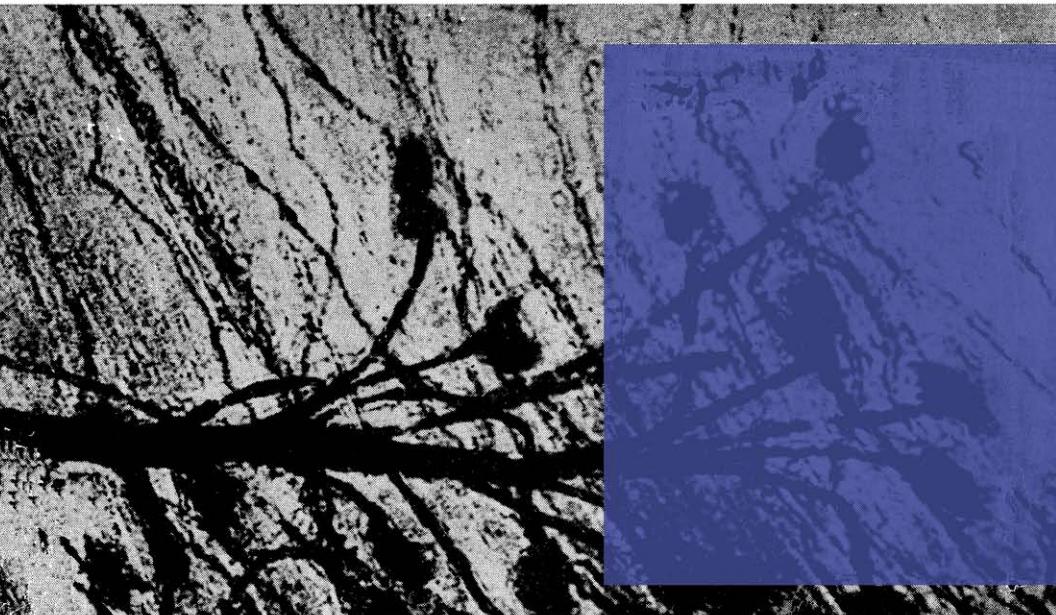


# **Vitamins in Endocrine Metabolism**

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University College  
University of Cambridge



William Heinemann Medical Books Limited

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## Preface

This book is intended to bridge the gap between scientists studying nutritional disease problems in the diverse fields of endocrinology, enzymology, pathology and vitamin research. Knowledge in each field is expanding so rapidly that workers in one subject who try to keep up with advances in their own science have very little time to look over their shoulders to see what is happening in the other subjects. In an age when the attention of outstanding scientists is focussed on the infinitely small detail of such matters as the haemoglobin crystal or the insulin molecule, there is still a need for a broad survey of the advancing frontiers of knowledge. The author, a pathologist, is fully aware of the dangers inherent in venturing on to the alien soils of biochemistry, physiology and cognate sciences—there are many pitfalls for the trespasser. However, the risk is well worth taking, if it encourages others to cross the interscience barriers, for there is much to gain from a study of unfamiliar knowledge and techniques.

Scientific papers on the subject of hormones, and on the subject of vitamins are available in vast quantities, quite beyond the power of any single worker to evaluate in a reasonable time. Material which deals directly with vitamin-hormone inter-relationships is exceedingly sparse and difficult to find. The author has been privileged to have the help of the medical literature analysis and retrieval system (MEDLAR) of the National Lending Library for Science and Technology in tracing those papers which span the gap between the various subjects. Nevertheless, a wide computer search of the literature brought forth only twenty-eight references for a five year period ending in 1967—a remarkably small number for such a wide field of research.

This present work therefore is inevitably biased by the author's experience in the field of nutritional disease, and by personal selection from the material available in each of the contributory subjects. Further bias has been introduced by the influence of the many scientists who come each year to Cambridge, to discuss advances in their own line of research with the staff and students of this university. To these, and to all the countless scientists whose work is discussed here, I extend my grateful thanks. Had it been possible to mention all these authors individually, the bibliography would have been much longer than the text. The references given at the end of each chapter have been chosen, sometimes because they contain important original work, sometimes because they are difficult to find, but more often because they provide excellent bibliographies in addition to their useful scientific content.

In preparing the text, I have had the invaluable assistance of Alan Wilson, University Lecturer in Chemical Pathology, and my thanks go to him for his careful reading of the manuscript and for many helpful suggestions on the biochemical aspects of the work. I am indebted to my husband for much constructive criticism, and to Mrs. Sally Roberts for typing the final draft. Miss Elizabeth McDowell kindly supplied the electron micrograph illustrating intracellular structures.

Isobel Jennings

University College,  
Cambridge, 1970.

## **Introduction**

The last twenty years have seen major developments in the sciences of endocrinology and enzymology, but the study of the vitamins has lagged far behind. The golden age of vitamin research took place between the two major world wars, since when there has been little in the way of advancement of knowledge and no major new discoveries, although it seems unlikely that all the essential food factors are known to us at the present time. In the realm of endocrinology there has been the recent discovery of two new hormonal systems, the prostaglandins and calcitonins. Enzymology as an exact science has advanced on a broad front, and large numbers of scientists are specializing in this important subject. As the biological sciences become more and more complex, the relationships between them tend to become forgotten. It is the new science of molecular biology which is drawing these various subjects together, so that workers studying widely separated areas of research are meeting on common ground, in the ultramicroscopic workings of the molecular components of the living cell. In the next few years there must surely be a renaissance in vitamin research, for vitamins are essential for the synthesis of hormones, for permitting their action to proceed and, in the case of the prostaglandins, the fatty acid vitamins are essential biological precursors of the hormones. Although many enzymologists are working with purified enzymes alone, some among them study the combined action of vitamins and enzymes, and it is to these scientists that we must look for advances in the understanding of biological processes.

In the living body, the enzymes are dependant for their action on the presence of the coenzymes, many of which contain vitamins in their complex molecular structure. The vitamins, enzymes and hormones are equal partners in the complex mechanisms which control the working of the cells of the animal body, and maintain the homeostasis which is necessary for health.

Fundamental research on the problem of control and communications within the cell has received a tremendous stimulus from the work of Jacob and Monod, on the way in which genes may be switched on or held in abeyance. Another major advance is the work of Sutherland and his colleagues, which led to the development of the second messenger hypothesis of cellular control. The determination of the structure of the DNA helix and the breaking of the genetic code are two more achievements in biological research which have contributed much to an understanding of the inner workings of the cell.

With all this new knowledge available we are now in a position to

make a fresh evaluation of the complex interrelationships between hormones, vitamins and enzymes. In the pages which follow, it is proposed to describe first of all the general principles governing the actions of the members of this triad, and then to examine in more detail the influence of variations in vitamin status on the various endocrine systems. The concluding chapters will deal with the interactions of vitamins and hormones in the initiation and control of tumours and in foetal dysmorphogenesis. It is hoped that this treatment of the subject will throw some light on the many deficiency diseases which are assuming greater importance in public health, as the major epidemic diseases are brought under control. The cure of a cretinous infant or an adult suffering from some obscure nervous disease by a simple alteration in the diet is a dramatic event, but the really important work lies in the field of preventive medicine, where so much can be done to improve the health of the population. Overt deficiency disease is all too common in the underdeveloped countries. Marginal deficiency disease is likely to increase as more and more of the food of the highly civilised nations is processed for convenience and storage. It is the marginal deficiencies which are so difficult to diagnose—they come in many guises and are often unrecognised. Scurvy, for example, is common enough in infants and old people in this country, and yet to our ears the very word sounds as old-fashioned as rabies and glanders, two of the many diseases which have been eliminated from our islands.

The pressing necessity then, is for automated estimation of the circulating vitamin and hormone levels in small samples of blood, or in biopsy specimens. Microtechniques for the estimation of metabolites are being developed in many laboratories and the day will surely come when these are all gathered together in one autoanalyser. We may look too for future developments in the preparation of nutrient media containing the correct vitamins and hormones, not only for cell and tissue growth, but also for organ growth for 'spare part' surgery. Such a concept lies in the realm of science fiction at the moment, but in perhaps ten or twenty years we may have sufficient knowledge to take primitive undifferentiated cells and apply to them the correct hormonal and nutritional stimuli, and the correct contact stimuli, to enable them to develop into a strand of muscle, a layer of skin, or even, dare one hope, a heart.

A not unimportant part of nutritional research for the future will be the provision of adequate diets for the astronauts who will eventually take off for journeys into space lasting a year or more. In such circumstances, a supply of material for the replacement of hormones, enzymes and vitamins is literally vital. These substances, although resembling inorganic catalysts in many ways, are not everlasting—they are continually being lost to the bodily economy in the normal turnover of cells, and by inactivation and excretion. In space travel, too, a knowledge

of the complex interactions between parathyroid hormone, calcitonin and vitamin D is essential in understanding and coping with the interference with calcium metabolism arising from weightlessness, which deprives living bone of one of the stimuli required for calcification. At the present moment, the control of calcium metabolism still holds many mysteries for the enquiring mind.

If this book provides greater understanding for the unravelling of such problems as prenatal death or deformity, or postnatal disease caused by nutritional deficiency, if it should in any way help to alleviate the human suffering associated with malnutrition, then it will have amply served its purpose.

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## *Chapter 1*

### **The Vitamins**

In animal tissues vitamins function, both in the structural parts of cells and in the fluid medium, in close collaboration with enzymes and hormones. Understanding of the complicated interactions of these substances is not yet at a very advanced stage, but an examination of our present knowledge of the subject may help to mark out the considerable areas in the field which are still available for exploration.

The vitamins are a heterogenous group of organic compounds required by most living organisms for normal growth and health. In this present context, however, only those required by the higher animals will be considered. Vitamins are essential food factors, of very high biological activity, and required in exceedingly small amounts. Nevertheless these minute quantities are able to maintain the normal structure and function of all the tissues of the animal body. In the absence of vitamins, metabolic defects arise which may be expressed as anatomical lesions, as mental disturbances with personality changes, or as biochemical changes, which may prove difficult to detect, particularly in the numerous cases of marginal deficiency. Vitamins normally must be provided in the diet, either in their active form, or in the form of their precursors. The status of certain substances as vitamins may vary with the species of animal for which it is required. For example vitamin C, although essential for domestic animals, is readily synthesized by them from carbohydrate sources, and need not be provided in the diet. For such animals, ascorbic acid is an endogenously synthesized essential metabolite, but is not strictly speaking, a vitamin. For man, subhuman primates and guinea pigs, ascorbic acid is a true vitamin.

Within the last twenty years, great advances have been made in determining the chemical structures of the vitamins. Inevitably this has led to a better appreciation of their mode of action, and to a deeper understanding of the pathological changes which occur in their absence. Broadly, they may be classified into two types, the fat-soluble vitamins and the coenzymatic vitamins. The fat-soluble group includes vitamins A, D, E and K. Of these, the first three are stored in the body in relatively large amounts. In contrast, the coenzymatic, water soluble vitamins B and C are not stored to any great extent in the body, and renewed supplies are required at fairly frequent intervals.

The fat soluble vitamins appear to function as integral parts of cell membranes and variations in availability of, for example vitamins A or

E may have a profound effect on the structure and properties of the membranes of cells and their contained organelles. The biochemical make-up of these membranes varies not only from one tissue to another, but also from one intracellular organelle to another. As a result, pathological effects resulting from deficiency or excess of vitamins are highly selective in their situation, whether in the tissues or within the cells themselves. The fat soluble vitamins in general are more akin to the steroid hormones, than to the vitamins which function as enzymic cofactors.

Coenzymes are complicated organic molecules, which, by virtue of their chemical constitution and configuration are able to accelerate enzymatic reactions, often as carriers of some particular chemical grouping. Thiamine, in its active form of thiamine pyrophosphate provides an example of this type of coenzyme. One of its functions is to assist in the transfer of carboxyl groups, and it is therefore known as a cocarboxylase. Other examples are the adenylic acid-containing coenzymes, nicotinamide adenine dinucleotide (NAD) and its phosphate (NADP) and coenzyme A, all of which contain vitamins of the B complex as part of their molecule. In some cases vitamin-containing organic molecules are so firmly attached to enzymes, that their removal from the enzyme results in loss of activity. These integral parts of enzyme molecules are known as prosthetic groups. They act as carriers of chemical groups from one substrate to another. The flavoproteins, which contain vitamin B<sub>2</sub> are examples of enzymes with such prosthetic groups. Many essential enzyme systems within the animal body are completely dependent on the presence of the cofactor vitamins which function as essential parts of coenzymes and prosthetic groups.

Although the general division into fat soluble and cofactor vitamins is valid in most cases, the distinction must not be taken as absolutely clear cut. Vitamin A, for example, in addition to its role in the activities of membranes, also functions, in the form of its aldehyde retinene, as a cofactor in the visual cycle.

### **Conditions affecting vitamin requirements**

Requirements for vitamins vary from species to species, from one individual to another, and during the lifetime of an individual in accordance with

1. Age
2. Physiological status
3. Inherited enzyme pattern
4. Modifying effect of other food stuffs in the diet
5. Availability of activators
6. Pathological status

1. **Age.** During periods of rapid growth, requirements for vitamins rise steeply; at some periods an adequate supply of vitamins is literally vital. The most important of these periods occur during prenatal life at the various stages of organogenesis. As each organ is formed it has requirements for essential food factors which must be met during a restricted period of time if malformation or even agenesis is not to occur. The endocrine organs, unlike some other tissues such as the lung, start working before birth. As each endocrine organ swings into action during prenatal life, and starts producing its own hormones, its vitamin requirements for hormone synthesis are in all probability, similar in quality if not in quantity, to those of the post-natal organ. Requirements are drawn from maternal sources, which must be adequate to support two sets of organs as gestation proceeds.

Early post-natal life is another period of rapid growth during which depletion in vitamin status can have a lasting effect, not only in growth but also in the production of irreparable pathological lesions. Of these last, perhaps arterial lesions are the most important in the so-called 'affluent' societies, epithelial and bone lesions in the underprivileged societies. Artificially fed infants are greatly at risk at this period, partly because of their predisposition to gastrointestinal disturbances with resulting malabsorption, and partly because dietary formulas may not be adequate. It would be arrogant to assume that we know, at the present day, all the vitamins and trace elements required by man and domestic animals in their first few months of life. The recent discovery of the entirely new series of hormones, known as the prostaglandins, derived from essential food factors, should be sufficient to induce a proper state of humility in the nutritional scientist, and to raise doubts about his ability to imitate nature.

During adolescence there is another spurt in growth which requires dietary support if growth is not to be retarded. The widespread occurrence of deficiency diseases in old age is probably a reflection of inadequate intake, rather than of increased requirements for essential food factors.

2. **Physiological status.** It goes without saying that during periods of physiological stress such as pregnancy and lactation, vitamin requirements are raised to compensate for the resources drained by the infant. Training for athletic competition too imposes its own demands on the body for a more rapid turnover of metabolites. It is no coincidence that the barrier of the first four minute mile was broken by a man with good knowledge of human nutrition.

3. **Inherited enzymic pattern.** Veterinary scientists familiar with vitamin deficiency disease are well aware of the remarkable variation in requirements for vitamins throughout a herd composed of animals of equal

age and size. Diets which prove quite adequate for some members of the herd may produce overt disease in others. To a certain extent this may be attributed to the genetic ability of some animals to produce the enzymes necessary for synthesis and metabolism of the active form of the vitamins concerned, and to the relative inability of others to produce the necessary enzymes. Species variations in inherited enzyme patterns are best illustrated by the inability of man and other primates to synthesize a particular enzyme which converts inactive vitamin C precursor to active vitamin C. Most other animals are able to undertake the conversion quite readily.

**4. Modifying effects of the diet.** Certain diets require the addition of larger than usual amounts of vitamins to replace loss due to oxidation of essential food factors. For example, loss of the antioxidant activity of vitamin E in diets high in polyunsaturated fats must be compensated for, by vitamin supplementation.

**5. Availability of enzymic activators.** The substances known as activators are usually simple electrolytes, which are essential for inducing a catalytically active state in some vitamin-assisted enzyme systems, but do not themselves take part in the reaction catalysed: examples are sodium, potassium, calcium, zinc, copper and cobalt. The lack of essential metallic activators is common enough in domestic animals grazing on soils deficient in the element in question. Supplementation of the defective diet with the related vitamin appears in some cases to improve the deficiency, perhaps by making the best use of the small amount of trace element available. However, complete cure of the deficiency depends on restoration of adequate amounts of the missing trace element.

**6. Pathological status.** In pathological states, requirements for some or all of the vitamins may be increased.

Hyperthyroidism, fevers and post-operative recuperation are examples of such states demanding supplementation. The diabetic patient is a special case. He is unable to convert vitamin A precursor into vitamin A, and must therefore get his quota of this vitamin from animal sources, as distinct from the vegetable sources which provide the precursor.

### Stress

The stress of modern life is much discussed nowadays in a rather abstract way. But stress has clearly identifiable effects on the animal body, ranging all the way from the physiological results of a small output of adrenalin to rapid death from shock. Many stress factors have the common primary effect of depleting bodily stores of vitamins,

and a secondary effect of reducing the capacity of the endocrine organs to respond adequately to physiological requirements. Such factors include:

### 1. *Physical stress factors*

- (a) Exposure to excessive heat or cold, or to ionizing radiation; exposure to high altitudes, or sudden changes in pressure or acceleration.
- (b) Normal physiological stress states such as rapid growth, pregnancy, lactation or strenuous muscular effort.
- (c) Inadequate food intake due to insufficient quantity, or following lack of appetite from any cause, including alcoholism and smoking.
- (d) Malabsorption following hepatic, pancreatic or gastro-intestinal disease.
- (e) Competitive demands of intestinal parasites.
- (f) Mechanical, electrical or other injuries, such as haemorrhage.

### 2. *Mental stress factors*

- (a) Prolonged fear or anger, overcrowding, imprisonment of man, caging of animals, are among the most stressful mental stimuli.
- (b) The most devastating stress is caused in animals by restriction of movement, and even such simple restriction in rats as binding up the limbs so as to immobilise them completely for only one or two hours causes acute stress. In such cases the adrenal steroids become depleted and there is bleeding into the stomach. This is a type of shock reaction.

There are in animals genetic strain differences in response to stress. Mice, which normally have high steroid levels in their blood plasma respond to stress by a prolonged rise in the outpouring of steroids. Mice with a normally low blood steroid level react only briefly to stress with a rise in plasma steroids (Levine and Treiman, 1964). It is probable that such differences exist also in man—there are individuals who evince an exaggerated response to stress, as well as individuals who take life calmly. Both man and animals may become unresponsive to stress through adrenal exhaustion, or they may gradually become adapted to stress. The depletion of the adrenal steroids in stress states is accompanied by depletion of vitamins of the A, B and C groups. In prolonged stress, a large excess of vitamins may be needed to replace the adrenal stores and to cope with the increased turnover in that organ.

### **Single, multiple and conditioned deficiencies**

It is not usual to find single vitamin deficiencies occurring naturally in man or animals. More commonly there is a state of multiple deficiency, and often in man this is associated with deficiency of the major food components. In kwashiorkor, which is usually attributed to general protein deficiency, signs of pellagra, beriberi, A deficiency and K deficiency can often be detected. Experimental single deficiencies have

been studied exhaustively in animals, and occasionally in man. Such studies can be of practical application clinically, as it often happens that in naturally occurring multiple deficiencies the symptoms and lesions expected of a single deficiency may predominate.

Deficiencies may be absolute or conditional. Conditioned deficiencies arise as a result of the operation of some predisposing factor. Dietary vitamin content may be quite adequate, but the availability of vitamins to the animal may be reduced or blocked entirely in various circumstances. These include:

1. Anorexia, arising from a multitude of causes. To mention one only, painful sores around the mouth caused by deficiency of B vitamins, lead to restricted food intake and thus cause the original condition to become worse.

2. Gastric disturbances. Lack of gastric intrinsic factor, whether as a congenital defect or after gastrectomy, blocks the absorption of vitamin B<sub>12</sub>. The presence of helminth parasites in the stomach wall of animals interferes with gastric digestion. The loss of food by vomiting is another cause of vitamin deficiency.

3. Lack of bile in hepatic disease and diminution of pancreatic enzymes in pancreatic disease reduce the intake of fat soluble vitamins.

4. Intestinal mucosal changes, causing diarrhoea, increase the excretion and reduce the intake of essential food factors.

5. Interference with the normal intestinal bacterial flora by the use of antibiotics in the food, or the use of sulpha-drugs, causes decreased production of vitamins of the B complex. Dietary composition is important in this respect. Protein-rich diets encourage the growth of proteolytic flora; carbohydrate-rich diets encourage the growth of the saccharolytic bacteria such as *Escherichia coli* and *Klebsiella aerogenes* which are said to synthesize biotin, riboflavin, pantothenic acid and pyridoxine, all members of the vitamin B complex. Malabsorption syndromes are said to follow the destruction in the intestine of fusiform and spore bearing anaerobic organisms, whereas healthy intestinal colonies of lactobacilli, sarcina and *E. coli* are said to compensate for dietary vitamin K deficiency.

In animals the synthesis of vitamins by gastric microflora (in poly-gastric species) and by intestinal microflora (in monogastric species) is well established as an important source of essential food factors. Whether vitamins synthesised in this way by microbial flora in man are available to the host is not yet clearly established, although there is some evidence that thiamine is available from this source. This would appear to be a most important field for research in man and animals.

