and Cain, 1947; Cain and Harrison, 1950), it would appear that, in the rat, vitamin A deficiency leads to some abnormality in the glucocorticoid activity of the adrenal cortical secretion, while the secretion of mineralocorticoids is unaffected. Lowe *et al.* (1953) pointed out that further study into this relationship is needed, taking into account the age of the rats, the duration and level of vitamin A administration, and the sodium chloride intake.

5. Vitamin A and Oxidative Mechanisms

The early work of von Euler and his coworkers (see von Euler and Ahlström, 1932) was very suggestive that vitamin A might function as an oxidative catalyst—at least it appeared that vitamin A was essential for the oxidation processes in the animal body.

Very little work seems to have been done in this field—in fact, von Euler's suggestions seem to have been forgotten. However, Ernster *et al.* (1950) showed that although fermenting yeast contained no vitamin A,

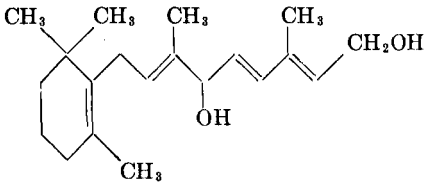
the same yeast made to respire by increased oxygen tension, did contain the vitamin (color test and ultraviolet absorption). This finding, if it were fully substantiated, would be of considerable interest and importance. The recent results of Blaizot and Serfaty (1955), who have shown that the O_2 uptake of diaphragms removed from vitamin A-deficient rats is greater than the uptake of diaphragms from control animals, are not encouraging for the oxidation catalyst role.

6. Vitamin A and Hyperkeratosis

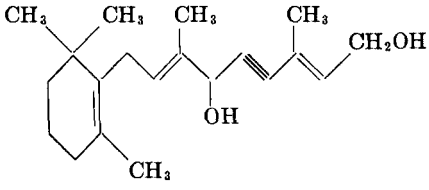
a. Metabolism of Sulfhydryl and Disulfide Groups. The current theory of keratin formation includes oxidation of cysteine sulfhydryl groups of adjacent polypeptide chains to produce cystine disulfide linkages, the covalent bonds of the latter grouping serving to hold the keratin molecule together and give it rigidity. Since vitamin A deficiency leads to excessive keratinization, the possibility that the vitamin may be concerned in the metabolism of sulfhydryl and disulfide linkages deserves scrutiny.

Flesch and Goldstone (1952) studied the reversible loss of hair induced by certain dimers used in the neoprene rubber industry. When painted on mice, guinea pigs, rabbits, and chicks, these substances produced a reversible total alopecia at the site of application. *In vitro*, the dimers inactivated free sulfhydryl compounds and sulfhydryl enzymes. Because of this, Flesch and Goldstone suggested that the depilatory action of the compounds was due to their unsaturated centers, through which they were able to unite with the sulfhydryl groups and thus prevent their conversion into disulfide linkages. Among other unsaturated compounds which they investigated, they found that vitamin A, when applied to the skin of mice in a corn oil-alcohol solution, also had a reversible local depliatory effect, but, *in vitro*, there was no interaction between vitamin A and sulfhydryl compounds. Reduction of the unsaturated compounds, including vitamin A, abolished the local depilatory activity. These are interesting findings, since alopecia is common in hypervitaminosis A (see Nieman and Klein Obbink, 1954), and various dermatoses characterized by hyperkeratosis (e.g. phrynoderma, hyperkeratosis follicularis, etc.) are now treated by giving *large* amounts of the vitamin. The work of Flesch and his colleagues throws new light on this use of vitamin A. Flesch (1952) thinks that it is not vitamin A but a degradation product which inhibits keratin formation and inactivates sulfhydryl groups. Later work (Flesch, 1953) indicated that in large amounts the methyl and phenyl ethers and the palmitic ester of vitamin A inactivated sulfhydryl groups. The synthetic products, hydroxenin and oxenin had the same

effect *in vitro* as the ethers, and the methyl ether caused loss of hair in mice on local application. The phenyl ether was, however, ineffective.



Hydroxenin



Oxenin

Montagna (1954) has also investigated the local effect of vitamin A on guinea pig skin. He found that vitamin A dissolved in linoleic acid, oleic acid, or ethanol caused hypertrophy of epidermis, but a similar hypertrophy was brought about by the fatty acids alone. Whereas the fatty acids, with or without vitamin A, seem slightly to retard keratinization, the vitamin dissolved in ethanol does not. Treatment of skin with vitamin A dissolved in oleic or linoleic acid, or indeed with the acids alone, decreased the histochemically demonstrable sulfhydryl groups, but massive doses of vitamin A in ethanol did not do so. Moreover, regardless of the amount of vitamin A or the type of vehicle employed, Montagna found no impairment of hair growth or hair loss in the guinea pig.

There is thus a sharp difference between the findings of Flesch and his coworkers on the one hand and Montagna on the other. Flesch has used mouse, rat, and rabbit and Montagna has used guinea pig, but species differences cannot readily account for the results.

Flesch considers that, in large quantities, vitamin A has "a non-specific direct drug-effect" upon the epidermis, probably connected with an interference in cutaneous sulfhydryl metabolism.

b. Vitamin A and Estrogens. The changes which occur in the vaginal epithelial cells of the rat during the estrus cycle result in the appearance of very many layers of fully keratinized cells when estrogen production is at its peak. The similarity between these changes and those brought about in epithelial tissues by vitamin A deficiency is so marked as to suggest a common denominator.

Clinical observations on morphological changes in skin said to be due to vitamin A deficiency have a bearing on this. Frazier *et al.* (1943) found the pathological changes in the pilosebaceous structures to be so correlated with age that the stage of sexual development is critical in conditioning the skin response to a deficiency in vitamin A.

There is also a "chemical castration" effect (Mason, 1933) which occurs in vitamin A-deficient male rats. Mayer and Truant (1949) com-

pared the effects of administering testosterone to vitamin A-deficient castrated and noncastrated male rats and found no difference in the hypertrophy induced in the accessory sex organs of the two groups. This implies that the castration-like effect produced by avitaminosis A is not due to lack of response of the target organs but rather to a lack of circulating androgens. On the basis of reports that blood cholesterol increases in the vitamin A-deficient rat and dog (Ralli and Waterhouse, 1933; Smith, 1934), Mayer and Truant suggested that their results might be interpreted in terms of vitamin A deficiency inhibiting the synthesis or release of sex hormones, possibly by preventing the conversion of cholesterol to testosterone. However, it should be noted that Sure *et al.* (1933) and, more recently, Green *et al.* (1955) failed to find any increase in blood cholesterol in vitamin A-deficient rats, so that the interpretation of Mayer and Truant is not necessarily correct.

That estrogens could induce hyperkeratosis was shown by Selye (1943) on the Rhino mouse, in which local application of estradiol caused an enormous increase in the production of keratin in skin. On the other hand, Montagna *et al.* (1949) recorded abundant cornification after local application of testosterone to rabbit ears. It could be argued that local application of hormones is unphysiological and the results dubious in significance. The objection is disposed of, however, by the work of Ebbing (1954), who measured the size of sebaceous glands and the thickness of the stratum germinativum during the estrous cycle in the albino rat and concluded that estrogens stimulate both the loss of sebaceous glands and the keratinization and subsequent loss of epidermal cells, quite independently of any possible changes in the rate of cell replacement.

Beyond doubt keratinization can be brought about in two ways, viz. the application of sex hormones and the withdrawal of vitamin A. The vagina of the vitamin A-deficient rat should be extremely keratinized and, in fact, the excessive cornification of vaginal epithelium in the rat, first noted by Evans and Bishop (1922), is a characteristic and early manifestation of vitamin A deficiency. It now becomes important to consider which of these two causes of keratinization is the more fundamental.

McCullough and Dalldorf (1937) saw three possible causes for keratinization, viz. estrone, vitamin A deficiency, and irritation. Vitamin A overcame the metaplasia induced by estrone, and all epithelial metaplasia and keratinization, at least in the rat, could be due to vitamin A inadequacy. Support for this idea came from Sherwood *et al.* (1937), who found that feeding fairly large doses of carotene suppressed the estrous vaginal smear in the normal rat. However, other workers found that high doses of vitamin A given orally (Burrill and Greene, 1941) or

by injection (Brody and Goldman, 1941) produced no inhibition of vaginal response. Kahn and Bern (1950) objected that, since most of the ingested and injected vitamin A merely increases the liver store, it is doubtful whether the methods used by Sherwood *et al.* (1937), Burrill and Greene (1941), and Brody and Goldman (1941) would actually result in a much increased supply of vitamin A to any specific target organ. Kahn and Bern (1950) overcame this difficulty by introducing the vitamin A directly into the vagina of ovariectomized rats which were estrogenized by subcutaneous implantation of estradiol pellets. Study of successive vaginal smears showed that the topically applied vitamin A resulted in a definite alteration of the keratinized picture produced by the estrogens (cf. McCullough and Dalldorf, 1937). Extending this work, Sabella *et al.* (1951) studied the effect of applying vitamin A, estradiol benzoate, and vitamin A plus estradiol benzoate to the shaved skin of ovariectomized rats. Histological study of the treated areas of the skin showed that the vitamin A treatment resulted in an increase in epidermal thickness, an increase in the extent of the stratum granulosum and a decreased rate of keratin formation. The effects were entirely local: moreover, the estrogens produced no observable changes in the epidermis, and, when applied with the vitamin A, did not counteract the stimulatory effect of the vitamin.

Tissue culture techniques have been used to study the interrelationship of vitamin A and estrogens. Hardy *et al.* (1953) were able to culture mouse vaginal epithelium by the hanging drop method and to show that estrogens can produce vaginal cornification *in vitro*. This technique was used by Kahn (1954), who not only confirmed the results of Hardy *et al.* (1953), but also found that his controls (without added estrogen) tended to undergo keratinization, albeit much more slowly than those to which estrogen had been added. This tendency might be due to lack of available vitamin A, for it has been shown by Fell and Mellanby (1953), using the tissue culture method on chick ectoderm explants, that vitamin A not only suppresses keratinization, but also induces the production of an actively ciliating, mucus-secreting membrane. Kahn reported, however, that a concentration of vitamin A which inhibits keratinization in normal medium did not prevent but only delayed cornification in the presence of estrogen.

The picture resulting from this work is complex, and it should be borne in mind that, although tissue culture is a useful research tool, it has uncertain relevance for the intact animal. Under normal conditions a balance may exist between the steroids (estrogens) and vitamin A (or vitamin A metabolite), disturbance of this "equilibrium" resulting in keratinizing metaplasia. Investigations of the sex hormone-vitamin A

relationship by the tissue culture technique promises well, and a study of lipid and protein metabolism within the epithelial cells under different conditions should, if practicable, give interesting results.

c. Hyperkeratosis in Cattle. At this point, it is convenient to consider X-disease or hyperkeratosis of cattle. This was first reported by Olafson (1947) and has caused serious losses throughout the U.S.A. The symptoms are in some ways similar to those of avitaminosis A, but Olafson found that an advanced case showed no response to repeated large doses of the vitamin. It is now known that the disease is due to ingestion of toxic materials, among which are certain processed feeds (Olafson and McEntee, 1951; Olson *et al.*, 1950; Olson and Cook, 1951; McEntee *et al.*, 1951), certain lubricants (Bell, 1952a), petroleum products (Sikes *et al.*, 1952), and wood preservatives (Hansel *et al.*, 1953). External application of an oil-based insecticide carrier also induced the condition (Hoekstra *et al.*, 1954a). Later work showed the chief causative agents to be chlorinated naphthalenes, and most (but not all) of the toxic feeds reported to produce X-disease have probably been contaminated with these naphthalene derivatives (Sikes *et al.* 1952; Bell, 1952b; Sikes and Bridges, 1952; Hansel *et al.*, 1953).

In the present context, the interesting point is that X-disease is accompanied by persistently low values of vitamin A in plasma. Adding vitamin A in large amounts to the diet raised the levels of vitamin A in plasma and liver, but when large doses of carotene were fed, the plasma carotene increased and the plasma vitamin A did not. This suggested the possibility that the hyperkeratosis-inducing agent interfered with the conversion of carotene to vitamin A (Hansel *et al.*, 1951). Bieri and Edwards (1955) claim, indeed, that octachloronaphthalene slightly impairs the conversion of carotene to vitamin A in rats when the carotene is fed, but not when it is injected. The problem is not so straightforward, however, because Hoekstra *et al.* (1954b) report that chickens, rats, and mice appear to be unaffected by feeds which induce hyperkeratosis in cattle. Schoettle *et al.* (1955) found, moreover, that a protein concentrate which brought about hyperkeratosis in cattle produced some cases of alopecia and mild hyperkeratosis with a depression of liver vitamin A stores when fed to rats. A commercial chlorinated naphthalene product (mixture of tetra-, penta-, and hexa-) also induced a few cases of alopecia (hyperkeratosis was not mentioned) and low levels of liver vitamin A in rats. When fed to hamsters, however, neither the protein concentrate nor the chlorinated naphthalene produced symptoms of hyperkeratosis, but some lowering of liver levels of vitamin A did occur. These varied findings offer a sharp warning that effects on one species may not reproduce those obtained on another species. If the hyperkeratosis results

from a failure to convert carotene to vitamin A, the beneficial effect of administering preformed vitamin A is not so marked as might have been expected. An alternative hypothesis has been advanced by Hove (1953), who noted low plasma tocopherol levels as well as low plasma vitamin A in several cases of X-disease. Pentachloronaphthalene is a prooxidant *in vitro*, and Hove suggests that the toxic chemicals catalyze oxidation of unsaturated lipids and fat-soluble vitamins.

The effect of protein on the toxicity to rats of hexachloronaphthalene has been studied by Engel *et al.* (1955). When hexachloronaphthalene (3–4 mg.) was given to rats for a period of one or two weeks on a 9% casein diet, there was an accumulation of fat in the liver and some liver hypertrophy. When the experiment was repeated using diets containing either 18 or 27% casein (proportionately less sucrose), however, there was significant protection against the induced fatty liver. The ability of estrogens and chlorinated naphthalene derivatives to cause hyperkeratosis, and the fact that the latter substances depress the plasma vitamin A, are very striking phenomena, but a full explanation cannot be offered yet.

d. Nucleic Acids. The effect of vitamin A deficiency on nucleic acid metabolism is the subject of a recent brief report. Niesar (1955) has found that the levels of both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in rat skin, testis, gut, kidney, adrenal, diaphragm, heart, brain, and thyroid fall in avitaminosis A; in the spleen and liver, however, the effect is reversed. When vitamin A palmitate (1,000 I.U. in water emulsion) was subcutaneously injected into normal rats, there was a rise in the RNA and DNA content of all the above mentioned organs (except the brain, where there was a slight fall in DNA, and thyroid was not mentioned). The rise in RNA was much more marked than the rise in DNA.

The deep-seated character of the changes in the epithelial cell during avitaminosis A has been stressed earlier in the present review. In the light of current ideas on the biological role of DNA, Niesar's report is significant. It needs to be confirmed and followed up.

e. Hyperkeratosis and Lipid Metabolism. The nature of lipid changes occurring during the process of epidermal keratinization was followed by Kooyman (1932) and Koppenhoefer (1936). Both workers found that the phospholipid content of the epidermis decreases greatly during keratinization. There is also a decrease in cholesterol, but it is not so marked. This means that the ratio phospholipid/cholesterol is much higher for the cells in the basal layers than it is for the keratin or horny layers. Kooyman found that the cholesterol in the unchanged cell was free, whereas in the keratinized cell it was in part esterified.

Attempts have been made to correlate lipid metabolism, keratinizing

metaplasia, and vitamin A. Monaghan and Schmitt (1932) demonstrated that *in vitro* small concentrations of vitamin A could for some hours completely inhibit the oxygen uptake of linoleic acid. Since much of the linoleic acid in the body occurs in phospholipids, it appeared that vitamin A might have a precise role in phospholipid metabolism. This is plausible, since lesions characteristic of vitamin A deficiency occur primarily in tissues rich in unsaturated phospholipid. Moreover, as Monaghan and Schmitt pointed out, a change from cuboidal or columnar to stratified cells, such as occurs in keratinizing metaplasia, might involve deep-seated modifications of cellular elements such as the Golgi apparatus or mitochondria, which contain phospholipid. Monaghan (1932) tested this hypothesis when she studied the effects of dietary deficiences (vitamin A, thiamine, riboflavin, vitamin D, and acute fasting) on phospholipid metabolism. She found a constant value for the phospholipid fatty acids in a given tissue of rats which were growing normally on an adequate diet, but, as soon as the growth rate fell, irrespective of the type of dietary deficiency, the phospholipid fatty acid content of the tissues decreased. Moreover, the iodine value of the acids indicated no difference in the degree of unsaturation of the vitamin-deficient tissues compared with the normal tissues. It would therefore appear that vitamin A is not concerned directly in the metabolism of fatty acids.

This view is supported by the work of Green (1934) on the utilization of fat by vitamin A-deficient rats compared with rats on a complete diet restricted in amount to equal that eaten by deficient animals. He found no evidence that the vitamin A-deficient rat failed to utilize its own fat reserves. At the terminal stages of vitamin A-deficiency, there was a rise in the iodine value of the liver fatty acids which coincided with a fall in the amount of fat in the liver. This effect is not peculiar to the vitamin A-deficiency state, as a similar increase in iodine number of liver fatty acids occurred in rats losing liver fat following either too little food or infection. Leutskii and Lyubovich (1954) claim a slight increase in unsaturation in fatty acids from liver, kidneys and lungs of vitamin A-deficient rats (Table VI). Unfortunately, their controls do not seem to have had the caloric restriction which Monaghan (1932) and Green (1934) found to be important.

As opposed to Monaghan (1932), Javillier *et al.* (1929) claimed a rise in total body phospholipids of mice in vitamin A deficiency and Leutskii and Lyubovich (1954) found an increase in the phospholipid content of kidney, lung, and brain of vitamin A-deficient rats (Table VI). Contradictory results here may depend on the type of controls and on analytical difficulties with phospholipids.

While examining the unsaponifiable fraction of liver lipid from vitamin A-deficient rats, Lowe *et al.* (1953) noted the presence of two substances which, at that time, had not been previously recorded as present in lipid unsaponifiable matter in the normal rat liver. These substances were characterized by the following absorption spectra (cyclohexane):

(1) $\lambda\lambda_{max}$ 275 mμ and 332 mμ with marked inflections at 233 mμ and 283 mμ.

(2) λ_{max} 275 mμ with much weaker absorption near 330 mμ and no inflection at 283 mμ.

TABLE VI

EFFECT OF VITAMIN A DEFICIENCY ON PHOSPHOLIPID AND UNSATURATED FATTY ACIDS OF RAT TISSUES

	Phospholipid (mg. per 100 g. dry wt.)		Iodine value per 100 g. crude tissue	
	Vitamin A-deficient	Control	Vitamin A-deficient	Control
Liver	290	290	12.28	9.77
Kidney	713	520	10.33	9.02
Lung	740.6	372	10.97	9.33
Muscle	194.5	182.1	not reported	
Brain	686.6	426.1		

Mean figures given in each case.
Modified from Leutskii and Lyubovich (1954).

Heaton *et al.* (1955) have extended this early work and have studied the unsaponifiable fractions of liver, kidney, small intestine, vagina, submaxillary gland, urinary bladder, testis, and skin from around the eye, taken from stock colony, control, and vitamin A-deficient rats. The unsaponifiable fractions from the different organs were chromatographed on weakened alumina and the new constituents were again observed, each member being characterized by its typical ultraviolet absorption both in cyclohexane:

Substance SA λ_{max} 272 mμ; λ_{min} 235 mμ, inflections 330 and 410 mμ.

Substance SC $\lambda\lambda_{max}$ 275 mμ; 330 mμ, inflections 233 and 283 mμ.

Both SA and SC are normal, although very minor, constituents of rat liver, kidney, and intestine; in fact, investigations into other species have shown that SA and SC are widely distributed, being found in the liver and kidney of pig, sheep, dog, and pullet; both are present in the human kidney (Cunningham *et al.*, 1955). When the rat is made vitamin A-deficient, the amount of SC present in the liver increases remarkably:

liver from xerophthalmic rats contains approximately 75 to 80 times as much SC as liver from stock rats (liver stores of vitamin A between 500 and 700 I.U./g.), and livers from rats on the vitamin A-free diet but supplied with 25 I.U. vitamin A per day contain about 40 times as much SC as the stock colony rats. There was a very slight rise in the intestinal content of SC during avitaminosis A, but the amounts present in the kidney appear to be independent of vitamin A status. The amount of SA found in vitamin A-deficient rat liver is also greater than that present in the livers of stock and control rats; once again, there appears to be little change in the kidney concentration of SA, although there is a rise in the amount present in the submaxillary gland. Fluctuations in the 7-dehydrosteroid content of the intestine have also been noted, there being approximately six times as much in the stock colony rats as in the deficients; in the vaginas, bladders, and submaxillary glands the effect is reversed, though not so markedly.

Some preliminary work on the structure of SA has been reported (Festenstein *et al.*, 1955), from which it would appear that SA is probably steroid in nature.

It is not easy to assess the significance of these changes in the unsaponifiable constituents of the different organs of the rat during avitaminosis A, and indeed, other species need to be studied. Lowe *et al.* (1953) have suggested that perhaps the best working hypothesis is that the increased amounts of substances SA and SC result from a lifting of restraint on the dehydrogenation of cholesterol or steroid hormones. The rather complex absorption spectrum of SC certainly requires some quite unsaturated molecule; in fact, the nearest approach to its spectrum is that of 2-naphthal acetone and its derivatives (see Wilds *et al.*, 1947). If SC does in fact prove to be steroid in nature, the relationship between the sex hormones and vitamin A will be brought much nearer to elucidation. The suggestion of Lowe *et al.* (1953) is in line with the findings of Leutskii and Lyubovich (1954) with regard to the increase in the degree of unsaturation of fatty acids during avitaminosis A.

On the basis of this tentative hypothesis, the results of Heaton *et al.* (1955) imply that cholesterol metabolism might be drastically deranged during vitamin A deficiency in the rat. Such a derangement could be pictured as a sequence of abnormal metabolites, of which only those exhibiting typical ultraviolet absorption would be detected by the method employed (i.e. ultraviolet spectrophotometry). Alternatively, the deranged metabolism might be quantitatively of little significance, in the sense that the proportion of steroid molecules affected would be rather small, although the new materials might be qualitatively sharply different, e.g. in their physiological effects. However, as previously mentioned,

there is no change in the plasma level of cholesterol during avitaminosis A (Green *et al.*, 1955). If, therefore, the substances SA and SC which accumulate in the livers of vitamin A-deficient rats are indeed dehydrogenation products of cholesterol or steroid hormones, then the amounts must be too small to make a measurable contribution to the total liver and plasma cholesterol levels. If the compounds are themselves toxic or the precursors of other toxic substances, then the amounts of the compounds formed need only be relatively small. Hence, the fact that the blood cholesterol level does not alter in vitamin A deficiency in the rat is not necessarily against the working hypothesis of Lowe *et al.* (1953). On the other hand, although the tendency to form increased amounts of 7-dehydrosteroid in the vaginas, bladders, and submaxillary glands supports the hypothesis, the marked decrease in the intestinal 7-dehydrosteroid content is not readily accounted for.

It is quite clear that much remains to be done in this particular branch of the field; nevertheless, when it is eventually shown that the increase in hepatic SA and SC is characteristic of vitamin A-deficiency and is not a general response to stress (e.g. starvation), this might prove to be a fruitful field of research.

IV. Conclusions

The present article has been concerned with vitamin A as an important catalyst in the animal economy, a catalyst which no doubt has its own peculiar mode of action but which cannot be viewed in isolation. Many other catalysts are obviously more or less directly concerned, and there would be some truth in the suggestion that to understand the mode of action of vitamin A much more must be found out about the roles of other vitamins and hormones. A truer analogy would be that we are at present in the position of a man who is inspecting a house from the outside. Without the key to the front door he can peer through the windows, he can guess at the layout of the cellars and the upper floors and he can climb up a tree to get a better view. The general trend of biochemical study leads us to think that there is a front door key to the biochemistry of vitamin A and that we have not found it.

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Regulation of Carbohydrate Metabolism in Isolated Tissues*

ALBERT E. RENOLD, JAMES ASHMORE, AND A. BAIRD HASTINGS

Department of Biological Chemistry,
Harvard Medical School, Boston, Massachusetts

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I. Introduction

The presence of an important regulatory mechanism for the normal functioning of carbohydrate metabolism has been apparent since Willis' discovery (1679) that the urine of patients affected by the strange and rapidly wasting disease already known as diabetes was sweet to the taste. Further *prima facie* evidence for the existence of such a regulation resulted from the observation of stable blood glucose concentrations in the face of pronounced alterations in its rate of accretion and in its rate of depletion. A complex regulatory system had to be postulated in order to insure adequate coordination between mainly glucose-producing and mainly glucose-utilizing tissues. Though nervous signals have been established, chemical signals appear to be chiefly responsible for this regulation of carbohydrate metabolism. This discussion will be restricted to the influence of hormones, of variations in the available substrates (such as changes in diet or fasting), and of changes in the intracellular and extracellular environment, such as those produced by variations in the absolute or relative concentration of ions.

The metabolic response to a hormone is determined by its chemical structure and by the specific responsiveness of individual tissues. The latter group of factors is best studied in systems providing adequate isolation of otherwise intact tissues. Major emphasis in this review will therefore be given to *in vitro* techniques utilizing tissue slices, cell suspensions, or perfused organs. Indeed, to emphasize this often neglected aspect of hormonal regulation, the material will be arbitrarily grouped according to tissues rather than according to hormones or other regulating factors. Effects obtained in preparations in which the architecture of the cell has not been preserved will not be discussed, nor will the effect of exogenous metabolic poisons, co-factors or inhibitors be presented.

Even though the subject has been thus limited, the area to be reviewed remains extensive indeed. The authors have availed themselves of the Editors' permission to prepare a review centered around their own experiments and recent interests, and as a result have perhaps given undue prominence to work from their laboratory. More recent observations have been given preference and attention has been particularly concentrated on studies where the isotopic labeling of substrates has permitted study of the quantitative metabolic relationships involved.

II. Carbohydrate Metabolism

In recent years, it has become customary to consider under the term carbohydrate metabolism not only the metabolism of carbohydrates *per se*, but also those intermediary metabolic products which are common to

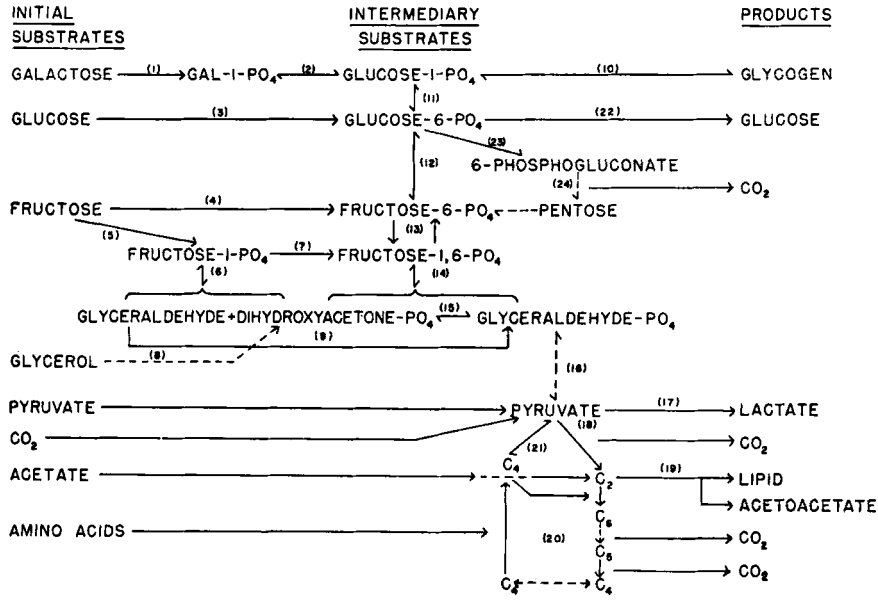

FIG. 1. Composite scheme of carbohydrate metabolism. Substrates participating in carbohydrate metabolism are listed at the left. Products of reactions are listed at the right. The individual reactions have been numbered and the enzymes and cofactors are given to facilitate identification of specific reactions in the text. The components of the tricarboxylic acid cycle are indicated by the symbols C_2 = acetyl, C_4 = dicarboxylic acids, C_5 = α-ketoglutaric acid, C_6 = tricarboxylic acids. The individual reactions are: (1) galactokinase—ATP, Mg, (2) waldenase—UDPG, (3) glucokinase—ATP, Mg, (4) hexokinase—ATP, Mg, (5) fructokinase, ATP, (6) fructoaldolase, (7) phosphofructokinase—ATP, (8) glycerolkinase ATP + α-glycerophosphate dehydrogenase, (9) triokinase ATP, (10) phosphorylase, (11) phosphoglucomutase—glucose-1,6-PO_4, (12) phosphohexoisomerase, (13) 6-phosphofructokinase, fructose-1,6-diphosphatase, (14) aldolase, (15) triose isomerase, (16) glycolytic reactions between glyceraldehyde phosphate and pyruvate (details omitted), (17) lactic acid dehydrogenase—DPN, (18) pyruvate decarboxylation, (19) active acetate condensation to acetoacetate, cholesterol and fatty acids—Co A, (20) tricarboxylic acid cycle (details omitted), (21) oxaloacetic decarboxylase—Mn, (22) glucose-6-phosphatase, (23) glucose-6-phosphate dehydrogenase—TPN, Mg, (24) phosphogluconate oxidation pathway (details omitted).

all organic compounds used by cells as metabolic fuels. When reviewing specific aspects of carbohydrate metabolism, it is necessary, therefore, to define the limits of the term as used in this particular instance. A scheme of carbohydrate metabolism, as understood in this review, is given in Fig. 1. It should be stressed that the scheme is a *composite* one and does not apply *in toto* to any single tissue except liver. It is given here to clarify the concept of carbohydrate metabolism and to serve as a basis for the

discussions of metabolism in specific tissues. The reactions which in the scheme lie above pyruvate are those which belong to carbohydrate metabolism in its stricter sense. Those below pyruvate form the connecting links among all metabolic fuels (Barron, 1943). The various steps have been numbered for future reference, either as single reactions or as groups of reactions. Some of the enzymes and co-factors involved are listed in the legend. Broken lines are used to indicate that steps in metabolism have been omitted.

The alternative pathway of glucose metabolism (reactions 23 and 24) first described by Dickens in 1936 has received a great deal of attention in the last few years. The enzymatic transformations involved in the metabolism of glucose by this pathway have been reviewed by Dickens (1953), Racker (1954), and Horecker and Mehler (1955). Glock and McLean (1954) and Kelly *et al.* (1955) have compared the activity of the first two enzymes of this pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) in various tissues. Several attempts have been made to evaluate the relative importance of the glycolytic and the phosphogluconate-oxidation pathways of glucose metabolism in intact animals as well as in isolated tissues, by comparing the metabolism of glucose-1-C^{14} with that of glucose-6-C^{14}. The incorporation of C^{14} from these substrates into fatty acids has been measured in isolated mammary tissue (Abraham *et al.*, 1954) and liver slices (Bloom and Stetten, 1955), and C^{14} incorporation into lactic acid has been measured in corneal epithelium (Kinoshita *et al.*, 1955) and lens (Kinoshita, 1955). The oxidation of carbons 1 and 6 of glucose to CO_2 has been measured in liver (Katz *et al.*, 1955), kidney, and diaphragm (Bloom and Stetten, 1955). These studies would indicate that the direct oxidation of glucose is an active process in lens, corneal epithelial, mammary, and liver tissue, but accounts for little if any of the over-all oxidation of glucose in kidney slices and diaphragm muscle.

III. Muscle

Muscle represents the main fuel-utilizing tissue of the mammalian organism and has been extensively studied. Various preparations incubated *in vitro* have been used in an attempt to isolate muscular metabolism. These preparations have included the diaphragm of the rat or mouse, the sartorius muscle of the frog, the gastrocnemius and abdominal muscles of the rat, heart and skeletal muscle slices of various species, and smooth muscle strips. A second group of preparations has been used, including the eviscerated dog and rabbit, the perfused hind limbs of the dog and rabbit, and the evaluation of data obtained by simultaneous analysis of arterial blood and of blood sampled by catheteri-

zation of the coronary sinus. While the latter preparations do not provide ideal isolation of muscular metabolism, they represent situations in which the general physiological relationships have been less disturbed than is the case for the *in vitro* studies.

Many of the reactions indicated in the composite scheme of carbohydrate metabolism (Fig. 1) occur only to a slight degree, or not at all, in muscle tissue. Galactokinase (reaction 1, Fig. 1) is not found in skeletal muscle (Cardini and Leloir, 1953; Bollman *et al.*, 1935). Villee *et al.* (1952) have found that glycerol is not utilized by isolated rat diaphragm. In experiments in which glycerol-α-C^{14} was incubated with rat diaphragm for two hours, all of the glycerol was recovered at the end of the experiment and its specific activity remained unchanged. Although all of the enzymes concerned with the utilization of fructose have been demonstrated in muscle (Slein *et al.*, 1950; Hers, 1955), fructose utilization by this tissue is considerably slower than glucose utilization, even when fructose is the only substrate. When equal quantities of glucose and fructose are incubated with diaphragm, fructose utilization is only about one-fourth that of glucose (Renold and Thorn, 1955), a situation strikingly different from that which occurs in liver. Recent experiments by Hers (1955) indicate that fructose metabolism by muscle is mostly by reaction 4 (Fig. 1)—i.e., directly phosphorylated to fructose-6-PO_4. Pyruvate is utilized by isolated rat diaphragm at approximately five times the rate of glucose (Villee and Hastings, 1949a,b; Villee *et al.*, 1952). Muscle contains no glucose-6-phosphatase (reaction 22, Fig. 1) (Cori *et al.*, 1938; Villee *et al.*, 1952), and the enzymes of the phosphogluconate oxidation pathway (reactions 23 and 24, Fig. 1) are absent in this tissue (Glock and McLean, 1954).

1. Insulin

In isolated tissues, just as in the intact organism, the main approaches to the study of the metabolic effects of a hormone are the observations of the changes which result from absence of the hormone and of the changes which result from its administration, either *in vivo*—before isolating the tissue—or *in vitro*. The effects of insulin on muscle have been studied mainly by comparing the metabolism of tissues removed from normal and diabetic rats, and the metabolism of tissues incubated with and without insulin. The methods most commonly used for the experimental production of diabetes mellitus have been the administration of alloxan and total or subtotal pancreatectomy. Although alloxan exerts widespread toxic effects on tissues other than the pancreas, these effects are mostly repaired within one or two weeks after administration of the compound. To date, no significant differences in carbohydrate metabolism have been found between these two types of experimental diabetes

(Mirsky, 1952; Renold *et al.*, 1953). Excellent recent reviews on the action of insulin have been published by Stadie (1951, 1954, 1955a,b), and alloxan diabetes was reviewed by Bailey in Volume 7 (1949).

When compared with normal muscle, muscle from diabetic animals has been shown to take up less glucose from the incubation medium; this is true for rat diaphragm (Krahl and Cori, 1947; Villee and Hastings, 1949a), for the isolated heart in heart-lung preparations (Cruickshank and Shrivastava, 1930; Cruickshank, 1936), and for the heart isolated *in situ* by coronary sinus catheterization in dogs (Ungar *et al.*, 1955) and man (Goodale *et al.*, 1951; Ungar *et al.*, 1955). Glycogen deposition in the presence of glucose is decreased in diaphragm from diabetic rats (Krahl and Cori, 1947; Villee and Hastings, 1949a). Whereas over-all oxygen consumption by the diaphragm is not significantly altered (Stadie and Zapp, 1947), the oxidation of glucose carbon to CO_2 is diminished in the presence of diabetes (Villee and Hastings, 1949a; Villee *et al.*, 1952). Decreased utilization of pyruvate and acetate by the diaphragm (Villee and Hastings, 1949b; Foster and Villee, 1954) and by heart muscle slices (Pearson *et al.*, 1949) of the diabetic rat, and decreased oxidation of pyruvate carbon to CO_2 (Villee and Hastings, 1949b) have also been reported.

In muscle the effects of insulin added *in vitro* have been extensively studied and represent one of the rare examples of significant, reproducible, and almost immediate hormonal effects which can be elicited by the addition *in vitro* of a hormone to an isolated tissue. Insulin increases glucose uptake by rat diaphragm (Gemmill, 1940; Krahl and Cori, 1947; Villee and Hastings, 1949a), by the perfused rat heart (Bleehen and Fisher, 1954), by the heart isolated *in situ* (Goodale *et al.*, 1951; Ungar *et al.*, 1955), by eviscerated preparations of dogs (Mann and Magath, 1923) and rabbits (Wick *et al.*, 1951), and by the isolated hind limb of the dog (Lundsgaard *et al.*, 1939). Insulin increases glycogen deposition in the presence of glucose (Gemmill, 1940, 1941; Gemmill and Hamman, 1941; Stadie and Zapp, 1947; Villee and Hastings, 1949a) and the incorporation of glucose carbon into glycogen and pyruvate (Villee *et al.*, 1952), as well as its oxidation to CO_2 (Wick *et al.*, 1951; Villee and Hastings, 1949a; Villee *et al.*, 1952). In diaphragms and in heart muscle slices obtained from diabetic rats, insulin increases pyruvate utilization toward normal (Villee *et al.*, 1952; Pearson *et al.*, 1949), as well as the oxidation of pyruvate carbon to CO_2 (Villee *et al.*, 1952), although the latter effect has not been consistently obtained (Foster and Villee, 1954). Furthermore, insulin increases phosphorus turnover in the rat diaphragm (Sacks, 1952) and leads to an increased phosphorylation in this tissue in excess of the loss in acid-labile phosphorus (Haugaard *et al.*, 1951). This observa-

tion has been interpreted as indicating an increased efficiency of oxidative phosphorylation, since no concurrent changes in oxygen uptake or respiratory quotient were noted (Stadie, 1955a). Insulin increases incorporation of alanine into rat diaphragm protein (Sinex *et al.*, 1952); an insulin effect on glucose-1-phosphate utilization but not on glycogen synthesis from that substrate has also been reported (Beloff-Chain *et al.*, 1953, 1955a).

Further support for the true hormonal nature of the effects of insulin on muscle preparations has come from the correlation which has been established between the amount of insulin "bound" to such preparations and the biological effect obtained (Stadie *et al.*, 1951, 1952, 1953). The amount of "bound" insulin was measured by utilizing S^{35}- or I^{131}-labeled insulin in which the labeling did not appear to affect the activity of the hormone. On this basis a metabolic activity per unit of bound insulin was calculated and was found to be of the same order of magnitude when insulin was added *in vitro* as when it was administered *in vivo* before removing the diaphragm (Stadie *et al.*, 1952). In perfused rat heart, insulin activity was found with physiological concentrations of insulin, and irreversible binding of insulin was not observed (Bleehen and Fisher, 1954).

2. Diabetogenic Hormone of the Pituitary

Many attempts have been made to assess the action of pituitary hormones on carbohydrate metabolism *in vitro*. Recognition of the hypophysis as an important factor in carbohydrate metabolism began with the experiments of Houssay (1929), in which it was demonstrated that removal of the hypophysis from diabetic animals resulted in decreased blood glucose levels and prevented loss of glucose in the urine. Fractionation of the anterior hypophysis in the search for a tropic hormone with diabetogenic properties has resulted in many extracts with varying potency. The diabetogenic hormone of the pituitary may be identical with the somatotropic or growth hormone, but this has not as yet been definitely established. We shall consider here the effects on carbohydrate metabolism of any fraction of the anterior pituitary which cannot be shown to exert its effects through either the thyroid or the adrenal gland.

The first attempts to determine at a biochemical level the effect of the hypophysis on carbohydrate metabolism of isolated muscle were made by comparing the metabolism of glucose and pyruvate by isolated diaphragm from normal, hypophysectomized, diabetic, and hypophysectomized-diabetic rats (Krahl and Park, 1948; Villee and Hastings, 1949a). This work established that hypophysectomy results in an increased glucose uptake which may be further increased by the addition *in vitro* of insulin. Furthermore, Stadie *et al.* (1953) demonstrated that in diaphragm ob-

tained from hypophysectomized rats, insulin has an increased metabolic activity per unit of bound insulin, a finding which correlates well with the enhanced sensitivity to insulin of hypophysectomized animals and patients with hypopituitarism.

Park *et al.* (1952) followed changes in blood glucose as well as in the metabolism *in vitro* of diaphragm after injection of pituitary fractions rich in growth hormone into hypophysectomized rats. Within two hours after injection of the pituitary extracts, blood glucose dropped 30 mg. per cent and glucose uptake by the isolated diaphragm increased. Similar results on glucose uptake by isolated diaphragms from hypophysectomized rats have been obtained by the addition *in vitro* of certain growth hormone preparations. Within three hours after injection of growth hormone, however, inhibition of glucose uptake by the isolated diaphragm was observed. This inhibition persisted for as long as 24 hours after injection of the hormone. The effect of various pituitary fractions on glucose uptake has been reviewed by Park (1952) and more recently by Krahl (1956).

The effects of growth hormone on muscle glycogen have been reviewed by Russell (1953). These are more pronounced when the hormone is administered *in vivo* than *in vitro*. In muscle obtained from fasting rats, addition of growth hormone to the incubation medium (phosphate buffer) diminished glycogen breakdown but did not influence the total amount of carbohydrate. When these experiments were repeated in bicarbonate buffer (with low phosphate content) no effect of growth hormone on muscle glycogen was observed. The respiratory quotient of glucose-fed rats is reduced by the injection of pituitary fractions (Greaves *et al.*, 1940).

These somewhat inconclusive results concerning the effects of growth hormone *in vitro* have recently been supplemented by the study of inhibitors of glucose uptake which are present in the serum of animals with intact pituitaries. Experiments of Tuerkischer and Wertheimer (1948) indicated that diaphragms from normal animals took up less glucose when incubated in sera from diabetic animals. Bornstein and Park (1953) confirmed these observations and extended them to sera from diabetic-hypophysectomized animals. In the latter case no inhibition of glucose uptake was observed, and the addition of growth hormone and of adrenocortical hormones was without effect when added *in vitro*. However, when both hormones were injected into hypophysectomized diabetic rats, sera then obtained from such animals inhibited glucose uptake by normal diaphragm. Bornstein (1953) found that the inhibitory substance was associated with the β-lipoprotein fraction of serum and that the inhibition could be reversed by the *in vitro* addition of insulin.

Krahl and Bornstein (1954) demonstrated that lipoprotein preparations from beef pituitaries inhibit glucose utilization by cell-free muscle extracts fortified with ATP and other components of the glucokinase system. Three to thirty micrograms of pituitary lipoprotein were sufficient to inhibit glucose phosphorylation. While growth hormone preparations varied in their ability to produce an inhibition of glucose phosphorylation (Colowick *et al.*, 1947), pituitary lipoprotein inhibited glucose phosphorylation in every muscle preparation tested (Krahl, 1955).

3. Thyroid Hormones

Most attempts to define the action of thyroid hormones on tissues have been concerned with their effects on oxygen uptake, electron transport mechanisms and oxidative phosphorylation. They have been recently reviewed by Barker (1951, 1955). The study of these and other effects on isolated tissues has been hampered by the characteristic lag which occurs between the administration of thyroxin or desiccated thyroid extracts and the onset of measurable changes. By incubation of isolated frog hearts with thyroxine for prolonged periods of time, pronounced increases in oxygen consumption were observed after 10 hours (Davis *et al.*, 1934). Thibault and Pitt-Rivers (1955) have recently observed that acetic acid analogues of thyroxine and triiodothyronine increase the oxygen consumption of kidney tissue *in vitro* without a latent period, suggesting that the delay in thyroxin action might be because it must undergo chemical alteration in order to be metabolically active.

Whereas studies in intact organisms strongly suggest the presence of thyroidal effects on carbohydrate metabolism (Houssay, 1948; Soskin and Levine, 1952), these effects have been little studied in isolated tissues. Thyroidectomy has been reported to decrease, and thyroxin to increase glucose uptake by the perfused rat heart (Ambrus, 1929). Thyroxin accelerated glycogen breakdown in the isolated rat auricle (Andrus *et al.*, 1930). These observations, however, may have been directly related to an increased rate of cardiac contractions. Wertheimer and Bentor (1953) have recently reviewed the effects of thyroid hormones, both administered *in vivo* and added *in vitro*, on rat diaphragm and on rat abdominal muscles. They measured glucose uptake and glycogen synthesis in the presence of glucose; these measurements were not altered by thyroidectomy two weeks before sacrifice. The administration of physiological doses of thyroxine and of thyroid-stimulating hormone a few hours before removing the diaphragm produced a very significant increase in glucose uptake and in glycogen synthesis, when muscle preparations were incubated in homologous serum; no effects were observed when incubation was carried out in Krebs-Ringer solution. Similar effects were pro-

duced by adding thyroxine *in vitro* in concentrations approaching physiological values (0.1 to 2.0 μg. per ml.). Again, the substitution of buffers for homologous serum led to disappearance of these effects of thyroxine. Larger doses of thyroxine either *in vivo* or *in vitro* exerted an irregular and sometimes opposite action. In the *in vivo* studies these effects on carbohydrate metabolism were maximal when the muscles were removed within one to four hours. They declined after six hours following the intravenous injection of the hormone, in contrast to the effects on oxygen consumption, which start later and last for several days. Wertheimer and Bentor (1953) suggested that this might represent a different type of thyroidal effect, perhaps one connected with the anabolic action which has been frequently postulated for thyroxine on the basis of general physiological considerations and which might well be associated only with small doses of the hormone. Kline (1949) reported that thyroidectomy decreases and thyroid administration increases the release of α-amino nitrogen by isolated diaphragm.

Although the effects of thyroid hormones on oxygen uptake alone are outside the scope of this review, the studies of Barker and Klitgaard (1952) and of Barker and Schwartz (1953) should be mentioned, since they provide an excellent example of the individual response of different tissues to hormonal agents. These authors isolated a number of rat tissues at varying time intervals after the administration of thyroid hormone and demonstrated that the oxygen consumption of some of these varied in a fashion approximately parallel to the increased oxygen consumption observed in the whole animal. A number of tissues, however, showed no change whatsoever in oxygen consumption, whereas the heart showed percentile changes greatly in excess of the increased metabolic rate of the intact organism.

4. Adrenocortical Hormones

The role of the adrenal cortex in the regulation of carbohydrate metabolism has been recently reviewed by Long (1953), Conn (1953), and Soskin and Levine (1952). It is generally accepted that hydrocortisone and corticosterone are the main carriers of this function of the adrenal cortex in the mammalian organism. Furthermore, the available evidence indicates that their main effect on carbohydrate metabolism is that of increasing hepatic gluconeogenesis. However, direct effects on extrahepatic tissues have been demonstrated. These effects are mainly related either to the mobilization of protein or to the utilization of glucose. Kline (1949) reported that adrenalectomy decreases the release of amino nitrogen by isolated diaphragm and that treatment with adrenal cortical extract restores the release of nitrogen to normal. It has also been established that adrenalectomy decreases (Ingle *et al.*, 1948; Bondy, 1949) and

the administration of adrenocortical extracts increases (Bondy, 1949) the amino acid concentration in the serum of hepatectomized animals, thereby confirming previous findings in intact animals (Engel *et al.*, 1949; Bondy *et al.*, 1949; Hoberman, 1950). In addition, the administration of carbohydrate-active steroids to eviscerated rats under carefully controlled conditions has been shown to decrease the rate of glucose removal from the blood stream (Ingle *et al.*, 1947, 1953). Previous observations in intact animals had shown that the glucosuria produced by large doses of carbohydrate-active corticoids is not entirely accounted for by increased protein catabolism (as evidenced by the increased excretion of urinary nitrogen) and had led to the assumption that adrenocortical steroids might interfere directly with the utilization of glucose. However, this has not as yet been substantiated by clear-cut and direct evidence. Indeed Wick *et al.* (1951) and Drury (1953) reported that the oxidation of C^{14}-labeled glucose to CO_2 is the same in eviscerated animals whether untreated, previously adrenalectomized, or given large doses of cortisone. Goldstein *et al.* (1950) have demonstrated that the "insulin-antagonism" which is exerted by carbohydrate-active steroids cannot be explained on the basis of interference with the effect of insulin on the permeability of cell surfaces to sugars such as galactose or glucose. The eviscerated adrenalectomized rat is not unduly sensitive to insulin (Levine *et al.*, 1949).

Contradictory evidence has been obtained in the isolated diaphragm. While Villee and Hastings (1949a) reported a slightly increased glucose uptake by diaphragm from adrenalectomized rats, Park (1952) and others (Koepf *et al.*, 1941a; Krahl and Cori, 1947; Li *et al.*, 1949) found no change in the glucose uptake by diaphragms from adrenalectomized or cortisone- and ACTH-treated animals when compared with normal tissues. However, Krahl and Cori (1947) reported a return to normal of the depressed glucose uptake in diabetic animals which were subsequently adrenalectomized. No consistently reproducible effects of carbohydrate-active corticoids added to muscle tissue *in vitro* have as yet been established. Definite effects *in vivo* and *in vitro* of the mainly electrolyte-active steroids deoxycorticosterone and aldosterone (Fluckiger and Verzar, 1954) and of related steroids including corticosterone have been described by Verzar and collaborators (1951). These effects have been related by these authors to their concept of the role of the adrenal cortex in facilitating phosphorylating processes.

Finally, it should be mentioned that observations are accumulating which would indicate an interdependence of the diabetogenic hormone of the pituitary and the carbohydrate-active steroids of the adrenal cortex in regulating carbohydrate utilization (Park, 1952; Krahl, 1955; deBodo

and Altszuler, 1955). This interdependence is sometimes antagonistic, sometimes synergistic, and has not as yet been clarified.

5. Glucagon and Epinephrine

The action *in vitro* of glucagon (hyperglycemic or HG factor) and epinephrine in regulating the carbohydrate metabolism of isolated tissue has been reviewed by Sutherland (1951a,b, 1952a,b) and by Cori (1956). No definite effect of glucagon on the metabolism of isolated muscle has been observed, although preliminary experiments by Candela (1953) indicate that addition of hyperglycemic factor *in vitro* prevents insulin stimulation of glucose uptake by isolated diaphragm. Similar conclusions were reached by Drury *et al.* (1954) in their studies on the effect of glucagon on glucose distribution in the eviscerated, nephrectomized rabbit.

When epinephrine is added *in vitro* to diaphragm incubated in phosphate buffer with glucose as the added substrate, glucose uptake is depressed, glycogen content is markedly decreased, and there is an increase in lactic acid production by the tissue (Riesser, 1947; Walaas and Walaas, 1950). These effects of epinephrine have been attributed to an increased rate of conversion of muscle phosphorylase from an inactive to an active form (Sutherland and Cori, 1951).

6. Composition of the Medium

When isolated rat diaphragm is incubated in serum, an increase in glucose utilization and glycogen synthesis is found over that obtained in an artificial physiological solution (Tuerkischer and Wertheimer, 1948; Groen *et al.*, 1952). These effects have been attributed to the insulin content of normal sera (Randle, 1955). Although liver rapidly loses its intracellular potassium when incubated in solution containing physiological concentrations of potassium, the rat diaphragm maintains a constant potassium content during four hours of incubation (Calkins *et al.*, 1954). Glycogen synthesis is optimal in the presence of low external potassium concentrations (Stadie and Zapp, 1947), although deposition of glycogen is accompanied by a net potassium uptake by the isolated diaphragm. In phosphate buffer, glucose utilization by rat diaphragm is increased over that found in bicarbonate buffer, but glycogen synthesis is virtually abolished (Villee *et al.*, 1949). There is a slight increase in Q_{O_2} accompanying the increased glucose utilization in phosphate buffer (Stadie and Zapp, 1947; Villee *et al.*, 1949). Further studies on the effect of ions on the metabolism of glucose by muscle have been reported by Beloff-Chain *et al.* (1955a), Torres (1952), and Clarke (1955).

7. *Composition of Diet*

The observation that fasting significantly affects glucose tolerance was made by Claude Bernard and has since been repeatedly confirmed (Lundback, 1948). It has further been shown that a similar effect can be obtained by feeding diets adequate in calories but low in carbohydrate content. Recent studies on isolated muscle have shown that at least part of the effect of carbohydrate-fasting on carbohydrate tolerance is due to changes in the metabolic activity of peripheral tissues. When rats were maintained for several days on a high fat, low carbohydrate diet, their diaphragms were found to utilize considerably less glucose and to synthesize less glycogen from glucose (Gilmore and Samuels, 1949; Hansen *et al.*, 1951) than diaphragms from rats maintained on a high carbohydrate diet. It is interesting to note that the diminished glucose utilization was not necessarily due to an impairment of glucose uptake and phosphorylation, since, in the absence of added substrate, the breakdown of glycogen previously stored was slowed to a similar degree, although oxygen consumption was unaltered. These observations were interpreted as providing evidence that endogenous substrates other than glucose were used preferentially by striated muscle of carbohydrate-starved animals, and it was suggested that this was due to the preferential oxidation of these substances in the tricarboxylic acid cycle. Effects of insulin *in vitro* were demonstrable when it was added to the isolated diaphragms from either carbohydrate-fed or carbohydrate-starved animals; the magnitude of the insulin effect was proportional to the activity originally shown by each tissue in metabolizing glucose.

8. *Summary*

The present interpretation of the main findings relating to the hormonal regulation of carbohydrate metabolism in muscle is summarized in Table I. Since quantitative data adequate for comparison between several laboratories were but rarely available, the summary has been limited to the indication of increases or decreases in the rates of certain reactions under the influence of the hormonal factors studied.

Three areas of definite hormonal effects on muscle can be distinguished. First, *glucose uptake* is a metabolic response which is rapidly and markedly increased by insulin, and this effect has been documented in so many different preparations and under such a variety of conditions that it may be reasonably considered as established. It also would seem that large doses of diabetogenic hormone of the pituitary, as well as a lipoprotein which is present in the serum of diabetic animals with intact pituitaries, decrease the rate of glucose uptake by muscle; this is in accord with the well-established physiological antagonism between the anterior

lobe of the pituitary and insulin. In addition, however, glucose uptake by the isolated diaphragm has been found to be affected to some degree by all of the hormones studied. While not unexpected, when one considers that glucose is the most generally usable fuel and that its over-all utilization may reflect a number of unrelated changes in the tissues examined, this observation suggests that caution is necessary when evaluating the importance of relatively small changes in glucose uptake. The second area where definite hormonal effects can be demonstrated is that of *protein catabolism*, which is markedly enhanced by adrenocortical hormones, moderately enhanced by thyroid hormones, and decreased by

TABLE I

REGULATION OF CARBOHYDRATE METABOLISM IN ISOLATED MUSCLE

	Insulin	Diabetogenic hormone of pituitary	Thyroid hormone	Adrenocortical hormones	Epinephrine	Glucagon
Glucose uptake	++	−	+	−	−−	−
Glucose to glycogen	++	−	+	−	−−	
Glucose to CO_2	++					
Glucose to lipid						
Pyruvate to CO_2	+					
Protein to amino acids	−	−	+	++		
Oxygen uptake			++			

insulin and by growth hormone, although the effect of growth hormone has been demonstrated only in the intact organism. Increased availability of amino acids for oxidation in the tricarboxylic acid cycle may account for part of the decreased over-all glucose utilization in the presence of carbohydrate-active adrenocorticoids. Third, *oxygen consumption* is increased by thyroid hormones, but is not significantly affected by insulin or other hormonal factors which have so far been studied.

It is quite apparent from a glance at Table I that much remains to be done to complete even such a simplified and qualitative survey of the changes in carbohydrate metabolism of muscle which can be brought about by hormone action.

IV. LIVER

Liver plays a central role in the carbohydrate metabolism of the mammalian organism. Although, in common with all other tissues, liver is able to utilize glucose, its most important function results from its ability to *produce* glucose from various precursors and either to store it as glycogen, or to liberate it into the blood stream. In the absence of exogenous

carbohydrate supplies, and after the relatively small amount of stored carbohydrate has been used, the circulating glucose which continues to be available to all tissues is produced by the liver from noncarbohydrate materials. A review of hepatic carbohydrate metabolism and of its regulation must therefore include not only glucose utilization, but also glucose production and factors affecting both functions.

The majority of studies concerned with the carbohydrate metabolism of isolated liver has been carried out on liver slices. It is recognized that slicing leads to marked trauma of some of the liver cells and that their metabolism is sufficiently disturbed to produce measurable derangements, such as the inability of liver slices to maintain adequate concentration gradients for potassium ions across the cell membrane (Flink *et al.*, 1950) and the non-homogeneous distribution of freshly synthesized glycogen (Deane *et al.*, 1947). However, the loss of intracellular potassium was partly circumvented by suitable adjustments in the incubation medium (Hastings and Buchanan, 1942; Hasting *et al.*, 1952), and liver slices have been shown to perform *in vitro* a number of the metabolic functions of the liver *in vivo*. Liver slices may perhaps be considered as representing the unresolved hepatocellular enzyme complex, preserving to some extent the intact spatial relationships between the metabolically coordinated enzymatic components.

All the reactions indicated on the composite scheme (Fig. 1) are known to occur in liver tissue. Glucose and glycogen are the major products of metabolism when substrates "above" pyruvate are incubated with liver slices. Although glycogen is formed from a wide variety of substrates incubated *in vitro* with liver slices, net glycogen synthesis has been achieved only in liver slices from fasted animals and only when incubation was carried out in a medium of intracellular cationic composition (Hastings and Buchanan, 1942; Buchanan *et al.*, 1949a,b). The effects of ions on liver metabolism will be discussed more fully in a later section. The relatively large amounts of glucose which are produced by liver slices result from both glycogenolysis and gluconeogenesis (Renold *et al.*, 1953). This is in accord with the function of liver *in vivo* as the organ chiefly responsible for the maintenance of blood glucose. Glucose is produced through the action of glucose-6-phosphatase on glucose-6-phosphate (Fantl and Rome, 1945). Although smaller amounts of this enzyme are found in kidney and intestinal mucosa, it is virtually absent from all other tissues (de Duve *et al.*, 1949). Changes in the activity of this enzyme in liver have been found in fasting (Weber and Cantero, 1954), alloxan diabetes (Ashmore *et al.*, 1954; Langdon and Weakley, 1955), and glycogen storage disease (Cori and Cori, 1952).

Glucose and glycogen formation by liver slices from carbon-14–labeled

glucose, fructose, glycerol, and pyruvate are illustrated in Fig. 2. Whereas, in the case of glucose, fructose and glycerol, 65 to 80% of the substrate utilized could be accounted for as glucose plus glycogen, only 35 to 40% of the pyruvate utilized was recovered as hexose. Carbon balance studies indicate that only 62 to 65% of the pyruvate carbon disappearing from

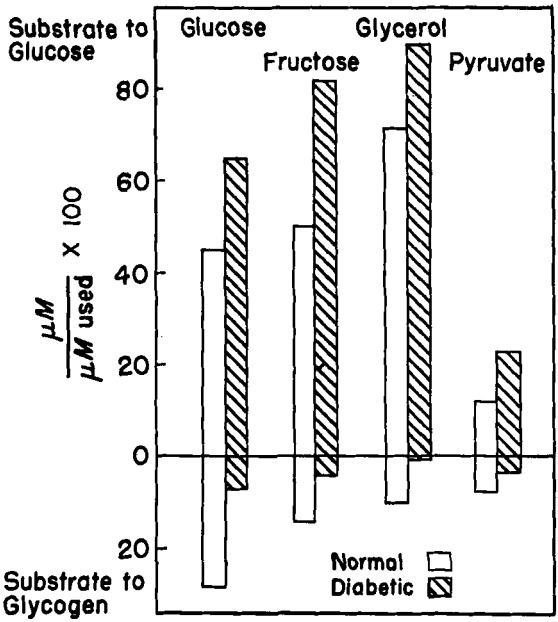

FIG. 2. Metabolism of glucose-U-C^{14} (20 μM per liter), fructose-U-C^{14} (20 μM per liter), glycerol-α-C^{14} (40 μM per liter), and pyruvate-2-C^{14} (40 μM per liter) by liver slices from normal and diabetic rats. The height of the bar above the line represents the micromoles of substrate per micromole used (×100) of the substrate that was incorporated into glucose during the 90-minute incubation period. The height of the bar below the line represents the micromoles of substrate per micromole used (×100) that was incorporated into liver glycogen. (Reprinted from Ashmore *et al.*, 1955.)

the medium could be accounted for in liver slice preparations. The fate of the other 35% remains obscure, since no appreciable activity was detected in phosphorylated intermediates, ninhydrin-labile CO_2, total lipid, or protein residue (Teng *et al.*, 1953).

Some of the detailed reactions involved in carbohydrate production and utilization have been studied quantitatively in liver slices *in vitro*. Topper and Hastings (1949) degraded the glycogen formed by liver slices from normal fasted rabbits after incubation with C^{14}-pyruvate. From the relative radioactivities of the individual carbon atoms of the glycogen

formed, they estimated the proportion of pyruvate molecules (a) directly converted to glycogen, (b) equilibrated with symmetrical dicarboxylic acids, and (c) converted to CO_2. Landau *et al.* (1955) applied these techniques to a study of the origin of glucose and glycogen carbons formed from pyruvate by liver slices from normal and alloxan-diabetic rats. These studies indicated that, for every molecule of pyruvate converted directly to glucose, 37 were equilibrated with symmetrical dicarboxylic acids before phosphorylation while 62 were being oxidized to CO_2. In diabetic livers a larger proportion of the pyruvate metabolized was converted directly to glucose than in normal liver.

Hers (1955) administered glucose-1-C^{14}, fructose-1-C^{14} and sorbitol-1-C^{14} to rats and degraded the liver glycogen formed. From the distribution of carbon 14 in the glucose isolated from glycogen it was concluded that glucose is incorporated directly into glycogen while fructose and sorbitol are first degraded to trioses and then reincorporated (reactions 5 and 6). Studies with differentially labeled glycerol indicate that it is incorporated directly into glucose by rat liver tissue without randomization of the carbon atoms (Landau, unpublished observations). Glycogen formation from lactate has been studied *in vivo* by Lorber *et al.* (1950). From the distribution of the carbon 14 in glucose, the ratio of lactate carbon incorporated directly into glycogen to that first entering the tricarboxylic acid cycle was obtained. This ratio was found to be 1:6.

Liver has been extensively used to study lipogenesis and fatty acid formation *in vitro*. Although relatively little carbohydrate is incorporated into fatty acids by liver slices, the pronounced influence of hormones on this process has made it the subject of many investigations. In comparison with other tissues, the liver produces less CO_2 from carbon-14–labeled carbohydrates (Olson, 1951). On the other hand, liver slices readily utilize $C^{14}O_2$ from the inorganic carbon of the incubation medium and incorporate it into carbons 3 and 4 of the glucose molecules synthesized (Hastings *et al.*, 1949; Topper and Hastings, 1949). About one-fourth of the carbon 14 from alpha-labeled pyruvate that was incorporated into glucose had first been oxidized to CO_2 (Landau *et al.*, 1955).

It is apparent from these studies that methods allowing for the estimation of the relative quantities of substrate metabolized by liver slices along alternative pathways have become available. The application of these and similar methods to tissues with abnormal carbohydrate metabolism should yield further insight into the functional aspects of the disturbance involved.

1. Insulin

A number of metabolic abnormalities have been demonstrated in the liver of the diabetic organism. Since the administration of insulin *in vivo*

results in a restoration of normal metabolism, these abnormalities have been ascribed to insulin deficiency. It is important to recognize, however, that diabetic metabolism represents not only the direct result of insulin deficiency but also the many secondary rearrangements which have occurred as an adaptation to the situation created by the primary defect. This distinction between primary and secondary effects becomes essential when the mechanism of insulin action is to be considered.

The uptake of glucose by liver slices from diabetic rats is less than that of normal liver slices (Hastings *et al.*, 1950; Renold *et al.*, 1953), whereas fructose (Renold *et al.*, 1954), glycerol (Ashmore *et al.*, 1955), and pyruvate (Renold *et al.*, 1953) are utilized at normal or nearly normal rates. Since glucose uptake is decreased, it is not surprising to find that glucose oxidation to CO_2 and glucose-carbon incorporation into glycogen, lactate, and fatty acids are similarly decreased. In addition, Chernick and Chaikoff (1951) demonstrated that, although oxidation of fructose to CO_2 by diabetic liver slices proceeded at a normal rate, fructose carbon incorporation into fatty acids was markedly depressed. It had previously been shown that the incorporation of pyruvate- and acetate-carbon into long-chain fatty acids was strikingly depressed in the presence of diabetes (Bloch and Kramer, 1948; Brady and Gurin, 1950; Osborn *et al.*, 1951). Similarly, it has been found that the incorporation of fructose-, glycerol-, and pyruvate-carbon into glycogen by diabetic liver was less than that by normal liver (Fig. 2). These findings suggested the presence in diabetic liver of defects other than that of glucose utilization.

Chernick and Chaikoff (1951), having established that liver slices from diabetic rats oxidize fructose but not glucose to CO_2 at a normal rate and that the enzymes necessary for the conversion of fructose to glucose appeared to be intact (Chernick *et al.*, 1951), deduced therefrom that a metabolic defect in diabetic liver was one of decreased hexokinase activity. This somewhat indirect interpretation (particularly in view of the recently established complex nature of the pathways of hepatic fructose metabolism) received recent support from more direct measurements of the activity of glucose conversion to glucose-6-phosphate by normal and diabetic liver. By incubating normal and diabetic liver slices with glucose and fructose simultaneously present in the medium, and by utilizing the role of glycogen as a continuous sampler of the glucose-6-phosphate pool, a quantitative estimate of the rate at which extracellular glucose is converted to intracellular glucose-6-phosphate has been obtained (Renold *et al.*, 1954). In diabetic liver this rate of glucose conversion was one-fifth to one-tenth of normal. Such a change could be brought about either by a diminished rate of glucose entrance into the liver cell or by diminished phosphorylation of intracellular glucose by hexokinase.

In addition to the various defects in substrate utilization which have been described, liver slices from diabetic animals have been shown to have a markedly increased gluconeogenetic activity (Hastings *et al.*, 1950; Renold *et al.*, 1953). The increased production of glucose by diabetic liver was shown to result from increased glycogen breakdown as well as from increased conversion of pyruvate, fructose (Renold *et al.*, 1954), glycerol (Ashmore *et al.*, 1955), and of unknown hepatocellular precursors to glucose. Since the final common step of these conversions is the liberation of glucose from glucose-6-phosphate by glucose-6-phosphatase action, the activity of glucose-6-phosphatase in diabetic liver was studied. This was found to be increased (Ashmore *et al.*, 1954). Although glucose-6-phosphatase was increased under these conditions, it was observed that an increase in gluconeogenesis preceded the increased activity of this enzyme (Ashmore *et al.*, 1956). Further metabolic abnormalities which have been described in the isolated diabetic liver include an increased conversion of acetate to cholesterol (Hotta and Chaikoff, 1952), a decreased incorporation of glycine into glutathione and of glycine and other amino acids into liver protein (Krahl, 1953, 1956), a decreased ability to phosphorylate thiamine (Siliprandi and Siliprandi, 1952), a decreased turnover rate for organic phosphates (Sacks, 1952), and a decreased efficiency of oxidative phosphorylation (Stadie, 1955b), although the last observation has not been confirmed (Parks *et al.*, 1955).

The oxidation of glucose via the phosphogluconate oxidation pathway appears to be decreased in the alloxan-diabetic rat. Glock and McLean (1955) have observed that the activity of liver glucose-6-phosphate dehydrogenase, the first enzyme of this pathway, was decreased in the diabetic liver. This has been confirmed in this laboratory. Bloom (1955), using differentially labeled glucose (glucose-1-C^{14} and glucose-6-C^{14}), also found that the metabolism of glucose by this pathway was reduced in liver slices from diabetic rats. Other data suggesting that this pathway of metabolism is under hormonal regulation are those of Agranoff *et al.* (1954), who found that oxidation of glucose via the phosphogluconate oxidation pathway was reduced in regenerating, fetal, fasting, and neoplastic liver, but was increased in maternal liver during pregnancy. Addition in *vitro* of growth hormone, glucagon, and insulin did not affect the activity of the first two enzymes of this pathway (Dickens, 1953).

For the effects of insulin in relation to metabolism in liver the findings are less clear-cut than was the case for muscle. The many attempts which have been made to demonstrate an effect of insulin added *in vitro* have been either unsuccessful or at least inconsistent. Thus, although an increased incorporation of acetate-carbon into long-chain fatty acids in the presence of insulin added *in vitro* has been reported (Bloch and

Kramer, 1948) and confirmed (Brady and Gurin, 1950; Haugaard and Stadie, 1953), this effect could be demonstrated in normal, but not in diabetic, liver slices and has furthermore been shown to be consistently present only in certain strains of rats (Bloch, 1948). Krahl (1953) reported an increased incorporation of glycine into glutathione by diabetic liver slices when insulin was added in the presence of glucose, although no such effect was obtained with normal liver slices. This effect could be demonstrated only when the liver slices had been obtained from animals whose diabetes had not been present more than 48 hours (Krahl, 1956). Effects of insulin added *in vitro* on glucose uptake, glucose phosphorylation, incorporation of glucose into glycogen, CO_2, or fatty acids have been repeatedly looked for, but positive effects have not been consistently observed (Renold *et al.*, 1955). Berthet *et al.* (1954) obtained an effect on glucose incorporation into glycogen in rabbit liver, but this was not associated with an increased uptake of glucose.

In contrast, insulin administered *in vivo* markedly affects the metabolism of the subsequently isolated liver, both in normal and in diabetic animals. The metabolic changes thus produced may be summarized as being the opposite of those which have just been described as resulting from the presence of diabetes. They include increased glucose uptake (Renold *et al.*, 1955), increased incorporation of glucose-carbon into glycogen (Teng *et al.*, 1952) and into long-chain fatty acids (Chernick and Chaikoff, 1950), increased oxidation of glucose to CO_2 (Chernick and Chaikoff, 1950), increased incorporation of fructose-, glycerol- and pyruvate-carbon into glycogen (Renold *et al.*, 1955; Ashmore *et al.*, 1955), and decreased gluconeogenesis (Renold *et al.*, 1955).

Whereas the administration of insulin to diabetic animals for 24 hours or more results in the correction of all metabolic abnormalities which have thus far been measured in diabetic liver, insulin administration for shorter periods does not (Chernick and Chaikoff, 1950; Renold *et al.*, 1955). This observation suggested that knowledge of the biochemical sequence of events during the correction of metabolic abnormalities in diabetes might aid in identifying the metabolic site or sites of insulin action. Experiments to this end were therefore carried out (Renold *et al.*, 1955). Alloxan-diabetic rats given insulin continuously were sacrificed at various times from 10 minutes to 48 hours after the beginning of insulin administration. Slices of their livers were incubated with C^{14}-labeled glucose, fructose, pyruvate and acetate, and their diaphragms with C^{14}-labeled glucose. Substrate utilization and oxidation as well as incorporation of substrate-carbon into glycogen, glucose, and fatty acids were measured. There was a striking discrepancy between muscle and liver in regard to the time required for insulin effects to become apparent.

Whereas the response of diaphragm to insulin was present and indeed maximal within 10 minutes of the intravenous administration of the hormone, insulin effects on liver were barely measurable six hours after the beginning of insulin administration, and only became well marked between 6 and 24 hours later. These effects of insulin on isolated liver and muscle, with respect to time, are illustrated by the curves in Fig. 3 and have been further compared to the levels of blood glucose at the time of

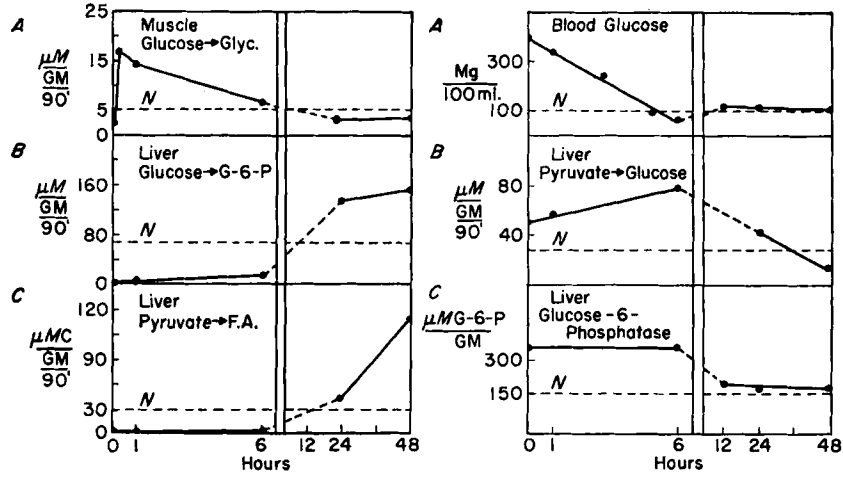

FIG. 3. Biochemical sequence of events after insulin administration to diabetic rats. On the left: Curve *A*, values expressed in micromoles of glucose equivalents per gram of muscle per 90 minutes. The dotted line represents the mean normal value. Glyc. = glycogen. Curve *B*, values expressed in micromoles of glucose per gram of liver per 90 minutes. G-6-P = glucose-6-phosphate. Curve *C*, values expressed in micromoles of carbon per gram of liver per 90 minutes. F.A. = fatty acids. On the right: Curve *A*, blood glucose in milligrams per 100 ml., Curve *B*, values expressed in micromoles of pyruvate-2-C^{14} incorporated into glucose per gram of liver per 90 minutes. Curve *C*, rat liver glucose-6-phosphatase activity as micromoles of G-6-P split per gram per 30 minutes at 30°. (Reprinted from Renold *et al.*, 1955; Ashmore *et al.*, 1956.)

sacrifice. Although all insulin effects on liver were delayed, it may be of some significance that glucose phosphorylation was the first parameter which showed significant changes.

It would appear, therefore, that the nature of the action of insulin on hepatic tissue may well differ from the action which the hormone exerts on muscle. The gradual effects of insulin on hepatic metabolism could be the result of a direct, though slow, hormonal action, but they could also be the result of a metabolic adaptation to the new demands created by the peripheral changes. An unequivocal direct effect of insulin on the

isolated liver has yet to be demonstrated (Lang *et al.*, 1954; Soskin and Levine, 1952).

2. Diabetogenic Hormone of the Pituitary

Attempts to demonstrate direct effects of pituitary extracts on hepatic carbohydrate metabolism have been mostly inconclusive (Russel, 1953). Renold *et al.* (1953) found that glucose uptake, glycogen deposition from glucose and pyruvate, glycogenolysis, and gluconeogenesis proceeded at essentially normal rates in liver slices from hypophysectomized animals. When hypophysectomy was superimposed on pre-existing diabetes, however, the overactive gluconeogenesis of the diabetic liver was markedly decreased and an increase in the previously depressed glucose uptake was noted. The latter change may reasonably be attributed to a direct pituitary effect, but not the former, since a return to normal of increased diabetic gluconeogenesis was also brought about by adrenalectomy. Hence, in the hypophysectomized diabetic animals, the return of gluconeogenesis to normal could be adequately explained by the occurrence of secondary adrenal atrophy. When growth hormone was administered daily to normal rats for five to seven days, no changes were noted in the ability of their livers to metabolize glucose, fructose, or pyruvate to glycogen, CO_2, or fatty acids (Ashmore and Herrera, unpublished observations).