6. Intestinal parasites compete with the host for essential food factors. Helminth parasites are reputed to abstract quantities of pantothenic acid from the intestinal contents and it is well established that the tapeworm *Diphyllobothrium latum* causes a B<sub>12</sub> deficiency.

7. Interference with transport across intestinal mucosal cells arises in many of the diseases affecting the intestinal mucosa. Where absorption is dependent on a hormonal stimulus, variations in the signal may cause variations in absorption.

8. Interference with utilization. The complicated interactions between nutritional deficiency and endocrine metabolism determines the extent of use of absorbed foodstuffs. The biological activity of vitamins is closely dependent on enzymic action. Decrease in the availability of enzymes, whether due to lack of hormonal stimulus, lack of protein for their manufacture, or the presence of defective genes for their synthesis may result in accumulation of unused vitamins or their precursors in the tissues.

The biosynthesis of an active vitamin from inactive precursors may require the presence of one or more other vitamins. In such a case the absence of the adjuvant vitamin leads to a conditioned deficiency of the vitamin in question. Such a situation arises in the synthesis of nicotinic acid from tryptophan, which requires the presence of pyridoxine.

Investigations into vitamin deficiencies have been concentrated mainly on animals, and the results have been extrapolated with more or less success to man. Broadly speaking, the principles of vitamin metabolism may be applied in this way, but when one comes down to fine detail, sources of error may arise. Each individual species has detailed differences in its inherited enzymic pattern, which may lead to errors in interpretation, if experimental results in one species are held to apply in another species.

The simplest and best determination of vitamin deficiency is the demonstration of consistent cure of a particular disease syndrome with a single vitamin. The pattern for investigation was laid down by James Lind in the first half of the eighteenth century, or to be exact, on the 20th May, 1747. Lind chose twelve sailors all suffering from scurvy, and all at a similar stage of the disease, with putrid gums and subcutaneous haemorrhages. He divided these twelve into six pairs. Two were given a quart of cyder daily, two had 25 guts (drops) elixir vitriol three times daily, two had vinegar three times daily, two had seawater to drink and two were given an electuary which included garlic, mustard seed, and Balsam of Peru in its composition. The remaining two men were given two oranges and a lemon each day 'These they eat with greediness . . . they continued but six days under this course having consumed the quantity that could be spared'. One of these men was fit for duty at the end of the six days. The other was 'deemed pretty well, was appointed nurse to the rest of the sick'. (Lind 1753) Forty years and twenty thousand scorbutic deaths later, their Lordships of the Admiralty made compulsory the issue of fruit juice to the Royal Navy, and scurvy became a thing of the past as far as naval personnel were concerned.

During the early part of this century, vitamin investigations were simple studies based on increase in weight of experimental animals or of their organs in the presence of vitamins, and lack of growth in their absence. Morphological studies of changes in organs and tissues followed and more recently studies at the sub-cellular level have brought us an understanding of those problems which our predecessors, working so brilliantly without previous knowledge to aid them, initiated. With modern techniques, including the use of radioactive tracers and the electron microscope we are able to determine the intracellular location of vitamins and their presumed site of action. We are able to work out the structure of individual vitamins, their conjugation into complexes with enzymes and their metabolic turnover under hormonal stimulus.

### **Antivitamins**

Substances with antivitamin activity occur naturally and can be synthesised chemically for experimental use. They may be specific enzymes, which destroy the related vitamin in food, or they may be compounds closely related chemically to individual vitamins. Enzymes with antivitamin activity include the thiaminase which is found in raw fish. Normally this enzyme is destroyed in cooking, but in some countries, notably the Scandinavian group, fish is often eaten raw. In cases where raw fish forms a large part of the diet, thiamine deficiency may occur. The major pathological effect is on the central nervous system but the adrenal gland reacts initially to deficiency by over-production of cortical steroids and subsequently by defective production of these hormones. Chastek paralysis of captive foxes and mink is a conditioned thiamine deficiency, caused by the ingestion of thiaminase in excessive amounts in the diet, and is curable in the early stage, before irreparable brain damage occurs, by supplementation of the diet with adequate quantities of the vitamin. Athiaminosis can also occur as a simple deficiency, as in the polyneuritis found in pigeons fed on polished rice. Bracken poisoning in horses is the result of excessive ingestion of thiaminase, and affected animals die of congestive heart failure. Bracken poisoning in cattle, on the other hand, has no element of athiaminosis because the ruminant alimentary flora are able to synthesize thiamine. In such animals the disease is characterised by multiple haemorrhages associated with thrombocytopaenia. The foregoing examples illustrate the fact that deficiencies may be simple or conditioned according to circumstances, and also that species reactions to deficiencies may be very varied indeed.

Many enzymes are highly specific in their action and for example will synthesize or degrade only one of a pair of specific stereoisomers. Presumably this is related to their spatial configuration—the situation is rather like wearing a right hand glove on the left hand. The glove will fit the fingers, but the thumb is not quite right. It seems that chemical

substances closely related to vitamins, whether stereoisomers or in some other way similar to vitamins, block the active site on the enzyme molecule, rendering it unfit to act as a catalyst in a specific reaction. Such substances are known for vitamin C and for members of the B group including folic acid, riboflavin, thiamine, pyridoxine and pantothenic acid and are called vitamin analogues.

These vitamin antagonists whether enzymes or stereoisomers are valuable research tools, used for rendering single vitamins unavailable to the experimental animal without the use of special dietary regimens. They may also be used clinically. The folic acid antagonists have been used in this way with temporary success in arresting the progress of acute leukaemia. Folic acid analogues are almost identical structurally with folic acid or its derivatives and apparently combine irreversibly with the single enzyme which normally converts folic acid in two distinct steps to the active folinic acid form. Rapidly multiplying cells such as leukaemia cells require folinic acid in large amounts for mitosis, and it is the substitution of the enzyme-antagonist complex for the active enzyme-vitamin complex which disrupts the process of mitosis at the point of action of the physiological analogue. Unfortunately, other body cells have this same folinic requirement in mitosis and side effects, particularly in children in whom cellular turnover is rapid, may become intolerable before the course of the disease is halted. The endocrine glands are not affected directly by toxic levels of folic acid antagonists, but there is a requirement for folinic acid as a cofactor in the rate limiting enzymatic step of synthesis of noradrenalin and adrenalin which has to be satisfied. If the antagonist level is kept within reasonable bounds the entire amount may be fixed by the mitosing leukaemic cells, leaving the remaining folic acid in the body free to assist in its various coenzymatic rôles (Jacobson, 1965).

### Synthetic vitamins

Vitamins for animal use are derived basically from plant life. Man is dependent on both plants and animals for his requirements, although in many cases synthetic vitamins are now available, which may be identical with the naturally occurring substance or only closely related. The close relations, although useful in many ways, pose some problems in that they may have only a fraction, whether large or small, of the biological activity of the natural product. They may substitute for several, but not all, of the functions of their natural counterparts, so that it is essential to use extreme care in their use. Even where the synthetic product is, as far as we know, identical with the natural product, there may be dangers in its use. It may be so inexpensive, and so easy to administer, that patients may be given an overdose, which although small in itself, is many hundreds of times the physiological dose. At this level it may well reach the toxic or even the lethal dose.

There is seldom any danger of ingesting toxic amounts of naturally occurring vitamins, because these are generally extremely well 'diluted' by the plant or animal substances in which they are found. The notable exception of course is the well publicised high content of vitamin A in the liver of the polar bear. This, no doubt, is extremely useful to and probably essential for the polar bear, but the liver is toxic to man even if eaten in reasonable quantities.

Daily requirements of vitamins for man and animals are known in many cases, and may be expressed by various authorities as the optimal amount necessary, or the minimum amount essential for health. As we have seen, the minimum requirements may be quite insufficient in many circumstances, both physiological and pathological. In some cases, stored vitamins, particularly the fat soluble ones, may be sufficient to allow the subject to 'tick over' comfortably throughout life. When stores are marginal, the amount available for the second generation may be too small, and infants may be born deformed, or fail to grow at a normal rate.

In general it may be said that the vitamins function as part of a complex series of reactions which require the presence together, at the correct time and the correct place, of the correct vitamins, enzymes and hormones. In the next chapter we shall examine some of the principles which govern the action of the enzymes, which may be regarded as the tools which do the actual work within the cells.

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## *Chapter 2*

### **Enzymes**

The animal organism may be likened to a fully automated factory, with its own generator powered by fuel from outside sources. Individual cells draw their supply of raw materials from the circulating blood, whose rate of flow is controlled by baroreceptors and whose oxygen tension is controlled by chemoreceptors. The control circuits are the hormones, directing the quantity and quality of cellular output, The enzymes are the machine tools, which have the ability to split complicated molecules apart at predetermined sites, and to assemble required metabolites by joining simpler atoms or molecules together. Output is shut off at the required level by means of a feedback system similar to that in an ordinary thermostat.

Enzymes therefore have a key position in cellular and somatic organisation. They resemble chemical catalysts in their ability to assist the mechanisms used in bodily metabolism without themselves being used up in the process. Examples of such biological catalysis are the hydrolysis of peptide linkages, the transfer of carboxyl groups, the splitting of carbon-to-carbon bonds, and the transformation of substances into their optical, positional or geometric isomeric forms. In all these and many other processes, enzymes may function alone, or with the assistance of cofactors. In the latter case the active enzyme, or holoenzyme, is a complex of two parts, the apoenzyme or protein part, and a smaller cofactoral element. The apoenzyme is powerless to act on its own. The cofactor may be a divalent or trivalent cation, when it is called an activator; or it may be a complicated organic molecule, usually a vitamin derivative, when it is customary to refer to it as a coenzyme. Either or both may be required for full enzymic action in specific instances.

#### **Isoenzymes**

Individual enzymes may be found in certain tissues in several different molecular forms, and these multiple forms are known as iso-enzymes. Several tissues in a single animal body may have different isoenzymic patterns; therefore pathological changes in a single tissue may be detected, by electrophoretic analysis, by determining the pattern of isoenzymes leaking out into the plasma from the damaged organ. Such patterns give a more exact guide to localisation of disease than does the determination of a single enzyme circulating in the plasma. One example

of an enzyme existing in multiple forms is lactic dehydrogenase, which occurs in at least five isoenzymatic forms, possibly originating from different intracellular organelles. The proportions of the five forms released from degenerating muscle fibrils in cases of myocardial infarction differs from the proportions released from degenerating liver cells. Furthermore, the amount of isoenzymes released in myocardial infarction is a guide to the extent of the damaged tissue, and repeated episodes of infarction are reflected in a higher level of released enzymes.

### **Pre-enzymes**

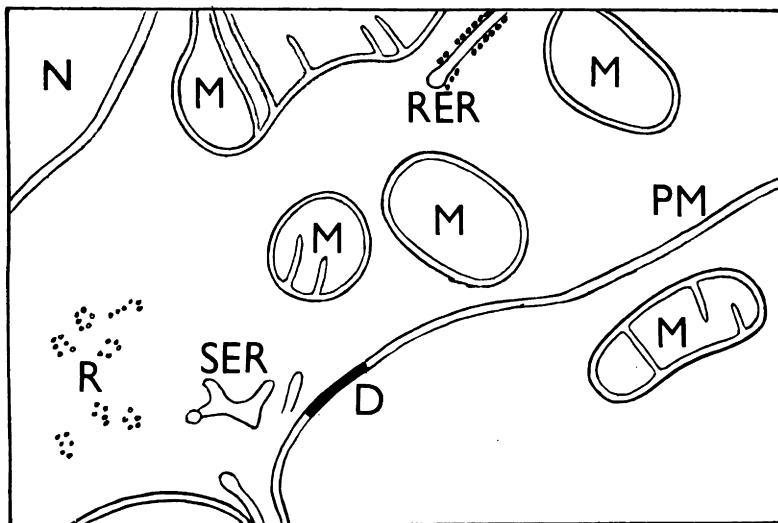
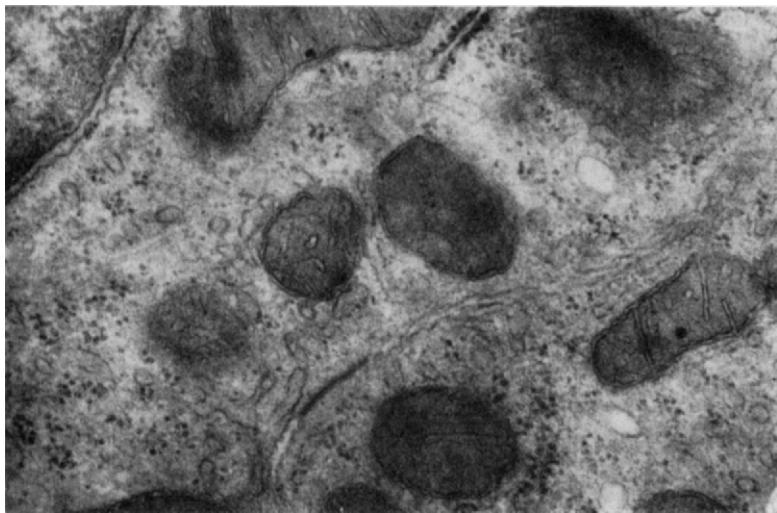
Certain proteins known as pre-enzymes, are secreted in an inactive form, and they must be converted to the active form, by catalytic action, before they are able to undertake their true enzymatic function. Trypsinogen, for example is converted to trypsin by an enzyme which splits six amino acids from each trypsinogen molecule, revealing in the process the active site of the enzyme protein.

### **Intracellular localization of enzymes**

Work on the intracellular distribution of enzymes is still in progress and contributions to our knowledge are coming in from electron microscopists, histoenzymologists, and organic chemists employing ultracentrifugation techniques for the separation of the various cellular organelles. The soluble enzymes of anaerobic glycolysis are found in the extramitochondrial part of the cell, while the enzymes concerned with energy production are associated with the mitochondria (Photomicrograph). On the outer layer of the mitochondrial membrane are found the enzymes responsible for the aerobic oxidation of metabolites, whereas the enzymes concerned with electron transport—the flavoproteins and the cytochromes—are arranged on the inner membrane of the double-layered mitochondrial wall. The fluid between the two layers is thought to provide the coenzymatic bridge required to link the two systems. The mitochondrial matrix contains enzymes catalysing the dephosphorylation of the high energy compound adenosine triphosphate.

Structurally, the mitochondria of some hormone-producing cells, such as those of the adrenal cortex, seem to have some peculiarities of their own, which may be related in some way to steroid synthesis. The enzyme which splits the side chain from cholesterol, before its conversion to pregnenolone, is found within the mitochondria, as is the 11- $\beta$ -hydroxylase which is essential for the conversion of progesterone to cortisol and aldosterone.

The lysosomes, which appear to be derived from the Golgi apparatus, contain the hydrolytic digestive enzymes which cope with particles arriving at or arising in the cell in an indigestible form. They are particularly important in phagocytic cells, in dealing with infective



Segments of two adjacent cells, showing nucleus (N), and several mitochondria (M) with double layered membranes and cristae. The rough-surfaced endoplasmic reticulum (RER) and the smooth-surfaced reticulum (SER) are seen in longitudinal and cross section. There are numerous clusters of ribosomes (R). The plasma membranes (PM) of the two cells are linked by a desmosome (D).  $\times 47,000$ .

(Photograph by Elizabeth M. McDowell)

particles and cell debris. Normally, of course, proteins, carbohydrates and fats are supplied to the cell in an easily assimilable form. The disposal of accumulations of fat in vitamin deficiency, or of excess of protein or glycogen, is the responsibility of the lysosomes, but excessive deposits of such material within the cell may be beyond their capacity to disperse. In such cases, the surplus material lies free in the cell sap.

The rough surfaced endoplasmic reticulum is the site of protein synthesis, and enzymes and protein hormones are assembled here, although there remains a possibility that mitochondria, which have their own complement of DNA and RNA are able to synthesize some of their own enzymes.

The smooth surfaced endoplasmic reticulum is thought to be the site of synthesis of triglycerides from fatty acids and glycerol. These combine with the proteins from the rough surfaced reticulum to form the lipoproteins for the cytoskeleton. It is here too that cholesterol is formed from acetate before being metabolised to form the steroid hormones. The smooth surfaced endoplasmic reticulum is a prominent feature in the cells of such steroid producing tissues as the interstitial cells of the testis.

Two of the steroid hydroxylases, the 17- $\alpha$  and the 21 hydroxylase, both of which require vitamins as cofactors for their activity, are associated with the microsomal fragments or the soluble fraction of cell homogenates. This does not rule out the possibility however, that in undamaged cells they may be adsorbed on the surface of the mitochondria.

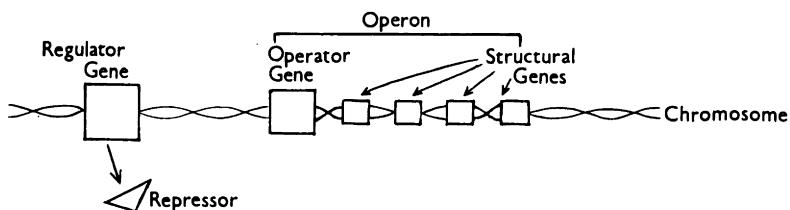
### **Enzyme synthesis**

As far as we know at present, all enzymes are protein in nature, and their synthesis involves the linking together of amino acids in correct sequence. Each animal carries within its body cells, in the DNA molecules, coded information for the building up of its own specific proteins. The genome, or total stock of instructions for synthesis of metabolites, determines the patterns of proteins to be produced and in the final analysis determines the form of each species and the inherited constitution of the individual within the species.

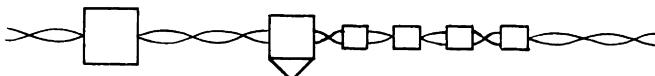
Enzymes are apparently synthesized not singly but as part of a sequence of the enzymes required for the successive steps in a metabolic pathway. A series of structural genes determines the molecular composition of the enzymes. From these genes, molecules of messenger RNA (ribonucleic acid) carry the transcribed list of instructions into the cell cytoplasm, where the ribosomes of the rough surfaced reticulum, with the assistance of transfer RNA assemble the individual amino acids into the required enzyme molecule.

Obviously, coordination and control of enzyme synthesis are essential for correct cellular function and at a given moment, most of the

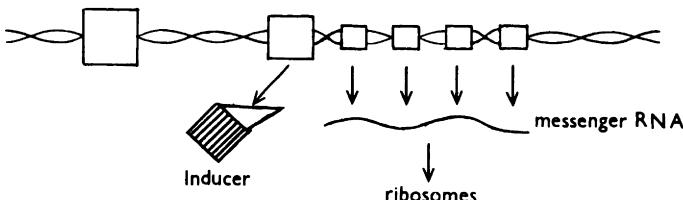
potentialities inherent in the genome must be inactive or repressed. Our present ideas on control are derived from the work of Jacob and Monod (1961) on the synthesis of bacterial enzymes. These scientists were the first to suggest that the genetic regulatory mechanism which they postulated as applicable to bacteria, might apply generally to cells in higher animals. It is interesting, therefore, to consider how their hypothesis might throw light on the mechanisms of mammalian enzyme synthesis and control. Briefly, they suggested that the rate of enzyme synthesis is under the control of regulator and operator genes, with a repressor molecule in the cell cytoplasm acting as a link between the two. There are two basic systems of control, the inducible system and the repressible system. In the inducible system, the repressor molecule is synthesised, under the coded instructions of a regulatory gene, and in its active form, it prevents the formation of specific proteins. To allow the formation of those proteins when they are required, the repressor is inactivated by combination with an inducer. As a result, an operator gene is allowed to switch on, setting in motion the transcription of a series of genes which code for single enzymes, or groups of related enzymes involved in a single metabolic pathway. The active



The regulator gene directs the synthesis of the repressor molecule.



The repressor binds to the operator, thereby inhibiting transcription of associated sequence of enzymes. The entire operon is in a state of repression.



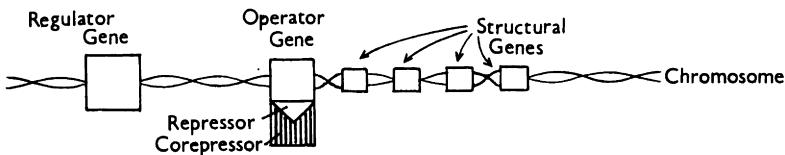
The inducing substrate, demanding a chain of enzymes for its metabolism, combines with and removes the repressor molecule, allowing transcription of a chain of genes to messenger RNA. The operon is now fully active.

*Fig. 1. Enzyme induction.*

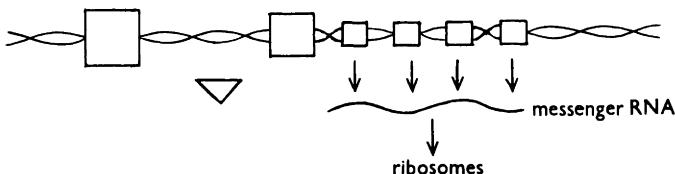
state of the repressor molecule may be visualised as negative control of enzyme synthesis or inhibition of enzyme induction.

This is the usual process for catabolic enzyme sequences. As a theoretical example, one might consider tyrosine as the inducing substrate for a series of enzymes required to convert it to noradrenalin. Increasing concentration of tyrosine induces increased concentration of enzymes for its conversion. When all available tyrosine is converted, repressor molecules are free to attach to the operator gene once more, and inhibit enzyme synthesis.

In the repressible enzyme system on the other hand, the repressor



Repressor molecule is combined with a corepressor to inhibit synthesis of protein.