Effects of hypophysectomy and of growth hormone on lipogenesis have been described. Tompkins *et al.* (1952) demonstrated a decrease in cholesterol synthesis from C^{14}-acetate in liver slices obtained from hypophysectomized rats. Furthermore, the administration of growth hormone has been reported to decrease the synthesis of long-chain fatty acids from C^{14}-labeled pyruvate (Greenbaum, 1955). The production of ketone bodies is increased in liver slices from animals treated with various pituitary fractions (Engel, 1955).

3. Thyroid Hormones

Studies of the effect of thyroid hormones on carbohydrate metabolism of isolated liver have been few. Studies in intact organisms as well as clinical observations suggest that thyroid hormones exert direct and perhaps specific effects on hepatic cells (Houssay, 1948; Soskin and Levine, 1952). One of the most striking effects described is that of decreased galactose tolerance in the presence of thyrotoxicosis (Lichtman, 1949), which occurs before any other definite evidence of hepatocellular damage can be shown. In view of the participation of a specific high-energy-phosphate carrier (uridine diphosphoglucose, UDPG) in the conversion of galactose-1-PO_4 to glucose-1-PO_4, the effects of thyrotoxicosis on this reaction may well deserve further study. An increase in glucose-6-

phosphatase in thyrotoxicosis has been reported by Maley and Lardy (1954). Spirites *et al.* (1953) found unaltered incorporation of acetate into long-chain fatty acids in liver slices of hyperthyroid rats. It may be seen from these observations that a definitive study of the effects of thyroid hormones on carbohydrate metabolism in liver is yet to be done.

4. Adrenocortical Hormones

Although carbohydrate-active adrenal corticoids may well exert a number of metabolic effects, the best documented one is that exerted on hepatic gluconeogenesis. The ability of adrenocorticoids to increase the deposition of liver glycogen in fasting organisms and to increase glucose excretion in phlorhizinized animals is well known. More recently it has been shown that cortisone increased glucose production in intact rats receiving constant infusions of C^{14}-glucose by a factor of seven, compared with only a twofold increase over normal observed in alloxan-diabetic animals (Welt *et al.*, 1952). As will be seen, similar effects have been observed in isolated liver.

In liver slices obtained from rats previously treated with cortisone, an increase in glucose production, but no decrease in glucose utilization, has been observed (Renold and Hastings, 1953). Previously Koepf *et al.* (1941b) had demonstrated increased glucose formation, in the presence of pyruvate, by liver slices from rats treated with adrenal cortical extracts. When the metabolism of C^{14}-labeled glucose and pyruvate was compared in liver slices from normal, adrenalectomized, diabetic and diabetic-adrenalectomized rats (Renold *et al.*, 1953), it was found that glucose utilization was the same in normal and adrenalectomized animals, and further that the diminished glucose utilization of the diabetic livers was also present in livers from diabetic adrenalectomized rats. On the other hand, glucose production by liver slices from diabetic adrenalectomized animals was approximately one-half that observed in liver slices from diabetic animals. This decrease in over-all glucose production was accounted for by decreased glucose formation both from pyruvate and from unknown precursors. That these may be of protein origin is suggested by the work of Roberts (1953), who found that liver slices from animals previously treated with adrenocortical hormones showed an enhanced release of proteins.

In addition to these effects of corticoids on gluconeogenesis, Brady *et al.* (1951) have observed that pretreatment of rats with cortisone markedly reduces incorporation of labeled acetate into fatty acids by liver slices obtained from such animals. Similarly, they have found that the impaired lipogenesis observed in liver tissue of diabetic animals was not found in liver from diabetic adrenalectomized animals.

Many attempts have been made to influence the carbohydrate metabolism of isolated liver by the addition *in vitro* of cortical hormone preparations (Teng *et al.*, 1952; Chiu, 1950; Chiu and Needham, 1950; Verzar, 1951). The changes reported have been relatively small and not uniformly reproducible. A study has been made of the time required for cortical

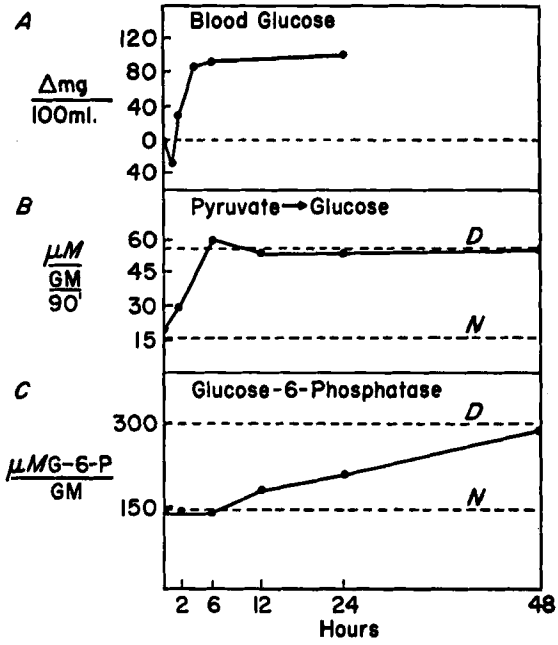

FIG. 4. Biochemical sequence of events after administration of adrenocortical hormone (5 mg. per 12 hours) to adrenalectomized diabetic rats. Curve *A*, change in blood glucose from time zero in milligrams per 100 ml. Curve *B*, values expressed in micromoles of pyruvate-2-C^{14} incorporated into glucose per gram of liver per 90 minutes. Curve *C*, glucose-6-phosphatase activity of rat liver as micromoles of G-6-P split per gram per 30 minutes at 30°. *N* = mean normal value; *D* = mean diabetic value. (Reprinted from Ashmore *et al.*, 1956.)

hormones to produce measurable changes in hepatic carbohydrate metabolism (Ashmore *et al.*, 1956). Hydrocortisone was administered to adrenalectomized diabetic rats for varying lengths of time before sacrifice, when their livers were removed, sliced, and incubated with C^{14} pyruvate. Blood glucose levels at time of death and incorporation of C^{14} into glucose, glycogen, and fatty acids by the liver slices were measured, as well as the activity of liver glucose-6-phosphatase. Diabetic adrenalectomized rats were used, since they represented particularly sensitive preparations. The results are shown in Fig. 4. Within two hours, measurable changes

occurred in blood glucose levels as well as in the incorporation of pyruvate-carbon into glucose, though maximal effects were not observed until six hours following the injection of the corticoid. A marked decrease in incorporation of pyruvate carbon into fatty acid (not shown in Fig. 4) also occurred during this period. However, maximal change in glucose-6-phosphatase activity was delayed until 48 hours after the beginning of the administration of the hormone. These studies would indicate that a few hours are required for hydrocortisone to exert a significant effect on gluconeogenesis by liver tissue, and this may explain in part the failure to produce consistent effects *in vitro* with adrenocortical hormones.

In summary, it would appear that the hormones of the adrenal cortex have no direct influence on hepatic glucose utilization but greatly accelerate hepatic glucose production and decrease hepatic lipogenesis. Regulation of carbohydrate metabolism *in vitro* by hormones of the adrenal cortex has not been conclusively established, although these hormones do cause measurable alterations in liver metabolism, measured *in vitro*, within two hours after their injection into intact rats.

5. Glucagon and Epinephrine

The hyperglycemic factor of the pancreas is a protein hormone probably elaborated by the alpha cells (Vuylsteke *et al.*, 1952) and perhaps by other tissues (de Duve, 1953a) and is quite distinct from insulin (Staub *et al.*, 1955) in its action, chemical composition, and physical characteristics. The chemistry and physiology of this substance has recently been reviewed by Best *et al.* (1955). The primary action of glucagon is to increase glycogenolysis in liver, and this action has been observed in intact animals and in liver slices. Glucagon presumably promotes glycogen breakdown by increasing the rate of conversion of phosphorylase from an inactive to an active form (Sutherland, 1951a,b, 1952a,b). It is of interest to note that, when liver slices are incubated with and without glucagon *in vitro*, an increase in the activity of phosphorylase can be observed when phosphorylase activity is measured by glucose formation in homogenates prepared from the slices, but not when phosphorylase is measured by the liberation of inorganic phosphate from glucose-1-phosphate (de Duve, 1953b).

In addition to its effects on glycogenolysis, glucagon appears to have a direct effect on fatty acid synthesis by liver slices when added *in vitro*. Haugaard and Haugaard (1954) have found that fatty acid synthesis from glucose, fructose, and acetate was decreased and that the production of ketone bodies was increased in the presence of glucagon. Similar effects of epinephrine on lipogenesis in liver slices have been observed (Haugaard and Stadie, 1953). Addition of epinephrine to liver slices in-

creases glycogenolysis (Sutherland and Cori, 1951). The action of this hormone has also been ascribed to an increase in the active form of phosphorylase in the tissue (Sutherland, 1951a,b; 1952a,b).

6. Composition of Medium

The inorganic composition of the incubation medium has a pronounced influence upon the formation of glucose and glycogen *in vitro* by liver slices incubated with a variety of substrates (Hastings *et al.*, 1952). From studies on glycogen formation (Hastings and Buchanan, 1942; Buchanan *et al.*, 1949a,b), it has been inferred that the normal intracellular ionic environment is better preserved in a potassium-rich medium than a sodium-rich medium, and that the maintenance of the normal potassium and sodium concentrations in the intracellular fluid has a decided influence on coordinated enzymatic activities of the liver cells *in vitro*. Measurements of changes in potassium and sodium concentrations in liver slices incubated in media of varying concentrations of these ions have demonstrated that the concentration of potassium ions in the incubation medium required to maintain a normal intracellular potassium concentration of liver slices appears to be about 78 millimoles per liter and that, with an extracellular concentration of less than 5 millimoles per liter, the intracellular potassium concentration decreases markedly and irreversibly (Flink *et al.*, 1950).

By using C^{14}-labeled glucose and C^{14}-labeled pyruvate as substrates, the utilization and formation of glucose and the formation and breakdown of glycogen have been studied in rat liver slices incubated in media of varying sodium and potassium concentrations (Hastings *et al.*, 1952). It was found that, as the potassium concentration in the medium was reduced from 110 to 5 m*M* per liter, the glycogen formed from pyruvate was reduced and the glucose formed from pyruvate was increased, the sum (glucose + glycogen) remaining essentially unchanged. When glucose-U-C^{14} was the substrate, decreased glucose utilization and decreased glycogen formation from glucose were observed. The diversion of metabolic reactions from glycogen synthesis to increased glucose formation observed with liver slices incubated in potassium-poor media is similar to the alterations observed in carbohydrate metabolism of liver slices from alloxan-diabetic rats (Hastings *et al.*, 1953).

These observations would indicate that the cation effects on glucose and glycogen formation must be on some enzymatic reaction above glucose-6-phosphate (Fig. 1). No effects of sodium and potassium ions on the activity of glucose-6-phosphatase (reaction 22) have been found in rat liver slices; it has been found, however, that after incubation in a sodium-rich medium, liver phosphorylase is two to four times as active

as that observed when slices have been incubated in a potassium-rich medium (Ashmore and Cahill, unpublished observations).

No effects of cations on pyruvate utilization by liver slices (Hastings *et al.*, 1952) have been observed, although phosphoenolpyruvic kinase is a potassium-dependent enzyme (Boyer *et al.*, 1942; Kachmar and Boyer, 1953). Similarly, no effects of cations on the oxidation of glucose or of pyruvate have been observed (Hastings *et al.*, 1952).

The divalent cations calcium and magnesium also exert a quantitatively significant influence on glycogen formation from both glucose and pyruvate. Indeed, it was found that for optimum glycogen formation by rat liver slices *in vitro* a medium of the following cation composition proved best: K = 110, Mg = 20, Ca = 10 m*M* per liter (Hastings *et al.*, 1952).

7. Composition of Diet

Although effects of changes in the composition of the diet on carbohydrate metabolism have been shown to occur in muscle, it is generally assumed that hepatic tissue is the main site of the alterations produced. Studies on isolated liver tend to support this view. Thus, fasting markedly affects the metabolic activity of liver slices, and it would appear that the resulting changes in hepatic metabolism are qualitatively similar to those found in diabetes, though quantitatively less marked. These effects include decreased glucose uptake, decreased oxidation of glucose to CO_2 and decreased incorporation of glucose-carbon into glycogen and fatty acids (Wyshak and Chaikoff, 1953; Renold *et al.*, 1953), decreased lipogenesis from acetate (Lyon *et al.*, 1952), and increased gluconeogenesis from pyruvate (Renold *et al.*, 1953), as well as increased ketogenesis. Similar changes have been produced by feeding diets with high fat but low carbohydrate content (Tepperman and Tepperman, 1955). One of the enzymatic changes known to occur during fasting is an increase in the activity of liver glucose-6-phosphatase (Ashmore *et al.*, 1954; Weber and Cantero, 1954). It is possible that some of the changes in liver carbohydrate metabolism that are observed in fasting may be due to the increase in activity of this enzyme.

A particularly interesting study has been carried out by Hill *et al.* (1954). Rats were fed adequate diets, the carbohydrate components being either glucose alone or fructose alone. The animals which had received the diet containing fructose exhibited a marked decrease in glucose tolerance, which was shown to be due entirely to a decreased ability of the liver to utilize glucose, a defect which was not shared by muscle and kidney. This observation was interpreted as suggesting the occurrence of adaptive changes in the enzymatic composition of the liver during the period of fructose feeding. Indeed, similar interpretations have also been

suggested to explain the changes occurring during fasting or during the feeding of high-fat diets, which have been previously discussed (Tepperman and Tepperman, 1955).

8. Summary

The studies of hepatic carbohydrate metabolism which we have just reviewed include a rather large number of measurements involving many substrates and many regulators. Data obtained by the authors and their colleagues on several aspects of carbohydrate metabolism in liver under different experimental conditions have been assembled in Table II. The following points emerge:

(1) *Glucose uptake and phosphorylation* is markedly decreased in diabetes mellitus and restored to normal by insulin administration for one or more days. Hypophysectomy in previously diabetic animals results in a return of the glucose uptake toward normal, whereas adrenalectomy or cortisone administration is without effect. Either fasting or a low carbohydrate diet results in a decreased glucose uptake by the subsequently isolated liver. In addition to these alterations in glucose uptake, it is not surprising to find that the incorporation of glucose-carbon into such products of metabolism as glycogen, fat, or CO_2 varies accordingly. In contrast to glucose, however, the uptake of all other substrates studied proceeds at unaltered rates under all conditions examined. The fact that the uptake of only glucose is altered with changing conditions serves to emphasize further the rather unique nature of this substrate in biological systems (Ross, 1951).

Whereas the uptake of substrates other than glucose (illustrated by pyruvate in Table II) proceeds at essentially fixed rates in a variety of conditions, striking differences exist in the relative amounts of these substrates which are metabolized along each of the pathways open to them. This presumably indicates that the enzymatic pattern which these compounds meet in hepatic intermediary metabolism may vary widely under the direct or indirect influence of hormones and other regulators. Substrate oxidation to CO_2 appears to be an exception and closely parallels in most situations the rate of substrate uptake. On the other hand, the rates of substrate-carbon incorporation into lipid, glycogen, and glucose vary independently.

(2) *The incorporation of substrate-carbon into long-chain fatty acids* appears to be particularly sensitive to variations in the hormonal environment. It is markedly decreased in the presence of diabetes, after a period of fasting or carbohydrate deprivation, or by treatment with cortisone. It is increased above normal by insulin administration or glucose feeding and, when previously depressed by the production of diabetes, it is returned toward normal by either adrenalectomy or fructose feeding, as

TABLE II

REGULATION OF CARBOHYDRATE METABOLISM IN ISOLATED LIVER

	Normal	Diabetic	Diabetic + Insulin	Diabetic, adrenal-ectomized	Diabetic, adrenal-ectomized + Cortisone	Diabetic, hypophys-ectomized	Normal, fasted	Normal, potassium depleted
Glucose uptake	100	50	114	40		72	54	47
Glucose phosphorylation	100	21	200					92
Glucose to glycogen	100	4	138	9		18	35	17
Glucose to CO_2	100	13	200					
Glucose to fatty acids	100	1	400					
Pyruvate uptake	100	104	88	95	105	95	100	97
Pyruvate to glycogen	100	33	15	90	50	57	65	26
Pyruvate to CO_2	100	83	85					87
Pyruvate to fatty acids	100	14	530	70	13			
Pyruvate to glucose	100	380	43	220	300	200	157	170
Glucose-6-phosphatase	100	230	110	110	197		183	100
Glucose production	100	200	90	110	210	115	58	140

well as by insulin administration. It has been suggested that the common denominator of all situations in which active lipogenesis occurs is the abundant supply of carbohydrate for glycolysis (Shaw and Gurin, 1953). Thus Haugaard and Stadie (1952) have shown a direct correlation between the glycogen content of the isolated tissue and lipogenic activity. The presence of higher levels of hepatic glycogen in fasted diabetic and fasted cortisone-treated rats, compared with fasted normal animals, would at first sight appear to represent exceptions to the correlation suggested by Stadie. It should be noted, however, that the higher levels of hepatic glycogen in these two situations (diabetes and cortisone administration) represent increased gluconeogenesis and hence a negative carbohydrate balance as far as hepatic tissue is concerned. The liver has to synthesize the glucose moieties from noncarbohydrate precursors and loses many of these to the environment.

(3) *The incorporation of substrate-carbon into glycogen* is markedly altered by changes in the ionic composition of the intracellular environment. Decreased intracellular concentration of potassium and increased concentration of inorganic phosphate lead to decreased glycogen synthesis and increased glycogenolysis. The presence of diabetes results in decreased glycogen synthesis and, since this was but one of several similarities in the metabolic pattern produced by insulin deficiency and by *in vitro* K depletion, the possibility of alterations of the intracellular ionic environment in diabetes has been considered. It would appear, however, that gross anomalies of the ionic composition of diabetic liver cannot be demonstrated (Tobian *et al.*, 1955). When liver slices are incubated simultaneously with two substrates, one labeled with C^{14} and one not labeled, under conditions leading to glycogen deposition, glycogen represents a continuous sampler of the glucose-1-phosphate and glucose-6-phosphate pools. Analysis of the glycogen, at the end of the incubation, can then be used to ascertain the relative contributions of the two substrates to the glucose-6-phosphate and glucose-1-phosphate pools. Thus, when normal and diabetic liver slices were incubated with glucose and pyruvate, alternately labeled with C^{14}, the very low ratio of glycogen-from-glucose to glycogen-from-pyruvate suggested the presence of inhibited glucose phosphorylation and of increased gluconeogenesis from pyruvate (Renold *et al.*, 1953). The only fully documented direct hormone effects on liver tissue *in vitro* are those of epinephrine and of glucagon, which both increase glycogenolysis within minutes of their addition to the medium surrounding liver slices.

(4) *The incorporation of substrate-carbon into glucose* has been used as an index of gluconeogenesis. Gluconeogenesis is increased by insulin deficiency or cortisone excess, and the increased gluconeogenesis characteris-

tic of liver slices from diabetic rats tends toward normal after adrenalectomy. Changes in the activity of liver glucose-6-phosphatase parallel the changes in gluconeogenesis. Among areas of intermediary metabolism which have been shown to be affected by hormonal regulation, but which are less directly related to carbohydrate metabolism, oxidative phosphorylation, ketogenesis, and cholesterol synthesis should again be mentioned.

(5) Finally, it should be stated that, with the exception of the effects of epinephrine and glucagon, none of the hormonal effects which have just been summarized has been consistently obtained by the addition of the hormone to isolated liver *in vitro*. Their *direct* action is often questionable and, indeed, it appears that their activities might be interpreted as secondary adaptations to changes in the concentrations and distribution of intracellular substrates. For example, adaptive changes in tryptophane peroxidase activity occur (Knox, 1951; Knox and Auerbach, 1955), both in response to tryptophan feeding and to cortisone and ACTH administration.

V. Other Tissues

When considering the regulation of carbohydrate metabolism in isolated tissues other than muscle and liver, the authors found that much interesting and painstaking work has been done. Although this portion of the review lacks completeness, it is felt that references to current work on some tissues other than muscle and liver should be included in order to bring out metabolic similarities, as well as contrasts. These are grouped together in the following section.

1. Kidney

The main effort in investigations of renal metabolism has been directed toward tubular excretion and reabsorption of water, electrolytes, and other solutes rather than toward its intrinsic carbohydrate metabolism. It is clear, however, that a properly functioning intrinsic energy metabolism is a prerequisite to the performance of the more specialized functions of the tubular cells, as has been so elegantly emphasized by the studies of Taggart and Forster (1950). The most important function of the kidney with relation to the carbohydrate economy of the intact organism is that of preserving blood glucose by tubular reabsorption. The mechanism of glucose reabsorption is still unknown, although it has been generally assumed that it involves phosphorylation with subsequent hydrolysis. The presence of glucose-6-phosphatase activity in renal tissue (de Duve *et al.*, 1949) and the inhibition of glucose reabsorption by phlorizin have

been considered supporting evidence. Some recent observations, however, represent a serious challenge to this interpretation (Mudge and Taggart, 1950; Keston, 1954).

Although renal carbohydrate metabolism has not been studied as extensively as hepatic carbohydrate metabolism, the available evidence indicates that the two tissues are qualitatively similar in this respect. The regulation of the carbohydrate metabolism of kidney slices has been mainly studied by Teng (1954a,b, 1955a,b). Kidney slices from alloxan-diabetic rats were found to utilize less glucose from the medium and to produce more glucose when incubated in the presence of pyruvate than kidney slices from normal rats. Insulin administration *in vivo* for one or more days before sacrifice corrected both anomalies. Adrenalectomy resulted in a decreased formation of glucose in the presence of pyruvate, whereas glucose uptake was unaltered. Hypophysectomy led to increased glucose uptake but did not affect glucose production. With the exception of this last observation, these results correlate well with the previously discussed results obtained in liver slices. Russell and Wilhelmi (1941) had demonstrated a decreased glucose production from alanine by kidney slices from adrenalectomized rats.

The effects of the ionic composition of the incubating medium on the metabolic activity of kidney slices has also been studied. Whereas Marsh and Miller (1953) reported that glycogen synthesis was optimal in a medium with a high potassium ion concentration—a situation similar to that which pertains in liver—Teng (1955b) found a higher rate of glucose, pyruvate, and glycerol metabolism in a medium with a high sodium ion concentration—an observation which differs from that in liver slices. Teng (1955b) suggested that the latter dissimilarity might be due to the different tenacity with which liver and kidney slices preserve their intracellular potassium concentrations when incubated in a medium poor in potassium ions.

2. Brain

Although brain preparations are able to oxidize a number of substrates *in vitro* (Elliott *et al.*, 1955), glucose appears to be the primary source of energy for this tissue under physiological conditions (Gibbs *et al.*, 1942). Thus, while respiration of preparations of brain *in vitro* can be supported by such substrates as fructose, mannose, galactose, pyruvate, lactate, and, to a lesser degree, by glutamate, acetoacetate, and ethanol, to date no substance other than glucose has been shown to relieve hypoglycemic symptoms in eviscerated, nephrectomized animals. This may result from the fact that brain contains a hexokinase similar to yeast hexokinase (Slein *et al.*, 1950) but no specific kinases for fructose and glycerol. The affinity of brain hexokinase for glucose is such that

equimolar concentrations of glucose completely inhibit the phosphorylation of fructose and mannose.

Isolated brain tissue utilizes glucose for a variety of synthetic purposes. Mouse brain incubated with glucose-U-C^{14} converted 1.87% to CO_2, 91.5% to acid soluble substances, 0.71% to protein and 1.28% to lipid (Rafelson *et al.*, 1949). C^{14} from glucose was also incorporated into all the essential amino acids except proline and threonine. Rat brain slices convert 60% of the glucose-U-C^{14} utilized to lactic acid, and 20% to CO_2 (Beloff-Chain *et al.*, 1955b). Most of the remaining 20% of the utilized glucose-C^{14} was found in the amino acid fraction of the tissue. Brain can store some glycogen, but in amounts insufficient to sustain the tissue for prolonged periods (Winzler *et al.*, 1952). The over-all metabolism of brain and nervous tissue has recently been reviewed by Elliott *et al.* (1955).

It is generally accepted that glucose utilization by brain is not affected by insulin *in vivo* (Kerr and Ghantus, 1936), and Le Baron (1954) observed that glycogen synthesis by slices of guinea pig cerebral cortex was not affected by the addition of this hormone *in vitro*. Similar observations by Beloff-Chain *et al.* (1955b) and Park and Johnson (1955) indicate that insulin has no effect on glucose utilization by the rat brain. It should be mentioned, however, that Weil-Malherbe (1950) and Stern (1954) have observed the presence of a factor in the plasma of certain diabetic patients which inhibits the rate of the hexokinase reaction in rat brain homogenates. This was not counteracted by the addition of insulin (Stern, 1954).

The effect of various steroid hormones, notably cortisone, desoxycorticosterone and testosterone, on brain metabolism has been repeatedly studied and recently reviewed (Gordon *et al.*, 1951). These studies, however, have been mostly concerned with oxygen consumption and are outside the scope of this review. In general, these hormones have been found to decrease oxygen consumption by brain slices incubated in the presence of glucose and other substrates. Gordon *et al.* (1951) suggested that the activity of steroids in depressing brain Q_{O_2} paralleled their anesthetic action. Although thyroid hormones increase oxygen consumption by many tissues, it would appear that this effect is not present in brain (Fazekas *et al.*, 1951; Barker and Klitgaard, 1952).

Changes in the ionic environment have been clearly shown to affect the metabolism of brain preparations *in vitro*. Ashford and Dickson (1935) and Dickens and Greville (1935) had already shown that a relatively high concentration of potassium ions increased oxygen consumption by slices of rabbit cerebral cortex in the presence of glucose, pyruvate, or lactate as added substrates. Racker and Krimsky (1945), working with

mouse brain homogenates, demonstrated that substitution of sodium ions for potassium ions in the medium greatly reduced glycolysis as measured by lactic acid production from glucose. This effect was not present when phosphorylated sugars were used as substrates. Utter (1950) similarly observed a stimulation of glycolysis by potassium and ammonium ions and a depression of glycolysis by sodium ions. The effects of ions on glycolysis of brain extracts has been recently reviewed by Stumpf (1954).

The ability of brain slices to preserve an adequate concentration gradient for potassium ions across the cell membrane is less easily damaged than is the case for liver. Interference with glycolysis by the addition of fluoride ions or by glucose depletion, however, results in an immediate loss of intracellular potassium (Dixon, 1949); addition of l-glutamate to the medium is partly effective in preventing potassium loss (Terner *et al.*, 1950).

3. Retina

Although it is difficult to prepare brain *in vitro* without extensive cell damage, the retina offers an excellent source of nerve tissue closely associated with brain and easily obtained relatively intact for study *in vitro* (Dickens and Greville, 1935). The glycolytic metabolism of this tissue has been studied by Kerly and his collaborators (Kerly and Bourne, 1940; Hoare and Kerly, 1954). They have shown that the ionic and coenzyme requirements for glycolysis in this tissue are the same as those for liver and muscle. Retinal extracts enriched with ATP phosphorylate glucose, fructose, mannose, and glucosamine. Magnesium, manganese, and cobalt activate this process.

The utilization of glucose by retina preparations incubated in serum is greater than that which occurs when incubation is carried out in Ringer solutions. Furthermore, the substitution of potassium for sodium ions resulted in an increased glucose utilization, although oxygen consumption was slightly decreased (Kornblueth *et al.*, 1953a). The utilization of glucose by rat retina incubated in serum was not altered in hypo- and hyperthyroid states, in diabetic, adrenalectomized, hypophysectomized, or castrated animals. Injection of insulin, adrenal cortical hormone, anterior pituitary extracts, or thyroxine into normal animals also failed to influence glucose utilization by the isolated retina (Kornblueth *et al.*, 1953b).

4. Erythrocytes

The chemistry and metabolism of the mammalian red blood cell has been reviewed by Granick (1949). Glucose metabolism mainly follows glycolytic pathways since, when glucose is incubated with red cells, the amount of lactic acid which can be isolated accounts for between 60 and

90% of the glucose utilized (Peters and Van Slyke, 1946). The red cell also stores some glucose as glycogen which may be detected histochemically (Wachstein, 1949; Gibb and Stowell, 1949). The mature red cell is normally unable to carry out complete oxidation of substrates to CO_2 and has a very low Q_{O_2}. Harrop and Barron (1928) observed that addition of as little as 5×10^{-5} M methylene blue to red cells increases their respiration tenfold. Later work by Nossal (1948) demonstrated that red cells could utilize fructose, glucose, mannose, galactose, and ribose as substrates for respiration in the presence of added methylene blue. The order of their activity in promoting respiration is as listed. Gibson (1948) suggested that methylene blue stimulates red cell respiration by serving as an electron acceptor for TPNH and thereby allowing glucose oxidation via the Dickens (1938) phosphogluconate oxidation pathway. Studies by Brin (unpublished observations) on the oxidation by human erythrocytes of differentially labeled glucose in the presence of added methylene blue indicates that this may be the case, and indeed the enzymes of the phosphogluconate oxidation pathway have been shown by Glock and McLean (1954) to be present in the erythrocyte.

5. Leucocytes

The carbohydrate metabolism of leucocytes has received increasing attention in the past few years, and recent advances in this field have been reviewed by Beck and Valentine (1953) and Martin *et al.* (1955). Wagner and co-workers have found white blood cells to be equipped with the same enzymes as liver cells insofar as carbohydrate metabolism is concerned, with the exception of glucose-6-phosphatase (Wagner and Yourke, 1955). Optimum hydrolysis of hexose phosphates by these cells occurred at pH 9, with minimum hydrolysis at pH 6 to 7. Thus, it appeared that alkaline phosphatase is the active hexose phosphatase in leucocytes since glucose-6-phosphatase has its optimum activity at pH 6.8 (Swanson, 1950). Similarly, Wachstein (1955) failed to demonstrate the presence of glucose-6-phosphatase histochemically in white blood cells. Stimulation of respiration of broken cells by the addition of tricarboxylic acid cycle intermediates indicates that, in contrast to red blood cells, this pathway of oxidative metabolism is present in leucocytes (Martin *et al.*, 1955).

Normal leucocytes contain glycogen, which has been observed to undergo a two- to sevenfold increase in children with the hepatic form of glycogen storage disease (Wagner, 1947). Since these cells do not contain the enzyme glucose-6-phosphatase, which has been implicated in this disease, and since in these patients plasma has a high glycogen con-

tent, the observed increase in leucocyte glycogen may well represent phagocytosis of plasma glycogen.

Hormones, particularly those of the adrenal cortex, exert an influence on the carbohydrate metabolism of leucocytes. An increase in leucocyte glycogen was found following administration of ACTH to rats (Robineaux *et al.*, 1951). White cells are able to glycolize glucose, fructose, mannose, and galactose to lactic acid (Levene and Meyer, 1913). Martin *et al.* (1955) have observed a decrease in lactic acid production by leucocytes within four hours after the injection of hydrocortisone and six hours after cortisone. Insulin, *in vitro*, increased lactic acid production in leucocytes from diabetic subjects.

The potential importance of leucocytes as a useful tool for metabolic studies has been pointed out by Martin *et al.* (1955) in the following words: "The neutrophilic leukocyte constitutes an active metabolizing cell. . . . since this cell responds to environmental changes, it may, in the future, occupy a unique position in offering an opportunity to study the regulation of cell metabolism in human disease."

6. Adipose Tissue

Previous concepts of the physiological activity of adipose tissue have been drastically revised as a result of the use of isotopes in biological investigations. Adipose tissue has been shown to represent an actively metabolizing tissue continuously transforming, storing, and releasing metabolic fuels. The newer concepts and their experimental basis have been extensively reviewed by Wertheimer and Shapiro (1948). The metabolism of isolated adipose tissue has mostly been studied in slices obtained from the perirenal or epididymal fat pads of the rat. Although studies concerned with the analysis of the enzymatic composition of adipose tissue have been few, it has been established that isolated adipose tissue can take up and release fatty acids (Shapiro *et al.*, 1952) and synthesize fatty acids (Shapiro and Wertheimer, 1948). Both acetate (Feller, 1954) and glucose (Hausberger *et al.*, 1954) can serve as precursors for the fatty acids formed. Whenever an attempt has been made to evaluate the metabolic activity of adipose tissue on the basis of fat-free weight or nitrogen content, the results have pointed to the surprising conclusion that the metabolic activity of adipose tissue is comparable or superior to that of liver (Feller, 1954; Hausberger *et al.*, 1954).

Observations on the regulation of carbohydrate metabolism in isolated adipose tissue have shown that slices obtained from the epididymal fat pad of diabetic rats oxidize less glucose to CO_2 and incorporate much less glucose carbon into long-chain fatty acids than do fat slices from normal rats. The administration of insulin to diabetic rats for one day

or more before sacrifice restored the fatty acid synthesis from glucose by adipose tissue slices to normal or greater than normal activity. Oxidation of glucose to CO_2 was also affected by previous insulin administration, but it is interesting to note that the effect of insulin on lipogenesis from glucose was much greater than that on glucose oxidation (Hausberger *et al.*, 1954). An effect of insulin added *in vitro* has been demonstrated by Krahl (1951), and a local effect of insulin on rat adipose tissue resulting in increased glycogen deposition and fat storage has been reported (Renold *et al.*, 1950). It has also been shown that previous fasting increases the uptake of fatty acids from serum by isolated adipose tissue (Shapiro *et al.*, 1952).

Observations made on the intact animal organism, as well as clinical observations, suggest that adrenal, pituitary, thyroid, and sex hormones, as well as nervous stimuli, modify the deposition of glycogen and fat in subcutaneous as well as "brown" adipose tissue (Wertheimer and Shapiro, 1948). It is evident that much work in isolated adipose tissue remains to be done and that interesting observations cannot but result from such studies.

7. Mammary Gland

Studies on isolated mammary tissue have been primarily concerned with the synthesis of fatty acids from various precursors. In 1949, Folley and French observed that slices from lactating mammary glands readily synthesized fatty acids in the presence of glucose and other substrates. These and related studies have been recently reviewed by Folley (1953). Slices from lactating rat mammary tissue have been shown to utilize glucose, fructose, glycerol, pyruvate, and acetate, as evidenced by the incorporation of C^{14} from these labeled substances into fatty acids and CO_2 (Hirsch *et al.*, 1954). In comparison with liver slices, mammary slices are more active in the formation of fatty acids from glucose, while fructose and glycerol represent rather poor precursors. Lipogenesis from acetate was found to be enhanced by the addition of glycerol to the incubation medium (Balmain *et al.*, 1952, 1953). This observation has been confirmed by Hirsch *et al.* (1954), who further noted that this stimulatory effect on lipogenesis from acetate was not peculiar to glycerol but was shared by glucose, fructose, or pyruvate. In addition, Popjak and Tietz (1954) observed that certain members of the tricarboxylic acid cycle, as well as glucose and pyruvate, have this effect in slices from lactating sheep mammary tissue. It is interesting to note that, when the ability of glucose to increase lipogenesis from acetate was measured both in rat mammary gland and in liver slices, it was found that the addition of glucose was without effect in liver under the same conditions which led to a tenfold increase in mammary tissue.

Balmain *et al.* (1950) and Balmain and Folley (1951) observed that the addition of insulin *in vitro* increased the oxygen consumption, the respiratory quotient, and the lactic acid production of mammary slices. These observations have been confirmed by Hills and Stadie (1952), who also reported insulin binding by the mammary slices since, after initial incubation with insulin, the increased respiratory quotient and increased fatty acid synthesis were not diminished by repeated washings of the slices. Hills and Stadie (1952) also noted that the synthesis of lactose from glucose by mammary tissue occurs *in vitro* but is not affected by insulin.

Using C^{14}-, C^{13}-, and T-labeled acetate and C^{14}-labeled glucose, Balmain *et al.* (1953) have demonstrated that addition of insulin increases lipogenesis from these substrates in lactating rat mammary slices. Balmain and Folley (1951) had previously noted that insulin has no effect on lipogenesis by mammary tissue obtained before parturition or after weaning. The addition of cortical hormones *in vitro* (Balmain *et al.*, 1954) has been reported to decrease lipid formation, and to prevent the effect of insulin when adrenocortical hormones and insulin were both added to the incubation medium. When the increase in lipogenesis observed with glycerol and with insulin were compared (Balmain *et al.*, 1953), it was found that addition of glycerol (0.2%) to the incubation medium increased lipogenesis to the same extent as insulin (1 I.U./ml.). Although this effect of insulin *in vitro* appears clear cut, it should be added that species differences have been noted. Thus, insulin promoted lipogenesis in mammary tissue of the rat but not in sheep (Balmain *et al.*, 1953), although glycerol was effective in increasing lipogenesis in both species Species differences have also been noted in the effects of adrenocortical hormones on lipogenesis (Balmain *et al.*, 1954): in the rat, cortisone and desoxycorticosterone decreased lipogenesis; in sheep, cortisone and hydrocortisone were without effect although decreased lipogenesis was observed with desoxycorticosterone and corticosterone.

8. Adrenal

Little is known of the carbohydrate metabolism of the adrenal gland and even less about its regulation *in vitro*. The existence of a glycolytic pathway of metabolism in adrenal tissue has been demonstrated (Haarmann, 1932; Peskina, 1940), and it is known that adrenal tissue can oxidize members of the tricarboxylic acid cycle (Potter, 1945; McShan *et al.*, 1947). Glock and McLean (1954) have demonstrated the presence of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in adrenal tissue. Kelly *et al.* (1955) have recently reported that the phosphogluconate oxidation pathway is very active in adrenal tissue, par-

ticularly in the adrenal cortex. All the known reactions of this pathway were shown to be present. In comparison with kidney, liver, muscle, brain, and testes, the adrenal proved to be the most active tissue studied with regard to glucose-6-phosphate dehydrogenase activity (Glock and McLean, 1954; Kelly *et al.*, 1955).

The addition of cortisone and deoxycorticosterone *in vitro* decreases respiration of adrenal slices (Sourkes and Heneage, 1952). This effect may be similar to that observed by Hayano and Dorfman (1951) in liver, kidney, and brain. Brummel *et al.* (1953) have observed that ACTH added *in vitro* to adrenal slices incubated in Krebs-Ringer buffer with glucose leads to an increased respiration of the slices. ACTH added to liver and kidney slices under identical conditions was without effect.

9. Male Accessory Organs

The seminal fluid of most species contains varying amounts of fructose, which is the product of one or the other accessory glands. Fructose formation in these tissues probably involves the conversion of glucose to fructose, since seminal fluid fructose concentration appears to be directly related to the blood glucose concentration. This conversion of glucose to fructose is an androgen-dependent metabolic activity. Castration or hypophysectomy leads to almost total disappearance of seminal fluid fructose, while testosterone administration to castrates restores fructose production to normal levels or better. This effect of androgens on fructose formation has been demonstrated in the transplanted rat coagulating gland which survives well in male or female subcutaneous tissue. Semen also contains very significant amounts of citrate, a product of accessory glands; this process is again markedly dependent on androgens. These and other aspects of the biochemistry of semen have been extensively studied and reviewed by Mann (1954).

VI. General Considerations

The foregoing review of experimental observations on carbohydrate metabolism in isolated tissues certainly does not settle the mode of action of any hormone, although it brings together some of the evidence which may help to identify the fruitful directions of future research. It has been the primary objective of the authors to assemble and order established facts regarding the behavior of tissues toward carbohydrates and other participants in carbohydrate metabolism under normal and abnormal conditions. From such an ordered assemblage, they hoped that the principles governing regulation of these aspects of metabolic activity would emerge. Such a utopian outcome naturally did not occur. What if any, are the more general conclusions which appear justified at present?

(1) Adequate techniques for the metabolic study of a number of isolated tissues have become available. The use of isotopically labeled substrates, singly or in pairs, has made it possible to quantitate alternative pathways of metabolism in a variety of physiological and pathological situations. These methods have been applied so far to only a small number of tissues and metabolic states.

(2) The individual responsiveness of each tissue is as important in determining the over-all physiological effectiveness of any given regulator as the chemical structure of the latter. Thus the same insulin molecule has an immediate effect on muscle, at best a delayed effect on liver, and no effect at all on brain. The basis for these differences in tissue responsiveness is not known, but it may be assumed to include differences in the enzymatic and physicochemical architecture of the cell. Such differences in the metabolic pathways open to glucose and other fuels have been discussed for each tissue. Tissues may also differ in their ability either to inactivate the hormonal agent or to metabolize it to further active products, a possibility which has particularly been considered in the case of thyroid hormones.

(3) The instances in which a direct, unequivocal metabolic effect on an isolated tissue can be demonstrated following the addition of a hormone *in vitro* to the incubation medium are few. They include the effects of insulin on muscle and adipose tissue and the effects of epinephrine and glucagon on liver. Although small in number, the theoretical importance of these well-documented hormonal effects is great, since they lend themselves to a more intimate analysis of the mechanism involved. Perhaps the best example is provided by the observations of Levine and collaborators (Levine *et al.*, 1950; Levine and Goldstein, 1955) in eviscerated preparations, Park and collaborators (Park *et al.*, 1955; Park and Johnson, 1955) in the isolated diaphragm, and Ross (1953) in the isolated rabbit lens, suggesting that insulin exerts an immediate effect on the passage of glucose across the surface of certain cells.

(4) In contrast to the direct effects *in vitro*, indirect hormonal effects on tissue metabolism, resulting from endocrine manipulation hours or days before isolation of the tissue, are many. Although metabolic changes of this type cannot be presumed to be the *primary* result of the hormonal deficiency or excess involved, their demonstration in individual tissues contributes greatly to our more detailed understanding of the complex over-all physiological situation in the intact organism. Furthermore, the sequence of metabolic events which follows the administration of a hormone can thus be studied and may ultimately yield insight into the orderly sequence and coordination of enzymatic effects.

From the standpoint of physiological regulation, the metabolic sys-

tem concerned is not to be regarded as a single enzyme or even a single enzyme system. Rather, the system being regulated is a cell and its environment. This physiologically significant unit is one which is metabolically susceptible to alterations in the composition of either the extracellular or intracellular environment, as well as to chemical and morphological alterations in intracellular enzyme systems.

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Experimental Hyperglycemic States Not Primarily Due to a Lack of Insulin

KENNETH H. SHULL* AND JEAN MAYER

Department of Nutrition, Harvard University School of Public Health, Boston, Massachusetts

I. Introduction

A variable number of abnormalities combine to make up the syndrome known as diabetes mellitus. The definition of exactly what constitutes the diabetic state is somewhat arbitrary and semantic, and is often viewed differently by the experimentalist and the clinician. Hyperglycemia is the one condition common to all definitions of diabetes. The commonest clinical type of diabetes mellitus and the diabetic states which have been most thoroughly investigated experimentally are caused by a primary lack or absence of insulin. It is the purpose of the present discussion to review those diabetic states occurring clinically and produced experimentally which are not due primarily to a lack of insulin. Some of these hyperglycemic states may subsequently depend upon a lack of insulin for their persistence, but their geneses depend upon other factors.

II. Pituitary Hyperglycemias

Two types of clinically occurring pituitary endocrinopathies, hypersecretion of the acidophilic cells and hypersecretion of the basophilic cells,

* Postdoctoral Research Fellow, National Heart Institute, National Institutes of Health, Bethesda, Maryland.

lead to hyperglycemic states. Hypersecretion of growth hormone from the acidophilic elements of the pituitary in the first two decades of life before epiphyseal closure causes gigantism. If it occurs after epiphyseal closure, it causes acromegaly (1). Acromegaly was first described by P. Marie (2, 3), who derived the name from the most apparent physical symptom, grossly enlarged extremities (from the Greek, $\alpha\kappa\rho o$ = extremity and $\mu\epsilon\gamma\alpha\sigma$ = large). One of the three acromegalies described by Marie had diabetes mellitus. Bailey and Davidoff (4) demonstrated that acromegaly is caused by adenoma of the anterior pituitary, the preponderance of whose elements in the active stage of the disorder show acidophilic granules. The first statistical evaluation of glycosuria due to hyperglycemia in acromegaly was done by Borchardt (5) in 1908, who found that 40% of 176 acromegalics displayed glycosuria. In 1927 Davidoff and Cushing (6) reported that 12% of a group of 100 acromegalics had diabetes mellitus and 25% were glycosuric. In 1938 Atkinson (7) reported glycosuria resulting from hyperglycemia in 33% of a group of 817 acromegalics. Coggeshall and Root (8) followed up the 100 acromegalics reported by Davidoff and Cushing plus 53 subsequent cases. Their findings indicated an incidence of frank diabetes mellitus of 17% and of glycosuria of 36%. An average of 9.2 years after the onset of acromegaly elapsed before the onset of diabetes mellitus. The incidence of hyperglycemia and glycosuria in gigantism is not as frequent as in acromegaly (9). Decreased sugar tolerance and resistance to insulin are characteristic of both gigantism and acromegaly (1).

Hypersecretion of the basophilic elements of the anterior pituitary leads to Cushing's disease. It is characterized by obesity, abnormal striae, purple facies, and hypergluconeogenesis. In 12 cases of pituitary basophilism described by Cushing (10) adenomas of the basophil cells were the causative agents. A high percentage of these patients displayed hyperglycemia and glycosuria. Since Cushing's original discussion of this disease, mixed cell adenomas of the pituitary and hyaline degeneration of the basophils have been found as causative agents. In some cases the anterior lobe shows no detectable abnormalities.

Acromegaly and gigantism are caused by a hypersecretion of growth hormone, while Cushing's disease is caused by a hypersecretion of corticotropin (11). The hyperglycemic states produced by injection of purified pituitary hormones, as well as crude pituitary extracts, form the subject of the next section. The part attributed to the pituitary in regulation of carbohydrate metabolism varied in two periods (12). From 1908 to 1930 the consensus was that most, if not all, of this pituitary regulation arose in the posterior or neurohypophysis. Since 1930 the emphasis has shifted almost entirely to the anterior or adenohypophysis.

1. Posterior Pituitary Hormones

In 1908 Borchardt (5), following the finding of a high percentage of glycosuria in acromegalics, injected posterior (infundibular) hypophyseal extracts into 5 rabbits and produced hyperglycemia and glycosuria in 2 to 6 hours with a maximum persistency of 24 hours. Similar treatment of 7 dogs with this pituitary extract did not consistently produce glycosuria. Cushing (13) reported in 1910 that intravenous administration of posterior pituitary extracts to dogs was followed, almost without exception, by hyperglycemia. The following year Goetsch *et al.* (14) found that dogs with posterior hypophysectomy showed an enduring augmentation in tolerance for sugars which could be drastically reduced by posterior pituitary therapy. Claude and Baudouin (15) were able to produce hyperglycemia with posterior lobe extracts but not anterior lobe extracts. Burn (16), using rabbits, and Magenta and Biasotti (17) and Geiling *et al.* (18), using dogs, demonstrated an antagonism between insulin and posterior pituitary extracts. The usual hypoglycemia induced in these animals by insulin could be abolished by simultaneous posterior pituitary administration.

Subsequent to the work of Borchardt and Cushing, the hyperglycemic effects of posterior pituitary extracts were reported in a variety of species by various laboratories. In 1928 Kamm *et al.* (19) fractionated posterior pituitary extracts into relatively pure oxytocic and vasopressor fractions. The availability of the posterior pituitary hormones following Kamm's work made it feasible to assay the effects of these hormones singly on the blood sugar of various animal species. Geiling and Eddy (20) found that both Kamm's vasopressor and oxytocic hormones raised the blood sugar when injected into rabbits or dogs. The vasopressin caused a greater hyperglycemia than the oxytocin. Likewise Draper and Hill (21) found that vasopressin was more potent in raising the blood sugar of dogs than oxytocin. Bacq and Dworkin (22) obtained a prompt rise in the blood sugar of cats which had been injected with either oxytocin or vasopressin. However, Bischoff and Long (23) found that doses of vasopressin which were effective in producing hyperglycemia in the rabbit were pharmacological, not physiological, and approached the lethal level. Holman and Ellsworth (24) demonstrated that the oxytocin of the posterior pituitary hormones was responsible for the hyperglycemic reaction in dogs. They concluded that the hyperglycemic effect of vasopressin in this species was probably due to a contamination with oxytocin. In a subsequent study Ellsworth (25) concluded, as did Bischoff and Long, that it was doubtful whether either of the posterior pituitary hormones was of significance in blood sugar regulation of the rabbit. The effects on blood

sugar of pure vasopressin and oxytocin as prepared by du Vigneaud have not yet been evaluated (26). It is the opinion of Waring and Landgrebe (27) that it is "improbable that the neural lobe [of the pituitary] is significant in carbohydrate metabolism," in view of the large doses required and the implication of oxytocin or vasopressin, depending upon the method of preparation and species used. The precocious and evanescent hyperglycemia produced by posterior pituitary extracts is clearly different from the hyperglycemia produced by anterior pituitary extracts, which is characterized by a two- to three-day latency period (28).

It has been established experimentally that the posterior pituitary extracts produce hyperglycemia through liver glycogenolysis. In 1926 Lambie (29) demonstrated that pituitrin (a posterior pituitary preparation) failed to prevent insulin hypoglycemia in the cat if the liver was excluded from the circulation. Following this Imrie (30) found that the posterior pituitary-induced hyperglycemia could be abolished by depleting the liver glycogen while maintaining the muscle glycogen. Geiling and Britton found that posterior pituitary extract has no consistent hyperglycemic effect in fasted animals (31). Houssay and Di Benedetto (32) corroborated these findings by demonstrating that posterior pituitary extracts produced no hyperglycemia in hepatectomized animals. They also reported that the adrenal medulla played a permissive role in this hyperglycemia.

2. Anterior Pituitary Hormones

The shift in emphasis from the posterior to the anterior pituitary in carbohydrate regulation was due mainly to Houssay and his colleagues. In 1925 Houssay and Magenta (33) and Houssay *et al.* (34) showed that extirpation of the pituitary in the dog and toad greatly increased the animals' sensitivities to insulin. Later Houssay and Potick (35) demonstrated that ablation of the anterior lobe only caused this increased sensitivity and that it could be better reversed with anterior rather than posterior pituitary extracts. In 1930 Houssay and Biasotti (36) discovered that diabetes in the toad produced by pancreatectomy could be prevented or greatly ameliorated by hypophysectomy and that reappearance of the diabetes could be effected by implantation of anterior pituitary extracts. Later in the same year these workers (37) observed this same phenomenon in the pancreatectomized-hypophysectomized dog.

The hyperglycemic effect of anterior pituitary extracts introduced into intact animals was first reported in 1927 by Johns *et al.* (38), who injected "protein-free" extracts of ox anterior pituitary into dogs and produced hyperglycemia and glycosuria in 24 hours. The hyperglycemia and glycosuria subsided gradually after withdrawal of the injections. In

1932 and 1933 several laboratories reported the hyperglycemic action of anterior pituitary extracts in the intact animal. In 1932 H. M. Evans *et al.* (39) produced hyperglycemia and glycosuria in two of four dogs by injections of crude hypophyseal growth hormone. After periods of eight and nine months the injections were stopped but the hyperglycemia and glycosuria persisted. In this same year Baumann and Marine (40) produced hyperglycemia and glycosuria in four rabbits by administration of anterior hypophyseal extracts for a period of six days. Houssay *et al.* (41) also observed hyperglycemia and glycosuria in the intact dog and rat after two or three days of injections of anterior pituitary extracts. In 1933 E. I. Evans (42) maintained hyperglycemia for a period of a week in 12 dogs by the injection of anterior hypophyseal extracts. Similar results were obtained with anterior pituitary injections in intact dogs by Barnes and Regan (43). In 1937 Young (44) confirmed and extended the observations of H. M. Evans *et al.* by producing permanent diabetes in the dog by administration of anterior pituitary extracts. In subsequent studies Young (45) found that the mouse, rat, and guinea pig were almost completely insensitive to the diabetogenicity of anterior pituitary extracts. With cats and rabbits, 25 to 50% of the cases displayed glycosuria and with dogs 100% of the normal animals gave a diabetic response when injected with anterior pituitary extracts. In 1936 Long and Lukens (46) reported that adrenalectomized-pancreatectomized cats failed to respond to the diabetogenic activity of anterior pituitary extracts. In contrast to this, Houssay and Leloir (47) reported that anterior pituitary extracts were diabetogenic in the adrenalectomized dog, and Houssay and Biasotti (48) found the adrenalectomized-pancreatectomized-hypophysectomized toad sensitive to the diabetogenicity of these extracts. Part of this discrepancy was undoubtedly due to the preponderantly "adrenotropic" content of the extract used by Long and Lukens. Such an extract would be expected to have little or no diabetogenicity in the adrenalectomized animal.

With the advent of purified anterior pituitary hormones in the 1940's it became possible to assay the individual hormones for diabetogenicity. Of the six anterior pituitary hormones, diabetogenicity has been regularly associated with only two, the corticotropic (ACTH) and growth hormones. A third one, prolactin, is diabetogenic in the partially pancreatectomized animal. In 1945 Ingle *et al.* (49) produced glycosuria in four out of five normal rats force-fed a high carbohydrate diet by administration of purified corticotropic hormone produced by the method of Li *et al.* (50). In 1947 Bennett and Li (51) found that the glycosuria of alloxan-diabetic or partially pancreatectomized rats is highly insensitive to the diabetogenic action of growth hormone (52). Engel *et al.* (53) produced con-

sistent hyperglycemia and glycosuria in rats force-fed a high carbohydrate diet by concomitant administrations of ACTH and growth hormone. Inconsistent or no diabetogenic responses were obtained with either hormone alone unless excessive diet was administered. A typical diabetogenic response to ACTH in humans was first observed in 1943 by Browne (54) in a normal male who had received this hormone for two days. In 1948 and 1949 Conn and colleagues in a series of communications (55, 56, 57) reported the effects of administering large daily doses of ACTH to a number of normal humans. In the majority of these cases they observed insulin-resistant hyperglycemias and glycosurias. In the dog and cat the diabetogenic response is less pronounced than that observed in rats and humans. Although Campbell *et al.* (58) have produced hyperglycemia and glycosuria in dogs with high doses of ACTH, the response was much less than with growth hormone. Houssay and Anderson (59) have also found the dog relatively refractive to the diabetogenicity of ACTH. This has also been found repeatedly in the cat by Reid (60).

Two types of highly purified growth hormone have been available for experimental use: the crystalline type prepared by the method of Wilhelmi *et al.* (61) and the amorphous type prepared by the method of Li *et al.* (62). In 1949 Cotes *et al.* (63) demonstrated that administration of growth hormone prepared by either method caused hyperglycemia and glycosuria in the intact adult cat. In 1950 Campbell *et al.* (58, 64) reported the production of both transient and permanent hyperglycemic states in dogs by administration of crystalline growth hormone prepared by the method of Wilhelmi *et al.* In contrast, the intact rat is highly refractive to the hyperglycemic effect of growth hormone. However, in rats partially pancreatectomized and fed a sucrose diet, marked increase in the glycosuria has been reported after administration of a partially purified growth hormone (65). Likewise, increased glycosuria after administration of partially purified growth hormone to alloxan-diabetic rats has been observed by Gaarenstroom *et al.* (66) but has failed of confirmation by Reid (52). Houssay and Anderson (59) have produced hyperglycemia in normal toads and frogs by administration of purified growth hormone.

Houssay and colleagues (59, 67) have shown that prolactin had a diabetogenic effect in dogs and cats in which the pancreases had been reduced. Most preparations of prolactin contain small amounts of ACTH, but diabetogenicity was also obtained with preparations of pure prolactin produced by the method of Cole and Li (68). In the partially depancreatized dog prolactin was found to be much less diabetogenic than growth hormone but more diabetogenic than ACTH. Foà and co-workers (69, 70) have shown that repeated intravenous injection of prolactin in

normal dogs is followed by hyperglycemia. In depancreatized dogs hyperglycemia is produced by a single dose.

The identification of diabetogenicity with either growth hormone and/or ACTH depends not only on the purity of these hormones but also on the species used as test animals. In addition, the responsiveness of the experimental animals depends upon their nutritional and physiological states and their ages.

Although separation of the diabetogenic from the growth-promoting principle of growth hormone has been repeatedly attempted, it has as yet not been achieved. The recent claim by Raben and Westermeyer (71) of having effected this separation does not appear to be based upon sound evidence (60). The recent work of Bell and colleagues (72), who have separated eight proteins of equally high corticotropin activity from "clinical" ACTH, casts serious doubt upon the purity of previously used ACTH preparations. The consensus at present favors the concept that the diabetogenicity of the anterior pituitary resides primarily in the growth hormone, with corticotropin and the adrenal glucocorticoid hormones acting synergistically and permissively.

The diabetogenicity of the anterior pituitary is dependent upon the species which is assayed and nutritional state of the animal at the time of assay. In the dog and cat hyperglycemic states can be produced and maintained with purified growth hormone alone. However, such responses are dependent on the animals' being in a fed state. The partially fed or fasted dog and cat respond poorly or not at all (43, 73). The intact rat is almost completely refractive to the diabetogenicity of anterior pituitary extracts (74). In this species a combination of growth hormone and ACTH plus force-feeding of a high carbohydrate diet is necessary to elicit a consistent diabetic response.

Finally, the age and physiological state of the animals affect their response to growth hormone. Young (75, 76) has demonstrated that diabetogenic growth hormone induces extra growth, not a diabetic state, in puppies and kittens. Young has further found that growth hormone does not elicit a diabetogenic response in the pregnant bitch (45) or pregnant cat (77).

The diabetic states produced by the injection of anterior pituitary extracts into susceptible species may be divided into two types: hypophyseal (idiohypophyseal) and permanent (metahypophyseal). The first type, with which we are especially concerned, is characterized by extreme insulin resistance and is temporary in nature. In the dog, the species most thoroughly investigated, there is a characteristic latency period of two to three days after the beginning of daily injections before a hyperglycemic state is attained (28). The hyperglycemia is usually of a moderate degree.

Houssay (12), in experiments with 300 dogs, obtained a fasting blood sugar of between 150 and 300 mg. per 100 ml. with a mean value of 178 mg. per 100 ml. In spite of continued daily injections the blood sugar usually reverts to normal or even to hypoglycemic levels in five to eight days. In addition to insulin resistance, ketosis, hyperlipemia, hyperphagia, and polyuria are typical of this type of diabetes. In favorable cases, by increasing the dosage when the blood sugar tends to revert to normal, it is possible to produce a permanent diabetic state. Examination of the pancreases of these permanently diabetic animals shows that the beta cells are vacuolated and hydropic to varying degrees. It is a diabetic state caused by a lack of insulin due to exhaustion of the beta cells of the islets of Langerhans (78, 79). Since this diabetic state is no longer associated with the hypophysis, Houssay has proposed the name metahypophyseal diabetes.

The mechanisms by which the anterior pituitary produces hyperglycemia have been given a variety of explanations by different experimentalists. In the 1930's the prevalent theories postulated to explain the hyperglycemic action of the anterior pituitary were by epinephrine, gluconeogenesis, and depressed oxidation. These theories as well as the investigators who proposed them should be judged in their proper historical context, as they do not necessarily indicate the views presently held by these investigators.

One of the first theories prevalent in the early 1930's proposed that the hyperglycemic states of anterior pituitary origin were produced by glycogenolysis due to sensitization of the liver to epinephrine. This was based largely on observations of the increased sensitivity of adrenal demedullated animals to insulin, of the decreased sensitivity of hypophysectomized animals to the hyperglycemic effect of epinephrine, and of the increased sensitivity of normal animals treated with anterior pituitary extracts to the hyperglycemic effect of epinephrine. In 1925 Lewis and Magenta (80), working with dogs, and in 1928 Britton *et al.* (81), working with cats, reported increased sensitivity to insulin in animals in which the secretion of epinephrine was precluded or suppressed. Subsequently Barnes *et al.* (82) reported that the insulin sensitivity of dogs with adrenal medullas almost completely destroyed could be reversed by epinephrine administration. Since the hypophysectomized animal had been shown to be hypersensitive to insulin by Houssay *et al.*, it was of interest when adrenal demedullated animals were also reported to be hypersensitive to insulin. The attenuation of the hyperglycemia produced by epinephrine was first reported by Aschner (83) in 1912 in hypophysectomized dogs. This effect was confirmed in hypophysectomized dogs by Houssay and Di Benedetto (84), Chaikoff *et al.* (85), and by Corkill *et al.* (86) in hypophysectomized rabbits. Houssay and Di Bene-

detto (87), experimenting with dogs, and Cope and Marks (88) with rabbits, found that animals treated with anterior pituitary extracts were more sensitive to the hyperglycemic action of epinephrine than their controls. Cope and Marks concluded that the anterior pituitary contains a substance which sensitizes the liver to epinephrine. From the observations discussed above, it was postulated that the anterior pituitary exerted its hyperglycemic effect by sensitizing the glycogenolytic processes of the liver to epinephrine. The absence of this sensitization in the hypophysectomized animal would lead to less liver glycogenolysis and hence a greater sensitivity to insulin. In the normal animal treated with anterior hypophyseal extracts the opposite effects, decreased insulin sensitivity and increased epinephrine sensitivity, would obtain. This theory fell into disfavor since certain observations upon which it was based were questionable. Re (89) and Lucke *et al.* (90) found no decreased sensitivity to the hyperglycemic action of epinephrine in hypophysectomized animals. Russell and Cori (91) found a somewhat decreased sensitivity to the hyperglycemic action of epinephrine in hypophysectomized rats when this hormone was given subcutaneously, but normal sensitivity when it was given intravenously. Thus, some of the previous observations on the decreased sensitivity of epinephrine in hypophysectomized animals were attributed to the use of the subcutaneous route for administration of this hormone. Likewise Zucker and Berg (92) found the insulin sensitivity essentially normal in bilateral adrenal demedullated dogs. This theory was further weakened by results obtained in the Houssay laboratory where hyperglycemia was produced in adrenalectomized dogs (47) and toads (48) by anterior pituitary extracts.

According to Houssay's school (93), working with dogs, and Long and Lukens (94, 95), working with cats, the production of extra sugar by anterior hypophyseal extracts was believed to be effected through gluconeogenesis from protein. Houssay based the theory of gluconeogenesis on observations from his laboratory as well as from other laboratories concerning the metabolism of hypophysectomized animals. Such animals when fasted develop a rapid hypoglycemia and show low nitrogen and creatinine excretion. Further, the hypophysectomized animal treated with phlorizin excretes smaller amounts of glucose and nitrogen than the intact phlorizinized animal. One of the most characteristic symptoms of the hypophysectomized animal is the hypoglycemic crisis which develops during fasting. This has been observed by Houssay (93) in the dog, by Corkill *et al.* (86) in the rabbit, by Long and Lukens (94) in the cat, and by Russell (96) in the rat. Braier, working in Houssay's laboratory, reported low nitrogen and creatinine excretions in fasted hypophysectomized dogs (97), toads (98), and rats (99). Further, diminished

excretions of nitrogen and glucose were observed by Houssay and Biasotti (100, 101) in hypophysectomized dogs treated with phlorizin. Such animals when fasted show much diminished glycosuria when compared to intact phlorizinized animals. The dextrose:nitrogen (D:N) ratio of fed phlorizinized-hypophysectomized dogs was much lower than that of intact phlorizinized dogs. A marked increase in nitrogen excretion and a high urinary D:N ratio was exhibited by the normal animals treated with phlorizin. A consideration of the altered nitrogen metabolism of fasted hypophysectomized animals, along with their apparent inability to convert protein to carbohydrate in response to phlorizin treatment or to maintain their blood sugar during fasting, led Houssay to believe that the anterior pituitary exerts its effect on carbohydrate metabolism through regulation of gluconeogenesis from protein. Likewise Long and Lukens concluded that alleviation of pancreatic diabetes by either adrenalectomy or hypophysectomy was due to a decrease in formation of glucose from protein, rather than resumption of normal carbohydrate metabolism. Soskin *et al.* (102) also postulated that the anterior pituitary exerted its hyperglycemic effect through gluconeogenesis, but from fat rather than protein. This was based on their findings that carbohydrate and protein maintained the blood sugar of hypophysectomized dogs while fat did not. Further, there was a lack of ketonuria in the hypophysectomized-depancreatized animal. Anterior lobe extracts, on the other hand, had been observed to promote ketogenesis by Anselmino and Hoffmann (103) and Black *et al.* (104). Russell, finding certain discrepancies in the "gluconeogenetic" theory, especially when applied to the rat, hypothesized that the anterior pituitary exerted an effect on carbohydrate metabolism by depressing glucose oxidation (105). In the hypophysectomized rat the nitrogen excretion was only moderately low or even normal (106). Furthermore, the nonprotein nitrogen of such operated animals was high (107) and the loss of body weight was considerable (108). In the rat, therefore, it did not appear that any defect in protein mobilization or catabolism existed. Further discrepancies had also been observed. Gaebler (109) actually observed nitrogen retention in normal dogs treated with anterior pituitary extracts. Regarding fat metabolism, Lee and Ayres (110) found that the hypophysectomized animal could dispose of large quantities of both exogenous and endogenous fat. Using hypophysectomized rats Russell and Bennett (111) observed an abnormal loss of carbohydrate, especially muscle glycogen, when these animals were fasted. Starting with like amounts of glycogen, fasted hypophysectomized rats lost more muscle glycogen and had higher respiratory quotients than the normal controls (106). Treatment of such animals with an alkaline extract of anterior pituitary restored not only the glycogen levels but

also the respiratory quotients to normal fasting levels (112, 113). Subsequently, Russell observed a fall in the oxidation of fed carbohydrate, not only in hypophysectomized rats which had been treated with anterior pituitary extracts, but also in normal rats which had been similarly treated (114). Working with rabbits, Greeley reported that the hypophysectomized animals required more carbohydrate to maintain their blood sugar than their normal controls (115). Russell interpreted these findings as showing that the anterior pituitary depresses the oxidation of carbohydrate. The extra carbohydrate might be stored as glycogen or appear as blood and tissue glucose. Houssay (12), in contrast, looked upon these findings as species differences and not necessarily contradictory to the gluconeogenetic theory. The theory of depressed oxidation mediated through the anterior pituitary has been further emphasized and extended by Young (116). He has observed in pregnant cats and dogs and growing puppies and kittens that neither anterior pituitary extracts nor growth hormone are diabetogenic. In both pregnancy and growth, sustained deposition of extra tissue is possible. The processes of laying down extra tissue and/or milk involve depressed oxidation of the substrates being used to synthesize these materials. Young concluded that when extra growth or lactation cannot be realized, the substrates which have escaped oxidation, such as glucose, appear in the blood. Under such conditions anterior pituitary extracts and growth hormone become diabetogenic.

Historically, the theories discussed above represented the first attempts at unification of experimental observations into coherent working hypotheses. Much of the experimental evidence upon which they were based has been corroborated many times in numerous laboratories. If a common fault were to be found in these theories, it lay in their lack of specificity. The *in vivo* research which formed their experimental basis did not elucidate the locus of action of the anterior pituitary. For this reason much interest centered around the report in 1945 from the Cori laboratory by Price *et al.* (117) that anterior pituitary extract [K fraction of Greaves *et al.* (118)], when added *in vitro* to various tissue extracts of the rat, inhibited the hexokinase reaction. It was further stated that this inhibition could be counteracted by insulin added *in vitro*. A further report by Price *et al.* (119) stated that adrenal cortical extracts greatly increased the inhibition of the hexokinase reaction brought about by anterior pituitary extracts. Colowick *et al.* (120) found that adrenal cortical extracts alone inhibited the hexokinase reaction in muscle extracts from diabetic rats. The hexokinase activity of extracts of normal rat muscle was not inhibited by adrenal cortical extract alone, but by the further addition of anterior pituitary extract, inhibition could

be obtained. *In vitro* addition of insulin reversed this inhibition. Following the reports from the Cori laboratory numerous experiments were conducted to investigate the effects of anterior pituitary and adrenal cortical extracts on cell-free hexokinase systems. Broh-Kahn and Mirsky (121) found only inconstant inhibition of muscle hexokinase by diabetogenic anterior pituitary extracts. Reid *et al.* (122) found that an anterior pituitary factor inhibited the hexokinase reaction of various tissue extracts. Although insulin reversed this inhibition, there was no correlation between this reversibility and the diabetogenicity of the factor *in vivo*. Stadie and Haugaard (123) were unable to demonstrate any inhibition of the hexokinase reaction in extracts of diabetic rat tissues to which adrenal cortical extracts were added. Christensen *et al.* (124) could find no alteration in the activity of hexokinase obtained from hemolyzed red cells by addition of adrenal cortical or anterior pituitary factors. An inhibition of the hexokinase system in cell-free preparations by anterior pituitary and/or adrenal cortical factors is at present *sub judice*.

The possibility that anterior pituitary-adrenal cortical inhibition of the hexokinase reaction existed but required cellular integrity for its demonstration was entertained and put to test by various investigators. In general the work of Hastings and colleagues has confirmed the "Cori hypothesis" of an insulin-reversible pituitary-adrenal inhibitor at the hexokinase level (125). Conducting investigations with the rat diaphragm, Villee and Hastings (126) found that glucose uptake and insulin sensitivity were significantly increased by hypophysectomy-adrenalectomy. Teng *et al.* (127) measured rat liver glycogen synthesis from radioactive labeled pyruvate and glucose. In the adrenalectomized diabetic animals with unopposed pituitary inhibition they found normal glycogen synthesis from pyruvate but decreased synthesis from glucose. This is what would be expected if the Cori hexokinase hypothesis is correct. Similar results were obtained by Renold *et al.* (128) with rat liver slices. Hypophysectomy superimposed upon diabetes caused glucose uptake to increase toward normal. Krahl and Park (129), working in the Cori laboratory, found that diaphragms from hypophysectomized rats had a significantly higher glucose uptake than diaphragms from normal rats. Subsequently Park and Krahl (130) found that the diaphragms of normal rats which had been treated three hours before sacrifice with anterior pituitary fractions had glucose uptakes similar to diaphragms obtained from diabetic animals. Concurrent adrenal cortical administration depressed the glucose uptake of diaphragms from anterior pituitary treated rats to an even lower level. Insulin caused a reversal of the pituitary-adrenal inhibition with a concomitant increase in glucose uptake. (See also the review of Renold *et al.* in this volume.)

Clarification of the nature as well as the mechanism of growth hormone inhibition of glucose uptake in muscle has been achieved by Bornstein and Park (131). They found that the serum from alloxan-diabetic rats inhibited the glucose uptake of normal diaphragms. However, the serum from either adrenalectomized or hypophysectomized diabetic rats did not cause inhibition. Injections of both growth hormone and cortisone into hypophysectomized diabetic rats were required to restore the inhibitory factor to the serum. The inhibition of glucose uptake produced by this serum factor could be reversed *in vitro* by insulin. Bornstein (132) reported that this inhibitor was associated with the $beta_1$ lipoprotein fraction of the serum. The insulin reversible inhibition caused by this lipoprotein factor appeared to be at the hexokinase level.

The experiments with isolated diaphragms and liver slices were *prima facie* evidence for an insulin-reversible pituitary-adrenal inhibition at the hexokinase level, with this phenomenon dependent on intact cells. There are still, however, two different kinds of dissent from this view; one group of workers questions the existence of this inhibition; another group accepts it but doubts the necessity of intact cell structure (133). In support of the first view are the observations of Stadie (134). In 1949 Stadie *et al.* (135) reported that diaphragms of rats dipped momentarily in insulin synthesized more glycogen from glucose than controls. They interpreted this phenomenon as due to binding of insulin in its physiological form. In subsequent work, Stadie *et al.* (136, 137) demonstrated that diaphragms from hypophysectomized rats dipped in insulin synthesized more glycogen than diaphragms from control animals. In adrenalectomized and hypophysectomized rats prior injections of both growth hormone and cortisone were required to diminish the glycogen synthesis in diaphragms which had been equilibrated with insulin. Since the diaphragms of hypophysectomized rats bound the same amounts of insulin as the control diaphragms, Stadie interpreted these results as evidence that a pituitary factor in conjunction with an adrenal cortical factor exerted a contra-insulin effect in the peripheral tissues. Regarding the necessity of intact cellular structure, Krahl (138) has recently reported inhibition of hexokinase in cell-free systems by the "Bornstein lipoprotein factor." Insulin was able to reverse this inhibition when added *in vitro* in only about 30% of the cases.

In spite of the persistence of some unsolved questions, it would appear likely that a pituitary inhibition does exist at the hexokinase level. The glucocorticoids, or ACTH acting through its tropic effect upon the adrenal cortex, probably play a permissive role in the formation of the active inhibitory factor. The reversibility *in vitro* of this inhibition by insulin appears less well established. The contra-insulin effect of pituitary factors

proposed by Stadie fails to explain the diabetogenicity of such factors in the insulin-free depancreatized hypophysectomized animal.

Although the mechanism of prolactin hyperglycemia is unexplained, Foà *et al.* (70) suggest that it may act by directly inhibiting glucose uptake by the tissues, as does growth hormone.

III. Adrenal Hyperglycemias

Hyperfunctions of both the adrenal cortex and adrenal medulla leading to hyperglycemic states occur in humans. The former is usually referred to as hypercorticoidism (Cushing's syndrome) and the latter hyperadrenalinism.

Although the morphologic cause of hypercorticoidism is somewhat confused, there is characteristically an augmented secretion of glucocorticoids (C-11-oxygenated steroids). The clinical manifestations of hypercorticoidism are similar to those of Cushing's disease in which there is increased secretion of ACTH, the tropic hormone of the adrenal cortex. There is characteristically hyperglycemia and glycosuria with a high incidence of diabetes mellitus (139). In a review of 55 patients with Cushing's syndrome by Lukens *et al.* (140), 44% showed glycosuria.

Hyperadrenalinism is caused by a pheochromocytoma, the only functioning tumor of the adrenal medulla. From a review of 50 patients with pheochromocytoma, Cook and Schneider (141) found transient hyperglycemia in 21 patients, 5 patients with clinical diabetes mellitus, and no carbohydrate disturbances in 24 of the patients.

1. Adrenal Cortical Hormones

Hyperglycemia and glycosuria have been produced in experimental animals and man by administration of either adrenal cortical extracts or hormones. In 1922 Régnier and Simonnet (142) reported an approximate doubling of the blood sugar of dogs given an alcoholic extract of the adrenal cortex. Britton and Silvette (143), conducting extensive investigations on adrenal cortical function in the early 1930's, reported the production of considerable hyperglycemia in cats, rats, and rabbits after the injection of adrenal cortical extracts. In rats the hyperglycemic effects of a single injection were still apparent after six hours. Thaddea (144) corroborated these results in the normal guinea pig.

In 1941 Ingle (145) maintained prolonged hyperglycemia and glycosuria in normal rats by the combination of force-feeding a high carbohydrate diet and daily administration of cortisone (17 hydroxy-11-dehydrocorticosterone). The hyperglycemia and glycosuria disappeared upon cessation of the hormone treatment. Hyperglycemia and glycosuria were also produced in normal rats force-fed a carbohydrate diet by the admin-

istration of corticosterone or 17-hydroxycorticosterone (146). Under these conditions 11-deoxycorticosterone is weakly diabetogenic (147). Administration of 11-oxygenated adrenal steroids to humans often causes hyperglycemia and glycosuria. The diabetic state produced by these corticoids is similar to that produced by ACTH (148). An increased secretion of the 11-oxygenated steroids occurs during pregnancy in both humans and animals. The hyperglycemia and glycosuria of prediabetic pregnant women is, according to Hoet (149), probably caused by the increased production of these corticoids. The adrenal cortical hormones active in producing hyperglycemic states are those possessing an oxygen function on carbon 11 of the steroid nucleus.

The hyperglycemic actions of the adrenal cortex were also inferred from investigations conducted on adrenalectomized and pancreatectomized animals. In 1908 Frouin (150) found that pancreatectomy produced less glycosuria in dogs which had the major portion of the adrenal glands removed. In 1929 Turcatti (151) demonstrated that extirpation of both adrenals and the pancreases from dogs was not followed by hyperglycemia. The adrenal cortex was implicated, since removal of one adrenal and demedullation of the other did not prevent hyperglycemia from developing in the depancreatized dog. Hartman and Brownell (152), reporting on a small number of adrenalectomized-pancreatectomized cats with short survival times, concluded that the adrenal cortex was necessary for maintenance of pancreatic diabetes. Conducting an extensive investigation on cats, Long (153) and Long and Lukens (94) found that marked amelioration of pancreatic diabetes in this species was obtained by adrenalectomy. The survival time of these doubly operated animals was much improved by these investigators. Long also showed that the diabetogenicity of the adrenal gland operated through the cortex and not the medulla in these animals. Similarly, Long and co-workers (154) were able to produce as great an amelioration of pancreatic diabetes in the dog by adrenalectomy as had been produced in the cat. In further investigations, Long *et al.* (155) demonstrated that adrenal cortical extracts intensified the diabetes of partially depancreatized rats or caused it to reappear in the subtotal pancreatectomized-adrenalectomized rat.

The mechanism of production of hyperglycemia by the adrenal corticoids is in part explicable by the effect of these hormones on gluconeogenesis. This was first inferred by Long *et al.* (155) from their data on rats. They found that when either a potent adrenal cortical extract or one of the C-11-oxygenated adrenal hormones was administered to fasted normal, hypophysectomized, or adrenalectomized rats, urinary nitrogen excretion was increased. Analyses indicated that 53 to 65% of the protein catabolized was converted to glucose. Likewise, the work of Ingle (145)

with rats force-fed a high carbohydrate diet indicated that adrenal corticoid administration brought about a concomitant increase in urinary nitrogen along with the hyperglycemia and glycosuria. The data of Wells and Kendall (156) demonstrated a marked impairment in the conversion of endogenous protein to glucose in the phlorizinized-adrenalectomized rat. With the availability of radioactive isotopes it became possible to prove more conclusively the effect of C-11-oxygenated adrenal steroids on gluconeogenesis. Welt *et al.* (157) injected C^{14}-glucose at a constant rate into rats and estimated gluconeogenesis by comparing activities of injected and excreted glucose. They found that cortisone administration resulted in a sevenfold increase in glucose production from noncarbohydrate materials.

Although the hyperglycemia produced by the adrenal steroids is in part due to gluconeogenesis, the totality of the hyperglycemic state cannot be explained on this basis. In the acute experiments of Long and co-workers and the chronic experiments of Ingle, in which carbohydrate was administered along with the adrenal cortical hormones, gluconeogenesis could account for only part of the extra carbohydrate. In addition, Ingle *et al.* (158) found extreme insulin-resistant hyperglycemias in their hormone-treated rats. Similar insulin resistance has been observed by Conn (148) in humans treated with adrenal cortical hormones. Thus, in addition to gluconeogenesis from protein, the added effect of inhibition of glucose utilization has been ascribed to the glucocorticoids. The nature of this inhibition is still being investigated. Mention has already been made of adrenal cortical inhibition at the hexokinase level. In addition to this, the adrenal cortical steroids may also exert an effect on fat synthesis. The work of Welt and Wilhelmi (159) with adrenalectomized rats indicated that ACTH or cortisone markedly inhibited lipogenesis from carbohydrate. Brady *et al.* (160) further demonstrated that liver slices from cortisone-pretreated rats exhibited a marked inhibition in the incorporation of acetate into fatty acids. This inhibition of fat synthesis from carbohydrate would have much the same net result as gluconeogenesis from fat, with which the adrenal cortical hormones have been implicated (161). The effects of the glucocorticoids leading to hyperglycemia are well summarized by Long (162): "When given in excess to intact animals, they not only present the organism with additional material to be converted to carbohydrate but, at the same time, diminish its capacity to dispose of this or dietary carbohydrate by the usual metabolic pathways. In consequence, the blood glucose may rise to levels at which glycosuria occurs."

2. Adrenal Medullary Hormones

The adrenal medulla secretes two hormones, epinephrine (adrenaline) and norepinephrine (arterenol, noradrenaline) both of which have hyper-

glycemic properties. Epinephrine was isolated in the form of metallic derivatives in 1898 by von Fürth (163) and as the benzoate in 1899 by Abel (164). The latter investigator gave the name epinephrine to the isolated adrenal medullary substance. Norepinephrine, which differs from epinephrine by the absence of the N-methyl group, was isolated from natural epinephrine in 1949 by Goldenberg *et al.* (165) and Tullar (166).

In 1901 Blum (167) noted the production of hyperglycemia and glycosuria when he introduced epinephrine into the circulation of dogs. In 1912 Garrod (168) observed glycosuria in humans given epinephrine clinically. Numerous other investigators have established the hyperglycemic effects of administered epinephrine in a variety of animal species (169). Experiments designed to test the hyperglycemic effect of norepinephrine indicate that it has one-fifth to one-eighth the hyperglycemic action of epinephrine (170, 171). The naturally occurring forms of both epinephrine and norepinephrine are the *l*-isomers. The *d*-isomers of these hormones are much less potent in their hyperglycemic actions than the *l*-isomers.

Although the hyperglycemic effect of epinephrine when introduced into human and animal systems has been uniformly confirmed, the source and the mechanism of production of the extra blood glucose have long been controversial issues. The clarification of the source of the extra blood glucose after epinephrine administration was achieved by the extensive work beginning in 1928 of C. F. Cori, G. T. Cori, and co-workers and of Sahyun and Luck. Although it had been postulated since the early 1900's that the liver glycogen supplied the extra glucose, evidence of a decrease in liver glycogen after epinephrine administration was inconclusive (172). On the other hand, there was more conclusive evidence for the disappearance of muscle glycogen under the influence of epinephrine. Lesser (173) experimenting with isolated frog muscles, Junkersdorf and Török (174) with dogs, Cori and Cori (175) with rats, Blatherwick and Sahyun (176) with rabbits, and Corkill and Marks (177) with cats all observed a decrease in muscle glycogen after epinephrine administration. However, since it was known from the work of Meyerhof (178) that the end product of anaerobic glycogenolysis in muscle was lactic acid, muscle glycogen was disregarded as the immediate source of the extra blood glucose resulting from epinephrine action. Sahyun and Luck (179) experimenting with rabbits, and Cori *et al.* (180) with rats, demonstrated that if analyses of liver glycogen were made in sufficiently short periods of time (<1.5 hours for the rabbit and <1.0 hour for the rat) after epinephrine administration, significantly less glycogen was found when compared with control values. After the initial decrease the liver glycogen increased until it invariably reached levels that were higher than those before the treatment. This occurred in fed (181), postabsorptive (172), or fasted

animals (175, 179). In contrast, the muscle glycogen decreased and remained low throughout the entire period of change in the liver glycogen. Blood lactic acid followed a course similar to the blood glucose. These changes were interpreted by Cori (169) as a balance between breakdown and resynthesis of glycogen. Epinephrine was viewed as causing glycogenolysis in both muscle and liver, the end product in the former being lactic acid and in the latter glucose. The excess blood lactic acid was used for synthesis of new liver glycogen. When this process exceeded glycogenolysis in the liver the glycogen level increased. However, the carbohydrate balance studies of Cori and co-workers indicated that the epinephrine hyperglycemias in rats (180) and rabbits (182) could not be accounted for solely on the basis of glycogenolysis.

Since this was the case it was assumed that epinephrine also inhibited the peripheral utilization of glucose. An inhibitory effect on glucose assimilation had been previously observed by Wiechmann (183) in humans given epinephrine. In a subsequent study Cori *et al.* (184) were able to demonstrate that epinephrine produced an inhibition of glucose uptake by measuring the arteriovenous glucose differences. After administration of this hormone the difference was much decreased. Soskin and co-workers (185, 186) disagreed with the conclusion of Cori *et al.* that epinephrine decreased glucose utilization in the peripheral tissues. Measuring blood flow and arteriovenous glucose differences in animals injected with epinephrine, they could find no evidence of a decreased peripheral utilization of glucose. They proposed that if the balance studies of Cori *et al.* were correct, then, in addition to its glycogenolytic effect, epinephrine must promote gluconeogenesis from fat in the liver. In spite of the reports to the contrary by Soskin and colleagues, most investigators have corroborated the observations from the Cori laboratory. Cohen (187) found that epinephrine inhibited glucose utilization in rat diaphragms. Similarly Somogyi (188), conducting a series of investigations on factors affecting the arteriovenous glucose difference in humans, found that epinephrine strongly depressed the rate of peripheral glucose assimilation.

Another factor entering into the increase in blood glucose and liver glycogen following epinephrine administration is ACTH. Vogt (189), Long and Fry (190), and Gershberg *et al.* (191) demonstrated that epinephrine causes the discharge of ACTH from the anterior pituitary. The resulting discharge of adrenal cortical hormones increased both liver glycogen and blood glucose levels through gluconeogenesis and decreased utilization of glucose. Munson and Briggs (192) more recently have shown that although epinephrine can stimulate the secretion of ACTH, it is not normally the direct effector substance. This hormone apparently acts

through some other mechanism at the hypothalamic level. The hyperglycemic state produced by epinephrine is, therefore, not due to this agent alone but has the added effects of the glucocorticoids superimposed upon it. The hormonal discharge from the adrenal medulla in effect brings into action the array of hormones from the gland as a whole.

The elucidation of the mechanism by which epinephrine promotes glycogenolysis has been due to the investigations of Sutherland and Cori (193, 194). These workers found that of the three enzymatic steps necessary for the production of glucose from glycogen in the liver, the phosphorylase step was the rate-limiting reaction. Two types of phosphorylase, an active and an inactive, have been identified in liver (195). Liver, as well as muscle and many other tissues, contains enzymes which catalyze the transformation of the active to the inactive form. Sutherland and Cori found that when epinephrine was administered either *in vitro* or *in vivo*, the concentration of active liver phosphorylase was increased. *In vitro* an accumulation of glucose-1-phosphate was also observed. A similar situation obtains in muscle as in the liver. Two forms of phosphorylase, a and b, have been isolated. Phosphorylase a, the active form, is converted to phosphorylase b, the inactive form, *in vivo* by stimulation of muscle to fatigue (196) or *in vitro* under the catalytic influence of muscle PR enzyme (197, 198, 199). The PR enzyme is the phosphorylase-rupturing enzyme of muscle. Phosphorylases a and b of muscle differ in physical properties from the active and inactive phosphorylases of liver. In addition, phosphorylase b of muscle is activated by adenylic acid while the inactive form of liver is not. In agreement with their findings in liver, Sutherland and Cori found that epinephrine increased the amount of active phosphorylase when added *in vitro* to rat diaphragms (194). From their data they postulated that epinephrine accelerated the resynthesis of active phosphorylase in both liver and muscle.

The major criticism of this postulate is that epinephrine accelerates only the breakdown and not the synthesis of glycogen. Since phosphorylase catalyzes the synthetic as well as the glycolytic phases, the reason for the exclusively glycolytic effect of epinephrine is obscure.

IV. Pancreatic Hyperglycemias

Following the successful extraction of biologically active insulin from the pancreases of dogs by Banting and Best in 1922 (200), Murlin *et al.* (201) reported that two substances were present in the aqueous extracts of pancreases. One of these was insulin, which lowered the blood sugar of normal and diabetic animals, and the other was a factor which raised the blood sugar of such animals. Kimball and Murlin (202) named this

hyperglycemic factor glucagon.* Following this, transient hyperglycemia was observed by various investigators when insulin preparations were administered intravenously. Extensive studies were conducted upon this aspect of insulin by Bürger and Kramer. In 1929 these investigators (203) reported that the intravenous injection of amorphous insulin was regularly followed in humans and dogs by an initial hyperglycemia which was followed in 40 to 60 minutes by hypoglycemia. After interruption of the portal circulation in dogs this hyperglycemic response was abolished. In subsequent experiments Bürger and Kramer (204) demonstrated that the hyperglycemia was dependent upon liver glycogen levels and that it was independent of the adrenal glands, since it was evoked in double adrenalectomized animals (205). Similar results were reported by Collens and Murlin (206), who observed hyperglycemia following the portal injection of insulin. Despite the earlier work of Murlin and colleagues, many investigators felt that the hyperglycemia observed with various insulin preparations was a property of this hormone and not due to a contaminant. However, after the successful crystallization of insulin by Abel in 1926 (207), it was shown by Geiling and deLawder in 1930 (208) that crystalline insulin did not produce hyperglycemia when administered intravenously. Similar results were reported in the same year by Bürger and Kramer (209). These results indicated that a hyperglycemic factor, presumably glucagon, was responsible for the initial hyperglycemia observed with amorphous insulin preparations. Although the crystalline insulin prepared according to the method of Abel was free of glucagon, crystalline insulin prepared by the use of zinc salts, a method developed by Scott in 1934 (210), was not free of glucagon. Thus in 1939 Lundsgaard *et al.* (211) found that a preparation of crystalline insulin supplied by Scott caused glucose production in livers perfused with it, while a preparation supplied by Abel did not. Since most of the pharmaceutical houses producing insulin adopted Scott's method, glucagon was again present in many insulin preparations. The problem was reopened in 1946 by deDuve *et al.* (212). They found that a sample of American insulin contained glucagon while a Danish preparation (Novo) was free of it.

Glucagon was first partially purified and studied by Bürger and Brandt (213) in 1935. They found that it was still active under conditions which inactivated insulin, *viz.* boiling in dilute alkali or treatment with cysteine. Their studies indicated that glucagon was proteinic in nature and had many physical properties similar to insulin. The most potent glucagon preparation prepared by Bürger and Brandt, when adminis-

* Glucagon is also referred to by many investigators as the hyperglycemic glycogenolytic factor (HGF).

tered intravenously to fasted rabbits in a dosage of 20 μg. per kg. body weight, raised the blood sugar 50% above the initial level in 40 to 60 minutes. In 1949 Sutherland *et al.* (214), starting with highly purified amorphous or crystalline zinc insulin as a source, achieved a tenfold purification of glucagon. In 1953 Staub *et al.* (215) succeeded in purifying and crystallizing glucagon. Their crystalline preparation, when administered intravenously to cats at a dose of 0.1 μg. per kg. body weight, produced a significant rise in blood sugar. In a subsequent report (216) these workers showed by end group and amino acid analyses that glucagon was a distinctly different substance from insulin. Their data supported the conclusion that glucagon is a straight chain polypeptide with histidine as the N-terminal amino acid and threonine as the C-terminal amino acid.

The cellular site of formation of glucagon has been a subject of considerable controversy. The distribution of glucagon in different tissues in various animal species was first studied by Sutherland and deDuve in 1948 (217). In pigs, sheep, and cattle, the pancreas was the only organ that gave active extracts of glucagon. In dogs and rabbits significant amounts of glucagon could also be extracted from the upper two-thirds of the gastric mucosa in addition to the pancreas. Sutherland and deDuve correlated the presence of glucagon in these two species with a certain type of argentophil cell. Since the alpha cells of the pancreatic islets were also found to stain with silver, it was possible that the distribution of glucagon in the dog and rabbit was due to an embryonic dispersion of a single cell type (218). Added evidence in favor of the pancreatic alpha cells being the site of formation of glucagon was the finding of Sutherland and deDuve that pancreases in which the beta cells were destroyed by alloxan did not show diminished amounts of glucagon. Pertinent to these observations were the findings of Van Campenhout and Cornelis (219) that the alpha cells of guinea pigs could be selectively destroyed by treatment of the animals with cobalt chloride. deDuve and Vuylsteke (220) reported that the glucagon content of the pancreases of guinea pigs thus treated was reduced to one-third of its normal value. In 1952 Audy and Kerly (221) found that the glucagon content of pancreatic extracts from rabbit, rat, cat, guinea pig, and ferret were similar. They found that in *Lophius piscatorius*, a species in which the islet tissue is separated from the rest of the pancreas, active glucagon extracts were obtained from the islet but not the acinar tissue. These results strongly suggest that the alpha cells are the site of formation of glucagon.

A number of observations on both experimental and human diabetes have indicated that glucagon is a normal hormonal secretion. Young (222) noted that dogs suffering from metahypophyseal diabetes required more insulin to control their glycosuria than depancreatized dogs. Dragstedt

et al. (223) also reported that dogs with diabetes due to subtotal pancreatectomy definitely required more insulin to control their diabetes than dogs suffering from diabetes due to total pancreatectomy. Thorogood and Zimmermann (224) found that alloxan diabetes in dogs was characterized by very severe glycosuria and a high insulin requirement. Using these criteria they concluded that alloxan diabetes is considerably more severe than that which follows total pancreatectomy. Similar results have been observed by Goldner and Clark (225) in humans who have diabetes as a result of total pancreatectomy. The insulin requirement of such diabetics is usually less than in human diabetics with intact pancreases. The work of Rodriguez-Candela (226) eliminated the possible role of the exocrine secretions of the pancreas in the lower insulin requirement of depancreatized animals. He found that ligation of the pancreatic ducts of alloxan-diabetic dogs did not alter their insulin requirement but subsequent removal of the sclerosed pancreases, which were composed almost entirely of alpha cells, significantly lowered the insulin requirement. These results suggest that the alpha cells normally secrete glucagon, which leads to the more severe diabetes in those conditions where this secretion is virtually unopposed, as compared to diabetes where both glucagon and insulin are absent.

Further evidence that the pancreas secretes glucagon has been obtained from the hypoglycemic states which result when the alpha cells are selectively destroyed by chemical means. Several chemical agents will produce such results. The first of such agents to be used was decamethylene diguanidine. This compound was first synthesized in 1926 by Frank *et al.* (227) and found to lower the blood sugar in partially depancreatized animals and human diabetics. It was produced commercially for a while as a substitute for insulin under the name of Synthalin. It has since been designated as Synthalin A and the less effective duodecamethylene diguanidine derivative as Synthalin B. The lack of understanding of the mechanism by which Synthalin A produced hypoglycemia, together with its toxicity, caused it to be abandoned as a therapeutic agent in human diabetes. In 1928 Bodo and Marks (228) reported that rabbits treated with sufficiently large doses of Synthalin A first became hyperglycemic and then hypoglycemic. Animals killed in the hypoglycemic phase had negligible amounts of liver glycogen. In 1952 Davis (229) made an extensive study of the metabolic and histologic effects of Synthalin A administration in rabbits. The effects on the blood sugar reported by Davis were similar to those described by Bodo and Marks. Histologic examination of the pancreases of the treated animals revealed gross hydropic degeneration of the islet alpha cells. Similar blood sugar changes have been observed in rabbits treated with sodium diethyl dithiocar-

bamate by Kadota and Midorikawa (230). Histological examination of the pancreases of animals in which hypoglycemia had persisted for 30 or more hours showed an almost complete absence of alpha cells. Recently Von Holt *et al.* (231) have reported the destruction of the pancreatic alpha cells in rabbits by the oral administration of "*p*-aminobenzolsulfonamid-isopropylthiodiazole." Severe hypoglycemia developed in the majority of the treated animals. When this compound was administered to alloxan-diabetic rabbits the hyperglycemias were ameliorated or completely eliminated. Conversely, when alloxan was given to animals already treated with *p*-aminobenzolsulfonamid-isopropylthiodiazole, the usual hyperglycemia did not occur, and the blood sugar remained within normal limits. In extreme cases the histological examination of the pancreases of doubly treated animals revealed only exocrine tissue. These experiments strongly indicate that glucagon is a hormone which is secreted by the alpha cells. Additional evidence for such a conclusion is furnished by the study of McQuarrie *et al.* (232) on a rare type of familial hypoglycemosis. The pancreases of individuals suffering from this condition were found to be almost totally devoid of alpha cells. Finally, a condition intensively studied in this laboratory, the hereditary obese hyperglycemic syndrome of mice, which is believed to be due to a hypersecretion of glucagon and of insulin will be discussed in Section VII. The mechanism by which glucagon produces hyperglycemia was found by Sutherland and Cori to be identical with that of epinephrine, i.e., both hormones increase the amount of active phosphorylase in the liver. In contrast to epinephrine, glucagon does not appear to accelerate the breakdown of muscle glycogen (233).

Many authors have called glucagon an "anti-insulin" factor (218). If the only criterion for such a definition is absolute effect on blood glucose level, it can doubtless be considered an insulin "antagonist." A less superficial analysis must needs take into account the mode of action of insulin and the possible influence of glucagon on this action (234). It is generally agreed that the essential role of insulin is to facilitate the entry of glucose into cells. For glucagon to be considered an insulin antagonist in the true sense of the word, it must inhibit the facilitating influence of insulin on glucose transfer. Rodriguez-Candela (235) did in fact report that glucagon decreased the glucose uptake of the isolated rat diaphragm. However, Young (235) could not confirm this finding. Tyberghein (236) infused glucagon intravenously into rabbits while administering repeated large doses of insulin. The animals at the end of 6 hours showed normal glycogen; they had not excreted excessive amounts of nitrogen, so that active gluconeogenesis appears unlikely. Tyberghein concluded that glucagon did exert an anti-insulin action both in the liver and in extra-

hepatic tissues. Drury *et al.* (237, 238), using radioactive glucose, reported a decrease in glucose uptake by eviscerated nephrectomized rabbits following glucagon administration, a result somewhat difficult to interpret because of the condition of the animals.

On the other hand, Root *et al.* (239) found that addition of an equal weight of glucagon to a glucagon-free insulin preparation did not affect the maximum insulin hypoglycemia in rabbits and mice. Ingle *et al.* (240) could find no effect of glucagon on the glucose tolerance of eviscerated rats. Van Itallie *et al.* (241, 242) examined the effect of glucagon on peripheral glucose uptake in man, using the capillary-venous glucose difference (Δ-glucose) as an index of glucose assimilation (243). They compared the hyperglycemias induced by intravenous glucagon with similar degrees of elevation of blood glucose resulting from intravenous glucose and oral glucose administration in combination with a small subcutaneous dose of epinephrine. While epinephrine markedly decreased Δ-glucose values, glucagon had no such effect; if anything, it appeared to have enhanced utilization. Van Itallie *et al.* (242) also found that, while venous blood levels fall toward normal during prolonged glucagon infusion, Δ-glucose values remain appreciable, indicating continued active peripheral glucose uptake. These findings were confirmed by Elrick *et al.* (244, 245) and Cardillo and Bondy (246). It would thus appear from these recent results that the hyperglycemic effect of glucagon is due to its glycogenolytic property and cannot be considered antagonistic to insulin. The fact that total pancreatectomy in diabetic patients often decreases insulin requirements may involve such complex interrelationships that it does not constitute a proof that glucagon is diabetogenic properly speaking. Permanent hyperglycemia can be caused in rats by administration of glucagon in oil (247), but this appears due to beta cell damage. On the other hand, if glucagon does accelerate glycogen turnover (as postulated in the obese-hyperglycemic syndrome, see Section VII) the requirements for insulin may be increased.

Results of work performed in Young's laboratory in England and in Foà's laboratory in the United States suggest that growth hormone might be tropic to glucagon. Bornstein *et al.* (248) at the former laboratory reported that a substance apparently identical with glucagon was present in the pancreatic vein blood of animals treated with growth hormone. Similar conclusions were reached by Foà *et al.* (249) using a cross circulation technique in which the pancreatoduodenal vein of a donor dog was anastomosed with the femoral vein of a recipient dog. Growth hormone injected into the donor dog was followed by hyperglycemia in the recipient dog, which was interpreted as a result of the release of glucagon in the donor dog. In opposition to this view, Best and his co-workers (250) sug-

gest, on the basis of some of their recent findings, that the hyperglycemic substance in the pancreatoduodenal vein of growth hormone-treated dogs is not actually glucagon but a substance with certain adrenergic properties.

V. Neural Hyperglycemias

Hyperglycemic states can be produced experimentally by certain neural lesions or stimuli. This was first demonstrated in 1849 by Claude Bernard (251), who induced hyperglycemia and glycosuria in unanesthetized animals by puncture (piqûre) of the floor of the fourth ventricle. A similar neurogenic hyperglycemia was produced in cats by decerebration by Mellanby in 1919 (252). Donhoffer and Macleod, conducting a series of studies on the nervous control of carbohydrate metabolism, found that decerebration of rabbits under Amytal or Luminal anesthesia produced hyperglycemia provided the pons was involved. With the same anesthetic conditions obtaining, piqûre of the floor of the fourth ventricle had no effect on the blood sugar (253). They further observed that in rabbits with low initial levels of glycogen the hyperglycemias could not be accounted for solely on the basis of glycogenolysis (254). In these animals double adrenalectomy or administration of atropine and section of both vagus nerves prevented hyperglycemia from developing after decerebration. However, in rabbits with high preoperative levels of glycogen, decerebration at the pons did cause hyperglycemia (255) under the above conditions. In 1878 Boehm and Hoffman (256) observed that painful or neural stimuli caused hyperglycemia and glycosuria in cats. Cats bound in restraining holders developed such a diabetic state (*Fesselungsdiabetes*) which continued for as long as the painful situation lasted. It was further observed that stimulation of the sciatic nerves of unrestrained cats produced the same effect.

The neurogenic hyperglycemias are generally temporary. Bernard found that hyperglycemia and glycosuria produced by piqûre lasted on the average five to six hours in the rabbit, with an upper limit of 24 hours. The extreme limit in the dog with piqûre of the fourth ventricle appeared to be 48 hours (257). In *Fesselungsdiabetes* the hyperglycemia subsides soon after the removal of the painful stimuli.

The neurogenic diabetes arising from piqûre or decerebration depends upon efferent impulses originating in the damaged areas and carried by the splanchnic nerves. Part of the hyperglycemic state may be accounted for by splanchnic stimulation of the adrenal medulla and the consequent release of epinephrine. Another part of the extra blood glucose which cannot be accounted for by glycogenolysis may come from gluconeogenesis (254). In doubly adrenalectomized animals which become hyperglycemic following decerebration, direct splanchnic stimulation of the

liver "glycogenolytic mechanism" probably plays a role. The hyperglycemia of *Fesselungsdiabetes* is probably mediated through increased secretion of epinephrine (258).

By contrast with the very short duration observed in piqûre diabetes, certain hypothalamic hyperglycemias appear to last for several days or even weeks. Aschner in 1912 (83) and later Camus and Roussy (259) and others after them described a variety of disturbances of carbohydrate metabolism arising out of hypothalamic lesions. An experiment of Camus *et al.* (260) is of particular interest. Two weeks after chemical cauterization of the supraventricular nuclei in dogs, glycosuria appeared in the experimental animals and lasted on the average 5 to 10 days and in one case up to 3 months. Barris and Ingram (261) working on cats obtained hyperglycemia in 42 out of 55 positions of lesions (done with the Horseley-Clarke stereotaxic instrument). When the lesions were in the anterior hypothalamus, and particularly in the paraventricular nuclei, temporary hyperglycemia was followed by permanent hypoglycemia. Davis *et al.* (262) observed an improvement of pancreatoprive diabetes following lesions in the tubes. On the other hand, Brobeck (263), in the rat, and Mayer *et al.* (264), in the mouse, could observe no disturbance in carbohydrate metabolism following electrolytic lesions in the ventromedial hypothalamus; any change in carbohydrate metabolism appeared entirely explained by the resulting hyperphagia (see Section VII). Similar observations were recorded when gold thioglucose was used to destroy the ventromedial area in the mouse (265–267). Thus, it cannot be considered that specific hypothalamic lesions have been unequivocally proved to cause hyperglycemia. A direct action of such lesions on the pancreas is not impossible (268).

Nevertheless, it appears that at least some of the hyperglycemic effects obtained by hypothalamic lesions could be the result of nonspecific stimulation of epinephrine secretion. This secretion appears to be in part under hypothalamic control (269). A more detailed discussion of the relation of obesity *per se* to diabetes will be found in Section VII.

VI. Thyroidal and Estrogenic Hyperglycemias

Hyperthyroidism in humans is often associated with hyperglycemia, especially after meals or the ingestion of glucose. From a survey of the literature Houssay (270) estimates that there are twice as many diabetics among hyperthyroid individuals as among the population as a whole. This investigator has shown that thyroid administration to normal rats and dogs is followed by a transitory hyperglycemia and glycosuria. In the dog a permanent diabetic state was produced by daily thyroid administration after the functioning pancreatic islet tissue had been reduced.

This diabetic state is due to a lack of insulin and does not depend upon the thyroid for its persistence; hence the term metathyroid has been given to this condition by Houssay (271). Thyroid hormone causes the oral glucose tolerance curve to be higher and more prolonged than normal. This is in part referrable to the increased rate of intestinal absorption of glucose brought about by this hormone (272).

Estrogenic hormones have been reported to be hyperglycemic in the normal force-fed and partially depancreatized rat by Ingle (273) and in the partially depancreatized ferret by Dolin *et al.* (274). Although the adrenal cortex appeared to be necessary for full manifestation of the diabetogenic action of diethylstilbestrol in the rat, an increased secretory activity of the cortex was **not** essential (275). In the partially depancreatized rat and ferret androgens in massive doses were only weakly diabetogenic. Up to now the mechanism of estrogenic diabetes has not been elucidated. Furthermore, Houssay's laboratory has reported (276) that, whereas short-term administration of estrogens to 95% subtotal pancreatectomized rats exacerbated the diabetes of these animals, long-term administration of estrogens tended to ameliorate the diabetes. These two opposite effects have not been reconciled.

Both the thyroid hormone and estrogens are weakly diabetogenic in some intact animal species. For their most potent diabetogenic effects reduction of the functional endocrine mass of the pancreas is necessary. However, under these conditions one is then dealing with hyperglycemias which depend upon a lack of insulin for their persistency.

VII. Obesity and Diabetes

The clinical association of obesity and diabetes has been known for a long time. Boulin (277) noted in 1924 that the decrease in the glucose tolerance of obese patients was frequently a direct function of the duration of their obesity, a result confirmed by recent observations of Beaudoin *et al.* (278). Joslin (279) insists on the probably etiological role of obesity in diabetes. This role is exemplified by the fact that 77% of diabetics are obese at the time of onset of the disease. Furthermore, in his large series, 78.5% of male patients and 83.3% of female patients have been overweight at one time before the onset of the disease, often with some alleviation of the degree of obesity at the time of onset (279, 280). The association between overweight and diabetes is also illustrated by life insurance statistics (281, 282, 283). Actually, if "juvenile" and "adult" diabetes are separated, the association of obesity with the adult form becomes even more remarkable. Joslin (279) finds that of the diabetic patients who were detected under the age of 10, only 19% were accompanied by overweight. Of the disease which started during the 20 to

30 year period, 71% were associated with obesity. The proportion rises to 89% for the 30 to 40 year decade, with all but 5% of diabetes associated with obesity above the age of 40. It thus seems that in the adult one can speak of an obese hyperglycemic syndrome, first distinguished as a clinical entity in the twenties by Labbé (284) under the name of *diabète gras*. Juvenile diabetes can rapidly lead to diabetic coma if not controlled by insulin. It is characterized by a blood sugar level which is very sensitive to this hormone. It is relatively resistant to dietary changes. By contrast, the obese diabetes shows very different characteristics. It can be endured for a long time without untoward complications, even in the absence of any insulin treatment. Diabetic coma, if it occurs, is usually the result of an additional stress, such as an infectious disease, not directly related to the diabetes. Blood sugar levels, while much more stable than in juvenile diabetes, are far more resistant to insulin. On the other hand, diet has a very marked effect on this condition: weight loss, if practiced early enough, is often sufficient to bring glucose tolerances back to near normal values (285, 286).

The association of obesity and hyperglycemia in the adult man has been tentatively explained in several different ways. Lawrence (287, 288, 289) speaks of "lipoplethoric diabetes," a term similar in its etiologic implications to Darnaud's *diabète par encombrement adipeux*, diabetes through fatty "hindrance" or "saturation" (290). According to this concept, the very accumulation of fat prevents disposal of carbohydrates in the obese patient. The idea that fatty infiltration of the liver is responsible for the degeneration of glucose tolerance has also been advanced (291, 292). A third thesis is that the increase in the work of the pancreas due to prolonged transformation of carbohydrates into fat eventually exhausts the islets of Langerhans (293). The situation is further complicated by the fact that in man diabetes may be associated with only certain types of obesity. A classification of obesities has been attempted by French clinicians (294, 295) and summarized in another review (296). Two main types have been differentiated: android and gynoid. Android obesity is characterized by an accumulation of fat on the upper half of the body and only secondarily on the abdomen, the epiploon, and the mesentery. By contrast, gynoid obesity is characterized by concentration of the fat on the hips and the lower part of the body. This differentiation can be quantified through the calculation of an index of male differentiation (I. D. M.) involving the determination of the thickness of a number of skinfolds. The relevant result, which seems statistically valid, is that only the android type is associated with diabetes (294, 295, 297–300). On the other hand, the explanation suggested by these authors that this obese hyperglycemic syndrome is the result of pituitary-adrenal hyperfunction still lacks unequivocal demonstration.

Hyperglycemia has been found to be associated with obesity in experimental animals. The most clear-cut association is the hereditary obese hyperglycemic syndrome of mice (296, 301, 302), a mendelian recessive syndrome characterized by extreme adiposity, hyperphagia, inactivity, a low oxygen consumption in proportion to size, hyperglycemia, hypercholesterolemia, and extreme sensitivity to cold, as well as increased lipogenesis and cholesterogenesis. The blood glucose is generally elevated (303) and can readily be increased by growth hormone (304–307), by epinephrine (303, 308), cortisone (307), and glucagon (306, 308). The islets of Langerhans are hypertrophied (309) and, in spite of beta cell degranulation, the insulin content of the pancreas is increased (310). An agent found to damage pancreatic alpha cells, diethyldithiocarbamate (230), reduces the blood glucose levels and considerably increases beta cell granulation (306, 311). Other agents which act on alpha cells also cause blood sugar levels to decrease, though in less striking manner (306, 312). It has been suggested that the primary lesion in these animals is a hypersecretion of glucagon, with a secondary hypersecretion of insulin (306, 313). This hypothesis is suggested by the finding of an increased glucagon content of the pancreas under the effect of growth hormone (314), as well as by increased liver glycogen turnover (315) and a very much increased hepatic phosphorylase activity (316). It is remarkable that the littermates of genetically obese mice made obese by gold thioglucose treatment (265) or by hypothalamic lesion (264) do not exhibit hyperglycemia or sensitivity of the blood sugar level to growth hormone; they also show normal liver phosphorylase activity (316). Anderson (317) has suggested, without bringing forth much experimental evidence, that the obese hyperglycemic syndrome of adult men may be mediated through similar endocrine abnormalities.

The obesity due to the injection or grafting of ACTH-secreting tumors (318, 319) is also frequently accompanied by glycosuria. The degree of fat accumulation is much smaller than in the hereditary obese hyperglycemic syndrome; hyperglycemia is also less frequent and marked. It is due to adrenal corticoid oversecretion, which also causes increased lipogenesis and cholesterogenesis (319, 320) and increased liver glucose-6-phosphatase activity (321). Again this increase in phosphatase activity is a specific finding; it is not observed in "regulatory" obesities (where hyperphagia is the result of a primary disturbance in the central regulation of food intake) as in gold thioglucose obesity (316). A similar association of obesity and hyperglycemia can be produced in guinea pigs by adrenalcorticoid administration (322).

Although diabetes is not the direct result of hypothalamic obesity, the prolonged hyperphagia displayed by these animals predisposes to the degeneration of the islets of Langerhans, as demonstrated in the monkey

by Ranson *et al.* (323). Hypothalamic lesions also cause diabetes in partially pancreatectomized rats. The hyperglycemia and glycosuria are prevented in these animals by dietary restriction (324). This phenomenon appears similar to the diabetes induced by Allen in the partially pancreatectomized, force-fed dog (325).

It appears that in experimental animals two general types of association between obesity and diabetes can be recognized: on the one hand, a number of specific associations, best illustrated by the hereditary obese hyperglycemic syndrome and also seen in ACTH tumor-bearing mice; on the other, a nonspecific effect which in turn may manifest itself in at least two ways: (1) a "lipoplethoric" state of adipose tissues which may retard lipogenesis, and all the more so as the animal reaches weight nearer the limiting weight for obese animals of the species to which it belongs (326); (2) overstimulation and eventual exhaustion of the islets of Langerhans. The last phenomenon may be made more acute by the increased rate of glucose absorption in obese animals (327). It appears highly likely that the clinical situation is at least as complicated and also entails both specific and nonspecific associations. Such a view would permit reconciliation of the apparent divergencies of opinion expressed in the literature.

VIII. Conclusion

It is perhaps a useful coincidence that in English the word diabetes is spelled like a plural. Certainly it ought to be considered as a collective noun, representing a class of very different conditions presenting the common characteristic of high blood glucose levels. Even in this regard differences exist between hyperglycemias as regards their degree and their dependence upon the nature and the amount of the diet concerned. Some of the results of hyperglycemia as such are common to all diabetes, at least within a given species: for example, it would appear that in the dog, maintenance of high blood glucose levels for any length of time, due to whatever cause, will eventually lead to exhaustion of the islets of Langerhans. Other results of hyperglycemia, such as the effect on lipogenesis, will depend on factors other than the blood glucose levels: an alloxan-treated hyperglycemic mouse will show inhibition of lipogenesis in spite of normal or hyperphagic levels of intake; by contrast, a mouse with the obese-hyperglycemic syndrome will show rates of lipogenesis increased over the normal, even under fasting conditions. As for possible causes, the foregoing discussion has shown that secretions from almost every endocrine gland, and other factors besides, may be associated with hyperglycemia. In contrast to the vast array of humoral agents causing increases in blood sugar only one, insulin, is regularly hypoglycemic. From a teleological standpoint this contrast may well attest to the mortal

danger of hypoglycemia. This review has probably not been inclusive of all forms of hyperglycemia recognized at this date not to be primarily due to a shortage of insulin.* Almost certainly, additional forms will be observed in the future. Their identification and the elucidation of their pathogenesis should prove highly fruitful in enhancing our understanding of the normal mechanism of the regulation of carbohydrate metabolism and our ability to correct its abnormalities.

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* For example, no attempt has been made to cover in this review "hunger" or "starvation" diabetes, or the related impairment of carbohydrate tolerance following carbohydrate-free feeding. Starvation diabetes is, of course, a condition known for a long time, but its discoverer, Claude Bernard and Hofmeister after him, recognized it as a transient condition which can affect any normal subject in these special dietary circumstances. These conceptions have been abundantly confirmed (328, 329, 330). While the mechanism of starvation hyperglycemia is complex [e.g., demonstration of the decrease in the blood-sugar–lowering effect of insulin after carbohydrate deprivation (331); increase in muscle phosphorylase activity under the same conditions (332)], there can be little doubt that reduction in pancreatic insulin secretion is an essential factor (333).

Similarly, no attempt has been made to cover such structures as the thymus or pineal gland, whose function in the mammalian organism remains to be elucidated. Pinealectomy in the rabbit has been recently reported to flatten the glucose tolerance curve and decrease urinary glucose excretion (334).

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Biochemical Studies on Insect Hormones

PETER KARLSON*

Max Planck-Institut für Biochemie, Tübingen, Germany

* Authorized translation by Hans E. Gruen, Biological Laboratories, Harvard University, Cambridge, Massachusetts.

I. Introduction

Our present knowledge of insect hormones can be compared with that of vertebrate hormones available at about 1920. The presence of hormones in the insect kingdom is generally recognized; their centers of production, the endocrine glands, and their physiological effects have been studied in detail by the classical methods of hormone research, i.e. by means of extirpation and implantation of endocrine glands and effector organs (see the review by Scharrer, 1955). Biochemical work has also been initiated; the isolation of the first insect hormone in pure form was recently reported (Butenandt and Karlson, 1954) and the first data on biochemical effects of hormones on enzyme systems and on metabolism have also been presented (Williams, 1951; Schneiderman and Williams, 1954a,b). The following survey deals primarily with the biochemical investigations. Results obtained by purely biological methods are only briefly considered, chiefly because excellent reports have appeared recently (Wigglesworth, 1954a; Scharrer, 1955; Bodenstein, 1954; Williams, 1953).

Normal Control of Growth and Metamorphosis in Insects

The growth of insects takes place, as is well known, in individual, sharply delimited, molting steps, and metamorphosis is characterized by molting to the pupa (or in some cases to a nymph), and to the imago (adult). Numerous experiments in extirpation, transplantation, and parabiosis have led to the recognition that these molts are induced by hormones. Their interaction is exemplified in the accompanying scheme (Fig. 1) which, though established for the development of Lepidoptera, is in principle valid for all insect orders.

Each molt is initiated by the brain, which produces a hormone, the prothoracotropic hormone, in special neurosecretory cells. This hormone acts on a second endocrine gland, the prothoracic gland, which is stimulated to produce the molting hormone. The hormone of the prothoracic gland (called "ecdysone") acts directly on the effector organs, especially the epidermis, where it initiates those histological changes which constitute the molting process.

Whether the molt thus initiated results in a larva, pupa, or imago is determined by the activity of a third endocrine gland, the corpus allatum (mostly formed as a paired organ). The hormone of the corpora allata promotes differentiation of the larva and inhibits that of the imago. A larval molt results when this hormone acts simultaneously with the hormone of the prothoracic gland on the epidermis. If the hormone of the corpora allata is missing, the molt will be regulated by the hormone of the

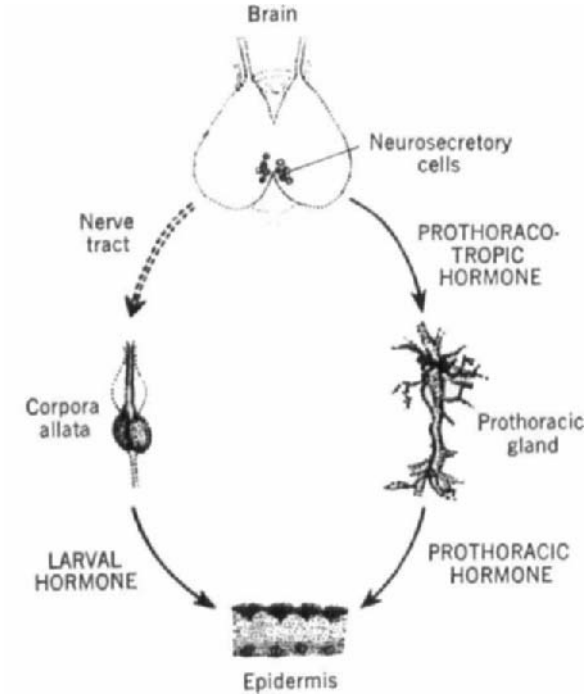


FIG. 1. Diagram of the action of hormones in the molting and pupation of Lepidoptera.

prothoracic gland in the direction of the imago; caterpillars will molt prematurely to pupae, and hemimetabolous insects to imagos or adultoid forms.

Table I shows some typical experiments which illustrate these relationships. Only the most important authors are listed, and the reader is referred to the accounts already cited. It can be seen from Table I that experimental operations often have considerable effects. It should be especially noted that holometabolous insects are forced to a nearly hemimetabolous mode of development by implantation of additional corpora allata. It follows that the holometabolous mode of development results from a special balance between hormones from the corpora allata and those from the prothoracic glands during the larval period, followed

TABLE I
THE EFFECT OF EXPERIMENTAL OPERATIONS ON INSECT DEVELOPMENT

Organ	Ligation	Extirpation	Implantation
Brain	Permanent larvae (Kühn and Piepho, 1936)	Permanent larvae (Kopec, 1922; Wigglesworth, 1934)	Pupation (imaginal molt) of permanent larvae. (Plagge, 1938; Wigglesworth, 1940)
	Permanent pupae (Williams, 1946)	Permanent pupae (Williams, 1946)	Imaginal development of permanent pupae. (Williams, 1946)
Prothoracic glands	Permanent larvae (Hachlow, 1931; Fraenkel, 1935; Wigglesworth, 1952)	Permanent larvae (Deroux-Stralla, 1948; Strich-Halbwachs, 1954)	Pupation (imaginal molt) of permanent larvae. (Fukuda, 1940; Wigglesworth, 1952)
	Permanent pupae (Williams, 1947)		Imaginal development of permanent pupae. (Williams, 1952)
Corpora allata	—	Premature pupation (imaginal molt) (Bounhiol, 1938; Fukuda, 1944; Pflugfelder, 1937)	Supernumerary larval molts. (Piepho, 1942; Pflugfelder, 1939; Wigglesworth, 1936)

by a marked change in this balance during pupation and imaginal differentiation. Hemimetabolous development, on the other hand, may be characterized by a gradual shifting of these quantitative relationships.

II. THE PROTHORACOTROPIC HORMONE OF THE BRAIN

1. Center of Production

The prothoracotropic hormone is produced by neurosecretory cells which are situated in the brain and are mostly united into groups. Such cells have been demonstrated in numerous insects, and generally much interest has centered on the phenomenon of neurosecretion, which has been well studied, particularly by B. and E. Scharrer (for review see Scharrer and Scharrer, 1954a,b; Symposium on Neurosecretion, 1954). Success was achieved in making secretions from these cells visible and in observing the migration along the nerve fibers (M. Thomsen, 1954; E. Thomsen, 1954a,b). Scharrer (1952) proved that the neurosecretion is transported into the corpora cardiaca, which were thus recognized as

storage organs for the neurosecretory substances. The experiments of Bodenstein (1953a) on the physiological function of the corpora cardiaca are in agreement with this.

2. Chemical Nature of the Neurosecretion and of the Brain Hormone

There is no doubt today that the visible and stainable neurosecretion is not the pure hormone. One is dealing probably with a high-molecular carrier material of lipid or lipoprotein character to which the active substance is bound (Scharrer and Scharrer, 1954b). In addition to storage, the corpora cardiaca probably have the function of liberating the active principle proper from the neurosecretion and releasing it into the blood stream. The prothoracotropic hormone has so far not been obtained as a cell-free extract, and nothing is known about its chemical nature.

L'Hélias (1955a) reported the finding that pteroylglutamic acid, or related compounds, constitute a part of the neurosecretion and also that the brain hormone itself possibly belongs to this class of compounds (see also page 256). Injection of folic acid or folinic acid (leucovorin) is said to shorten the intermolt period of allatectomized *Carausius* (L'Hélias, 1955b). This is no evidence for a prothoracotropic activity, however; it may be the result of a general stimulation of metabolism. In a conclusive test, viz. in brainless diapause pupae of *Platysamia*, pteroylglutamic acid is inactive. Moreover, pteroylglutamic acid antagonists do not interfere with metamorphosis when injected in intact pupae (Williams, personal communication). Therefore, the hypotheses of L'Hélias will not be discussed in detail.

3. Physiological Effects of the Brain Hormone

The effect is exhaustively characterized by the name "prothoracotropic hormone," because the brain hormone acts on the prothoracic glands and stimulates them to hormone production. All hitherto known effects of the brain hormone are brought about indirectly by way of the prothoracic glands. According to our present knowledge the tropic hormone itself has no direct effect on the epidermis or on other effector organs. Wigglesworth (1952) in particular has pointed out that the first recognizable cellular change in the epidermis, the "activation" (see page 246), is not directly induced by the brain hormone, but results from effects of the hormone of the prothoracic gland.

Two subjects which have been thoroughly studied are available for detecting the prothoracotropic hormone: *Rhodnius prolixus* (Hemiptera) and *Platysamia cecropia* (Lepidoptera). Decapitated *Rhodnius* larvae molt after brain implantation (Wigglesworth, 1940); diapausing *Platy-*

samia pupae from which the brain has been extirpated are brought to development by implantation of active (cooled) brains (Williams, 1946). The same effects should be obtainable by injection of hormone-containing extracts. Since development is induced in both cases through the prothoracic gland, which becomes active under the influence of the tropic hormone and produces ecdysone, the extract must be tested for the absence of ecdysone. This can be done on the isolated abdomen of the same object or in the *Calliphora* assay (see page 241).

In *Platysamia*, ecdysone itself seems to have a certain prothoracotropic effect (see also page 247) which may introduce errors when extracts are injected (Karlson and Williams, unpublished; compare also Williams, 1952; Karlson, 1954a). On the other hand, in *Rhodnius* there is no histological evidence that the thoracic gland is activated by injected ecdysone, and a larger dose of ecdysone is needed to induce molting in the decapitated insect (with thoracic glands) than in the isolated abdomen (Wigglesworth, personal communication).

Some insight into the mechanism of neurosecretory activity has been gained in *Platysamia*, where a diapause normally occurs in the pupal stage and imaginal development begins only after a resting period. Williams (1946) showed that stimulation by chilling is necessary in order to terminate the diapause and that after such chilling the brain is able to secrete the prothoracotropic hormone. From this time onward a higher temperature (25°) promotes hormone production.

The diapausing brain is not only endocrinologically inactive, but also electrically "silent." Microelectrodes do not record any nerve impulses in the brain; apparently general depolarization of the brain neurons takes place. This fact can be accounted for by the virtually total absence of brain cholinesterase. Indeed, the cholinergic substrate of this enzyme is present only as a trace in the inactive brain. When the pupa is exposed to low temperature, the cholinergic substrate is progressively synthesized and accumulated. This substrate, judging from assays on the heart of the mollusk *Venus*, may be acetylcholine itself. This is the only change that appears to result from chilling.

When the insect is then placed at room temperature, a prompt and apparently inductive synthesis of cholinesterase occurs. Simultaneously, the cholinergic substrate falls to an intermediate level and the brain becomes active both electrically and endocrinologically (Van der Kloot, 1954, 1955). These changes are peculiar to the brain and are not encountered in any other ganglia. It is suggested that the entire brain is "shut down" in order to prevent the neurosecretory cells from being driven to activity by any nerve impulses.

4. Concluding Remarks

The activity of the neurosecretory cells has a decisive significance in regulating normal development. This is particularly clear in cases where the molting rhythm is not fixed but depends on external factors. In the case of *Platysamia* discussed above, chilling is necessary to promote hormone production and to initiate imaginal development via the prothoracic glands. In *Bombyx* afferent stimuli induce the secretion cycles of the brain which then initiate pupation and imaginal development in rapid sequence. *Rhodnius* larvae begin their molting cycle after a full blood meal, and nervous perception of the degree of abdominal swelling determines initiation of the molt, which can be prevented by nerve sectioning (Wigglesworth, 1934). A similar mechanism could be effective in numerous butterfly larvae with obligatory feeding periods (Bounhiol, quoted by Lees, 1955). In all these cases the brain receives the nervous stimuli and then regulates development by means of hormones. The significance of the neurosecretory processes resides in this conversion of nervous to hormonal stimuli (Scharrer and Scharrer, 1954a), which may be analogous, or even related, to the release of neurohumors.

III. The Hormone of the Prothoracic Gland (Ecdysone)

1. General Survey

a. History. It is remarkable that chemical investigations of this hormone yielded noteworthy results even before the physiological significance of the prothoracic glands was recognized. Chemical experiments began with the observation of Fraenkel (1935) that puparium formation can be induced in ligated *Calliphora* larvae by blood transfusion. Continuing these experiments, Becker and Plagge (1939) developed the *Calliphora* test, prepared effective extracts, and indicated the first purification steps. The purely chemical work has been continued since 1943 by Butenandt and Karlson. At first it was intended only to isolate the substance active in the *Calliphora* test, for which initially *Calliphora* pupae and later those of the silk moth, *Bombyx mori*, were used as starting material.

Completely independently, Fukuda (1940, 1944) and Williams (1947, 1949) rediscovered the prothoracic glands and recognized their importance as endocrine organs, in the sense of the scheme in Fig. 1. Proof that the substance active in *Calliphora* is identical with the hormone of the prothoracic gland was given only after its isolation in pure form (Williams, 1954).

b. Nomenclature. The hormone of the prothoracic gland acts on the peripheral organs, especially on the epidermis, where it initiates the molting process. For this reason it has been termed the "molting hormone" (Wigglesworth, 1934, 1940). It determines pupal molt or imaginal molt in absence of the juvenile hormone, and is therefore identical with the earlier found "pupation hormone" (Kühn and Piepho, 1936) and the "imaginal differentiation hormone" (Vogt, 1943). Scharrer (1948a) introduced the term "growth and differentiation hormone" after the identity of the three hormones became clear. The name ecdysone, derived from the Greek *ecdysis* = molt, and pointing to the significance of the active substance for all molting processes, was proposed (Karlson, 1955a,b) for the pure, chemically defined, substance after isolation of the prothoracic gland hormone. This name will be used in the following when the pure compound or extracts containing this compound are involved. The old names may well be retained for biological experiments where it is not clear whether ecdysone or an as yet unknown hormone of the prothoracic gland is involved.

c. Center of Production. The glands in the various insect orders show large anatomical differences and in consequence have been given very different names. The prothoracic gland of the Lepidoptera is a paired, much branched, lobulated organ which extends along several tracheal trunks from the head into the prothorax, in part into the mesothorax. It shows very large cells, connected only loosely with one another (Fukuda, 1944; Lee, 1948). In cockroaches the glands are considerably more compact (Bodenstein, 1953a; Scharrer, 1948b); the same is true in *Rhodnius* (thoracic glands, Wigglesworth, 1952a). In some Hemimetabola they were called ventral glands or ventral head glands, because they lie ventrally at the labial segment (for details see Scharrer, 1948b; Pflugfelder, 1952; Wigglesworth, 1954a). In Diptera they are fused with the corpora allata and corpora cardiaca into the ring gland, and form the large lateral cells, or peritracheal glands (Possompès, 1953). Extirpation is possible only in exceptional cases [Deroux-Stralla, 1948; Strich-Halbwachs, 1954; the operations reported by Piepho (1948), and by Chadwick (1955) may have been incomplete].

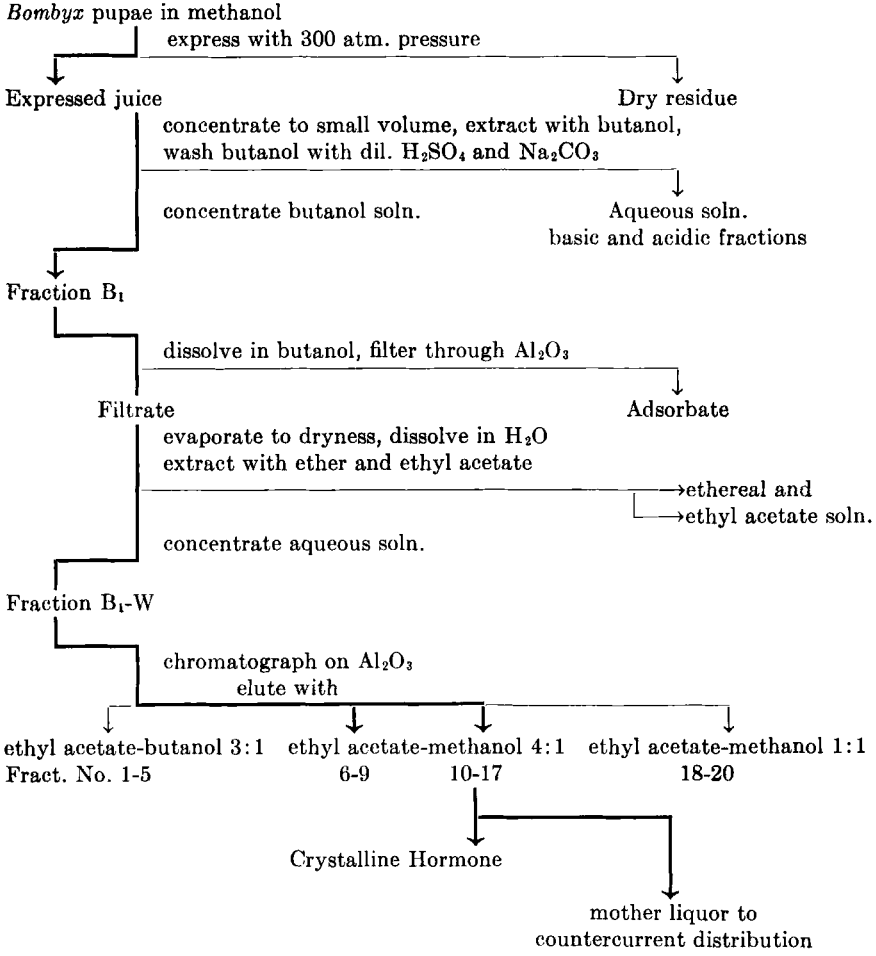
2. Concentration and Isolation of the Ecdysones

a. Preparation of Crude Extracts and of Hormone Concentrates. Methanol is the best solvent for insect material, and it can serve simultaneously as a preservative when material is collected over longer periods. Ethanol and acetone also yield active extracts. The extracts are made by first squeezing out the tissues under high pressure or by centrifugation. This is followed by evaporation under reduced pressure. Since insects contain

about 75% water, an aqueous solution results, which is extracted with butanol. The butanol solution containing the hormone is washed with dilute acid, dilute sodium carbonate, dilute acetic acid, and water and then evaporated to dryness (see Chart I).

CHART I

ISOLATION OF PROTHORACIC GLAND HORMONE



The crude concentrate is in most cases accompanied by toxic substances and is further purified by dissolving the dry residue, a red-brown viscous oil, in dry butanol, filtering through ten times its weight of alumina (activity V), and then washing with butanol. The butanol solution containing the active principle is evaporated, the dry residue taken

up in water, and the aqueous solution extracted several times, first with ether and then with ethyl acetate. The aqueous solution is evaporated to dryness, leaving behind as a yellow-brown oil a hormone concentrate which is already highly active, and if a hormone-rich starting material like *Bombyx* pupae was used, it contains one *Calliphora* unit (see page 242) in about 1 μg. Extracts from *Calliphora* pupae are mostly less active.

b. Isolation of Pure Crystalline α-Ecdysone. The procedure described so far can be carried out as a standard method without determining the activity. However, further concentration of the hormone by means of chromatography requires biological testing of the individual fractions by means of the *Calliphora* assay.

The adsorbent used is alumina of activity IV, according to Brockmann and Schodder (1941), since oxide of higher activity destroys the hormone. The eluting solvents are first ethyl acetate-butanol, and then ethyl acetate-methanol mixture. In the most favorable cases ecdysone can be crystallized directly from the highly active fractions; in other cases further purification is carried out by means of Craig countercurrent distribution in the solvent system butanol 4:cyclohexane 6:water (see below). Isolation of crystalline ecdysone succeeds only if a large amount of insect material is worked up, and if the oily concentrate contains not less than about 10% ecdysone. We obtained the first crystals by working up 500 kg. of *Bombyx* pupae. For crystallization the oil is ground with ethyl acetate and mixed with a few drops of methanol or dissolved in a little hot water and allowed to stand for some time. The pure hormone, which we call α-ecdysone, crystallizes in thin needles of melting point 235–237°. Countercurrent distribution has established that the material is a single substance (Butenandt and Karlson, 1954).

c. Detection, Separation, and Isolation of β-Ecdysone. It became clear during fractionation of the extract from *Bombyx* pupae that it must contain at least two substances active in the *Calliphora* assay. These two substances could be separated by countercurrent distribution in the above-mentioned system. Figure 2 shows the distribution diagram of a fraction rich in the second substance. While the weight curve is typical for the distribution of a rather complex mixture, the physiological activity shows two sharply defined maxima which correspond well to the theoretical curves within the limits of error of the biological assay. To judge from the curves, the partition coefficients are very different and permit a separation of the two substances with as few as 30 transfers.

The active substance was eventually obtained in crystalline and apparently pure form (Karlson, 1955b). Countercurrent distribution of the crude crystals, carried out with only 5 mg., indicates strongly that it is a single substance. We propose the name β-ecdysone for this hormone

to distinguish it from α-ecdysone, the first to be isolated. Crystallization of β-ecdysone is very much more difficult than of α-ecdysone, and the yield is less, only 2.5 mg. crude crystals and 0.33 mg. of a recrystallized, apparently pure product being obtained from 500 kg. of *Bombyx* pupae.

d. Occurrence of the Ecdysones in Different Animal Groups. Ecdysone has so far been isolated in crystalline form only from *Bombyx* pupae. It may be noted that imagos also yield active extracts but contain much less hormone (possibly only residues).

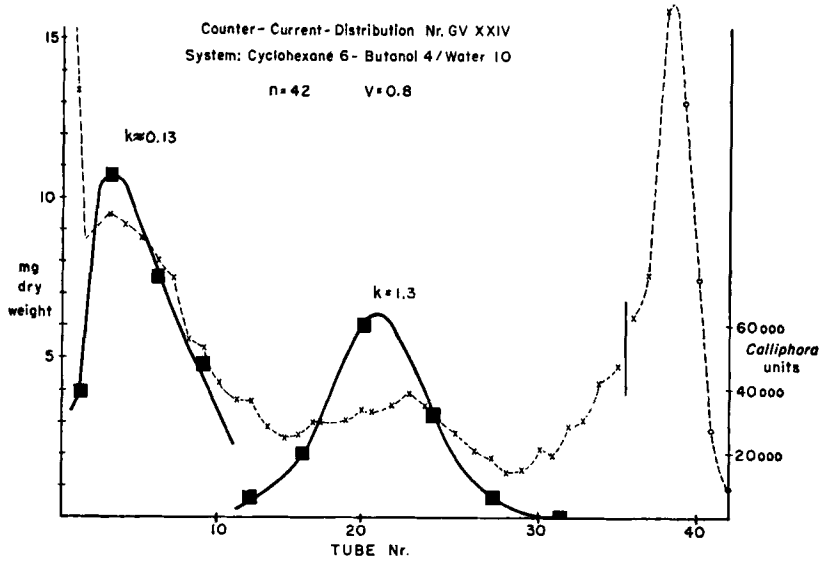


FIG. 2. Distribution diagram of a purified *Bombyx* extract showing two peaks of activity. x -------x Weight curve (left ordinate). ■———■ Distribution of activity expressed in Calliphora units per fraction (right ordinate).

We have also studied *Calliphora* pupae, the material first used by Becker for the preparation of extracts. A highly purified extract, containing according to the biological test 75 μg. ecdysone, was obtained from about 15 kg. In this concentrate α-ecdysone could be identified by paper chromatography (Karlson and Matussek, unpublished). The extracts also contained β-ecdysone as shown by the distribution of activity in the countercurrent apparatus.

Investigations by Gabe (1953), Arvy *et al.* (1954), and Echalier (1955) show that the molting cycle of Crustacea, as in insects, is induced by hormones. These investigations made it promising to test extracts of Crustacean material for ecdysone activity. An extract from shrimps, *Crangon vulgaris*, fractionated according to the above method indeed

proved active when tested on *Calliphora* larvae (Karlson, 1955a). It would be premature to conclude that the molting hormones of crayfish and insects are chemically identical, since the active principle must first be isolated in pure form, but the possibility of interchanging the hormones has been demonstrated. This finding makes it probable that ecdysone, or a related hormone, is present in all Arthropods.

3. Physical and Chemical Properties of the Ecdysones

a. Physical Constants. The available physical data for both ecdysones are assembled in Table II. Apart from the melting points, the differences

TABLE II
PHYSICAL CONSTANTS OF THE ECDYSONES

	α-Ecdysone	β-Ecdysone
Melting point	235–237°C.	177–178°C.
Ultraviolet spectrum	λ max = 244 mμ	λ max = 242 mμ
	ε = 7,100*	ε = 6,400*
Optical rotation	$[\alpha]_D^{20°} = +58.5° \pm 2°$	—
(in ethanol)	$[\alpha]_{546\ m\mu}^{21°} = +82.3° \pm 2°$	—
Distribution	in cyclohexane 6:butanol 4:water	
coefficient *K*	1.3	0.13
	in ethyl formate 9:butanol 1:water	
	0.75	0.2
Biological activity	133,000 C.U./mg.	66,000 C.U./mg.

* Calculated for molecular weight 310.

are primarily in the partition coefficients in different solvent systems. β-Ecdysone is the more soluble in the aqueous phase. On the other hand, the absorption spectra in the ultraviolet (Fig. 3) and infrared (Fig. 4) are very similar. Hence it can be presumed that the two substances are chemically related; perhaps differing only by different substitution of the basic structure.

The behavior of α-ecdysone on heating under the microscope is very characteristic. At 162–164°C. a change in crystal shape, which can be associated with partial melting, is observed. Recrystallization then takes place. Probably a loss of water of crystallization is involved. The changed crystals melt at 235–237°C. as determined under the microscope. α-Ecdysone is optically active, $[\alpha]_D = +58.5°$; it is easily soluble in methanol, ethanol, higher alcohols, and dioxane; less soluble in acetone. At room temperature the saturated aqueous solution contains about 0.45 mg./ml. as determined spectroscopically, and the solubility in ethyl acetate is probably not much greater. β-Ecdysone, on the other hand, is easily

soluble in water, and does not crystallize from aqueous solvents. It is slightly soluble in ethyl acetate and in ether.

b. Chemical data are not yet available for β-ecdysone; so far only α-ecdysone has been analyzed. The probable formula $C_{18}H_{30}O_4$ or $C_{18}H_{32}O_4$ can be deduced from the analytical data (Table III), and the

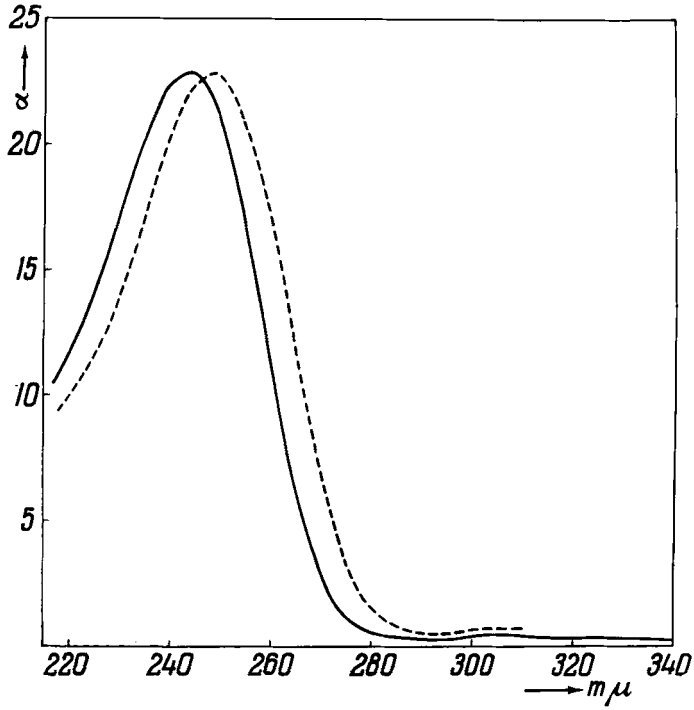


FIG. 3. Ultraviolet absorption spectrum of α-ecdysone: ——— in ethanol, ------- in water. Ordinates are absorption coefficients $\alpha = \frac{1}{c \cdot d} \cdot \log \frac{I_0}{I}$; used because of uncertainty in the molecular weight.

crystals presumably contain about 2 moles of water which are given off during drying. The spectra (Figs. 3 and 4) lead to the assumption of an α,β-unsaturated keto group as a structural element, which possibly undergoes chelate bonding to a vicinal hydroxyl group. No further details of the chemical structure can yet be given, since the amounts so far isolated are insufficient for determining the structure.

α-Ecdysone is relatively stable in air and resistant to heat; brief, dry heating to 170–180° does not cause inactivation. However, rapid loss of activity with yellowing of the solution takes place both in acid and in alkaline solution. After heating with hydrochloric acid a substance can

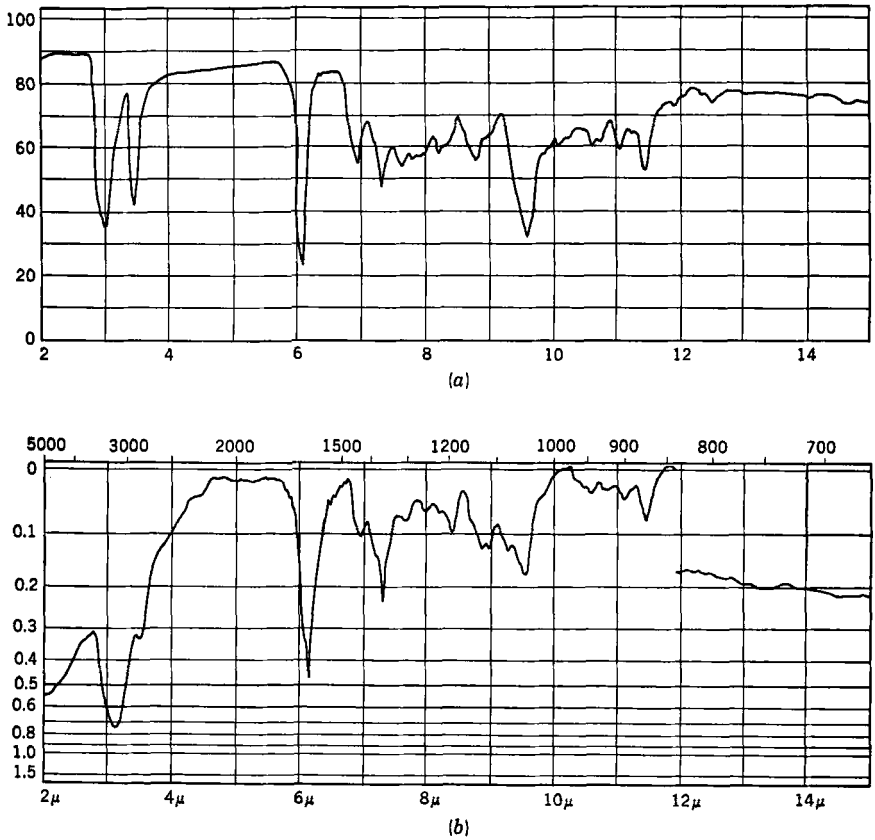


FIG. 4. Infrared absorption spectra. Samples pressed in KBr. *A*. α-Ecdysone. *B*. β-Ecdysone.

be isolated which is characterized by a second absorption maximum at 295 mμ. A rapid change in the spectrum also occurs in alkaline solution even at room temperature.

TABLE III

ANALYTICAL DATA OF α-ECDYSONE

	Experimentally determined		Calculated for	
	Sample 1	Sample 2	$C_{18}H_{30}O_4$	$C_{18}H_{32}O_4$
C	69.58	68.84	69.64	69.19
H	9.62	10.57	9.74	10.32
O	21.17	—	20.62	20.49
Mol. weight	296; 305	—	310.4	312.4

c. Detection of Ecdysones by Paper Chromatography. Experiments designed to separate ecdysone-containing extracts by paper chromatography remained unsuccessful for a long time because it was not possible to detect the ecdysone on paper. After isolation of the pure substance and determination of its properties, detection became possible by means of the photoprint method, which makes use of the strong absorption in ultraviolet at 250 mμ. In this way 10–12 μg. of ecdysone can be localized (Karlson and Matussek, unpublished). An even more sensitive test is the one with concentrated sulfuric acid reported by Reich *et al.* (1950) for 11-oxysteroids; this depends on development of a blue fluorescence which is visible with as little as 2 μg. ecdysone. The cyclohexane-butanol mixture (6:4) already used in countercurrent distribution proved useful as solvent system. The R_f value for α-ecdysone is 0.81, that for β-ecdysone 0.57. Interference is caused by kynurin (4-oxyquinoline) which often accompanies the product (Butenandt *et al.*, 1951) and which has an R_f value of 0.79. Kynurin can be separated by paper chromatography in butanol-saturated water, wherein α-ecdysone has an R_f of 0.71, and kynurin 0.50.

Even though paper chromatography is a micromethod, biological detection of ecdysone with the *Calliphora* assay is about a hundred times more sensitive. It is true, however, that paper chromatography permits separation of α- and β-ecdysone when only small amounts are present.

4. Physiological Effects of the Ecdysones; Assay Methods

a. In Diptera. Puparium formation in Diptera is induced by ecdysone produced in the ring gland. This does not involve a pupal molt proper, but a specialized molt to the prepupa. The hormone can be kept away from the abdomen by means of ligation (Fraenkel, 1935), and the abdomen of a *Calliphora* larva isolated by a ligature is a very useful object for the biological test of ecdysone activity (Becker and Plagge, 1939).

The *Calliphora* test (see Figs. 5 and 6), improved to yield a quantitative assay (Karlson and Hanser, 1952), was used during fractionation of the *Bombyx* extracts to follow the purification of ecdysone. Adult larvae of the blow-fly *Calliphora erythrocephala* serve as experimental animals. Other genera, such as *Lucilia* and *Phormia*, are unsuitable. The larvae are ligated in the anterior third of the body. Twenty-four hours later those animals are selected for the experiment which have formed a puparium in the anterior portion but not in the abdomen. The remaining ones are discarded. On the average only 10% of the ligated animals react in the desired manner, and thus many animals have to be ligated each time. The usable animals are kept a further 24 hours for observation in

order to eliminate those which react spontaneously. The anterior part is then cut off, and 0.01 ml. of the test solution are injected through the cut surface. A suitable second ligature is made to close the cut surface. Puparium formation is checked 24 hours after injection, and animals are graded according to an empirical scale as complete, marked, or slight pupation. These are assigned values of 1, 0.75, and 0.5. As the *Calliphora* unit we define that amount of hormone which, according to this scale,

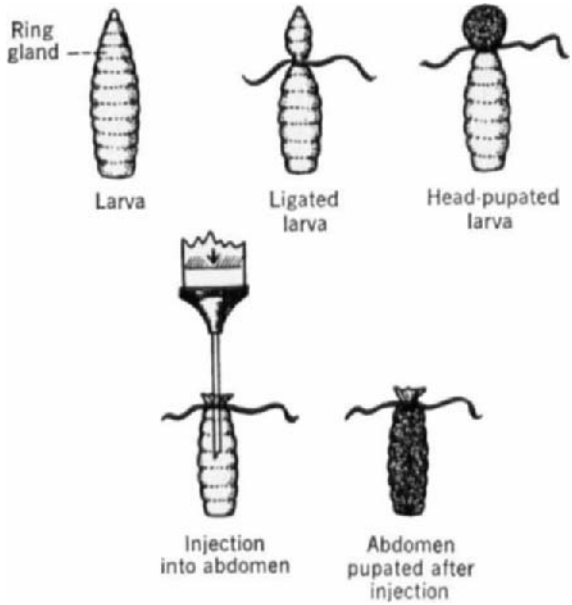


FIG. 5. Schematic representation of the Calliphora assay.

gives 50–70% pupation in a group of 15 animals.* The *Calliphora* assay is very reproducible despite this arbitrary division. The good results are probably due to the fact that selection of the animals pupated in their anterior portion guarantees a very uniform material of defined physiological age, and hence of defined sensitivity. A full dose-response curve has not yet been established, but the dose dependence is manifest from the results of several tests with crystalline, pure α-ecdysone given in Table IV.