The absence of the corepressor inactivates the repressor, allowing synthesis of protein.

*Fig. 2. Enzyme repression.*

molecule is considered to be active only when combined with a co-repressor, and it is the absence of the corepressor which initiates new enzyme synthesis, in a process known as derepression.

This is the usual system for anabolic enzymes used in synthesis of metabolites. Corepressors are generally the final products of the enzyme sequence. To illustrate this system, the synthesis of tyrosine, one of the many amino-acids in insulin, may be considered. If there is sufficient tyrosine in the synthesizing cell, then the enzymes for its synthesis are unnecessary, and the genes coding for its synthesis are repressed by the active repressor/tyrosine complex. If the tyrosine is absent, the repressor, unaided by its corepressor, becomes inactive, and allows transcription of the genes coding for the enzymes needed for tyrosine synthesis.

Enzyme induction and derepression both involve new synthesis of protein, and both are inhibited by drugs which block protein synthesis, such as actinomycin D. In contrast, activation of a preformed enzyme is not affected by such drugs.

Both induction and repression of enzymes are usually highly specific. Inducers are generally the specific substrates for enzymic action or their analogues. Repressors are generally the products of the enzymic action, or their analogues. Repression of an enzyme denotes inhibition of its synthesis, not of its activity. Feedback inhibition, on the other hand, denotes inhibition of the activity of the first enzyme in a series, by the end-product of the biosynthetic chain which it initiates.

Alteration of the genetic code for synthesis of an enzyme, by genetic mutation, may modify the structure of that enzyme in such a way as to cause partial or complete loss of activity. If the resulting defect is not lethal, it becomes transmissible to succeeding generations, giving rise to an inherited metabolic defect. It is possible that this is the way in which primates originally lost their ability to synthesize vitamin C and were therefore placed at some disadvantage when separated from a source of that vitamin for long periods. Guineapigs are even more susceptible to deprivation of vitamin C than are primates, and develop scurvy in about three weeks if they are not provided with ascorbic acid, simply because they are unable to synthesize the last enzyme in the metabolic pathway to ascorbate synthesis. However, if a large enough population of guineapigs is examined, it will be found that most members are completely dependent on outside sources of vitamin C supply, others are much less dependent, and there may be one or two completely independent. It is a matter for debate as to whether the aberrant guineapigs are survivors of original stock possessing the gene for correct enzyme synthesis, or whether they have developed a mutant gene which will endow them, and their progeny, with the ability to complete the metabolic pathway leading to ascorbate synthesis. It is very likely that such variations in ability to synthesize enzymes occurs too in human populations. If we cast our minds back to the long journeys of exploration in the past, we may recall that the great sailing ships were brought back home by the survivors of epidemics of scurvy in which their companions died or became totally incapacitated. Is it possible that some of these men were able to synthesize the missing enzyme?

### **Enzyme structure**

The active form of an enzyme is built up of a chain of amino acids joined in defined linear order by peptide linkages. This arrangement is known as the primary structure. Portions of the chain are drawn together by bonds, such as hydrogen bonds or disulphide bridges, giving rise to a three dimensional secondary structure. Further coiling of secondary structures or the intertwining of several secondary structures gives rise to a more compact arrangement known as the tertiary structure. The bonds holding the tertiary structure in position are weaker than those bridging gaps in the secondary structure, and may be broken by treatment with acid or alkali. Such treatment causes

denaturation of the protein and inactivation of the enzyme. The aggregation of several sub-units, in the tertiary form, gives rise to a quaternary structure. Interference with the molecular arrangement at any stage of the construction may result in loss of activity. This happens occasionally when a mutation in the genetic code causes alteration in the primary structure and thus alters the entire configuration of the molecule.

The enzyme in its tertiary arrangement is a globular protein, which according to present thought, bears on its surface one or more active sites, which may be composed of only a short chain of aminoacids. These active or catalytic sites attract the substrate molecules in chemical linkage, under correct conditions of concentration, temperature and pH. In a catalytic reaction the substrate molecule forms a complex with the enzyme, before being broken down into the products of the reaction. When the enzyme releases the products, it is ready for further action. In the transfer of chemical groups from one substrate to another the two reactants are brought together in close proximity on the enzyme surface, so that the energy required for the reaction to take place is minimised. The function of the enzyme is not to initiate a reaction, but to facilitate and accelerate that reaction. Some enzymes are able to undertake this function without help. Others require the assistance of cofactors, before the correct linkages between enzyme and substrate become possible.

### **Enzyme cofactors**

Most enzymes have nonprotein components on which catalytic activity depends. Those cofactors which are not normally isolated with the apoenzyme are known as *coenzymes*. It has become customary to describe cofactors which are tightly bound to the apoenzyme, and which cannot be removed without denaturation of the protein molecule as *prosthetic groups*. A third type of cofactor is a simple inorganic ion, known as an *activator*. Cofactors are non-specific for substrates; the specificity for substrate is a function of the protein apoenzyme.

**Coenzymes.** Coenzymes are complicated organic molecules, which in general are less specific than the enzymes which they assist. They usually act as essential carriers of the products of the reaction. Coenzymes themselves may be changed in the reaction, but are commonly regenerated for further use. An example is the enzyme lactic dehydrogenase, for which the coenzyme is nicotinamide adenine dinucleotide (NAD). In the dehydrogenation of lactate to pyruvate, NAD accepts hydrogen and itself becomes reduced. NAD is a common hydrogen acceptor for catalytic reactions, but some enzyme systems require the phosphate derivative nicotinamide adenine dinucleotide phosphate (NADP), which likewise becomes reduced in dehydrogenase reactions.

Such a reaction takes place in the conversion of pregnenolone to progesterone for the synthesis of adrenal steroids, in which the enzyme  $\beta$ -hydroxysteroid dehydrogenase requires the assistance of the coenzyme NADP. Reoxidation of the reduced coenzyme is achieved by the transfer of the accepted hydrogen along a chain of hydrogen acceptors, ultimately to form water by combination with molecular oxygen.

NAD and NADP are both derivatives of nicotinic acid, one of the vitamins of the B complex. Other vitamins found in coenzymes are pantothenic acid, ascorbic acid, tetrahydrofolate, pyridoxal phosphate and thiamine pyrophosphate. Vitamins with possible coenzyme functions are  $B_{12}$  which is associated with tetrahydrofolate in some of its functions, and vitamin A, which is said to function, in the form of its aldehyde retinene, as a cofactor in the visual cycle.

**Prosthetic groups.** Enzymes with a 'built-in' cofactor include the flavoproteins and some biotin and pyridoxine containing enzymes. The flavoproteins are metal-containing enzymes which transfer hydrogen from coenzymes such as reduced NAD to their own prosthetic group. In this case the prosthetic group is flavin adenine dinucleotide (FAD) a derivative of the vitamin riboflavin. Having accepted hydrogen, the reduced flavin is reoxidised in its turn by coenzyme Q in the next stage of the hydrogen transport chain. Biotin is thought to function as a cofactor in the synthesis of long chain fatty acids, in the fixation of carbon dioxide which is an essential preliminary to the build up of long chains of two-carbon moieties. Theoretically, therefore, biotin can be expected to function in the synthesis of the hormones known as prostaglandins, which are derived from essential fatty acids. Pyridoxal phosphate, containing the vitamin pyridoxine, is the prosthetic group of dopa decarboxylase, the enzyme responsible for catalysing an essential step in the metabolic pathway to adrenal medullary hormone synthesis.

**Activators.** Ionic cofactors form important parts of many enzyme systems, being vital to the attainment of the catalytically active state. They may be simple electrolytes such as sodium or potassium, divalent cations such as magnesium, manganese, calcium, zinc or copper, or trivalent cations such as aluminium. Ascorbate oxidase for example requires copper for the conversion of ascorbic acid to dehydroascorbic acid, a process which is required for the formation of noradrenalin in the adrenal medulla and elsewhere. Some metallic ions such as silver, mercury and lead are toxic to nearly all enzymes. Others are inhibitors for some enzyme systems and activators for others. Into this category comes magnesium, which inhibits the adenosine triphosphatase activity occurring in myosin, but activates nucleosidephosphatase, the enzyme which converts nucleoside diphosphate to nucleotides. Some

adenosine triphosphatases (ATPases) are activated by both calcium and magnesium, others by magnesium alone. In the case of the ATPases, substrate specificity appears to depend on the activating divalent cation, as well as on the presence of the monovalent cations sodium and potassium.

### **Enzyme specificity**

Most, if not all, metabolic reactions require the assistance of enzymes, and these by their nature are generally highly specific in their action. In many cases a specific enzyme will catalyse only one step in a complicated metabolic pathway, involving only one substrate. Other enzymes are less demanding and will catalyse reactions involving a group of closely related substances. Others again are able to undertake the transfer of a particular chemical grouping from a variety of substrates. The enzymes with the broadest spectra of substrates are the digestive ferments such as trypsin and chymotrypsin, which split peptide linkages with a specific configuration on each side of the susceptible bond. Thus the Enzyme Commission (*vide infra*) specifies the reactions mediated by trypsin as hydrolysis of peptides, amides, esters etc., at bonds involving the carboxyl groups of L-arginine or L-lysine. The chymotrypsins are said to hydrolyse the same substrates, especially at bonds involving the carboxyl groups of aromatic L-amino-acids. Pepsin is even less demanding in its specificity, being able to hydrolyse peptides generally, including those with bonds adjacent to aromatic or dicarboxylic L-amino acid residues. However, these broad-spectrum hydrolytic enzymes are in the minority, and each cell must undertake the synthesis of very many enzymes for normal existence. Fortunately the potential ability of the genome to code for different enzymes is almost without limit, although in practice perhaps only a few thousand enzymes are concerned in the everyday business of living.

There are examples of enzymes which interconvert the two optical isomers (mirror images) of their substrates, and of enzymes which can act on several of the geometric (or stereo-) isomers of their substrates, but the majority of mammalian enzymes are specific for one isomer only. In the case of the amino acids, there are very few exceptions to the rule that enzymes act on the L-isomers only in mammalian metabolism. This preference for L-isomers is a hereditary quirk which has not yet been explained, although it is known that some forms of animal life have an equal avidity for the D-forms of amino acids.

### **Rate of enzyme reactions**

Following a short time lag, during which initial inertia is overcome, a specific enzyme accelerates the speed of the catalysed reaction. Under optimal conditions, a constant rate is attained and may be maintained

for many hours, until lack of substrate or accumulation of end-products slows down the reaction. Excess substrate may inhibit an enzymic reaction—a situation similar to the pro-zone phenomenon familiar to immunologists. It is thought that in such cases, the excess substrate molecules may bind or block active sites on the enzyme molecules. Until all the active sites are freely available the initial rise in the rate of the reaction may be quite slow. Acceleration takes place when sufficient substrate molecules are catalysed to reduce substrate concentration to the optimum for the enzyme concerned.

Low substrate concentration and low enzyme concentration both reduce the speed of the reaction, because there is less opportunity for the formation of the enzyme-substrate complexes which are essential for the working of the catalytic process. Under optimal conditions reaction rate is proportional to the concentration of the enzyme, so that high concentrations of both enzyme and substrate result in a rapid turnover of the products of the reaction.

Enzymic reactions, like chemically catalysed reactions, are reversible. Normally, within the living cell, the reaction is a one way process, because the products are generally removed by the next enzyme in the metabolic pathway concerned. In pathological states, however, such as vitamin deficiencies, in which essential coenzymes are missing, there may be a buildup of reaction products, or even a reversal of the reaction.

The rate of enzyme reaction is dependent on the temperature of the medium in which the reaction takes place. Most enzymes are denatured by high temperatures, although there are some thermophilic bacteria which elaborate enzymes capable of withstanding temperatures quite close to 100°C. Each enzyme has an optimal temperature range, and for the mammalian enzymes this approximates closely to the temperature of the blood. Avian enzymes, in accordance with the higher body heat of birds, react best at a slightly higher temperature than do mammalian enzymes. The enzymes of cold-blooded animals act best at a lower temperature. Temperatures below the optimum slow down the rate of reaction—a factor of some importance to hibernating animals. Testicular enzymes in the higher animals probably require a relatively low temperature for optimal function; it is known that the higher body heat to which the undescended testis is exposed causes sterility in animals, as does exposure of the normally descended testis to high temperatures, in animals moved to unaccustomed tropical climates.

Most enzymes too have their favoured pH values for optimal activity, and even moderate change towards alkalinity or acidity inhibits the rate of reaction by dissociation of either enzyme or substrate or both. Thus, most enzymes in mammalian metabolism have their peak activity between pH values of 5·0 and 9·0, with the range of activity of a single enzyme seldom extending over more than one or two pH

units. Alkaline phosphatase, for example, reacts in the pH range 9.2–9.6, and acid phosphatase in the range 5.3–5.6. The gastric juice enzyme, pepsin, on the other hand can and does act at a pH of 1.8–3.8, a degree of acidity which could inactivate other enzymes by a process of denaturation analogous to that suffered by most proteins exposed to excessive acidity. Denaturation follows the breakdown of the tertiary configuration of the protein concerned, by rupture of the weak ionic bonds responsible for maintaining the linkage between amino acids in the secondary structure. Pepsin, like trypsin, provides us with an example of autocatalysis, a process in which it converts its precursor pepsinogen into active pepsin, by splitting off the fraction of the pepsinogen molecule which normally keeps the precursor in an enzymatically inactive state.

### **Enzyme inhibition**

Enzymes, being protein in nature, are subject to denaturation or inactivation from many causes. Inhibitors may be specific, acting against a specific enzyme, or non-specific, having the ability to inhibit small or large groups of catalysts. In either case their effect may be disastrous, as loss of activity of a single enzyme may result in complete holdup in a complicated chain of enzymatic reactions.

Ionizing radiations may inactivate enzymes already present in cells by forming organic peroxides within the cells. These peroxides destroy enzymes by a process of oxidation. High energy sources of radiation may also interfere with the intracellular synthesis of enzymes by damaging the genes which code for specific enzymes. In this way, ultraviolet rays, X-rays,  $\beta$ -rays or  $\gamma$ -rays may have long term effects on the animal body.

Denaturation may also occur as a result of high temperatures as in burning accidents, or acidity as in the leakage of gastric juice from perforating ulcers.

There are many examples of inhibition of enzymes by poisons. Perhaps the most dramatic is the effect of cyanide, which inhibits cytochrome oxidase, with complete cessation of the chain of aerobic oxidation. If the concentration is sufficient—and very little is needed—death results in a few minutes. Both cyanide and carbon monoxide are known as respiratory poisons because of their ability to inhibit cell respiration. It is thought that cyanide combines, in a stable complex, with the heavy metals, copper and iron which are essential for the functioning of the cytochrome oxidase system.

Nerve gases and the organophosphorus compounds owe their pathogenicity to their ability to inactivate esterases, in particular cholinesterase, the enzyme responsible for hydrolysis of acetylcholine in the parasympathetic and voluntary nerve endings. Substances with this

ability are known as anti-cholinesterases. They are rather non-specific and may inactivate other ester hydrolases, such as carboxyl-esterase, acetylesterase and glycerol ester hydrolase. The organophosphorus compounds have a fairly wide range of antienzymatic activity, being able to neutralise, in addition to the esterases, several peptide hydrolases, including such important ones as thrombin, trypsin and chymotrypsin.

Salts of the heavy metals, such as silver, lead, copper or mercury, precipitate proteins generally, and if the proteins happen also to be enzymes, they are simultaneously inactivated. This form of inhibition can be prevented by administration of a chelating agent, such as ethylene diamine tetra-acetate (EDTA) which combines with and thus detoxifies the heavy metal.

**Antienzymes.** The protein nature of enzymes confers on them the property of antigenicity. Antienzymes may be formed in the animal body by parenteral injection of the relevant enzyme. Leakage of digestive or other enzymes into the body cavities or the circulation, if not lethal, may stimulate the production of antibodies which, on reacting with their antigens, precipitate in the tissues, giving rise to some of the manifestations of autoimmune disease. The antibody to an enzyme may combine with its specific antigen in such a way as to mask the active site or to leave it uncovered. In the first case, the enzyme is inactivated, in the second it remains active. It is said that lecithinase is inhibited by its antibody. In the case of alkaline phosphatase, it has been shown that the antigenic and the catalytic sites in the enzyme studied were not identical, since inactivation of either site left the remaining site functionally active (Schlamovitz, 1957).

There are cases of inherited lack of a specific enzyme in man which can now be treated by injection of the missing substance, but antibodies eventually form against the enzyme, which is usually prepared from animal sources. Screening of the missing enzyme by a semipermeable membrane, from the antibody forming apparatus is the subject of research at the present time.

**Enzyme nomenclature.** The naming of enzymes was until recently in a chaotic state. Some order has been created by the setting up of an international commission to study the nomenclature and standardization of enzymes and enzymatic units. The commission has classified enzymes into six groups.

1. *Oxidoreductases*, which catalyse oxidoreductions between two substrates, and include enzymes known formerly as oxidases, reductases, dehydrogenases and peroxidases.

2. *Transferases* catalyse the transfer of a chemical group, such as an aldehyde, ketone or hydroxyl group between two substrates.

3. *Hydrolases* catalyse the hydrolysis of various bonds, including peptide linkages and ester bonds.

4. *Lyases* remove groups from their substrates (but not by hydrolysis) leaving double bonds, or alternatively add groups to double bonds.

5. *Isomerasases* are the enzymes which catalyse the interconversion of geometrical, optical or positional isomers.

6. *Ligases* catalyse the binding together of two molecules coupled with the breakdown of a pyrophosphate bond in adenosine triphosphate or a similar energy-rich triphosphate.

Each group has numerous divisions and subdivisions so that each individual enzyme may have its own number. As an example, one enzyme concerned in steroid hormone synthesis is steroid 11- $\beta$ -hydroxylase. This is an oxidoreductase (class 1), and acts on paired donors with incorporation of oxygen into one donor (sub-class 14). One of the donors is the coenzyme known as reduced nicotinamide adenine dinucleotide phosphate, a vitamin containing molecule. This brings the enzyme into sub-sub-class 1. It is the sixth enzyme to be named in this sub-sub-class and therefore its full number is 1.14.1.6. Enzyme numbers are commonly preceded by the initials E.C. for Enzyme Commission (Florkin and Stotz, 1965).

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## *Chapter 3*

### **Hormones**

The higher animal organism is a wonderfully complex arrangement of numerous interdependent organs, each composed of many different tissues, none of which is able to function in isolation. Obviously, smooth working of such a complicated mechanism is not easily achieved. Control must be exercised over each individual organ and tissue so that the animal may adjust to the everchanging nature of its external environment and to the constant and inexorable demands of its tissues for nourishment. The motivation for these demands lies in the inborn impulse for survival and less urgently in the biological desire to perpetuate the species. A major part of the control of bodily function is exercised by the nervous system which directs organs or tissues to carry out certain processes voluntarily or involuntarily, such as contraction of muscle, outpouring of glandular secretion or even storage of information. Supplementary assistance in the execution of these orders is given by the hormones. These are highly specialized chemical messengers, essential to normal life, which modify the activity of their target organs or cells in such a way as to alter their pattern of behaviour. They are able to exert their influence even when present in exceedingly small concentration. In many respects they resemble the vitamins, and in fact the steroid hormones are more closely related chemically and functionally to the fat-soluble vitamins than they are to the protein hormones. It is quite rational to think of hormones as endogenous vitamins, or to think of vitamins as exogenous hormones. The functions of the two overlap, and as far as we know their modes of action may be quite similar. In the case of vitamin C, which is synthesized from simple precursors by domestic animals, but not by man, it would be quite appropriate to refer to it as a vitamin for man, and a hormone for domestic animals. Hormones resemble vitamins too in that deficiency, however caused, results in recognisable disease, and excessive amounts cause symptoms of toxicity. The margin between deficiency and excess is a narrow one.

The hormones of course are secreted by the endocrine glands or tissues. A single gland may produce several different hormones, and the histochemist is often able to recognise the particular groups of cells which assemble individual hormones within such a gland. Scattered foci of endocrine tissue within organs, such as the Islets of Langerhans in the pancreas, have long been granted the status of endocrine organs.

Although hormones are required in very small amounts, they are literally vital and may be needed at very short notice. As a rule, stored hormone is limited to the amount which can be contained in the secretory granules of the synthesizing cells. A notable exception is the thyroid, which accumulates secretion in follicles within the gland. Less well prepared for emergency are the cells of the adrenal cortex, which store hormonal precursors rather than the fully active hormones.

The ability of endocrine cells to secrete their particular hormones depends equally with other bodily cells, on a correct supply of nutrients, and of these, a correct supply of vitamins and trace elements is of prime importance. Demand for nutriment varies according to the output of the endocrine organ and this in turn is decided by the magnitude of variation in external and internal environment. Response to an external stimulus, such as low temperature, demands increased thyroid secretion, response to fear demands adrenalin. Response to changes in internal environment may require, for example, insulin for control of the level of glucose in the circulating blood, or aldosterone for the control of the sodium level. In addition to such normal adaptive responses to circumstances, there is often a rhythm of secretion, either diurnal or seasonal. There is the well recognised daily ebb and flow in the tide of adrenalin secretion. The oestrogenic and androgenic hormones too are subject to physiological cycles of varying length depending on the species and sex concerned. In the domestic animals at least the seasonal onset of the physiological sexual cycle is dependent on the length of the hours of daylight, and in the case of the larger animals, the cycle is adjusted so that young are born in the spring time, when available food is at its best and the newly born can look forward to several months of good nourishment before the lean days of winter set in.