In 2400 assays carried out since 1943 we have observed a slight systematic variation in sensitivity of the animals. This is due to a small variation in physiological age and can be taken into consideration by determining the percentage of wholly pupated animals (i.e. in both the anterior

* In our previous publication a *Calliphora* unit called for 60–80% pupation, but the definition has since been changed as indicated.

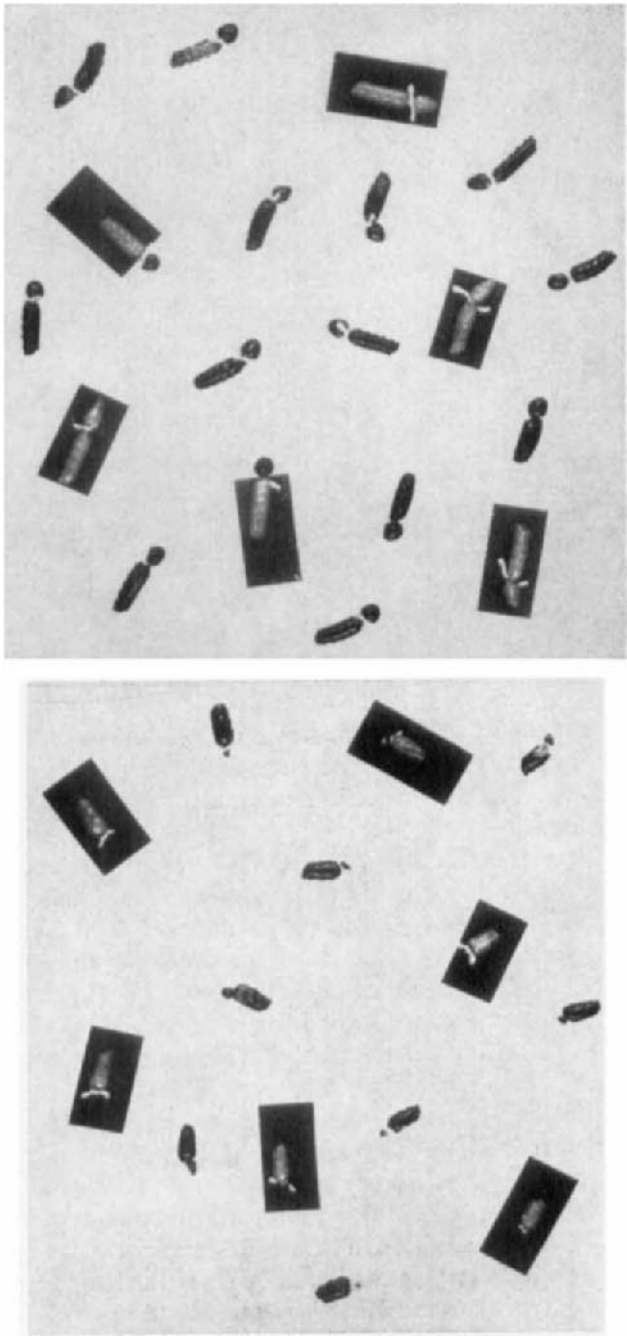


FIG. 6. *Calliphora* assay. *A*. Group of animals 24 hours after ligation. Two animals show cephalic pupation. *B*. Group of isolated abdomens 24 hours after injection of a hormone solution.

and the posterior part). We distinguished three age groups: 0–30%, 30–60%, and over 60% total pupation, which we designate as early ligation, medium ligation, and late ligation. A more precise comparison is only possible within series of experiments belonging to the same age group.

TABLE IV

BIOASSAY OF α-ECDYSONE IN LIGATED *Calliphora* LARVAE

Date of injection	Number	Dose γ	Effectiveness, %	Number pupated			Number not pupated	Number dead	Number surviving	Number of positives
				Complete	Marked	Slight				
3/25/54	2060	0.002	3.6%	—	—	1	13	—	14	0.5
3/25/54	2059	0.005	46%	4	2	2	6	1	14	6.5
3/25/54	2062	0.005	41%	5	1	—	8	—	14	5.75
4/28/54	2080	0.0075	70%	8	2	2	3	—	15	10.5
4/29/54	2089	0.0075	69%	7	1	1	3	2	12	8.25
3/25/54	2061	0.01	80%	10	1	1	2	—	14	11.25
4/28/54	2079	0.015	100%	15	—	—	—	—	15	15

The food supplied to the larvae has a definite effect on the mode of reaction. Feeding with liver proved very unfavorable, since it leads to large variations and to a decrease in readiness to react. The cause of this effect is not clear.

As can be seen from Table IV, the *Calliphora* assay is very sensitive; 0.0075 μg. α-ecdysone produces the effect of a *Calliphora* unit. β-Ecdysone is only half as effective, 0.015 μg. corresponding to a *Calliphora* unit. The high sensitivity of *Calliphora* larvae may be related to the special physiological condition of the ligated animals pupated in the anterior part. Abdomens of animals which do not form a puparium in their anterior part require 5 to 20 times the amount of hormone (Karlson and Hanser, 1953). Whether injection of ecdysone into the ligated *Calliphora* abdomen can produce imaginal differentiation as well as puparium formation has not yet been determined. Surgical removal of the ring gland has the same effect as ligation, namely that puparium formation does not take place (Burtt, 1938). Possompès (1953) experimentally confirmed this finding in *Calliphora*, and in addition showed the same endocrine relations between brain, peritracheal glands (lateral cells of the ring gland, homologous to the prothoracic glands), and corpus allatum, which have been described for Lepidoptera on page 229. Hence *Calliphora* also is thoroughly studied biologically.

Calliphora larvae with their ring glands removed can be brought to pupation by injecting ecdysone-containing extracts (Karlson and Hanser, 1953). This proved that the hormone detectable in the *Calliphora* test is actually formed in the ring gland and disproved the contrary assumption of Becker (1941).

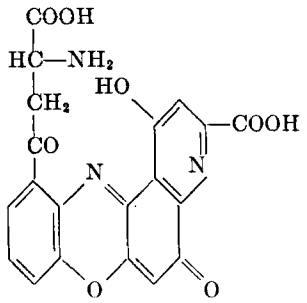
Some genetical interest attaches to experiments with the *lgl* strain of *Drosophila melanogaster* which is characterized by defective ring glands (Scharrer and Hadorn, 1938). The homozygous *lgl*-larvae pupate late or not at all. This genetic defect can be partly relieved either by implantation of normal ring glands (Hadorn, 1937) or by injection of ecdysone-containing concentrates (Karlson and Hanser, 1952). Animals thus treated form a puparium, but no further development takes place since the imaginal discs have degenerated (Hadorn, 1938). A relationship between the *lgl* gene and tumor genes in *Drosophila* was found by Burdette (1954a,b), and is probably traceable to hormone deficiency in the *lgl*-strain. From this arise interesting aspects for the cancer problem.

b. In Lepidoptera. The physiology of pupal molting in Lepidoptera has been studied mainly by Kühn (1938), Kühn and Piepho (1936, 1938), and Piepho (1942). Permanent larval abdomens can be obtained by ligature just as in *Calliphora*. Injection of ecdysone or ecdysone-containing extracts induces pupation phenomena in the isolated abdomen of *Ephestia* (Hanser and Karlson, unpublished; compare Karlson, 1954a). These phenomena, however, are limited to small epidermal regions with the doses so far used. *Ephestia* appears to be a particularly unsuitable object for these experiments, since relatively high doses are needed to obtain a visible effect.

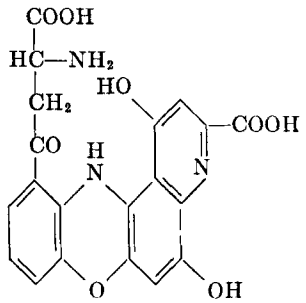
Far more interesting results were achieved with *Cerura vinula*. Caterpillars of this species undergo a characteristic color change (Bückmann, 1953). Feeding caterpillars show a dorsal red rhomboid design which is delimited from the remaining green parts by a white band (stage 0). Shortly before spinning—usually 12 days before pupation—the entire epidermis turns red within 12 hours (stage I). During the following two to four days red pigments also appear in the intestine and the fat bodies (stage II). Thereafter both the red color and the white border of the rhomboid design fade again (stage III). Pupation takes place only four to six days later (stage IV).

The red pigments of *Cerura* caterpillars belong to the ommochromes which are widely distributed in insects (Butenandt *et al.*, 1954a,b,c; Butenandt and Beckmann, 1955b; Butenandt and Neubert, 1955a). Xanthommatine (formula I) was found in the rhomboid design in stage 0. The red epidermal pigment in stage I is the dihydroxanthommatine (formula II); in addition rhodommatine, probably a metabolic product

of xanthommatine, and ommatine C are present in stage III and IV (Bückmann, Biekert, and Neubert, unpublished). These chemical transformations of the ommochromes are of interest in relation to the biochemical effects of the prothoracic gland hormone (see page 251). In ligated caterpillars the color change takes place only in the thorax, not in the abdomen, and it was therefore likely that the color change was induced by the prothoracic gland hormone, ecdysone (Bückmann, 1952, 1953). This was confirmed recently when it was found that ligated abdomens of *Cerura vinula* caterpillars change color on injection with ecdysone or ecdysone-containing extracts (Karlson and Bückmann, unpublished).



I



II

The effect is strongly dependent on the dose; small amounts (13–66 *Calliphora* units) induce the first stage of the normal color change, the formation of dihydroxanthommatine in the epidermis; a single injection of a larger dose at once induces stage II, that is, formation of pigments in the fat body and intestine; and high doses cause an atypical pupal molt.

The color change occurs normally five to six days before the known histological changes in the epidermis. These can be briefly characterized as follows: the epithelium thickens, the nuclei round up, numerous mitoses take place, and simultaneously the epidermis begins to loosen from the cuticle. This period, coinciding in *Cerura* with the end of color change, that is, with the end of stage III, can be designated as the "activation and cell-division phase" in contrast to the subsequent "secretion phase" when the new cuticle is secreted and the old one is dissolved by molting fluid from the exuvial space. The color change is evidently a particularly good indicator for the processes occurring in the preparatory phase—before cell division—under the influence of ecdysone. These are apparently induced by very small hormone concentrations.

Becker (1941) had supposed that two hormones act together in regulating the pupal molt, one presumably initiating the activation and cell-division phase, the other the secretion of the cuticle and its sclerotiza-

tion. We have discussed this question in an earlier publication (Karlson and Hanser, 1953) and at that time preferred the view that two different active substances act successively on the epidermis. However, the experiments on which Becker's hypothesis is based are inconclusive, as has been shown recently (Hanser and Karlson, 1956), and the results obtained in *Ephestia* and *Cerura* show that an assumption of two hormones is unnecessary, since it is possible to induce all hitherto known processes of pupal molting with α-ecdysone. Whether α- and β-ecdysone act together in a definite way in the normal process, and influence the individual phases differently, remains an open question. There may, of course, be differences in the physiological effects, as is the case with the hormones of the adrenal cortex. So far no experiments in this direction have been found feasible.

It is interesting that the time course of cellular activities in the epidermis of *Cerura* depends on the hormone concentration. This effect should be studied more closely, and it is to be hoped that it may contribute to a more precise analysis of the effect of ecdysone on the epidermis.

The results so far discussed concern the transformation of caterpillar to pupa. Williams was able to show in an impressive way in *Platysamia cecropia*, a species undergoing a diapause in the pupal stage, that the hormone of the prothoracic gland is also necessary for the development of the butterfly inside the pupa. Many extirpation, implantation, and parabiosis experiments demonstrated that termination of the diapause, i.e. the beginning of imaginal development, is induced by the brain through hormonal action by way of the prothoracic glands (Williams, 1952). It could be clearly proved through knowledge of these relationships that the active substance isolated by Butenandt and Karlson was identical with the hormone of the prothoracic gland, because isolated abdomens of *Platysamia cecropia*, and of the related species *Samia walkeri* (*Cynthia*), developed after injection of crystalline ecdysone (Williams, 1954). The minimum dose required to induce development is 10 μg. (weight of the animal: 2 g.). Complete development can be achieved with 20 μg. Brain-excised pupae react to hormone injection in a similar manner, though with even greater sensitivity, the minimum dose being 6.2 μg. In this case the prothoracic glands of the experimental animal are stimulated to hormone production (prothoracotropic effect, see page 232). With sufficient ecdysone, complete butterflies develop. Large overdoses lead to excessively rapid and atypical development, the new cuticle being secreted before the bristles, scales, or the primordia of the genitalia are fully developed. This agrees largely with the findings in *Cerura* which have already been discussed.

The two preparations from diapausing pupae, i.e. isolated abdomens

and brainless pupae, of *Platysamia cecropia* or *Samia walkeri* have the advantage as test objects that their mode of reaction is well known from biological experiments and gives clear results. They require, however, relatively large amounts of hormone, which is a decisive disadvantage when extensive series of experiments are planned.

c. Test on Spermatocyte Cultures. Schmidt and Williams (1953) developed a test for the prothoracic gland hormone whereby the development of explanted spermatocytes is observed. If these are cultured in the blood of resting pupae they survive without differentiating. But in the blood of developing *Cecropia* pupae there is rapid development of spermatocytes in culture. Stimulation in the latter case is ascribed to the prothoracic gland hormone which is present in the blood of developing pupae. Closer examination of the blood factor showed that it has the characteristics of a protein. It can be separated electrophoretically as a uniform band. A volatile factor is also involved (Ketchel and Williams, 1955).

In contrast to this protein, neither pure ecdysone nor ecdysone-containing concentrates have any activity in the spermatocyte test. According to our present knowledge it is very probable that the active material is a protein-bound form of ecdysone. It is conceivable that the hormone becomes bound to a specific protein when injected into the living animal but not in a tissue culture, and that protein binding is necessary for activity. An alternative explanation would be that the spermatocyte test detects another hormone present only in the developing pupa, such as a special sexual hormone. However, experience so far favors the former alternative (Williams, personal communication).

d. In Hymenoptera. Only preliminary results from Williams (1954) are available on the effects of ecdysone on Hymenoptera. The larvae of the sawfly, *Cimbex americana*, normally go through a diapause before they pupate. Injection of ecdysone removes the diapause, and the larvae molt immediately, giving rise to a larva. Hence a supernumerary larval instar is introduced, probably because there is still much corpus allatum hormone present in the hemolymph. A minimum dose cannot yet be given.

e. In Hemiptera. The physiology of larval molting and metamorphosis of hemimetabolous insects has been studied in detail by Wigglesworth in the bug *Rhodnius prolixus* (see Wigglesworth, 1952a,b; 1954a). Endocrine control by the brain, thoracic glands, and corpora allata corresponds completely to the scheme in Fig. 1. More recently Wigglesworth (1955a,b) was able to show that apparently the hemocytes are also involved in some way, but their role is not yet clarified, and is probably not in the main line of endocrine events.

It is probable that the thoracic glands of *Rhodnius* produce the same

hormone as those of the Lepidoptera. At any rate pure crystalline ecdysone is active in *Rhodnius,* provoking a molt in the isolated abdomen which becomes an imaginal molt because the corpora allata are missing. About 0.5 to 0.75 μg. are required for a fourth stage *Rhodnius* larva (Wigglesworth, 1955a,b). β-Ecdysone is also active (Wigglesworth, unpublished). This result is important, since it shows that the same hormones are effective in hemimetabolous insects as in holometabolous butterflies and flies, despite their different mode of development.

f. Concluding Remarks. Table V summarizes the effects of ecdysone as known to date, arranged according to species. Even though our knowledge shows many gaps, it becomes clear that the hormone has many functions in postembryonic development and especially participates decisively in the regulation of molting and differentiation processes. The possibility cannot be excluded that the "thoracic differentiation center" demonstrated in embryonic development acts by mediation of the same hormone.

It can further be seen from Table V that the principle of species nonspecificity of hormones is as valid in the insect kingdom, probably even in the Arthropods as a whole, as in the vertebrates. This conclusion is also justified for orders not yet examined, because, on the one hand, biological experiments not mentioned here have shown a similar system of endocrine glands in all insects, and, on the other hand, substances with ecdysone-like activities occur even in Crustacea (see Section III, 2d).

Calliphora is most suitable for the bioassay of ecdysone, at any rate as far as routine control of the chemical purification is concerned. The assay is rapid, simple to carry out, reliable, and very sensitive. For exact demonstration of the activity of prothoracic gland hormone the isolated abdomen of *Platysamia* or *Rhodnius* is preferable, because these objects have been more thoroughly examined biologically. The other effects merit special biological examination but are unsuitable as assay methods.

5. Biochemical Effects of the Prothoracic Gland Hormone

It should be made clear that the changes in enzyme content to be discussed below were established in animals in defined stages of normal development. Since complicated systems embracing living tissues are nearly always involved, enzyme-chemical data can be correlated with the hormone as inducing factor only indirectly, by using our knowledge of normal development. Only the most important findings are considered, and no complete literature coverage is attempted.

a. In Diptera. Browning and sclerotization of the cuticle are the basis of the above-mentioned *Calliphora* test. According to the work of Fraenkel and Rudall (1940, 1947), Dennell (1947), Pryor (1940), Pryor *et al.*

TABLE V

THE ACTION OF α-ECDYSONE IN VARIOUS INSECTS

Order	Species	Preparation	Type of action	Dose necessary	
				In Calliphora units	In μg. crystalline α-ecdysone
Diptera	*Calliphora erythrocephala*	Ligated larval abdomen	Puparium formation	1	0.0075
	Drosophila melanogaster	*lgl* larvae (defective ring gland)	Puparium formation	3	(0.0225)*
Lepidoptera	*Ephestia kühniella*	Ligated larval abdomen	Pupation	>50	>0.375
	Cerura vinula	Ligated larval abdomen	Color change	13–66	0.1–0.5
	Platysamia cecropia	Brainless diapause pupae	Initiation of development	825–1330	6.2–10
	Samia walkeri	Isolated abdomen of diapause pupae	Initiation of development	1330	10
Hymenoptera	*Cimbex americana*	Diapause larvae	Initiation of development	<660	<5
Hemiptera	*Rhodnius prolixus*	Decapitated larvae	Molting	66–100	0.5–0.75

* Value calculated from *Calliphora* units; experiments performed with purified amorphous hormone concen trate.

(1946, 1947), Hackman *et al.* (1948), Hackman (1953), and Mason (1955), among others, this consists in tanning of cuticle proteins by quinones resulting from tyrosine metabolism. Tyrosine yields dopaquinone and indolequinone through tyrosinase activity, according to Raper's scheme. Both substances could react with proteins either directly or after further degradation.

The central problem in Diptera, and probably also in other orders, is the following: tyrosine, tyrosinase, and oxygen are present in the adult larva. Nevertheless there is no reaction at first, but only at the time of pupation, i.e. probably through the intervention of ecdysone. Why?

Analysis of this problem has proved very difficult. The simplest explanation would be that ecdysone activates tyrosinase. However, this has been tested experimentally (Karlson and Schmid, 1955) with the result that ecdysone has no effect on the *in vitro* activity of tyrosinase.

Continuing earlier experiments of Dennell (1949), we then studied the activity of several enzymes during the last larval stage, and during pupation, as far as is possible with the method of selective poisoning. It was found that tyrosinase activity and cytochrome respiration change in opposite directions during this period. Thence was derived a working hypothesis according to which both enzyme systems are interrelated through dopaquinone (Karlson and Wecker, 1955). Proof of this hypothesis is not yet available and awaits further experimentation. According to our hypothesis the role of the hormone consists in influencing the cytochrome oxidase. Decreased activity of this enzyme during pupation has also been observed in other objects (in *Drosophila* by Bodenstein and Sacktor, 1952; in *Popilia japonica* by Ludwig, 1953; in *Platysamia* by Schneiderman and Williams, 1954a,b).

Another possible explanation of the behavior of tyrosinase which merits serious discussion derives from the finding of Horowitz and Fling (1955) that tyrosinase in *Drosophila* is present in an inactive condition, and is activated through an autocatalytic process. A precise analysis of this process is still lacking, especially with regard to the participation of ecdysone.

b. In Lepidoptera. Recently Faulkner (1955) found an enzyme in *Bombyx* caterpillars which dehydrogenates malate and transfers hydrogen to triphosphopyridine nucleotide. Changes with age of the caterpillars, in line with the above-mentioned decrease in cytochrome oxidase, were observed. The appearance of reduced quinone pigments (ommochromes) in *Cerura* also indicates a change in redox potentials.

The clearest results concerning the influence of the prothoracic gland hormone on respiratory enzymes were obtained in *Platysamia* (Williams, 1951; Schneiderman and Williams, 1954a,b) by comparing the metab-

olism of diapausing pupae with that of pupae which have begun imaginal development under the influence of ecdysone produced by the prothoracic gland.

The diapausing pupa is remarkably insensitive to different respiratory poisons, especially cyanide, carbon monoxide, pilocarpine, and diphtheria toxin (Williams, 1951; Pappenheimer and Williams, 1952). These facts permit the conclusion that respiration of the diapausing pupa is not mediated by the easily inhibited cytochrome-cytochrome oxidase system. However, this does not apply to the metabolism of muscles, which is catalyzed also in these animals by cytochrome $a + a_3$. In the other tissues, cytochrome b_5 has been identified as the enzyme mediating the cyanide-insensitive respiration of diapausing pupae (Shappirio and Williams, 1953; Pappenheimer and Williams, 1953, 1954). Cytochrome b_5 can not only reduce cytochrome c and thus establish a shunt bypassing the Slater factor, but can also react with oxygen and thus act as terminal oxidase (Chance and Pappenheimer, 1954; Keilin and Hartree, 1955); the enzyme presumably has this latter function in the diapausing pupa.

The breakdown of the cytochrome system ensues very rapidly after the pupal molt; within five to eight hours the cytochrome b and c content of the nonmuscular tissues has decreased by 50%, and within 24 hours these cytochromes can no longer be detected. The cytochrome-cytochrome oxidase system is thus blocked by the complete absence of the components b and c, though small amounts of cytochrome oxidase continue to be present (Shappirio, 1954).

If pupae with facultative diapause, just ready to begin development on their own, are prevented from developing by brain extirpation, then the metabolism rapidly adapts to the low level characteristic for diapause (Williams, 1952).

Completely different conditions exist in the developing pupa. Here cytochrome oxidase is the most important respiratory enzyme; cyanide is deadly, and the other respiratory poisons are strongly inhibitory. The respiratory system corresponds to the sequence found also in other animals: cytochrome $b \rightarrow c \rightarrow a \rightarrow a_3 \rightarrow O_2$. This metabolism is absolutely necessary for morphogenesis, just as it is for growth. Blockage of the cytochrome system, e.g. by carbon monoxide, leads to an artificial diapause, as a result of which the prothoracic gland hormone cannot exert its morphogenetic activity. Elaboration of the complete cytochrome-cytochrome oxidase system, obviously through *de novo* synthesis of the b and c components, constitutes one of the most important accomplishments of the tissues in the first week of development.

However, new formation of the cytochrome system is apparently not the first effect of ecdysone, but an indirect one. As far as the causal rela-

tion between morphogenesis and metabolism is concerned, there is no doubt that the degree of morphogenesis determines the metabolism and not the reverse. This can be very nicely demonstrated in wound-healing processes, which cause a marked and long-lasting increase in respiration; the total respiration is not less than during the first days of development (Schneiderman and Williams, 1953a,b). Nevertheless there are no signs of even the beginning of differentiation. Lees (1955) pointed out that the intracellular enzyme system is adapted to whatever the requirements of the organisms are, and that there are also insects whose diapause is not characterized by the absence of cytochrome oxidase. It is apparent that the hormone plays a significant role in regulating the enzyme system, but the details of this process remain to be clarified.

IV. The Hormones of the Corpora Allata and Corpora Cardiaca

The corpora allata and corpora cardiaca are accessory glands of the brain. They are paired in most insects but can be fused to form unpaired organs. Their anatomy and histology have been thoroughly studied (Cazal, 1948; see also Pflugfelder, 1952 for further literature). The corpora allata are under nervous, possibly even neurosecretory, control of the brain (E. Thomsen, 1952).

1. The Juvenile Hormone

The most important function of the corpora allata in developmental physiology consists in secreting a hormone which guarantees the persistence of larval characters in larval molting. For this reason it has been designated the juvenile hormone (Wigglesworth, 1940), larval hormone (Karlson and Hanser, 1953), status-quo hormone (Williams, 1953), or inhibition hormone (Wigglesworth, 1934; Piepho, 1942; this designation is unfortunate). Recently Wigglesworth (1954a) proposed the name "neotenin."

Extirpation and transplantation experiments yielded the first important evidence of the role of the corpora allata. After their removal from butterfly caterpillars the next molt is already a pupal molt (Bounhiol, 1938). In *Carausius* (Pflugfelder, 1937) and *Leucophaea* (Scharrer, 1946a) an adultoid terminal stage is reached through two molts. On the other hand, implantation of corpora allata causes supernumerary larval molts in nearly all insects.

Individuals showing both larval and pupal, or imaginal, characteristics after molting have frequently been observed in implantation experiments (Piepho, 1942, 1950b; Wigglesworth, 1940, 1954a; Radtke, 1942; Williams, 1953). The type of differentiation shown by the epidermis at molting is determined by the relative quantities of juvenile hormone and

of ecdysone acting on the tissue. This can also be demonstrated by skin implants (Piepho, 1938, 1939; Piepho and Meyer, 1951; Wiedbrauck-Meyer, 1953).

The clearest experimental proof for the interaction of ecdysone and juvenile hormone has been given by C. M. Williams (unpublished). Two pairs of corpora allata were implanted in an isolated abdomen of a *Cecropia* pupa and 25 μg. ecdysone were injected. The preparation molted to a pupa; the molt was induced by ecdysone but modified into the juvenile direction by the corpora allata.

The corpus allatum hormone is not order-specific, as can be demonstrated by transplantations (Piepho, 1950a; Wigglesworth, 1954b). It has not yet been studied biochemically. No suitable test has as yet been worked out, nor has the hormone been successfully extracted. Thus the prerequisites for isolation work by biochemists remain to be provided. Biological effects of neotenin sufficiently well studied to merit consideration for a testing method are: the local formation of larval cuticles in the imaginal molt of *Rhodnius* (Wigglesworth, 1940) or in the pupal molt of *Galleria* (Piepho, 1942), or the formation of neotenic forms in *Carausius* (Pflugfelder, 1952) or *Locusta* (Joly, 1952). Implantation of killed glands has so far been unsuccessful (personal communications of Piepho and Joly), probably because little hormone is stored in the corpora allata. It is not improbable that all physiological effects so far observed, especially the channeling of a molt in the larval or pupal direction, are only obtainable with comparatively large amounts of hormone. Positive results may be more easily achieved by implantation of living glands, which produce hormone continuously, than with a single injection.

A characteristic effect of implanted corpora allata on the instinctive behavior in cocoon construction by *Galleria* caterpillars has been described by Piepho (1950c). Different stages in the social life of honey bees are accompanied by changes in size of the corpora allata (Pflugfelder, 1952). A similar phenomenon is found in termites (Kaiser, 1955).

2. The Yolk-Formation Hormone (Gonadotropic Hormone) and Its Effect on Metabolism

In many orders the corpora allata are necessary for normal maintenance of sexual functions in the female animal. After allatectomy egg maturation ceases (Wigglesworth, 1936; Scharrer, 1946b; E. Thomsen, 1952; see Wigglesworth, 1954a and Pflugfelder, 1952 for literature); it can be restored by implantation. Hence one can conclude that the internal secretion of the corpora allata acts on the gonads. It is debatable whether this hormone is identical with the juvenile hormone.

Although allatectomy interferes with egg maturation in *Calliphora* imagos (E. Thomsen, 1952), it has no effect when carried out on *Calliphora* larvae; the females developing from these larvae produce normal eggs (Possompès, 1955).

Altmann (1950, 1952; see also Koller, 1954) found that injection of corpora allata extracts leads to the development of ovaries in bees. The worker bees are, as is well known, females with suppressed sexual activity, but under certain conditions they begin egg development and oviposition. According to Altmann this condition of drone production can be induced by injection. Extracts from bee heads and also from ovaries, as well as the "royal jelly," the food of queen larvae, are effective. The active principle, possibly a protein, is reported to be water soluble, thermolabile, and of high molecular weight.

Drone production in bees is a very complex phenomenon because adaptation to the social life plays a part, and because the "queen substance" (see page 257) intervenes. It could be hoped, nevertheless, that a sensitive test object for the corpora allata hormone was on hand, just because of the lability of the bee workers. We have therefore reexamined these results, but we found no activity in "Royal jelly" or in extracts from heads and ovaries. Moreover, in some of our control series different stages of drone production also occurred (Hanser and Karlson, unpublished). It remains to be seen under which special conditions the results of Altmann are reproducible.

Several authors (see Scharrer, 1955 for literature) have expressed the opinion that yolk formation is dependent on a general stimulation of the metabolism. After allatectomy, disturbances can be observed, especially in fat metabolism; the fat bodies hypertrophy and store abnormally large amounts of reserve materials which normally serve for yolk production. Whether this is cause or effect of the absence of egg production cannot yet be decided.

3. Color Change Hormones

In some insects, such as *Locusta migratoria* (Joly, 1952), a color change can be observed which is hormonally controlled by the corpora allata. In this species color and behavior are coupled: the animals in the migration phase are yellow-brown and live together in swarms; in the solitary-living phase they are green. This green color can be obtained in the variety *migratoides* by implantation of corpora allata. However, this effect was observed only when implantation was carried out shortly before molting. Animals provided with implants at a longer period before molting show the effect of the juvenile hormone, but no color change. An explanation of this experiment must remain hypothetical. According to

Joly (private communication) the color change could be used as biological test for the corpus allatum hormone.

In *Carausius*, the color change is predominantly under the hormonal control of the brain and the corpora cardiaca (Dupont-Raabe, 1954; L'Hélias, 1955a). The animals acquire a silver-grey color after extirpation of the corpora cardiaca. Injection of extracts of corpora cardiaca, or of the brain, leads to melanization. Extracts of corpora allata are only weakly active.

According to L'Hélias, pteroylglutamic acid and related compounds are present in the brain and corpora cardiaca; they can be detected in histological preparations after ultraviolet irradiation, or in extracts of these organs by means of paper electrophoresis, paper chromatography, and the *Lactobacillus casei* test for pteroylglutamic acid. The activity of the extracts is ascribed to these compounds or their metabolites, namely xanthopterine and a pteridine-carboxylic acid (L'Hélias, 1955b). On injection of synthetic pteroylglutamic acid, or of pterines, into *Carausius* individuals without corpora cardiaca, the animals are reported to darken just as on injection of extracts. This could not be confirmed by Dupont-Raabe (1955), who pointed out that the test is not valid, and by De Lerma *et al.* (1955). Moreover, pterines are found widely distributed in insects, and it seems difficult to accept the view that they have hormonal functions.

The chromatophore-active substance of the insect head (Hanström, 1940), which causes contraction of the pigment cells in shrimps, such as for example *Crangon* or *Leander*, is apparently not a folic acid derivative. It is a base existing in part free, in part bound to a protein (Carlisle *et al.*, 1955), and is probably identical with the amine present in Crustacea, the concentration of which has already proceeded very far (Fänge and Östlund, 1955). The substance from shrimp eyestalks, when purified by column chromatography and paper electrophoresis, is active at 0.01 μg. per shrimp. The role of this material in insects is unknown.

4. Myotropic Effects of the Corpora Allata and Corpora Cardiaca

Extracts from corpora allata and corpora cardiaca have a myotropic effect, increasing the frequency of movements of the intestine, the Malpighian tubes, and the heart. A first chemical characterization of the active substance has been given by Cameron (1953). It has the property of an *o*-diphenol but isolation in pure form has not yet been carried out. Altmann (1953; see also Koller, 1954) observed in addition an increase in reabsorption of dyes in the Malpighian tubes, and an effect on the water balance of bees. The methods used have been criticized (Schreiner, 1955). According to Altmann the substance is heat stable and has a low

molecular weight, hence cannot be identical with the yolk-formation hormone.

5. Concluding Remarks

The endocrinology of the corpora allata and corpora cardiaca is far from being clearly understood. Numerous hormonal effects of these organs have been described, and it should also be recalled that the corpora cardiaca function as storage organs for the prothoracotropic hormone of the brain. It remains an open question whether the other hormones of color change and metabolism, supposedly present in the corpora cardiaca, are also products of neurosecretory cells.

The effect on developmental physiology is the one most thoroughly studied among the activities of the corpora allata. It has been discovered in all insect orders examined. There can be no doubt concerning the existence of the juvenile hormone, even though no hormonally active extract has yet been prepared.

The second effect of the corpora allata, the gonadotropic effect, has not been observed with the same regularity. It is absent in Lepidoptera. In Diptera the situation is not clear, and experiments of Possompès (1955) conflict with those of E. Thomsen (1952). The important question whether the gonadotropic effect is due to the juvenile hormone or to an independent hormone can only be answered by biochemical research.

The same is true for the various hormonal effects ascribed to the corpora cardiaca and the neurosecretory cells, i.e. the chromatotropic, myotropic, and metabolic effects. These last have been discussed by Bodenstein (1953b). It should be made clear, however, that these activities are not related to the prothoracotropic hormone of the brain.

V. Ectohormones

The rather unfortunate term "ectohormone" comprises those active substances which are given off externally by the organism and affect other individuals of the same species. The author considers that these substances do not fall into the category of hormones, and that it would be better to create a neutral name for them. The substances involved serve in the first place as social substances of correlation between individuals of colony-building insects, and in the second place as sexual attractants in the pairing of males and females of numerous butterfly species.

1. The Queen Substance of Bees

It has long been known that the worker bees of a society always know whether or not the queen is present, and behave accordingly. If the queen is lacking, brood cells of special shape are built, and the workers begin egg development and oviposition (drone production). Butler (1954)

found that communication between bees takes place by means of a specific substance produced by the queen and distributed by the workers to one another. The substance is present on the cuticle of the abdomen and of the thorax, and is taken up orally. Distribution probably takes place with the food, and it is remarkable how rapidly many thousands of bees can be informed by this method.

In addition to the construction of queen cells, the development of ovaries in colonies lacking a queen is suitable as test method. The substance under consideration suppresses egg development (De Groot and Voogd, 1954). Voogd (1955) obtained the substance in the form of extracts. The active principle is soluble in alcohol and acetone and behaves like a fatty acid.

Pain (1955) describes an attractant present on the cuticle of the queen bee, which may be identical with Butler's "queen substance." It can be detected by the fact that worker bees search out and lick a piece of elder pith containing this substance. The substance is soluble in ether and chloroform. No weighable amounts have yet been obtained. The absorption spectrum of the solution shows a maximum at 240 mμ which was not present in a control extract obtained from worker bees. From this observation the conclusion has been drawn (though it may not be justified) that this maximum belongs to the active substance. The assignment of this substance to the steroids or waxes (Pain, 1955), or the fatty acids (Voogd, 1955), is based only on the solubility properties, and the chemical nature of the substance must still be regarded as unclear.

2. The Social Substance of Termites

The facts suggesting the presence of a social substance in termites resemble the findings discussed for bees. Instead of brood cell construction and drone production, supplementary reproductives are produced here as soon as the king or queen is removed from the colony (Grassé and Noirot, 1946). Supplementary reproductives originate from larvae or nymphs by one molt which is induced by the absence of functional reproductives and differs from a normal molt. The molt giving rise to supplementary reproductives can be determined within 24 hours (Lüscher, 1952a,b).

Functional reproductives must produce an inhibitory stimulus because no molting to supplementary reproductives takes place when the former are present in a colony. The material nature of this stimulus was demonstrated by various experiments. The substance is secreted by the abdomen, possibly through the intestine, and is taken up orally by the larvae (Lüscher, 1955). Light (1944) showed that feeding with reproductives or extracts from such animals inhibits production of supplementary re-

productives. These extracts probably contained the so-called "social hormone."

The action of this substance must almost certainly be accomplished by way of the endocrine glands which control molting. According to Lüscher there is probably an influence on the neurosecretory cells which leads to a shift in the quantitative relationship between ecdysone and juvenile hormone. (See also Kaiser, 1955 for a hypothesis concerning the origin of castes in termites.)

3. The Sexual Attractants of Lepidoptera

In numerous butterfly species mutual detection of the sexes and the mating behavior is facilitated, or made possible, by specifically active odors (for review see Götz, 1951). These substances are active in very small amounts and can be included among the ectohormones even though —contrary to the other known hormones—they are species-specific, or at least genus-specific. In particular the attractant of the silkmoth, *Bombyx mori*, has been studied chemically in detail (Butenandt, 1939; Butenandt and Hecker, unpublished). *Bombyx* is particularly suitable as laboratory animal. The attractant is produced by the female in special scent glands (sacculi laterales), from which it can be extracted with petroleum ether. It is very volatile, a fact to be kept in mind during chemical investigations. Even during concentration of ether solutions appreciable amounts can pass over into the distillate. *Bombyx* males characteristically respond to the stimulus of this substance by performing a whirling dance. This behavior is the basis of the biological test which is carried out in the following manner: the tip of a glass rod is dipped into a solution of the substance and brought within a few centimeters of the antennae of the male. A positive reaction can be recognized by the beating of wings of the animals. One "attractant unit" is that amount of substance which, when dissolved in 1 ml. of solvent (low-boiling petroleum ether), will give a positive reaction in at least 10 out of 20 animals. The test is not entirely satisfactory in a quantitative sense since only differences in concentration of 1:10 can be detected with certainty, but it is usable for controlling chemical fractionation and purification.

Chemical purification begins with the extraction of severed abdominal tips which contain the sacculi laterales. Petroleum ether is used as solvent. The sexual attractant is found in the unsaponifiable portion of the lipid extract. Esterification demonstrated that it is an alcohol. The esters are inactive in the biological test, and the active principle can be recovered after saponification. The alcohol fraction, containing one attractant unit in 0.01 μg., was esterified with *p*-nitroazobenzenecarboxylic acid, and the

resultant ester mixture separated by chromatography and countercurrent distribution. The individual fractions were tested biologically after saponification (Butenandt and Hecker, unpublished). A 100-fold enrichment was achieved in this way. A chromatographically uniform ester was obtained recently by means of repeated counter-current distribution, partition chromatography, and chromatography on Al_2O_3. This represents the first preparation of a derivative of a pure sexual attractant (Butenandt, 1955). The amount which can be isolated is extremely small; only 5.3 mg. of the pure ester were obtained from 313,000 scent glands. The small yield is associated with a high activity; the alcohol obtained by saponification contains one attractant unit in less than 10^{-5} μg.

Perception of the material takes place through the antennae. Schneider (1955) has demonstrated action potentials in the antennae which indicate the perception. Schwinck (1954) presents an analysis of the biological mode of action. It acts as a stimulating material which indicates only proximity of the female. The direction of movement of the *Bombyx* male is essentially determined by the air current, in the sense of an orientation against the direction of the current. This should apply also to free-flying butterflies.

The use of an air current in combination with the electrophysiological demonstration of action potentials constitutes an assay method of 1000-fold increased sensitivity (Schneider and Hecker, 1956).

In comparison with the *Bombyx* attractant the odors of other butterfly species are chemically only partially determined. Experiments with several cotton pests (Flaschenträger and Amin, 1950) showed that these species also produce volatile substances which are in part alcohols, in part carbonyl compounds (aldehydes). The attractant of the Gypsy moth has also been obtained as extract and was concentrated by chemical methods (Acree, 1953).

Another substance which will be mentioned here is found in the tropical water bug *Belostoma indica* and only in one of the two sexes. This is a water-clear secretion produced in tubular organs, having a characteristic cinnamon-like odor. It is used as a spice in Southeast Asia. Up to 0.02 ml. of this liquid can be obtained from one animal. The chemical structure of the odoriferous compound was very recently found to be Δ^2-hexenol-1-acetate, $CH_3—CH_2—CH_2—CH{=}CH—CH_2O—CO—CH_3$, as could be demonstrated by degradation and synthesis (Butenandt and Tam, 1955). The biological significance of this substance has not yet been studied, but presumably its function is similar to that of the sexual attractant in Lepidoptera. It is remarkable that its chemical structure has a recognizable relationship to the probable structure of the butterfly attractant (Butenandt, 1955).

4. Concluding Remarks

The work discussed in the preceding section shows that biochemical research on the substances classified here as ectohormones has progressed rapidly. On further development of biological testing methods additional substances in this category will probably be isolated in pure form. The work on *Bombyx* shows, however, that large quantities of insect material will be required, and for that reason great difficulties will be experienced, particularly in connection with research on the social hormones of termites.

VI. The So-Called Gene Hormones

Eye color mutants known in *Drosophila* (Beadle and Ephrussi, 1935) and in *Ephestia* (Kühn, 1932; Caspari, 1933) have been thoroughly studied biochemically. It appeared that the effect of the wild alleles v^+ and cn^+ (*Drosophila*), or a^+ (*Ephestia*), can be replaced in the mutants by implantation of wild-type tissue, or by injection of extracts. (For review see Caspari, 1949; Karlson, 1954b.) Because of the possibility of humoral transfer, these substances have been called "gene hormones." We know today that no hormones are involved, but only the precursors of the ommochrome pigments, namely kynurenine and hydroxykynurenine (Butenandt *et al.*, 1943, 1949). The term gene hormone should therefore be eliminated from the literature, at any rate as far as processes of pigment formation are concerned. Their discussion falls outside the topic of this communication. It will be only mentioned that the chemical structure of some of the eye pigments has been elucidated. They are phenoxazones resulting from the oxidation and condensation of two molecules of hydroxykynurenine (Butenandt *et al.*, 1954a,b,c). One of them, xanthommatine, is also present in *Cerura* (see formula on page 246).

VII. General Considerations and Future Problems

A comparison of what is known at present about insect endocrinology with the endocrinology of vertebrates brings to light some similarities but also some differences. In both groups of animals we find a highly developed system of endocrine glands, and the principle of a dominant hormone, as we know it from the hypophysis of vertebrates, has its counterpart in the neurosecretory cells of insects.

The great importance of insect hormones for morphogenesis is striking. Developmental physiology in insects is particularly closely interrelated with hormones. Our knowledge of the hormonal regulation of development is more extensive than that of other endocrine effects, because the former was the first to be discovered and has been studied

more thoroughly. It is only in the area of morphogenesis that studies of insect hormones have reached the biochemical level.

Isolation in 1954 of the prothoracic gland hormone in pure crystalline form will now make possible a more precise comparison between insect and vertebrate hormones with regard to chemical constitution and physiological effects. Work on the elucidation of the chemical structure is being carried out. So far, no distinct effects of vertebrate hormones have been obtained in insects. The reverse experiment has not yet been attempted, but might be feasible in the near future. Studies on the biochemical basis of development, i.e. the relationship between morphogenesis and enzyme activity, have yielded the first noteworthy results. Both are under endocrine control. This area of research will also profit by the availability of pure ecdysone.

The role of the juvenile hormone, and its interaction with ecdysone, deserves special attention from the point of view of developmental physiology. The juvenile hormone alone appears to be without effect; its controlling function becomes evident only in conjunction with ecdysone. Many questions remain to be answered in this connection, both from the biochemical and from the physiological point of view. We do not know, for example, which of the two hormones is the first to be secreted and to act on the epidermis. Does the juvenile hormone subsequently modify the effect of ecdysone, or does it prepare the cell for the larval molt which is then induced by ecdysone? These and other questions will be answered only when the juvenile hormone can be obtained in the form of active extracts or as a pure substance. This represents a biochemical problem. The chemical identification of the prothoracotropic hormone also remains to be carried out.

In contrast to the problem of developmental physiology in insect endocrinology, other problems are of lesser importance. Their biological basis is not yet sufficiently understood. In striking contrast to the well-known hormones of the vertebrate gonads, no special sexual hormones have as yet been clearly demonstrated in insects.

The active substances designated temporarily as social hormones are found only in insects. They represent special adaptations to the social life of colony-building insects and give rise to speculations concerning the phylogenetic origin of these mechanisms. It will undoubtedly be interesting to study these problems in terms of comparative biochemistry and to determine whether these insects have utilized available substances in the service of their social mode of life, or whether new mechanisms have been evolved. The sexual attractants demonstrated in Lepidoptera are possibly phylogenetic precursors of the social hormones.

In view of the advances made during the last few years, especially the

work on endocrine control of enzyme systems and the isolation of the prothoracic gland hormone, we may hope that the near future will bring us closer to a solution of many unsolved problems.

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Glucuronide Metabolism, with Special Reference to the Steroid Hormones

G. A. LEVVY

Rowett Research Institute, Bucksburn, Aberdeenshire, Scotland

I. Introduction

1. Steroid and Other Glucuronides

Provided that there is a hydroxyl group in the molecule, any steroid hormone or its metabolite would appear to be capable of undergoing conjugation with glucuronic acid in the animal body, with subsequent appearance of the glucuronide in the urine. Only a few steroids have actually been isolated as the glucuronides (for references, see Marrian, 1946; Brooksbank and Haslewood, 1950), but it is generally assumed that a large number of the other steroids in urine are present, to some extent at least, in combination with glucuronic acid. This assumption is justified by the successful use of the enzyme β-glucuronidase for the hydrolysis of urinary steroid conjugates.

Conjugation with glucuronic acid is by no means confined to the steroids. A very large number of alcohols and phenols are excreted as glucuronides after administration to animals or man (for reviews, see Williams, 1947, 1951). Stilbestrol (4,4′-dihydroxy-α,β-diethylstilbene) and some other synthetic estrogens have been shown to behave in this way. Unlike the great majority of other glucuronidogenic alcohols and phenols, the steroid hormones and their metabolites are truly endogenous products, and only in their case would the excretion of the conjugated glucuronides in the urine appear to be an indisputably physiological process.* The incorporation of the glucuronide residue in polysaccharides (polyuronides) is, however, of equal physiological importance. In animals the glucuronide residue is found in the acid mucopolysaccharides, such as hyaluronic acid, chondroitin, and heparin. It has also been found in certain bacterial immunopolysaccharides, and in plants it is present in many of the gums and in hemicellulose.

2. Physiological Significance of Glucuronides

Conjugation of glucuronic acid with compounds foreign to the body is often described as a "detoxication mechanism," and there are several other processes similarly labeled, such as combination with sulfuric acid

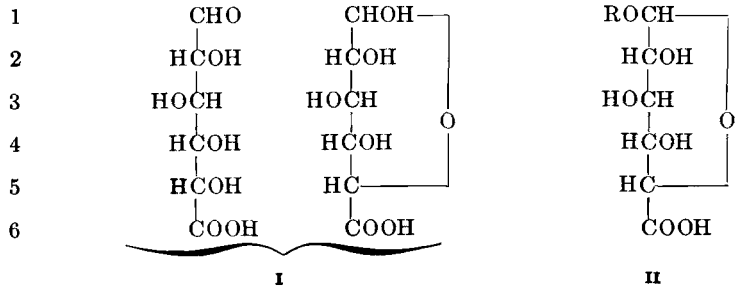
* The formation of glucuronides, of a rather different type, from branched-chain fatty acids (Kamil *et al.*, 1953) may also be a normal metabolic process.

or acetic acid. At one time, such reactions were regarded as deliberate attempts by the body to convert foreign compounds into innocuous and readily excreted derivatives. Although the old misnomer is retained, the underlying theory is no longer widely held, in the sense that the mechanism concerned is reserved solely for this purpose. Formation of, for example, a glucuronide from a foreign alcohol or phenol is now believed to be an accident of metabolism, in which the foreign compound has become entangled in a normal process. The result may or may not be of advantage to the animal.

So far as the steroid hormone glucuronides are concerned, the idea of "detoxication" lingers on, inasmuch as they are often thought of as inactive and readily excreted forms of the hormones, although this idea may not be explicitly stated. In the present state of knowledge, however, one might be equally justified in regarding the glucuronide as the active form of the hormone, and this view has gained some acceptance of late (Fishman, 1951; Szego and Roberts, 1953). It is true that estriol glucuronide is an estrogen. On the other hand, its activity does not exceed that of free estriol, and hydrolysis would appear to be an essential preliminary to its action (Odell *et al.*, 1937). It may also be significant that no free estriol is excreted in the urine (Clayton and Marrian, 1950).

3. Chemistry of Glucuronic Acid

Before discussing the enzymes concerned in their metabolism, one must briefly consider the chemistry of the glucuronides. Glucuronic acid (I) is a reducing sugar, resembling glucose in the configuration of the secondary hydroxyl groups, but differing from it at C-6, where the $-CH_2OH$ of glucose is replaced by a carboxyl group. Substitution at C-1 in the cyclic form of glucuronic acid gives rise to the glucuronides, such as are found in urine (general formula, II; R = aglycone residue). They are not reducing compounds, but retain the acidic properties of glucuronic acid. A few compounds of this type have also been isolated from certain plants. All the naturally occurring glucuronides appear to have the β-glucopyranuronide structure shown in II. Glucuronic acid has not been found free in nature.



I II

4. Occurrence of β-Glucuronidase

β-Glucuronidase is a group-specific enzyme that catalyzes the hydrolysis of the β-glucuronides to the aglycones and free glucuronic acid. The first record of the decomposition of a glucuronide by an animal tissue preparation appears in Röhmann (1908). The enzyme responsible, β-glucuronidase, was identified in a comprehensive study by Masamune (1934). Subsequent work from many quarters has shown it to be present in practically every animal tissue. The activity of the enzyme may be relatively high, as in liver, kidney, and spleen, or low, as in blood and brain. Excretion of the enzyme in the urine can occur. β-Glucuronidase is a constituent of the crude hyaluronidase complex, acting on some of the oligosaccharides produced during the degradation of hyaluronic acid and chondroitin (Hahn, 1947; Meyer *et al.*, 1951; Linker *et al.*, 1955). So far as other sources of the enzyme are concerned, it is sufficient for present purposes to say that this or other similar enzymes have been found in many pathogenic and nonpathogenic microorganisms, and in certain plants, insects, and mollusks, as well as in the contents of the alimentary tract from herbivores, carnivores, and omnivores (for references, see Marsh *et al.*, 1952; Levvy, 1954).

5. Assay of β-Glucuronidase

For the assay of β-glucuronidase activity, several different glucuronides have been employed, in conjunction with crude or partially purified tissue homogenates. At one time, glucuronic acid liberated from menthol glucuronide was measured by means of its reducing power. The lack of specificity in the determination of reducing sugar in tissue preparations was, however, a serious drawback, and this method has been superseded by others in which the free aglycone is determined by a specific color reaction. Two substrates that have been extensively used for this purpose are phenol glucuronide (Kerr *et al.*, 1948) and phenolphthalein glucuronide (Talalay *et al.*, 1946). The phenolphthalein glucuronide method is the better of the two and leaves little to be desired, but the fluorimetric method recently described by Mead *et al.* (1954) may prove to have certain advantages.

6. Physiological Significance of β-Glucuronidase

Since β-glucuronidase hydrolyzes the steroid glucuronides *in vitro*, there is reason to believe that it may play an important part in their metabolism and action *in vivo*, and extensive studies have been made of the behavior of the enzyme in animal tissues. Much of this work in its earlier stages was instigated by Prof. G. F. Marrian, F.R.S. The interesting discovery was made by Fishman (Fishman, 1940; Fishman and

Fishman, 1944) that the β-glucuronidase activity of certain tissues can be raised by administering estrogens and other compounds to animals. Subsequent work by the author and his collaborators (Levvy *et al.*, 1948; Kerr *et al.*, 1949, 1950) suggested that a rise or fall in the β-glucuronidase activity of a tissue reflects a change in its state of proliferation. Contradictory evidence from various sources, culminating in the discovery that genetic factors determine not only the β-glucuronidase activity of a tissue (Law *et al.*, 1952), but also the response of the enzyme to extrinsic agents (Fishman and Farmelant, 1953), makes it necessary to revise previous theories on the significance of changes in β-glucuronidase activity *in vivo*. Cancer tissue has a high β-glucuronidase activity (Fishman and Anlyan, 1947), and the assay of the enzyme in vaginal fluid has been recommended as a clinical test in uterine carcinoma (Odell and Burt, 1950). Also of possible diagnostic value is the assay of blood β-glucuronidase activity in toxemia of pregnancy (Odell and McDonald, 1948).

7. Mechanism of Glucuronide Synthesis

The origin of glucuronic acid in the body and the mechanism of glucuronide formation are problems that have attracted attention for at least seventy years, but it is only of recent years that any substantial progress has been made. For a period, there was wide acceptance of the view that glucuronides are formed by the condensation of free glucuronic acid with alcohols and phenols in the presence of β-glucuronidase (Fishman, 1940). A great deal of evidence has been accumulated, however, to prove that the enzyme responsible for glucuronide synthesis in the intact animal or in tissue preparations is quite distinct from β-glucuronidase (Levvy, 1952a; McGilvery, 1953). It has now been shown by the brilliant work of Storey and Dutton (1955) that the compound that reacts with alcohols and phenols in liver homogenates to form glucuronides is an "active" complex of uridine diphosphate and glucuronic acid. Radioactive tracer techniques have recently been employed in whole animal experiments by different groups of workers, and it has been shown that glucose, but not glucuronic acid, can act as a precursor in glucuronide synthesis.

8. Measurement of Glucuronide Synthesis

For the study of glucuronide synthesis in tissue preparations, a sensitive method of measurement was essential, and such a method was found by the use of *o*-aminophenol as the aglycone (Levvy and Storey, 1949): *o*-aminophenol glucuronide can be diazotized and coupled with naphthylethylenediamine to give a red dye under conditions that exclude interference by the free aglycone. This method has been extensively employed in various connections, and by substituting *m*-aminophenol for *o*-amino-

phenol it has been applied to the related problem of sulfate synthesis by tissue preparations (Bernstein and McGilvery, 1952).

It is believed that all the germane literature has been consulted in preparing the following account of β-glucuronidase and the glucuronide-synthesizing enzyme system, but no attempt has been made to compile an exhaustive bibliography. Certain features of the biochemistry of β-glucuronidase are more thoroughly discussed in an earlier review by the present author (Levvy, 1953), and a recent review by Teague (1954) deals more fully with chemical and pharmacological aspects of glucuronide metabolism.

II. β-Glucuronidase and Changes in Its Activity *in vivo*

1. Theoretical Background

Changes in β-glucuronidase activity *in vivo* in response to extrinsic agents were first observed by Fishman (Fishman, 1940, 1947a; Fishman and Fishman, 1944). To explain his results, Fishman put forward a theory based on the fact that certain of the compounds he had studied were known to be glucuronidogenic. The complete theory required three distinct postulates: (1) that *in vivo* β-glucuronidase catalyzes the condensation of alcohols and phenols with free glucuronic acid to form glucuronides; (2) that in this synthetic role the enzyme, by analogy with some bacterial enzymes, responds adaptively to the presence of excess aglycone; (3) that the synthesis of estrogen glucuronides occurs only in sex organs, and of nonestrogen glucuronides in nonsex organs. [This theory has of late tended to assume a more nebulous character, in which glucuronide formation is imagined to be an essential preliminary to the action of the steroid hormones (Fishman, 1947a, 1951a, 1951b).]

Leaving aside the major question of whether anything analogous to bacterial enzyme adaptation can occur in the mammalian cell,* Fishman's theory, on his own evidence alone, contained several anomalies. The chief objection to his theory came, however, from existing evidence (Lipschitz and Bueding, 1939) that quite a different enzyme system was at work during glucuronide synthesis by surviving tissue slices, and that glucuronic acid was not involved as such in the process. These facts have now been established beyond any reasonable doubt. Fishman himself has never demonstrated the synthesis of a glucuronide by β-glucuronidase (see Section II, 13). In general, reversal of the action of a hydrolytic enzyme, although it has been demonstrated in a few instances *in vitro*, requires such drastic conditions as to make the process an unlikely one *in vivo* (McGilvery, 1953).

*For an excellent discussion of this topic see Knox (1951) who reports adaptation by the tryptophan-oxidizing enzyme in liver.

The action of extrinsic agents on β-glucuronidase activity *in vivo* was reinvestigated by Levvy and his collaborators (Levvy *et al.*, 1948; Kerr *et al.*, 1949, 1950) and was found to be unrelated to their ability or inability to form glucuronides. Their action appeared to depend upon tissue damage or a direct stimulation of tissue growth, and these factors determined the site of action on the enzyme activity. The effects of steroid hormones on the enzyme were not confined to the sex organs. In general, it was postulated, a rise or fall in the β-glucuronidase activity of a tissue was an index of a change in its state of proliferation, irrespective of the function of the enzyme in the body. This theory reconciled all the existing evidence at that time.

No causal relation between the β-glucuronidase activity of a tissue and its state of proliferation could be suggested, and it was impossible to decide whether a rise in enzyme activity was essential for growth or merely reflected some incidental alteration in metabolism. Recent work from several sources, however, has revealed so many instances of a lack of correlation between enzyme activity and growth, and such complex control of the enzyme, that the second explanation is clearly the correct one.

2. Action of Menthol in Liver, Kidney, and Spleen

In his first experiments, Fishman (1940) fed mice twice or thrice daily with the glucuronidogenic compound, menthol. After five days, when the total administered had reached about 10 g./kg., the mice were killed and organs were removed for enzyme assay by hydrolysis of menthol glucuronide. Statistical analysis of the results revealed that in comparison with untreated mice there was a rise in β-glucuronidase activity in the liver, kidney, and spleen, but not in the uterus and other sex organs. Essentially similar results were obtained in dogs fed repeatedly with borneol, another glucuronidogenic compound. These results were explained by Fishman in terms of adaptation by the enzyme in liver, kidney, and spleen, acting synthetically, to the presence of excess substrate.

In a reinvestigation of the effects of menthol on β-glucuronidase activity in mice (Levvy *et al.*, 1948), the compound was given by a single intraperitoneal injection of rather a small dose (333 mg./kg.), and the enzyme was assayed by the phenol glucuronide method. One day after the injection of menthol, the β-glucuronidase activity of liver rose to more than three times the normal level, and pathological changes were observed in the organ. Injection of menthol glucuronide had similar effects. No rise in kidney β-glucuronidase activity was observed until a few days after the injection of menthol, by which time delayed damage was apparent in this organ. Spleen β-glucuronidase was unaffected by menthol in these experiments.

These results suggested that the action of menthol on liver and kidney β-glucuronidase activity might have nothing to do with its glucuronidogenic properties, but might be secondary to its toxic action. It was considered likely that the rise in enzyme activity was associated with tissue repair, rather than the preceding injury. On this view, there was no difficulty in reconciling the effects of menthol with those of, for example, estriol (Section II, 5), nor of either with those of its glucuronide.

3. Effects of Poisons in Liver and Kidney

Nonglucuronidogenic poisons, such as chloroform, carbon tetrachloride, mercuric nitrate, yellow phosphorus, and uranyl acetate, after administration to mice produced rises in β-glucuronidase activity in liver or kidney or both, according to the site of injury (Levvy *et al.*, 1948). By way of contrast, the glucuronidogenic compound pregnanediol (333 mg./kg. by intraperitoneal injection) had no effect. In both organs the rise in enzyme activity was delayed by increasing the severity of poisoning, in agreement with the view that it was a manifestation of repair rather than injury. No significant changes in spleen β-glucuronidase activity were observed. The effects of chloroform on kidney β-glucuronidase activity reflected the sex-linked nature of the susceptibility of this organ to chloroform necrosis in the mouse (Eschenbrenner and Miller, 1945), which in turn reflects differences in the structure of Bowman's capsule (Crabtree, 1941), and only the enzyme in male-type kidney was affected (see also Kerr *et al.*, 1949). Fishman's theory of enzyme adaptation afforded no explanation of the effects of nonglucuronidogenic agents on the β-glucuronidase activity of an organ.

4. Growing and Regenerating Tissues

After partial hepatectomy in mice (Levvy *et al.*, 1948; Kerr *et al.*, 1949, 1950), liver β-glucuronidase activity was elevated during the period of hypertrophy of the remaining lobe. High β-glucuronidase activities were observed in infant mice, compared with adults, in liver, kidney, spleen, uterus, and lung (Levvy *et al.*, 1948; Kerr *et al.*, 1949; Karunairatnam *et al.*, 1949). Colchicine, itself without effect on β-glucuronidase activity in normal adults, reduced the liver enzyme activity in young mice to the adult level, in a dose which arrested growth (Kerr *et al.*, 1950; Table I): litter-mate controls showed the usual figures for their age.

These findings were interpreted in terms of an association between β-glucuronidase activity and tissue growth, but more recent work has not always supported this generalization. The fall in tissue β-glucuronidase activity with increasing age in mice was confirmed in later work by

Walker and Levvy (1951), Walker (1952), and Mills (1952), but not by Fishman (1951) and Morrow *et al.* (1951). The discrepancy may result from differences in the strains of mice employed (see Section II, 8), or from other uncontrolled factors. In rats, Mills *et al.* (1953) found that liver β-glucuronidase activity is much lower in infants than in adults, and that liver hypertrophy after partial hepatectomy is not correlated with increased enzyme activity. These results for rats have been confirmed in their essential features by Walker and Levvy (1953). It is evident that

TABLE I

EFFECT OF COLCHICINE ON LIVER β-GLUCURONIDASE ACTIVITY IN YOUNG MICE

Subcutaneous injection of 1 mg. colchicine/kg. in 0.9% NaCl solution every other day. Controls (litter-mates) received NaCl solution only. One G.U. (glucuronidase unit) liberates 1 μg. phenol from 0.015 *M* phenol glucuronide in 1 hr. at 38° and pH 5.2.

Expt. no.	Age of mice (days)		Colchicine	Increase in body wt. (%)	G.U./g. liver
	At start	At end			
1	9	18	+	−3	347 ± 31
			−	65	673 ± 49
2	10	15	+	1	310 ± 17
			−	14	1043 ± 62

From Kerr *et al.*, 1950.

there is a species difference between rats and mice in the behavior of the enzyme during tissue growth. Hollinger and Rossiter (1952) found that the β-glucuronidase activity of cat sciatic nerve increased nearly forty-fold during Wallerian degeneration after nerve section or nerve crush, but the rise in enzyme activity appeared to be subsequent to the phase of rapid cell proliferation, as determined from the deoxyribonucleic acid content of the tissue.

5. Effects of Steroids on the Uterus and Other Sex Organs

After his experiments with menthol and borneol, Fishman (Fishman and Fishman, 1944; Fishman, 1947a) examined the effects on the enzyme of repeated injection of ovariectomized mice with estrone, estradiol-17β, estriol, estriol glucuronide, stilbestrol, progesterone and pregnanediol. The estrogens, natural and synthetic, caused a rise in β-glucuronidase activity in uterus, but not in liver, kidney, and spleen. Progesterone and pregnanediol had no effect on the uterine enzyme, and estriol glucuronide produced an increase in activity. Ovariectomy itself led to a fall in the enzyme activity in uterus compared with intact controls. These results, like those for menthol, were explained in terms of adaptation by the

enzyme, acting synthetically, to the presence of excess aglycone, with the proviso that the enzyme is specific in its response to different substrates according to its location in the body. There was no evidence, however, and there is none now, for any such difference in the specificity of the enzyme in different organs in its hydrolytic action *in vitro*. In fact, Fishman assayed the uterine enzyme by hydrolysis of menthol glucuronide, and in earlier work (Fishman, 1939) he described the hydrolysis of estriol glucuronide by a spleen enzyme preparation. The effect of estriol glucuronide administration on the enzyme activity of uterus was another anomaly in the theory of enzyme adaptation.

It seemed possible that the effects of estrogens on uterine β-glucuronidase activity could be better explained in terms of uterine growth, but one point required elucidation. Fishman (1947a) found that testosterone propionate (100–1000 μg.) did not antagonize the action of estradiol benzoate (1–2 μg.) on the enzyme, and this was said to argue for "a unique type of specificity of action by estrogen." In the doses used, however, the androgen did not entirely abolish the action of the estrogen on uterine weight, and given alone it caused a rise in weight and enzyme activity. The failure to observe antagonism between estrogen and androgen could thus have been due to the use of too much of the latter.

This point was examined by Kerr *et al.* (1949, 1950), with the results shown in Table II. A dose of testosterone, itself too small to influence uterine weight or β-glucuronidase activity, antagonized the effects of estrone on both. In a slightly larger dose, the androgen by itself caused slight increases in uterine weight and enzyme activity, comparable to the effects of a small dose of estrone. Progesterone also antagonized the action of estrone on uterine weight and enzyme activity, although itself quite inactive. These results were consistent with the view that changes in uterine β-glucuronidase activity were manifestations of changes in growth.

Subsequent papers from various quarters on the effects of the sex hormones on the β-glucuronidase activity of uterus and other sex organs present a very confused and contradictory picture and do not always confirm the earlier observations on points of detail. Thus, for example, although estrogens always stimulated uterine β-glucuronidase activity in ovariectomized rats and mice,* the increase was subsequent to the rise in uterine weight (Leonard and Knobil, 1950), and the enzyme in vagina was unaffected (Harris and Cohen, 1951). Progesterone was variously found to antagonize the action of estrogens on uterine weight, without

* One must note, however, the remarkable observation of Fishman and Farmelant (1953) that estrogen has no effect on uterine β-glucuronidase activity in mice of the C_3H strain (see Section II, 8).

significantly altering the effect on the enzyme (Harris and Cohen, 1951; Knobil, 1952), or to antagonize the action on the enzyme, but not on the weight (Beyler and Szego, 1954a). Many interesting new phenomena were studied, for example the stimulation of the β-glucuronidase activity of the prostates and seminal vesicles in orchiectomized rats by estradiol benzoate (Knobil, 1952), but the results as a whole did not bear out the general conclusions arrived at in the earlier work of Fishman or of Levvy and his co-workers. The response of the enzyme to sex hormones in any one tissue appeared to be complicated by the actions of pituitary and

TABLE II

EFFECTS OF STEROID HORMONES ON UTERINE WEIGHT AND β-GLUCURONIDASE ACTIVITY, FOUR DAYS AFTER INJECTION INTO OVARIECTOMIZED MICE

Hormones given by single subcutaneous injection of solution in olive oil. One G.U. (glucuronidase unit) liberates 1 μg. phenol from 0.015 *M* phenol glucuronide in 1 hr. at 38° and pH 4.5.

Treatment	G.U./g. uterus	Uterine weight (mg.)
Intact controls	333 ± 53	234 ± 56
Ovariectomized controls	166 ± 21	36 ± 9
Oestrone (1.7 mg./kg.)	524 ± 56	210 ± 11
Oestrone (0.3 mg./kg.)	226 ± 34	102 ± 15
Testosterone (3.3 mg./kg.)*	260 ± 14	101 ± 19
Testosterone (2.0 mg./kg.)	154 ± 31	39 ± 9
Testosterone (2.0 mg./kg.) + estrone (1.7 mg./kg.)	205 ± 31	47 ± 7
Progesterone (5.4 mg./kg.)	189 ± 7	36 ± 4
Progesterone (1.8 mg./kg.)	167 ± 10	41 ± 4
Progesterone (1.8 mg./kg.) + estrone (1.7 mg./kg.)	174 ± 13	32 ± 3

* One-day figure; effect had gone by fourth day.
From Kerr *et al.*, 1949, 1950.

adrenal hormones (Knobil, 1952; Szego and Roberts, 1953), and probably also by genetic factors (Section II, 8).

It would no longer seem possible to adhere to any idea that a rise in β-glucuronidase activity is an adaptive response to glucuronidogenic compounds or a nonspecific response to growth stimuli. What the results do suggest is that there is direct endocrine control of the activity of the enzyme, of a complicated character and bearing no simple relation to any other known physiological or morphological process in the tissue concerned.

6. Extraovarian Estrogen and Liver Regeneration

In the course of their experiments described above, Kerr *et al.* (1949, 1950) studied the effects of estrogens on the enzyme in nonsex organs,

and of liver and kidney poisons on the uterine enzyme. The purpose was to test the specificity of the response by the enzyme in different organs. No evidence was obtained for the classification of active agents into "estrogens" and "nonestrogens," according to the site of action (Fishman and Fishman, 1944; Fishman, 1947a). On the contrary, two unexpected phenomena were discovered: (1) stimulation of uterine growth and β-glucuronidase activity after liver damage in ovariectomized mice, in absence of extrinsic estrogen; (2) stimulation of liver β-glucuronidase activity by estrone (Section II, 7). Tenuous though the link may be, these findings indicate that alterations in the activity of the enzyme may on occasion point to unsuspected changes in tissue proliferation.

Seven days after the injection of ovariectomized mice with chloroform or carbon tetrachloride, increases in uterine β-glucuronidase activity and weight were observed, although no estrogen had been administered. The kidney poison, mercuric nitrate, had no effect on uterus. The effects of chloroform and carbon tetrachloride were not due to a direct action of the toxic agent on uterus, but were secondary to liver damage, since partial hepatectomy produced similar changes. Rises in uterine weight during liver regeneration were similar to those caused by a small dose (0.3 mg./kg.) of estrone (Table II), and the vaginal smears resembled those obtained with subeffective doses of estrogens. On histological examination of six uteri, metestrus, proestrus and, in one case, full estrus were observed, compared with diestrus in ovariectomized controls. Testosterone and progesterone antagonized the effects of partial hepatectomy on uterine weight and β-glucuronidase activity, without influencing the rise in liver enzyme activity (Table III).

It was considered that these results could only be explained in terms of increased potency of an extraovarian estrogen. This observation, which awaits confirmation, has an obvious bearing on certain experiments in which an increase in the effects of administered or ovarian estrogen has been found to follow liver damage (see, for example, Roberts and Szego, 1947): these experiments are usually interpreted as demonstrating a derangement of the normal metabolism of the exogenous or ovarian hormone in the liver. Furthermore, toxicity for liver may explain the actions of some compounds with feebly estrogenic properties.

7. Effects of Steroid Hormones on Nonsex Organs

Kerr *et al.* (1949, 1950) discovered that the effects of secondary sex hormones on β-glucuronidase activity are not confined to the enzyme in sex organs. Estrone had a stimulant effect on liver β-glucuronidase activity in ovariectomized mice, and this effect was antagonized by testosterone and progesterone; neither of the latter had any effect by itself.

This action of estrone was also seen in normal and castrate males, but was absent in intact females. Estriol and estradiol did not affect liver β-glucuronidase activity, but it was considered not impossible that some action might have been revealed under different experimental conditions. These experiments are summarized in Table IV: no significant changes were observed in the kidney enzyme.

Estrone caused no liver damage, but it did stimulate mitotic activity in the tissue, except in intact females. Testosterone was seen to antagonize the effect on cell division. The histological findings did not, however,

TABLE III

THE STIMULATION OF UTERINE GROWTH BY PARTIAL HEPATECTOMY IN OVARIECTOMIZED MICE AND THE ANTAGONISTIC EFFECTS OF TESTOSTERONE AND PROGESTERONE

The dose of the steroid hormone was divided between two subcutaneous injections, one 6 hr. after partial hepatectomy, the other 3 days later. The mice were killed 8 days after partial hepatectomy. One G.U. (glucuronidase unit) liberates 1 μg. phenol from 0.015 *M* phenol glucuronide in 1 hr. at 38° and pH 5.2 (for liver) or 4.5 (for uterus).*

Treatment	G.U./g. liver	G.U./g. uterus	Uterine weight (mg.)
None	270 ± 18	166 ± 21	36 ± 9
Partial hepatectomy	559 ± 49	351 ± 30	111 ± 13
Partial hepatectomy + testosterone (4.0 mg./kg.)	572 ± 48	247 ± 27	43 ± 7
Partial hepatectomy + progesterone (3.6 mg./kg.)	509 ± 36	186 ± 16	38 ± 5
Testosterone (4.0 mg./kg.)	283 ± 17	195 ± 15	32 ± 4
Progesterone (3.6 mg./kg.)	282 ± 25	174 ± 21	35 ± 2

* In different organs, the enzyme may show an optimum at pH 4.5 or 5.2 or both. This probably reflects combination of the protein with different anions (Levvy, 1953) but has no apparent influence on the response of the enzyme to extrinsic agents (Kerr *et al.*, 1949).

From Kerr *et al.*, 1950.

completely parallel the changes in enzyme activity, the effect of estrone on cell division being relatively slight in intact males, whereas estriol and estradiol caused some stimulation of liver mitosis in intact males and castrate females.

Bullough (1946), after studying the action of estrone on mitotic activity throughout the female mouse, concluded that "those substances which have come to be called oestrogenic or female sex hormones are in fact general mitosis stimulators." Estrone had no effect on liver in his experiments, which were confined to the intact female. Stimulation of liver growth in rats by estrogens was noted by Teague (1941, 1942).

Harris and Cohen (1951) were unable to confirm the effect of estrone on liver β-glucuronidase activity in the ovariectomized mouse, but prog-

esterone together with estrone depressed the liver enzyme activity in their experiments. Tuba (1953) found that estradiol stimulated liver β-glucuronidase activity in orchiectomized rats, but not in normal males.

Fishman and Farmelant (1953) have reinvestigated and extended the observations of Kerr *et al.* (1949, 1950) on the effects of sex hormones on nonsex organs, using inbred mice. Liver β-glucuronidase activity was elevated by stilbestrol in intact and castrate male and female mice of the

TABLE IV

THE EFFECTS OF STEROID HORMONES ON MOUSE LIVER β-GLUCURONIDASE ACTIVITY AT PERIOD OF MAXIMUM RESPONSE

Hormones given by single subcutaneous injection of solution in olive oil. One G.U. (glucuronidase unit) liberates 1 μg. phenol from 0.015 *M* phenol glucuronide in 1 hr. at 38° and pH 5.2. M = male, F = female, c = castrated.

Hormone	G.U./g. liver			
	*F**	*cF**	*M*†	*cM**
None	334 ± 48	270 ± 18	273 ± 14	301 ± 18
Estrone (1.7 mg./kg.)	267 ± 26	562 ± 33	879 ± 98	562 ± 58
Estradiol (6 mg./kg.)	—	287 ± 20	292 ± 19	—
Estriol (5 mg./kg.)	—	279 ± 26	281 ± 17	—
Testosterone (3.3 mg./kg.)	272 ± 40	251 ± 48	—	311 ± 36
Testosterone (2.0 mg./kg.) + estrone (1.7 mg./kg.)	—	279 ± 20	261 ± 20	—
Progesterone (5.4 mg./kg.)	—	272 ± 19	—	—
Progesterone (1.8 mg./kg.) + estrone (1.7 mg./kg.)	—	289 ± 22	—	—

* Fourth-day figures.
† First-day figures.
From Kerr *et al.*, 1949, 1950.