The special requirements of the endocrine glands for vitamins and trace elements for hormonal synthesis at times of physiological stress, as in growth and reproduction, must be met in full if adverse effects are to be avoided. Acute conditions of stress may lead to such an outpouring of hormone that the animal goes into a state of shock which may even lead to death. In both physiological and pathological stress, the animal with an adequate supply of nutriment has a distinct advantage over the poorly nourished animal.

At one time it was thought that the pituitary gland was the leader in the hierarchy of endocrine glands. However it is now known that the hypothalamus exercises control over the pituitary by means of its corticotropin releasing factors, and it is suspected, but not confirmed, that it may produce releasing factors for the other anterior pituitary hormones. In addition the hypothalamus is known to be the source of the hormones oxytocin and vasopressin, which were originally attributed to the posterior pituitary gland. The latter situation is merely

the storage site for these two hormones and it is from this part of the pituitary (the pars nervosa) that they are released into the blood stream from the nerve endings.

The anterior pituitary gland produces at least six hormones which direct the activity of their target organs and tissues in various parts of the body. These are the tropic hormones, namely the two gonadotropic hormones, adrenocorticotrophic hormone, thyroid stimulating hormone, lactogenic hormone and somatotropin (growth hormone).

The suffix -tropin derives from a Greek word meaning turning and implies that the relevant hormones direct themselves towards their targets. This they do in a manner calculated to arouse the admiration of a guided missile expert. It is quite usual to find these hormones referred to in the literature as trophic (nourishing) hormones, but in no sense do they nourish their target cells—rather do they cause depletion.

The pars intermedia of the pituitary is the source of intermedin, or melanocyte stimulating hormone. The other major hormones of the body include the thyroid and parathyroid hormones, calcitonin, the adrenal cortical and medullary hormones, insulin and glucagon from the islet cells of the pancreas, and the gonadal hormones. There is no unity in the chemical make-up of these substances. They are as diverse in nature as are the vitamins. They can be classified into three major groups as steroids, proteins of simple or complex molecular structure, and amine derivatives. The steroid hormones include the mineralocorticoids and glucocorticoids of the adrenal cortex, and the oestrogens and androgens. All are derived basically from the cholesterol molecule. The protein hormones range in size from the small octapeptides oxytocin and vasopressin, through the larger polypeptides ACTH, calcitonin, glucagon and insulin, to the very large protein molecules of thyroid stimulating hormone, prolactin and growth hormone. The gonadotropins are glycoproteins of high molecular weight, containing hexosamine. The parathyroid hormones A and B are composed of amino acids but also seem to contain a lipid fraction of a steroid nature. The amine derivatives are the catecholamines, adrenalin and noradrenalin, and the thyroid hormone which is a compound of tyrosine with iodine.

The regulatory effect of endocrine systems often seems to be the result of a natural balance between different hormones. Insulin, for example, is opposed in its action by glucagon, and parathyroid hormone is opposed by calcitonin. In these two cases, the insulin and parathyroid hormones appear to be responsible for major adjustments in glucose and calcium homeostasis respectively, and their opposing hormones are responsible for minor but opposite adjustments. The complicated interactions of the major hormones constitute in the normal animal a delicately balanced mechanism. Any disturbance of this balance may have far-reaching effects. The endocrine system may be compared to a

a spider's web, in which a slight pull on any single strand alters the tension on every other strand in the whole complex structure.

Alteration, in the form of excess or deficiency, may arise during the formation of a hormone, and this may be the result of nutritional imbalance or it may be genetically determined. Thus the inherited constitution of an individual may predispose to endocrine disease. For example an inherited tendency to diabetes may be averted by proper dietary management, or encouraged by dietary mismanagement; diets leading to obesity are recognised as increasing the risk of diabetes. Hormonal imbalances may be secondary to local or systemic disease, or a natural result of variations in the physiological status of the host. Or again, alterations in output of a given hormone may be induced by specific stimulation or inhibition by other endocrine organs. Unwanted accumulation of hormone may result from failure of the body to utilise the product, failure to excrete it, or failure to detoxify it.

Failure of a target endocrine organ to react to its stimulatory hormone, leads to failure of its negative feedback mechanism. In such a case, the stimulatory hormone is produced to excess. Conversely, excessive production of hormone by a target gland shuts off the supply from the stimulatory organ. Prolonged depression of hormonal synthesis in this way may continue until the animal organism is incapable of synthesizing the stimulating hormone. Such a situation arises when hormones, such as corticosteroids are used unwisely as therapeutic agents. Long continued excessive administration results in involution or even disuse atrophy of the cells responsible for steroid synthesis, and withdrawal therapy must be practised over many months before the patient is able once again to synthesize his own steroids. In some cases the ability is never regained. A further danger in hormone therapy resides in the fact that many hormones are protein in nature and therefore immunogenic. For medical use they are generally prepared from animal tissues, and although there is much similarity between hormones from various species there is not always complete identity. Crossing of the interspecies barrier brings with it the probability that injected hormone sooner or later will stimulate the production of antibodies in the tissues of the host. The immune reaction to insulin in man is rather weak and cases of insulin resistance are fortunately rare. When the condition does occur, injected insulin forms a complex with its antihormone. In experimental animals it has been shown that the antigen-antibody complex precipitates in the renal glomeruli. This is thought to provide an explanation of the pathogenesis of diabetic glomerulosclerosis. It seems however that immunological inactivation of the protein hormone by its antibody does not necessarily involve biological inactivation, so that diabetic patients apparently well controlled with insulin may nevertheless develop renal lesions.

The steroid hormones, although not immunogenic themselves, may

be rendered so by complexing with a carrier protein. It might be thought that the stimulation of immunity to hormones would have a practical application in the treatment of cases of excessive hormone production, but so far this principle has not been used with success in any disease.

Hormonal imbalance is more detrimental to vitamin deficient patients than to those on a properly balanced diet. Therefore a knowledge of the vitamins concerned in hormonal synthesis is essential for the re-establishment of normal function in the endocrine glands after systemic or local disease, whether hormonal therapy has been used or not.

Before we leave this subject of hormonal imbalance, a short reference to a specific type of dietary interference with hormone production may not be out of place. It is well known that thyroid hyperplasia develops in cattle and sheep fed on certain Brassica crops such as rape or kale. This is apparently due to the effects of a metabolite of the toxic alkaloid linamarin, present in the foodstuff. Animals eating such crops develop goitre, as do their unweaned calves or lambs. Furthermore it has been shown that the thyroid glands of human beings drinking milk from cows which have eaten these crops are unable to abstract labelled iodine from the circulating blood for the synthesis of thyroxine. Cabbages and kohlrabi too contain a substance which is broken down by intestinal bacteria to yield a goitrogenic compound.

### **Methods of investigation**

The investigation of hormonal action is pursued at many levels, ranging from the clinical to the ultramicroscopic. In the past, basic knowledge of endocrine action was acquired mainly by the physician, and it might be said that the science of endocrinology was founded by Addison when he correlated clinical findings with post-mortem findings in his series of cases involving destruction of the adrenal cortex. Since then, clinical syndromes relating to underactivity or overactivity of all the major endocrine organs have been described. It is now the task of the present generation to explain exactly why these clinical effects happen so that endocrine diseases may be prevented or at least alleviated.

The histologist, the enzyme histochemist and the electron microscopist all have their parts to play in detecting the cellular changes in postmortem material from clinical or experimental cases of endocrine disease. Unless disease is well established, investigations with the light microscope are limited in their usefulness. The electron microscope however can reveal alterations in mitochondria suggestive of membrane damage, and alterations in quantity of rough or smooth surfaced endoplasmic reticulum, which give some idea of the metabolic state of the individual endocrine cell.

The great advances in microscopical resolution in the past decade give us hope that in the near future we may be able to examine the

molecular structure of cell membranes. In the meantime studies of hormonal action at the molecular level depend more on deduction than on direct observation. Advances on a broad front therefore depend on cooperation between scientists studying widely divergent aspects of the subject.

Cell and tissue cultures provide valuable information about the *in vitro* reactions of cells in response to increase or decrease of hormone content in the nutrient medium, in the presence or absence of specific vitamins or trace elements. In general this work is tedious and time consuming, though rewarding, and much remains to be done. The use of radioactively labelled hormones in the nutrient medium permits the path of the hormone through the cell to be traced. Thus, labelled triiodothyronine is seen to appear first in the nucleoli, before moving through the nucleus and out into the cytoplasm. This particular hormone, according to Siegal and Tobias (1966) causes an increase in the number of nucleoli in cultured kidney cells. In contrast, heart cells are induced by the hormone to differentiate from their normal polygonal shape and compact colonial arrangement to fibroblast-like cells, with spaces between individual cells crossed by thin cytoplasmic bridges. These and many other similar experiments demonstrate that the nature of reaction to hormonal activity is a function of the target cell rather than of the hormone. They also demonstrate that adhesiveness of cells may be influenced by hormonal action. Whether such adhesiveness is or is not related to hormonally induced changes in the protein content of the cell membrane has not yet been resolved, but the subject is of major importance in the understanding of the growth of malignant tumours, which in general lose their property of contact inhibition and multiply unrestrained by the presence of their neighbours.

Although cell culture has provided us with a great amount of knowledge its usefulness is still limited by the annoying tendency of cells to de-differentiate if they are not supplied constantly with the impossibly complex nutritive supply to which they are accustomed. Ideally this should contain the correct hormones and vitamins in correct quantity, varying from one minute to another, as in the animal body, and should in addition have some means of removal of unwanted metabolites. Such conditions can only be approached by perfusion of cells with a living blood stream.

### **Hormonal Assay**

There are many methods for the assay of hormones in current use in clinical and experimental trials. Immune assay and radioimmunoassay are delicate methods for studying variations in circulating levels of protein hormones, while gas and paper chromatography can be used for assay of steroid hormones. Isotopic labelling of both hormones and vitamins is extremely useful to the experimentalist but can be

prohibitively expensive. On a less exact scale reactions to endocrine imbalance can be reflected in changes in blood pressure and in the electrocardiogram. Behavioural changes often indicate endocrine disorder and may vary from slight irritability to complete mental collapse. Changes in weight of experimental animals, or in the weight of their target organs are sometimes used as a measure of endocrine function, either with or without corroborative histological examination of the tissues.

The chemical assay of excreted metabolites has been much used as an index of endocrine function, but results are not always easy to interpret. The amount of excreted metabolite is not necessarily related to the amount of the original hormone formed. It may be influenced by the efficiency or otherwise of the detoxifying mechanism, and by variations, caused by renal malfunction, in the renal threshold for the metabolite.

The relative accuracy of the methods used in hormonal assay, and the appreciation of the numerous modifying factors operating in the animal body have to be taken into account in assessing the validity of published experimental work.

### **Mode of action of hormones**

The mechanisms whereby hormones exert their effect on cells and tissues are still the subject of much debate. Several theories developed during the last few years have so far stood the test of time. Others have failed to find much support and have been quietly dropped. An examination of the more enduring of these hypotheses should provide a basis for understanding some of the vitamin-hormone interactions. The basic problem is to determine which component of a target cell acts as a receptor for specific hormone action. It is very unlikely that all hormones have a common receptor site, bearing in mind their great diversity in structure and in size.

Theories acceptable at the moment relate to hormonal action on the genetic apparatus, on the synthesis of protein at ribosomal level, on direct activation of single enzymes, and on modification of cell and organelle membranes.

The theory of hormonal action by gene activation is explicable on the basis of the Jacob and Monod theory referred to in the previous chapter. The hormone may be visualised as the inducer molecule which combines with the repressor molecule to allow transcription of messenger RNA to proceed. The latter then codes for the synthesis of a single enzyme or a group of enzymes responsible for a single metabolic series of operations. In support of this theory several hormones have actually been traced to the nucleus by radioactive techniques. Labelled cortisone for example has been injected into animals and thereafter has been recovered from the nuclei. Fractionation of the nuclei shows

that a considerable amount of the label is attached to the histone component, and none to the DNA or RNA. The histones are a group of simple proteins, strongly basic in character, which combine with the deoxyribonucleic acid of the cell. It has been suspected, but not as yet proved, that histones are the substances which repress cellular DNA, and the finding of histone associated with cortisone in this system provides encouraging support for the Jacob-Monod theory. Once within the cell nucleus, cortisone induces synthesis of a series of enzymes, mainly those required for the synthesis of glucose from amino acid precursors. The effect is inhibited by previous treatment of the cells with actinomycin, a drug, which by forming a complex with DNA, renders it unable to take part in the synthesis of messenger RNA. Actinomycin treatment of cells allows cell respiration to proceed but allows no protein synthesis after the existing complement of messenger RNA is used up. The drug is used extensively to provide confirmatory evidence that hormones act at the gene level.

The growing list of hormones which can be shown to increase messenger RNA synthesis includes growth hormone, thyroid hormones, oestrogens, androgens and insulin. However both cortisone and insulin have a wide variety of actions, and some of these are mediated by modifications of cell membranes. The protein synthesis which is induced in the adrenal gland by ACTH appears to be gene activated, but steroid synthesis is not. It can be demonstrated that steroid synthesis may proceed even in actinomycin treated cells.

A combination of gene activation and membrane alteration is displayed by the hormone aldosterone, which can be found in the nuclei of susceptible cells shortly after injection. Following a delay period of one to two hours, the cell membrane becomes increasingly permeable to sodium and other cations. The membrane change is thought to be dependent on new protein synthesis because it is inhibited by previous treatment of the cells with puromycin, a substance which inhibits protein synthesis at the translational level i.e. at the stage of protein synthesis by the ribosomes, according to the instructions brought by messenger RNA from the nucleus. Hormones have a restraining action on enzyme synthesis as well as a stimulatory action. Their restrictive action on protein synthesis might be attributed to the induction of a specific messenger RNA coding for repressor molecules, although there is as yet no proof for this theory.

A further contribution to the understanding of the mode of action of hormones has been provided by Monod, Changeux and Jacob (1963) in their account of what has come to be known as the allosteric theory of control of biological activity. These authors suggest that hormones exert their influence by causing a conformational alteration or 'allosteric transition' in certain key enzymes responsible for protein synthesis. This they do by binding to the enzymes, not at the catalytically

active site, but at some other site. In so doing, they alter the tertiary structure of the enzyme, so that its catalytic activity is modified. The control exerted may be stimulatory or inhibitory, depending on the nature of the effector. The effect is fully reversible by removal of the allosteric effector. This concept is useful in providing an explanation for the negative feedback mechanism which exerts a controlling influence on hormonal levels in circulating blood.

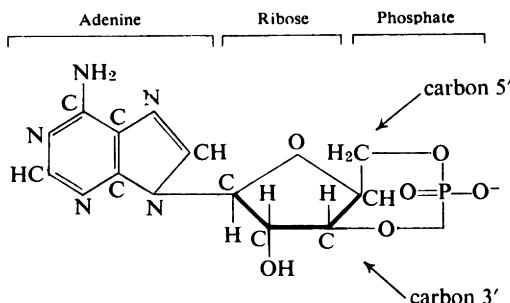
Monod *et al.*, feel that only by considering hormones as allosteric effectors can they account for their specificity, and their capacity simultaneously to exert an activating or an inhibiting influence on a wide variety of metabolic processes in a range of tissues. In developing their theory they were principally concerned with hormones of small molecular weight. It is not suggested that this is the sole way in which these hormones work; only that many of the manifestations may be accounted for in this way. Practical proof of the allosteric theory has not yet accumulated to a great extent but at least one non-steroid hormone (thyroxine) and several steroid hormones (oestrogens and androgens) have been shown to act as allosteric effectors on glutamic dehydrogenase. In the presence of both oestrogens and androgens the activity of this enzyme is inhibited, but it acquires instead the function of an alanine dehydrogenase, thus demonstrating both the quantitative and qualitative effects of an allosteric regulator.

It is not yet known whether any hormones exert their activity by inducing coding for the RNA polymerase which catalyses the synthesis of messenger RNA; or whether hormones have any specific activating or inhibiting effect on the RNA polymerases once they are formed. It is conceivable that they may exert indirect control by monitoring the blood level of the various cations, calcium, magnesium and manganese, which activate or inhibit specific RNA polymerases.

In many cases it can be shown that hormones exert a specific effect by activation of a single enzyme. In the liver, adrenalin, and to a lesser extent glucagon, both activate an enzyme, phosphorylase kinase, which converts glycogen phosphorylase from an inactive to an active form. Glycogen phosphorylase (2.4.1.1.) is a vitamin-containing enzyme which catalyses the rate limiting step in the metabolic pathway for the oxidation of glycogen to pyruvate and lactate. A similar activation of phosphorylase by a kinase occurs in the cells of the adrenal cortex, but here the hormone concerned is ACTH. The adrenal cell, unlike the liver cell, oxidises glucose by the pentose phosphate pathway, the enzymes for which are present in the extramitochondrial part of the cell. In this pathway (also known as the direct oxidative pathway) nicotinamide adenine dinucleotide phosphate (NADP) functions as the hydrogen acceptor. As a result, oxidation of glucose in this way generates the reduced NADP which is required for many of the steps in steroid hormone synthesis.

The kinases mentioned above are themselves stimulated to action by the cyclic nucleotide 3'5' adenylic acid, a derivative of adenosine monophosphate (AMP) (Figure 3). It was the discovery of the increase in cyclic AMP following adrenalin and glucagon stimulation of glycolysis in the liver which suggested to Sutherland, Øye and Butcher (1965) the outlines of the two-messenger concept of the action of hormones on their target cells.

Briefly, it was postulated that a hormone—the first messenger—interacts with a component of the target cell membrane, to initiate



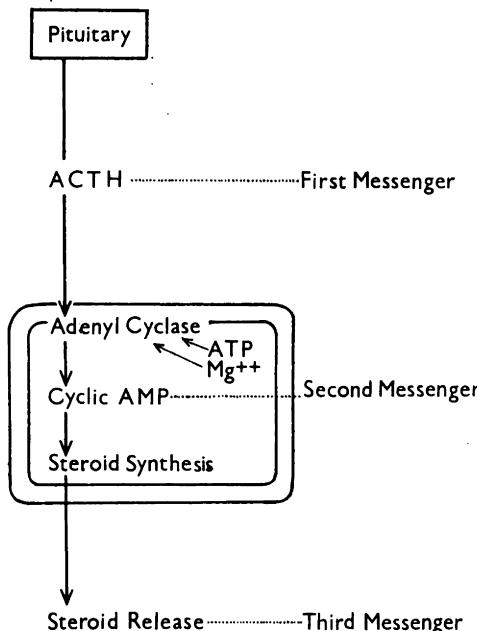
*Fig. 3. Cyclic 3' 5' adenosine monophosphate (cyclic AMP), a mono-nucleotide of adenylic acid. The phosphate group is diesterified at carbons 3' and 5' of the ribose moiety.*

increased accumulation of a non-specific mediator—the second messenger—which acts upon components of the effector cell to produce a specific result. The result may be alteration in enzymic activities, changes in permeability barriers, or the production or release of a third messenger, as for example a steroid hormone.

To date, the majority of the work confirming this theory has been done on the system involving adenyl cyclase, and this may be used as example to illustrate the action of ACTH on the adrenal cortex. Stimulation of the pituitary gland produces the hormone ACTH (the first messenger) which circulates in the blood and, provided it is not inactivated, eventually reaches the adrenal circulation where it comes in contact with the cell membrane. Here it activates the enzyme adenyl cyclase, probably on the inner surface of the cell membrane. Activated adenyl cyclase then splits cyclic AMP from ATP in the presence of magnesium ions. It is this cyclic AMP, the second messenger, which stimulates the physiological response within the cell. The nature of the response depends on the biochemical pattern of the effector cell. In the case we are considering, the response to increased cyclic AMP in the adrenal fasciculata cell is the release of corticosteroid, which may be visualised as the third messenger (Figure 4). Confirmation of

the role of cyclic AMP as a second messenger is provided by the observation that addition of exogenous cyclic AMP to target cells mimics the effect of ACTH in inducing increased corticosteroid production.

The adenyl cyclase system may be blocked by inactivation of the hormone before it reaches its target cell. It may also be blocked by inactivation of cyclic AMP within the target cell by a phosphodiesterase, specific for 3'5' bonds, which is apparently associated with both the



*Fig. 4. The adenyl cyclase system, illustrating the release of steroid from an adrenal cell by ACTH activation of adenyl cyclase in the adrenal cell wall.*

particulate and supernatant fractions of cell homogenates. Although investigations so far have been concentrated on 3'5' cyclic AMP it is possible that other second messengers exist, which may or may not be cyclic nucleotides. 3'5' guanylic acid has already been identified and may prove to form the basis of other second messenger systems.

The list of tissues which respond to hormonal stimulation by increased adenyl cyclase activity is a lengthy and growing one. Sutherland *et al.*, list the brain, skeletal muscle, heart, liver, lungs and spleen as responding to catecholamine stimulation, and the corpus luteum responding to luteinising hormone in this way. More recently Chase and Aurbach (1968) have shown that adenyl cyclase in rat renal cortex is activated by parathyroid hormone, and that the same enzyme in the renal

medulla is activated by vasopressin. Evidence is accumulating too that parathyroid hormone may release cyclic AMP in bone, and that calcitonin exerts an inhibitory effect on the process. Care and Gitelman (1968) recently demonstrated that in the pig, activation of the adenyl cyclase system within the thyroid cells was followed by calcitonin release in the presence of hypercalcaemia. There was no response to cyclic AMP when the circulating blood was hypocalcaemic. This finding is analogous to the fact that stimulation of insulin secretion by cyclic AMP is not detectable when glucose is absent or in low concentration in the circulating blood.