C_3H strain, but only in intact females of the 129 strain (see Table V); testosterone did not antagonize these effects. In mice of both strains and sexes, intact and castrate, testosterone increased kidney β-glucuronidase activity, and this effect was antagonized by stilbestrol. The enzyme activity of urine parallels the effects of the androgen in the kidneys (Riotton and Fishman, 1953). Variable effects of androgen and estrogen were seen in spleen.*

8. Hereditary Factors

Morrow and his co-workers (Morrow *et al.*, 1949, 1950; Law *et al.*, 1952) discovered that certain inbred strains of mice (notably the C_3H strain) have remarkably low β-glucuronidase activities in liver, kidney, and spleen, in comparison with most other strains, and they have shown

* The doses employed by Fishman and Farmelant (1953) were very much in excess of those used by Kerr *et al.* (1949, 1950).

TABLE V

STATISTICALLY SIGNIFICANT ($P \not> 0.01$) CHANGES IN β-GLUCURONIDASE ACTIVITY IN VARIOUS ORGANS AFTER ADMINISTERING STILBESTROL AND TESTOSTERONE TO MICE OF PURE STRAINS

M = male, F = female, c = castrate; ↑ = rise in enzyme activity compared with appropriate control group, ↓ = fall in activity, s denotes relatively small effect, 0 = no *significant* change in activity; + = antagonism between androgen and estrogen, − = no antagonism (several small synergist or additive effects are included under "−"). Weight of mice, 20 g.; 0.5 mg. stilbestrol or testosterone propionate or both injected subcutaneously (?) in peanut oil every other day for 14 days; controls received oil alone. The figures for normal mice are μg. phenolphthalein/mg. moist tissue, liberated from 0.001 *M* phenolphthalein glucuronide in 1 hr. at pH 4.5 and 38°.

Treatment	Kidney				Liver				Spleen				Uterus		Seminal vesicle	
	F	cF	M	cM	F	cF	M	cM	F	cF	M	cM	F	cF	M	cM
Strain 129 ("high β-glucuronidase") mice																
Normal mice and effect of castration	1.4	0	1.5	0	3.2	0	4.1	0	7.7	↑s	6.9	0	2.8	↓	3.9	↑
Androgen	↑	↑	↑	↑	↑s	0	0	↑s	0	↓s	0	↑s	↓s	0	0	↓
Estrogen	0	0	0	0	↑	0	0	0	↑	0	0	↑	0	↑	↑	↓
Estrogen + androgen	+	+	+	+	−	−	−	+	+	−	−	+*	+	−	+*	↑†
Strain C_3H ("low β-glucuronidase") mice																
Normal mice and effect of castration	0.3	0	0.3	0	0.5	0	0.5	0	1.4	0	1.2	0	1.0	0	0.1	↑
Androgen	↑	↑	↑	↑	0	0	0	0	0	0	0	0	↓s‡	↓s	0	↓
Estrogen	0	0	0	↑s	↑	↑	↑	↑	0	0	↑s	↑s	0	0	↑	↓
Estrogen + androgen	+	+	+	+	−	−	−	−	−	−	−	−	+	+	+	−

* No statistical data given by Fishman and Farmelant.

† Figure higher than in controls.

‡ Figure significantly different from estrogen + androgen, but not from controls.

Adapted from Fishman and Farmelant, 1953.

that a "high" β-glucuronidase activity is dependent upon the presence of a single dominant gene. Mice of unspecified ancestry hitherto had been employed in practically all work on β-glucuronidase.

Cohen and Bittner (1951) found that the ancestry of the animals determined the β-glucuronidase activity of normal and cancerous mouse mammary tissue, the C_3H strain having the lowest values: in every instance the mammary tumor had a higher activity than the neighboring healthy tissue (see Section II, 9).

Fishman and Farmelant (1953) compared the effects of an androgen and an estrogen on the β-glucuronidase activities of sex and nonsex organs in "high β-glucuronidase" and "low β-glucuronidase" strains of mice. Table V presents a personal attempt to summarize their results. In the original account of this most important work, the statements in the text are sometimes at variance with the figures in the tables, and there appear to be clerical errors. In the main, however, there is little disagreement on major points. The principal findings with regard to the nonsex organs have already been discussed (Section II, 7). With regard to the sex organs, the most interesting finding is that neither castration nor subsequent estrogen administration had any effect on uterine enzyme activity in C_3H mice: one would expect enzyme adaptation to be most marked in a "low β-glucuronidase" strain.

To save space, the values for normal mice in Table V are given only to the first decimal place. They do demonstrate, however, the fact that there was no significant difference between the enzyme activity of male and female kidney in mice of either the C_3H or the 129 strain. In this respect, these strains contrast with others in which male kidney has a higher β-glucuronidase activity than female (Morrow *et al.*, 1951; Fishman and Farmelant, 1953).*

The difference in the activity of the enzyme in the livers of "high β-glucuronidase" and "low β-glucuronidase" strains of mice was not due to any detectable difference in its physical properties (Morrow *et al.*, 1950; Sie and Fishman, 1953).

It is clear that heredity governs not only the general level of tissue β-glucuronidase activity, but also the effects of sex and castration on the enzyme in individual organs, and the response of the enzyme to extrinsic agents. The genetic control of β-glucuronidase activity appears to extend to cancer tissue. It would be interesting to see some of the earlier work on, for example, the effects of menthol administration or of age on tissue β-glucuronidase activity repeated in experiments in which genetic factors are controlled. Minor discrepancies in experiments with animals of a sin-

* A higher β-glucuronidase activity in male kidney than in female was detected histochemically by Campbell (1949), using mice of unspecified strain.

gle strain suggest, however, that there may yet be variables to be discovered, such as the effects of diet or climate.

9. Cancer Tissue

It has been well established for humans (Fishman and Anlyan, 1947; Odell and Burt, 1949; Fishman and Bigelow, 1950), and confirmed for mice (Karunairatnam *et al.*, 1949; Kerr *et al.*, 1950; Cohen and Bittner, 1951), that the β-glucuronidase activity of malignant tumors is high in comparison with that of most normal organs, and usually much higher than that of the surrounding healthy tissue. The solitary reported exception comes from Smith and Mills (1951), who found that hepatomas induced in rats with *p*-dimethylaminoazobenzene had a lower β-glucuronidase activity than normal rat liver, but the changes wrought in rat liver with this agent are not always malignant (Orr, 1940).

Odell and Burt (1950) suggested that abnormally high figures for β-glucuronidase activity in vaginal fluid (see Section II, 10b) might have diagnostic significance in uterine cancer; they appear to have particular statistical significance in premenopausal women (Kasdon *et al.*, 1953). Blood β-glucuronidase levels (see Section II, 10a) do not reflect cancerous changes in organs (Fishman, 1947b; Anlyan *et al.*, 1950). Urine has a high β-glucuronidase activity in cancer of the bladder, and the activity is depressed by stilbestrol administration, but not by removal of the bladder tumors (Boyland *et al.*, 1954).

The high β-glucuronidase activity of cancer tissue was readily explained as another instance of the association between changes in activity and tissue growth. It is interesting to note that there is a slight possibility that "low β-glucuronidase" strains of mice (see Section II, 8) may have a peculiarly high incidence of spontaneous hepatomas (Morrow *et al.*, 1950) or leukemia (Fishman and Farmelant, 1953).

10. Body Fluids in Women and Clinical Applications

In the last few years, a confusing literature has arisen on the subject of changes in the β-glucuronidase activity of body fluids in women, in relation to the menstrual cycle, pregnancy, the menopause, and cancer. The following are some of the more interesting relevant findings. Occasional observations have been made on healthy human sex tissues, but since they add little to information obtained in animal experiments (Section II, 5), they are mentioned only insofar as they bear on the effects in the body fluids.

a. Blood. The greater part of the enzyme in blood is in the leucocytes and lymphocytes and the remainder in the plasma (Fishman *et al.*, 1948b):

the activity is high in the infant, compared with the mother, but low in the fetus, and these differences are reflected in the urine (Odell *et al.*, 1948).* During pregnancy, the β-glucuronidase activity of blood rises, and it falls after parturition (Fishman, 1947b): McDonald and Odell (1947) observed abnormally high values in pre-eclampsia, but not in hypertensive toxemia of pregnancy, and they suggested that the enzyme assay might be of diagnostic value in the former condition. It seems possible that these abnormally high values are due to partial failure of renal excretion of the enzyme. Administration of estrogen prevents the fall in blood β-glucuronidase activity after parturition (Fishman *et al.*, 1950b) and under other circumstances elevates the activity (Cohen and Huseby, 1951; Fishman *et al.*, 1951). It has been suggested (Fishman *et al.*, 1950b; Fishman *et al.*, 1951) that these elevated figures are due to the stimulation of enzyme activity in the liver by estrogen (Kerr *et al.*, 1949), followed by leakage of the enzyme into the blood (for some relevant work in the rat, see Bernard and Odell, 1950). It is difficult, however, on this reasoning, to see why high figures for cancer tissue are not likewise reflected in the blood β-glucuronidase activity (see Section II, 9). There is no consistent correlation between blood β-glucuronidase activity and phases of the menstrual cycle (Fishman *et al.*, 1951).

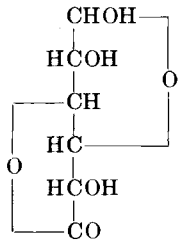
b. Vaginal Fluid. A great deal of attention has been paid to this fluid in healthy subjects, with a view to defining the limits for Odell and Burt's diagnostic test for uterine cancer (see Section II, 9). In normal subjects, the activity of the enzyme in vaginal fluid appears to be under ovarian control. It rises after the menopause, natural or artificially induced (Fishman *et al.*, 1950a), in spite of the fall in enzyme activity in uterine and vaginal tissue (Fishman and Anlyan, 1947), and it is subsequently depressed by administration of estrogen (Fishman *et al.*, 1951). The healthy uterus does not appear to contribute to the enzyme activity of vaginal fluid. The latter is not depressed below normal values by hysterectomy (Fishman *et al.*, 1950a), and during the menstrual cycle it is at its lowest midway between menses (Fishman *et al.*, 1951), at which time the activity in uterine endometrium is at its highest (Odell and Fishman, 1950).

11. Inhibition and Activation

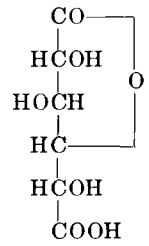
Under this heading, brief mention will be made of only those substances that have been employed in attempts to elucidate the physiological function of β-glucuronidase, or that might conceivably influence its

* Most of the work on urine has been of a sporadic nature, and systematic studies of the renal excretion of the enzyme are required. Urine β-glucuronidase activity is high in patients with renal tuberculosis (Boyland *et al.*, 1954).

activity *in vivo*. The enzyme has been shown to be unaffected by many of the better-known enzyme poisons, such as fluoride, iodoacetate, cyanide, and azide. It is inhibited by glucuronic acid (I; Spencer and Williams, 1951), which is of course a product of its hydrolytic action, but not by the 3,6-lactone, glucurone (III). By far the most powerful and most useful inhibitor so far discovered for the enzyme is saccharo-1,4-lactone (IV).



III



IV

a. Saccharo-1,4-lactone. Karunairatnam and Levvy (1949) discovered that β-glucuronidase is powerfully inhibited by saccharate solutions, and it was later shown (Levvy, 1952b) that the inhibitory agent is not saccharate ion, but saccharo-1,4-lactone (IV), formed in small amounts under the conditions of enzyme assay. Saccharo-1,4-lactone, which has a very high affinity for the enzyme, acts competitively. At a given lactone concentration, therefore, the degree of inhibition varies inversely with the concentration of the substrate and the affinity of the substrate for the enzyme. The lactone is unstable under physiological conditions, but it is more stable at a pH of 6 or less. It has been employed (in the form of saccharate solution) in critical studies of histochemical tests for β-glucuronidase (Campbell, 1949; Burton and Pearse, 1952), and to arrest the enzymic hydrolysis of estriol glucuronide that occurs in labor urine* (Clayton and Marrian, 1950), a process that explains the variable appearance of free estriol in urine at labor. For further instances of the use of the lactone in the study of urinary steroids see Section II, 14.

b. Endogenous Inhibitor. Animal tissues contain an unidentified, thermostable, nondialyzable inhibitor for β-glucuronidase, the effect of which can be overcome by adding the nonionic surface-active agent Triton X-100 (Walker and Levvy, 1953). Enzyme from different sources varies in its susceptibility to inhibition by this substance or group of substances, the enzyme from mouse organs, for example, being relatively unaffected, although mouse tissues contain appreciable quantities of inhibitor as

* The anionic surface-active agent Teepol XL (Walker and Levvy, 1951) might also be used to prevent hydrolysis of glucuronides in urine.

tested on rat enzyme. The effect of a single inhibitor preparation on rat enzyme varied with the age of the animal and the tissue from which the enzyme was prepared. This inhibitor is apparently similar to that detected in blood plasma by Fishman *et al.* (1948a). It would appear to be of minor importance with regard to the changes in β-glucuronidase activity that occur *in vivo*.

c. Deoxyribonucleic Acid. Bernfeld and Fishman (1950) found that highly purified β-glucuronidase was activated by deoxyribonucleic acid and tentatively suggested that the latter might be a dissociable co-enzyme for β-glucuronidase. Activation was seen only at high dilutions of the enzyme in pure solution and has subsequently been shown to be far from a specific effect of deoxyribonucleic acid (Smith and Mills, 1953; Bernfeld *et al.*, 1954): several other substances, e.g. albumin, are more efficient. There is no doubt that the enzyme is fully active in the usual type of preparation employed for comparative tissue assays, or for the hydrolysis of urinary conjugates (Section II, 14).

12. Location in the Mammalian Cell

From studies of the distribution of β-glucuronidase activity in different kinds of tissue homogenates (Kerr and Levvy, 1951; Walker and Levvy, 1951, 1953; Walker, 1952), it was concluded that, whatever the activity of the tissue, nearly all the enzyme in the cell was present in the cytoplasmic granules and was spread over granules of all sizes.* Very little, if any, was free in the cytoplasm. When the integrity of the granules was maintained in isotonic media, the enzyme within them was not freely accessible to substrate in the medium. It is thus quite possible that permeability changes within the cell may play as important a part in regulating the action of the enzyme *in vivo* as changes in the enzyme concentration. Infant mouse-liver homogenates in isotonic media were, however, more active than adult.

It is not practicable to carry out accurate comparative measurements of β-glucuronidase activity in isotonic media, and water homogenates of the tissues, with or without partial purification, have always been employed for purposes of assay. On homogenizing a tissue in water, there are osmotic changes in the granules, and the greater part of the enzyme passes into the surrounding medium, while the fraction remaining in the granules becomes completely accessible to substrate. The enzyme in the water homogenate of a tissue thus displays its full potential activity (ignoring possible complications due to endogenous inhibitor—Section II, 11b), and changes in the β-glucuronidase activity of such a preparation

* See also Gianetto and Duve (1955).

cannot possibly represent alterations in the cellular permeability of an organ (Beyler and Szego, 1954b). The β-glucuronidase activity of a tissue can increase with surprising speed, but there is no evidence to suggest that this is due to anything other than a real change in the amount of active enzyme protein, which could of course occur through the combination of existing large molecules, as an alternative to complete synthesis.

Histochemical experiments on the localization of the enzyme in the cell agree with the biochemical evidence in some respects (Campbell, 1949), but not in others (Campbell and Levvy, 1950); but the existing histochemical methods for β-glucuronidase leave a lot to be desired (Burton and Pearse, 1952; Rutenburg and Seligman, 1953).

13. Catalysis of Condensation in vitro

The only workers to obtain evidence for the condensation of an aglycone with free glucuronic acid in the presence of β-glucuronidase *in vitro* were Florkin *et al.* (1942). Condensation was very slight, and the authors did not consider that their results had any bearing on the problem of glucuronide synthesis *in vivo*. In their experiments, borneol (saturated solution) and glucuronic acid or glucurone* (0.01 M) were incubated with beef spleen β-glucuronidase at pH 4.5. At the end of the incubation period, free glucuronic acid was removed by precipitation with copper sulfate and calcium hydroxide, and glucuronic acid in the filtrate was determined by the Tollens color reaction. The figure thus obtained was assumed to represent glucuronic acid in the form of borneol glucuronide. Even after several days incubation, only a small fraction (never more than 5%) of the glucuronic acid was found in this fraction. When the sensitive and specific color reaction for *o*-aminophenol glucuronide was introduced (Levvy and Storey, 1949), attempts were made to demonstrate the condensation of *o*-aminophenol with glucuronic acid in the presence of β-glucuronidase preparations, but without success (Karunairatnam and Levvy, 1949; *cf.* Section III, 4a).

It is perhaps significant that no other role than a hydrolytic one has been envisaged for the glucuronide-decomposing enzyme in microorganisms, although in certain instances the enzyme appears to be adaptive (Buehler *et al.*, 1949; Barber *et al.*, 1951). Every method for the assay of β-glucuronidase, or histochemical method for its localization in tissue sections, is based on the hydrolytic action of the enzyme.

* The confusion that has resulted from regarding solutions of glucurone (III) and glucuronic acid (I) as identical in their properties is dealt with by Spencer and Williams (1951).

14. Use in the Hydrolysis of Urinary Steroid Conjugates

β-Glucuronidase has been extensively employed as a reagent for the hydrolysis of urinary steroid conjugates (see, for example, Bayliss, 1952; Cox and Marrian, 1953a; Venning *et al.*, 1953: numerous references to this topic will also be found in Pincus, 1954). As compared with acid hydrolysis, enzymatic hydrolysis may give increased yields of aglycone and avoid artifacts. The specificity of the enzyme having been fairly well defined (Levvy and Marsh, 1952), hydrolysis of a conjugate by β-glucuronidase affords substantial proof of the β-glucopyranuronide structure. It must first be established, however, that hydrolysis is in fact due to β-glucuronidase and not to some other enzyme present as impurity in the preparation. Selective inhibition of hydrolysis by saccharo-1,4-lactone (Section II, 11a), in the form of saccharate solution, has been found useful in this connection (Cox, 1952; Cox and Marrian, 1953a,b).

Several workers have encountered difficulties in using the enzyme as a hydrolytic agent, some of which might perhaps have been avoided if limitations in the action of the enzyme had been appreciated. It is proposed to discuss these only in general terms (see also Levvy and Marsh, 1954).

The activity of β-glucuronidase added to urine may be drastically reduced, so far as any one substrate is concerned, by inhibitory substances present in the urine. As pointed out in Section II, 11b, the enzyme may, according to its origin, be subject to the variable action of an inhibitor in animal tissues, and it is possible that the latter may appear in urine. It seems probable, however, that a more important cause of the inhibition of hydrolysis of any one particular steroid conjugate (or group of steroid conjugates) is the presence in urine of other steroid or nonsteroid glucuronides. These being alternative substrates will in effect behave as competitive inhibitors for the enzyme. Inhibition will depend on the relative concentrations of "substrate" and "inhibitor," and on their relative affinities for the enzyme. It should also perhaps be noted that the relative affinities of two compounds for the enzyme are no criterion of their relative rates of hydrolysis by it.* Until such interfering substances have been eliminated, it is a mistake to rely on rapid hydrolysis of a selected glucuronide in urine by an enzyme preparation.

It is also a mistake to expect, even in the absence of interfering substances, complete hydrolysis by the enzyme in a given time. It is true that, *in vitro* at least, the equilibrium point in a reaction catalyzed by

* There is real need for more data on the affinities of known steroid glucuronides for the enzyme, and on their relative rates of hydrolysis by a standard enzyme preparation.

β-glucuronidase lies far over in favor of hydrolysis, but to attain the equilibrium point requires complete control of the experimental conditions. The reaction always follows an exponential course in its later stages. If the substrate concentration is high, the reaction is zero order to begin with, a fixed weight of substrate hydrolyzing in unit time. Incidentally, enzyme assays are done under these conditions. It is only when the enzyme ceases to be saturated with substrate that the reaction becomes exponential. By this time the enzyme may have lost considerable activity. Without control of the substrate concentration (and this includes all substrates present), one cannot therefore ensure that any given percentage will be hydrolyzed in unit time. This factor introduces difficulties into the use of the enzyme for the comparative assay of steroid glucuronides in urine.

15. Conclusions

Quantitative changes in the β-glucuronidase activity of animal tissues do not necessarily provide qualitative evidence on the function of the enzyme. Apparently healthy animals can show wide variations in the β-glucuronidase activity of a single tissue, and in the response of the enzyme to extrinsic agents (Section II, 8). β-Glucuronidase activity may be an incidental property of a structural protein, in which case alterations in activity could be devoid of physiological significance. It seems more probable, however, that they do indicate quantitative differences in some specialized aspect of metabolism.

So far as the possible function of β-glucuronidase is concerned, there is nothing on which to base an opinion except its hydrolytic action on steroid and other glucuronides. The purpose of the enzyme may very well be to release steroid hormones from their glucuronides in target organs. β-Glucuronidase may also participate in the catabolism of the acid mucopolysaccharides, in which the β-glucopyranuronide residue is one of the major constituents. As discussed in Section III, 6, provision of free glucuronic acid for transformation into other essential molecules may also be an important function of β-glucuronidase. By analogy with some other hydrolytic enzymes, it seems not impossible that β-glucuronidase might, under certain circumstances, act on existing glucuronides as a transferase for the glucuronide residue, but there is as yet no hint that it can act in this way.* The enzyme certainly plays no such part in the glucuronide-synthesizing system of liver (Section III).

The β-glucuronidase activity of all body tissues appears to be under

* Packham and Butler (1955) observed no transfer of the glucuronide residue, labeled with C^{14}, from injected naphthol glucuronide to orally administered *o*-aminophenol in the rat (*cf.* Section III, 5). Fishman and Green (1956) have recently reported glucuronide transfer by β-glucuronidase using propylene glycol (15%) as acceptor.

endocrine control (Section II, 5). It does not follow that an alteration in enzyme activity produced by a hormone is an essential feature of the metabolism of that hormone, nor that it is in any sense an adaptive response. The fact that an effect on growth is another frequent manifestation of hormone action probably explains the correlation that has been observed between the β-glucuronidase activity of a tissue and its state of proliferation, but the relationship is not an invariable one.

It would appear that little further information on the function of the enzyme is to be gained from comparative measurements of tissue β-glucuronidase activity. On the other hand, even on a purely empirical basis, such measurements may prove to be of increasing value for diagnostic purposes in medicine (Section II, 10). In healthy individuals, deviations from the mean in the β-glucuronidase activity of an organ or body fluid may reflect variations in endocrine constitution, and in the extreme case peculiar susceptibility to degenerative disease. Under suitably controlled conditions, the measurement of tissue β-glucuronidase activity in animals might be of value for the assay of steroid hormones (*cf.* Beyler and Szego, 1954b), since the effect on enzyme activity is usually rapid, and can easily be measured with considerable accuracy.

III. Glucuronide Synthesis

1. Theoretical Background

It would appear that a single mechanism is responsible for the production of all steroid and nonsteroid glucuronides in the animal body (Isselbacher and Axelrod, 1955; Dutton, 1955). Whether the mechanism that links glucuronide residues to *N*-acetylhexosaminide residues to form acid mucopolysaccharides is an identical one is less certain. A key point in this subject is whether free glucuronic acid is a precursor of the glucuronides, or whether it arises only after the hydrolysis of the latter. This point could have an important bearing on the problem of ascorbic acid synthesis in living tissues, if, as has been suggested by Horowitz and King (1953) and Isherwood *et al.* (1954), glucuronolactone is an intermediate in this process (see also Mosbach *et al.*, 1950).

Different schemes that have been put forward at one time or another for the biosynthesis of glucuronides are summarized below. According to Fishman's theory (Section II, 1), scheme (2) is the correct one, and β-glucuronidase the enzyme involved. It is possible, but unlikely, that some other enzyme in tissues could catalyze this condensation. β-Glucuronidase could also conceivably act in scheme (3), transferring the glucuronic acid residue from an 'active' derivative to the aglycone, but it would seem to be more probable that a different enzyme would be re-

quired for this reaction. The immediate precursor in scheme (3) could be formed from glucuronic acid itself* or from glucose, without fission of the 6-carbon chain, or from 3-carbon intermediates in carbohydrate metabolism.

$$\text{Glucose} + \text{aglycone} \xrightarrow{-H_2O} \beta\text{-glucoside} \xrightarrow[-2H]{O} \beta\text{-glucuronide} \quad (1)$$

$$\text{Glucuronic acid} + \text{aglycone} \xrightarrow{-H_2O} \beta\text{-glucuronide} \quad (2)$$

$$X\,CH\,(CHOH)_3CH\,COOH + \text{aglycone} \xrightarrow{-HX} \beta\text{-glucuronide} \quad (3)$$

Earlier work on glucuronide synthesis, largely involving whole animal studies, has been admirably reviewed by Williams (1947), and it is not proposed to discuss it here. It was difficult to draw any firm conclusions from this work, except on one point, namely that glucosides of alcohols and phenols are not oxidized to glucuronides in the animal body. Scheme (1) is thus excluded. On the whole, the earlier work tended to suggest that glucuronides are not formed directly from glucose, but from intermediary products of carbohydrate metabolism. Of late, further whole animal experiments have been carried out, this time using radioisotopes, with a view to establishing the origin of the precursor in scheme (2) or (3).

Recent remarkable progress on the mechanism of glucuronide synthesis could be said to date from the work of Lipschitz and Bueding (1939), who initiated the study of this process in surviving tissue slices. Their results suggested that free glucuronic acid is not the immediate precursor. This finding, which has been supported by other workers, excluded scheme (2). Substantial evidence against the participation of β-glucuronidase in glucuronide synthesis was obtained by Levvy and co-workers (Karunairatnam and Levvy, 1949; Karunairatnam *et al.*, 1949). Dutton and Storey (1951, 1954) were able to reconstitute the system in homogenates, and established scheme (3) as the correct one. The immediate precursor has been identified (Dutton and Storey, 1953; Storey and Dutton, 1955), and the steps in its formation from glycogen have been established (Strominger *et al.*, 1954).

The aglycones that have been commonly employed in studying the mechanism of glucuronide synthesis by tissue preparations are menthol, borneol, *o*-aminophenol and *m*-aminophenol. In the first two compounds, the glucuronide was determined by the Tollens color reaction after extraction with ether or ethyl acetate, and in the last two it was determined directly by the method of Levvy and Storey (1949). Crépy (1947) has

* As in the synthesis of glycogen from glucose, phosphorylation could be an essential preliminary to the utilization of glucuronic acid in glucuronide synthesis.

demonstrated the synthesis of estrogen glucuronides by liver slices: butyl alcohol was used for extraction, followed by determinations by the Kober and Tollens color reactions.

2. Distribution of the Synthetic System in the Body

It was found by Lipschitz and Bueding (1939) that the glucuronide-synthesizing system in tissue slices is present only in liver and, to a much smaller extent, kidney. Crépy (1947) could observe synthesis of estrogen glucuronides with no other tissue than liver. Karunairatnam *et al.* (1949) showed that the ability of tissues to synthesize glucuronides is quite unrelated to their β-glucuronidase activity. Tissues other than liver or kidney, for example spleen or malignant tumors, although high in β-glucuronidase activity, were devoid of synthetic power. The activity of the synthetic system in mouse liver was practically nil at birth, and only reached its ultimate value when the mice were one month old: this was the reverse of the change seen in liver β-glucuronidase activity in mice from the same colony (*cf.* Section II, 4). Measures leading to changes in adult mouse-liver β-glucuronidase activity, such as partial hepatectomy, or menthol or estrone administration (Sections II, 2,4, and 7), had no effect of any kind on the activity of the synthetic system. The "glucuronyl-transferase" of Dutton and Storey (1954; see Section III, 4c) is apparently confined to liver.

The observation that the ability to synthesize glucuronides is low in young mice may have a bearing on the suggestion that has frequently been made that glucuronic acid is a dietary growth factor (Robinson *et al.*, 1939; Almquist *et al.*, 1940; Deichmann *et al.*, 1952).

3. Inhibition

a. Saccharo-1,4-lactone. Saccharo-1,4-lactone, a powerful inhibitor of β-glucuronidase (Section II, 11a), had no effect whatsoever on the synthetic system in mouse-liver slices (Levvy, 1952b), although Campbell (1949) obtained convincing histochemical evidence for the penetration of intact mammalian cells by the lactone.* The lactone did not inhibit the reconstituted glucuronide-synthesizing system in liver homogenates (Dutton and Storey, 1954). At the neutral pH employed in measuring glucuronide synthesis, saccharo-1,4-lactone is unstable, but sufficient was added to maintain a concentration adequate to inhibit β-glucuronidase throughout all these experiments. It is therefore evident that β-glucuronidase does not participate in glucuronide synthesis.

* The lactone was fully effective against the intracellular β-glucuronidase in a suspension of intact microorganisms from the rumen of the sheep (Marsh, 1955).

b. Saccharic Acid. Saccharate ion, which does not lactonize in neutral solution, had a feeble inhibitory action in high concentration on glucuronide synthesis by liver slices (Karunairatnam and Levvy, 1949). This was considered to be a nonspecific effect on the metabolism of the slices since other dicarboxylic acids acted likewise. Essentially similar results for tissue slices were obtained by Storey (1950), but the reconstituted synthetic system in homogenates was unaffected by saccharate (Dutton and Storey, 1954). Sie and Fishman (1954) have provided further confirmation of this nonspecific action of saccharate on glucuronide synthesis by liver slices, and have shown that ethereal sulfate synthesis by the slices is similarly affected.

c. Glucurone and Glucuronic Acid. Glucurone (glucuronolactone, III), after treatment with one equivalent of bicarbonate solution, caused considerable inhibition of glucuronide synthesis by liver slices (Storey, 1950). It was believed that inhibition was due to glucuronate formed from the lactone in the presence of alkali, but conversion under such conditions must have been far from complete. It was subsequently shown that sodium or potassium glucuronate had no effect whatsoever on synthesis by slices or homogenates (Dutton and Storey, 1954). Sie and Fishman (1954) have confirmed that glucuronide synthesis in liver slices is inhibited by glucurone, but not by glucuronate (see also Touster and Reynolds, 1952). β-Glucuronidase is inhibited by glucuronate, but not by glucurone (Section II, 11).

d. Interruption of Cell Metabolism. Oxidation and phosphorylation are essential for glucuronide synthesis by liver slices, since inhibition is produced by anaerobiosis, cyanide, iodoacetate, fluoride, azide, and dinitrophenol (Lipschitz and Bueding, 1939; Crépy, 1947; Storey, 1950). Disruption of the cell is followed by complete, or almost complete, cessation of glucuronide synthesis, unless the preparation is fortified with essential factors and co-factors. When the thermostable precursor of Dutton and Storey (Section III, 4c) is added to homogenates, synthesis is not inhibited by anaerobiosis or cyanide, showing that oxidation is no longer an essential step.

e. Sulfate. Sulfate ion, which has no action on β-glucuronidase, was found to inhibit the synthesis of *o*-aminophenol glucuronide by mouse liver slices, by stimulating the competitive formation of *o*-aminophenol sulfate (Storey, 1950). Sulfate ion did not have this effect on the reconstituted synthetic system in mouse liver homogenates (Dutton and Storey, 1954), which was evidently deficient in at least one of the requisite factors for ethereal sulfate synthesis. Using rat liver slices, De Meio and Tkacz (1952) observed stimulation of phenol sulfate synthesis and depression of phenol glucuronide synthesis by sulfate ion. Sie and Fish-

man (1954) found no depression of *m*-aminophenol glucuronide synthesis by rat liver slices when synthesis of the ethereal sulfate was stimulated by addition of sulfate ion. It seems possible that this failure to observe competition between the two modes of conjugation may have resulted from the use of too much *m*-aminophenol.

4. The Mechanism of Synthesis in Tissue Slices and Homogenates

a. Glucuronic Acid. Lipschitz and Bueding (1939) found that glucuronic acid did not stimulate glucuronide synthesis by liver slices. This finding has been repeatedly confirmed, both for slices and homogenates (see, for example, the references in Section III, 3c). Glucuronate failed to restore glucuronide synthesis by slices under anaerobic conditions (Storey, 1950). Contrary to the statement of Sie and Fishman (1954), the only point that this type of observation can prove is that glucuronic acid is not the immediate precursor in glucuronide synthesis, although it could still conceivably be a remote precursor.* Glucuronate also failed to overcome the fall in glucuronide synthesis produced in mouse liver slices by sulfate ion (Storey, 1950), and in guinea pig liver slices by starving the animals (Lipschitz and Bueding, 1939).

b. Glucuronic Acid-1-phosphate. On theoretical grounds, Storey (1950) suggested that the immediate precursor in glucuronide synthesis might be glucuronic acid-1-phosphate, but when methods for the synthesis of the α- and β-anomers of this compound had been devised, neither was found to stimulate synthesis in tissue slices or homogenates (Levvy and Marsh, 1952; Touster and Reynolds, 1952; Dutton and Storey, 1954).

c. Derivatives of Uridine Diphosphate. A very important advance was made when Dutton and Storey (1951, 1954) obtained a thermostable factor from liver, addition of which to mouse liver preparations and free aglycone led to the synthesis of *o*-aminophenol or menthol β-glucuronide.† On subsequent purification, this factor was shown to be a glucuronic acid derivative of uridine diphosphate (UDP), in which glucuronic acid-1-phosphate and uridine-5′-phosphate (uridylic acid) were linked by a pyrophosphate bond (Dutton and Storey, 1953; Storey and Dutton, 1955). In the presence of the enzyme, "glucuronyl-transferase," the glucuronide

* De Meio and Arnolt (1944) found that glucuronate overcame the depression of phenol conjugation produced in liver slices by iodoacetate, although this was not confirmed in later experiments (De Meio and Arnolt, 1945). It was subsequently shown (Levvy and Storey, 1949; see also De Meio and Tkacz, 1952) that their method of estimation did not include phenol conjugated as the glucuronide, but only as the sulfate.

† The same system has been shown to accomplish the synthesis of the β-glucuronides of phenolphthalein, thyroxine, and two corticosteroids (Isselbacher and Axelrod, 1955).

residue was transferred from the factor to the aglycone (ROH), according to the equation:

$$\text{UDP-glucuronide} + \text{ROH} \rightarrow \text{UDP} + \text{R-glucuronide}$$

It will be noted that water is not involved in this reaction. The pH optimum was at 7.6 to 7.9, and the reaction was apparently irreversible. Glucuronyl transferase has been found only in liver, in which it was present in the cytoplasmic granules. Unlike β-glucuronidase, the transferase was destroyed by treatment with acetone or ammonium sulfate and rapidly lost activity on storage, even at low temperatures. The transferase was not inhibited by saccharo-1,4-lactone (Section II, 11a), and it could not be replaced in the above reaction by purified β-glucuronidase.

Dutton and Storey (1954) could not detect the factor UDP-glucuronide in any other animal tissue than liver, but there is evidence for its presence in Flexner-Jobling carcinomas (Schmitz *et al.*, 1954). The factor was not a substrate for β-glucuronidase, and this would seem to suggest that the glucuronide residue in it had the α-configuration,* since β-glucuronidase has been shown to hydrolyze β-, but not α-glucuronic acid-1-phosphate (Levvy and Marsh, 1952). Walden inversion would therefore appear to have occurred at C-1 in the glucuronide residue during the transfer from UDP to the aglycone. This point awaits confirmation by the identification of the glucuronic acid-1-phosphate anomer formed when UDP-glucuronide is degraded with dilute acid (Storey and Dutton, 1955).

Smith and Mills (1954) could observe no formation of UDP-glucuronide when uridine triphosphate (UTP) was incubated with α- or β-glucuronic acid-1-phosphate in the presence of a liver preparation. The following analogous reaction for α-glucose-1-phosphate, on the other hand, is well established:

$$\text{UTP} + \alpha\text{-glucose-1-phosphate} \rightleftharpoons \text{UDP-glucoside} + \text{pyrophosphate}$$

The intermediate steps linking glucuronide (and glucuronic acid) synthesis with glycogen metabolism were established when it was discovered (Strominger *et al.*, 1954) that liver contained an enzyme capable of oxidizing UDP-glucoside to UDP-glucuronide in the presence of diphosphopyridine nucleotide (DPN). This enzyme, which was apparently free in the cytoplasm, was acetone-stable. For each mole of UDP-glucoside oxidized, two moles of DPN were reduced. When glucuronyl-transferase and *o*-aminophenol were added to the system, *o*-aminophenol glucuronide

* In conformity with the probable structure of UDP-glucoside (Paladini and Leloir, 1952).

was formed. The complete process can therefore be summarized in terms of known reactions as follows:

$$\text{Glycogen} \overset{H_3PO_4}{\rightleftharpoons} \alpha\text{-glucose-1-phosphate}$$

$$\alpha\text{-Glucose-1-phosphate} + \text{UTP} \rightleftharpoons \text{UDP-glucoside} + \text{pyrophosphate}$$

$$\text{UDP-glucoside} \xrightarrow[\text{DPN}]{+O,\ -2H} \text{UDP-glucuronide}$$

$$\text{UDP-}\alpha\text{-glucuronide} + \text{ROH} \xrightarrow[\text{inversion}]{\text{Walden}} \text{UDP} + \text{R-}\beta\text{-glucuronide}^*$$

It can be seen that conversion into α-glucose-1-phosphate is an essential preliminary to the utilization of any other carbohydrate or carbohydrate precursor in glucuronide synthesis. The glucuronic acid residue makes its first appearance in the combined form. Free glucuronic acid may arise in two ways, either by the hydrolysis of a β-glucuronide by β-glucuronidase, or through degradation of UDP-glucuronide. The latter has been shown to be unstable in the presence of liver homogenates (Storey and Dutton, 1955) and to be attacked by phosphatase preparations (Dutton and Storey, 1954), while Levvy and Marsh (1952) have demonstrated hydrolysis of α-glucuronic acid-1-phosphate by a liver phosphatase.

5. Experiments with Radioactive Carbon

A great many experiments have been done in which various possible precursors of glucuronic acid, labeled with C^{14}, were administered to animals, along with a glucuronidogenic compound. After the isolation of the glucuronide from urine, determinations of C^{14} in the glucuronic acid moiety were carried out. In some instances, the results were at first in apparent conflict with those obtained with tissue preparations (Section III, 4), but these difficulties have been resolved by later work.

Glucose, glycerol, lactate, and pyruvate were all apparently equally effective as glucuronide precursors in the body (Mosbach and King, 1950; Packham and Butler, 1952; Eisenberg and Gurin, 1951; Doerschuk, 1952). As Eisenberg and Gurin (1952) pointed out, this follows from the fact that these compounds are in equilibrium with each other in the body. It also follows that glucose, uniformly labeled from C-1 to C-6, could give rise to uniformly labeled glucuronic acid (Mosbach and King, 1950), with or without preliminary fission of the hexose chain into three-carbon fragments. In fact, the same result was obtained after administering uniformly labeled lactate (Eisenberg and Gurin, 1951). Glucose la-

* Figures ranging from 50–100% have been variously reported for the efficiency of glucuronide transfer in this reaction.

beled only at C-1, however, was converted into a glucuronide labeled predominantly in the same position (Eisenberg and Gurin, 1952), proving that a large part of the hexose had been utilized without fission. Other workers (Douglas and King, 1953a; Bidder, 1952) have obtained similar results with asymmetrically labeled glucose. This finding is perfectly consistent with the mechanism for glucuronide synthesis described in Section III, 4, since glucose, like pyruvate, lactate, and glycerol, can be converted into α-glucose-1-phosphate *via* glucose-6-phosphate.

The incorporation of C^{14} into the glucuronide molecule after the administration of labeled glucurone or glucuronic acid has been studied by two groups of workers. Douglas and King (1952, 1953b) found that the administration of uniformly labeled glucurone was followed by the excretion of a glucuronide in which the glucuronic acid residue was unevenly labeled. Carbon atoms 1 to 3 were more radioactive than carbon atoms 4 to 6, indicating that at least some of the glucuronide was formed from three-carbon compounds, with preferential utilization of one half of the glucurone molecule. The same uneven labeling was observed in glucose from the liver glycogen of the glucurone-treated animals, suggesting that all the C^{14} utilized in glucuronide synthesis had first been converted into glucose or glucose phosphate. Final proof that the glucuronic acid molecule is not a glucuronide precursor was obtained by administering glucurone labeled only at C-6. There was almost complete loss of C^{14} as exhaled CO_2 or as free glucuronic acid in the urine, and only traces were found in the conjugated glucuronide.*

Packham and Butler (1952, 1954) observed that the percentage incorporation of C^{14} into naphthol glucuronide was much greater after injecting labeled sodium glucuronate than after administering labeled lactate, pyruvate, or glucose, and they concluded that glucuronic acid condenses directly with the aglycone. Given orally, however, sodium glucuronate was no more effective than any other compound. The sodium glucuronate employed in these experiments was prepared from naphthol glucuronide isolated from urine after feeding labeled lactate or starch, and it subsequently transpired (Butler and Packham, 1955) that it was contaminated with unhydrolyzed naphthol glucuronide. After injection, the glucuronide was largely excreted unchanged.† When labeled sodium glucuronate prepared by chemical synthesis was injected, very little of

* See also Eisenberg *et al.*, 1955.

† Garton and Williams (1949) have shown that there is much greater hydrolysis of a conjugated glucuronide in the body after administration by mouth than after injection (see also Odell *et al.*, 1937). This probably explains the difference between "sodium glucuronate" given by mouth and by injection in the earlier experiments of Packham and Butler (1952).

the C^{14} was utilized for glucuronide synthesis, and, accordingly, it was concluded that glucuronic acid does not participate in this process.

The experiments with uniformly labeled glucurone (Douglas and King, 1952, 1953b) provide quantitative information on the fate of this compound in the body. One day after intraperitoneal injection of a glucurone solution, 50% of the C^{14} was excreted in the urine as free glucuronic acid and 35% was exhaled as CO_2. There was little fixation of C^{14} in the tissues, apart from liver which contained up to 4% of the total dose. Simultaneous administration of a glucuronidogenic compound had no appreciable effect on these figures, but about 5% of the C^{14} was excreted as conjugated glucuronide.*

Although glucurone and glucuronic acid are interconvertible in aqueous solution, opening of the lactone ring is slow at physiological pH. When labeled glucuronic acid (Butler and Packham, 1955) was injected intravenously or intraperitoneally, more was excreted unchanged than after injection of glucurone. Much less C^{14} appeared as CO_2 and conjugated glucuronide. It seems probable that the fate of glucuronic acid in the body is determined largely by the rates of absorption and excretion, and that oral administration or subcutaneous injection of sodium glucuronate would favor oxidation of the glucuronic acid and utilization of the metabolites. There was as much exhalation of $C^{14}O_2$ after oral administration of impure, labeled sodium glucuronate (Packham and Butler, 1952) as after injection of glucurone. Any apparent differences in the fates of glucurone and sodium glucuronate in the body are likewise more probably due to differences in excretion rate than to qualitative differences in metabolism, although Butler and Packham (1955) favor the latter alternative.

6. Conclusions

It is clear that there are at least two distinct enzyme systems in the body concerned with the metabolism of the conjugated glucuronides. One of these, β-glucuronidase, is present in practically every tissue, and its action *in vivo* is entirely hydrolytic. The other enzyme system, a more complex one, is responsible for the synthesis of glucuronides and is found only in liver and, to a smaller extent, kidney. These conclusions were arrived at by Karunairatnam *et al.* (1949), who put forward the following views regarding the functions of the two enzyme systems. "Taken together, the two enzyme systems may provide a mechanism for regulating the transport, action and excretion of physiologically active, glucuronidogenic compounds, such as oestriol. Alternatively, their function may be to provide free glucuronic acid or a transformation product for building

* Traces of metabolized glucurone may appear in the urine as L-xylulose (Touster *et al.*, 1955).

up into more complex molecules." These views and conclusions would still appear to be substantially correct.

Now that the mechanism of glucuronide synthesis from glycogen has been elucidated, it can be seen that the glucuronic acid residue makes its first appearance when the glucose derivative of uridine diphosphate is oxidized in the penultimate stage of the synthesis. The resulting glucuronic acid derivative of uridine diphosphate reacts with alcohols and phenols in the presence of the appropriate transferase to form conjugated glucuronides. In contradiction of Fishman's theory (Section II, 1), neither β-glucuronidase nor free glucuronic acid participates in the process at any stage. In fact, the only endogenous source of free glucuronic acid in the body would appear to be hydrolysis of a β-glucuronide by β-glucuronidase, or degradation of the glucuronic acid derivative of uridine diphosphate.* Although the complete glucuronide-synthesizing system is confined to liver and kidney, it does not follow that individual components of the system may not be found in other tissues. With regard to the site of conjugation of steroids (and other alcohols and phenols) with glucuronic acid in the body, the determinant feature will be the distribution of the glucuronyl transferase of Dutton and Storey (1954). This has so far been found only in liver, but it is probably also present in small amounts in kidney.

The remaining outstanding problem in the field of glucuronide metabolism at the present time is the incorporation of the β-glucuronide residue into the molecules of acid mucopolysaccharides, such as hyaluronic acid, a process that is by no means confined to liver and kidney. One possibility (Smith and Mills, 1954; Storey and Dutton, 1955) is that the glucuronide-synthesizing system in liver provides disaccharides containing a β-glucuronide moiety for acid mucopolysaccharide synthesis in other tissues. It has still to be shown, however, that the glucuronyl transferase in liver can utilize a sugar as acceptor, or if it does that the right type of disaccharide will be produced. Sucrose (Leloir and Cardini, 1953) and trehalose (Leloir and Cabib, 1953) can be synthesized from the glucose derivative of uridine diphosphate, but both of these are nonreducing disaccharides. For acid mucopolysaccharide synthesis, on the other hand, 1,3- or 1,4-linked, reducing disaccharides would be required. It may very well be found that a distinct enzyme system, widely distributed in the body, is responsible for the formation of the β-glucuronide linkage in acid mucopolysaccharides, and that the system hitherto studied in liver is specific for the synthesis of heteroglucuronides, such as those derived from steroids.

* Derangement of glucuronide synthesis might thus affect the production of ascorbic acid in those animals that can synthesize this vitamin (*cf.* Mosbach *et al.*, 1950; Horowitz and King, 1953; Isherwood *et al.*, 1954).

IV. Summary

The enzyme β-glucuronidase is practically ubiquitous in the animal body. It catalyzes the hydrolysis of the naturally occurring glucuronides of steroid and nonsteroid alcohols and phenols, and also acts upon degradation products of chondroitin and hyaluronic acid. The activity of the enzyme in sex and nonsex organs is under endocrine control and is also governed by hereditary factors.

A more complex enzyme system, present only in liver and kidney, accomplishes the synthesis of the conjugated glucuronides from glycogen and the aglycones. Neither β-glucuronidase nor free glucuronic acid participates in this process at any stage. Whether the same synthetic system is involved in the production of chondroitin and hyaluronic acid is as yet unknown.

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Bioassay of Pituitary and Placental Gonadotropins in Relation to Clinical Problems in Man

JOHN A. LORAINE

Clinical Endocrinology Research Unit (Medical Research Council), University of Edinburgh, Scotland

I. Introduction

In 1927, Aschheim and Zondek demonstrated that the blood and urine of pregnant women contained a gonad-stimulating substance which, when injected subcutaneously into intact immature female mice, produced follicular maturation, luteinization and hemorrhage into the ovarian stroma. In the years immediately following this discovery there was much speculation regarding the sources of the various gonadotropic factors in man. It soon became clear that gonadotropins could be classified according to their site of production, and that both the pituitary and placenta were capable of elaborating these substances.

The original view put forward by Aschheim and Zondek (1927) that the gonadotropic factor in pregnancy urine was derived from the pituitary was soon found to be untenable, and in the early 1930's a large amount of experimental evidence was presented by numerous investigators to show that the gonadotropic principle which floods the body during pregnancy emanates from the placenta and not from the pituitary (Engle, 1929; Philipp, 1930; Collip, 1930, 1934, 1935; Collip *et al.*, 1933; Hamburger, 1933; Deanesly, 1935). Probably the most cogent evidence for the placental origin of the hormone was the demonstration that the gonadotropin was produced *in vitro* in placental tissue culture and that this property was exhibited by the chorionic villi, being especially marked in the Langhans cells (Sannicandro, 1934; Gey *et al.*, 1938; Jones *et al.*, 1943; Stewart *et al.*, 1948). Confirmatory evidence for this view was obtained by histochemical studies (Wislocki and Bennett, 1943; Zilliacus, 1953) and by intraocular transplantation experiments (Kido, 1937; Stewart, 1951). Accordingly the substance, which was originally called "pregnancy prolan" or "anterior pituitary-like substance" (APL) became known as "human chorionic gonadotropin" (HCG). Purified preparations of HCG have been made by Gurin *et al.* (1939, 1940), Katzman *et al.* (1943), Claesson *et al.* (1948), and Landgrebe *et al.* (1954). A potency of 6,000 to 8,000 International Units per milligram has been claimed. The gonadotropin is thought to be a glycoprotein, the molecule of which contains carbohydrate in the form of galactose (Gurin, 1942). HCG has a predominantly luteinizing action but differs to some extent in its chemical and biological properties from pituitary interstitial-cell–stimulating hormone.

It is generally believed that pituitary extracts from sheep and swine prepared by techniques involving fractional precipitation with organic solvents and inorganic salts combined with pH adjustment contain two distinct gonadotropic principles (Fevold *et al.*, 1931; Van Dyke and Wallen-Lawrence, 1933; Bates *et al.*, 1934; Jensen *et al.*, 1939; Shedlovsky *et al.*, 1940; McShan *et al.*, 1954). These substances have been termed the "follicle-stimulating hormone" (FSH) and "interstitial-cell–stimulating" or "luteinizing hormone" (ICSH or LH). Claims have been made regarding the "biological" and "chemical" purity of these substances. ICSH preparations have been obtained which, when administered to hypophysectomized immature female rats, had no effect on the follicles but produced interstitial cell repair. Conversely, highly purified FSH preparations have been shown to cause enlargement of the ovarian follicles without any significant effect on the interstitial tissue. Two independent laboratories have stated that pituitary ICSH has been obtained in a pure state and that the preparation when subjected to electrophoretic, ultracentrifuge and solubility tests behaves as a homo-

geneous protein (Li *et al.*, 1940; Shedlovsky *et al.*, 1940). According to Li *et al.* (1940), sheep ICSH has a molecular weight of 40,000 and an isoelectric pH of 4.6. The hormone is insoluble in 66% acetone and contains carbohydrate in its molecule. Swine ICSH, on the other hand (Shedlovsky *et al.*, 1940), has a molecular weight of 100,000 and an isoelectric pH of 7.45. It is also insoluble in 66% acetone and contains carbohydrate in its molecule. Although highly purified preparations of pituitary FSH have been made, it has not yet been claimed that the hormone has been isolated in the pure state. Sheep FSH is believed to be a glycoprotein with a molecular weight of 70,000 and an isoelectric pH of 4.5. The properties of pig FSH are very similar.

It has been suggested that pituitary extracts contain a third gonadotropic principle which in rats is responsible for the maintenance of activity of corpora lutea and for the secretion of progesterone (Astwood, 1941, 1953; Evans *et al.*, 1941). This substance has been termed "luteotropin" and is believed to be identical with the lactogenic hormone prolactin. There is, however, no definite evidence that luteotropin is excreted in human urine and its physiological function in man remains obscure.

The assumption has been made that human nonpregnant urine contains two gonadotropic substances, FSH and ICSH, which are derived from the pituitary and are similar in their chemical and biological properties to the hormones isolated from animal pituitaries. However, it has not so far been possible to separate human urinary FSH from human urinary ICSH. Indeed it has been suggested recently (Segaloff *et al.*, 1954) that human nonpregnant urine contains a single gonadotropic substance with both follicle-stimulating and luteinizing activities.

Estimations of HCG were first performed in the clinical field as a means of pregnancy diagnosis. The effect of the hormone on the ovaries of mice formed the basis of the Aschheim-Zondek test. Other pregnancy tests depended on ovulation in rabbits (Friedman and Lapham, 1931), ovulation in the South African clawed toad, *Xenopus laevis* (Hogben *et al.*, 1931; Landgrebe, 1948), and release of spermatozoa by male toads (Galli-Mainini, 1947). An early diagnostic application of HCG assay was its demonstration in very high titer in conditions such as hydatidiform mole, chorionepithelioma, and certain types of testicular tumor where actively proliferating chorionic tissue is present (Zondek, 1929, 1937, 1942; Crew, 1936; Hamburger, 1943). The diagnostic and prognostic significance of urinary and serum estimations of HCG has also been studied in cases of hyperemesis gravidarum (Schoeneck, 1942), pre-eclamptic toxemia (Smith and Smith, 1939; Loraine and Matthew, 1950), essential hypertension in pregnancy (Watts and Adair, 1943; Loraine and Matthew, 1950), and pregnancy complicated by diabetes (White, 1946;

Loraine and Matthew, 1954). It has not, however, been conclusively demonstrated that HCG assays are of any great help in the management of patients suffering from these diseases.

Assay of pituitary gonadotropins by the mouse uterus test has been of considerable value to clinicians in differentiating primary gonadal failure from gonadal failure secondary to lesions of the pituitary. In conditions such as ovarian agenesis (Turner's syndrome) or sclerosing tubular degeneration of the testes (Klinefelter's syndrome) the excretion of gonadotropins is abnormally high, while in panhypopituitarism the quantities found in urine are extremely small. Assays have also been performed in a wide variety of medical and gynecological conditions including acromegaly, Cushing's syndrome, various types of infantilism and precocity, amenorrhoea, and dysfunctional menstrual irregularities, but the diagnostic value of such estimations has not yet been definitely established. It has been claimed that the gonadotropin excretion may be of considerable prognostic significance in patients suffering from mammary carcinoma and that high titers are generally associated with a good prognosis (Segaloff *et al.*, 1954). This interesting finding requires further confirmation. It is well known that estrogens depress the titer of urinary gonadotropins. The suggestion has been made that in postmenopausal patients the potency of various estrogens may be compared by their ability to depress the urinary excretion of pituitary gonadotropins (Tokuyama *et al.*, 1954).

Many investigators in recent years have attempted to increase the accuracy and specificity of gonadotropin assays. The introduction by the League of Nations in 1939 of an International Standard for HCG made it possible to express assay results in International Units and to eliminate the wide variety of "animal units" which had previously been employed. The precision of HCG assays using different indices of response has been studied by Gaddum (1950), Diczfalusy (1953, 1954), Bukovics and Wohlzogen (1953), and Diczfalusy and Loraine (1955). No International Standard preparation is yet available for comparative assay of human nonpregnant urinary gonadotropins. Recent work from this laboratory has shown, however, that it is a valid procedure, as far as statistical criteria are concerned, to assay gonadotropins from normal men and from normally menstruating women in terms of human menopausal gonadotropin (HMG)* (Loraine and Brown, 1955).

Many investigators have attempted to devise assay methods specific

* Human menopausal gonadotropin (HMG): this term will be used to designate the gonadotropic activity in the urine of menopausal and postmenopausal women. Such urine extracts contain both FSH and ICSH activity. The term HMG was introduced at the first meeting of the Gonadotrophin Club in Geneva (August, 1953).

for pituitary FSH and pituitary ICSH respectively. According to Evans *et al.* (1939) the test depending on microscopically detectable follicular enlargement in hypophysectomized female rats is specific for FSH and is not appreciably affected by ICSH. On the other hand, Greep *et al.* (1942) claim that the enlargement of the ventral lobe of the prostate in hypophysectomized male rats provides a test for ICSH which is unaffected by the presence of FSH in the solutions used. In the majority of estimations performed for clinical purposes a nonspecific assay method depending on the enlargement of the uterus in intact immature mice has been employed.

This article deals with assay methods for FSH, ICSH and HCG, with the excretion of these hormones in normal subjects, and with the diagnostic value of such estimations when applied to a study of pathological conditions in man. Luteotropin and pregnant mares' serum gonadotropin are not discussed, nor is any attempt made to review the physiological and chemical properties of the various gonadotropins. For a fuller discussion of these topics the reader is referred to publications by Fevold (1937), Fosco (1943), Thayer (1946), Li and Evans (1948), Evans and Simpson (1950), Gaddum and Loraine (1950), Loraine (1954a), Noble and Plunkett (1955), and Morris (1955).

II. Methods of Assay of Pituitary Gonadotropins in Urine

1. General

It is generally believed that men, nonpregnant women and children excrete gonadotropins with both follicle-stimulating and luteinizing activities and that these hormones are elaborated by the anterior pituitary. The urinary titer is relatively high at and beyond the menopause, after castration, and in various types of gonadal failure, but is low in children, in men, and in women during reproductive life. At present no available assay method is sufficiently sensitive to detect FSH and ICSH activity with any regularity in blood and accordingly the great majority of studies have been conducted on urine extracted and concentrated by a variety of procedures. The test animals employed have generally been rats or mice, which are more sensitive to stimulation by gonadotropic hormones than are, for example, rabbits, guinea pigs or amphibia. Ideally, assays should be performed in hypophysectomized animals in order that the endogenous production of gonadotropins does not interfere with the results.

2. Standard Preparations

One of the great difficulties in the estimation of pituitary gonadotropins in urine is the fact that no International Standard preparation is available for comparative assay of these substances. It has therefore

been necessary to express results of clinical assays in arbitrary "rat" and "mouse" units, a unit being defined as that quantity necessary to produce a given effect. Arbitrary units employed in intact and hypophysectomized rats have included the doses necessary to cause vaginal cornification (Katzman and Doisy, 1934), to produce histological changes in the ovaries (Frank and Salmon, 1935), and to cause 50–100% increase in weight of the ovaries (Wallen-Lawrence and Van Dyke, 1931), of the uterus (Evans and Gorbman, 1942), of the seminal vesicles (Fevold, 1939), and of the ventral lobe of the prostate (Greep *et al.*, 1942). In intact immature mice most investigators have adopted as a "unit" the quantity of extract producing 100–200% increase in uterine weight (Levin and Tyndale, 1937; Varney and Koch, 1942; Catchpole *et al.*, 1942; Smith *et al.*, 1943; Klinefelter *et al.*, 1943). Lloyd *et al.* (1949) have proposed a "mouse hyperemia unit" and defined it as the dose necessary to cause hyperemia in 25% of eight ovaries.

Trevan (1927) was among the first to note that the threshold dose of a substance varies enormously even when an animal colony is kept under relatively constant environmental conditions. It is now generally recognized that the error of estimations expressed in "animal" units without reference to a standard preparation may be threefold or more and that results calculated in this way have little quantitative significance.

Recent work in this laboratory has shown that the urinary gonadotropins from men and from normally menstruating and postmenopausal women can be assayed in terms of HMG (Loraine and Brown, 1955). The standard preparation (HMG 20) was prepared by Messrs. Organon (Newhouse, Scotland) from the urine of menopausal and postmenopausal women. The kaolin-acetone method of extraction was employed, and the crude material was treated by tricalcium phosphate in order to reduce toxicity. Two bioassay methods were used, one depending on the enlargement of the uterus in intact immature mice, and the other on the enlargement of the ventral lobe of the prostate in hypophysectomized immature rats. Pooled urine was collected from normal male subjects, from normally menstruating women during the follicular and luteal phases of the cycle, and from postmenopausal subjects. The method of extraction of the gonadotropins from urine was similar to that adopted for the preparation of the standard. A total of 16 four-point assays was performed in which the various gonadotropins were compared with HMG 20. The weighted mean index of precision (λ) was 0.127 for the mouse uterus test and 0.109 for the ventral prostatic weight test. Statistical calculations by the techniques recommended by Finney (1952) and by Gaddum (1953a,b) showed that there was no significant difference between the slopes of the various log dose-effect curves or in the results given by the two methods.

It has recently been suggested (Gaddum, 1955) that data of this type can be expressed in terms of the "index of discrimination." This is equal to the ratio of the two results which would be obtained if one of the substances were used to assay an unknown extract containing the other. The figures obtained in the present investigation are shown in Table I.

TABLE I

COMPARISON OF URINARY GONADOTROPINS FROM MALES AND FROM NORMALLY MENSTRUATING AND POSTMENOPAUSAL WOMEN WITH HMG 20 USING DIFFERENT ASSAY METHODS

Urinary extract administered	Ratio of activities, standard/unknown uterine wt. test (mice)	Ratio of activities, standard/unknown ventral prostatic wt. test (rats)	Mean index of discrimination, uterus/ventral prostate
Male	54/1	33/1	
	36/1	36/1	1.30
Follicular phase female	114/1	80/1	
	110/1	91/1	1.31
Luteal phase female	125/1	87/1	
	68/1	80/1	1.15
Postmenopausal female	3.0/1	2.8/1	
	6.8/1	4.8/1	1.28

It is evident that with all four types of urinary extract the index was approximately equal to unity. This suggests that the active principle measured by the two tests is identical and provides further evidence that there is no qualitative difference among the various nonpregnant gonadotropins when extracted from urine by the kaolin-acetone method. Walter (1955) has recently claimed that the index of discrimination may vary with the method of extraction used.

3. Tests Claimed to Be Specific for FSH

Most investigators agree that any specific assay method for FSH must employ hypophysectomized animals and that in intact animals the secretion of gonadotropins from the animal's own pituitary will interfere with the response of the end organs. In general, methods have depended on observations of the ovaries and testes of rats after hypophysectomy. Recently, however, an assay method for FSH has been described using the ovarian weight test in intact immature rats primed with relatively large doses of HCG.

Three assay methods for FSH will be considered:

(a) Follicular growth in hypophysectomized immature female rats (Evans *et al.*, 1939).

(b) Increase in testicular weight in hypophysectomized immature male rats treated with an excess of HCG (Paesi *et al.*, 1951).

(c) Increase in ovarian weight in intact immature rats treated with HCG (Steelman and Pohley, 1953).

a. Follicular Growth in Hypophysectomized Immature Female Rats. This test has been used to estimate the FSH content of sheep, pig, and human pituitaries (Evans *et al.*, 1939; Greep *et al.*, 1942; Bahn *et al.*, 1953a,b,c). In the technique described by Evans *et al.* (1939) hypophysectomized immature rats were injected subcutaneously once per day for 3 days. The animals were killed 72 hours after the first injection and the ovaries examined histologically. A "unit" was arbitrarily defined as the minimum quantity of extract necessary to produce healthy nonatretic follicles with small antra. This method is claimed to be highly specific and sensitive but no information is available about its accuracy. It has the additional advantage that the presence of ICSH in pituitary extracts can be detected by its effect on the ovarian interstitial tissue.

So far assays by this technique have been mainly restricted to pituitary tissue and few attempts have been made to estimate FSH activity in human urine using hypophysectomized female rats. Leathem and Levin (1941a,b) prepared highly concentrated extracts from pooled male urine and assayed their potencies in hypophysectomized female rats. The extracts produced follicular stimulation without luteinization. Evans and Gorbman (1942), using similar material, produced follicular stimulation in hypophysectomized rats with total dosages equivalent to 500–1300 ml. of original urine per animal. Bahn *et al.* (1953a,c) extracted pooled urine from males and from normally menstruating women and found that follicular stimulation occurred with a total dose per animal of concentrate equivalent to 1200 ml. of original urine.

Studies in most laboratories with extracts prepared from the urine of menopausal and postmenopausal women have generally indicated that the female hypophysectomized rat is relatively insensitive to stimulation by pituitary gonadotropins. It is therefore necessary to prepare highly concentrated extracts from urine which, in dosages required to produce follicular growth in the ovaries, are frequently toxic to the experimental animals. Accordingly, there is little likelihood that this assay method will prove of much value for the estimation of FSH activity in clinical practice.

b. Increase in Testicular Weight in Hypophysectomized Immature Male Rats Treated with Excess of HCG. This test was described by Paesi *et al.* (1951) and was used to estimate the FSH content of rat pituitaries. Hypophysectomized immature male rats were implanted intra-abdominally with pituitary tissue from castrated male and female rats. The right testis

was removed on the day of implantation. All animals received 10 I.U. of HCG per day for seven days. They were killed on the eighth day and the left testis was weighed. The difference in weight between right and left testes was taken as an index of the FSH activity of the implanted pituitaries.

This assay method was employed by Diczfalusy (1953) to determine whether placental extracts contained demonstrable quantities of FSH, but the technique has not so far been employed to assay the FSH content of human urine extracts. The method is laborious and tedious and its specificity is somewhat questionable in view of the finding of Simpson *et al.* (1944) that purified preparations of both FSH and ICSH are capable of causing testicular growth in hypophysectomized rats.

c. Increase in Ovarian Weight in Intact Immature Rats Treated with HCG. This method depends on the observation that HCG will augment the action of FSH on the rat ovary. It has been used by Steelman and Pohley (1953) to assay FSH in pituitary glands. Neal *et al.* (1954) using rats, and Brown (1955) using mice, have adapted the method for studies on human urine. In the procedure described by Steelman and Pohley (1953) intact immature rats were injected subcutaneously thrice per day for 3 days with a purified preparation of swine FSH and with HCG. The animals were killed 72 hours after the first injection, at which time the ovaries were dissected out and weighed. A total dose of 20 I.U. of HCG per animal was found to produce a satisfactory degree of augmentation of ovarian weight and thereby to increase markedly the sensitivity of the animals to exogenous FSH. Over the dosage range used there was a linear relationship between the dose of injected FSH and the ovarian weight. Other pituitary hormones, including a purified preparation of swine ICSH, did not interfere with the response. In hypophysectomized female rats the method was less sensitive and less accurate.

This test appears promising as a simpler means of determining FSH activity in human urine. Further information should be accumulated regarding its accuracy, specificity and sensitivity.

4. Tests Claimed to be Specific for ICSH

As for FSH, most assays for ICSH have to be conducted on hypophysectomized animals. The following methods will be considered:

(a) repair of the interstitial tissue in the ovaries of hypophysectomized immature female rats (Evans *et al.*, 1939);

(b) enlargement of the prostate in hypophysectomized immature male rats (Greep *et al.*, 1942; McArthur, 1952; Loraine and Brown, 1954, 1955);

(c) enlargement of the seminal vesicles in hypophysectomized immature male rats (McArthur, 1952; Loraine and Brown, 1954);

(d) increase of activity of prostatic alkaline phosphatase in hypophysectomized immature male rats (Schaffenburg and McCullagh, 1951).

a. Repair of Interstitial Tissue in Hypophysectomized Immature Female Rats. This test was used by Evans *et al.* (1939) to estimate the ICSH content of sheep pituitary extracts. The injection schedule was similar to that described for the assay of FSH except that injections were given intraperitoneally instead of subcutaneously. ICSH has a specific action in repairing the degenerated interstitial cells resulting from hypophysectomy. This is indicated by the reappearance of a normal nuclear pattern and by the disappearance of the characteristic "wheel cells" in the ovaries. The test is claimed to be highly specific for ICSH, but no information is available about its accuracy. The assay method when applied to the estimation of ICSH in human urinary extracts has been shown to be insensitive. Fraenkel-Conrat *et al.* (1940), using histological changes in the ovaries of hypophysectomized female rats, were able to demonstrate both ICSH and FSH activities in urinary extracts derived from menopausal and postmenopausal women. Evans and Gorbman (1942) extracted pooled male urine by alcohol precipitation with dialysis. Interstitial cell repair and follicular stimulation were demonstrated with total dosages per animal equivalent to 350–1330 ml. of original urine. The minimal doses for follicular stimulation and interstitial cell repair were approximately equal. These results are at variance with those of Bahn *et al.* (1953a,c), who extracted pooled male and female urine by the kaolin method and found that the minimal dosage for interstitial cell repair was much greater than for follicular stimulation.

It is evident that this assay method for ICSH, like that for FSH depending on follicular growth, is too insensitive and laborious for routine use in the clinical field. It is possible, however, that the sensitivity of both tests could be increased, e.g. by priming the animals with dosages of HCG insufficient in themselves to cause demonstrable histological changes in the ovaries.

b. Enlargement of the Prostate in Hypophysectomized Immature Male Rats. According to Greep *et al.* (1942) this is a unique test in that it is specific for ICSH and is not affected by the presence of FSH. The assay method has been used to estimate ICSH activity in sheep pituitary extracts (Greep *et al.*, 1942), in swine pituitary extracts (Simpson *et al.*, 1943), in the urine of normal men and women (McArthur, 1952; McArthur *et al.*, 1955; Bahn *et al.*, 1953a,c; Loraine and Brown, 1955), and in urine from menopausal and postmenopausal women (Greep and Jones, 1950; Loraine and Brown, 1954; Borth *et al.*, 1954). The design of the assay has varied somewhat in different laboratories. Greep *et al.* (1942) commenced injections on the third day after hypophysectomy, while

McArthur (1952) and Loraine and Brown (1954) employed a five-day rest period between hypophysectomy and the start of injections. It has been found that the method is more sensitive and more accurate with a four- or five-day rest period than with a seven-day rest period (Loraine and Brown, 1954). Most workers inject the animals subcutaneously once or twice per day for four days and kill them 96 hours after the first injection. Simpson *et al.* (1943) claim that the sensitivity of the assay is increased when injections are made intraperitoneally instead of subcutaneously. The precision of the method is greater than when the ventral lobe of the prostate, rather than the total gland, is weighed. In studies on menopause urine Loraine and Brown (1954) found a weighted mean index of precision (λ) of 0.174 for the ventral prostate and 0.182 for the total prostate. In four-point assays in which male and nonpregnant female gonadotropins were estimated in terms of HMG, the weighted mean figure for λ was 0.109 for the ventral prostate and 0.140 for the total prostate.

McArthur (1952) extracted pooled urine from normally menstruating women by the kaolin method with subsequent alcohol precipitation. Concentrates of as little as 3 hours' output of urine showed significant ICSH activity by the ventral prostate test. Over the dosage range used there was a linear relationship between the log dose and the effect. Loraine and Brown (1954) demonstrated that pooled urine from menopausal and postmenopausal subjects is a relatively rich source of ICSH activity. Crude extracts were prepared by the kaolin-acetone method and were subsequently treated by tricalcium phosphate in order to reduce toxicity. Mean increases in ventral prostatic weight of 65, 155 and 265% over untreated control animals were obtained with total doses per rat equivalent approximately to 15, 30 and 60 ml. respectively of original urine. Quantitatively similar results were obtained by Borth *et al.* (1954) who used the same preparation of HMG without preliminary treatment by tricalcium phosphate.

More recent studies (Loraine and Brown, 1955) have indicated that ICSH activity can be detected by this method in pooled male urine and in pooled urine obtained during the follicular and luteal phases of the normal menstrual cycle. Treatment of the crude kaolin extracts by tricalcium phosphate made it possible to administer to hypophysectomized rats total doses equivalent to approximately 1 liter of original urine. Pooled male urine was found to contain approximately four times more gonadotropic activity than pooled follicular and luteal phase urine. When extracts of normal male and female urine were assayed against HMG using a four-point design, the slopes of the dose-response curves did not show any significant deviation from parallelism.

This assay method is sufficiently sensitive to estimate ICSH activity in clinical conditions in man. Although the technique is too tedious and too laborious for routine studies, it is probable that valuable information will be obtained by its use in selected patients. Recently Segaloff (1955) has stated that prolactin sensitizes the prostate to the action of ICSH and that the assay method may not be as specific as previously believed. This interesting observation requires further study.

c. Enlargement of the Seminal Vesicles in Hypophysectomized Immature Male Rats. This method has been used to estimate ICSH activity in nonpregnent female urine (McArthur, 1952) and in menopause urine (Loraine and Brown, 1954). The technique of assay is similar to that described for the prostate. This test is less sensitive than that depending on prostatic weight and is therefore not to be recommended for the assay of urinary ICSH in man.

d. Increase in Activity of Prostatic Alkaline Phosphatase in Hypophysectomized Immature Male Rats. This method was described by Schaffenburg and McCullagh (1951) and used by McCullagh *et al.* (1953) to estimate urinary ICSH activity in cases of eunuchoidism. Animals were injected twice per day for three days and were killed four days after the first injection. Prostatic alkaline phosphatase was estimated by the technique of Huggins and Talalay (1945). It was concluded that the response was specific for ICSH and was not affected by FSH or prolactin. Results of the assay were expressed in terms of the international standard for HCG. As described by the authors the method is at best semiquantitative and probably does not satisfy the criteria of a valid bioassay.

5. Nonspecific Assay Methods for Pituitary Gonadotropins

Such tests generally depend on observations in intact immature rats and mice. Assays can be divided according to whether the effect is primary or secondary. In the primary group the gonads themselves are inspected, while in the secondary group the effects result from the liberation of estrogens or androgens from the gonads. Some of the tests proposed by various investigators are shown in Table II.

Many of these assay methods are not sufficiently sensitive to measure gonadotropic activity in human nonpregnant urine and will not be discussed by the reviewer. Consideration will be given to the following three methods: (a) the mouse uterus test, (b) the ovarian hyperemia test in rats and mice, (c) the test depending on the expulsion of spermatozoa in *Xenopus laevis*.

a. Mouse Uterus Test. This assay method has gained wide popularity for the estimation of pituitary gonadotropins in human urine. It is a highly sensitive test and gives positive results with the majority of urine

TABLE II

METHODS OF ASSAY OF PITUITARY GONADOTROPINS IN INTACT ANIMALS

Primary Group				Secondary Group			
Animal	Index of response	Material administered	Reference	Animal	Index of response	Material administered	Reference
Immature rat	Ovarian weight	Sheep pituitary extracts	Wallen-Lawrence and Van Dyke (1931)	Immature rat	Uterine weight	Rat pituitary extracts	Heller *et al.* (1938)
Immature rat	Formation of corpora lutea	Bovine pituitary extracts	Janssen and Loeser (1930)	Immature rat	Vaginal opening	Ox, sheep and swine pituitary extracts	Wallen-Lawrence and Van Dyke (1931)
Immature rat	Ovarian hyperemia	Human urine	Farris (1946)	Immature rat	Vaginal cornification	Sheep pituitary extracts	D'Amour and D'Amour (1938)
Immature mouse	Histological changes in ovary	Sheep pituitary extracts	Thomopoulou and Li (1954)	Immature rat	Seminal vesicle weight	Sheep pituitary extracts	Fevold (1939)
Immature mouse	Ovarian hyperemia	Human urine extracts	Lloyd *et al.* (1949)	Immature mouse	Uterine weight	Human urine extracts	Levin and Tyndale (1937)
Immature mouse	Ovarian weight	Human urine extracts	Levin and Tyndale (1937)				
Estrus rabbit	Ovulation	Ox pituitary extracts	Hill *et al.* (1934)				
Ring dove	Testicular weight	Ox and Dove pituitary extracts	Riddle and Flemion (1928)				
Xenopus laevis	Expulsion of spermatozoa	Ox pituitary extracts Human urine extracts	Hobson and Landgrebe (1954)				

extracts. Most centers have used the technique described by Klinefelter *et al.* (1943) or some modification thereof. Intact immature mice weighing 8–10 g. are injected subcutaneously once or twice per day for three days and are killed approximately 72 hours after the first injection. Results of clinical assays are expressed in "mouse uterine units" of "FSH" per 24 hours, a unit being defined as that quantity necessary to produce a given effect—usually 100–150% increase in uterine weight. The disadvantages of expressing results in "animal units" has already been mentioned. In addition, it should be emphasized that the mouse uterus test measures a mixture of FSH and ICSH activities and should not be regarded as in any way specific for urinary FSH.