Generally, it is supposed that where the adenyl cyclase system operates, hormones which produce a physiological effect stimulate a rise in cyclic AMP level. If the hormones have no physiological effect on a particular tissue, they are unable to elicit a rise in cyclic AMP. As Sutherland points out this implies that the adenyl cyclase systems of various tissues are different in their molecular configuration, but this is not difficult to accept as several enzymes are already known to exist in multiple forms. It is thought that even very slight variations in the molecular configuration of the adenyl cyclase system may account for the specificity of hormone and tissue interaction.

The elucidation of the mode of action of hormones on cell membranes and on the membranes of intracellular organelles depends to a large extent on developments in knowledge of the molecular structure of membranes. A brief resumé of this subject viewed from several angles may provide some insight into the mechanics of the process. Cell membranes are visualised by the electron microscopist as consisting of two protein layers separated by a space. Intracellular membranes are known to be thinner than the outer limiting membrane. The histochimist demonstrates that some membranes have a carbohydrate component. The biochemist suggests that the two protein layers of membranes are separated by a lipid layer. He is able to detect the presence of varying amounts of phospholipids in membranes. These phospholipids vary from one tissue to another and from one intracellular organelle to another. The composition of each phospholipid varies with respect to the degree of saturation and the chain length of its constituent fatty acids. Other lipid compounds known to be present are cholesterol and its esters, and the fat soluble vitamins A, D, E and K, all of which probably form part of the membrane structure. The cholesterol adds rigidity to the structure. The concentration of vitamin E is possibly related to requirements of the cell membrane or the cell contents for antioxidant activity, for lipid peroxidation causes membrane damage with resulting disruption of cell function. Abnormal variations in content of vitamins A and E interfere with membrane integrity. Vitamin A excess disrupts cell membranes and vitamin E deficiency allows damage by lipid peroxides and free radicals. In both cases

accumulation of autoxidised lipid (lipofuscin) within cells interferes with normal metabolism. The functions of vitamins D and K as membrane components are not known, although there is some evidence that vitamin D reduces the permeability barrier to calcium uptake. The sterol hormones are found associated with cell membranes but it is likely that the attachment is transient and purely for the purpose of activating the adenyl cyclase resident on the inner surface of the target cell membrane. The peptide hormones, insulin and vasopressin are thought to attach more firmly to the target cell surfaces by S-S linkages. Enzymes present in cell membranes may be firmly or loosely attached and these vary with the cell structure, as do their activating monovalent and divalent cations. All these biochemical variations in membrane composition help to explain why hormonal specificity lies not in the hormone itself but in the chemical composition of its target cell.

The physicist sees cell membranes as bimolecular leaflets of polar lipids, incorporating protein molecules and having numbers of aqueous channels, lined by protein polar groups, piercing the membranes. The interactions of the electrical charges on the protein side-chain groups lining these channels are thought to determine the diameter of the pores, and consequently the ease or otherwise of passive transport of metabolites into the cell. Variations in the structures and properties of the cell membranes can be induced by dietary alterations involving lipids, proteins or vitamins; or they may be provoked by injection of exogenous hormone. Vasopressin, for example, increases cell permeability, possibly by enlarging channels within membranes. It is thought that calcium ions tighten up membranes by linking up negatively charged groups across the channels. Conversely lowered  $\text{Ca}^{++}$  concentration may have the opposite effect. Parathyroid hormone, therefore, by maintaining adequate serum calcium levels may have an indirect effect on stabilizing cell membranes.

Cortisol and cortisone appear to protect membranes from various damaging agents including excessive vitamin A, but the nature of this action is not known. The steroid hormones may induce some of their effects by altering membrane permeability and thus modifying the rate at which substrates are allowed to enter the cell for processing by intracellular enzymes. Cortisol, for example, is known to increase the permeability barrier to calcium uptake, in direct opposition to the increased permeability induced by vitamin D.

From the foregoing, it is obvious that we are only on the threshold of an understanding of the mechanics of hormonal action. Our present knowledge suggests that hormones exert their effect by activating or inhibiting enzyme systems, or by altering membrane permeability, and thus coordinating metabolic processes, so that normal growth and development may continue in the presence of constantly changing external and internal environments.

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## *Chapter 4*

### **Vitamin A**

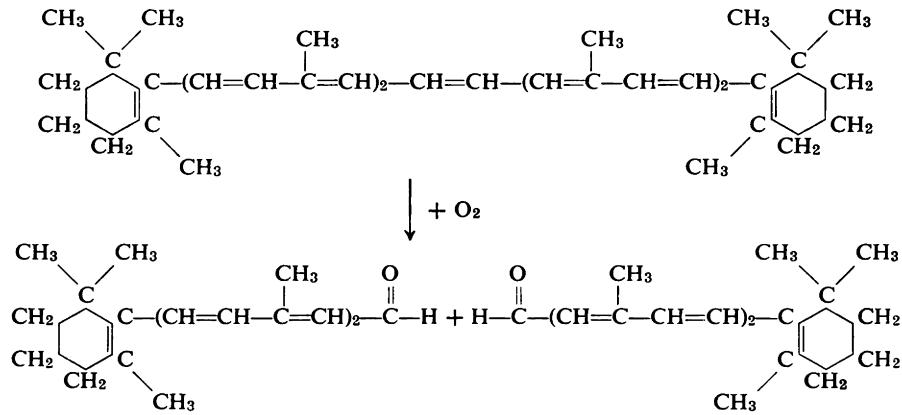
In order to understand the functional disorders of the endocrine glands associated with vitamin A deficiency or excess, we must first consider the various forms in which the vitamin occurs in nature and the relative usefulness of these forms in nutrition in man and animals.

Vitamin A is a term used loosely to cover the alcohol, aldehyde, acidic and esteric forms of vitamins A<sub>1</sub> and A<sub>2</sub>. In the case of A<sub>1</sub> these are known respectively as retinol, retinal, retinoic acid and the retinyl esters. Vitamin A<sub>2</sub> or dehydroretinol occurs in the aldehydic form known as dehydroretinal, and as dehydroretinyl esters. The biological activities of the various forms are by no means identical.

Carnivorous animals and man acquire their quota of the vitamin by eating animal tissues containing the preformed vitamins and their precursors. The livers of marine fish have a high concentration of retinyl esters, which they are able to build up from the carotenoids and vitamin A of marine invertebrates. The livers of fresh water fish contain dehydroretinyl esters. Both forms of the vitamin, the retinyl esters and the dehydroretinyl esters are found in fish which grow in fresh water and migrate to salt water.

Herbivorous animals and man are able to synthesize vitamin A from the provitamins or carotenoids, which occur in green leafy vegetables, in vegetable oils, in milk and in egg yolk. At least nine carotenoids are known and of these the most important biologically are  $\alpha$ ,  $\beta$  and  $\gamma$  carotene and the cryptoxanthines,  $\beta$ -carotene being the most active. Vitamin A alcohol represents approximately one half of the  $\beta$ -carotene molecule and theoretically at least, the precursor may be split and oxidised at the central double bond, to form vitamin A aldehyde which is subsequently reduced to the alcohol. The potential yield then is two molecules of vitamin A from one molecule of  $\beta$ -carotene (Figure 5). The alpha and gamma carotenes and cryptoxanthin yield only one molecule of vitamin A from each precursor molecule. The conversion may be by stepwise beta-oxidation of the terminal double bonds. However the metabolic pathway between the various precursors and vitamin A still remains debatable, and it must be admitted that no enzyme systems capable of undertaking the conversion have been discovered as yet.

The transformation of the carotenes to vitamin A takes place mainly in the small intestine in animals, but in man, the liver is thought to be



*Fig. 5. Production of two molecules of retinal from one molecule of  $\beta$ -carotene*

the only organ capable of undertaking the conversion. Carotene from vegetable sources is not well absorbed, and the amounts taken in are generally limited to day-to-day requirements—preformed vitamin A is more easily ingested and stored. Infants and persons suffering from various forms of gastroenteritis, both acute and chronic, have a much reduced ability to undertake the conversion. In diabetes and in hypothyroidism the conversion is almost completely blocked. Cretins, lacking a functional thyroid gland, are quite unable to convert carotene to vitamin A.

The efficiency of intestinal absorption of vitamin A from dietary fats is dependent on the presence of bile salts and therefore on the state of the liver cells. The numerous nutritional, bacterial, viral and other causes of fatty change or necrosis in liver cells reduce availability of all the fat-soluble vitamins, and add to the already existing stress on the animal body. It is possible too that pancreatic enzymes are required for absorption of vitamin A esters, as there is reduced absorption in pancreatic disease.

From the lining cells of the intestine the vitamin is carried by way of the lymphatic vessels to the blood stream where it circulates as retinol, bound to a high density protein, mainly in the  $\alpha_1$  globulin fraction, but partly with the  $\alpha_2$  globulins. The carotenoids circulate with the lipoprotein fractions.

Storage of the vitamin appears to be mainly in the parenchymal cells of the liver, to which it is passed from the blood by the Kupffer cells lining the liver sinusoids; much of the vitamin is in the form of retinyl palmitate. A high concentration is found in the zona fasciculata of the adrenal gland, and in the interstitial cells and corpora lutea of the ovary, where it probably has some physiological function. It is present in the interstitial cells of the lungs and kidney, in the pleura, pericardium, peritoneum and meninges, and in fat cells generally. Most importantly, it is present in the retina and pigment layer of the eye. In the rat, it has been detected by fluorescence microscopy in the pars intermedia of the pituitary, and in the ovary and testes. By histological methods, the distribution can be shown to be similar in rats and man. In neither case is vitamin A demonstrably present in the gastrointestinal tract (except during absorption), in the bronchial or renal pelvic epithelium, in the cornea or in the enamel organs of the teeth. These structures however are the first to show gross and microscopical changes in vitamin A deficiency.

Stores of vitamin A are large in the adult and may take many months to deplete, either by reduced intake, or by excessive loss in disease conditions. Foetal vitamin A and carotene stores, and the neonatal level of vitamin A are lower than the maternal levels and depend to a certain extent on maternal intake.

Depletion occurs in severe exertion or in conditions of prolonged

stress. Popper and Greenberg (1941) have studied the various stages of the process in the rat. They found that in the young animal depletion might be complete in 9–15 days. The retina, however, even in the severe deficiency state, managed to retain some vitamin A; this was an unexpected finding in view of the fact that in man, night blindness is one of the first signs of deficiency. The adrenal cortex of adult rats took rather a long time to deplete. It retained large amounts of vitamin A even when the liver, kidney and lung stores were exhausted. The cells of the middle third of the zona fasciculata retained their supply the longest, with the inner and outer thirds giving up their stores first. Restoration to the normal state was best achieved by feeding rather than injecting supplies of vitamins. Repletion started in the lamina propria of the intestine and progressed to the Kupffer cells of the liver. Then the cells of the adrenal cortex were restocked and finally the endothelial cells of the renal cortex and the lung rebuilt their stores.

#### **General pathology of vitamin A deficiency in man and animals**

All vertebrates require exogenous provitamin or vitamin A for the maintenance of health of their epithelial tissues, for normal development of their bones, and for their ability to see. Xerophthalmia, a specific A-deficiency state, is responsible for thousands of cases of blindness in the Middle and Far East, and numerous cases of infantile death must be attributed to the same cause. In the underprivileged countries the animal sources of the vitamin, which are the most easily absorbed form, are scarce and expensive, and the population has to rely on vegetable sources. Even these sources are not sufficient in quality or quantity in arid desert countries, and it follows that those most susceptible to deficiency, the newly born and the young children, must show in a greater or lesser degree, signs of damage attributable to A deficiency.

In almost all cases there is associated general malnutrition, and most of the affected children show the typical signs of kwashiorkor, a protein-calorie deficiency syndrome. As a result, there may be deficiency of the vitamin A transport proteins. As in kwashiorkor, the disease at its most virulent attacks the newly-weaned child. It is often precipitated by an attack of measles or gastroenteritis. The resulting lack of inclination to eat can only add to the already existing deficiency state. Xerophthalmia in man is generally accompanied by significantly lowered vitamin E levels. This is presumably due to lowered intake, and it follows therefore that the protective antioxidant effect of vitamin E on the meagre supply of vitamin A is lost.

In animals, vitamin A deficiency is responsible for defective reproduction and foetal resorption, as for example, in the rat. In this animal, the placenta is affected before the foetus, in contrast to the

condition in vitamin E deficiency, when the foetus suffers first. Congenital malformations are seen in both pig and the rat, and include eye defects, displaced kidneys, harelip, cleft palate and abnormalities of the heart and larger blood vessels. The retarded bone growth in infancy fails to keep pace with normal nervous tissue growth, so that there is herniation of brain or cord, and compression of nerves passing through the cranial and intervertebral foramina.

Microscopically, A-deficiency is associated with keratinising squamous metaplasia of mucous epithelia—that is, mucous membranes change from a single layer of mucin secreting and ciliated epithelium to multiple layers of epithelial cells, with overlying keratin, resembling those of the skin. The increase in thickness of the necks of the underlying mucous glands plugs their orifices and seals off their secretion. Obstruction of pancreatic ducts by keratotic plugs blocks the release of pancreatic enzymes into the small intestine. At the subcellular level, there is disruption of lysosomal and other cellular membranes both in deficiency and in excess of vitamin A, and it is probable that an optimal amount of vitamin A for the species is essential for the integrity of pericellular and intracellular membranes. (Fell, 1964.)

In evaluating signs and symptoms of naturally occurring and experimental A deficiency, it should be remembered that in animal experiments there is usually no concurrent deficiency of protein or of other essential food factors. Results may therefore not be strictly comparable with those found in man, in whom the condition seldom occurs as a single deficiency. It is important to remember too, that serum levels of vitamin A may not correlate with liver levels, either in the normal or the undernourished subject. It is possible to have large stores of the vitamin, with little in the circulation, or to have a normal serum level in association with a depleted liver.

**Pituitary gland (or hypophysis).** Although the hypophysis is undoubtedly affected in A-deficiency, it is not yet known whether the changes seen histologically in the A-deficient animal are a primary local effect of the deficiency, or whether they are the result of over-stimulation of the gland following pathological changes in target organs. It is well established that there is an inverse relationship between the circulating level of target endocrine gland hormones (Figure 6), and the blood levels of the pituitary hormones which control these target organs—the so-called negative feedback mechanism. Thus in adrenocortical necrosis in man the loss of cortical steroids causes an increase in the large basophils of the pituitary which are said to secrete ACTH, and there is an increased level of circulating ACTH. We shall see later that adrenocortical failure may be caused by one of several vitamin deficiencies, including A deficiency, as well as by acute stresses of various kinds, bacterial diseases and rapidly growing tumours.

Increased numbers of basophil cells are seen also in severely A-deficient ruminants and rats, suggesting increased demand for the production of adrenocorticotrophic and other hormones.

In the young growing A-deficient animal, in which bony growth outpaces nervous tissue growth, the pituitary may be misshapen and compressed by ingrowths of the wall of the *sella turcica*. This does not seem to affect the histological appearance of the gland, apart from some slight increase in connective tissue in the compressed area; nor does it

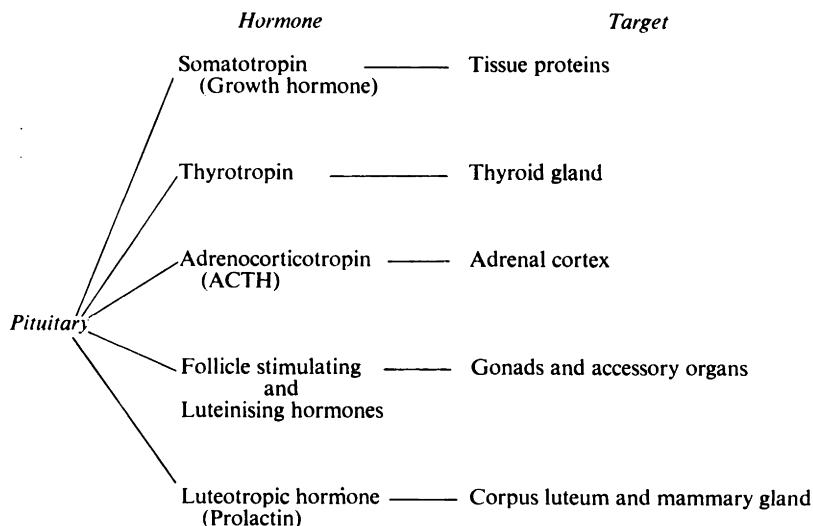


Fig. 6. The hormones of the anterior pituitary gland and their target organs and tissues.

affect the hormonal output, except in those cases in which there is marked hydrocephalus, and cystic dilatation of the gland.

The adult central nervous system contains very little vitamin A and depletion after maturity is unlikely to cause any direct pituitary damage.

**The pituitary hormones.** (1) Adrenocorticotrophic hormone. The sequence of amino acids in the major, active part of this polypeptide hormone appears to be identical in man, pigs and ruminants, although there are species variations in the remainder of the molecule. ACTH is probably stored in protein bound form, but there is some evidence to show that, under stress, it may be released from the cells in a free, low-molecular-weight form. ACTH controls the activity of the adrenal cortex and is involved in the synthesis of the necessary enzymes for steroid production. The ability of the adrenal to respond to ACTH stimulation is closely linked with its content of vitamin A.

(2) Growth hormone—this is another polypeptide hormone, and therefore its site of production may be located by fluorescent antibody techniques. It is formed in the acidophilic cells of the adenohypophysis, and hormones from human and animal sources appear to be species specific.

There is no direct evidence of an association with vitamin A, but high doses of growth hormone given experimentally to animals apparently increase requirements for the vitamin and accentuate deficiencies, presumably because of the general stimulus to growth induced by the hormone.

(3) Thyrotropic (or thyroid stimulating) hormone (TSH). This glycoprotein hormone assists in maintaining the growth and function of the thyroid in normal animals, but in the A deficient animal there is inhibition of this effect. The hormone originates in the pituitary basophil cells; histological changes can be induced in these particular cells by the injection of thyroxine, the endocrine secretion of its target cells.

TSH stimulates the uptake of iodide by the thyroid gland, and activates the iodinase (E.C. 1.11.1.8.) which converts iodide to an active form of iodine, so that it may unite with the tyrosine residues in thyroglobulin. It is also thought that TSH activates the coupling enzyme which unites monoiodotyrosine and diiodotyrosine to form triiodothyronine, or two molecules of diiodotyrosine to form thyroxine (tetraiodothyronine). TSH is difficult to purify and has not yet been characterized chemically. However, it is known to contain about 1% of sulphur in its molecule. Vitamin A is thought by some authors (although this is denied by others), to be concerned in the incorporation of sulphur into the naturally occurring sulphate esters such as chondroitin sulphate, corneal hyaluronic acid and the steroid sulphates. This may be due to the fact that it is required for the synthesis of activated adenosine 3' phosphate 5' phosphosulphate. Whether the vitamin is directly concerned in TSH hormone synthesis, however, has not yet been proven.

Severely A deficient cattle and sheep show marked degenerative changes in the pituitary basophils, which produce TSH, an indication that TSH production might be deranged. However, in a series of experiments on pigs, Palludan (1966) felt that, in this species at least, the inhibiting action of A deficiency on thyroid function was of a direct rather than indirect nature.

(4) The gonadotropins. (a) Interstitial cell stimulating hormone (ICSH) or luteinising hormone. In normal animals this gonadotropic hormone has little effect on seminiferous tubules or spermatogenesis. Its function is to stimulate androgen production by the Leydig cells of the testis. If it is injected into an animal, it also stimulates an increase in the number of Leydig cells. Interstitial cell stimulating hormone restores the atrophic prostate and seminal vesicles of A-deficient rats

to normal. This effect is achieved by the direct action of the gonadotropin on the interstitial cells of the testis; the output of testosterone is increased, with resulting benefit to the secondary sex organs. In A-deficient animals as in normal animals, ICSH causes an increase in the number of Leydig cells although many of these are abnormally nucleated and in various stages of degeneration.

Degeneration of the male target cells in moderate A deficiency induces a reciprocal increase in production of ICSH, which can be detected histologically by an increase in the parent basophilic cells in the pituitary. In severe A deficiency, the increase in circulating gonadotropin may approach the level found in the castrated animal.

(b) Follicle stimulating hormone (FSH). This hormone which has as its target cells the ovarian graafian follicles and the testicular spermatogenic cells is a glycoprotein hormone, with, in the pig at least, a high cysteine and cystine content. Therefore, being sulphur containing it may have the same requirement for vitamin A in its synthesis, as does the thyrotropic hormone. However, ovarian function may often be quite unimpaired in the deficient female animal, although spermatogenesis is regularly decreased in the male. It is not an easy matter to deplete the female experimental animal of vitamin A, unless it is deprived of the vitamin from the time of weaning.

(5) Prolactin (luteotropic hormone, LTH). The function of LTH is to activate the corpus luteum and to stimulate the secretion of progesterone, in experimental animals at least. It is a protein hormone composed of a single peptide chain, and contains disulphide bridges linking parts of the molecule. Rupture of these links inactivates the hormone. In man, there is immunological cross reaction between growth hormone and LTH, which suggests that at least part of the polypeptide chain is common to both hormones. We have no information on the effect of vitamin A deficiency on the synthesis or the action of this hormone.