Few estimates are available in the literature regarding the accuracy of this method. In studies on menopause urine Loraine and Brown (1954) found a weighted mean index of precision (λ) of 0.133, and in four-point assays in which urinary extracts from males and from nonpregnant females were compared with HMG the weighted mean figure for λ was 0.127 (Loraine and Brown, 1955). In all four-point assays there was no significant difference between the slopes of the dose effect curves for HMG on the one hand, and for male and cyclic female gonadotropins on the other. These results support the view that HMG can be used as a standard preparation for all assays on nonpregnant human urine.

b. Ovarian Hyperemia Test in Rats and Mice. This technique in rats has been used both for the diagnosis of pregnancy and for the estimation of urinary and serum HCG throughout normal pregnancy (Zondek *et al.*, 1945; Albert and Berkson, 1951). Farris (1946) has suggested that the test may also be employed in nonpregnant women to determine the time of ovulation. Unextracted urine (total dose 2.0 ml.) was administered subcutaneously to intact immature rats and the ovaries were examined for hyperemia two hours later. In normal subjects positive reactions were found on three or four successive days of the cycle at a time at which ovulation generally occurs. Other investigators, however, have been unable to confirm these results. Lloyd *et al.* (1949) claim that the mouse is 10–20 times more sensitive than the rat and have used the mouse hyperemia test as an assay method for urinary ICSH in nonpregnant individuals. Intact immature mice were injected subcutaneously with various dilutions of urine extracts prepared by alcohol precipitation, and the ovaries examined for hyperemia after an interval of seven hours. "Hyperemia-producing material" was found in relatively large quantities at the time of ovulation. Males excreted less "hyperemia-producing material" than normal females and the titer was also relatively low in castrate women.

Tests depending on hyperemia can be criticized mainly on the grounds

of specificity. Spontaneous reactions are relatively frequent (Zondek *et al.*, 1945), and the success of the method appears to be largely conditioned by the strain of animal employed. It has been shown (Payne, 1951; Smith, 1955) that cortisone and hydrocortisone interfere significantly with the hyperemia response to HCG, and a similar interference may well occur in the case of pituitary gonadotropins. Finally, the technique depends on an all-or-none (quantal) and not a graded response. Gaddum (1933) and Perry (1950) have shown that for any given degree of accuracy, quantal assays require approximately twice as many observations as assays depending on graded effects.

c. Expulsion of Spermatozoa in Xenopus laevis. Although amphibia are now widely used in the diagnosis of pregnancy, few studies on nonpregnant urine have so far been reported. Recently Hobson and Landgrebe (1954), using male *Xenopus laevis*, have demonstrated gonadotropic activity in an extract of menopausal urine prepared by the kaolin-acetone method. Further observations with this assay technique in male and cyclic female urine will be awaited with interest.

III. Extraction of Gonadotropins from Human Nonpregnant Urine

1. General

Many methods have been proposed for the preparation of gonadotropins from human urine. The procedures differ considerably in nature and complexity. Usually, however, they suffer from the common disadvantages that they are laborious and tedious, that considerable loss of gonadotropic activity may occur during their performance, and that the final extracts obtained are often toxic to the experimental animals. A discussion of various methods follows.

2. Alcohol Precipitation

This technique, with various modifications, has been used by numerous investigators including Zondek (1931), Albright *et al.* (1935), Heller and Heller (1939a), Nathanson *et al.* (1941), Varney and Koch (1942), Klinefelter *et al.* (1943), Smith *et al.* (1943), Howard *et al.* (1950), and Easterling *et al.* (1951). In the procedure described by Heller and Heller (1939a) the pH of the urine was adjusted to 4.0–6.0, aliquots of 500 ml. were precipitated with 4 vols. of 95% alcohol and were left to stand in the refrigerator overnight. On the following morning the supernatant fluid was removed, the residue centrifuged, and the precipitate washed with ether. The powder was dried and was then eluted three times with tap

water, the suspension being centrifuged each time and the supernatant fluid retained. In studies conducted on pooled menopausal and postmenopausal urine, Heller and Heller (1939a) and Varney and Koch (1942) found that the yield of gonadotropic material was greater with alcohol than with tannic acid precipitation. These workers also showed that crude extracts prepared by alcohol precipitation were less toxic to experimental animals than those prepared by the tannic acid or benzoic acid methods.

In general, the alcohol precipitation technique has given satisfactory results in high titer urines such as are found at the menopause and in various types of ovarian and testicular failure. However, in low titer urines, e.g. in males and normally menstruating women, the crude alcohol extracts are frequently too toxic for routine use. Various attempts have been made to decrease toxicity. Heller and Heller (1939a) and Heller and Chandler (1942) suggested that inorganic salts, particularly sodium chloride, were responsible, and claimed that by dialysis of the final solution against tap water toxicity was reduced and the yield of gonadotropin unaffected. This modification has been widely employed in clinical studies in man (Klinefelter *et al.*, 1943; Heller and Nelson, 1945a,b, 1948; Jungck *et al.*, 1947). Varney and Koch (1942) found, however, that aqueous extraction of the crude alcohol precipitate followed by dialysis and reprecipitation caused a loss of activity of approximately 50%. This loss could be attributed to the dialysis rather than to the extraction or the reprecipitation. They state that extraction of the crude material with 50% alcohol and reprecipitation of the gonadotropin from the solution by the addition of 4 vols. of alcohol is a more efficient purification step than dialysis and that extracts prepared in this way give better yields and are less toxic than those in which the dialysis step is included.

3. Acetone Precipitation

This procedure has been employed for estimations on nonpregnant human urine by Frank and Salmon (1935) and Drips and Osterburg (1938). Investigators in the United Kingdom have generally preferred acetone to alcohol as a precipitating agent for gonadotropins for reasons of economy (Loraine, 1949a, 1950a,b; Dekanski, 1949). The technique of extraction is very similar to that described for the alcohol method. In clinical studies satisfactory results are obtained when the excretion of gonadotropins is high, but in low titer urines extracts prepared by simple acetone precipitation are frequently toxic to the experimental animals. Attempts to reduce toxicity, e.g. by dialysis, may seriously interfere with the yield of gonadotropins.

4. Tannic Acid Precipitation

Levin and Tyndale (1936) and Thomsen and Pedersen-Bjergaard (1936) reported that gonadotropins could be extracted quantitatively from human urine by precipitation with tannic acid. In the method described by Levin and Tyndale (1936) the pH of the urine was adjusted to 5.0, aliquots of 1000 ml. were precipitated with 20 ml. of an aqueous 10% tannic acid solution and were centrifuged after standing. The bulky precipitate was extracted repeatedly with 80–95% alcohol and was washed several times with acetone. From each liter of original urine 100–200 mg. of "crude tannate powder" was obtained containing 75–100% of the original gonadotropic activity. The crude material could be purified by extraction 4 to 5 times with a barium hydroxide-barium acetate mixture at pH 9.0–10.0 followed by acetone precipitation. Finally the powder was dissolved in 60% alcohol at pH 9.0–10.0, the pH was brought to 6.5, and the precipitate which formed was washed several times with 60% alcohol. The brown alcoholic solution and the washings were combined and were precipitated by an equal volume of acetone. The precipitate was washed with acetone and dried. The final powder obtained (10–15 mg. per liter original urine) was claimed to be entirely soluble in water, to contain 80–100% of the original gonadotropic activity, and to be completely nontoxic when administered to intact mice and hypophysectomized rats.

Extracts prepared by tannic acid precipitation have been used in clinical studies by Pedersen-Bjergaard (1936), Rothermich and Foltz (1940), Levin (1941), Pedersen-Bjergaard and Tønnesen (1948), and Funnell *et al.* (1951). Other investigators, however, have not obtained satisfactory results with this technique. Heller and Heller (1939a), using menopausal urine, were unable to obtain quantitative yields of gonadotropin by tannic acid precipitation and found that crude tannate extracts were too toxic for routine use; various purification steps did not reduce toxicity to any marked extent. These observations were confirmed by Varney and Koch (1942) and by Smith *et al.* (1943). In the opinion of the reviewer the tannic acid method is more laborious and tedious than those depending on alcohol precipitation or kaolin adsorption and therefore cannot be recommended as a routine procedure for the extraction of gonadotropins from nonpregnant urine.

5. Benzoic Acid Adsorption

In the procedure described by Katzman and Doisy (1934), chilled urine was acidified by pH 5.0 and for each liter 75 ml. of acetone saturated with benzoic acid was added. The precipitate was collected on

a Buchner funnel, washed with a saturated aqueous solution of benzoic acid and dissolved in cold acetone. The acetone-insoluble material containing the gonadotropins was washed with acetone and dissolved in dilute NaOH. Any insoluble material was removed by centrifugation. The extract was further concentrated by adding 10 vols. of cold acetone; the precipitate was dried and dissolved in the desired volume of water. It was claimed that this technique concentrated the urine approximately 200-fold.

Katzman (1937) and Varney and Koch (1942), using menopausal urine, compared the alcohol precipitation and benzoic acid adsorption techniques. In general the yield of gonadotropins obtained by the benzoic acid method was considerably lower than that by alcohol precipitation. It is evident that this procedure cannot be recommended for the extraction of gonadotropins from human nonpregnant urine when quantitative assays are required. The method is, however, simple and rapid and is well adapted to industrial conditions where it may be necessary to prepare large quantities of active material.

6. Kaolin Adsorption with Acetone Precipitation

In 1941 Scott described a method for the extraction of HCG from pregnancy urine. The hormone was adsorbed on kaolin at pH 4.0 and eluted by $N/10$ NaOH. However, extracts prepared by the original kaolin method are not usually sufficiently concentrated, to permit the estimation of gonadotropins in nonpregnant urine. Accordingly various workers, including Bradbury *et al.* (1949), Loraine (1949a, 1950a,b), and Dekanski (1949), have, after adsorption and elution, precipitated the gonadotropins by acetone or alcohol. Extracts prepared by the kaolin-acetone or kaolin-alcohol techniques have proved suitable for administration to intact mice and to intact or hypophysectomized rats (Loraine, 1950a,b; McArthur, 1952; Prunty *et al.*, 1953; Loraine and Brown, 1954, 1955).

The kaolin-acetone method has recently been investigated by a series of recovery experiments in which HMG was added to pooled male urine (Loraine and Brown, 1954). Extracts were assayed biologically by the mouse uterus test. It was shown that accurate control of pH was essential at both adsorption and elution stages of the procedure. The critical pH for adsorption of HMG was 4.0: when the pH fell to 3.5 or rose to 4.5 approximately 40% of the material was not adsorbed. For elution a pH of 11.0–11.5 was critical: losses of approximately 50% were encountered at pH's of 10.0 and 12.0. For the step involving precipitation with 5 vols. of acetone, however, accurate control of the pH was not essential and satisfactory yields of HMG were obtained over a pH range from 3.0 to 6.0. In subsequent studies gonadotropins were extracted from pooled

male urine and from urine collected during the follicular and luteal phases of the normal menstrual cycle (Brown and Loraine, 1955). A similar series of recovery experiments were undertaken in which these extracts were added to pooled male urine and their biological activity determined at different stages of the kaolin-acetone procedure. The importance of accurate control of the pH was again emphasized and it was shown that the optimal pH's for adsorption and elution of male and cyclic female gonadotropins were very similar to those for HMG.

Kaolin-acetone powders from low titer urines are sometimes toxic to experimental animals, especially hypophysectomized rats, since they contain relatively large quantities of inactive substances which are sparingly soluble in water. Further purification of these extracts is therefore necessary, and such a procedure has recently been described by Brown and Loraine (1955). It was shown that, by eluting the crude powders with several volumes of water at pH 8.0, all the gonadotropic activity could be extracted in a conveniently small volume of water and the main bulk of the inactive material could be discarded. The extract obtained was then further purified by treatment with tricalcium phosphate in the manner described by Loraine and Brown (1954). These purification steps did not result in any significant loss of gonadotropic activity and made it possible to administer the equivalent of approximately 1 liter of original urine to an intact mouse or an hypophysectomized rat.

In the opinion of the reviewer the kaolin-acetone method with accurate pH control is the procedure of choice for the routine extraction of gonadotropins from human nonpregnant urine. In low titer urines purification of the crude material by treatment with tricalcium phosphate may be necessary. A quantitative extraction method for estimating the gonadotropin content of nonpregnant urine based on these findings has been developed in this laboratory. The technique is less tedious and laborious than the majority of published methods and gives better yields of gonadotropins than those obtained by tannic acid precipitation and benzoic acid adsorption. In addition, extracts prepared in this way are generally less toxic to experimental animals than those prepared by simple alcohol or acetone precipitation.

7. Aluminum Hydroxide Adsorption

This technique (Malburg and Goodman, 1954) is similar in many respects to the kaolin-acetone method. The urinary gonadotropins were adsorbed on 10% aluminum hydroxide at pH 4.0 and were eluted by normal NaOH at pH 10.0. The pH of the eluate was adjusted to 5.5–6.0 and the hormones were precipitated by adding 5 vols. of cold acetone. The precipitate was separated by centrifugation, washed with ether, and

taken up in water. The method was found to be simple and rapid and gave yields of gonadotropins almost identical with those obtained by the alcohol precipitation-dialysis technique. Further studies with this procedure will be awaited with interest.

8. Permutit Adsorption

Katzman *et al.* (1943) described a chromatographic procedure for the preparation of HCG from pregnancy urine in which the hormone was adsorbed onto permutit and eluted by ammonium acetate in alcohol. Johnsen (1955a,b) has slightly modified the method and has applied it to the extraction of gonadotropins from pooled postmenopausal urine. The method is both simple and rapid, and highly active extracts of low toxicity were obtained. In the present form the technique is not suitable for the quantitative extraction of 24-hour specimens but may be of value for the large-scale preparation of active material.

9. Ultrafiltration

Gorbman (1945) described a method in which urinary gonadotropins were ultrafiltered through a collodion membrane under pressure. When filtration was completed the membrane, along with the protein hormone residue which had not passed through the pores of the membrane, was removed from the filter and placed in an alcohol-ether solution in a centrifuge tube. The alcohol-ether dissolved the collodion membrane and precipitated the gonadotropins. The solution was then centrifuged, the supernatant fluid discarded, and the residue washed repeatedly with alcohol and ether. Before bioassay the dry precipitate was taken up in water or saline. Van Gilse (1955) has recently investigated the role of adsorption in the ultrafiltration procedure and has estimated that 80–90% of the gonadotropic activity is adsorbed on to the collodion membrane.

Jungck *et al.* (1947) compared the ultrafiltration and alcohol precipitation-dialysis methods for the recovery of gonadotropins from human nonpregnant urine. The methods of assay depended on the enlargement of the ovary and uterus in intact immature rats. They concluded that the ultrafiltration method was preferable to the alcohol precipitation-dialysis technique in that it was less complex, less laborious, and less expensive. They also found that the yields of gonadotropic activity were very similar by the two methods. Kaesenaar and Paesi (1951) extracted pooled urine from patients with Klinefelter's syndrome and Turner's syndrome by the ultrafiltration method and were able to demonstrate both FSH and ICSH activity in their extracts.

IV. Separation of Different Gonadotropic Factors by Tricalcium Phosphate

In a series of papers Crooke, Butt, and their collaborators have claimed that it is possible to separate and estimate the individual urinary gonadotropins by a chromatographic technique (Crooke and Butt, 1952, 1953; Butt and Crooke, 1953; Ingram *et al.*, 1954; Crooke *et al.*, 1954). These workers extracted the urine by the kaolin-acetone method and fractionated the extracts on tricalcium phosphate columns. The eluting agents used were disodium hydrogen phosphate (Na_2HPO_4) and trisodium phosphate (Na_3PO_4). The material eluted by disodium hydrogen phosphate was referred to as "Gonadotropin A" (GA) and that eluted by trisodium phosphate as "Gonadotropin B" (GB).* Assays of the eluates by biological methods showed that GA was excreted predominantly at the menopause and also at certain times during the normal menstrual cycle, while GB was the main gonadotropin in the urine of pregnant women. Using the ovarian weight test in intact immature mice to measure FSH activity and the prostatic weight method in hypophysectomized immature rats to estimate ICSH, Crooke *et al.* (1954) have also claimed that GA prepared from pooled luteal phase urine by chromatography on tricalcium phosphate columns had FSH activity and that ICSH activity had been removed by passage through the column. This observation was based on the fact that the GA fraction prepared from luteal phase urine produced no significant increase in ventral prostatic weight over untreated control animals, while material prepared by the kaolin-acetone method from the same source but not put through the tricalcium phosphate procedure showed ICSH activity as indicated by a small but statistically significant (43 %) mean increase in ventral prostatic weight over untreated controls. In view of these findings it was suggested that the tricalcium phosphate technique might be a means of separating human urinary FSH from human urinary ICSH.

Loraine and Brown (1954) have recently studied the tricalcium phosphate procedure of Butt and Crooke (1953) as a means of separating HMG from HCG and human urinary FSH from human urinary ICSH. It was found that the adsorption of gonadotropins on tricalcium phosphate was influenced markedly by the pH of the solutions. HMG was not adsorbed to any appreciable extent on tricalcium phosphate at pH's above 6.0, and HCG was adsorbed completely below pH 7.0. The adsorption of HCG on tricalcium phosphate decreased as the pH increased and no adsorption occurred at pH 10.0 and above. These results indicated that the separation of HMG from HCG was possible by differential adsorption on

* "GA" and "GB" bear no relationship to "prolan A" and "prolan B" and should not be confused with them.

tricalcium phosphate. It is possible that this technique might prove of value in testing for the presence of pituitary gonadotropins in pregnancy urine and in assessing whether the pituitary contributes significantly to the very high gonadotropin titer in human urine in the first trimester of normal pregnancy and to the abnormally high excretion of gonadotropin found in some patients with pre-eclamptic toxemia and pregnancy complicated by diabetes. Various workers, including Hasenbein (1952), Lyon *et al.* (1953), and Lajos *et al.* (1953a), have claimed that human pregnancy urine contains FSH activity. This interesting observation requires further investigation.

Regarding the separation of urinary FSH from urinary ICSH the results obtained by Loraine and Brown (1954, 1955) were at variance with those reported by Butt and Crooke (1953). In this laboratory it was found that the GA fraction prepared from HMG treated with tricalcium phosphate either batchwise or chromatographically contained considerable ICSH activity as estimated by the ventral prostate test in hypophysectomized immature rats. ICSH activity was also present when the GA fraction from pooled male urine and pooled follicular and luteal phase female urine were assayed in the same way. It was therefore concluded that treatment of urine extracts by tricalcium phosphate did not effect any separation of urinary FSH from urinary ICSH. It was found, however, that such treatment significantly reduced the toxicity of crude kaolin-acetone extracts and made it possible to administer total doses per animal equivalent to approximately 1 liter of original urine.

V. Chemical Estimation of Gonadotropins

Many investigators have emphasized the desirability of estimating urinary gonadotropins by chemical means in order to avoid laborious and time-consuming bioassay procedures, and attempts have been made to find some chemical property which consistently parallels biological activity. For example, Bowman (1941) studied the oxidation-reduction characteristics of HCG. He found that the hormone became biologically inactive when treated with oxidizing agents and that the activity could be restored by reducing agents; it was suggested that the reducing capacity of HCG might be used as the basis of a chemical test for the diagnosis of pregnancy. Maxwell and Bischoff (1935) showed that reagents known to react with amino groups cause partial or complete inhibition of the biological activity of pituitary gonadotropins. This finding was confirmed by Li (1949). All gonadotropins contain a hexose in their molecule and this is probably important in relation to biological activity. Stran and Jones (1954) and Crooke *et al.* (1954) have attempted to estimate gonadotropins by testing urinary extracts for proteins and sugars.

Stran and Jones (1954) extracted morning specimens of urine by the kaolin method and studied the behavior of HCG and HMG using paper electrophoresis. For identification of the hormones on paper the protein stain bromphenol blue was found to be the most reliable: unsatisfactory results were obtained with ninhydrin, which stains proteins with free amino groups, and with orcinol and *p*-anisidine, which are polysaccharide stains. Techniques depending on ultraviolet fluorescence and spectrophotometry were also unsuitable. Studies on HMG showed that only one component could be identified on paper. The technique was not found to be capable of separating HMG from HCG, however, and is therefore inferior in this respect to that involving tricalcium phosphate adsorption. It would therefore appear that at present paper electrophoresis cannot be regarded as an efficient method for separating the various gonadotropic factors in human urine.

Crooke *et al.* (1954) have claimed that it may be possible to estimate gonadotropic activity in human urine by chemical means and that since there is at present no known chemical reaction specific for gonadotropins it is justifiable to prepare purified extracts and to measure these extracts by nonspecific tests. Urine was extracted by the kaolin-acetone method and chromatographed on tricalcium phosphate columns to obtain GA and GB fractions. Chemical tests employed for GA included the ninhydrin reaction for free amino groups, the orcinol reaction for hexoses, and an estimation of the cysteine content by polarography. Using these techniques the GA excretion has been studied in a variety of normal and pathological conditions in man, and the effect of steroids on the GA level has been investigated (Crooke and Butt, 1953; Crooke *et al.*, 1954). Although the chemical results obtained are of interest, it should be emphasized that, in the absence of parallel assays performed by both chemical and biological methods in the same individuals in normal and diseased states, there is little or no evidence to support the view that the GA excretion as measured by these nonspecific reactions for protein and sugars does actually reflect variations in the titer of urinary gonadotropins.

Until chemical methods of assay become more specific, it is unlikely that they will replace biological techniques in the estimation of urinary gonadotropins for clinical purposes.

VI. Excretion of Gonadotropins in Normal Nonpregnant Subjects

1. General

This topic has been reviewed by Klinefelter *et al.* (1943), Pedersen-Bjergaard and Tønnesen (1948) and Evans and Simpson (1950) and will be discussed only in outline in the present paper.

Existing methods of estimation of pituitary gonadotropins for clinical purposes are very unsatisfactory and take the form of qualitative tests rather than quantitative assays. Results are expressed in "animal units"—usually "mouse uterine units"—and no attempt can be made to compare results from different laboratories. The great variability from center to center makes it at present impossible to quote normal figures in units for the excretion of these hormones in nonpregnant subjects. It is probable, however, that, with the introduction of HMG as a standard preparation for nonpregnant urinary gonadotropins, assays of these substances will become more accurate and reliable.

2. Children

All investigators agree that the gonadotropic activity in the urine of children and adolescents is very low (Saethre, 1933; Frank, 1935; Catchpole *et al.*, 1938; Nathanson *et al.*, 1941; de Sario, 1954). One of the most detailed studies in the literature is that of Nathanson *et al.* (1941). These workers extracted gonadotropins from urine by alcohol precipitation, and their assay method depended on the production of follicular stimulation in the ovaries of intact immature mice. In boys no activity was detected before the age of 13 but in girls positive results were generally obtained at the age of 11. It was noted that in girls activity was often detected approximately one year before the onset of menstruation. In a study of the gonadotropin excretion in young and adolescent boys Catchpole *et al.* (1938) concluded that the titer was related to developmental status rather than to chronological age.

3. Normal Men

Gonadotropic activity has been detected in normal male urine by numerous investigators including Saethre (1933), Harris and Brand (1934), Leathem and Levin (1941a,b), Varney and Koch (1942), Klinefelter *et al.* (1943) and Jones and Bucher (1943). The amount excreted is generally higher than in normally menstruating women. Few reports are available in the literature of the excretion of gonadotropins by men at different ages but there is no evidence to suggest that the output rises significantly with advancing years. The most careful study is probably that of Schou (1951), who found that the titer remained relatively constant in normal men between the ages of 39 and 63 and concluded that in the male there was no equivalent of the female climacteric.

On the basis of assays involving histological changes in the ovaries of hypophysectomized immature rats it has been stated that male urine is predominantly follicle-stimulating in nature (Fraenkel-Conrat *et al.*, 1940; Jones and Bucher, 1943; Bahn *et al.*, 1953c). Recent work in this laboratory using the prostate test in hypophysectomized immature rats

has shown that a relatively large amount of ICSH activity is also present (Loraine and Brown, 1955). It is evident that further work is necessary before any figure can be quoted for the ratio of FSH to ICSH activity in normal male urine.

4. Normally Menstruating Women

The gonadotropin excretion during the normal menstrual cycle has been studied by numerous investigators including Kuzrock *et al.* (1934), Smith and Smith (1936), Frank *et al.* (1937), Drips and Osterburg (1938), Heller (1941), Levin (1941), Werner (1941), Klinefelter *et al.* (1943), Main *et al.* (1943) and Pedersen-Bjergaard and Tønnesen (1948). It is generally believed that the titer is low in the follicular and luteal phase and that a peak of excretion occurs at or about the time of ovulation. D'Amour (1943) attempted to determine the time of ovulation in 20 normal cycles by a variety of tests including basal temperature records, vaginal smears, and the excretion of estrogens, pregnanediol and gonadotropins. He concluded that the gonadotropin peak was the most reliable index that ovulation had occurred. Other workers, however, have been unable to obtain such clear-cut results. Pedersen-Bjergaard and Tønnesen (1948) found no systematic rhythmic fluctuations in gonadotropin titer, and Main *et al.* (1943) reported the presence of two peaks in a portion of their cases. In a detailed study of 22 normal cycles Heller (1941) found that the level of excretion was generally low, that fluctuations in titer usually occurred at different phases of the cycle, but that the pattern of excretion was extremely variable from one patient to another. In further studies on 28 women on whom bilateral salpingo-oöphorectomy and hysterectomy were performed, Heller *et al.* (1944a) concluded that the stage of the ovarian cycle and the time of ovulation could not be predicted by estimating the titer of urinary gonadotropins. It is obvious that much work is still required with improved methods of extraction and more accurate bioassay techniques before the pattern of excretion of gonadotropins during the normal menstrual cycle can be established.

Female gonadotropins resemble male gonadotropins in their biological properties and most workers have stated that they are predominantly follicle-stimulating in nature. More recent work by McArthur (1952) and by Loraine and Brown (1955) using the prostate test in hypophysectomized rats has demonstrated that normal female urine contains significant amounts of ICSH activity.

5. Menopausal and Postmenopausal Women

It is generally agreed that the gonadotropin titer in blood and urine is elevated at and beyond the menopause (Fluhmann, 1929; Zondek, 1930; Mazer and Hoffman, 1931; Jeffcoate, 1932; Osterreicher, 1932). This is

believed to represent an attempt on the part of the pituitary to stimulate unresponsive ovaries; a similar effect occurs after castration in men and women. Heller and Heller (1939b), using assay methods depending on the enlargement of the uterus and ovaries of intact immature rats, found that menopausal urine was approximately twenty times more active than urine obtained from normally menstruating women. Evidence is conflicting on the relationship between the gonadotropin titer and the development of climacteric symptoms. This subject has been reviewed by Fluhmann (1944). It has been suggested (Albright, 1936; Lawrence and Moulyn, 1941) that menopausal symptoms, particularly flushes, may be caused directly by the overproduction of pituitary gonadotropins. This view has been challenged by Heller *et al.* (1944b) who, in a careful and detailed investigation, showed that there was no apparent correlation between the height of the urinary gonadotropin titer and the severity of symptoms, and that patients with menopausal symptoms could obtain relief from estrogen therapy without concomitant depression of the excretion of urinary gonadotropins.

Few studies have so far been undertaken of the gonadotropin excretion in postmenopausal subjects. The most comprehensive is probably that of Heller and Shipley (1951). These workers were unable to show any correlation between gonadotropin titer and chronological age, but found that a relationship existed between the amount excreted and the duration of time since the onset of the menopause. In patients who were less than 25 years beyond the menopause the mean excretion was significantly higher than in those in whom the menopause occurred more than 25 years before the investigation. The titer in the second group, however, was still considerably higher than that found in women during reproductive life.

Much experimental work has been done on the biological properties of menopausal gonadotropin. In intact and hypophysectomized female rats the hormone is a potent stimulator of follicular growth without much effect on the interstitial tissue, and this has led to the assumption that the material is predominantly, if not entirely, follicle-stimulating hormone (FSH). Borth *et al.* (1954) and Loraine and Brown (1954), using the prostate test in hypophysectomized rats, have recently shown that menopause urine is, in addition, a relatively rich source of ICSH activity.

VII. Excretion of Pituitary Gonadotropins in Pathological Conditions

1. Disorders of Menstruation

Borth and de Watteville (1952) and Hecht-Lucari (1952) have recently discussed the importance of hormone assays in gynecological conditions,

and little has been added to the literature since their articles were written. Gonadotropin assays have until now proved of little diagnostic value to the gynecologist in the elucidation of problems associated with abnormal menstruation. This has been due mainly to the lack of methods sufficiently sensitive to detect activity in such low-titer urines. The development of such assay methods is urgently required in order to obtain more accurate information on the pattern of hormone excretion in such patients and thereby to create a sounder basis for substitution therapy.

a. Amenorrhea. Various workers have attempted to assay gonadotropins in patients with amenorrhea. Fluhmann (1929) found activity in the blood of 6 out of 17 such patients. In the 10 patients reported by Frank *et al.* (1937), 4 showed abnormally high levels and in 6 no activity could be detected. Similar results have been obtained by Béclère and Simmonet (1949) and by Käser (1949). One of the most intensive investigations is that of Pedersen-Bjergaard and Tønnesen (1951), who extracted gonadotropins from urine by precipitation with tannic acid and used as their bioassay endpoint the enlargement of the uterus in intact immature mice. In 110 patients with primary amenorrhea, 35% had abnormally high readings and in 65% the excretion was within or below the normal range. In 221 patients with secondary amenorrhea the corresponding figures were 34% and 66%. From these and other studies it is evident that the gonadotropin titer may be of value in indicating whether amenorrhea results from failure of the ovary or failure of the pituitary. If due to the former the urinary excretion is often abnormally high, if to the latter the titer is generally so low that no activity can be detected by current assay methods.

b. Dysfunctional Menstrual Irregularities. Few investigations have been reported on the excretion of FSH and ICSH in such patients, but there is no evidence at present to suggest that with existing assay techniques estimations would aid significantly in the management of these cases. This is borne out by the findings of Pedersen-Bjergaard and Tønnesen (1951), who, in a study of 222 patients with various types of menstrual anomaly other than amenorrhea, showed that in the majority of cases the gonadotropin excretion was within the normal range.

2. Various Types of Gonadal Failure

The vast majority of pituitary gonadotropin assays in the clinical field are conducted in order to differentiate primary gonadal failure from gonadal failure secondary to lesions of the pituitary. Such a classification may be of value from a therapeutic point of view. If possible, gonadotropin assays should be performed in parallel with estrogen or 17-ketosteroid estimations in order that an adequate appraisal can be made of

ovarian or testicular function. Important additional information in these cases can often be obtained by attempting to correlate the pattern of hormone excretion with vaginal smears or with the findings on testicular biopsy.

a. Primary Gonadal Failure in the Male. In 1942 Klinefelter *et al.* described a group of patients in whom the presenting features were gynecomastia and sterility in association with varying degrees of impairment of androgenic function. Testicular biopsy showed a diminution or absence of spermatogenesis with destruction of the Sertoli cells; on the other hand the interstitial cells, though sometimes showing degenerative changes, were generally increased in number. The syndrome was subsequently termed *sclerosing tubular degeneration.* In such patients the 17-ketosteroid excretion is within or below the normal range, but the urinary gonadotropin titer as measured by the mouse uterus test is considerably increased. The cause of the condition is at present unknown, and none of the theories suggested for the endocrine abnormalities in such patients are entirely satisfactory. A similar syndrome without gynecomastia has been reported by Heller and Nelson (1945a,b), while del Castillo *et al.* (1947a) have described a group of patients in whom absence of spermatogenesis without impairment of Leydig cell function was associated with normal levels of urinary gonadotropins. In a detailed study of 142 cases with varying types of testicular deficiency, Howard *et al.* (1950) found that the gonadotropin excretion was below normal in 26%, normal in 28% and abnormally high in 46%. For a more detailed discussion of hormone assay in cases of male hypogonadism the reader is referred to articles by Nelson (1947, 1948), Werner (1947), and McCullagh (1948).

b. Primary Gonadal Failure in the Female. This is exemplified by the condition known variously as "ovarian agenesis," "ovarian dwarfism," or "Turner's syndrome." Approximately 100 cases have so far been reported in the literature. Since the original description of the disease by Turner (1938) the clinical features and hormone excretion in such patients have been studied by numerous investigators including Albright *et al.* (1942), Varney *et al.* (1942), Wilkins and Fleischmann (1944), del Castillo *et al.* (1947b), Lisser *et al.* (1947), Greenblatt and Nieburgs (1948), Chavez-Montes (1952), and Prunty *et al.* (1953). It is believed that the hormone pattern in typical cases consists of (i) diminished output urinary estrogens, (ii) diminished output of 17-ketosteroids, and (iii) abnormally high level of urinary gonadotropins. In some patients the urinary gonadotropin titer resembles that in menopausal and postmenopausal women.

Little or no information is at present available on the biological nature

of the gonadotropins excreted in Turner's syndrome, since most assays have been made with the mouse uterus test, which is not specific for either FSH or ICSH activity. Studies on hypophysectomized male and female rats will be awaited with interest.

c. Secondary Gonadal Failure in Males and Females. All investigators are agreed that in *panhypopituitarism* the excretion of gonadotropins is abnormally low. Frequently no activity can be detected in highly concentrated urine extracts, even with the mouse uterus test. The largest series of cases reported is probably that of Klinefelter *et al.* (1943), who, in 14 patients, found that the mean excretion of urinary gonadotropins was significantly lower than that obtained in normal men or in normally menstruating women of a comparative age group. Similar results have been obtained by Easterling *et al.* (1951). It has been rightly emphasized that the diagnosis of panhypopituitarism should only be made if no gonadotropic activity is detectable by the mouse uterus test.

VIII. Pituitary Gonadotropins in Urine—Conclusions

1. Urinary gonadotropins from normal men and from normally menstruating women can be assayed in terms of human menopausal gonadotropin (HMG) using assay methods depending on the enlargement of the uterus in intact immature mice and enlargement of the prostate in hypophysectomized immature rats. It is proposed that HMG be used as a standard preparation for the assay of human nonpregnant urinary gonadotropins.

2. For routine clinical assays of urinary gonadotropins from nonpregnant individuals the nonspecific mouse uterus test is recommended. Results should be expressed in terms of HMG. The prostatic weight test in hypophysectomized immature rats is sufficiently sensitive to estimate ICSH activity in clinical conditions but is probably too tedious and laborious for routine studies.

3. In the opinion of the reviewer the kaolin-acetone method with accurate pH control is a suitable procedure for the extraction of gonadotropins from human nonpregnant urine. Treatment of the crude kaolin extracts by tricalcium phosphate makes it possible to administer without toxic effects the equivalent of approximately 1 liter of original urine to intact mice and hypophysectomized rats.

4. The separation of HMG from HCG can be effected by differential adsorption on tricalcium phosphate. This technique should make it possible to test for the presence of pituitary gonadotropins in pregnancy urine.

5. Chemical methods of assay of gonadotropins are relatively nonspecific and should not at present replace biological techniques.

6. Both FSH and ICSH activity are present in postmenopausal urine, in normal male urine, and in urine from normally menstruating women. It is not yet clear whether the effects are due to two separate substances or to a single substance with both activities.

7. The diagnostic value of pituitary gonadotropin assays is discussed in relation to various pathological conditions in man.

IX. Methods of Assay of Human Chorionic Gonadotropin (HCG)

1. General

An international standard is available for the assay of HCG and all results should now be expressed in International Units. By definition 1 I.U. is the activity contained in 0.1 mg. of the standard preparation. The main source of uncertainty in assays of HCG has always been the fact that each colony of animals is liable to differ in sensitivity from other colonies. This error is eliminated by the use of a standard preparation. There is no longer any excuse for expressing results of HCG assays in "animal units" and it is regrettable that publications still appear in which "rat" and "mouse" units are employed.

Assay methods for HCG are very numerous. The subject has been reviewed by Emmens (1939), Thayer (1946), Loraine (1952, 1954b), and Diczfalusy (1953, 1954). For quantitative assays intact immature rats and mice have been favored as test animals, but for pregnancy diagnosis studies rats, mice, rabbits and amphibia have been widely used. As for pituitary gonadotropins, it is possible to classify tests according to whether the effect is *primary* or *secondary*. Tables III and IV, which are

TABLE III

Accuracy of Assay Methods for HCG—Primary Effects

Index of response	Indices of precision		Reference
	λ	L^*	
Ovarian wt. (rat)	0.139	1.19	Sealey and Sondern (1940)
Ovarian wt. (rat)	0.230	4.36	Diczfalusy and Loraine (1955)
Ovarian hyperemia (rat)	0.450	2.21	Albert and Berkson (1951)
Expulsion of spermatozoa (toad)	0.120–0.176	5.65–8.32	Wohlzogen (1953)

* Woolf's L is the reciprocal of λ. It has recently been claimed (Woolf, 1953) that in assays depending on graded responses this quantity should be used instead of λ since, when the total number of observations in each experiment is large, the distribution of L is approximately normal with variance $1/S(x - \bar{x})^2$. Gaddum (1953a) has suggested that, in addition to other indices of precision such as λ and g, different assay methods should be compared in terms of their mean value of L.

TABLE IV
ACCURACY OF ASSAY METHODS FOR HCG—SECONDARY EFFECTS

Index of Response	Indices of Precision		Reference
	λ	*L*	
Uterine wt. (rat)	0.329	3.06	Delfs (1941)
Uterine wt. (rat)	0.149	6.70	Dorfman and Rubin (1947)
Uterine wt. (rat)	0.240	4.18	Sealey and Sondern (1940)
Vaginal smears (rat)	0.276	3.60	Loraine (1950)
Vaginal smears (Vitamin B-deficient rat)	0.115	8.70	Heard and Winton (1939)
Total prostatic wt. (rat)	0.139	7.19	Loraine (1950b)
Total prostatic wt. (rat)	0.100	10.00	Loraine (1955)
Ventral prostatic wt. (hypophysectomized rat)	0.150	6.70	Diczfalusy *et al.* (1950)
Seminal vesicle wt. (rat)	0.200	5.00	Watts and Adair (1943)
Seminal vesicle wt. (rat)	0.195	5.14	Loraine (1950b)
Seminal vesicle wt. (hypophysectomized rat)	0.495	2.00	Diczfalusy *et al.* (1950)
Total accessory reproductive organs (rat)	0.210	4.75	Diczfalusy (1953)
Total accessory reproductive organs (rat)	0.105	9.50	Diczfalusy (1954)

largely based on data calculated by Diczfalusy (1954), show some of the methods used and compare their relative accuracy in terms of Gaddum's λ and Woolf's *L*.

2. Primary Group

Many of these methods have considerable disadvantages when applied to the quantitative assay of HCG in the blood and urine of patients. The method based on the formation of *corpora lutea* in the rat ovary is relatively sensitive but is very inaccurate and is little better than a qualitative test. This was the technique used by Smith and Smith (1934, 1939, 1941, 1948) in studies of the serum concentration of HCG in pre-eclamptic toxemia and also by White (1947, 1949) in similar estimations in pregnant diabetics. *Ovarian weight in rats* is now little used as an assay method because it is both inaccurate and insensitive (Diczfalusy and Loraine, 1955). Using this technique Evans *et al.* (1937) constructed one of the earliest curves for the urinary HCG concentration throughout normal pregnancy. Methods depending on *ovulation in rabbits* and in *toads* have generally proved reliable as pregnancy tests but are too insensitive to be of great value in quantitative assays.

Within the last five years the two methods which have been most

employed in the clinical field are those depending on (a) *ovarian hyperemia in rats* and (b) *expulsion of spermatozoa in amphibia.*

a. Ovarian Hyperemia in Rats. The ovarian hyperemia test was introduced by Zondek *et al.* (1945) for the diagnosis of pregnancy and has been made the basis of a clinical bioassay of HCG by Albert and his co-workers (Albert, 1948; Albert and Berkson, 1951). This is a sensitive method but is relatively inaccurate (Table III). Its chief advantage is the fact that the total time for the assay is only four hours; this is in marked contrast to most other procedures involving multiple injections into rats and necessitating 72- to 96-hour injection periods. The main disadvantages of the test are as follows:

(i) The success of the assay appears to depend greatly on the strain of animal employed. In certain strains spontaneous reactions are relatively frequent (Zondek *et al.*, 1945). In the author's and other laboratories the technique could not be made the basis of a satisfactory quantitative assay for HCG.

(ii) Relatively large numbers of animals are required because the method depends on a quantal and not a graded response.

(iii) The specificity of the test is doubtful in view of the finding that cortisone and hydrocortisone will affect the response of the ovary to injected HCG (Payne, 1951; Smith, 1955). It is possible that blood and urine contain other substances which may also interfere with the reaction.

b. Expulsion of Spermatozoa in Amphibia. In 1947 Galli-Mainini described a new pregnancy test depending on spermiation in the male toad, *Bufo arenarum.* Since then it has become evident that several other male amphibia can be used for pregnancy diagnosis and there is now a large literature on this subject. For fuller information the reader is referred to papers by Robbins and Parker (1948), Hinglais and Hinglais (1948), Haines (1948), Schockaert *et al.* (1948), Mello (1949), Klopper and Frank (1949), Bhaduri and Bardhan (1949), Thorborg (1950), and Hobson (1952). More recently attempts have been made to adapt the method for the quantitative assay of HCG in blood and urine using *Rana pipiens* (Haskins and Sherman, 1949, 1952), *Bufo Bufo* (Thorborg and Hansen, 1951), *Rana esculenta* (Lajos *et al.*, 1951), *Bufo marinus* (Wannan, 1952), *Xenopus laevis* (Hobson, 1952), and *Bufo viridis* (Wohlzogen, 1953; Bukovics and Wohlzogen, 1953).

The main advantages of the test are as follows:

(i) The method is both simple and rapid; quantitative estimations of HCG potency can be obtained in 24 hours or earlier.

(ii) The test is said to be relatively accurate (Table III). With 20 animals per dose level of standard and unknown preparations, Landgrebe

et al. (1953), using *Xenopus laevis*, found that the error was approximately 20%.

(iii) The same animal can be used repeatedly. Most authors state that a week's rest is sufficient after a positive response.

The chief disadvantages of this assay method are:

(i) Amphibia are relatively insensitive. This may necessitate concentration of urine extracts and the use of large quantities of the standard preparation in order to elicit positive responses.

(ii) The response of the animals to HCG is subject to marked seasonal modification. It is generally believed that the sensitivity is at a maximum late in the winter and in the early spring and is at a minimum in the autumn (Thorborg, 1950).

(iii) Relatively large numbers of animals are required because the assay depends on a quantal rather than a graded response.

3. Secondary Group

In this group the majority of assays have been conducted in *intact immature rats*. The following indices of response will be considered:

(a) uterine weight
(b) vaginal smears
(c) prostatic weight
(d) seminal vesicle weight
(e) weight of total accessory reproductive organs.

a. Uterine Weight. According to Emmens (1939) this test is sensitive but inaccurate. The technique has been used by Jones *et al.* (1944) to estimate the serum concentration of HCG throughout normal pregnancy and by Dorfman and Rubin (1947) for studies on pregnancy urine. Diczfalusy and Loraine (1955) found the test relatively accurate but showed that the effect of HCG on the uterine weight in rats was increased approximately twofold when the hormone was dissolved in serum or plasma instead of saline. They concluded that the method was unsuitable for HCG assays in untreated serum or plasma. In estimations on pregnancy urine the estrogens will interfere with the response and it is necessary to remove them, e.g. by alcohol or acetone precipitation, prior to bioassay.

b. Vaginal Smears. This method was used by Browne and Venning (1936) and by Venning (1948) to study the urinary excretion of HCG during normal pregnancy and was employed by Bruner (1951) to determine the distribution of the hormone in the mother and fetus at different stages of normal pregnancy. It is a relatively accurate and reliable method (Table IV) but has the disadvantage of depending on a quantal rather

than a graded effect. In Edinburgh the test was found to have a larger error than that depending on the prostatic weight (Loraine, 1950b). According to Heard and Winton (1939) the error of the test can be reduced by using adult aneurine-deficient rats, which are probably more uniform in response than are intact immature animals. For studies on pregnancy urine the vaginal smear tests resembles that depending on uterine weight in that the estrogens will interfere with the response and must be removed prior to bioassay.

c. Prostatic Weight. This method (Loraine, 1950b) has been shown to be both accurate and convenient for the quantitative assay of HCG in urine, serum, and placentas of patients. The technique used in Edinburgh is as follows: intact immature rats are injected subcutaneously once per day on three consecutive days at approximately 24-hour intervals. They are killed on the fourth day, at which time the prostates are dissected out from the surrounding structures and fixed in Bouin's solution for 24 hours. On the fifth day the organs are freed from fat, dried between pieces of filter paper and weighed on a torsion balance. The results are expressed as milligrams of prostatic weight per 100 g. rat weight. Assays are made in comparison with the international standard for HCG. The assay design used for estimations on body fluids has been either four-point (two doses of the standard versus two doses of the unknown) or three-point (two doses of the standard versus one dose of the unknown). The weighted mean index of precision (λ) in the original experiments was 0.139 and in more recent assays 0.100 (Loraine, 1955). The method has a sensitivity comparable to that of ovarian hyperemia. The estrogens in pregnancy urine do not interfere with the test and untreated urine can be employed for the assays. The effect of HCG on the prostatic weight of intact and hypophysectomized rats is not increased significantly when the hormone is dissolved in serum or plasma instead of saline, and accordingly this technique can be used to assay HCG in untreated serum or plasma (Diczfalusy and Loraine, 1955).

d. Seminal Vesicle Weight. This test is sensitive but is less accurate than that depending on prostatic weight (Loraine, 1950b) or on the weight of the total accessory reproductive organs (Diczfalusy, 1954). It has been used by Watts and Adair (1943) to estimate the urinary excretion of HCG in cases of pre-eclamptic toxemia and essential hypertension in pregnancy. As in the case of the prostatic weight method, the estrogens in pregnancy urine do not interfere with the response and untreated urine can be injected.

e. Weight of Total Accessory Reproductive Organs (Total Prostate + Seminal Vesicles). Diczfalusy (1954) found that in his strain of animals this test was slightly more accurate than that depending on prostatic

weight alone. He used it to estimate the placental concentration of HCG at different stages of normal pregnancy.

X. Extraction of HCG from Urine

The methods used are very similar to those already described for pituitary gonadotropins and include alcohol precipitation (Zondek and Aschheim, 1928), acetone precipitation (Frank and Salmon, 1935), benzoic acid adsorption (Katzman and Doisy, 1934), tungstic acid precipitation (Katzman and Doisy, 1933), kaolin adsorption (Scott, 1941), kaolin adsorption with acetone precipitation (Loraine, 1950b) and permutit adsorption (Milton, 1946).

The end-point of the bioassay largely determines whether or not extraction methods for HCG should be employed. With relatively insensitive techniques, e.g. those using male and female amphibia, it is often necessary to prepare concentrated extracts in order to elicit a biological response. In the more sensitive assays, depending on vaginal smears and uterine weights in rats, the estrogens must be removed from pregnancy urine prior to bioassay. This is usually done by precipitation of the urine with alcohol and acetone. As noted above, the great advantage of the assay method depending on the prostatic weight in rats is the fact that untreated urine can be injected.

XI. HCG Assays in Human Serum

Many investigators have estimated the serum concentration of HCG in normal and abnormal pregnancy using a variety of bioassay techniques. Recently it has been shown that in such estimations on untreated serum the end-point of the test must be selected with care in order to obtain valid assays. Maddock and his collaborators reported that the effect of HCG on the rat uterus and ovary was increased 5- to 10-fold when the hormone was administered in human plasma instead of in saline (Maddock and Leach, 1952; Maddock *et al.*, 1953; Leach *et al.*, 1954). In this series of papers, however, no attempt was made to calculate the error of the assays, and it is doubtful if the results have much quantitative significance. Diczfalusy and Loraine (1955) also found that serum and plasma increased the effect of HCG on the rat uterus but reported only an approximate doubling of potency. From these results it is apparent, however, that bioassays of HCG in serum and plasma by the uterine weight method, as recommended by Jones *et al.* (1944) and by Dorfman and Rubin (1947), will most probably give an overestimate of the real potency and will thus yield erroneously high serum levels.

In certain types of assay an underestimate of potency has been observed when HCG was dissolved in human serum. Thus Salvatierra and

Torres (1952), using the assay method depending on spermiation in *Rana esculenta*, reported that three times as much HCG was necessary to produce a positive response when the hormone was dissolved in serum instead of in saline. Similarly, Albert and Berkson (1951) noted that the response obtained in undiluted serum in the ovarian hyperemia test in rats was only one-third of that obtained with the standard HCG in saline. However, the possibility that the ovarian hyperemia reaction may give an overestimate when the assay is conducted on diluted but untreated serum must also be considered. Such an assumption would seem to be supported by the discrepancy in the figures for the renal clearance of HCG reported by Gastineau *et al.* (1949) on the one hand and by Loraine (1950c) on the other. Using the ovarian hyperemia reaction, the former group of workers found that the serum concentration of HCG was approximately three times that found in the urine, whereas Loraine (1950c), employing the prostatic weight method, reported that the concentration of HCG in the serum was approximately equal to that in urine.

Diczfalusy and Loraine (1955) have shown that the effect of HCG on the weight of the accessory reproductive organs of immature male rats is not influenced appreciably when the hormone is dissolved in human serum or plasma instead of saline. They concluded that for clinical bioassays on human serum, satisfactory results from a quantitative point of view can be obtained if the prostatic weight, seminal vesicle weight, and weight of the total accessory reproductive organs are used as indices of response.

XII. Clinical Applications of HCG Assays

1. General

HCG estimations are of great value to the clinician in the *diagnosis of pregnancy*, and the demonstration of large quantities of the hormone in urine forms the basis of most pregnancy tests, including the widely used Aschheim-Zondek, Friedman, Hogben, and Galli-Mainini tests. As shown by Zondek (1929, 1937, 1942), Aschheim (1930) and many others, very high levels of HCG in blood and urine are usually encountered in cases of *hydatidiform mole* and *chorionepithelioma*, and the urinary excretion in such patients may reach 1,000,000 I.U./24 hours. Similarly, in *chorionepithelioma of the testis* the body is flooded with enormous quantities of gonadotropic material. This hormone is predominantly luteinizing in nature and is qualitatively indistinguishable from HCG.

An extensive literature already exists on the relative value of the various pregnancy diagnosis tests and on the estimation of HCG in patients with mole and chorionepithelioma, and these subjects will not be discussed by the reviewer. In recent years the quantitative assay of

HCG in body fluids has become of interest in other clinical states, some of which are considered below.

2. Normal Pregnancy

Normal curves for HCG throughout pregnancy are available in international units both for the urinary excretion (Loraine, 1950b; Albert and Berkson, 1951) and for the serum concentration (Jones *et al.*, 1944; Wilson *et al.*, 1949; Haskins and Sherman, 1952; Loraine and Matthew, 1955). The curves are of the same general shape as those obtained by previous investigators who expressed results in "rat" and "mouse" units.

Using the prostatic weight method of assay, it has been shown that in the first trimester of normal pregnancy very high readings, e.g. 20,000–50,000 I.U., are obtained for the urinary excretion (I.U./24 hours) and for the serum concentration (I.U./liter). In the second and third trimesters, however, the urinary excretion and serum concentration are in the range 4,000–11,000 I.U. ($P = 0.99$). Estimations of HCG consistently outside this range must be regarded as pathological.

3. Pre-eclamptic Toxemia

Numerous workers have estimated HCG in the blood and urine of toxemic women, but results have generally been expressed in "animal units" and in many investigations the assay methods used have been inaccurate and insensitive. From the clinical point of view interest has been focussed on the possible value of serum and urinary HCG estimations as a prognostic index in such cases.

Smith and Smith (1934, 1939, 1941), using the corpora lutea assay method in rats, were the first to note that a proportion of pre-eclamptic patients (over 70% of their series) showed abnormally large quantities of HCG in the serum when compared with normally pregnant women. The rise in serum HCG was often observed some weeks before the development of pre-eclampsia and the Smiths suggested that this finding might serve as a warning of impending toxemia. In later work, however, they were forced to the conclusion that the elevation of the serum HCG bore little or no relationship to the severity of the disease and was therefore of little value in prognosis. Taylor and Scadron (1939), employing the same method of assay as that used by the Smiths, reported high concentrations in the urine and serum of some pre-eclamptic cases, but in many toxemic women the readings were entirely normal throughout the period of investigation. Similar results were obtained by Lajos *et al.* (1953b), using the assay method for HCG depending on explusion of spermatozoa in *Rana esculenta*, and by Eichenberger and Käser (1942) using the Aschheim-Zondek test. Watts and Adair (1943) performed assays of HCG and estrogens in the urine of pre-eclamptic and eclamptic patients and of

cases of essential hypertension in pregnancy. The method of assay of HCG depended on the enlargement of the seminal vesicles in intact immature rats, and the data was subjected to careful statistical analysis. It was shown that the mean excretion of HCG in pre-eclamptic toxemia and eclampsia was significantly higher than in normal pregnancy, but that cases of essential hypertension in pregnancy also excreted significantly greater amounts of HCG. These observers therefore concluded that abnormalities in HCG *per se* did not play an important role in the etiology of pre-eclamptic toxemia. In this and other papers on the subject little attempt was made to classify cases on a clinical basis.

A re-investigation of the problem was undertaken by Loraine and Matthew (1950) using the prostatic weight method to assay HCG. Observations were made in 29 patients with pre-eclampsia, 9 with essential hypertension in pregnancy, and 5 with essential hypertension with superimposed toxemia. The pre-eclamptic patients were further subdivided into three groups—severe, moderate, and mild. The assessment of the severity of the toxemia was based mainly on the rapidity of evolution of the disease and on its response to therapeutic measures. Cases were said to be mild if the symptoms subsided under treatment and the patient proceeded uneventfully to term. The disease was regarded as moderate when there was no appreciable change in the condition in spite of therapy. In the severe group the course of the disease was fulminating, in some eclamptic fits occurred, and in all of them there was an urgent indication for the interruption of pregnancy. For a diagnosis of essential hypertension in pregnancy it was necessary to have the history of hypertension prior to the onset of pregnancy or from the earliest weeks of pregnancy. Another group presented features of both essential hypertension and pre-eclamptic toxemia. In those patients with a history of hypertension there were added the criteria of pre-eclamptic toxemia arising after the twentieth week. The classification was made without knowledge of the results of the assays. It was found that in severe pre-eclamptic toxemia the mean urinary excretion and serum concentration of HCG were significantly higher than in normal pregnancy ($P = 0.99$), but in none of the other groups of patients was this observed. No correlation could be found between the high readings of HCG and any particular clinical feature, e.g. blood pressure, albuminuria, or edema. It was concluded that at the present time routine assay of the urinary and serum HCG would not be of great assistance to the clinician in the management of these cases.

4. Hyperemesis Gravidarum

It has been claimed that patients with hyperemesis tend to show abnormally large quantities of HCG in blood and urine (Anselmino and

Hoffman, 1936; Brindeau *et al.*, 1939; Rakoff, 1940; Schoeneck, 1942). The most detailed study is probably that of Schoeneck (1942), who attempted to adapt the Friedman test for quantitative assay of HCG. He reported that patients with hyperemesis excreted significantly larger quantities of HCG than normally pregnant women and that a positive correlation existed between the severity of the vomiting and the HCG titer. These results were not confirmed in Edinburgh (Loraine, 1949a), where it was found that the HCG excretion in such patients lay within the normal range for the duration of pregnancy.

5. Diabetic Pregnancy

The subject of hormone assay in pregnancy associated with diabetes has been recently reviewed (Loraine and Matthew, 1954), and the reader is referred to this paper for a more detailed survey of the present position.

Although the introduction of insulin greatly reduced the maternal mortality rate in diabetes mellitus, the fetal mortality has not shown a corresponding decline, and the high fetal loss rate remains the great problem of obstetrical diabetes. In some series of cases the fetal mortality has reached 50%. It is not surprising, therefore, that considerable interest has been aroused by the claims made by White and her collaborators at the Joslin Clinic (White, 1947, 1949, 1952; White and Hunt, 1943) that a very significant reduction in the fetal mortality rate in diabetic pregnancy (from 50% to approximately 10%) can be obtained with hormonal therapy. The therapy for White's patients consisted of the parenteral administration of estrogens and progesterone; these substances were given to correct a so-called "hormonal imbalance" characterized by an abnormally high serum concentration of HCG and an abnormally low excretion of urinary pregnanediol. HCG was assayed by the method depending on the demonstration of corpora lutea in the ovaries of immature female rats. Results were expressed in "rat units." Urinary pregnanediol was estimated at first by the method of Venning (1937) and subsequently by that of Astwood and Jones (1941).

White (1952) has recently published her experience with this therapy in 525 pregnant diabetics studied over a 15-year period. Stilbestrol and progesterone (proluton) were administered parenterally in gradually increasing dosage throughout pregnancy to those patients showing "hormonal imbalance." The dosage schedule employed was similar to that used by Smith and Smith (1939, 1941, 1948) in the treatment of patients with pre-eclamptic toxemia. Approximately 10,000 estimations of serum HCG were made in the 525 cases. From these studies White (1952) drew the following conclusions:

(i) 91.0% of the patients showed an abnormally high serum HCG concentration.

(ii) Estimation of the serum HCG was of considerable diagnostic and prognostic value in these patients because a rise in serum HCG often preceded obstetrical accidents by some weeks.

(iii) Only in patients with an abnormally high serum HCG concentration did obstetrical accidents and complications occur. In 47 patients in whom the serum level was within normal limits, the fetal survival rate was 96.0% and the incidence of premature delivery and pre-eclamptic toxemia was nil.

(iv) Stilbestrol and progesterone corrected the so-called "hormonal imbalance" and restored the serum HCG and urinary pregnanediol levels to normal. When hormonal therapy was employed the fetal survival rate rose dramatically and the incidence of premature delivery and pre-eclamptic toxemia fell correspondingly.

In 98 patients showing "hormonal imbalance" and not receiving therapy the fetal survival rate was only 52.0% and the incidence of premature delivery was 33.0% and of pre-eclamptic toxemia 33.0%. In 380 patients in whom the "hormonal balance was corrected" by substitution therapy, however, the fetal survival rate rose to 90.0%, premature delivery occurred in 17.0% and the incidence of pre-eclamptic toxemia fell to 7.0%.

Keltz *et al.* (1950) estimated the serum HCG concentration in 30 pregnant diabetics. The method of assay was the same as that employed by White (1952). Over a two-and-a-half-year period, 2,086 estimations were made. Many of the patients in whom hormone assays were undertaken received therapy by oral stilbestrol and intramuscular progesterone in gradually increasing dosage throughout pregnancy. Although these workers observed a reduction in the fetal mortality rate in the treated patients compared with a control series, they were unable to show that the serum HCG concentration bore any relationship either to the occurrence of fetal death or to the development of pre-eclamptic toxemia. The mean serum HCG concentration in the third trimester of pregnancy in patients delivering a viable fetus (111 estimations in 20 cases) did not differ significantly from that obtained in patients with whom fetal death occurred (56 estimations in 9 cases). In addition stilbestrol and progesterone did not appear to influence the serum concentration of HCG in the third trimester of diabetic pregnancy. These authors therefore concluded that estimation of the serum HCG was of little value as a guide to hormone therapy.

A study of hormone levels in 52 pregnant diabetics has been made in Edinburgh over the past five years (Loraine and Matthew, 1954). Alto-

gether 511 estimations were made of the urinary excretion of HCG and 245 of the serum concentration. The method of assay depended on the enlargement of the prostate in intact immature rats. In the earlier stage of this investigation oral stilbestrol was administered to those diabetics showing an abnormally high urinary excretion of HCG. During this time 6 of 14 patients were so treated. Of the subsequent 38 patients, in only one was hormone therapy given. This was commenced at the eleventh week; the serum concentration of HCG was high, the urinary excretion was normal, and intrauterine death of the fetus occurred at the thirty-sixth week.

The results obtained in Edinburgh did not agree in certain respects with those of White and her collaborators. It was noted that a much smaller proportion of diabetic patients showed abnormally high HCG readings, i.e. levels of urinary and serum HCG consistently above 11,000 I.U. in the second and third trimesters of pregnancy. Of the 49 patients in whom estimations of the urinary HCG were undertaken only 14 (28.5%) showed abnormally high figures, while of the 37 patients in whom the serum concentration of HCG was estimated only 10 (27.0%) were above the normal range.

In the total series of 52 patients high levels of HCG either in urine or serum or both were found in 17 cases (32.7%). The series was analyzed to show the relationship of HCG levels to obstetric complications. In 13 patients (25.0%) the fetus was lost from a variety of causes, but in only 4 of these 13 (30.8%) was a high level of HCG noted. In the 39 patients with surviving babies high levels of HCG were noted in 13 (33.3%). Pre-eclampsia was present in 8 patients of the original 52 (15.4%). In this group 7 showed normal HCG readings and only 1 was associated with high levels. Hydramnios was noted in 22 out of the 52 patients (42.3%). Of these 22 instances 15 (68.2%) were associated with normal HCG readings and 7 (31.8%) with high readings. It is of interest to note that the mode of delivery in this series, excluding seven cases of intra-uterine death, was by caesarean section at or about the thirty-sixth week in 71.0% of the cases. It is apparent that in this series of patients fetal death appeared just as likely to occur in pregnant diabetics with normal HCG readings as in those with abnormal readings.

In the experience of the author stilbestrol produced only an evanescent fall in the 24-hour excretion of HCG in normal and diabetic women (Loraine, 1949b, 1953). Consistent depression of the titer by prolonged estrogen therapy was not obtained even when large doses of stilbestrol (100–200 mg./day) were exhibited. The initial depression was soon followed by an "escape" phenomenon, the readings tending to climb back to their original level while the patient was still receiving therapy.

It can only be assumed that the differing results obtained by the various investigators for the HCG levels in pregnant diabetics must depend to a large extent on the methods of assay employed. Mention has already been made of the grave disadvantages of the rat corpora lutea test as used by White (1946, 1952) and by Keltz *et al.* (1950). The method depends on a quantal response, and is relatively inaccurate. Furthermore, in the assays conducted by these investigators the relative potency was not expressed in terms of the international standard for HCG but in "rat units"/100 ml. of serum. It is doubtful if any quantitative significance can be attached to results expressed in this way.

XIII. Renal Clearance of HCG in Normal and Abnormal Pregnancy

As noted previously, the urinary excretion and serum concentration of HCG fluctuate characteristically throughout normal pregnancy. This fluctuation might represent alterations in the rate of formation, in the rate of destruction, or in the rate of excretion of the hormone. In an attempt to elucidate this problem Gastineau *et al.* (1949) studied the renal clearance of HCG in four normally pregnant women, in a patient with hydatidiform mole, and in a patient with testicular tumor. The method of assay of HCG depended on the ovarian hyperemia response in rats; renal clearance was calculated by the formula UV/B and expressed as milliliters per minute. These workers reported a mean clearance in normal pregnancy of 0.36 ml./min. and also found that the mean clearance in the first trimester of normal pregnancy did not differ significantly from that obtained in the second and third trimesters. From available data Gastineau *et al.* (1949) calculated that the renal clearance in a series of normal patients studied by Smith and Smith (1939) was 0.85 ml./min., and that obtained by Taylor and Scadron (1939) was 0.44 ml./min. Using the method of assay for HCG depending on spermiation in *Rana esculenta*, Salvatierra (1954) found the clearance in normal pregnancy to be approximately unity. Such very low figures for clearance are typical of proteins in general.

Loraine (1950c), using the prostatic weight method of assay, estimated the HCG clearance in 12 normally pregnant women, 29 patients with pre-eclampsia, 7 pregnant diabetics, 9 patients with essential hypertension in pregnancy, and 5 patients with essential hypertension with superimposed toxemia. In all types of case the mean clearance was found to be less than 1.00 ml./min. In normal pregnancy the mean figure was 0.90 ml./min. In normal and diabetic pregnancy consistent figures for clearance were obtained in the three trimesters. Accordingly, it was concluded that variations in the HCG level during pregnancy probably

represented alterations in the rate of production or destruction of the hormone and were not the result of differences in the renal excretion.

The pre-eclamptic patients were classified on a clinical basis into three categories—mild, moderate, and severe (Loraine and Matthew, 1950). In the mild and moderate cases the mean clearance did not differ significantly from that in normal pregnancy. Similar results were obtained in patients with uncomplicated essential hypertension and in patients with essential hypertension with superimposed toxemia. In the severe pre-eclamptic group, however, the mean clearance (0.74 ml./min.) was significantly lower than that in normal pregnancy ($P < 0.01$). This observation is probably another index of the renal damage frequently found in severe cases of pre-eclamptic toxemia. The majority of these patients showed pronounced oliguria at the time the assays were performed.

In the studies conducted in Edinburgh a close quantitative similarity was noted between the urinary and serum concentration of HCG. In normal pregnancy the mean urine/serum concentration ratio was 0.95, in diabetic pregnancy 0.94, in mild pre-eclampsia 1.26, in moderate pre-eclampsia 1.03, in severe pre-eclampsia 1.08, in essential hypertension in pregnancy 1.08, and in essential hypertension with superimposed toxemia 0.86. In the series of patients studied by Gastineau *et al.* (1949), the figures obtained for the urinary excretion of HCG were quantitatively very similar to those obtained by Loraine (1950c) but their serum concentrations were much higher, averaging 3.06 times the concentration in the urine. It has been suggested (Diczfalusy and Loraine, 1955) that this discrepancy might result from the different assay methods employed by the two groups. The precision of the ovarian hyperemia test as used by Gastineau *et al.* (1949) is relatively low and it is difficult to exclude the possibility that the figures obtained by this method for the concentration of HCG in pregnancy serum may be erroneously high. Such a criticism does not appear to apply to results obtained using the prostatic weight technique.

XIV. Concentration of HCG in the Placenta in Normal and Abnormal Pregnancy

Smith and Smith (1935), using relatively inaccurate assay methods, estimated the HCG content of placentas from 10 normally pregnant women, 9 patients with pre-eclamptic toxemia and 5 eclamptics. It was found that placentas from toxemic patients contained a significantly greater concentration of HCG than those from normally pregnant women. Various workers, including Augustin (1941) and Bickenbach (1941), have demonstrated a considerably higher concentration of HCG

("units"/g.) in placentas obtained during the early weeks of gestation as compared with those examined in late pregnancy. By far the most careful and detailed study of the HCG concentration in normal human placentas is that of Diczfalusy (1953). This worker, who expressed results in international units, estimated the HCG content of 40 placentas at various stages of normal pregnancy. The hormone was extracted from placentas by phosphate buffer at pH 7.8, and the extract was then precipitated by 8 vols. of acetone at pH 4.0. Two different assay methods were used, one depending on the increase in weight of the total accessory reproductive organs in intact immature male rats and the other based on the formation of corpora lutea in the ovaries of intact immature mice. Great attention was paid to the question of assay design and the results were subjected to careful statistical analysis. It was found that the placental concentration of HCG (I.U./g. wet weight) was maximal in the second and third months of pregnancy (corresponding to the peak period in blood and urine), fell rapidly in the third and fourth months and from the fifth month was relatively constant, remaining below 20 I.U./g.

In a recent study Loraine and Matthew (1953) estimated the placental concentration of HCG in 27 normally pregnant women delivered by the vaginal route, 12 patients delivered by caesarean section for obstetric indications, 17 patients with pre-eclamptic toxemia, 14 pregnant diabetics, and 11 patients in whom various other complications occurred during pregnancy. A simple saline method of extraction of HCG from placentas was employed, and this technique gave a yield of the hormone comparable with that obtained by methods involving lyophilization and acetone precipitation. In all cases the prostatic weight method of assay was used. It was found that the placental HCG concentration (I.U./g. wet weight) did not differ significantly between normal patients delivered by the vaginal route and patients delivered by caesarean section for purely obstetrical indications. In patients with moderate and severe pre-eclamptic toxemia and in diabetic pregnancy, however, the mean figure for the HCG concentration was significantly higher than that in the control series ($P < 0.001$), but in mild pre-eclamptic toxemia and in otherwise complicated pregnancy the mean figure did not differ significantly from that in the control series.

XV. Human Chorionic Gonadotropin—Conclusions

1. The method of assay of HCG depending on the enlargement of the prostate in intact immature rats is a convenient and relatively accurate test which can be used for the quantitative assay of HCG in serum and urine. Results of the assay should be expressed in I.U.'s.

2. Care is necessary in the selection of assay methods for estimating

HCG in pregnancy serum to avoid either an overestimate or an underestimate of activity.

3. In severe pre-eclamptic toxemia the mean figures for the urinary excretion and serum concentration of HCG are significantly higher than those in normal pregnancy, but in mild and moderate pre-eclamptic toxemia and in essential hypertension in pregnancy the figures do not differ significantly from those in normal pregnancy. There is no apparent correlation between the HCG levels and any clinical feature of the disease.

4. In diabetic pregnancy approximately 30% of patients show abnormally high readings of HCG in serum and urine. There is no correlation, however, between the urinary and serum levels of HCG on the one hand and the medical and obstetrical findings on the other. Stilbestrol administered to normal and diabetic women produces an evanescent fall in the urinary excretion of HCG.

5. In all cases the mean renal clearance of HCG is less than 1.0 ml./min. In normal and diabetic pregnancy constant figures are obtained in the three trimesters of pregnancy. The mean clearance in severe pre-eclamptic toxemia is significantly lower than in normal pregnancy.

6. There is no significant difference between the placental concentration of HCG of normal patients delivered by the vaginal route and patients delivered by caesarean section for obstetric indications. In moderate and severe pre-eclamptic toxemia and in diabetic pregnancy the mean figure is significantly higher than in the control series.

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Microbiological Transformations of Steroids and Their Applications to the Synthesis of Hormones

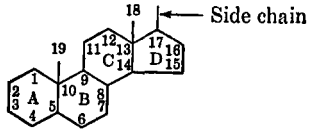
S. H. EPPSTEIN, P. D. MEISTER, H. C. MURRAY, AND D. H. PETERSON

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan

I. Introduction, Historical, and Background

The earliest record of a microbiological transformation of a steroid is that of Mamoli and Vercellone, in 1937, dealing with the reduction of a ring ketone with yeast (89). This work was soon confirmed by Kim (71, 72) using a ketocholanic acid.

Mamoli and Vercellone (87, 88, 117, 156) later demonstrated that some yeasts and bacteria could modify certain steroids through reduction of double bonds or conversion of hydroxyl groups to carbonyl groups (and *vice versa*) on the steroid nucleus (see formula I).



I

Such studies were intensified during the next five years by various groups of workers. However, in the following decade, microbiological studies on steroids were relatively quiescent. During the latter period, Turfitt (154) observed the degradation of the side chain and the cleavage of ring A of cholestenone, while Krámli and Horváth reported dehydrogenation (64) as well as hydroxylation (75) of cholesterol.

During the last few years, interest in this field has been reactivated. In 1949, Hench *et al.* (59) demonstrated the dramatic effect of cortisone and, later, cortisol (hydrocortisone) in the treatment of rheumatoid arthritis. These events greatly stimulated research aimed at an economical synthesis of these hormones. A major obstacle in this effort was the introduction of the biologically necessary oxygen at carbon 11. For this purpose, microbiological methods have proved to be outstandingly successful. In addition, various other microbiological transformations have been discovered, producing many new steroids, some of which exhibit interesting physiological activity. Several reviews of the micro-

biological transformations of steroids have appeared recently, notably Finch (39), Florey (41), Fried *et al.* (49), Hanč and Riedl-Tůmova (53), Peterson (127), Stanley and Hickey (143), and Wettstein (164).

By deep fermentation techniques[1] adopted from other commonly used industrial processes, the steroids have been altered in many different ways. Carbon-carbon double bonds or carbonyl double bonds have been reduced or introduced; hydroxyl groups have been introduced at many positions of the nucleus and to a limited extent on the side chain; carbon-carbon scission has occurred (rupture of ring D sometimes results); steroid esters and glycosides have been hydrolyzed. In addition, various combinations of these reactions have often taken place.

The ability to dissimilate steroids is widespread, especially among the fungi. Most molds are generally active in this regard. The action of bacteria on steroids is less well known, e.g. the dehydrogenation reactions being the major and more clearly defined ones so far.

The transformation of various steroids, some readily available from abundant plant sterols, can now be easily accomplished with conversion to the naturally occurring adrenocortical and gonadal hormones as well as derivatives of these two groups. These transformations have been accomplished directly by certain fermentation procedures or by a combination of microbiological and organic chemical methods.

Several aspects of steroidal transformations have been omitted in this chapter. We have completely avoided the published work on simple microbial hydrolyses of esters and glycosides inasmuch as these involve no alteration in the basic steroid nucleus. Nor have we discussed in detail the earlier work of Mamoli, Kim, and others because adequate reviews of this work are available (40, 53). These oxidation and reduction reactions, however, have been included in the tables.

The shift of the double bond from the 5–6 to the 4–5 position on oxidation of 3-hydroxysteroids to 3-ketosteroids we have considered to be nonenzymatic—an artifact in the fermentation-isolation procedure[2] These reactions, therefore, are listed as simple oxidations in Table I. In transformations with steroid esters as substrates, we have assumed that hydrolysis preceded the transformation, with one notable exception (cf. page 397), and thus have considered only the parent alcohol in Table I

[1] The term "fermentation" as used broadly pertains to chemical activities of microorganisms. Most of the reactions are not true fermentations but aerobic dissimilations. Wherever possible the expressions "bioconversion" and "conversion" will be used in this review.

[2] Since the writing of this article, P. Talalay and V. S. Wang (149a) have clearly shown that mammalian tissue and a *Pseudomonas* can carry out the isomerization of 3-keto-Δ^5-steroids to 3-keto-Δ^4-steroids enzymatically. In the case of the *Pseudomonas*, at least, the enzyme is induced (adaptive).

and, for the most part, in Table II. We hope that we have not wielded Occam's razor too enthusiastically.

In the discussion of microbial oxygenation of steroids no attempt has been made to be all-inclusive; rather, typical reactions have been cited. A comprehensive listing of all proved microbiological modifications of steroids (except for hydrolytic changes) is summarized in Table II. In many of the microbial dissimilations minor constituents can be demonstrated by paper chromatography. Unless those constituents have been isolated they have been omitted from the tables and, with few exceptions, from the discussion. Considerable material has been included which has been taken from unpublished work done in our laboratory and in that of Fried and co-workers at the Squibb Institute for Medical Research. We recognize that a review article should cover only material whose experimental details are completely available to the reader. To follow this dictum in the present case would greatly detract from the interest of the chapter. And so, with apologies, we have drawn on unpublished experiments.

An explanation of the two major tables is in order. Table I lists in outline form the various known types of microbiological transformations of steroids with an example of each. The cross-reference numbers to Table II show which transformations fall in each category. Obviously, some of the products are the result of several separate reactions. But since in most cases the sequence can only be conjectured, we have elected to consider only the over-all reaction. Three products have been put in a miscellaneous reactions category. They are 3β,11α-dihydroxy-5-pregnene-7,20-dione, 17β-hydroxyandrostane-3,6-dione, and 11α,17a-dihydroxy-17a-methyl-D-homo-4-androstene-3,17-dione. They were so classified for the following reasons: (1) Thus far they are unique. (2) Two of them involve reactions (rearrangements) not at all comparable to the "common or garden variety" of microbial reactions to be discussed. (3) All are possibly artifacts. Thus, the 3β,11α-dihydroxy-5-pregnene-7,20-dione found by Meister *et al.* (98) and Murray and Peterson (114) can be produced by a nonenzymatic oxidation of 3β,7β,11α-trihydroxy-5-pregnen-20-one, also isolated from the same fermentation, since this allylic 7-hydroxyl should be very easily oxidized. The 17β-hydroxyandrostane-3,6-dione, reported by Eppstein *et al.* (29) can easily be formed by rearrangement of 6β-hydroxytestosterone (also isolated from the fermentation). Although we have no evidence that the hydrogen ion concentrations conducive to this rearrangement occurred during either the fermentation or the isolation, the compound is suspect. Also suspect is the 11α,17a-dihydroxy-17a-methyl-D-homo-4-androstene-3,17-dione isolated by Fried *et al.* (46). Fried *et al.* (47) have informed us that this isolation is repeatable, but, of course, should the environment be

favorable for the rearrangement[3] of 11α,17α-dihydroxyprogesterone to the D-homosteroid this is to be expected.

Table II puts emphasis on the products of the microbial dissimilations. The products are grouped by molecular formulas and are arranged alphabetically within groups and by position sequence of the affected carbons, i.e. 6β, 7α, 7β, etc. The various steroid starting materials for a given product are listed in the next column. (Esters have not been listed separately from the free alcohols, as already noted.) The last column gives the microorganisms involved, together with the bibliographical references. Column one contains a numerical assignment for each reaction for cross reference to Table I.

To avoid the frequent use of long chemical names, we propose to use, in many cases, trivial names, some of which are not accepted by the Committee of the International Union of Applied Chemistry, Division of Chemistry and Chemical Technology, National Research Council. We wish to emphasize that we have no desire to perpetuate new trivial names; however, the exact, but cumbersome, chemical terminology does not readily lend itself to purposes of discussion. The derivatives of these steroids, then, will be named either by attaching the appropriate prefix to the trivial name or in accordance with strict chemical terminology, depending upon which is more suitable. Thus, a derivative of pregnenolone (3β-hydroxy-5-pregnen-20-one) is named 3β,11α-dihydroxy-5-pregnene-7,20-dione, but a similar derivative of 19-nortestosterone (17β-hydroxy-4-estren-3-one) is called 11α-hydroxy-19-nortestosterone. The list of new or unaccepted trivial names which we have used together with their chemical names is given below.

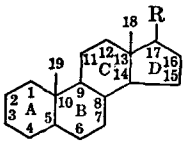
Trivial Name Used	*Chemical Name*
Androstadienedione	1,4-Androstadiene-3,17-dione
Androstenedione	4-Androstene-3,17-dione
Cholic acid	3α,7α,12α-Trihydroxycholanic acid
Corticosterone	11β,21-Dihydroxy-4-pregnene-3,20-dione
Cortisol	11β,17α,21-Trihydroxy-4-pregnene-3,20-dione
11-Deoxycortisol	17α,21-Dihydroxy-4-pregnene-3,20-dione
Methyltestosterone	17β-Hydroxy-17-methyl-4-androsten-3-one
19-Nortestosterone	17β-Hydroxy-4-estren-3-one
Pregnenolone	3β-Hydroxy-5-pregnen-20-one
Testololactone	3-Keto-13α-hydroxy-13,17-seco-4-androsten-17-oic acid lactone

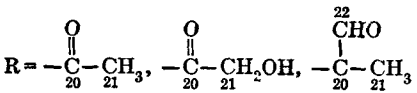
[3] Conceivably the 11α-hydroxyl could promote this reaction. Thus, Florey and Ehrenstein (42) have shown an activation of carbon no. 17 by the 11α-acetoxyl group. When they converted 6β,11α-diacetoxyprogesterone to 6α,11α-diacetoxyprogesterone, the equilibrium between the 17β-acetyl and the 17α-acetyl side chain was shifted more in favor of the 17α-acetyl side chain than would be the case on equilibration of the 11-deoxysteroid under like conditions; hence an appreciable amount of the 17α-progesterone derivative was formed.

TABLE I

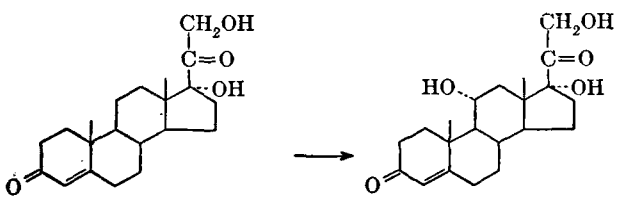
MICROBIOLOGICAL REACTIONS ON STEROIDS WITH TYPICAL EXAMPLES

Basic Steroid Structure





Simple Reactions

	Example	Cross Reference to Examples in Table II
I. Oxidation A. Oxygenation 1. Monohydroxylation a. Ring		6β: 3, 10, 24, 39, 56, 69, 77.1, 78, 79, 84.1, 108.1 7α: 57, 70 7β: 91, 102, 104 7ξ: 120 8ξ or 9ξ: 58, 71, 85 10ξ: 4 11α: 5, 11, 25, 40, 46, 50, 59, 72, 78A, 80, 86.1, 95, 97.1, 100.1, 101 11β: 61, 66, 81, 83.1 14α: 12, 26, 62, 73, 88 15α: 13, 27, 63, 74 15β: 64, 75 16α: 14, 28.1, 65, 76 17α: 51, 51.1

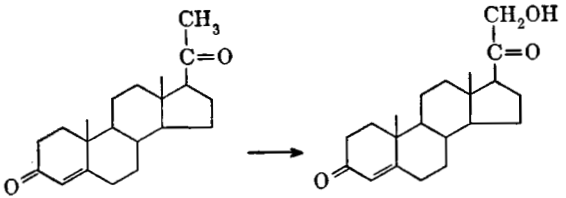
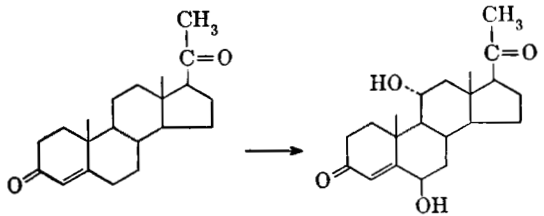
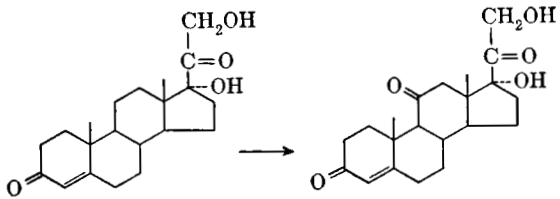
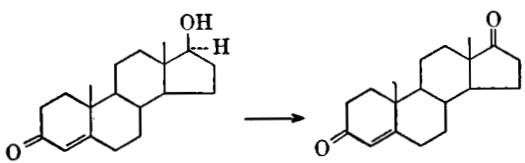
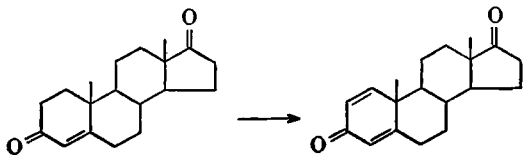
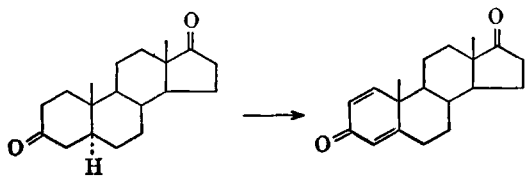
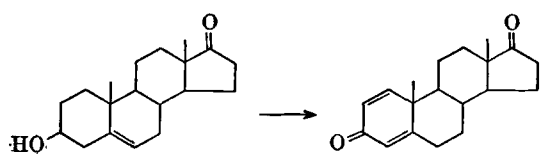
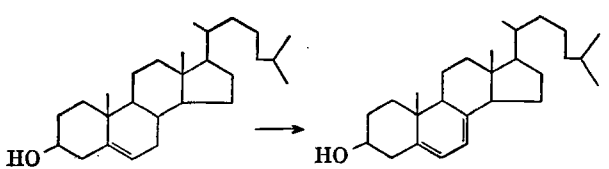
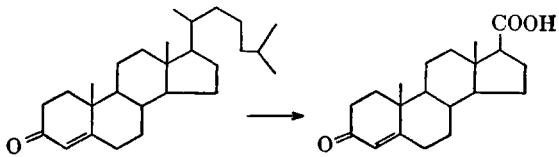
b. Side chain		21: 37, 48, 54.1, 66.1, 67.1, 69.1, 72.1, 73.1
2. Dihydroxylation		6β,11α: 77 6β,17α: 84 7β,11α: 99 11β,14α: 87 11α,17α: 80.1, 86
3. Ketone formation—direct		51.2
B. Dehydrogenation 1. Oxidation of a ring hydroxyl		1, 8, 8.3, 8.5, 17.1, 19, 22.1, 22.4, 38, 44, 53, 54, 109, 110, 111, 112, 114, 115, 118, 119, 122, 123

TABLE I.—(*Continued*)

	Example	Cross Reference to Examples in Table II
2. Formation of a 1,4-dien-3-one chromophore a. From a 3-keto-Δ^4-steroid		6.2, 41, 42, 43, 45, 45.1, 47, 49, 52
b. From a saturated steroid		6.1
c. From a 3β-hydroxy-Δ^5-steroid		6.5
3. Introduction of a double bond in ring B		117

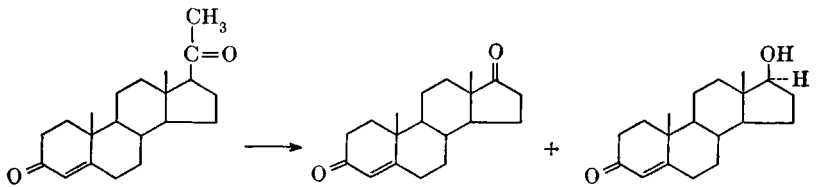
C. Rupture of carbon-to-carbon linkages

1. Partial side-chain degradation with formation of an acid



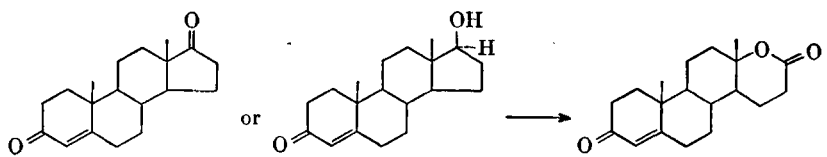
36, 36.1

2. Side-chain oxidation with formation of a ketone or hydroxyl group



8.1, 8.2, 8.4, 12.1, 17, 19.1, 28

3. Ring D rupture with formation of a lactone



16, 16.5

4. Ring A rupture with formation of an acid

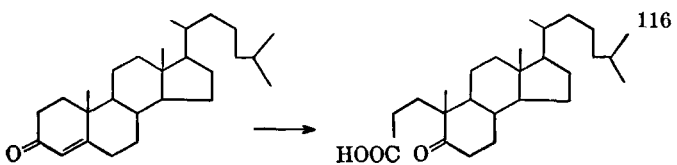


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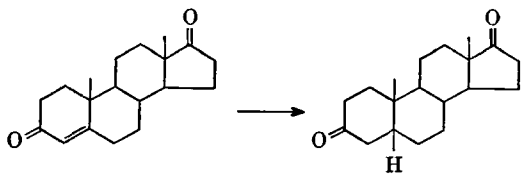
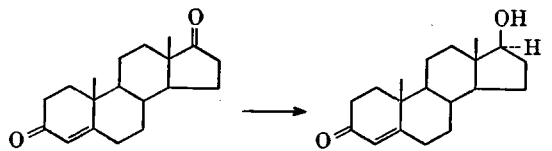
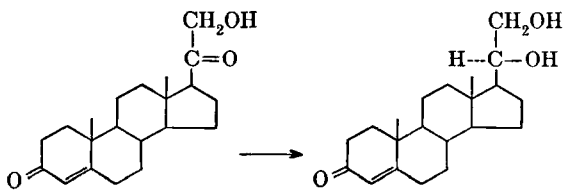
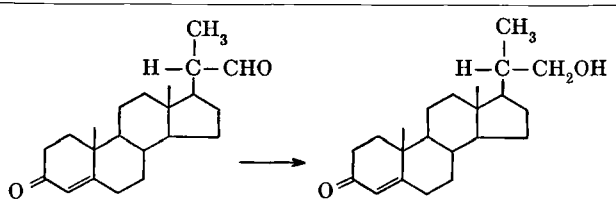
TABLE I.—(*Continued*)

	Example	Cross Reference to Examples in Table II
II. Reduction A. Ring double bonds		18, 32, 33.2, 90, 97
B. Carbonyls 1. Ring ketones		2, 15, 20, 21, 22, 22.2, 29, 29.1, 30, 31, 33.1, 34, 35.1, 35.2, 94, 96, 101.1, 113, 121
2. Side chain a. Ketones		89, 92, 93
b. Aldehydes		105

Mixed Reactions

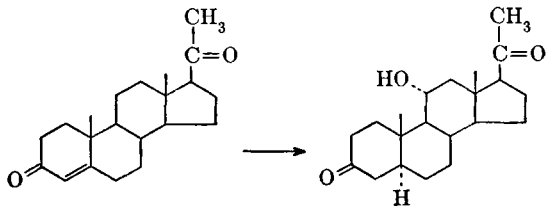
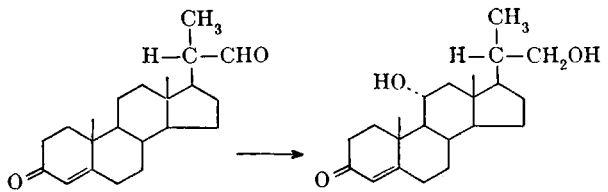
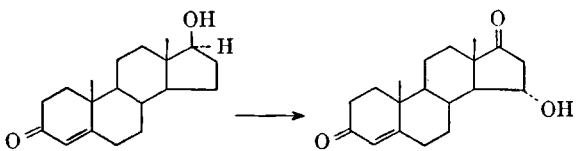
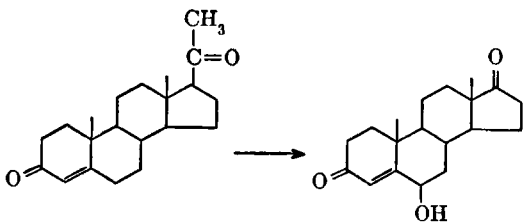
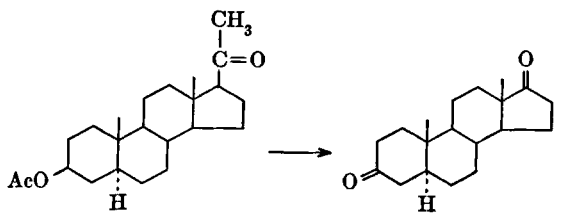
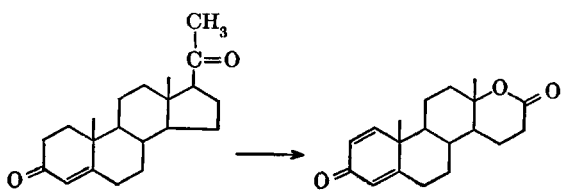
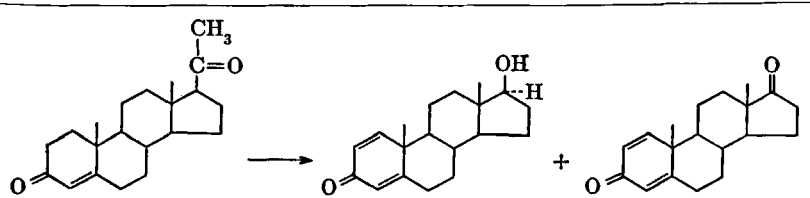
I. Hydroxylation A. With reduction 1. Of ring double bonds		60, 95.1, 98, 100
2. Of carbonyl groups		106, 107, 108
B. With oxidation of pre-existing hydroxyl groups		10.2, 13.1
C. With side-chain degradation (cf. II, A, 1)		10.1

TABLE I.—(*Continued*)

	Example	Cross Reference to Examples in Table II
II. Rupture of carbon-to-carbon linkages A. Oxidation of side chain 1. With ring hydroxylation (cf. I, C)		
2. With oxidation of pre-existing hydroxyl groups		17.2
3. With formation of a 1,4-dien-3-one chromophore a. With lactone formation in ring D		7.0, 7.1
b. With formation of a 17β-hydroxyl or 17-ketone group (1) From a 3-keto-Δ^4-steroid		6.3, 6.7, 9

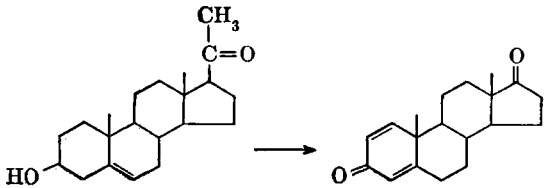
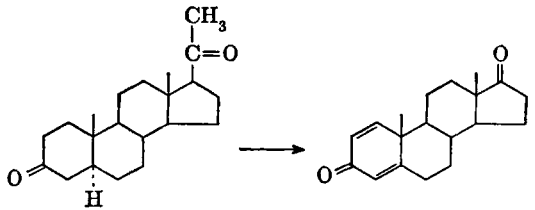
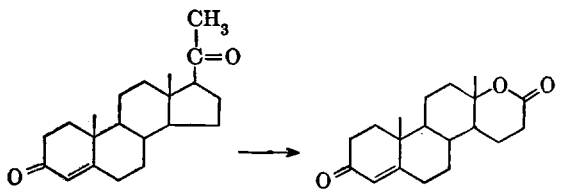
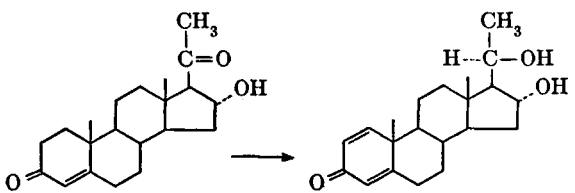
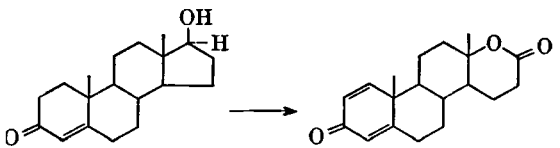
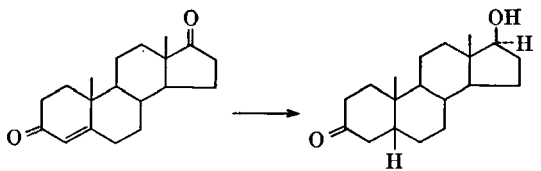
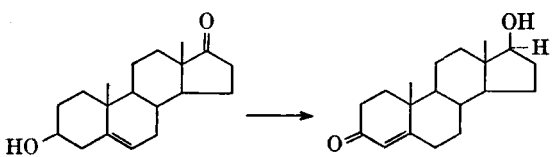
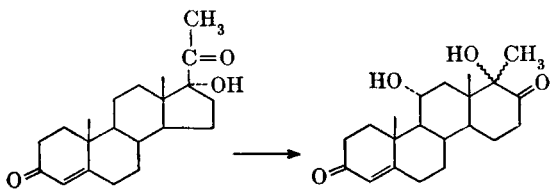
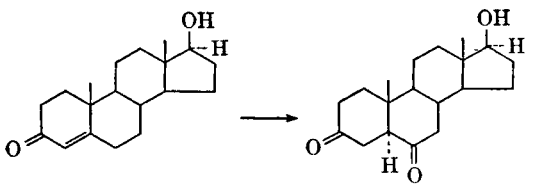
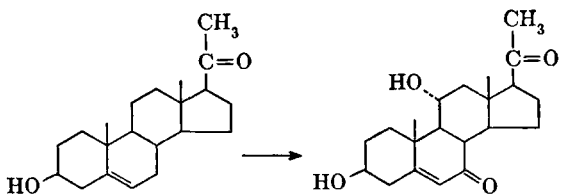
(2) From a 3β-hydroxy-Δ⁵-steroid		6.6
(3) From a saturated steroid		6.0, 6.4
4. With lactone formation in ring D		16.1, 16.2, 16.3, 16.4
III. Formation of a 1,4-dien-3-one chromophore with other reactions A. With concomitant reduction of a pre-existing carbonyl group		55

TABLE I.—(*Continued*)

	Example	Cross Reference to Examples in Table II
B. With formation of a ring D lactone		7.2
IV. Reduction reactions A. Reduction of a ring double bond and a ring ketone		33, 34.1, 34.2, 34.3, 34.4, 35, 35.3, 103
B. Oxidation of a hydroxyl and reduction of a ketone group		22.3

Miscellaneous Reactions[a]

I.		82
II.		23
III.		68

[a] See page 362 for the discussion of this classification.

TABLE II

MICROBIOLOGICAL TRANSFORMATIONS OF STEROIDS

No.	Product	Substrate	Microorganism and References
	$C_{18}H_{22}O_2$		
1.0	Estrone	17β-Estradiol	*Corynebacterium mediolanum*[a] (*Flavobacterium dehydrogenans*, *Micrococcus dehydrogenans*) (5, 6, 8) *Streptomyces albus* (*Actinomyces albus*) (162) *Pseudomonas* sp. (146) *Proactinomyces erythropolis* (Nocardia) (154)
	$C_{18}H_{24}O_2$		
2.0	17β-Estradiol	Estrone	Yeast (79, 163) *Pseudomonas* sp. (146)
	$C_{18}H_{26}O_3$		
3.0	6β-Hydroxy-19-nortestosterone	19-Nortestosterone	*Rhizopus nigricans* (121)
4.0	10ξ-Hydroxy-19-nortestosterone	19-Nortestosterone	*Rhizopus nigricans* (121)
5.0	11α-Hydroxy-19-nortestosterone	19-Nortestosterone	*Rhizopus nigricans* (121)
	$C_{19}H_{24}O_2$		
6.0	Androstadienedione	Allopregnane-3,20-dione	Fusaria (160)
6.1		Androstane-3,17-dione	Fusaria (160)
6.2		Androstenedione	Fusaria (160)
6.3		Deoxycorticosterone	Fusaria (160)
6.4		3β-Hydroxyallopregnan-20-one 3-acetate	Fusaria (160)
6.5		3β-Hydroxy-5-androsten-17-one	Fusaria (160)
6.6		Pregnenolone	Fusaria (160)
6.7		Progesterone	*Streptomyces lavendulae* (48) Fusaria (160)
	$C_{19}H_{24}O_3$		
7.0	1-Dehydrotestololactone	11-Deoxycortisol	*Cylindrocarpon radicicola* (48)

7.1		Progesterone	*Cylindrocarpon radicicola* (48) *Fusarium caucasicum* (31)
7.2		Testosterone	*Cylindrocarpon radicicola* (48)
	$C_{19}H_{26}O_2$		
8.0	Androstenedione	5-Androstene-3β,17β-diol	*Corynebacterium mediolanum*[a] (5, 35, 157) Flavobacteria (36) *Proactinomyces erythropolis* (153) Yeast (74)
8.1		Deoxycorticosterone	Aspergilli, Penicillia (129)
8.2		11-Deoxycortisol	Aspergilli, Penicillia (129)
8.3		3β-Hydroxy-5-androsten-17-one	*Corynebacterium mediolanum*[a] (4, 80) *Flavobacterium carbonilicum* (109) *Proactinomyces erythropolis* (153) *Acetobacter pasteurianum* (74)
8.4		Progesterone	Gliocladia, Aspergilli, Penicillia (129)
8.5		Testosterone	*Pseudomonas* sp. (147, 148) *Proactinomyces erythropolis* (153)
9.0	17β-Hydroxy-1,4-androstadien-3-one $C_{19}H_{26}O_3$	Progesterone	*Streptomyces lavendulae* (48)
10.0	6β-Hydroxyandrostenedione	Androstenedione	*Rhizopus nigricans* (29) *Aspergillus niger* (47)
10.1		Progesterone	*Gliocladium catenulatum* (129)
10.2		Testosterone	*Fusarium* sp. (98)
11.0	11α-Hydroxyandrostenedione	Androstenedione	*Rhizopus nigricans* (29)
12.0	14α-Hydroxyandrostenedione	Androstenedione	Mucors, *Helicostylum piriforme* (96, 97)
12.1		14α-Hydroxyprogesterone	*Penicillium lilacinum* (96, 97)
13.0	15α-Hydroxyandrostenedione	Androstenedione	*Fusarium* sp. (98)
13.1		Testosterone	*Fusarium* sp. (98)
14.0	16α-Hydroxyandrostenedione	Androstenedione	*Streptomyces roseochromogenus* (152)
15.0	17β-Hydroxy-4-androstene-3,11-dione	4-Androstene-3,11,17-trione	Yeast (60)
16.0	Testololactone	Androstenedione	Aspergilli, Penicillia (129)

TABLE II.—(*Continued*)

No.	Product	Substrate	Microorganism and References
16.1		Deoxycorticosterone	Aspergilli, Penicillia (129)
16.2		11-Deoxycortisol	Aspergilli, Penicillia (129)
16.3		17α-Hydroxyprogesterone	Aspergilli (129)
16.4		Progesterone	Aspergilli, Penicillia (48, 129) *Mucor mucedo, Penicillium chrysogenum* (129)
16.5		Testosterone	Aspergilli, Penicillia (48, 129)
	$C_{19}H_{28}O_2$		
17.0	Androstane-3,17-dione	Allopregnane-3,20-dione	Fusaria (160)
17.1		Androsterone	*Pseudomonas* sp. (149)
17.2		3β-Hydroxyallopregnan-20-one 3-acetate	Fusaria (160)
18.0	Etiocholane-3,17-dione	Androstenedione	*Bacillus putrificus*[b] (85)
19.0	3β-Hydroxy-5-androsten-17-one	5-Androstene-3β,17β-diol	*Pseudomonas* sp. (147)
19.1		Pregnenolone	*Fusarium solani* (160)
20.0	17β-Hydroxy-1-androsten-3-one	1-Androstene-3,17-dione	Yeast (18)
21.0	17β-Hydroxy-3,5-cycloandrostan-6-one	3,5-Cycloandrostane-6,17-dione	Yeast (20)
22.0	Testosterone	Androstenedione	Yeast (87) *Pseudomonas* sp. (147)
22.1		5-Androstene-3β,17β-diol	*Acetobacter pasteurianum* (74) *Corynebacterium mediolanum*[a] (36) Flavobacteria (109) *Proactinomyces erythropolis* (153)
22.2		5-Androstene-3,17-dione	Yeast (88)
22.3		3β-Hydroxy-5-androsten-17-one	Yeast (74)
22.4	Testosterone 17-benzoate	5-Androstene-3β,17β-diol 17-benzoate	*Proactinomyces erythropolis* (153)

	$C_{19}H_{28}O_2$		
23.0	17β-Hydroxyandrostane-3,6-dione	Testosterone	*Rhizopus nigricans* (29)
24.0	6β-Hydroxytestosterone	Testosterone	Rhizopi (29)
25.0	11α-Hydroxytestosterone	Testosterone	Rhizopi (29)
26.0	14α-Hydroxytestosterone	Testosterone	Mucors (96, 97)
27.0	15α-Hydroxytestosterone	Testosterone	Fusaria (98)
28.0	16α-Hydroxytestosterone	16α-Hydroxyprogesterone	*Streptomyces lavendulae* (152)
28.1		Testosterone	*Streptomyces roseochromogenus* (152) *Streptomyces* sp. (123, 155)
	$C_{19}H_{30}O_2$		
29.0	5-Androstene-3β,17β-diol	3β-Hydroxy-5-androsten-17-one	Yeast (89) *Bacillus putrificus*[b] (85)
29.1	5-Androstene-3β,17β-diol 3-acetate	3β-Hydroxy-5-androsten-17-one 3-acetate	Yeast (79)
30.0	Androsterone	Androstane-3,17-dione	Putrefactive bacteria (86) *Pseudomonas* sp. (149)
31.0	3β-Hydroxyandrostan-17-one	Androstane-3,17-dione	*Bacillus putrificus*[b] (85) Putrefactive bacteria (86)
32.0	17β-Hydroxyandrostan-3-one	Testosterone	Putrefactive bacteria (86)
33.0	17β-Hydroxyetiocholan-3-one	Androstenedione	*Bacillus putrificus*[b] (85)
33.1		Etiocholane-3,17-dione	Putrefactive bacteria (33, 85)
33.2		Testosterone	*Bacillus putrificus*[b] (85) Putrefactive bacteria (33)
	$C_{19}H_{32}O_2$		
34.0	Androstane-3β,17β-diol	Androstane-3,17-dione	*Bacillus putrificus*[b] (85) Yeast (156)
34.1		1-Androstene-3,17-dione	Yeast (19)
34.2		5-Androstene-3,17-dione	Yeast (87, 88)
34.3		17β-Hydroxy-1-androsten-3-one	Yeast (19)
34.4		Testosterone	Putrefactive bacteria (86)

TABLE II.—(*Continued*)

No.	Product	Substrate	Microorganism and References
35.0	Etiocholane-3α,17β-diol	Androstenedione	*Bacillus putrificus*[b] (85)
35.1		Etiocholane-3,17-dione	Putrefactive bacteria (33)
35.2		17β-Hydroxyetiocholan-3-one	Putrefactive bacteria (33)
35.3		Testosterone	*Bacillus putrificus*[b] (34) Putrefactive bacteria (86)
	$C_{20}H_{28}O_3$		
36.0	3-Keto-4-etienic acid	4-Cholesten-3-one	*Proactinomyces erythropolis* (154)
36.1		3-Keto-4-cholenic acid	*Proactinomyces erythropolis* (154)
37.0	19-Nordeoxycorticosterone	19-Norprogesterone	*Aspergillus niger* (166)
	$C_{20}H_{30}O_2$		
38.0	Methyltestosterone	17α-Methyl-5-androstene-3β,17-diol	*Corynebacterium mediolanum*[a] (6, 82)
	$C_{20}H_{30}O_3$		
39.0	6β-Hydroxymethyltestosterone	Methyltestosterone	Rhizopi (29)
40.0	11α-Hydroxymethyltestosterone	Methyltestosterone	Rhizopi (29)
	$C_{21}H_{26}O_5$		
41.0	1-Dehydrocortisone	Cortisone	*Fusarium solani* (158) *Corynebacterium simplex* (118)
	$C_{21}H_{27}O_5F$		
42.0	9α-Fluoro-1-dehydrocortisol	9α-Fluorocortisol	*Corynebacterium simplex* (118)
	$C_{21}H_{28}O_2$		
43.0	1-Dehydroprogesterone	Progesterone	*Calonectria decora* (158)
44.0	17β-Hydroxy-17-ethinyl-4-androsten-3-one	17-Ethinyl-5-androstene-3β,17β-diol	*Corynebacterium mediolanum*[a] (5, 6)
	$C_{21}H_{28}O_3$		
45.0	1-Dehydrodeoxycorticosterone	Deoxycorticosterone	*Calonectria decora* (158)
45.1	1-Dehydrodeoxycorticosterone 21-acetate	Deoxycorticosterone acetate	*Corynebacterium simplex* (118)

46.0	11α-Hydroxy-6-dehydroprogesterone $C_{21}H_{28}O_4$	6-Dehydroprogesterone	*Rhizopus nigricans* (134)
47.0	1-Dehydrocorticosterone	Corticosterone	*Calonectria decora* (158) *Corynebacterium simplex* (118)
48.0	11-Dehydrocorticosterone	11-Ketoprogesterone	*Ophiobolus herpotrichus* (105) *Aspergillus niger* (166)
49.0	1-Dehydro-11-deoxycortisol	11-Deoxycortisol	*Fusarium solani* (158) *Corynebacterium simplex* (118)
50.0	11α-Hydroxy-16,17-oxidoprogesterone $C_{21}H_{28}O_5$	16,17-Oxidoprogesterone	Rhizopi (130)
51.0	Cortisone	Corticosterone	*Trichothecium roseum* (105) *Cephalothecium roseum* (102)
51.1		11-Dehydrocorticosterone	*Cephalothecium roseum* (102) *Trichothecium roseum* (105)
51.2		11-Deoxycortisol	*Cunninghamella blakesleeana* (55) Curvulariae (138)
52.0	1-Dehydrocortisol $C_{21}H_{30}O_2$	Cortisol	*Corynebacterium simplex* (118)
53.0	Progesterone	Pregnenolone	*Corynebacterium mediolanum*[a] (5, 35) Yeast (*Corynebacterium mediolanum*) (80) Streptomycetes, *Phycomyces blakesleeanus*, *Aspergillus niger*, *Penicillium chrysogenum*, *Eremothecium ashbyii*, *Ustilago zeae* (122)
	$C_{21}H_{30}O_3$		
54.0	Deoxycorticosterone	3β,21-Dihydroxy-5-pregnen-20-one 21-acetate	*Corynebacterium mediolanum*[a] (81)
54.1		Progesterone	*Ophiobolus herpotrichus* (105) *Aspergillus niger* (166)
55.0	16α,20β-Dihydroxy-1,4-pregnadien-3-one	16α-Hydroxyprogesterone	*Streptomyces lavendulae* (50)
56.0	6β-Hydroxyprogesterone	Progesterone	*Streptomyces aureofaciens* (43) *Penicillium urticae* (111, 120)

TABLE II.—(*Continued*)

No.	Product	Substrate	Microorganism and References
57.0	7α-Hydroxyprogesterone	Progesterone	*Phycomyces blakesleeanus* (44, 50)
58.0	8- or 9-Hydroxyprogesterone	Progesterone	*Streptomyces aureofaciens* (43)
59.0	11α-Hydroxyprogesterone	Progesterone	*Rhizopi* (131, 132) *Rhizopus* sp. (70, 90) Aspergilli (46, 112) *Pestalotia foedans* (141)
60.0	11α-Hydroxy-17α-progesterone	16-Dehydroprogesterone	*Rhizopus nigricans* (100)
61.0	11β-Hydroxyprogesterone	Progesterone	Curvulariae (138) *Cunninghamella blakesleeana* (54)
62.0	14α-Hydroxyprogesterone	Progesterone	Mucors, *Helicostylum piriforme* (96, 97) *Bacillus cereus* (151)
63.0	15α-Hydroxyprogesterone	Progesterone	*Colletotrichum antirrhini* (50) Gibberellae, Fusaria (98) *Penicillium urticae* (111, 120)
64.0	15β-Hydroxyprogesterone	Progesterone	*Phycomyces blakesleeanus* (50)
65.0	16α-Hydroxyprogesterone $C_{21}H_{30}O_4$	Progesterone	*Streptomyces* sp. (124)
66.0	Corticosterone	Deoxycorticosterone	Curvulariae (138) *Cunninghamella blakesleeana* (92)
66.1		11β-Hydroxyprogesterone	*Aspergillus niger* (166)
67.0	11-Deoxycortisol	Deoxycorticosterone	*Cephalothecium roseum* (95) *Trichothecium roseum* (105)
67.1		17α-Hydroxyprogesterone	*Ophiobolus herpotrichus* (105)
68.0	3β,11α-Dihydroxy-5-pregnene-7,20-dione	Pregnenolone	*Rhizopus arrhizus* (98, 114)
69.0	6β,21-Dihydroxy-4-pregnene-3,20-dione	Deoxycorticosterone	Rhizopi (30) *Trichothecium roseum* (105)
69.1		6β-Hydroxyprogesterone	*Aspergillus niger* (166)

70.0	7α,21-Dihydroxy-4-pregnene-3,20-dione	Deoxycorticosterone	Curvulariae, *Peziza* sp. (106)
71.0	8,21-Dihydroxy-4-pregnene-3,20-dione[c]	Deoxycorticosterone	Mucors, *Helicostylum piriforme* (97, 98, 115) *Neurospora crassa* (145)
72.0	11α,21-Dihydroxy-4-pregnene-3,20-dione	Deoxycorticosterone	Rhizopi (30) Aspergilli (31, 46, 112)
72.1		11α-Hydroxyprogesterone	*Aspergillus niger* (166)
73.0	14α,21-Dihydroxy-4-pregnene-3,20-dione	Deoxycorticosterone	Mucors, *Helicostylum piriforme* (96, 115) *Cunninghamella blakesleeana* (92)
73.1		14α-Hydroxyprogesterone	*Aspergillus niger* (166)
74.0	15α,21-Dihydroxy-4-pregnene-3,20-dione[d]	Deoxycorticosterone	Gibberellae[d] (98, 106) Fusaria (98)
75.0	15β,21-Dihydroxy-4-pregnene-3,20-dione[d]	Deoxycorticosterone	*Lenzites abietina*[d] (106)
76.0	16α,21-Dihydroxy-4-pregnene-3,20-dione	Deoxycorticosterone	*Streptomyces* sp. (152, 159) *Streptomyces roseochromogenus* (152)
77.0	6β,11α-Dihydroxyprogesterone	Progesterone	Rhizopi (132) *Aspergillus niger* (46)
77.1		11α-Hydroxyprogesterone	*Cunninghamella blakesleeana* (31)
78.0	6β,16α-Dihydroxyprogesterone	16α-Hydroxyprogesterone	*Aspergillus nidulans* (45)
78A[e]	11α,16α-Dihydroxyprogesterone	16α-Hydroxyprogesterone	*Aspergillus niger* (45)
79.0	6β,17α-Dihydroxyprogesterone	17α-Hydroxyprogesterone	Rhizopi (101)
80.0	11α,17α-Dihydroxyprogesterone	17α-Hydroxyprogesterone	Rhizopi (101), *Aspergillus niger* (46)
80.1		Progesterone	*Cephalothecium roseum* (102)
81.0	11β,17α-Dihydroxyprogesterone	17α-Hydroxyprogesterone	Curvulariae (138)
82.0	17a-Methyl-D-homo-4-androstene-11α,17a-diol-3,17-dione $C_{21}H_{30}O_5$	17α-Hydroxyprogesterone	*Aspergillus niger* (46)
83.0	Cortisol	Corticosterone	*Cephalothecium roseum* (102) *Trichothecium roseum* (105)

TABLE II.—(*Continued*)

No.	Product	Substrate	Microorganism and References
83.1		11-Deoxycortisol	*Streptomyces fradiae* (25) *Cunninghamella blakesleeana* (55) Curvulariae (138) *Coniothyrium* sp. (150)
84.0	6β,17α,21-Trihydroxy-4-pregnene-3,20-dione	Deoxycorticosterone	*Cephalothecium roseum* (102)
84.1		11-Deoxycortisol	Rhizopi (128)
85.0	8,17α,21-Trihydroxy-4-pregnene-3,20-dione	11-Deoxycortisol	Mucors, *Helicostylum piriforme* (97, 110)
86.0	11α,17α,21-Trihydroxy-4-pregnene-3,20-dione	Deoxycorticosterone	*Cephalothecium roseum* (102)
86.1		11-Deoxycortisol	*Rhizopus nigricans* (128), *Rhizopus* sp. (70) *Aspergillus niger* (46, 110) *Aspergillus nidulans* (47) *Helicostylum piriforme* (97)
87.0	11β,14α,17α-Trihydroxy-4-pregnene-3,20-dione	17α-Hydroxyprogesterone	*Curvularia lunata* (140)
88.0	14α,17α,21-Trihydroxy-4-pregnene-3,20-dione $C_{21}H_{32}O_2$	11-Deoxycortisol	Mucors, *Helicostylum piriforme* (96, 97) *Cunninghamella blakesleeana* (91)
89.0	20β-Hydroxy-4-pregnen-3-one	Progesterone	*Streptomyces lavendulae* (48)
90.0	Pregnane-3,20-dione $C_{21}H_{32}O_3$	Progesterone	*Bacillus putrificus*[b] (85)
91.0	3β,7β-Dihydroxy-5-pregnen-20-one	Pregnenolone	*Rhizopus nigricans* (98)
92.0	16α,20β-Dihydroxy-4-pregnen-3-one	16α-Hydroxyprogesterone	*Streptomyces lavendulae* (50)
93.0	20β,21-Dihydroxy-4-pregnen-3-one[f]	Deoxycorticosterone	*Streptomyces* sp. (126)
94.0	3α-Hydroxyallopregnane-11,20-dione	Allopregnane-3,11,20-trione	Yeast (22)
95.0	11α-Hydroxyallopregnane-3,20-dione	Allopregnane-3,20-dione	*Rhizopus nigricans* (32)

95.1		Progesterone	*Rhizopus nigricans* (132)
96.0	3α-Hydroxypregnane-11,20-dione	Pregnane-3,11,20-trione	Yeast (22)
97.0	11α-Hydroxypregnane-3,20-dione	11α-Hydroxyprogesterone	Anaerobe (unknown) (116)
97.1		Pregnane-3,20-dione	*Rhizopus nigricans* (32)
98.0	16α-Hydroxypregnane-3,20-dione $C_{21}H_{32}O_4$	Progesterone	*Streptomyces* sp. (123, 124)
99.0	3β,7β,11α-Trihydroxy-5-pregnen-20-one $C_{21}H_{32}O_5$	Pregnenolone	*Rhizopus arrhizus* (98, 113)
100.0	11α,17α,21-Trihydroxypregnane-3,20-dione	11-Deoxycortisol	*Rhizopus nigricans* (128)
100.1	 $C_{21}H_{34}O_3$	17α,21-Dihydroxypregnane-3,20-dione	*Rhizopus nigricans* (128)
101.0	3β,11α-Dihydroxyallopregnan-20-one	3β-Hydroxyallopregnan-20-one	*Rhizopus nigricans* (98)
101.1		11α-Hydroxyallopregnane-3,20-dione	Yeast (22)
102.0	3β,7β-Dihydroxyallopregnan-20-one	3β-Hydroxyallopregnan-20-one	*Rhizopus arrhizus* (98, 110)
103.0	3α,11α-Dihydroxypregnan-20-one $C_{21}H_{34}O_4$	11α-Hydroxyprogesterone	Anaerobe (unknown) (116)
104.0	3β,7β,21-Trihydroxyallopregnan-20-one $C_{22}H_{34}O_2$	3β,21-Dihydroxyallopregnan-20-one	*Rhizopus* sp. (70)
105.0	3-Ketobisnor-4-cholen-22-ol $C_{22}H_{34}O_3$	3-Ketobisnor-4-cholen-22-al	*Penicillium lilacinum* (133)
106.0	3-Ketobisnor-4-cholene-11α,22-diol	3-Ketobisnor-4-cholen-22-al	*Rhizopus nigricans* (99)
107.0	3-Ketobisnor-4-cholene-15α,22-diol $C_{22}H_{34}O_4$	3-Ketobisnor-4-cholen-22-al	*Rhizopus nigricans* (98)
108.0	3-Ketobisnor-4-cholene-6β,11α,22-triol	3-Ketobisnor-4-cholen-22-al	*Rhizopus nigricans* (99)
108.1		3-Ketobisnor-4-cholen-11α,22-diol	*Cunninghamella blakesleeana* (99)

TABLE II.—(*Continued*)

No.	Product	Substrate	Microorganism and References
	$C_{24}H_{34}O_5$		
109.0	3,7,12-Triketocholanic acid	Cholic acid	*Alcaligenes faecalis* (137) *Proactinomyces erythropolis* (154)
	$C_{24}H_{36}O_3$		
110.0	3-Keto-4-cholenic acid	3β-Hydroxy-5-cholenic acid	*Proactinomyces erythropolis* (153)
	$C_{24}H_{36}O_5$		
111.0	3α-Hydroxy-7,12-diketocholanic acid	Cholic acid	*Alcaligenes faecalis* (137)
112.0	7ξ-Hydroxy-3,12-diketocholanic acid	Cholic acid	*Escherichia coli* (51)
	$C_{24}H_{38}O_4$		
113.0	3α-Hydroxy-12-ketocholanic acid	3,12-Diketocholanic acid	Yeast (71, 83)
	$C_{24}H_{38}O_5$		
114.0	3α,12α-Dihydroxy-7-ketocholanic acid	Cholic acid	*Alcaligenes faecalis* (137)
	$C_{26}H_{38}O_5$		
115.0	Nor-4-cholestene-3,25-dione	3β-Hydroxynor-5-cholesten-25-one	*Corynebacterium mediolanum*[a] (5, 6)
	$C_{26}H_{44}O_3$		
116.0	Windaus' keto acid	4-Cholesten-3-one	*Proactinomyces erythropolis* (154)
	$C_{27}H_{44}O$		
117.0	5,7-Cholestadien-3β-ol	Cholesterol	*Azotobacter* sp. (64)
118.0	4-Cholesten-3-one	Cholesterol	*Proactinomyces erythropolis* (153, 154) *Mycobacterium* sp. (142) *Flavobacterium maris* (7, 10, 26) *Azotobacter* sp. (64) *Acetobacter xylinum* (74) *Proactinomyces* sp. (75, 76)
	$C_{27}H_{46}O$		
119.0	Coprostan-3-one	Coprostanol	*Proactinomyces erythropolis* (153)

	$C_{27}H_{46}O_2$		
120.0	5-Cholestene-3β,7ξ-diol	Cholesterol	*Proactinomyces* sp. (75, 76)
	$C_{27}H_{48}O$		
121.0	Cholestanol	Cholestan-3-one	Yeast (83)
	$C_{29}H_{46}O$		
122.0	4,22-Stigmastadien-3-one	Stigmasterol	*Proactinomyces erythropolis* (153)
	$C_{29}H_{48}O$		
123.0	4-β-Sitosten-3-one	β-Sitosterol	*Proactinomyces erythropolis* (153)

[a] *Corynebacterium mediolanum* is reported to be identical to *Flavobacterium dehydrogenans* and *Micrococcus dehydrogenans* (84, 108).

[b] *Bacillus putrificus* Bienstock has not been accepted as a name for an anaerobe due to confusion in its description (56) (cf. page 395).

[c] For a discussion of this compound see pages 388–389.

[d] The orientation of the hydroxyl at carbon 15 in these compounds has been corrected in conformity with the proof offered by Fried *et al.* (47) (cf. page 391).

[e] After the completion of this manuscript, it was found that this compound had been inadvertently omitted.

[f] The configuration of the hydroxyl at carbon 20 in this compound was previously designated to be "α" (144, 126). However, Caspi and Dorfman (23) have recently proved by molecular rotational comparisons that this configuration is "β".

II. Simple Oxygenation of the Steroid Rings

The announcement in 1952 from these laboratories (131) that *Rhizopus arrhizus* could introduce a hydroxyl group into the 11α-position of the progesterone molecule marked a new phase of microbiological transformations of steroids. The report had a threefold significance: (1) 11-Oxygenation was, until that time, the major stumbling block in the synthesis of the adrenocortical hormones, cortisone and cortisol. (2) Hydroxylation of the steroid nucleus by microorganisms was indicated to be a commercially feasible process. (3) This was the culmination of a deliberate attempt to bring about a specific hydroxylation of specific steroids by a microbiological procedure. While it is true that Krámli and Horváth (75) had earlier reported the conversion of cholesterol to a 7-hydroxycholesterol by means of a microorganism, theirs was an incidental discovery—the investigators had not set out to make 7-hydroxycholesterol from cholesterol. In our case, we wanted a method for 11-oxygenation of specific steroids and sought to accomplish this by microbiological means. From the technological point of view this is, perhaps, the most interesting aspect of the work.

This early success and the rapid expansion of the field were greatly aided by the development of the techniques of paper chromatography. Application of these techniques enables investigators to examine a wide variety of steroids and microorganisms relatively quickly and inexpensively. Promising leads can then be developed on a larger scale for isolation and characterization of products.

The first paper on 11α-oxygenation was soon followed by a report by Perlman *et al.* (124) on 16α-hydroxylation of progesterone by an unidentified actinomycete. (This culture has since been identified as a *Streptomyces* (123).) Hydroxylation in the 11α-position by *Aspergillus niger* was reported by Fried *et al.* (46) in 1952. The widespread ability of the fungi to hydroxylate and otherwise modify the steroid skeleton has since been amply demonstrated in numerous publications. The steroid substrate and the species and strain of microorganism used greatly influence the course of these reactions. For example, the pattern of hydroxylation products of a particular substrate is often radically altered by using a different strain of the same species of microorganism; or the same microbial culture will attack different steroids to form distinctly different types of products. These aspects of microbial transformations will be amplified later. Oxygenation at carbon no. 11 will be discussed in greater detail in its appropriate section.

1. Oxygenation at Carbon No. 6

Hydroxylation of steroids at carbon no. 6 is one of the commonest reactions of the fungi. We first observed it in the transformation of

progesterone by *Rhizopus arrhizus*[4] where, besides 11α-hydroxyprogesterone, 6β,11α-dihydroxyprogesterone was found (132). Fried *et al.* (46) found the same compound as a product of the dissimilation of progesterone by *Aspergillus niger*.

Hydroxylation in the 6β-position of various steroid substrates has been observed in other genera of the order of Mucorales besides *Rhizopus*, i.e. *Mucor* (31), *Helicostylum* (97), *Cunninghamella* (31); it has also been shown to take place with species of *Penicillium* (120), *Aspergillus* (46, 47), *Streptomyces* (43), *Gliocladium* (129), *Fusarium* (98), and *Cephalothecium* (102). Although most molds which hydroxylate progesterone in the 6β-position do so in combination with other reactions such as hydroxylation or side-chain cleavage, *Penicillium urticae* (120) and *Streptomyces aureofaciens* (43) are notable in that they formed 6β-hydroxyprogesterone in isolable amounts. From a preparative point of view, chemical methods of synthesis (3, 9, 42) will, in some cases, be the procedure of choice, but in many cases, where fermentation yields are high and where other functional groups on the steroid molecule could cause complications in a chemical synthesis, the microbiological approach is superior (e.g. 6β-hydroxydeoxycorticosterone (6β,21-dihydroxy-4-pregnene-3,20-dione) from deoxycorticosterone).

2. Oxygenation at Carbon No. 7

Besides the report of Krámli and Horváth (75, 76) on the formation of 7-hydroxycholesterol[5] from cholesterol, several authentic instances of 7-oxygenation have been found. Kahnt *et al.* (70) isolated 3β,7β,21-trihydroxyallopregnan-20-one from a fermentation of 3β,21-dihydroxyallopregnan-20-one by a *Rhizopus* sp. We isolated 3β,7β-dihydroxyallopregnan-20-one as a dissimilation product of 3β-hydroxyallopregnan-20-one by *R. arrhizus* (98, 110). When we fermented pregnenolone with *R. arrhizus*, 3β,7β,11α-trihydroxy-5-pregnen-20-one and 3β,11α-dihydroxy-5-pregnene-7,20-dione were isolated (97, 114). This latter compound possibly resulted from a nonenzymatic oxidation of the 3β,7β,11α-trihydroxy-

[4] In this article, microorganisms will be identified only by generic and specific names when discussing published material; when unpublished data is drawn upon, we shall endeavor to identify the organisms further, when practicable, by giving their American Type Culture Collection (A.T.C.C.) number the first time reference is made to that particular reaction. To avoid confusion, when discussing *strains* of a specific microorganism, we shall distinguish them by A.T.C.C. number or other description. Because we shall contrast *Rhizopus arrhizus* Fischer (A.T.C.C. 11145) and *R. nigricans* Ehrb. (A.T.C.C. 6227b) many times to illustrate organism specificity, and since strain differences often are very great, the names *R. arrhizus* and *R. nigricans* will always refer to the two strains identified in this note unless otherwise stated.

[5] In neither of their communications do these authors offer any proof of the identity of their product.

compound as mentioned earlier (page 362). From a similar fermentation with *R. nigricans*, we isolated *inter alia* the monohydroxylated derivative, 3β,7β-dihydroxy-5-pregnen-20-one (98).

Here we have several instances wherein oxygenation by Rhizopi[6] produced not the expected 6β-hydroxysteroids (leaving out of the discussion 11α-hydroxylation) but 7β-hydroxy derivatives. With pregnenolone one can possibly ascribe this shift in point of attack to the unsaturation at carbon no. 6, but with the allopregnanes this shift is clearly the result of the 3β-hydroxyl group, since with neither the 3-ketopregnanes nor 3-ketoallopregnanes was there evidence of 7-hydroxylation (31, 32). It would be of great interest to examine the various microbiological fermentations of steroids having the 3α- and the 3β-hydroxyl in combination with the pregnane and the allopregnane configuration for influence on the course of the enzymatic dissimilations. Such a study might help to clarify the mechanism of the enzymatic action.

Hydroxylation at carbon no. 7 has been achieved with other microorganisms and without recourse to 3-hydroxysteroids. Meystre and co-workers (106) have employed several species of *Curvularia* as well as a *Peziza* sp. to effect 7α-hydroxylation of deoxycorticosterone. Fried and co-workers (50) isolated, in addition to 15β-hydroxyprogesterone, a 7ξ-hydroxyprogesterone from the dissimilation of progesterone by *Phycomyces blakesleeanus*.[7]

3. Oxygenation at Carbon No. 8 (or 9)

The dissimilation of deoxycorticosterone by *Helicostylum piriforme* allowed us to isolate not only 14α-hydroxydeoxycorticosterone but a second tertiary-hydroxylated deoxycorticosterone, presumably the 8β-hydroxy compound (97). Fermentation of 11-deoxycortisol with this organism likewise yielded a second tertiary-hydroxylated derivative besides the 14α-hydroxy-11-deoxycortisol (14α,17α,21-trihydroxy-4-pregnene-3,20-dione). From our studies by paper chromatography we have assigned to these two new compounds the structures of 8β-hydroxy derivatives of deoxycorticosterone and 11-deoxycortisol, respectively. Stone *et al.* (145) have recently isolated the same tertiary-hydroxylated

[6] The plural form of the nonitalicized generic name to denote a number of species of that genus is commonly employed in the case of some microorganisms, i.e., Penicillia and Aspergilli; after consultation with a taxonomist we have extended this principle to cover all the genera discussed in this article.

[7] Meystre *et al.* (106) assigned the α orientation to their 7-hydroxydeoxycorticosterone on the basis of molecular rotational comparison with saturated steroids without regard to any possible vicinal effects of the 3-keto-Δ^4-grouping. If this argument is valid, then on the same basis the 7ξ-hydroxyprogesterone of Fried *et al.* (50) is 7α-hydroxyprogesterone.

deoxycorticosterone from a fermentation of deoxycorticosterone with *Neurospora crassa* and assigned to this compound the structure of 8 (or 9)-hydroxydeoxycorticosterone. Fried *et al.* (43) isolated from a fermentation of progesterone with *Streptomyces aureofaciens*, besides 6β-hydroxyprogesterone, either the 8 (or 9)-hydroxyprogesterone. Since the latter compound and ours have different side chains, direct comparison cannot be made. Comparison of the contribution to the molecular rotation produced by the tertiary hydroxyl in the hydroxydeoxycorticosterone and in the hydroxyprogesterone leads to the conclusion that different positions are involved.

$$\Delta([M]_D^{\text{Hydroxydeoxycorticosterone}} - [M_D^{\text{Deoxycorticosterone}}) = -33$$
$$\Delta([M]_D^{\text{Hydroxyprogesterone}} - [M]_D^{\text{Progesterone}}) = +34$$

If we assume that configuration at carbon no. 8 and carbon no. 9 is retained, then one of the compounds is an 8β-hydroxysteroid and the other a 9α-hydroxysteroid.

4. Oxygenation at Carbon No. 10

In our laboratories (121) fermentation of 19-nortestosterone with *Rhizopus nigricans* has led to the isolation, among other things, of a small amount of 10-hydroxy-19-nortestosterone. The stereochemical configuration of the new hydroxyl group has not been established.

5. Oxygenation at Carbon No. 11

Hydroxylation at carbon no. 11 by Rhizopi has already been mentioned. In general, we have found that *R. nigricans* is among the best for this purpose, allowing high yields of product. For testosterone and methyltestosterone, *R. reflexus* is, perhaps, a shade better (29). A number of *Rhizopus* organisms are about as good as *R. nigricans* (110). Many of the Aspergilli efficiently hydroxylate steroids in the 11α-position (31, 46, 112). An interesting constituent found by Fried *et al.* (46) in the conversion of 17α-hydroxyprogesterone to 11α,17α-dihydroxyprogesterone by *Aspergillus niger* was 11α,17a-dihydroxy-17a-methyl-D-homo-4-androstene-3,17-dione. This is the only instance known of D-homosteroid formation in a microbiological conversion. We have already discussed the possibility that this is a nonenzymatic rearrangement.

Hydroxylation of steroids in the 11α-position is performed by many other fungi than those mentioned, but usually the yield is low or the fermentation is unsatisfactory in that complicating reactions take place, e.g. 6β,11α-dihydroxylation. An interesting case is the discovery (102) that *Cephalothecium roseum* can introduce either the 11α- or the 17α-hydroxyl group, or both. Thus, in the fermentation of deoxycorticosterone one

finds 11-epicortisol (11α,17α,21-trihydroxy-4-pregnene-3,20-dione) as well as 11-epicorticosterone and 11-deoxycortisol (95).

Hydroxylation in the 11β-position by microorganisms was first noted in these laboratories. Colingsworth *et al.* (25) isolated cortisol in small yield from a fermentation of 11-deoxycortisol with *Streptomyces fradiae*, and Hanson *et al.* (55) obtained excellent yields of cortisol (and some cortisone) by the fermentation of 11-deoxycortisol with *Cunninghamella blakesleeana*. The cortisone produced in this fermentation is either formed directly from 11-deoxycortisol, or is a further oxidation of the cortisol-enzyme complex, since cortisol itself is not oxidized to cortisone by this organism (54). A partial control of the cortisone formation was achieved by addition of various alcohols or phenols (93). More recently, Shull *et al.* (138, 140) have reported 11β-hydroxylation using several species of *Curvularia*. Thoma *et al.* (150) employed a *Coniothyrium* sp. to carry out 11β-hydroxylation. 11β-Hydroxylation by microorganisms is not unique, but, to the extent of our present knowledge, is much rarer than 11α-hydroxylation.

6. Oxygenation at Carbon No. 14

In the order of Mucorales, we have found that various species of *Mucor*, *Cunninghamella*, and *Helicostylum* carry out 14α-hydroxylation, the efficiency varying with the steroid substrate and organism (97). Thoma *et al.* (151) have utilized a bacterium, *Bacillus cereus*, to introduce the 14α-hydroxyl group into progesterone. As Fried *et al.* (49) have pointed out, this is one of the few cases where bacteria have been found useful in reactions other than hydrogenation and dehydrogenation. Shull *et al.* (140) have reported on the isolation of 11β,14α,17α-trihydroxyprogesterone from the fermentation of 17α-hydroxyprogesterone by *Curvularia lunata*. It is interesting to note that the two molds which are efficient 11β-hydroxylators are also 14α-hydroxylators, whereas the 11α-hydroxylators, in general, are not 14α-hydroxylators (the only one known to us is *Helicostylum piriforme*, which is not a good 11α-hydroxylator, making less than 1% of 11-epicortisol from 11-deoxycortisol).

7. Oxygenation at Carbon No. 15

Fried *et al.* (50) isolated 15β-hydroxyprogesterone (in addition to 7ξ-hydroxyprogesterone) from the fermentation of progesterone with *Phycomyces blakesleeanus*. By the action of *Colletotrichum antirrhini* on progesterone, Fried and co-workers (50) were able to isolate 15α-hydroxyprogesterone. We isolated this compound as a dissimilation product of progesterone by *Penicillium urticae* (120). Recently, Meystre and co-workers (106) reported the formation of 15β-hydroxydeoxycorticoster-

one (15β,21-dihydroxy-4-pregnene-3,20-dione) from deoxycorticosterone by *Gibberella baccata* and of 15α-hydroxydeoxycorticosterone from deoxycorticosterone by *Lenzites abietina*. However, we had found that a number of species of *Fusarium*[8] and of *Gibberella*[8] (98) convert C_{19}–C_{21} steroids to what we considered to be the corresponding 15α-hydroxysteroids (not 15β- as reported by Meystre *et al.*). The configurational assignments at carbon no. 15 made by us and the Squibb Institute group of workers are in agreement. Fried (47) has presented, in elegant manner, the arguments in favor of reversing the orientations at carbon no. 15 as given by the Swiss workers. In Table II we have taken the liberty of correcting this error and have named the two 15-hydroxylated deoxycorticosterones in conformity with the orientations established by Fried and co-workers and by us.

8. Oxygenation at Carbon No. 16

Perlman *et al.* (124) reported the isolation of 16α-hydroxyprogesterone after the addition of progesterone to a *Streptomyces* culture. Vischer *et al.* (159) have reported on the 16α-hydroxylation of deoxycorticosterone by various species of *Streptomyces*.

9. Oxygenation at Carbon No. 17

Mention has already been made (cf. 11-oxygenation) of the 17α-hydroxylation carried out by *Cephalothecium roseum*. In our hands this microorganism hydroxylated in the 17α-position with or without concomitant hydroxylation in the 6β- or the 11α-position. Progesterone yielded 11α,17α-dihydroxyprogesterone, deoxycorticosterone yielded 11-epicortisol and 6β-hydroxy-11-deoxycortisol. In a simultaneous publication, Meystre *et al.* (105) reported 17α-hydroxylation *without* 11α-hydroxylation by *Trichothecium roseum* [a synonym for *Cephalothecium roseum* (2)]. Indeed, these authors stated that the 11α-hydroxyl group inhibited the 17α-hydroxylation. This difference between our results and those of the Swiss workers can possibly be ascribed to strain difference as noted by Fried (49). In order to avoid a taxonomical discussion, we shall continue to describe these two cultures as *Trichothecium roseum* and *Cephalothecium roseum*. With one organism it is thus possible to overcome two of the most difficult steps in the synthesis of the adrenocortical hormones—11- and 17-oxygenation.

[8] For example, *Fusarium orthoceras* (A.T.C.C. 10082), *F. sulphureum* (A.T.C.C. 7642), *F. avenaceum* (A.T.C.C. 8150) and a *Fusarium* sp. (our strain F_{18}); *Gibberella baccata* (Wallbr.) Sacc., *G. cyanea* (Sollm.) Wr., *G. fujikuroi* (Saw.) Wr., var. *subglutinans* Edw., *G. saubinettii* (Mont.) Sacc.

10. Oxygenation at Carbon No. 21

Meystre *et al.* (105) have reported that *Ophiobolus herbotrichus* [sic!][9] will hydroxylate the 21-methyl group. Progesterone, 11-ketoprogesterone, and 17α-hydroxyprogesterone yielded deoxycorticosterone, corticosterone, and 11-deoxycortisol, respectively. No mention was made of the influence of the 11-hydroxyl group (in α or β orientation) on the course of the 21-hydroxylation. Zaffaroni *et al.* (166) later showed that *Aspergillus niger* (A.T.C.C. 9142) could 21-hydroxylate progesterone or a number of its derivatives (19-norprogesterone, 11-ketoprogesterone, 11α- and 11β-hydroxyprogesterone, 6β-hydroxyprogesterone, 14α-hydroxyprogesterone).

It is obvious that one can now transform relatively cheap and abundant steroids into the most important (from the medical point of view) adrenocortical hormones in a very few steps by a combination of microbiological dissimilations with, perhaps, one or two chemical reactions. These sequences will be discussed in the section on hormone synthesis.

III. Polyoxygenation of Steroids by Microorganisms

Besides the simple oxygenation (that is, the introduction of a single hydroxyl group with no other changes), in many cases more than one oxygen function is added. We have already pointed out that species of *Rhizopus* and *Aspergillus* can hydroxylate in the 6β- and 11α-positions. Depending upon the species, strain, substrate, and fermentation conditions, different types of products will result. In some cases, this leads to the isolation of dihydroxylated derivatives of the steroid substrate, for example, 6β,11α-dihydroxyprogesterone from progesterone by *Rhizopus* or 11-epicortisol from deoxycorticosterone by *Cephalothecium roseum*. In general, high yields of such double hydroxylations are rare, the introduction of one or the other hydroxyl group usually being favored.

Sometimes oxygenation is accompanied by other transformations such as dehydrogenation, hydrogenation, side-chain removal, etc. These will be discussed under the section on mixed reactions.

IV. Side-Chain Removal and Formation of $\Delta^{1,4}$-Steroids

These reactions will be discussed together, since many of the molds which do the one also do the other. The 1-dehydrogenation with or without side-chain degradation is of great importance, as will be amplified in the discussion on hormone synthesis.

[9] Presumably a typographical error for *Ophiobolus herpotrichus* (Fr.) Sacc. Henceforth, we shall refer to this species as *herpotrichus*.

In simultaneous publications, Fried *et al.* (48) and Peterson *et al.* (129) reported on side-chain degradation by a number of microorganisms. Several species of *Penicillium* and *Aspergillus* were found by us to remove the acetyl, α-ketol or dihydroxyacetone types of side chain of C_{21}-steroids to yield androstenedione and testololactone. *Gliocladium catenulatum* degraded progesterone to androstenedione and also formed 6β-hydroxyandrostenedione. Presumably the 6β-hydroxylation followed the side-chain removal (or the 6β-hydroxyl activated side-chain removal) since no 6β-hydroxyprogesterone was detected. Fried *et al.* obtained testololactone from the fermentation of progesterone with *Penicillium chrysogenum*, *Aspergillus flaviceps*, or *Mucor mucedo*. With *Cylindrocarpon radicicola* these workers found that progesterone, testosterone, and 11-deoxycortisol were converted to 1-dehydrotestololactone. From the fermentation of progesterone with *Streptomyces lavendulae* Fried *et al.* isolated 1-dehydrotestosterone (17β-hydroxy-1,4-androstadien-3-one) and androstadienedione, which may readily be aromatized to estradiol and estrone, respectively. On the basis of these bioconversions, Fried *et al.* offered the suggestion that progesterone, 1-dehydrotestosterone, and androstadienedione might be intermediates in the mammalian synthesis of estrogens.[10]

Shortly thereafter Vischer and Wettstein (160) reported on the removal of the side chain with the dehydrogenation of ring A of progesterone and deoxycorticosterone by *Fusarium solani* and *F. caucasicum* to produce, in good yield, androstadienedione. Allopregnane-3,20-dione rapidly lost its side chain to form androstane-3,17-dione, which, in turn was converted slowly and in poor yield to androstadienedione. 3β-Hydroxy-5-androsten-17-one (epidehydroandrosterone) was slowly converted to androstadienedione, as was pregnenolone—apparently *via* 3β-hydroxy-5-androsten-17-one. Continued incubation of progesterone, deoxycorticosterone, androstenedione, pregnenolone, 3β-hydroxy-5-androsten-17-one, or androstadienedione with the mold led to the formation of a new more highly oxygenated material, the same in each case. This material, although not further characterized by the authors, is probably 1-dehydrotestololactone, if we may extrapolate from the results obtained with *Cylindrocarpon radicicola*, Penicillia and Aspergilli (as discussed above) and from our experience with *Fusarium solani* and *F. caucasicum* (98). Thus the terminal oxidation product of the organisms capable of side-chain degradation (with or without 1,2-dehydrogenation) appears

[10] In this connection the contribution by Meyer is most interesting. Having shown that androstenedione was converted to 19-hydroxyandrostenedione by adrenal tissue (104), he was then able to show that the latter compound was converted to estrone by human placental tissue or bovine ovarian follicular fluid (103). If androstadienedione was at all converted to estrone, it was at a much slower rate.

to be in all cases the ring D lactone. The formation of the 17-hydroxyl[11] group from the acetyl side chain as well as the formation of the lactone ring from the 17-ketone group is reminiscent of the action of peracids on ketones. The terminal degradation product, i.e. the lactone ring, appears to be the result of successive steps of enzymatic "peroxidations." Therefore, favorable yields of 1-dehydrotestosterone and androstadienedione appear to depend on the successful interruption of this sequence.

Vischer and Wettstein were unable to detect side-chain degradation of steroids having more than 21 carbons (methyl 3-ketobisnor-4-cholenate, nor-4-cholene-3,22-dione, 3β-hydroxynor-5-cholen-22-one and 3β-acetoxy-27-norcholesten-25-one). From 11-deoxycortisol and from cortisone no C_{19}-steroids were obtained, but one or more unknown materials resulted in each case. The authors concluded that the presence of the 17α-hydroxyl group prevents the side-chain degradation; degradation of the side chain of 20-ketopregnanes or pregnenes occurs rapidly; the A-ring dehydrogenation also is rapid when the Δ^4-3-ketone grouping is present, but occurs more slowly with Δ^5-3β-hydroxysteroids and 3-keto or 3β-hydroxyallosteroids.

In a subsequent publication, Vischer *et al.* (158) have characterized the fermentation products of 11-deoxycortisol and of cortisone by *Fusarium solani* as being the corresponding 1-dehydro-derivatives, i.e. 17α,21-dihydroxy-1,4-pregnadiene-3,20-dione and 17α,21-dihydroxy-1,4-pregnadiene-3,11,20-trione. In this article the authors state that organisms of the genera *Calonectria*, *Ophiobolus*, and *Alternaria* introduce the 1,2-double bond but carry out little or no side-chain degradation. (As discussed earlier, this group of workers had found that *Ophiobolus herpotrichus* carried out 21-hydroxylation with no side reactions.) From progesterone, deoxycorticosterone, and cortisone they isolated, respectively, 1-dehydroprogesterone, 1-dehydrodeoxycorticosterone, and 1-dehydrocortisone using *Calonectria decora* as the transforming microorganism.

Very recently, Nobile and co-authors (118) have reported on 1-dehydrogenation without side-chain degradation by means of *Corynebacterium simplex*. A considerable number of steroids were dissimilated by this organism, among others cortisol, cortisone, and 9α-fluorocortisol, yielding in all cases the corresponding 1-dehydrosteroid.

V. Microbial Reduction of Steroids

From the earlier work on this subject only a few remarks for purposes of orientation will be made. The oxidation of hydroxyl functions to

[11] Vischer and Wettstein (160) did not find 1-dehydrotestosterone as a dissimilation product of steroids by *Fusarium solani*. We have usually found this steroid in these reactions.

ketone functions at positions 3 or 17 of C_{19}-steroids as mediated by several species of bacteria and Actinomycetes had been demonstrated. As a rule, yeasts were found to be incapable of reducing conjugated ketones of steroids, although 1-androstene-3,17-dione was shown by Butenandt *et al.* (19) to be reduced by yeast to androstane-3β,17β-diol (isoandrostanediol). The presence of a side chain inhibited yeast reductions. In contrast to yeast, anaerobic bacteria were found capable of reducing both the double bond and the carbonyl of conjugated ketones. The crude culture of Mamoli and Schramm (86) could reduce androstenedione to both androstane and etiocholane derivatives. Purification of the culture, however, yielded an anaerobe which reduced the 3-keto-Δ^4-androgens only to 3-keto- and 3α-hydroxyetiocholanes. The culture was identified by Mamoli *et al.* (85) as *Bacillus putrificus* Bienstock. Hartsell and Rettger (56) had previously shown that *B. putrificus* is *nomen dubium*. According to Zimmermann and May (167) the *B. putrificus* culture was lost in 1944.

We have repeated Mamoli's reduction using a crude culture from an aqueous mixture of lubricating oil containing a badly decomposed frog. This culture reduced 11α-hydroxyprogesterone to both 11α-hydroxypregnane-3,20-dione and 3α,11α-dihydroxypregnan-20-one (116). We have had no success with pure cultures of anaerobes including *Clostridium lentoputrescens* Hartsell and Rettger.

Although the earlier work with yeasts led to the belief that a side chain prevented reduction, several exceptions are described in the recent work of Camerino and co-workers (22), who fermented with yeast pregnane-3,11,20-trione, allopregnane-3,11,20-trione, 11α-hydroxypregnane-3,20-dione, and 11α-hydroxyallopregnane-3,20-dione. Both the first two steroids surprisingly yielded the corresponding 3α-hydroxysteroids; the third steroid was not attacked, whereas the fourth yielded the expected 3β-hydroxyderivative. (This effect on the stereochemistry of the reduction is discussed in greater detail under substrate specificities.) The authors found that the presence of a 17α-hydroxyl or 21-hydroxyl group prevented reduction of the 3-ketone: thus, there was no reaction with 17α-hydroxypregnane-3,20-dione, 21-hydroxypregnane-3,20-dione, 17α,21-dihydroxyallopregnane-3,11,20-trione 21-acetate, or 11β,17α,21-trihydroxyallopregnane-3,20-dione 21-acetate. Acetylation of the 11α-hydroxyl group of 11α-hydroxyallopregnane-3,20-dione prevented the reduction by yeast.

A great step toward understanding the enzymatic processes in the bacterial dehydrogenation reactions was made in the recent contributions of Talalay and co-workers (94, 147, 148, 149). These workers succeeded in preparing two active cell-free dehydrogenases from a *Pseudomonas* adapted to growth on testosterone. The enzymes catalyze the reversible

dehydrogenation of hydroxyls at positions 3 and 17 stereospecifically. A detailed account of this notable work is given in the discussion under enzyme mechanisms.

Reduction of the ketone at carbon no. 20 has been accomplished by several molds. Fried *et al.* isolated 20β-hydroxy-4-pregnen-3-one as a dissimilation product of progesterone (48) and 16α,20β-dihydroxy-4-pregnen-3-one as a dissimilation product of 16α-hydroxyprogesterone (50) using *Streptomyces lavendulae* in both cases. Peterson and Murray (126) isolated 20β,21-dihydroxy-4-pregnen-3-one as a conversion product of deoxycorticosterone by a *Streptomyces* sp.

VI. Mixed Reactions

Since many of the molds have several enzyme systems capable of acting on steroids, a competitive situation exists in which the substrates may have undergone several alterations. The genus, species, and strain of microorganism as well as the condition of fermentation (composition of medium, aeration, pH, etc.) and the steroid substrate can all affect the course of reaction.

1. Oxygenation with Side-Chain Degradation

6β-Hydroxyandrostenedione was formed from progesterone by *Gliocladium catenulatum* (129).

2. Oxygenation with Reduction of Nuclear Double Bonds

Rhizopus nigricans acting on progesterone makes mainly 11α-hydroxyprogesterone with a small amount of 6β,11α-dihydroxyprogesterone; besides these two products 11α-hydroxyallopregnane-3,20-dione is formed in very small amounts (0.5% to 2%) depending upon fermentation conditions (132). In contrast to this, the minor by-product in the bioconversion of 11-deoxycortisol to 11-epicortisol by *R. nigricans* (128) is the corresponding pregnane compound (11α,17α,21-trihydroxypregnane-3,20-dione). From these two examples, it appears that the side chain influences the stereochemistry of the hydrogenation. From 16-dehydroprogesterone this same organism (100) makes in good yield 11α-hydroxy-17α-progesterone, the hydrogenation of the double bond proceeding in such a manner as to yield the unnatural (and thermodynamically unstable) configuration of the side chain.

As a minor constituent in the fermentation of progesterone to 16α-hydroxyprogesterone by a *Streptomyces* sp., Perlman *et al.* (124) isolated 16α-hydroxypregnane-3,20-dione.