**The thyroid gland.** The thyroid is under the direct control of pituitary thyroid stimulating hormone, and reflects in its structure and output, changes in that organ. There is, generally speaking, a balance between the amount of TSH released and the amount of thyroid hormone (TH) stored and released. However, at a certain blood level, thyroid hormone both inhibits the production of TSH, and limits directly the release of more TH.

The output of the gland in experimental animals depends on the vitamin A status of the body. At non-physiological levels, both increase and decrease of vitamin A may interfere with thyroid function and the serum concentration of protein-bound iodine.

Clinically, hyperthyroidism increases the need for vitamin A, and lowers the serum level of the vitamin and its precursor.

In clinical hypothyroidism there is decreased need for the fully formed vitamin in accordance with the lowering of the basal metabolic rate.

Thyroidectomised rabbits on a normal diet tend to develop xerophthalmia, a dry scaly condition of the scleral conjunctiva, which is prevented by vitamin A but not by carotene. This suggests that in the absence of the thyroid, carotene is not metabolised to vitamin A, and indeed in hypothyroidism the rise in blood carotene following ingestion of food containing the vitamin A precursors causes a yellow staining of the skin which can be prevented or cured by altering the diet. The human cretin, being hypothyroid or athyroid, is totally unable to change carotene to vitamin A. In the hypothyroid state in man known as myxoedema, there is reduced excretion of 17-ketosteroids, which require vitamin A for their synthesis—another indication of conversion failure. Thyroid antagonists too, such as thiouracil and thiourea interfere with utilisation of dietary carotene, and if preformed vitamin A is not supplied along with these drugs, then stores of the vitamin in liver and kidneys gradually become depleted. If thyroxine is supplied, to oppose the action of the antagonists, then assimilation of carotene and conversion to vitamin A revert to normal.

There seems to be a sex difference in rats, in that A-deficiency produces thyroid hypertrophy in the female, but atrophy in the male. Combined deficiency of iodine and vitamin A give rise to hyperplasia of thyroid epithelium, while A-deficiency alone causes a specific type of degeneration in these cells.

The rate of thyroidal uptake of iodine is markedly reduced in A-deficient animals. Thus, in a carefully controlled series of experiments, Palludan (1966) showed that thyroid secretion as expressed in terms of thyroxine level was markedly reduced in severely A-deficient boars and gilts. In the case of the boars, levels dropped in some cases to 25% of the values of normal animals. In the case of the gilts the experiments were performed first when the A-status of the animals was normal and later when the animals were almost depleted. Again the thyroxine level was markedly reduced in deficiency. The blood concentration of protein bound iodine, however, although reduced in A-depletion, did not become markedly so until the terminal phase of the deficiency. A-hypervitaminosis, extending over two weeks, was found to reduce thyroid secretion by 40–50% in pigs.

**The pancreatic islets.** As has already been mentioned, the diabetic patient is unable to convert carotene to vitamin A. Human patients have high blood carotene levels as compared with normal controls. In rats, in which conversion normally takes place in the intestinal wall, experimental diabetes can be produced by the administration of alloxan. Such animals show a much diminished conversion of the provitamin

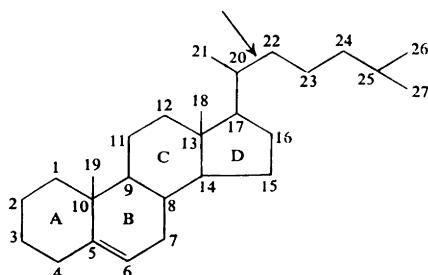
to the vitamin. In man, the conversion is thought to take place in the liver, and it is here that the defect must reside. The mechanism of the defect is not known as yet. Animal experiments are misleading in this particular area of research, not only because of the different anatomical sites of conversion noted above, but also because insulin secretion is induced in the various species by different metabolites. In dogs, for example, insulin is secreted in response to aceto-acetate or 3-hydroxybutyrate. This presumably is a device to prevent acidosis in animals which normally do not consume much carbohydrate. Insulin secretion in ruminants occurs in response to the short-chain fatty acids, such as butyrate and propionate, which are produced from the cellulose of the food by the ruminal bacterial flora. Glucose is a less potent inducer of insulin secretion in ruminants.

We are not aware of any research on A deficiency as an initiator of diabetes, but it is reasonable to think that A deficiency might exacerbate the pathological lesions. The chronic pyelonephritis and necrotising renal papillitis which often complicate diabetes in man may have their origin in epithelial hyperplasia of the collecting tubules, which is a common feature of vitamin A deficiency in animals.

**The adrenal gland.** The importance of vitamin A metabolism in the adrenal gland has long been recognised. Numerous experiments have shown that A-deficiency reduces resistance to stress, lowers insulin tolerance, and impairs glucogenesis. By histological techniques, the vitamin can be demonstrated in high concentration in the cells of the zona fasciculata, suggesting its importance for glucocorticoid synthesis. Adrenal sections often show masses of 'lipochrome' pigment in the cells of the zona reticularis. This is a carotenoid pigment and may represent a store of vitamin A precursor. The pigment is seldom, if ever, seen in the medulla, and its presence in the cortex lends support to the view that it participates in steroid synthesis.

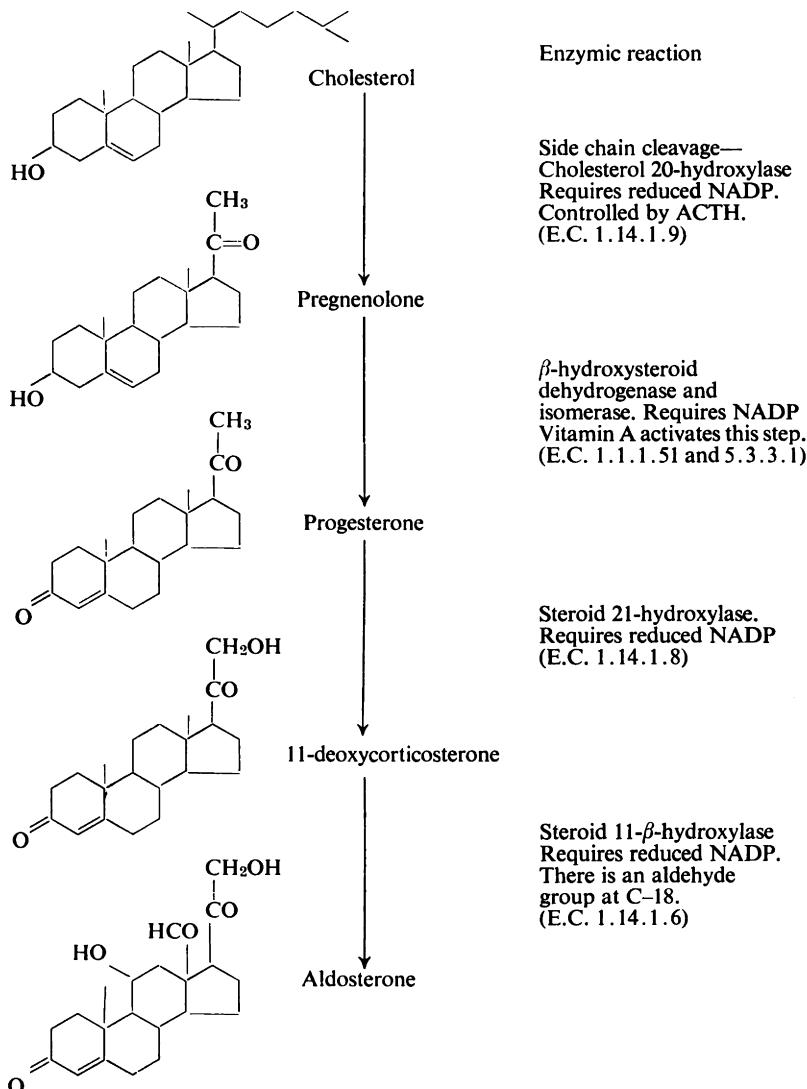
Cholesterol is the common precursor for the steroid hormones formed in the cells of the adrenal cortex and in the interstitial cells of the testis and ovary. It is a substance of ill-repute nowadays, and food-stuffs known to contain cholesterol are avoided by those worried about the state of their arteries. Without cholesterol however, we would be in no position to enjoy life, liberty or the pursuit of happiness, particularly the last. It is fortunate therefore that many cells of the body are able to synthesize all the cholesterol we need, from simple precursors. The liver is the main site of synthesis and builds up the molecule from acetyl-coenzyme A. (CoA, it will be remembered, contains pantothenic acid, one of the B vitamins.) Acetyl-CoA is converted by way of mevalonate to the isoprenoid units which form the basis of the cholesterol skeleton. The acetate to mevalonate series of reactions is unaffected by A deficiency, but the mevalonate to cholesterol conversion is inhibited in severe A deficiency.

The accompanying diagram shows the structure of the cholesterol molecule, the conventions for referring to the rings, and the various positions where changes take place in the molecule to form the different steroids. Those who are intimidated by chemical formulae should not feel daunted as the diagrams are intended only to simplify the description of the various steps catalysed by the enzymes and their vitamin containing cofactors, which alter the basic cholesterol molecule to form the steroid hormones (Fig. 7).



*Fig. 7.* The numbering of the carbon atoms in the cholesterol molecule and the lettering of the rings. The side chain is split between carbon atoms 20 and 22 (arrow) before steroid synthesis starts. Specific hydroxylases, requiring reduced NADP and oxygen for their action, catalyse the addition of hydroxyl groups at positions 11, 17 and 21 for the production of the various hormones. (In cyclic structures, the angle of a ring represents a carbon atom with as many hydrogens as are necessary to saturate it, unless otherwise shown.) The biological activity of many hormones is determined by their stereochemical configuration. If a substituent is located, in relation to the plane of the ring system, on the same side of the cholesterol molecule as the methyl groups at C-10 and C-13 it is in the *beta*- or *cis*-configuration; if on the other side, in the *alpha*- or *trans*-configuration. This latter (*α*) is indicated by a broken line in the diagrams. For convenience, the methyl group at C-10 is regarded as being above the plane of the ring. In the steroids the C-13 methyl group is usually on the same side as the C-10 methyl group.

The adrenal cortical hormones are generally divided into glucocorticoids and mineralocorticoids, but the distinction between the two is rather an artificial one. During stress the glucocorticoids have marked effects on mineral and water metabolism, overlapping in this respect the functions of the mineralocorticoids. On a weight for weight basis, the mineralocorticoid aldosterone has anything from 25% to 100% of the biological activity of the glucocorticoids, depending on the species source of the sample. However, aldosterone circulates at a plasma level of only 0.03 micrograms per 100 ml., while cortisol circulates at a level of 10 µg/100ml., and corticosterone at approximately 1 µg/100 ml. Normally, therefore the glucocorticoid activity of aldosterone is negligible in comparison with that of cortisol. The amount of vitamin



*Fig. 8. Postulated route of synthesis of the mineralocorticoid aldosterone, so-called because of the presence of an aldehydic group at C-18. Progesterone is the common precursor of the adrenal glucocorticoids and mineralocorticoids. Its formation from pregnenolone involves dehydrogenation of the 3-hydroxy group, and isomerisation in which the double bond migrates from 5:6 to 4:5 (or  $\Delta^5$  to  $\Delta^4$ )*

A needed for glucocorticoid synthesis in all probability exceeds that for mineralocorticoid synthesis by a factor of 30 to 300 times. This may be the reason why aldosterone synthesis is not affected until the very latest stages of vitamin A depletion. It would also explain why vitamin A has not been detected histologically in the zona glomerulosa of the adrenal, where aldosterone is synthesized, the quantity required being too minute for detection by such a procedure.

There has been a suggestion that aldosterone synthesis may even rise in A deficiency, with resulting increased plasma sodium content and decreased plasma potassium. If this is so, then the compensatory hypertrophy in the zona glomerulosa may represent an attempt to restore glucose homeostasis, with plasma sodium changes arising as an unwanted side effect of raised aldosterone synthesis. The probable route of synthesis of aldosterone from cholesterol is shown in diagrammatic form in figure 8. It is known that the 11-oxycorticosteroids have a more marked effect on carbohydrates than those without an oxygen atom at C-11, such as deoxycorticosterone. The most potent glucocorticoids are those oxygenated at C-11 and C-17, namely cortisol and cortisone. Aldosterone, being oxygenated at C-11 has quite powerful glucocorticoid action when given in therapeutic doses.

Figures 9 and 10 show the postulated routes of synthesis of the main glucocorticoids in man, and in the more commonly used experimental animals. The first steps in the synthesis of the glucocorticoids from cholesterol are the same as for aldosterone, namely conversion to pregnenolone and then progesterone. If there is, for any reason such as a lack of the necessary enzymes for further conversion, an accumulation of pregnenolone, then further production of pregnenolone from cholesterol is inhibited, by a negative feedback mechanism. The cholesterol to pregnenolone step requires nicotinamide as a cofactor and is under the control of ACTH.

In A-deficient animals there is hypoplasia of the adrenals and marked diminution of the total quantity of progesterone elaborated by the glands. The pregnenolone to progesterone conversion requires two enzymes,  $\beta$ -hydroxysteroid dehydrogenase (E.C. 1.1.1.51) and isomerase (E.C. 5.3.3.1). Grangaud, Nicol and Delaunay (1958), in a series of experiments carried out both *in vivo* and *in vitro* have shown that vitamin A aldehyde (retinal) activates these enzymes. Vitamin A acid (retinoic acid) and vitamin A alcohol (retinol) are equally effective in accelerating the enzyme activity. The actual concentration of a vitamin is an important factor in steroid hormone synthesis. In this particular reaction, however, Grangaud *et al.*, found that the concentration might vary within fairly wide limits, in marked contrast to the narrow limits essential in sex hormone synthesis (*vide infra*). This observation implies that an optimal concentration for oestrogen or androgen synthesis might be quite ineffective for progesterone synthesis.

$\beta$ -hydroxysteroid dehydrogenase is present in almost all the steroid producing glands of vertebrates and is apparently the rate limiting

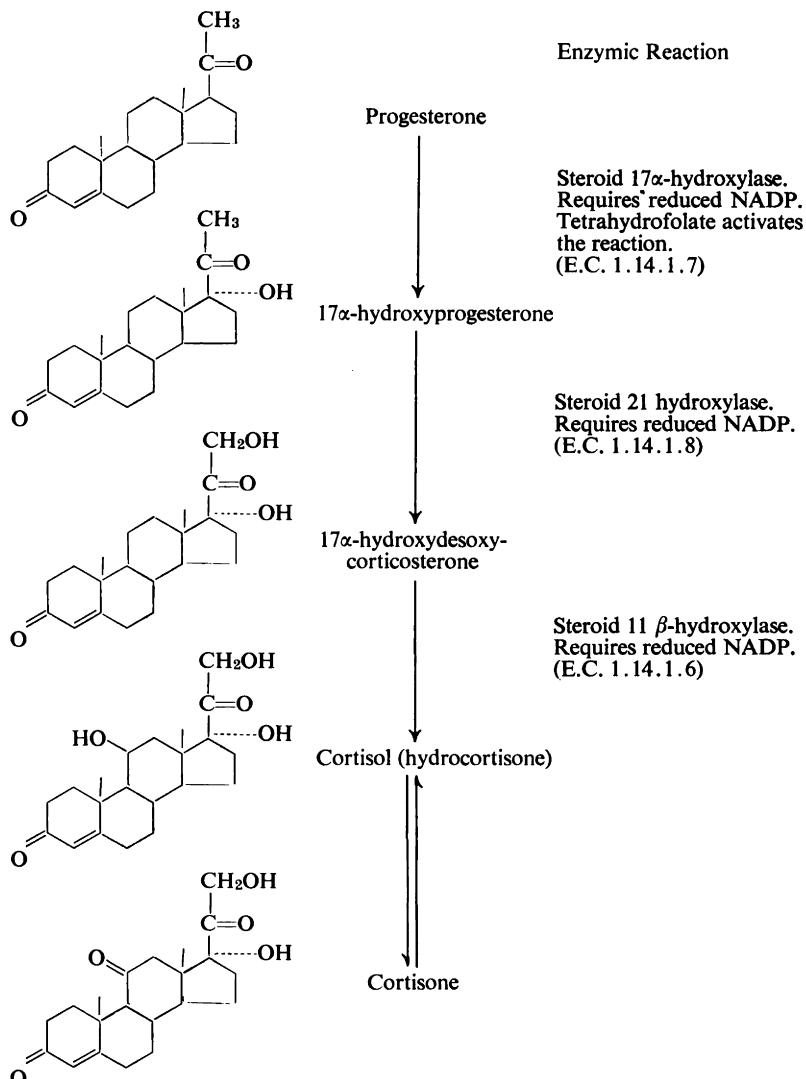
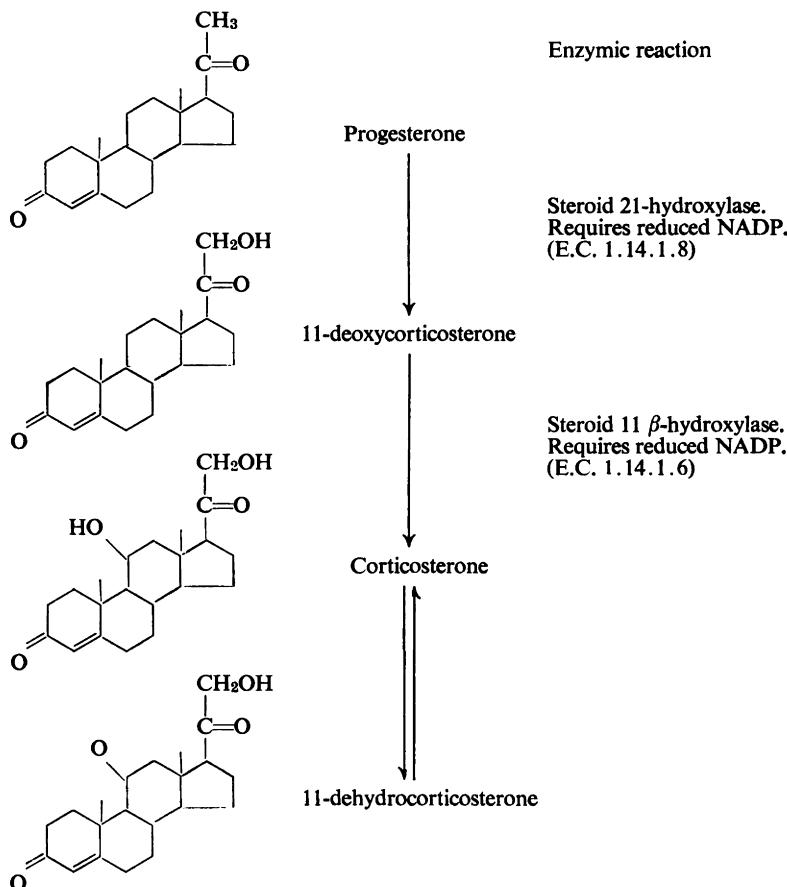


Fig. 9. The synthesis of cortisol and cortisone, the main corticosteroids in man.

enzyme in steroid synthesis. Therefore vitamin A has a key role to play in hormonal production. This can be demonstrated in *in vitro* systems, where the activity of the enzyme can be shown to be depressed in

material from A deficient male and female rats. Restoration of the enzyme to full activity can be achieved by giving vitamin A to the



*Fig. 10.* Synthesis of corticosterone, which exists in equilibrium with 11-dehydrocorticosterone. These are the main glucocorticoids in the rat and rabbit, in which the 17  $\alpha$ -hydroxylating enzyme is missing or present at an extremely low concentration.

deficient animals 24 hours before the tissues are taken for enzyme studies (Juneja, Murthy and Ganguly, 1966).

Levine, Glick and Nakane (1967) carried out an interesting series of experiments in newly born rats. They found that there is a period between the third and the eighteenth day of life, during which the young rats failed to respond to stress by release of corticosteroids. The injection of ACTH during these fifteen days was not followed by plasma

steroid release, although it was clearly shown that the adrenal content of corticosterone started to rise as early as the third day of life and continued to rise. This seems to show that there is a clear distinction between the synthesis and the release of adrenal steroids in the newborn rat. The effective stimulation of adrenals by ACTH in these experiments caused parallel depletion of corticosterone and vitamin A, suggesting that the ability of the adrenal to respond to ACTH by hormone synthesis is closely related to the vitamin A concentration in the gland.

In very mild A depletion, only the deoxycorticosterone to corticosterone step appears to be inhibited. Several authors have shown that in the severely depleted rat, many steps in steroid synthesis are inhibited, including mevalonic acid to cholesterol, cholesterol to progesterone, cholesterol to deoxycorticosterone and deoxycorticosterone to corticosterone. At this late stage, which can be detected by the inability of ACTH injections to restore glucogenesis, it is possible that the A deficiency has caused irreversible degeneration of adrenocortical cells, and in fact this can be confirmed by histological examination. Severe A-deficiency can be considered to be a chemical adrenalectomy as far as glucocorticoid synthesis is concerned.

One of the major effects of A deficiency is the reduced rate of glycogen synthesis in the liver; this is the only effect of A-deficiency which can be restored to normal by cortisone. Glycogenesis stops early in the A-deficient animal, at the same time as weight gain ceases. No enzymatic defect in the liver has been found to account for this. The enzyme systems for the synthesis of glucose from triose are unaffected, and there is no lack of high energy phosphate. Acetate, lactate, and glycerol are incorporated into glycogen normally, and ability to incorporate glucose into liver glycogen is equal in both normal and A deficient rat tissue. However, the injection of cortisol or cortisone into deficient rats restores glycogen synthesis from acetate to normal. Deoxycorticosterone does not. This again suggests a possible block in  $\beta$ -hydroxylation in A deficiency.