3. Oxygenation Accompanied by Reduction of a Carbonyl

Incubation of 3-ketobisnor-4-cholen-22-al with *Rhizopus nigricans* yields predominantly 3-ketobisnor-4-cholene-11α,22-diol (99).

4. Reduction of a Carbonyl Accompanied by 1-Dehydrogenation

On incubation of 16α-hydroxyprogesterone with *Streptomyces lavendulae*, Fried *et al.* (50) isolated, among other things, 16α,20β-dihydroxy-1,4-pregnadien-3-one.

5. Reduction of a Carbonyl Accompanied by Hydrogenation of a Double Bond

Mamoli *et al.* (85) isolated 3α,17β-dihydroxyetiocholane as the product of a reduction of androstenedione by an anaerobic culture, *Bacillus putrificus* (cf. page 395).

6. Other Mixed Reactions

In the section on side-chain degradation, several mixed reactions were discussed: *Dehydrogenation at Carbons 1, 2 Accompanied by Side-Chain Degradation; Dehydrogenation at Carbons 1, 2 with Side-Chain Degradation and Ring D Rupture;* and *Side-Chain Degradation and Ring D Rupture.*

VII. Discussion

1. Esterases

The esterase activities of these various molds are very high. In our own experience (30) on hydroxylations with 21-acetoxysteroids, if the fermentation sequence is stopped well before completion, we have found small amounts of the original steroid substrate, a larger amount of de-esterified substrate and a considerable amount of unesterified conversion product—never have we been able to detect the 21-acetoxy conversion product. This can only mean that the rate of ester hydrolysis is much more rapid than the rate of hydroxylation. It might also indicate that the oxidative reaction either does not occur on the esterified molecule or occurs only at a greatly reduced rate, since otherwise one would expect to find traces, at least, of esterified product. Recently, a microorganism has been reported by Nobile and co-authors (118) which apparently is low in esterase activity. This microorganism, *Corynebacterium simplex*, is not a filamentous fungus, and its dissimilation of steroids consists mainly in the dehydrogenation of carbons, not in hydroxylation (cf. page 394), so that it constitutes no exception to these observations on hydroxylating fungi. It is most interesting that *C. simplex* will dehydrogenate deoxycorticosterone acetate to 21-acetoxy-1,4-pregnadiene-3,20-dione. It is uncertain from the published article whether this was the sole product or if the deacetylated diene also was formed. A comparison of yields in the two cases [(1) steroid acetate → dehydrosteroid acetate; (2) steroid (free alcohol) → dehydrosteroid (free alcohol)] would be of interest to see if the 21-acetoxyl offers any impediment to the reaction.

2. *Species Specificity*

The effect of species and even strains on the course of steroid bioconversions by microorganisms must always be kept in mind. The 6β-hydroxylating ability of the Rhizopi, already mentioned, serves as an excellent example. We will consider progesterone as the substrate. *R. arrhizus* (A.T.C.C. 11145) will form 6β,11α-dihydroxyprogesterone at such a rate that from about 10% to 25% of the total bioconversion product is this dihydroxyprogesterone, the remainder being 11α-hydroxyprogesterone. We have never found any 6β-hydroxyprogesterone. Shaken-flask experiments, evaluated only by paper chromatography, indicate that if 11α-hydroxyprogesterone is at all converted to 6β, 11α-dihydroxyprogesterone it must be only at a very slow rate. It seems probable, therefore, that the 6β-hydroxylation occurs first,[12] and that the 6β-hydroxylated product is further oxygenated to the 6β,11α-dihydroxyprogesterone. Since at most only about 25% of this product is found, the 11α-hydroxylation must be proceeding at a rate of about four times that of the 6β-hydroxylation. *R. nigricans* (A.T.C.C. 6227b), in contrast, produces only about 2% of dihydroxyprogesterone; hence, in this case 11α-hydroxylation is occurring at a rate of about 50 times that of 6β-hydroxylation. Other strains of *R. nigricans* vary in their ability to hydroxylate at carbon no. 6, some, indeed, doing as well as *R. arrhizus*.

3. *Substrate Specificity*

The picture becomes more complicated when the effects of substrate specificity are considered. Again, the *Rhizopus* cultures offer a good illustration. The course of the bioconversion is surprisingly affected by rather small changes in structure of the steroid substrate. If we use deoxycorticosterone (21-hydroxyprogesterone) as the steroid substrate (30) instead of progesterone, we find that *R. arrhizus* converts this predominantly to 6β-hydroxy-11-deoxycorticosterone, making only minor amounts of the 11α-hydroxyderivative (11-epicorticosterone). Fried *et al.* (47) report a similar case of substrate specificity in the hydroxylation of 11-deoxycortisol and 16α-hydroxyprogesterone by *Aspergillus nidulans*, the first substrate yielding 11-epicortisol but the latter substrate predominantly yielding the corresponding 6β-hydroxyderivative. Such a complete reversal in rates by the two competing enzymes in *R. arrhizus* is truly remarkable, since the *R. nigricans* culture is not thus affected by

[12] Dulaney *et al.* (28) (cf. page 401) clearly show that in *Aspergillus ochraceus* the 6β,11α-dihydroxyprogesterone is a further transformation product of 11α-hydroxyprogesterone. This does not appear to be the case with the Rhizopi we have studied. Whether our experiments are of 24 or 72 hours duration, the absolute amounts of dihydroxyprogesterone and of 11α-hydroxyprogesterone remain constant.

the change in substrates—this organism produces 11-epicorticosterone almost exclusively. Omission of the side chain on the steroid substrate also affects the course of the hydroxylation reactions discussed here, but to a lesser extent. When testosterone or androstenedione were the substrates, *R. nigricans* and *R. arrhizus* yielded practically identical bioconversion patterns, about 20% of the isolated product being the 6β-hydroxylated derivative and 80% the 11α-hydroxylated derivative (29).

In an earlier discussion (30) of the qualitative and quantitative differences elicited by changing the species of *Rhizopus* and the steroid substrate, two mechanisms were postulated as possibly operative. (1) Two different enzymes are involved, differing in relative proportions in the two species; a given steroid structure is more suitable for attachment or attack by one or the other enzyme. (2) One enzyme is involved; the different substituents on the steroid affect the spatial relations of enzyme-substrate complex so that different positions of the steroid nucleus are activated. But if this second alternative is the case, then one must postulate that considerable difference exists in the structures of the enzyme in these two species of *Rhizopus* to account for their different behavior. The first explanation seems the more plausible. The second mechanism also might possibly be operable in other cases. The shift from 6β-hydroxylation to 7β-hydroxylation by the Rhizopi without appreciable effect on 11α-hydroxylation when allopregnanolone or pregnenolone are the substrates might well be such an example.

Changes or additions of other functional groups on the steroid substrate also can affect the course of the enzymatic reactions. The presence of the 17α-hydroxyl group favors the introduction of the 8-hydroxyl over the 14α-hydroxyl by *Helicostylum piriforme*. From deoxycorticosterone this mold makes primarily the 14α-hydroxyderivative with relatively small amounts of the other tertiary hydroxyderivative. But from 11-deoxycortisol the reverse is true, the 8-hydroxysteroid being the major dissimilation product.

The presence or absence of the 4,5-double bond has little influence on hydroxylation, if the reactions of the *Rhizopus* group (32) can serve as a pattern. However, dehydrogenation in the 1,2-position as carried out by *Fusarium solani* is profoundly affected by the absence of the Δ^4-function. Dehydrogenation of 3-keto, or 3β-hydroxyallosteroids, or Δ^5-3β-hydroxysteroids proceeds with difficulty, as shown by Vischer and Wettstein (160). Similar steroids of the 5β, or normal, series were not investigated so that it cannot be concluded from their data whether or not the impairment is due only to the lack of the double bond. Yet, since the Δ^4-steroids and allosteroids are essentially planar (in contrast to the normal steroids), it is difficult to explain the difference in reactivity between the former

two classes other than as the effect of the 4,5-unsaturation. This functional group is not necessary for side-chain degradation, *F. solani* performing this reaction equally well on Δ^4-steroids, Δ^5-steroids, or allosteroids (again, the effect of the nonplanar steroids of the 5β-series is unknown).

In contrast to this, the side-chain degradation, according to the Ciba group (160), is completely inhibited by the 17α-hydroxyl function: *Fusarium solani* converted cortisone and 11-deoxycortisol to the corresponding 1-dehydro derivatives (and additional uncharacterized material, in the case of 11-deoxycortisol) but did not degrade the side chain. This is contrary to our findings (129) with several species of *Penicillium* and *Aspergillus*, which degraded acetyl, α-ketol, and dihydroxyacetone side chains with equal ease. It is also contrary to our findings (116) with *Hypomyces haematococcus* (Berk. et Br.) Wr. [identical to *Fusarium solani* (Mart.) App. et Wr.] and with *Fusarium caucasicum* (Let.), both of which could degrade 17α-hydroxysteroids.

An oxygen function at carbon no. 11 has a most startling effect on the enzymatic reduction of the 3-ketone of saturated steroids, according to Camerino *et al.* (22). These workers reduced pregnane-3,11,20-trione with yeast to obtain the expected 3α-hydroxypregnane-11,20-dione. When allopregnane-3,11,20-trione was the substrate, the product isolated was 3α-hydroxyallopregnane-11,20-dione. (This result contrasts with the chemical reduction, which in the allo series yields the 3β-hydroxyl.) But when 11α-hydroxyallopregnane-3,20-dione was the substrate, the product was the 3β,11α-dihydroxyallopregnan-20-one. No reduction was detected when 11α-hydroxypregnane-3,20-dione was the substrate. Identification of products was by melting point, mixture melting point, and rotation, as well as precipitability by digitonin and preparation of acetates. For such effects to be truly convincing one would like a more thorough characterization of products, including infrared spectra and paperstrip mobilities. How these results conform to the studies of Talalay and co-workers (94) (cf. page 403) is uncertain.

VIII. Mechanism of Enzyme Action and Related Studies

1. Hydroxylation

A most interesting example of organism-substrate specificity has been recently discovered by Bloom and Shull (11).[13] The interest lies not only in the reaction itself—the first case of microbiological epoxidation—but

[13] This work was announced at too late a date for the products to be included in our tables. The two epoxides, 17α,21-dihydroxy-9β,11β-oxido-4-pregnene-3,20-dione and 17α,21-dihydroxy-14α,15α-oxido-4-pregnene-3,20-dione should be included in Table II and in a new reaction category, epoxidation, in Table I.

in the theoretical implications of the enzymatic processes. These workers found that when *Cunninghamella blakesleeana* or *Curvularia lunata* (both good 11β-hydroxylators) were incubated with 17α,21-dihydroxy-4,9-pregnadiene-3,20-dione they could isolate, among other products, 17α,21-dihydroxy-9β,11β-oxido-4-pregnene-3,20-dione. Incubation of 17α,21-dihydroxy-4,9-pregnadiene-3,20-dione with *Rhizopus nigricans* or other 11α-hydroxylating organisms did not produce any trace of the corresponding 9α,11α-epoxide. When *Helicostylum piriforme*, *Mucor griseocyanus*, *Curvularia lunata* or other 14α-hydroxylating agents were incubated with 17α,21-dihydroxy-4,14-pregnadiene-3,20-dione, the corresponding 14α,15α-epoxide was formed. However, microorganisms lacking the 14α-hydroxylating system could not effect this epoxidation. The results of these admittedly preliminary experiments were correlated by the authors as follows: "A microorganism capable of introducing an axial hydroxyl function at C_n of a saturated steroid also effected the introduction of an epoxide grouping 'axial' at C_n in the corresponding unsaturated substrate." Since in this reaction none of the possibly expected hydroxylated derivative was found (e.g. the 11β-hydroxysteroid from fermentation of the Δ^9-steroid by *Curvularia lunata*), this would indicate that dehydrogenation to form a carbon-carbon double bond cannot be a step in the mechanism for the axial hydroxylation studied. Indeed, this experiment implies that the two axial hydroxylations (11β- and 14α-) proceed by a mechanism quite different from that for the equatorial 11α-hydroxylation (since no epoxide was formed in the case of 11α-hydroxylators).

The concept that the hydroxylations do not occur *via* a dehydrogenation followed by a hydration is strengthened by the studies of Fried *et al.* (47). These authors added 17α,21-dihydroxy-4,9-pregnadiene-3,20-dione to a culture of *Aspergillus nidulans* (an 11α-hydroxylator) but could isolate no 11-epicortisol. Similarly, when they added 16-dehydroprogesterone to a culture of their 16α-hydroxylating streptomycete (A.T.C.C. 11009) no 16α-hydroxyprogesterone was found. This is in agreement with the findings of Hayano and Dorfman (57) on bovine adrenal enzymes, and quite in contrast to the results of Miescher *et al.*, (107) who alone claim to have demonstrated the conversion, by adrenal tissue, of 17α,21-dihydroxy-4,9-pregnadiene-3,20-dione into cortisol.

Dulaney and co-workers (28) have recently published a study of *Aspergillus ochraceus*, which, like *Rhizopus*, converts progesterone to 11α-hydroxyprogesterone and 6β,11α-dihydroxyprogesterone. In contrast to our interpretation of the *Rhizopus* mechanism, however, *A. ochraceus* produces the dihydroxyprogesterone by a second hydroxylation of 11α-hydroxyprogesterone, as the authors clearly demonstrate. When the

organism was grown on a zinc-deficient medium, the dihydroxyprogesterone formation did not take place. Having shown that Zn^{++} is not required directly in the 6β-hydroxylation, the authors postulate that in the zinc-deficient medium the metal (which is a growth requirement for the mold) is present in amounts sufficient for growth with just enough extra to allow for limited protein synthesis. When adapted to progesterone, the 11α-hydroxylating enzyme is formed, but no additional protein synthesis for the next adaptive enzyme, the 6β-hydroxylator, can take place. This hypothesis carries hidden implications: (1) that protein synthesis requires Zn^{++}; (2) that the Zn^{++} is "used up," i.e. made nonavailable, in the process of protein synthesis. Perhaps both of these implications are true, but they are unproved. Dulaney *et al.* state that both the 11α-hydroxylating enzyme and the 6β-hydroxylating enzyme are adaptive enzymes, but nowhere do they offer any proof of this statement. If it is true and if this finding carries over to the other fungi and other steroid-transforming enzymes, it has far-reaching implications.

2. Ring Dehydrogenation

The mechanism whereby a carbon-carbon double bond is introduced into steroids by microorganisms is completely unknown. To speculate that it occurs by introduction of a hydroxyl on carbon no. 1, followed by a loss of water from the thus-formed β-hydroxyketone, as Vischer *et al.* (158) have done, is ill advised, especially since their reason for so doing is "by analogy to the many known hydroxylations" (our translation).[14] Until more is known concerning the biochemistry of the steroid-transforming microorganisms, it is best to abjure speculation on the enzyme mechanisms. Continued studies along the path indicated by Bloom and

[14] Wettstein (164a) has since elaborated on this enigmatic statement. He offers two alternatives, mechanism 1, as above, supported by "the analogy with the many known microbiological hydroxylations" and 2, "the typical aerobic dehydrogenase mechanism." Arguments by analogy such as these are dangerous (viz., the analogy between microbial hydroxylation of steroids and of succinate). The β-ketonic hydroxyls as required in mechanism 1 are probably not so unstable that they would spontaneously dehydrate under the conditions of fermentation-isolation without leaving appreciable amounts of the parent 1-hydroxysteroids—which should certainly be apparent in chromatograms. The same mechanism, presumably, should be operative for the introduction of the Δ^4 function; no 3-keto-5-hydroxysteroids have been reported for the pertinent microbial reactions. Yet 3-keto-5α-hydroxysteroids (which should certainly be no more stable than 1-hydroxy-3-keto-Δ^4-steroids) can withstand a fair amount of manipulation (168, 169, 170). One, therefore, has to postulate a second and extremely efficient enzymatic mechanism to dehydrate the hydroxysteroid intermediates. There is no evidence to warrant this. Nor does our knowledge warrant us to say that the alternative mechanism is the "typical aerobic dehydrogenase" system.

Shull will, perhaps, be of aid. Analysis of the bioconversion products obtained by varying the functional groups and their orientation at certain carbons, seemingly influencing the reactions under study, will help (e.g. 3α- and 3β-hydroxyderivatives of both allopregnane and pregnane; no oxygen function in or near the side chain; variations in configuration at the ring junctures other than at carbon no. 5).

3. Hydroxyl Dehydrogenases

Cell-free systems are now available for studying the bacterial oxidation-reduction reactions of steroidal oxygen functions. Talalay and co-workers (147, 148, 149) have isolated in fairly clean preparations two dehydrogenases from a *Pseudomonas* culture. Both enzymes are adaptive (functional) and both require DPN as co-enzyme. The 3α-dehydrogenase, as its name implies, is specific with respect to position and stereochemical arrangement. The β-dehydrogenase is stereospecific but will activate oxygen at either carbon no. 3 or carbon no. 17 (and, to a lesser extent, at carbon no. 16). Neither enzyme will activate a ketone in conjugation with a double bond. The substrate is limited to steroids devoid of a side chain. The stoichiometry of the reactions is known:

3α-dehydrogenase: androsterone + $DPN^+ \leftrightharpoons$ androstanedione + DPNH + H^+
β-dehydrogenase: testosterone + $DPN^+ \leftrightharpoons$ androstenedione + DPNH + H^+

Marcus and Talalay (94) have conducted a comprehensive study of the β-dehydrogenase. At higher substrate concentrations the results with this enzyme deviate markedly from the Michaelis-Menten equation. The authors very nicely show that the data are compatible with the formation of a *bis*(substrate)-enzyme complex, $(S)_2E$, which competes with the enzymatic reaction: $E + S \rightleftarrows ES \rightleftarrows E +$ product. The firmness of attachment between the enzyme and various substrates was calculated and was found to be: $\Delta^{1,3,5}$-steroid > Δ^4- or Δ^5-steroids or allosteroids (5α-) > normal steroids. Neither the presence of a 6β-hydroxyl nor the absence of carbon no. 19 affect the reaction, although an 11-hydroxyl, α- or β-, interferes markedly. Oxidation of the 17β-hydroxyl is completely inhibited by the presence of an oxygen (ketone or α- or β-hydroxyl) at carbon 16. Conversely, oxidation of 16β-hydroxyl is stopped by the presence of the 17β-hydroxyl. With convincing logic Marcus and Talalay postulate that a single apo-enzyme is involved, attaching itself to the "back" of the steroid by multiple attractions, and having two reactive centers, one in the vicinity of carbon no. 3 and one in the vicinity of carbon no. 17. The authors make an interesting attempt to correlate the structure, enzyme-binding, and biological activity of the steroids studied.

IX. Application of Microbial Transformations to the Synthesis of the Naturally Occurring Steroidal Hormones and Their Newer Modifications

The microbial transformations which we have discussed above are not only helpful in the preparation of the adrenal steroids from simple key intermediates such as progesterone but they are equally important in the conversion of these key intermediates to the androgenic and estrogenic hormones. With the advent of the 1-dehydrocorticosteroids, the microbiological transformation of the steroid nucleus has again proved to be a tool equal or superior to conventional chemical approaches. Furthermore, the facile introduction or modification of functional groups by microbial dissimilation has produced numerous new steroid compounds. A thorough biological evaluation of these new steroids will undoubtedly bring to light new or improved biological properties of a limited number of compounds.

The discovery of the microbial 11α-hydroxylation of progesterone and of 11-deoxycortisol made these two compounds the key intermediates for the synthesis of many steroids. Both intermediates are available to an unlimited extent from plant sterols such as diosgenin, ergosterol, and stigmasterol. Thus, the microbiological process, besides solving the key problem of 11-oxygenation, effected a shift with respect to the sources of raw material: The bile acids, the rather limited and expensive starting material of preceding synthetic procedures, could be replaced by the cheap and plentiful plant sterols.

The presence of functional groups in the side chain of many sterols greatly facilitated the design of short and efficient chemical synthesis of the above-mentioned key intermediates. Perhaps because there was little economic incentive to duplicate these degradations by microbiological reactions, no practicable microbial side-chain degradation of sterols has been reported. However, conversions of this type do, indeed, occur, as is indicated by Turfitt's observation of the conversion of cholestenone to 3-keto-4-etienic acid (154).

1. Corticosteroids

The publications by Fried *et al.* (44) on the 9-halocorticosteroids and by Herzog *et al.* (61) on the 1-dehydrocorticosteroids demonstrated that the intrinsic biological properties of cortisone and cortisol could be potentiated through modification of their structure. This section, therefore, deals first with the preparation of the naturally occurring corticosteroids, and then with the preparation of some of the structurally modified corticosteroids.

a. Naturally Occurring Corticosteroids. Levy *et al.* (78), in their investi-

gations *in vitro* of the adrenal enzymes, have found that these enzymes are not able to oxygenate the 17-position in the presence of a 21-hydroxyl group. In striking contrast to this, the microbial enzymes exhibit almost complete freedom of choice with respect to the sequence of oxygenation. In the light of the present knowledge, one can legitimately claim absolute freedom. Six sequences of stepwise oxygenation are possible for the preparation either of 11-epicortisol or of cortisol. The sequences thus far carried out will be discussed in this section. Some of the sequences have not yet been completed as described in the paragraph on 21-deoxysteroids (cf. page 411). However, present information permits the conclusion that these missing gaps may soon be filled.

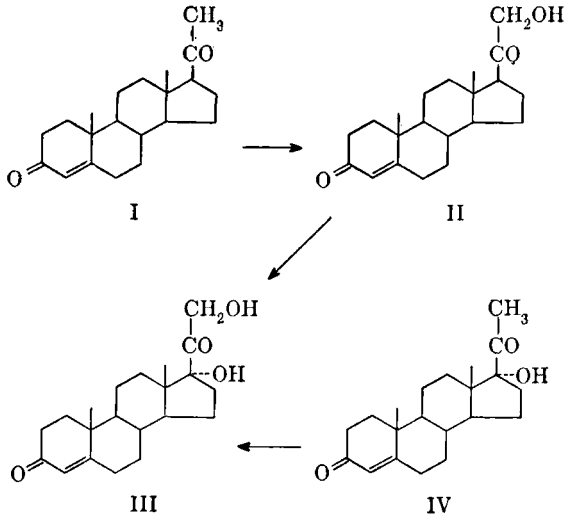


FIG. 1.

(*1*) *Introduction of the functional groups in positions 11, 17, and 21 by microbiological procedures.* (*a*) *Deoxycorticosterone and 11-Deoxycortisol* (Fig. 1). The usefulness as well as the limitations of microbiological reactions can be amply demonstrated by a critical examination of the pathways leading from progesterone to the various adrenocortical steroids. In the conversion of progesterone (I) to deoxycorticosterone (II), *Ophiobolus herpotrichus* (105) produces a 60% yield of II. No yields have been given for this step when carried out with *Aspergillus niger* (A.T.C.C. 7142) (166). This simple oxygenation compares favorably with the more elaborate, though successful, chemical preparation of II from pregnenolone by Julian (69). The presence of 25% of unconverted starting material (I) in the product of the *Ophiobolus* fermentation necessitated a chromatographic separation of I and II. It might prove

possible, however, to increase the yield of II through proper changes of the fermentation conditions. A low concentration of unused substrate in the final product would not be deleterious to a direct isolation of deoxycorticosterone, and in this event the preparation of the latter compound by the microbial procedure would be superior to the chemical process.

The 17α-hydroxylation of deoxycorticosterone (II) with *Trichothecium roseum* gave 11-deoxycortisol (III) in 30% yield. The presence of 6β,21-dihydroxy-4-pregnene-3,20-dione in 10% yield, besides some remaining substrate, again called for a chromatographic separation (105). The relatively low yield and the elaborate isolation procedure make it unlikely at the present time that this procedure is competitive with the well-developed chemical syntheses of III by Julian and co-workers (69) and Gallagher and co-workers (73). The 21-hydroxylation of 17α-hydroxyprogesterone (IV) by *Ophiobolus herpotrichus* has been described, giving deoxycortisol (III) in an unspecified yield (105).

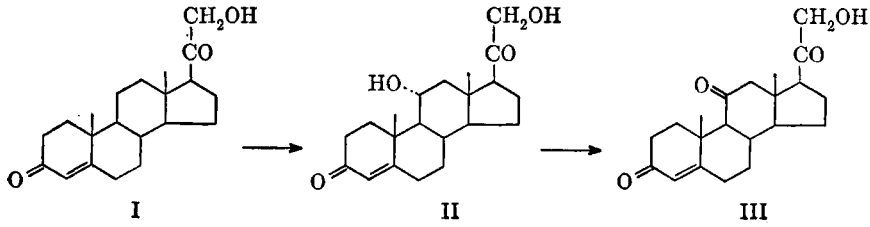


FIG. 2.

(*b*) *11-Dehydrocorticosterone* (Figs. 2, 3, and 4). 11-Dehydrocorticosterone (Fig. 2, III) can be prepared in a number of different ways.

Route 1: The first method starts from deoxycorticosterone (Fig. 2, I), which is hydroxylated to 11-epicorticosterone (Fig. 2, II). Of the various 11α-hydroxylating organisms, *Aspergillus niger* (Wisconsin strain 72–2) (46) and *Rhizopus nigricans* (30) probably give the best yields of II (approximately 60 to 70%). The *Aspergillus* conversion is free of undesirable by-products, whereas *Rhizopus* often gives varying amounts of a dihydroxy derivative of I.

11-Epicorticosterone (Fig. 2, II) is then acetylated at the 21-position with 1 mole of acetic anhydride and oxidized at position 11 with chromium trioxide to give the 21-acetate of 11-dehydrocorticosterone (Fig. 2, III). The 45% yield reported for these two reactions (30) is low because of the small scale of the experiment. No yields were given by Fried *et al.* (46) or by Kahnt *et al.* (70). If desired, the 21-acetate of compound III can be deacetylated chemically in a number of ways. If it is to be used as a substrate for a further microbiological conversion, the 21-acetate

group can be left intact to be deacetylated by the esterases present in most of these fungi.

Route 2: In an alternative route to 11-epicorticosterone (Fig. 3, III) the sequence of 11α- and 21-hydroxylation is reversed. Progesterone (Fig. 3, I) is converted first to 11α-hydroxyprogesterone (Fig. 3, II). Besides *Rhizopus nigricans*, which gives an almost quantitative yield, other 11α-hydroxylating organisms (cf. page 389) will unquestionably respond favorably to the development of bioconversion conditions, thus giving II in excellent yields.

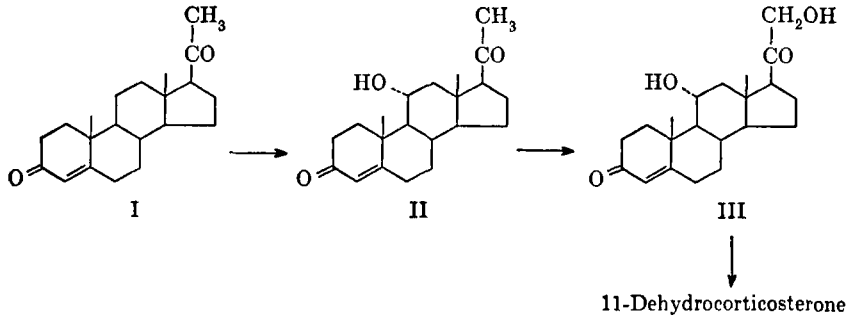


FIG. 3.

The next step involves 21-hydroxylation of 11α-hydroxyprogesterone (II). When the latter compound was incubated with *Aspergillus niger* (A.T.C.C. 9142) epicorticosterone (Fig. 3, III) was identified as the main product (166). The yield of III and the extent to which more polar conversion products were present were not specified.

Route 3: A third variation of these syntheses passes through 11-ketoprogesterone (Fig. 4, IV) as the key intermediate; this is readily accessible from both 11-hydroxyprogesterones (Fig. 4, II and III) by chromium trioxide oxidation.

In going from I to IV via II the yield is practically quantitative for each step. With III as the intermediate, nothing definite can be said concerning yields, since in the preparation of III from I by *Curvularia lunata* no yield was given (138). (Four minor by-products were present which might present difficulties in the isolation of desired product.) Presumably III could be oxidized to IV as efficiently as can II, but this reaction is not recorded in the literature.

The 21-hydroxylation of IV to 11-dehydrocorticosterone (V) has been accomplished on a small scale with *Ophiobolus herpotrichus* in 50% yield (105). The yield for this reaction with *Aspergillus niger* (A.T.C.C. 9142) (166) was not revealed, although the presence of more polar transformation products was reported. Assuming yields of 90, 90, and 50% for the

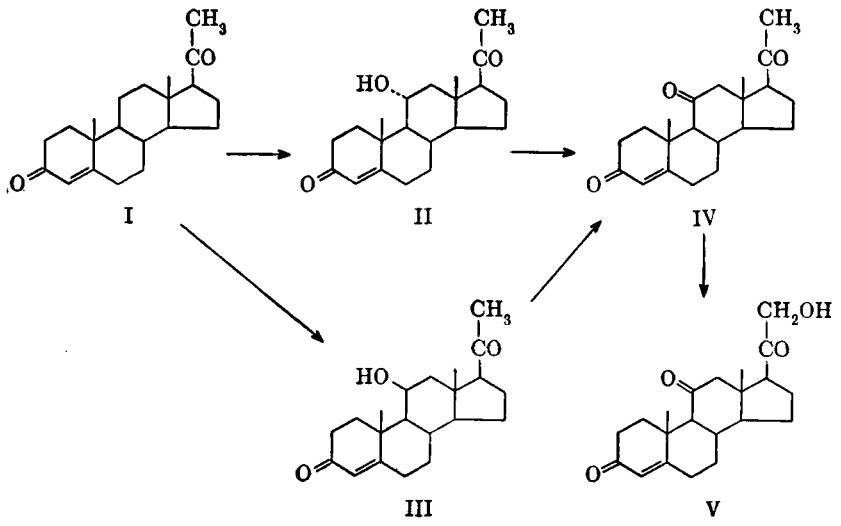


FIG. 4.

three transformations involved in route 3, it would appear that this process for the preparation of 11-dehydrocorticosterone is the most satisfactory of the microbial syntheses from a point of view of over-all yields.

(*c*) *Cortisone* (Fig. 5). Variations in the sequential microbiological oxygenation of positions 11, 17, and 21 again make different approaches to the synthesis of cortisone possible.

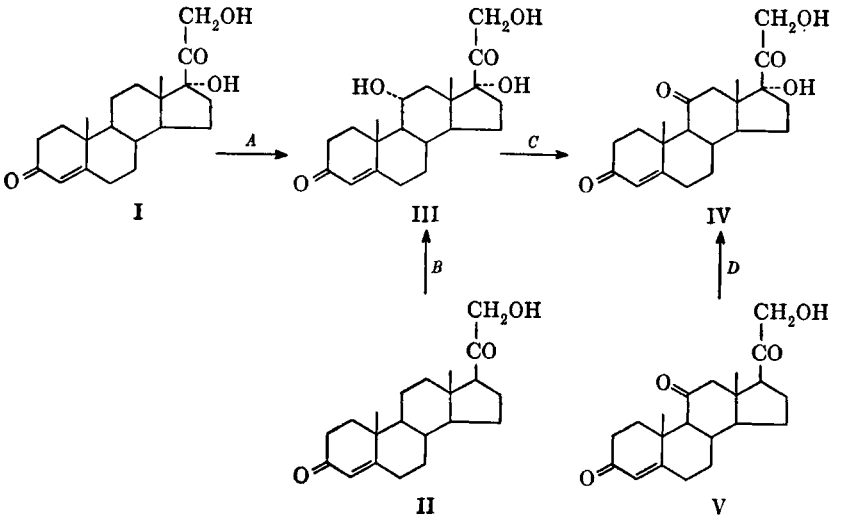


FIG. 5.

Step A: Best explored is the 11α-hydroxylation of 11-deoxycortisol (I) to 11-epicortisol (III). The use of various molds such as *Aspergillus niger* (Wisconsin Strain 72–2) (46) or *Rhizopus nigricans* (128) for this transformation, although giving reasonably good yields of III, has the disadvantage of producing by-products in yields of 5 to 20%.

Step B: Interesting from a theoretical point of view is the dihydroxylation of deoxycorticosterone (II) to 11-epicortisol by *Cephalothecium roseum* (102). This conversion demonstrates the possibility of introducing two of the three crucial oxygen functions in one step. However, as pointed out above (cf. page 392) dihydroxylations in general are rarely high-yielding processes and this specific conversion was complicated by other side reactions.

Step C: The preparation of cortisone acetate is then completed chemically by chromium trioxide oxidation of the 21-monoacetate of III. A yield of 70% for these two steps was reported by Fried *et al.* (46).

Step D: In an alternative route the 17α-hydroxyl group is introduced in the last step. 11-Dehydrocorticosterone (V) is converted to cortisone (IV) by *Trichothecium roseum* (105). Since no information regarding the yield of the latter bioconversion is available, a final appraisal of the various oxygenation procedures is not possible. At present the combination of steps *A* and *C* is likely to be the most fruitful one. Also, cortisone is a by-product of the 11β-hydroxylation of 11-deoxycortisol (cf. page 410).

(*d*) *Corticosterone and Cortisol* (Fig. 6). The approaches to the synthesis of corticosterone and cortisol follow the same pattern as

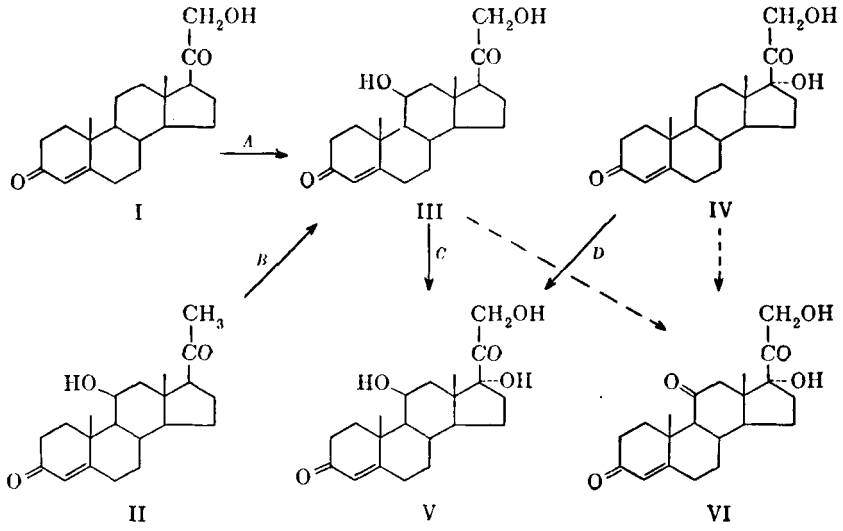


FIG. 6.

described for the preparation of their 11α-epimers, i.e. 11-epicorticosterone and 11-epicortisol (see Figs. 2, 3, and 5), insofar as there is almost absolute freedom of choice in the consecutive oxygenation of positions 11, 17, and 21.

Step A: Two organisms, i.e. *Curvularia lunata* (138, 139) and *Cunninghamella blakesleeana* (92), which are known to introduce the 11β-hydroxyl group into deoxycorticosterone (I), yield 28% and 12% corticosterone (III), respectively. In the transformation by *Cunninghamella blakesleeana*, compound III was the by-product and 14α,21-dihydroxy-4-pregnene-3,20-dione was the major oxygenation product (46%). Mann *et al.* (91, 92) have shown that, upon addition of the antibiotic Actidione (cycloheximide) in a concentration of 100 μg/ml. or more to the broth, a 45% yield of corticosterone is achieved with practically no 14α-hydroxylated steroid being present.

Step B: Corticosterone is, furthermore, available from 11β-hydroxyprogesterone by a 4- to 6-day incubation with *Aspergillus niger* (A.T.C.C. 9142) (166). Lack of information regarding the yield of Step *B* does not allow for a comparison with Step *A*. However, all the available evidence points to the superiority of the sequence progesterone → deoxycorticosterone → corticosterone.

Step C: Corticosterone (III) can then serve as a substrate for the conversion to cortisol (V) which is brought about with *Cephalothecium roseum* (102) or with *Trichothecium roseum* (105). *Trichothecium roseum* afforded an approximately 30% yield of isolated cortisol, with some material remaining in a mixture of cortisol (V), cortisone (VI), and an unknown steroid. The formation of cortisone (VI) was also observed with *Cephalothecium roseum*. Unless the formation of by-products is suppressed by one means or another, the preparation of cortisol from corticosterone will not be of practical significance.

Step D: When the microbiological syntheses of corticosteroids came to the fore, 11-deoxycortisol (IV) was already available in considerable quantities at a relatively low price. Therefore, the preparation of cortisol (V) from 11-deoxycortisol has been developed into a large scale process.

Useful, from a preparative point of view, are *Cunninghamella blakesleeana* (55) and *Curvularia lunata* (138), which produce the desired compound in 60 to 70% and 40% yield, respectively. Both fungi also give rise to varying concentrations of cortisone (VI). Although 11β-hydroxylation is a much rarer phenomenon than 11α-hydroxylation, various groups of investigators seem to have their preferred organism. Unfortunately, not much is known about some of these fungi and their efficiency in 11β-hydroxylation.

(*e*) *Intermediates Potentially Useful for the Synthesis of Cortisone and*

Cortisol. In the course of the investigations on the 11-oxygenation of steroids, a number of compounds have been prepared which are of the 21-deoxy type. Although their transformation to cortisone or cortisol by microbiological means has never been completed, such a step could be feasible, indeed, in view of the recent results on 21-hydroxylation. Therefore, the preparation of 21-deoxycortisol and 21-deoxycortisone is briefly discussed here.

21-Deoxycortisol (Fig. 7, II) can be prepared in 33% yield from 17α-hydroxyprogesterone (I) by *Curvularia lunata* (138).

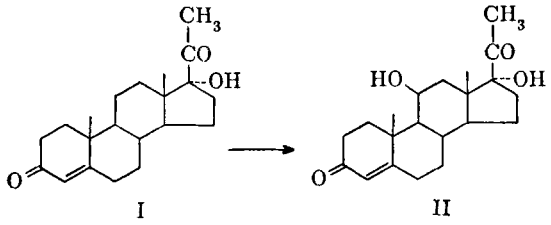


FIG. 7.

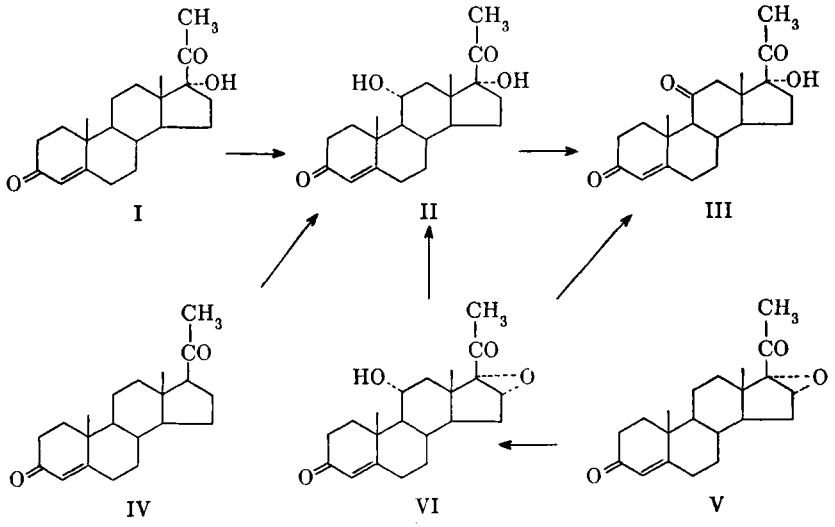


FIG. 8.

21-Deoxycortisone (Fig. 8, III) can be prepared in various ways. Three of the four possible routes employ 11α,17α-dihydroxyprogesterone (II) as an intermediate which can be oxidized to III with chromium trioxide (37, 46, 101). Compound II has been prepared in the following ways:

11α-Hydroxylation of 17α-hydroxyprogesterone is best carried out with *Rhizopus nigricans* in 75% yield (101).

11α,17α-Dihydroxylation of progesterone with *Cephalothecium roseum* is not a very profitable way of preparing II in view of the side reactions produced by this organism (102).

11α-Hydroxylation of 16α,17α-oxidoprogesterone (V) by *Rhizopus nigricans* gave VI in 70 to 80% yield (37, 130).

Compound VI can be converted to II by lithium aluminum hydride reduction of the 3,20-bis(ethylene diketal) of VI followed by regeneration of the carbonyl group. A yield of 18% was reported for these three steps (130).[15]

A slightly different but very efficient procedure was used by Ercoli *et al.* (37) for the transformation of VI to III. Oxidation of VI with chromium trioxide-pyridine gave 16α,17α-oxido-4-pregnene-3,11,20-trione. Opening of the oxide ring with hydriodic or hydrobromic acid, followed by dehalogenation with Raney nickel, resulted in 64% over-all yield of III.

From a preparative standpoint, many of the transformations presented above are at present of little value for various reasons. Either they give low yields of the desired product or the enzymes of the microorganism generate one or more wasteful products. But, fortunately, such results cannot be considered to be final until various modes of attack have been tried out to modify these results. A detailed study of the optimal bioconversion conditions involving controlled stepwise changes of some or all variables will often give a remarkably different picture. As already pointed out, the choice of a different strain of organism can improve the yield of the desired dissimilation product. Addition of cofactors or of enzymatic inhibitors may serve to alter appreciably the course of steroid dissimilation.

Most pertinent to the reactions discussed above are the results of studies to improve the yield of cortisol obtained from 11-deoxycortisol by *Cunninghamella blakesleeana* (Fig. 6, IV $\rightarrow$ V).

When the originally reported yield (33%) of cortisol was increased to higher levels, the concentration of cortisone (Fig. 6, VI) was also raised. Although cortisone is valuable *per se*, the increased concentration of cortisone necessitated complex isolation and separation procedures. A significant increase in over-all steroid recovery from 64 to 80% as well as a decrease in cortisone formation from a level of 25 to 30% down to

[15] Ercoli *et al.* (37) converted the 11-acetate of VI to the 11-acetate of II by the procedure which they used for the conversion of 16α,17α-oxido-4-pregnene-3,11,20-trione to III. No mention was made of the hydrolysis of the 11α-acetoxyl group in the final product.

16% was achieved when approximately 10% ethanol was added to the fermentation medium. Similar results were obtained with other simple alcohols and phenol (93). Equally effective were some changes in the fermentation medium. Substitution of dextrin for sucrose in the Czapek-Dox medium or the addition of fatty acid esters to Czapek-Dox medium gave consistently high yields of cortisol with low yields of cortisone (119).

These examples, therefore, suggest that through changes of the environmental conditions, the unsatisfactory results of many of the above outlined transformations can be improved. Furthermore, it should be possible to modify the metabolic make-up of an organism by mutation and thus to achieve a more selective action of the microorganism upon the steroid substrate.

(*2*) *Combined chemical and microbiological syntheses.* Soon after the discovery of the 11α-hydroxylation by *Rhizopus* and *Aspergillus* a number of chemical syntheses of cortisone and cortisol appeared in the literature; these syntheses were principally concerned with the transformation of 11α-hydroxyprogesterone to the corticosteroids. Later communications described the chemical transformation of 11-epicortisol to cortisol. A detailed discussion of these chemical transformations is not within the scope of this review, particularly since they have been adequately reviewed elsewhere (39, 49, 125). It is worthwhile, however, to point out that in a recent communication by Hogg *et al.* (63) a new approach to the synthesis of cortisol and cortisone from 11α-hydroxyprogesterone was devised. This scheme differs from previous syntheses in that it does not require the hydrogenation of the 4,5-double bond prior to the elaboration of the dihydroxy acetone side chain and the reintroduction of the double bond at the end of the reaction sequence.

b. Modified Corticosteroids. Fried's discovery of the 9-halosteroids (44) drastically widened the concept of the relationship between structure and adrenocortical activity. It served to emphasize the point that the activity of adrenocortical steroids can be modified. Although no microbiological methods were employed in the preparation of this group of steroids, it is interesting to note that the discovery of the 9-halosteroids resulted directly from the effort of the Squibb group to convert a microbiological conversion product, i.e. 11-epicortisol into cortisol. Therefore, the microbiological process serves to create the raw material for the preparation of an important group of corticosteroids.

More recently, the process of microbial dissimilation has been successfully applied by the Schering group to the preparation of 1-dehydro corticosteroids. The 1,2-double bond was introduced by this group of investigators with *Corynebacterium simplex* (118). A number of fungi have been claimed by the Ciba group to perform the same reaction.

Experimental details, available only for *Corynebacterium simplex* and *Calonectria decora* (158), make the former appear to be the higher yielding organism. With these organisms 11-deoxysteroids as well as 11-oxygenated steroids can be dehydrogenated in the 1,2-position to give the 1,4-dien-

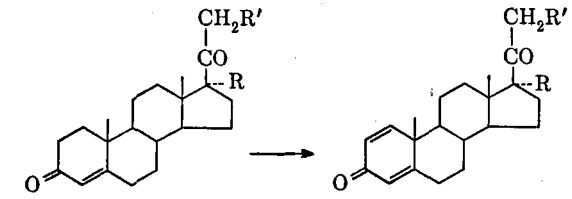


FIG. 9.

3-one chromophore (Fig. 9). The three 11-deoxysteroids, progesterone (I), deoxycorticosterone (III), and 11-deoxycortisol (V) are converted to the corresponding 1-dehydrosteroids, II, IV, and VI. The presence of an 11-oxygen function does not seem to inhibit the enzymes responsible

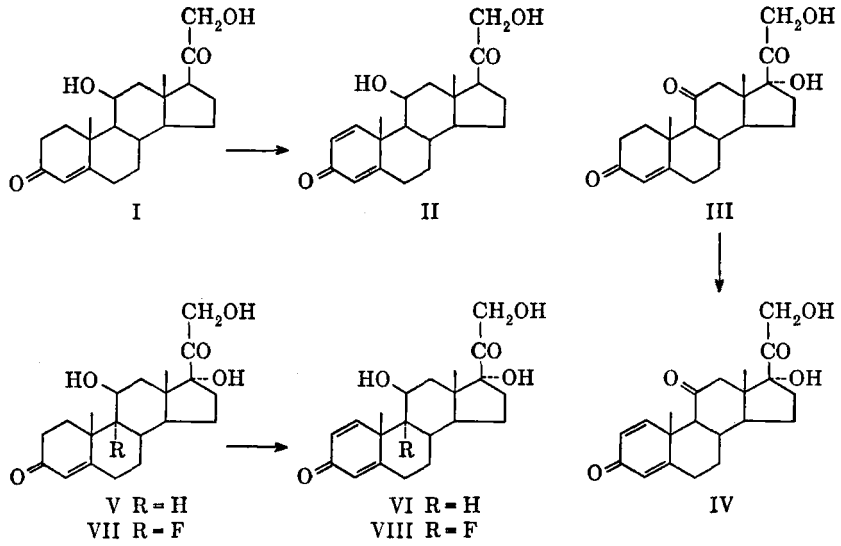


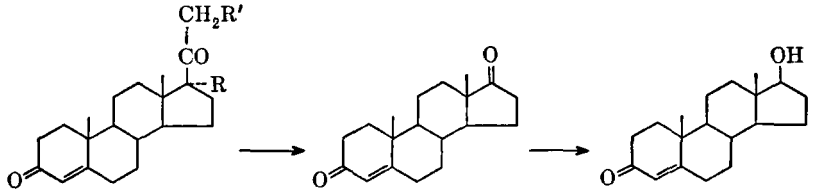
FIG. 10.

for this reaction. Thus, 1-dehydrocorticosterone (Fig. 10, II) and 1-dehydrocortisone (IV) are available from corticosterone and cortisone, respectively. The introduction of the 1,2-double bond into cortisol (V)

or 9α-fluorocortisol (VII) to give the corresponding 1-dehydrosteroids VI and VIII has been reported only with *Corynebacterium simplex.*

2. Androgens and Estrogens

The simultaneous publications from three different groups of investigators opened up new avenues to the preparation of C_{19}-steroids. We described the degradation of C_{21}-steroids (Fig. 11, I-IV) to androstenedione (V) by a number of Aspergilli, Penicillia, and Gliocladia (129).



I R = R' = H
II R = R' = OH
III R = H; R' = OH
IV R = OH; R' = H

V

VI

FIG. 11.

Although several by-products, notably 6β-hydroxy-4-androstene-3,17-dione and testololactone, were observed, this transformation can be carried out in excellent yield. This, together with the reduction of V to testosterone (VI) by yeast (87), constitutes a very efficient synthesis of the male hormone (shorter than any chemical transformation of C_{21}-steroids to testosterone thus far devised).

Various organisms and numerous C_{21}-steroids have been used for microbial side-chain degradation coupled with introduction of the 1,2-double bond (48, 160). Of all the substrates (cf. pages 393–394), progesterone (Fig. 12, I) is, from a practical point of view, the most economic starting material. *Fusarium solani* appears to be the most practicable organism because it gives androstadienedione (III) in excellent yield (160). In our hands, *Hypomyces haematococcus* (*Fusarium solani*) yielded 1-dehydrotestosterone (II) and androstadienedione (III) in a combined yield of 50% (116). This, together with the results on *Streptomyces lavendulae* (48) indicates that II and III can be produced by the same mold. These transformations, in conjunction with the pyrolysis of steroids possessing the 1,4-dien-3-one chromophore to ring A phenols by Inhoffen (68), provide attractive two-step syntheses of estradiol (IV) and estrone (V) from progesterone or pregnenolone.

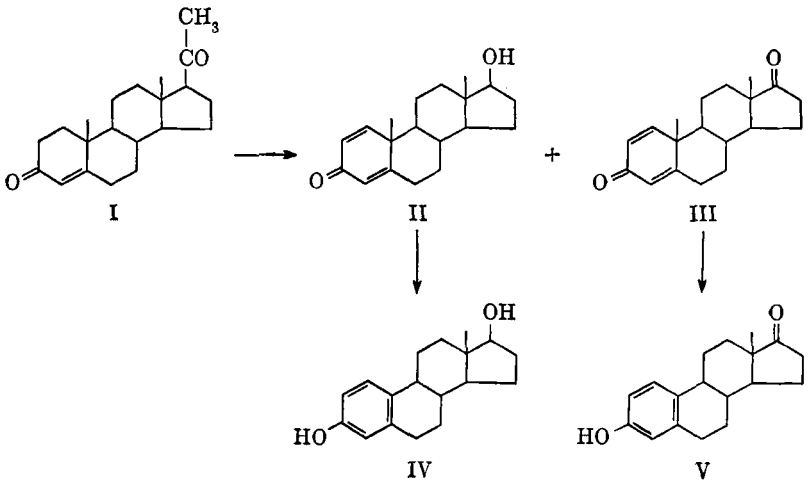


FIG. 12.

3. *Miscellaneous Steroids*

As Table II shows, a host of new steroid compounds has been produced by microbiological dissimilation of one kind or another. The list could obviously be greatly enlarged if all the compounds were included which directly resulted from chemical transformation of the bioconversion products. Thus, for most groups of epimeric monohydroxylated compounds there is the corresponding ketone, which is usually prepared for purposes of structure proof. However, with the notable exception of some simple 11-oxygenated steroids (65, 66, 67), of the 1-dehydrosteroids (61) and of 14α-hydroxycortisol (1), very few of these new steroids have been indicated in published reports to possess any significant biological activity. Nevertheless, some of them might, on closer examination, display new types of activity, as evidenced by the antigonadal activity of 11α-hydroxyprogesterone (21).

Although many of the compounds prepared by microbiological dissimilation appear to be of limited value, in some instances the newly introduced functional groups provide attractive points of attack for further chemical transformations which otherwise could only be achieved by more laborious methods. A few examples not yet discussed will illustrate this point:

The 7-hydroxylation described by Fried *et al.* (50) or by Meystre *et al.* (106) facilitates the preparation of 6-dehydrosteroids, as exemplified by the alkaline dehydration of 7ξ-hydroxyprogesterone (Fig. 13, I) to

4,6-pregnadiene-3,20-dione (II) (50) or the formation of 6-dehydrodeoxycorticosterone acetate (IV) from 7α,21-diacetoxy-4-pregnene-3,20-dione (III) upon alumina chromatography (106). Similar transformations are feasible at other positions of the steroid molecule. As the preparation of epoxides from the corresponding unsaturated compounds, e.g. 14α,15α-oxido-11-deoxycortisol from 14-dehydro-11-deoxycortisol by microbial oxidation shows, a large group of new steroids can be expected to result from this observation (11).

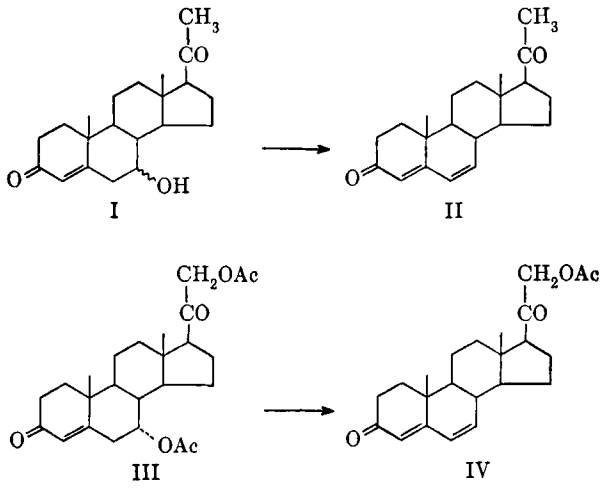


FIG. 13.

The great variety and the many combinations of microbiological reactions very often present the solution to a problem in an unexpected form. When 4,16-pregnadiene-3,20-dione (Fig. 14, I) was exposed to *Rhizopus nigricans* 11α-hydroxy-17α-progesterone (II) was formed instead of the expected 11α-hydroxy-4,16-pregnadiene-3,20-dione (VI) (100). The latter compound was synthesized in an elegant manner by Fried *et al.* (47). Progesterone (III) was hydroxylated to 16α-hydroxyprogesterone (IV) with a *Streptomyces* species. Incubation of IV with *Aspergillus niger* gave 11α,16α-dihydroxyprogesterone (V). When compound V was oxidized with aluminum *tert*-butoxide, 11α-hydroxy-4,16-pregnadiene-3,20-dione (VI) was obtained.

Finally, the synthetic work on inactive adrenal corticosteroids and urinary metabolites might in time profit from certain reactions. For instance, the microbial reduction of the ketone group at position 20 might be used for the preparation of the adrenal companion steroids which possess a 20-hydroxyl group. The advantage of the microbial reduction

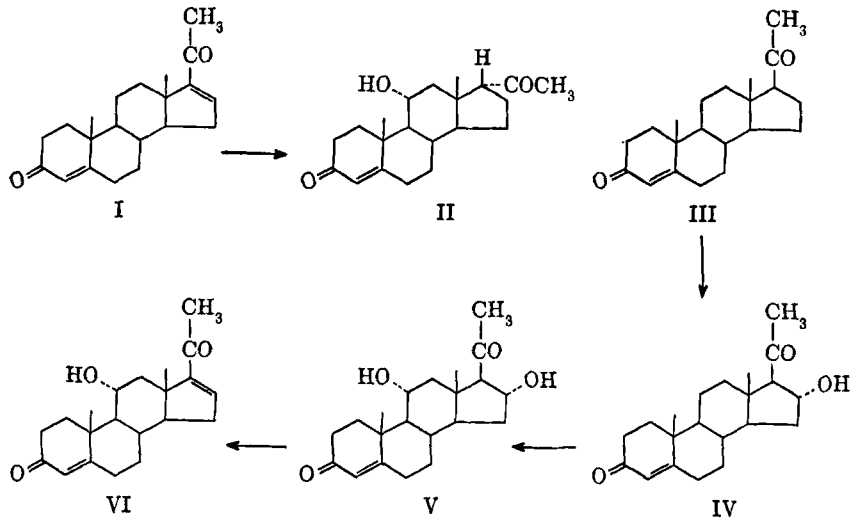


FIG. 14.

over a chemical reaction lies in the stereochemical specificity of the former.

X. METHODS OF STRUCTURE PROOF

In most instances, the structure proof of microbial dissimilation products has presented no involved problem because the correlation of an unknown bioconversion compound to a known steroid was possible with simple operations such as oxidation, dehydration, and hydrogenation. In some cases background information obtained from chemical work was sufficiently extensive to allow for easy clarification of the position of a newly introduced functional group. For instance, the investigations of Ehrenstein *et al.* (9, 42) on the chemical synthesis of 6β-hydroxysteroids had established a large body of criteria characteristic of the 3-keto-Δ^4-6β-hydroxy chromophore. As a result, any new compound bearing this group was readily recognized as such. Similarly, the structure of the 16α-hydroxylated derivatives obtained by two *Streptomyces* cultures (cf. page 391) was derived mostly from molecular rotation increments and from chemical reactions typical for the 16-hydroxyl group. Unambiguous proof for the stereochemistry, however, came from the synthetic work of Hirschmann and co-workers (62) and of Cole and Julian (24). In certain compounds, however, the application of special techniques was required for the clarification of the structure.

1. Microbial Reactions as a Tool in Structure Proof

Infrared and ultraviolet spectroscopy, molecular rotation differences, paper chromatography, and the concepts of stereochemistry have been extremely helpful for identification and structure proof. Since extensive reviews have appeared which have discussed these tools and thereby covered the whole steroid field, the discussion will be limited to a conspicuously simple tool of structure proof, i.e. the application of microbial reactions.

6β,11α-Dihydroxyprogesterone was readily identified as a 6β-hydroxysteroid through a comparison of its physical properties to the known 6β-hydroxysteroids of Ehrenstein (*vide supra*). The 11α-position of the second hydroxyl group was ascertained by incubation of 11α-hydroxyprogesterone with *Cunninghamella blakesleeana* which produced 6β,11α-dihydroxyprogesterone (31). [Fieser's (38) proof of structure proceeds in the opposite direction insofar as the allylic hydroxyl group is removed reductively with zinc and acetic acid.] The structure of 6β,11α,22-trihydroxybisnor-4-cholen-3-one obtained from *Rhizopus* conversions of 3-ketobisnor-4-cholen-22-al was elucidated in a manner similar to that used for 6β,11α-dihydroxyprogesterone (99).

Since the 6β-hydroxyl group present in 6β,11α-dihydroxyprogesterone and 6β,11α,22-trihydroxybisnor-4-cholen-3-one was easily recognized by its physical properties, only one reaction was necessary to establish the presence of the 11α-hydroxyl group. One can easily visualize a procedure whereby the structure of an x,y-dihydroxysteroid could be established largely by means of microbial transformations. The success of such a procedure hinges on several factors:

(1) Preliminary information about the possible location of x and y is necessary and can be deduced from various sources.
   (a) The monohydroxyderivatives obtained from the microorganism which produces x,y-dihydroxyprogesterone usually give some clues concerning this.
   (b) Substantial information may be derived from the physical properties of the dihydroxyderivative (notably, mobility on paper chromatograms and the molecular rotation difference).
   (c) The chemical behavior of the dihydroxyderivative in oxidation and acetylation reactions yields further insight into the nature of the two hydroxyl groups.
(2) Microorganisms must be available which introduce stepwise the two hydroxyl groups under consideration. Since usually the species

producing the x,y-dihydroxyprogesterone often generates a monohydroxyderivative, e.g. the x-hydroxysteroid, only one microorganism oxygenating position y is necessary.

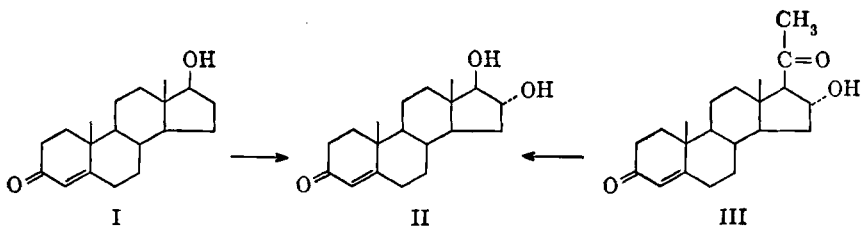
(3) Since the introduction of a functional group into a molecule might possibly influence the position of microbial attack (e.g., cf. page 399 for the influence of the 17α-hydroxyl group on *Helicostylum piriforme*), a double sequence of stepwise oxygenation is often necessary to prove unambiguously the location of the two hydroxyl groups in the x,y-dihydroxysteroid.

Let us call x' and y' the two positions suspected to be hydroxylated in x,y-dihydroxysteroid. Then, supposedly, x'-hydroxysteroid $\xrightarrow{y'\text{-hydroxylator}}$ x',y'-dihydroxysteroid and y'-hydroxysteroid $\xrightarrow{x'\text{-hydroxylator}}$ y',x'-dihydroxysteroid. If the x',y'-dihydroxysteroid is identical with the y',x'-dihydroxysteroid, we know that no shift in the positions of hydroxylation by these two microorganisms has occurred. *This is a necessary condition for the structure proof.*

Now, if the x',y'-dihydroxysteroid is identical with, the x,y-dihydroxysteroid, our proof is complete. But if the x',y'-dihydroxysteroid is not identical with the x,y-dihydroxysteroid, our original assumption concerning the positions of x or y or both was fallacious, and the structure proof has to be carried out in a different manner.

Microbiological side-chain degradation was helpful in two instances. 14α-Hydroxyprogesterone was degraded to 14α-hydroxyandrostenedione by *Penicillium lilacinum* (96). This reaction correlated the former compound with several other 14α-hydroxysteroids which had been converted by chemical oxidation to 14α-hydroxyandrostenedione. Thus, the microbial degradation of 14α-hydroxyprogesterone obviated a tedious chemical conversion.

In the second case, 16α-hydroxyprogesterone (Fig. 15, III) was degraded to 16α-hydroxytestosterone (II) by *Streptomyces lavendulae* (50). Compound II had been prepared from testosterone (I) by *Streptomyces*



I II III

FIG. 15.

roseochromogenus (152). The degradation of compound III to II establishes the α-configuration of the 16-hydroxyl group in II unless inversion of the hydroxyl group at carbon 16 is assumed to have taken place. There is no precedent for such an assumption.

XI. General Methods

1. Microbiological

a. Selection of Cultures. Research workers in the field of synthetic chemistry are frequently unfamiliar with the use and the availability of microbial cultures. In general it is advisable to work with pure cultures which may be obtained in a number of ways.

Some investigators interested in biochemical studies of this nature isolate their own cultures from soil or other sources which are known to contain the desired type of microorganism. The specific details of isolation technique are described in manuals of mycology or bacteriology. Often, individual investigators or laboratories have accumulated their own culture collections as a result of their research in specific fields of microbiology. Certain rare and unusual cultures may be obtained only from the investigator who isolated them.

The purchase of identified cultures from one of the large repositories of microbial cultures is probably the most convenient, rapid, and economical way of obtaining microorganisms. The best known collections of this kind are:

The American Type Culture Collection, Washington, D. C.; and
the Centraalbureau voor Schimmelcultures, Baarn (Holland)

The *Centraalbureau* is probably the world's most complete collection of filamentous fungi with an auxiliary yeast collection. The American Type Culture Collection covers bacteria, yeast, and fungi.

In the selection of cultures caution should be exercised, since not only bacteria but some of the filamentous fungi have been implicated in various human diseases. Supposedly nonvirulent fungi such as certain Mucorales have been found to precipitate mycoses. Manuals on clinical mycology will indicate the specific pathogenic cultures. Likewise, plant pathogens (identified as such in manuals of plant pathology) should be treated with proper concern for the possibility of spreading the plant disease.

b. Fermentation. Specific techniques such as submerged culture have been developed for the production of antibiotics and have been applied with very minor modifications to the steroid transformations. Although

details of these techniques have been adequately discussed in manuals on industrial microbiology by Prescott and Dunn (135) and by Underkofler and Hickey (155) certain points of experience can be summarized for the benefit of those readers who are not familiar with the microbiological part of steroid transformations.

c. Media. Numerous media useful for the culture of microorganisms have been described. Their composition ranges from a synthetic "inorganic" medium containing only a hexose as a carbon source [Czapek-Dox (135)] to a "natural" medium such as white bread, bran mash, etc. However, a medium suitable for steroid dissimilation should fulfill three major requirements: (a) It should sustain relatively rapid growth of the organism; (b) it should be adaptable to submerged culture; and (c) it should not interfere with the recovery of the steroidal products.

Points (a) and (b) can be satisfactorily fulfilled with a large number of liquid media that contain carbon and nitrogen sources in a form which can easily be assimilated. Often, good growth of the microorganism provides satisfactory steroid dissimilation. As Perlman *et al.* (123) have observed, the composition of the medium has hardly any effect on the over-all steroid-oxidizing ability of their *Streptomyces* culture. We have made a similar observation with our *Rhizopus* culture. A medium composed of 1.2% (w./v.) corn steep liquor solids and 1% (w./v.) dextrose (technical) has been used by us for a large number of diverse bioconversions. The latter medium is very satisfactory with respect to requirement (c), inasmuch as the extraction of the steroid products is not complicated by emulsification or by the extraction of nonsteroidal material. Certain media, e.g. those containing yeast extract, will often cause severe foaming during the fermentation or will give rise to emulsions during extraction. The foaming can frequently be controlled by the addition of antifoam; however, since the antifoam might interfere with the isolation procedure, its use should be avoided whenever possible.

It should be pointed out here that in some instances the composition of the medium has changed the ratio of the dissimilation products (28, 119).

d. Inoculation. Thus far nothing has appeared in the literature which would indicate that the type, preparation, amount, or age of the inoculum has any perceptible effect on the course of the bioconversion. Usually an inoculum grown for 24 hours on $\frac{1}{20}$ the volume of the fermentation medium furnishes rapid growth. The preparation of the inoculum of a very slow growing organism may take several days.

e. Growth and Substrate Addition. The older method of surface culture has been applied in a few instances and shown to be very slow and inefficient. The growth in submerged culture is most luxuriant if the standard procedure of aeration and agitation is used. It is advisable to

increase the effect of agitation by introducing baffles into the container. There are various methods of calibrating the fermentation vessel, i.e. of determining the effect of aeration or agitation or both upon the available oxygen supply. The relative merits of the various methods are still under discussion and need not concern us here. Since a change in the rate of aeration-agitation is often desirable, some indicator for such a change is necessary. For this purpose, the method of Cooper *et al.* (27) has proved, in our hands, to be satisfactory for small-scale equipment.

Two methods of substrate addition are used. Perlman *et al.* (123) add the steroid in chloroform solution prior to the sterilization of the medium. However, most workers introduce in acetone, alcohol, or propylene glycol (28) solution after appreciable growth has occurred (usually 17–48 hours).

Since the sensitivity of microorganisms to antiseptics varies from species to species, no general rule can be made for the allowable concentration of solvents. At the beginning it may be advisable to keep the concentration as low as 1 to 2% of the volume of the medium. Later experiments may serve to establish the maximum tolerance, which in the instance of *Cunninghamella* has been found to be above 10% with respect to alcohol (93).

Similarly, the highest noninhibitory concentration of steroid varies from substrate to substrate as well as from species to species. Therefore, each case has to be investigated individually. Generally speaking, higher concentrations of progesterone (0.5 to 1 g./liter) than of deoxycorticosterone, of 11-deoxycortisol, or of the androgens (0.1 to 0.25 g./liter) can be applied.

The above-discussed variables and their influence on the result of the steroid dissimilation are profitably studied on a 100-ml. scale. Aeration and agitation is provided in this case by shaking on a rotary shaker at 250 rpm. To increase the aeration, baffling is necessary. This can be accomplished in various ways, e.g. by making two to four indentations into the sidewall of 250-ml. Erlenmeyer flasks.

f. Examination of the Course of Steroid Dissimilation. Various methods are available to check on the satisfactory progress of a culture. With fungi, a fruity odor characteristic for each organism is a conspicuous phenomenon of good noncontaminated growth. Contamination by bacteria manifests itself very often in a sour or "evil" odor. A more accurate measure of the reproducibility of a fermentation is obtained by determination of the pH at the time of inoculation, substrate addition, and extraction. The change of pH is the result of many factors, notably the composition of medium (particularly with respect to carbohydrate concentration), the aeration and the metabolic preferences of the organism. However, under constant conditions the change of pH is quite reproducible.

The majority of filamentous fungi show a rise in pH after the carbohydrate has disappeared from the medium. To avoid bacterial contamination during the growth phase, an initial pH 5 is advantageous. If the medium contains 1% of carbohydrate, the pH will then drop after 17 to 24 hours to 4.5, and after an additional 24 hours will rise to 6 or 7. Bacterial contamination is often indicated by an excessive rise of the pH after 17 to 24 hours.

Furthermore, the final mass of the fungal mycelium or the bacterial cells can be used as a measure of reproducibility. This is determined either by weighing the dried mass or by measuring the volume of the centrifuged cell mass.

The rate of steroid conversion can be followed very readily by a roughly quantitative papergram analysis of aliquots at various intervals.

2. Extraction and Purification of Steroids from Fermentations

Most of the steroids are very soluble in chlorinated solvents. Methylene chloride, ethylene dichloride, and chloroform are generally used to extract them from beers. We have preferred the use of methylene chloride, which has a low toxicity and a low boiling point. Other solvents, such as ethyl or amyl acetate, may be used; but the steroids are less easily recovered from these ester solvents. When the extraction procedure is complicated by emulsification, chlorinated solvents have been found useful because they are less readily emulsified.

From small-scale fermentations (up to 25 liters) the steroids are readily recovered by extracting the beer with methylene chloride three or four times with one-quarter the volume of beer. If the final cell mass has to be determined, mycelium and beer are extracted separately. The mycelium is first dried twice with acetone and then extracted twice with methylene chloride. The combined acetone washings and methylene chloride extracts are added to the beer extracts. At this point, removal of a small aliquot for paper-chromatographic analysis is helpful, since a decision can then be made on the choice of isolation procedure.

The steroid content of the solvent-free extracts is generally very high. In extracts which contain a single product, this may be recovered in good yield and relatively high purity by triturating with ethyl ether, ethyl acetate, etc. The 11α-hydroxyprogesterone produced by *Rhizopus nigricans* is in a good condition after being worked up in this way.

When several steroids are present in the extract, as indicated by paper chromatography, more laborious methods of isolation are needed.

Chromatography over alumina, silica gel, magnesium silicate, carbon, etc., (161) have been used for separations of the products. If the products have a 17-hydroxyl group, the alkaline nature of the alumina column

may be unsatisfactory and silica gel or magnesium silicate have to be used. With alumina as the adsorption medium, a steroid:alumina ratio of 1:50 is used, with silicates a ratio of 1:80. Correspondingly higher ratios are in order, if the fermentation has produced a high concentration of nonsteroidal metabolites (as expressed by the weight ratio of substrate to final extract).

For the alumina chromatogram, the following solvent sequence is used: benzene, ether, chloroform, acetone, and methanol and mixtures thereof. [For the exact sequence of solvents see various papers (105, 132, 159).] Essentially the same solvents can be applied to the silica and silicate chromatograms. In our hands a system composed of magnesium silicate and ethylene chloride-acetone mixtures has been very useful.

The fractions are collected in measured aliquots from which the solvents are evaporated and the residues are weighed. A curve may be prepared of these weights. For convenience, paper chromatograms of the "peak" aliquots are obtained, which leads to decisions for further pooling and recrystallization of products.

Some marvelous equipment has been devised to make the operation nearly effortless, with time control, ultraviolet cut-off points, etc. Usually, such elaborate equipment is not necessary.

For purification, the fractions may be recrystallized several times from solvents. Many polar steroids have been recrystallized from a 50–50 mixture of methanol and chloroform.

If a separation is difficult or impossible by conventional means of adsorption chromatography, it may be achieved in a number of other ways. Acetylation (or esterification) or formation of ketone derivatives sometimes affords better separation by chromatography. If the mobility on a paper chromatogram of the two components is sufficiently different, the particular paper chromatogram system can be adapted to a partition column for which cellulose or some other inert material is used as a support.

3. Paper Chromatography of Products from Steroid Fermentations

Undoubtedly, the great advances in the detection and studies of microbial changes of the steroids have been due to the use of paper chromatography.

Zaffaroni (165) and Bush (14) have developed this tool specifically for studies with the corticosteroids. With variations, these two general methods have been used in microbial studies on steroids.

Voigt *et al.* (161), Bush (15, 16, 17), Savard (136), and Heftmann (58) have critically reviewed the paper chromatography of steroids. A comprehensive survey of the application of these techniques to microbial

dissimilation of steroids is not yet available. However, the references cited here furnish criticisms and descriptions of the methods which are applicable, with but slight extension, to steroid fermentation.

There has been some discussion of the merits of the Zaffaroni versus the Bush systems. In our work, we have preferred to take advantage of both in determining the identity or novelty of a bioconversion product. If unknown samples are mixed with known controls and applied to several different chromatography systems, it is possible to identify most dissimilation products.

A system of diethylene glycol monoethyl ether (Carbitol) as a stationary phase and methyl cyclohexane saturated with Carbitol as a mobile phase is useful in separating progesterone, androstenedione, and similar less polar steroids. Isolation and structure proof by physical and chemical methods is the final criterion for identification of a fermentation product. The definitive separations possible by using several chromatographic systems have justified the extra work necessary to maintain simultaneously a number of solvent systems.

For analysis of the differentiated components, an ultraviolet scanner (52) will indicate steroids with a conjugated ketone; a triphenyltetrazolium reagent or Tollens' reagent (12, 165) will indicate retention or production of the formaldehydogenic side chain; dinitrophenylhydrazine reagent may be used to indicate the presence of steroid ketones; the phosphomolybdic acid reagent of Kritchevsky and Kirk (77) is of aid in detecting many hydroxysteroids, especially the 3-hydroxy-Δ^5-steroids. These reactions are used both to locate the material and also to gain some knowledge of the chemical configuration of the product.

We feel that by a combination of paper-chromatographic separation on several systems with the application of color tests only very few groups of steroids will resist differentiation. However, for a few isolated cases it might prove helpful to prepare derivatives from the material in the "spot" and to rechromatograph these derivatives after the methods of Zaffaroni *et al.* (165, 166). The latter procedure is more definitive but also more laborious than the former.

Paper chromatography of steroids may be used in quantitative estimations of the bioconversion products. This can be done in one of two ways: Visual estimation of the ultraviolet absorption or the intensity of color spots is fairly accurate when the spots are compared to reference standards of known concentration. Progesterone, for example, is readily estimated in less than 25-μg. quantities if the sample is chromatographed side by side with graded amounts of the control. For routine studies, this visual method is labor-saving and, frequently, entirely sufficient.

A more precise but involved method requires the elution of the

material from the paper strip and its determination by spectrophotometric or colorimetric means. The accuracy of this procedure is sometimes not so much better than that of the visual method because of errors inherent in the sampling technique and because of background absorption eluted from the paper.

4. Fermentation Example

The preceding discussion is a condensation of published details and the authors' (partly unpublished) experience. For the detailed description of a fermentation, the paper by Peterson *et al.* (132) should be consulted. The procedure described therein is readily adaptable to most fungal fermentations. For the design of small scale studies concerned with the parameters of steroidal bioconversions, the papers by Perlman *et al.* (123) or by Dulaney *et al.* (28) are helpful.

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