The biological effects of the corticosteroids are mediated in many, if not all cases, by their activation of specific enzymes. The amount of increase in enzyme concentration after steroid administration suggests that new stores of enzyme are produced as a result of derepression of the genome unit which codes for their synthesis. One of the more interesting effects of cortisone in the normal animal is a fall in liver vitamin A reserves, and a rise in the level of circulating vitamin A esters.

**The gonads and accessory organs.** The effects of deficiency or excess of vitamin A on the gonads and accessory organs depend on the age of the animal, and its general nutritional status. The older animals are not readily affected by deficiency. High protein diets which induce rapid growth call for increased amounts of vitamin A, as do diets deficient in

vitamin E, which protects the vitamin A in food from destructive oxidation. It is possible that male animals have greater need for the vitamin, because they become more quickly depleted than do females, and are less rapidly restored to normal by correction of the deficiency.

The synthesis of testicular hormones is impaired in A-deficiency, and this is followed by atrophic changes in the male accessory organs (prostate and seminal vesicles). Testicular androgen production is itself under anterior pituitary control, so it can be seen that the development

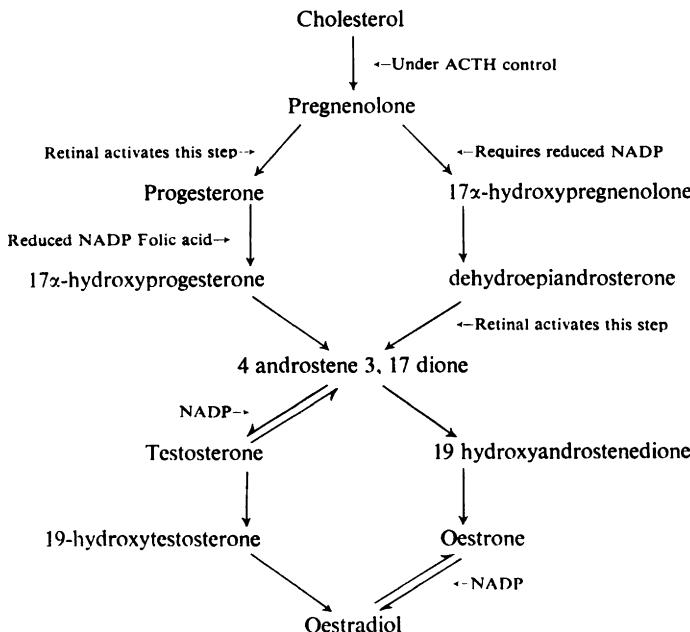


Fig. 11. Possible biosynthetic pathways for androgens and oestrogens, showing cofactoral requirements for vitamin A (retinal) and members of the B complex (nicotinamide and folic acid).

and functioning of the accessory organs is dependent on a complex series of reactions.

The possible pathways of oestrogen and androgen synthesis are indicated in Figure 11. Retinol or its esters are essential for reproduction in the rat, and the form in which the vitamin is supplied is important. Both male and female rats maintained on retinoic acid fail to reproduce, although they may be otherwise apparently healthy. This finding is at slight variance with *in vitro* tests which show that a loss in production of androstenedione from dehydroepiandrosterone can be reactivated by both retinol and retinoic acid, but only at the mild stage of deficiency. The concentration of vitamin required to activate the reaction is

restricted to a very narrow range. Progressive inhibition of enzymic conversion in testes and ovaries of dehydroepiandrosterone into androstanedione and its derivatives occurs as A-deficiency becomes more severe. However, parenteral injection of retinol, retinal or retinoic acid into the animal 24 hours before the hormonal assay counteracts the effects of deficiency on these reactions.

Advanced A-deficiency in the male rat is equivalent virtually to a chemical castration, and is associated with degenerative changes in the germinal epithelium of the testis and the production of abnormal spermatozoa. The response of the accessory organs to injections of testosterone remains normal, indicating that the deficiency interferes with the synthesis or release of androgens, and not with the state of receptivity of the target cells.

Palludan has shown that injection of retinol or retinal, but not retinoic acid into the deficient boar testis restores spermatogenesis around the site of the injection. It is possible that retinoic acid may be more demanding in its need for correct conditions in its role as effector of enzymic reactions. The temperature, pH, or concentration of substrate may be vital to its action.

The presence of carotenoid pigments similar to those in adrenal zona reticulata in the ovary and corpora lutea of mammals suggests a local requirement for vitamin A in oestrogen production. The epithelial squamous metaplasia which develops in the female genital tract of A-deficient rats appears to be dependent, at least in the uterus, on the presence of oestrogen, as it fails to appear in the ovariectomised A-deficient animal. However, keratinization of vaginal epithelium in A-deficiency is not apparently under oestrogenic control.

Reproduction is upset in heifer (i.e. young female) calves fed on diets low in carotene content. Corpora lutea fail to regress and ovarian follicles become atretic. Sexually mature animals may have sufficient stores of vitamin A to permit normal reproduction.

The mode of action of vitamin A in maintaining the integrity of mucous membranes is not yet known, but it is suggested that its action in inhibiting the conversion of cysteine to cystine and the oxidation of sulphhydryl groups prevents the excessive keratinization which results from cross linking of newly formed disulphide bonds. The importance of vitamin A in steroid production is likely to become more apparent in the future as more support is gained for the idea that steroid sulphates are not merely waste products of steroid metabolism, but important intermediary metabolites. It may be that the role of vitamin A in sulphur metabolism is its most important aspect.

**Vitamin A excess.** The effect of excessive doses of vitamin A is even more damaging than deficiency, and may involve necrosis and sloughing of the entire male germinal epithelium. Present evidence suggests that its toxic effect is exerted on lysosomal membranes, with the uncontrolled

release of proteolytic and lipolytic enzymes into the tissues. The glucocorticoids tend to counteract this effect of vitamin A and to stabilise both lysosomal and mitochondrial membranes. It is likely that both hormones and vitamin A are required in strictly balanced ratio for membrane integrity in living cells.

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## *Chapter 5*

### **The Vitamin B Complex**

Metabolic reactions at the molecular level are controlled by enzymes, and in many cases these reactions cannot proceed without the assistance of substances known as cofactors. The cofactors combine with the enzyme in some way to activate it or provide suitable conditions for the enzyme to become catalytically active. They may be simple inorganic ions such as sodium, potassium, copper or cobalt, and in this case are known as activators. Another type of cofactor known as a coenzyme is a complicated organic molecule, often acting as a carrier of some chemical group. Similar organic compounds, more firmly bound to the enzymic protein are designated as prosthetic groups. One major function of the B vitamins is to serve as the operative part of specific coenzymes or prosthetic groups for the oxidation of proteins, carbohydrates and fats.

The chemical composition of most vitamins of the B complex is known. They vary widely in their molecular structure, but all have in common their solubility in water and their ubiquitous distribution in animal cells. The B-vitamins, or their precursors can be found in bacteria, yeasts and higher forms of plant life. The intestinal bacteria occurring naturally in vertebrate species are able to synthesize most members of the group, presumably for their own use. However, under certain conditions, these vitamins become available to the animal host. It follows therefore, that if these intestinal bacteria are killed off by antibiotics or sulphonamides or are lost as a result of enteritis, or if the natural balance of intestinal organisms is upset, the animal host may suffer the symptoms of deficiency of one or all of the B vitamins to a greater or lesser degree, depending on the level of dietary intake of the vitamin concerned.

The B compounds, being water soluble, are readily excreted from the animal body, and are not liable to occur in excess.

The following compounds are included in the vitamin B complex:

(1) *Thiamine*—the biologically active form is thiamine pyrophosphate or cocarboxylase, which functions as the prosthetic group of various enzymes.

(2) Riboflavin is present in the co-enzymes flavin mononucleotide and flavinadenine dinucleotide.

(3) *Nicotinic acid* (niacin), and its amide are present in nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide

phosphate (NADP) which act as coenzymes for oxidative enzymes (dehydrogenases).

(4) *Pyridoxine* and its derivatives and their phosphates (the B<sub>6</sub> group) act as coenzymes in amino acid metabolic reactions.

(5) *Pantothenic acid* is a component part of Co-enzyme A which is involved in numerous carbohydrate, lipid, and amino acid reactions.

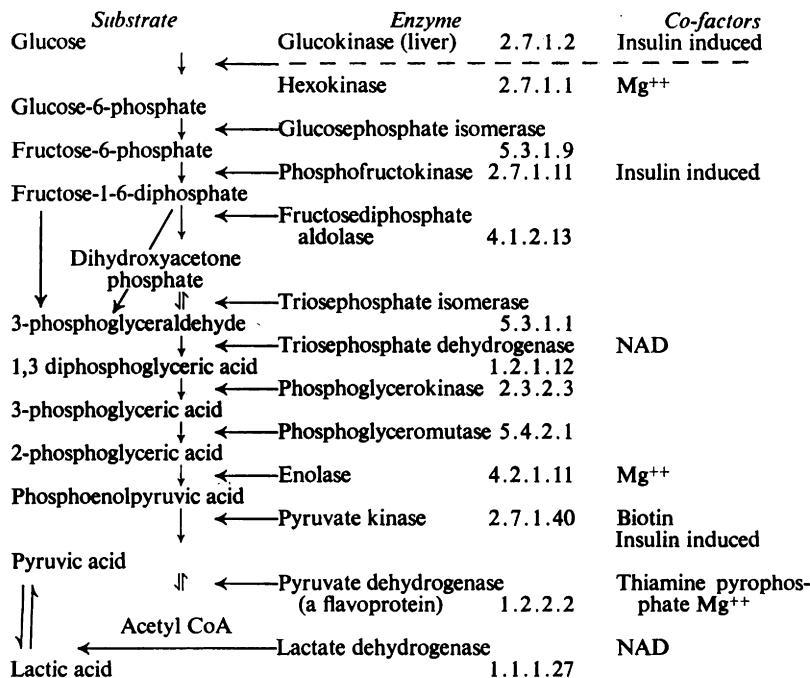


Fig. 12. The glycolytic pathway, showing the enzymes and cofactors in each reaction.

(6) *Biotin* functions as a co-enzyme in several enzyme systems involving transfer of carboxyl groups.

(7) *Folic acid* derivatives act as co-enzymes in the intermediate metabolism of purines and pyrimidines. Tetrahydrofolate is an acyl group carrier.

(8) *Cyanocobalamin* or vitamin B<sub>12</sub>—the metabolically active form, coenzyme B<sub>12</sub>, in which the cyano-group is replaced by an adenine nucleoside, is concerned in amino acid metabolism.

At least 5 vitamins of the B group are concerned in the breakdown of glucose for the production of energy. This process takes place in three stages.

1. In the stage known as anaerobic glycolysis (because it can proceed in the absence of oxygen), glucose is broken down to pyruvic acid.

During the process chemical energy is generated in the form of adenosine triphosphate (ATP). The procedure takes at least eleven steps, each catalysed by a specific enzyme. Several steps require B vitamins as cofactors, and three steps are catalysed by insulin induced enzymes. These latter three steps are irreversible. Synthesis of glucose in the reverse direction from non-carbohydrate sources (gluconeogenesis) can be undertaken only by cells which provide alternative pathways to by-pass these reactions (Fig. 12). All the enzymes concerned are found in the soluble fraction of the cell, not in the mitochondria.

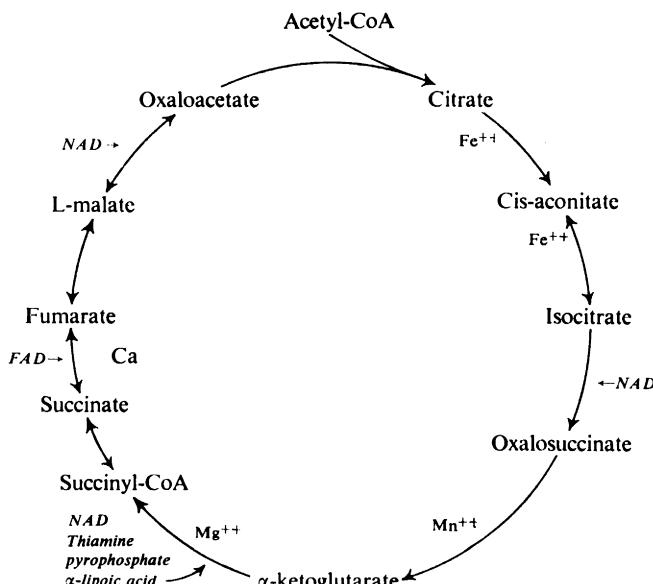


Fig. 13. Requirements of the citric acid cycle for coenzymes derived from vitamins of the B complex. Acetyl-CoA and succinyl-CoA contain pantothenic acid as part of the CoA molecule. NAD contains nicotinamide and FAD contains riboflavin.

The conversion of pyruvate to acetyl CoA is a complicated series of reactions requiring the intervention of thiamine pyrophosphate, alpha-lipoic acid, nicotinamide adenine dinucleotide, and of course pantothenic acid as part of the CoA molecule. Biotin is probably also necessary, and magnesium ions are essential for the reaction.

2. The second stage is known as the Krebs or citric acid cycle and requires the presence of oxygen. Acetyl CoA, derived from anaerobic glycolysis, or from fats or amino acids, combines with oxaloacetic acid, to form citric acid. An ensuing series of reactions, shown in Fig. 13,

regenerates oxaloacetic acid, so that the cycle may continue. The acetyl CoA is finally oxidised to carbon dioxide and water and the energy liberated is used to form molecules of ATP. The enzymes involved in the citric acid cycle are present in the mitochondria.

3. Electrons removed from lactate, pyruvate and several stages in the Krebs cycle are passed along a respiratory chain of hydrogen carriers and electron carriers, activating adenosine diphosphate and phosphate to form energy-rich adenosine triphosphate, and finally reducing oxygen to water. The electron transport chain has not been completely worked out in detail, but a provisional scheme would include nicotinamide adenine dinucleotide, flavin adenine dinucleotide and probably quinone (coenzyme Q) as the primary hydrogen carriers, and the cytochromes B, C, A and A<sub>3</sub> as the electron carriers which complete the chain.

Normally oxidation along this line of carriers is firmly coupled to phosphorylation, but antibiotics, poisons and lack of ADP may 'uncouple oxidative phosphorylation', that is, the oxidation may proceed without the generation of ATP. It is thought that thyroxine is able to uncouple oxidation from phosphorylation so that excess food may be broken down without the formation of chemical energy. The reactions of the electron transport chain take place within the mitochondria.

Mild deficiency of any of the vitamins concerned in the above three stages affects the rate of the reactions, and severe deficiency may seriously interrupt the metabolism of the carbohydrates, fatty acids and amino-acids which are oxidised in this manner. Parenchymal cells of endocrine organs, in common with those of other organs, are therefore directly dependant on adequate supply of B vitamins for their energy requirements.

Although the effects of single B deficiencies can be studied experimentally, such situations rarely occur naturally either in animals or in man. Multiple deficiencies are more usual. However, the symptoms expected of a single deficiency may predominate in some cases of multiple deficiency.

### **Thiamine or Vitamin B<sub>1</sub>**

Thiamine is an essential food factor for all animal species, in which it functions in its co-enzyme form, thiamine pyrophosphate (TPP), also known as cocarboxylase (Fig. 14). The vitamin circulates in the animal body either as free thiamine or as thiamine monophosphate, and the biologically active form appears to be synthesised at the site of catalysis. The holoenzyme depends for its activity on the presence of magnesium ions. It is concerned mainly in carbohydrate metabolism, where it functions as part of the multienzyme sequence required to perform oxidative decarboxylation of pyruvic acid and  $\alpha$ -ketoglutaric acid. In

its absence, pyruvate may build up to a damaging level in the tissues of the body.

TPP functions as a co-enzyme in all enzymic decarboxylations of  $\alpha$ -keto acids, and in the transketolase reaction which is part of the direct oxidative pathway of glucose metabolism in the cytoplasm of adrenal cells and testicular Leydig cells. This metabolic route, the hexose monophosphate shunt, provides an alternative pathway for glucose oxidation in tissues which are active in synthesising steroids or fatty acids.

Requirements for thiamine are high in hyperthyroidism, in which condition there is increased excretion of the vitamin as well as increased

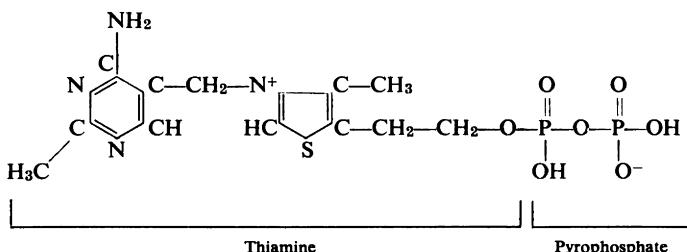


Fig. 14. Cocarboxylase, the pyrophosphate of thiamine.

utilisation. High carbohydrate diets demand increased amounts of thiamine, but dietary fat has a thiamine sparing action.

The tissues suffering most from deficiency are those most dependent on carbohydrate for energy, that is, the cardiovascular system and nervous tissue.

The lowering of testicular hormone output with consequent inadequate functioning of accessory sex organs in thiamine deficiency is apparently not a direct effect on testosterone-forming cells. It seems to be rather the result of failure of the hypothalamo-hypophyseal axis to stimulate androgen secretion. A contributing factor may be the need for TPP in reduced NADP synthesis, the latter being required for hydroxylation of steroids.

In B<sub>1</sub> deficiency in man (Wernicke's encephalopathy) and in animals, distinctive lesions are found in the hypothalamus, and particularly in the corpora mammillaria. These take the form of symmetrical areas of congestion and small, petechial haemorrhages. There is swelling of vascular endothelium, which with blockage of some capillaries, gives rise to compensatory congestion in others. Thickening of capillary walls renders the vasculature more prominent in histological sections. Neuronal damage appears to be secondary to vascular disturbance. Microglial and astrocytic proliferation contribute to the increased cellularity of the lesions. The reasons for the particular sensitivity of

capillaries in restricted areas of the brain to thiamine deficiency have, so far, not become apparent. The location of the lesion tends to vary with the species affected.

The initial adrenal reaction to loss of the hypothalamo-pituitary stimulus is one of over-production, particularly in the zona fasciculata, followed by reduction, and then depletion of the elements necessary for steroid production.

Atrophy of the thymus occurs in thiamine deficiency. The cause of this is not known, but it may be due to impaired ability to synthesize proteins.

### Riboflavin or Vitamin B<sub>2</sub>

This vitamin, which owes its name to its content of ribose, and its yellow colour, occurs in the free form, or as a component of the co-factors flavin adenine dinucleotide and flavin mononucleotide (Fig. 15).

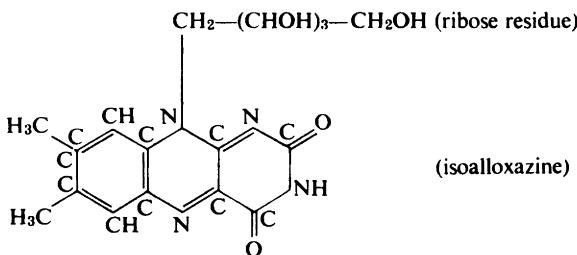


Fig. 15. Riboflavin, a derivative of isoalloxazine.

The flavin nucleotides, unlike the nicotinic nucleotides, are firmly bound to the apo-enzyme, and cannot be removed by dialysis, and are therefore known as prosthetic groups. The enzymes, together with their prosthetic groups are known collectively as the flavoproteins. Copper, iron and molybdenum flavoproteins are known to occur. Metal-containing enzymes catalyse the transfer of hydrogen from reduced nicotinic acid containing enzymes to their own prosthetic groups, thus regenerating the co-enzymes for further activity. The displaced hydrogen is carried through a series of reactions involving the iron-porphyrin cytochromes, finally to react with molecular oxygen to form water. The simultaneous generation of metabolic energy as adenosine triphosphate molecules gives this chain of reactions its name of oxidative phosphorylation.

Dietary riboflavin is phosphorylated in the intestinal wall, whence it circulates to be stored in the heart, liver and kidneys. Evidence of the relationship between riboflavin and endocrine metabolism so far has come indirectly from the pathological effects, in man and animals, of its insufficiency. In man, riboflavin deficiency is made evident by skin

and mucosal changes. In animals, in which a thorough *post-mortem* examination can be made at the various levels of deficiency, fat is found to accumulate in adrenals, as well as in liver, kidney and arterial walls. Riboflavin is required as an integral part of the prosthetic group of acyl-CoA dehydrogenase, (1.3.99.3.) an enzyme which catalyses  $\beta$ -oxidation of fatty acids. ( $\beta$ -oxidation is the principal method by which fatty acids are oxidised, and involves the removal of two carbon atoms at a time from the carboxyl end of the fatty acid molecule.) Complete dissimilation of fat *via* acetyl CoA and the Krebs cycle requires the presence of flavoprotein at the succinate to fumarate stage (See figure 13). Riboflavin deficiency may have the effect of slowing up the dissimilation at either stage, and causing accumulation of products of fat metabolism in adrenal and other cells. Rats on a high fat diet are unable to tolerate riboflavin deficiency for as long as rats on a high carbohydrate diet. Massive accumulation of fat in the adrenals with resulting adrenocortical failure appears to be part of the severe deficiency syndrome.

In the deficiency state there is marked depletion of both liver and muscle glycogen, and lowered concentration of fasting blood glucose.

Liver glycogenesis as a response to stress in riboflavin deficient rats was the subject of an interesting series of experiments performed by Forker and Morgan (1955). The particular stress factor used by these investigators was exposure to anoxia by simulated high altitude conditions. Normal rats responded to this stress with increased production of liver glycogen. Animals maintained for about 8 weeks on riboflavin deficient diet failed to do so. The correct response was restored by supplying the deficient rats, before the final test, with either cortisone or riboflavin.

The rôle of cortisone in permitting or initiating glycogenesis suggested adrenocortical failure as part of the deficiency syndrome. However, the immediate repair of the metabolic defect by riboflavin alone, implied unimpaired secretory ability of the cortex, and presumably also of the anterior pituitary, which stimulates the cortical response. Forker and Morgan thought therefore that the impediment must lie in the stimulus of the pituitary-adrenal axis. To confirm this view they tested pituitary function in riboflavin deficient rats, by enucleating the adrenal substance from within the capsule. It is known that the presence of a functioning pituitary is necessary for adrenal regrowth. Deficient animals successfully regenerated their adrenals, thus demonstrating that the pituitary gland was still functional, in this respect at least.

The adrenal glands were proved to have retained their ability to function by responding to all the tests by which ACTH potency is measured. So the fault was not due to inability of the pituitary to produce ACTH or to inability of the adrenal to respond to ACTH.

The authors thought that some riboflavin-containing coenzyme must be involved in the primary hypothalamic stimulation of the pituitary.

So it seems that adrenal failure to respond to stress in riboflavin deficiency must lie in the trigger mechanism for ACTH release, the hypothalamic corticotropin releasing factor. Thus the possibility again arises that the high degree of dependence of nervous tissue on carbohydrate metabolism, which in turn depends on vitamin B-containing cofactors, may cause secondary failure of endocrine metabolism.

**The Pancreatic Islets.** Insulin is broken down in the liver, which contains a proteolytic 'insulinase' system. Riboflavin deficient rats have impaired ability to inactivate insulin, which may partly account for the increased formation and decreased breakdown of fats in these animals.

**Gonads.** According to Mann, Watson, McNally and Goddard (1952) gonadal function is depressed in the riboflavin deficient monkey. Histological examination of the animal shows the main changes to occur in the endocrine glands. In particular the thyroid and adrenal fail to show evidence of secretory activity.

#### Nicotinic acid (Niacin)

Nicotinic acid is the  $\beta$ -carboxylic acid of pyridine, and occurs in the free state and in the amide form (Fig. 16). Nicotinamide finds its most

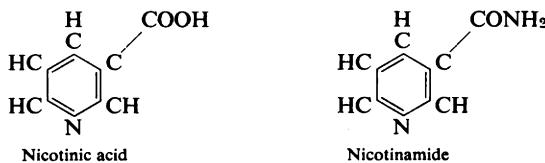


Fig. 16. Nicotinic acid and its amide.

important rôle as a constituent of the pyridine nucleotide coenzymes, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). These function in the removal of hydrogen from substrates as the co-enzymes of the dehydrogenases, and in the transfer of hydrogen which is the first link in the hydrogen transport chain of reactions. The co-enzymes themselves are non-specific, the apoenzymes are specific. The various dehydrogenases vary in their affinity for co-enzymes, so that a coenzyme concentration suitable for one dehydrogenase might be quite insufficient to permit the functioning of another.

The pyridine nucleotides are required by all living cells. They are intimately concerned in the metabolism of carbohydrates, proteins and fats and in cell respiration. They are essential for the maintenance of health of all tissues of the body, including the endocrine glands.

Niacin deficiency in man causes pellagra. It was suggested by Handler and Dann in 1942 that in canine 'black-tongue', the analogous disease in dogs, death occurs as a result of dehydration and electrolyte imbalance. The authors were able to prolong the life of niacin deficient dogs for 180 days by correcting the dehydration and salt loss. There is an obvious implication here of adrenocortical insufficiency. It is now known that nicotinamide adenine dinucleotide phosphate is an essential cofactor in the 21-hydroxylation and the 11-hydroxylation of progesterone to aldosterone, the salt-regulating corticosteroid.

The administration of nicotinic acid to rats is said to cause a significant rise in the level of plasma corticosterone, the response being apparently dependent on an intact pituitary-adrenal axis. In this connection it should be remembered that nicotinic acid is not a vitamin for the rat, which is able to synthesize it in sufficient quantity from its precursor tryptophan, provided that adequate quantities of thiamine, riboflavin and pyridoxine are present. Pellagra in man is usually associated with diets low in both niacin and tryptophan.

**Adrenal Cortex.** The importance of the pyridine nucleotides in the synthesis of the adrenal steroids is indicated in Figs. 8, 9 and 10. The enzymes responsible for aldosterone biosynthesis are located in the mitochondrial fraction of cortical homogenates and require as co-factors reduced nicotinamide adenine dinucleotide phosphate and magnesium ions.

The reversible conversion of cortisone to the much more effective hydrocortisone is catalysed by an NAD or NADP co-enzyme activated reaction. The equilibrium of this reaction may be affected in nicotinamide deficiency with a shift in the balance to the less powerful corticosteroid.

The physiological importance of hydrocortisone, the main glucocorticoid, depends not so much on its effect on carbohydrate metabolism as on its homeostatic effect on mineral and water metabolism, particularly under conditions of stress. The NAD catalysed reaction therefore is of vital importance in the maintenance of circulation, fluid balance and renal function in shock.

**Adrenal Medulla.** Combined niacin and tryptophan deficiency in the rat causes a marked drop in catecholamine production. The rationale of this change is not immediately obvious, but the simple explanation of the marked dependence of tissues of neuroectodermal origin on carbohydrate metabolism may hold here.

**Gonads.** Glucose-6-phosphate dehydrogenase (1.1.1.49), the first enzyme in the direct pathway for oxidation of glucose in the adrenal gland, has NADP as its cofactor. It is twice as active in females and

castrates as in males, which suggests that testosterone may repress its synthesis.

Nicotinamide co-enzymes are known to be required for the oxidation of testosterone to androstenedione in the liver and kidney. This reaction is one link in the chain leading to the synthesis of the oestrogens. Deficiency therefore may be expected to result in lowered oestrogen production.

### Pyridoxine or Vitamin B<sub>6</sub>

This vitamin occurs in animal tissues as pyridoxal and pyridoxamine, and in plant tissues it is present in the form of pyridoxol (Fig. 17). The active forms of the vitamin are the phosphates of these three compounds which act as co-enzymes for the transaminase reactions,

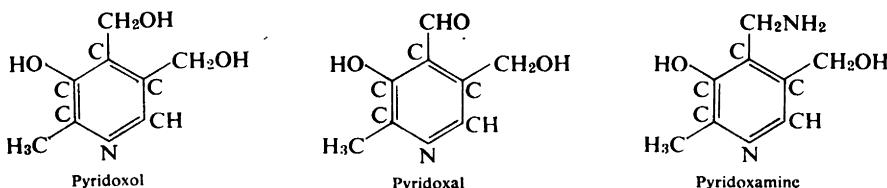


Fig. 17. The three forms of pyridoxine.

and as such they are important in the synthesis of amino-acids, many of which are used to build up enzymes and hormones.

The co-enzymes function also in decarboxylation and desulphuration reactions in amino-acid metabolism. The active forms of the vitamin appear to be bound more closely to the transaminases than to the decarboxylases, which may in part explain the selective inhibition of some enzyme systems, and the variation in clinical syndromes found in various animal species at progressive levels of deficiency.

It is thought also that pyridoxine is involved in the synthesis of arachidonic and hexaenoic acids from linoleic and linolenic acids, and it may therefore have a part to play in the formation of the prostaglandins (Chapter 9).

Generally speaking, pyridoxine requirements are raised in hyperthyroidism, and when dietary protein levels are high. Requirements are increased too in infancy, in pregnancy (to cope with the parasitic demands of the foetus) and during the treatment of tuberculosis by isonicotinic acid hydrazide, which is a specific antagonist. In view of its importance in amino-acid metabolism it is to be expected that various defects in polypeptide hormone synthesis may arise in pyridoxine deficiency. The decreased synthesis of RNA found in the deficiency state may also contribute to impaired hormonal synthesis. The thymic

atrophy of the deficiency state may be the result of impaired protein synthesis.

**Pituitary.** Most of the experimental work on the pituitary in B<sub>6</sub> deficiency has been conducted in the rat. In this animal the pituitary takes up tritiated pyridoxine avidly, and the content of the vitamin in that gland is relatively high compared with the amount in the liver and other tissues. Deficiency can be relatively rapidly induced and brings with it a marked decrease in growth hormone output. There is also a drop in blood glucose and increased sensitivity to insulin, leading to a drop in insulin secretion and output. Pyridoxine is thought to have a rôle in the synthesis of growth hormone, and some amelioration of the deficiency state occurs if growth hormone is given. In the normal rat growth hormone stimulates release of insulin by the pancreas, and the administration of insulin also has an ameliorative effect on the deficiency.

**Adrenals.** The association of pyridoxine deficiency with arteriosclerosis has been noted in some animals. Adrenal blood vessels are among those affected and in cases of long standing deficiency their thickened walls may provide some impediment to the free passage of nutrients from the blood to the adrenal cells for hormonal synthesis.

B<sub>6</sub> deficiency is associated with depletion of zona fasciculata cells of lipids, cholesterol esters and cholesterol as well as ascorbate. This would explain the impaired resistance to stress of deficient animals. Both lipids and stress resistance are restored to normal by administration of pyridoxine.

Pyridoxine and glucocorticoids have a synergistic action on gluconeogenesis, that is, the formation of glucose from non-carbohydrate sources. This is the result of increased activity of alanine aminotransferase (2.6.1.2), for which pyridoxal phosphate is the coenzyme, in the conversion of amino-acid to glucose.

The medullary hormones too may be affected in B<sub>6</sub> depletion. The production of noradrenalin from tyrosine occurs in three steps, and pyridoxal phosphate has a coenzymatic function in the second step, that is the decarboxylation of L-dihydroxyphenylalanine (DOPA) to dihydroxyphenylethylamine (dopamine) shown diagrammatically in Fig. 22. However, this does not seem to be the rate-limiting step in noradrenalin and adrenalin synthesis. Experimentally, it can be shown that DOPA given to B<sub>6</sub> deficient rats is not converted to dopamine as effectively as in normal rats. It is likely that all aminoacid decarboxylases are pyridoxal-phosphate dependent. Other neurohormones with this requirement for their synthesis are serotonin,  $\gamma$ -aminobutyric acid and possibly histamine; there seems to be some debate as to whether the conversion of histidine to histamine is B<sub>6</sub> dependent. In pyridoxal deficiency the serotonin content of the brain and other tissues is much

reduced.  $\gamma$ -aminobutyric acid, a normal constituent of brain tissue, is formed by decarboxylation of glutamic acid. The pyridoxal phosphate dependent enzyme (4.1.1.15), normally present in the brain, is apparently unable to act in  $B_6$  deficiency. It is thought that the occurrence of convulsions in deficient rats or human infants is the result of defective synthesis of  $\gamma$ -aminobutyric acid with resultant failure of regulation of neuronal activity. In swine,  $B_6$  deficiency causes demyelination of peripheral nerves and degeneration of their axons.

**Thyroid.** Hyperthyroidism increases requirements for  $B_6$ , and daily injections of the vitamin are said to ameliorate the muscle weakness usually associated with this condition.  $B_6$  deficiency is thought to lower the basal metabolic rate and the respiratory quotient. Thyroxine appears to have an antagonistic effect on the vitamin and to decrease the liver content of both pyridoxine and pyridoxal phosphate. In contrast, enucleation of the thyroid causes a rise in pyridoxal phosphate content in the liver.

Decreased uptake of radio-iodine by the  $B_6$  deficient thyroid suggests that this vitamin has a role in thyroid hormonal metabolism, either by direct action on the thyroid or indirectly by interfering with thyrotropin synthesis or release.

**Gonads.** During pyridoxine deficiency the pituitary apparently accumulates gonadotropic hormones, indicating some interference with the mechanism for their release. This may be the cause of the abortion which is common in  $B_6$  deficient rats since the administration of follicle stimulating hormone and progesterone restores gestational ability to normal.

#### Pantothenic acid

Pantothenic acid is a compound of pantoic acid and  $\beta$ -alanine (Fig. 18). It occurs in nature as the free acid, and is widely distributed in plant

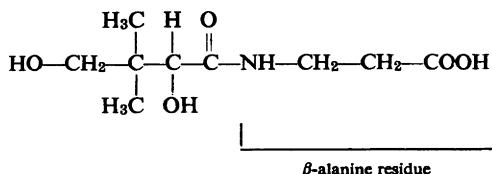


Fig. 18. Pantothenic acid.

and animal tissues. In its active form it is a constituent of the dinucleotide known as co-enzyme A (CoA) and is concerned with reactions involving the active forms of organic acids. Folic acid and biotin are apparently necessary for the utilisation of this vitamin.

Although not much is known about pantothenic acid deficiency in

man, it is certainly an essential food factor for animals, and in its absence adrenal insufficiency and failure lead inevitably to inflammation and ulceration of the gastric and intestinal mucosa at an early stage. The human infant is supplied with an abundance of the vitamin in the maternal milk, no doubt an indication of its essentiality for man. Low blood levels are found in pellagrins and in those suffering from beri-beri and ariboflavinosis.

Acetyl-CoA is the starting point for the Krebs cycle, the main energy producing system of the body. The result of defective CoA synthesis is a reduction in energy as cells switch to the glycolytic system—an alternative which produces much less power. The key position of the Krebs cycle in metabolism entails, in this situation, a deceleration in many other metabolic reactions, including the synthesis of other co-enzymes of the vitamin B complex.

The involvement of CoA in amino-acid metabolism introduces the possibility of peptide hormone defects. Its requirement for the synthesis of the phospholipids, including the lecithins and cephalins may be a major factor in the neural demyelination which is a feature of the deficiency in animals.

CoA has a rôle to play too in the phosphorylations coupled to electron transport, although this seems to be in maintenance of the reaction rather than in stimulatory effect.

As part of the acyl carrier protein, pantothenic acid is involved in the biosynthesis of fatty acids, and may therefore have a function in prostaglandin metabolism.

**Adrenal.** The most damaging effect of pantothenic acid deficiency appears to be on the adrenal. Mild depletion leads to accumulation of fat in zona fasciculata cells—more severe depletion leads to breakdown of cell membranes, fusion of fat filled cells, and eventually the affected cells become necrotic. If the condition is acute, there is adreno-cortical haemorrhage as well as necrosis; this is the pathological picture which develops in acute shock of many types, whether anaphylactic, psychogenic or induced by overwhelming bacterial or viral infection. Pantothenic acid given in excess also causes adrenal damage, with haemorrhage in the cortex.

In progressive pantothenic acid deficiency, the adrenal cortex becomes completely depleted of hormone—a not unexpected result in view of the fact that cortical hormones are synthesised from active acetate (acetyl CoA) *via* cholesterol. Breakdown of cell walls may be attributed partly to deficiency of structural phospholipids, and partly to anoxic change arising from interference with blood flow through the organ caused by the obstruction provided by cells grossly swollen with accumulated fat. Release of proteolytic enzymes from damaged lysosomes may initiate the necrosis of adrenal parenchymal cells.

**Thyroid.** The suggestion has been made that thyroxine may be important in the conversion of pantothenic acid to coenzyme A, but this remains to be confirmed. As with the other B vitamins, hyperthyroidism increases requirements.

**The Pancreatic Islets.** The pantothenic acid deficient rat has impaired ability to break down insulin, an indication of its possible requirement for the hepatic insulinase system.

**Gonads.** Sterility in the pantothenate deficient mouse is associated with testicular oedema followed by degeneration and disappearance of testicular interstitial cells. In the female, sterility is associated with the presence in the ovary of large numbers of atretic follicles and few corpora lutea. In advanced deficiency there may be degeneration of granulosa lutein cells and of the cells of the theca interna folliculi.

#### Biotin

Biotin is a sulphur containing, monocarboxylic acid, derived from valeric acid, which is one of the rather uncommon fatty acids containing

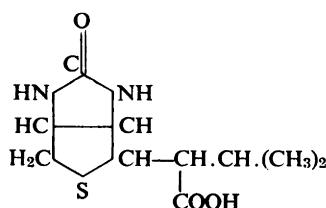


Fig. 19. Biotin.

an odd number of carbon atoms (Fig. 19). Intestinal bacterial synthesis provides useful amounts of the vitamin to both man and animals. In its metabolic rôle it is closely allied to pantothenic acid, and both are concerned, together with other B vitamins, in the synthesis of nicotinic acid.

A marked rise in blood cholesterol in biotin deficiency denotes breakdown in the process of oxidation of the cholesterol sidechain in the liver. This is apparently due to an enzyme defect, involving propionyl CoA carboxylase (6.4.1.3.) which is a biotin containing enzyme. In an enzymatic reaction, the cholesterol sidechain is normally broken off to form isocaproic acid, which is then metabolised in a series of steps through propionate and propionyl CoA to methyl-malonyl CoA before entering the citric acid cycle for oxidation. It is the step from propionyl CoA to methylmalonyl CoA which is inhibited in biotin deficiency. The accumulation of reactants in the chain of reactions has

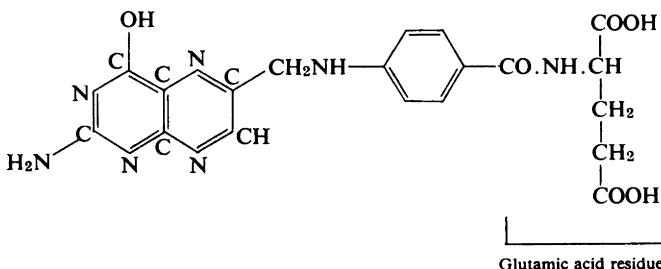
an inhibitory effect on the initial reaction in the chain, which is the cholesterol sidechain cleavage.

Several carboxylation and decarboxylation reactions are affected in biotin deficiency. Acetyl CoA carboxylase (6.4.1.2), essential for the biosynthesis of fatty acids, is a biotin containing enzyme, as is pyruvate carboxylase (6.4.1.1). The reduction in pyruvate oxidation and tissue respiration in deficiency can be expected to cause biochemical defects similar to those initiated by thiamine deficiency.

In biotin deficient rats, deficiency of male sex hormones causes degeneration of testes with delayed spermatogenesis and abnormal spermatocytes.

### Folic acid

Folic acid is the term used to denote a group of pteroylglutamic acids: in particular it is used for pteroylmonoglutamic acid (Fig. 20). It is



*Fig. 20. Folic acid (pteroylmonoglutamic acid).*

sparingly soluble in water, and like other B vitamins, is synthesized by the intestinal flora of man and domestic animals. This source appears to be available for use by man and some animal species, under normal conditions, but may be lost when intestinal bacterial populations are destroyed or markedly changed by sulphonamides, or in any mal-absorption syndrome. Monkeys, guineapigs and some birds are apparently fully dependent on dietary supply. Pteroyltriglutamic acid and pteroylheptaglutamic acid, containing 3 and 7 glutamic acid residues respectively, can be absorbed from the intestine and are then split to the monoglutamic acid by a folic acid conjugase in the blood.

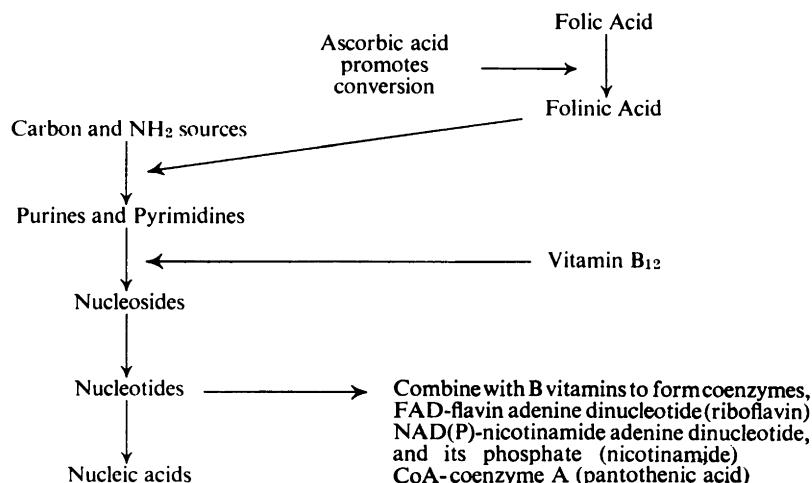
Conversion to the active form, known as folinic acid (tetrahydropteroylglutamic acid, tetrahydrofolic acid and citrovorum factor are synonyms) occurs in the liver in the presence of ascorbate. The conversion is reduced in ascorbic acid deficiency (scurvy).

Folic acid co-enzymes are concerned in a number of methylation reactions, including conversion of ethanolamine to choline, homocysteine to methionine, and nicotinamide to 1-methyl nicotinamide,

the latter being a detoxication reaction. The co-enzymes function in the intermediate metabolism of the purines and the pyrimidines in nucleic acid synthesis, and in certain metabolic functions of the nucleoproteins (Fig. 21).

Folinic acid can be demonstrated in the chromosomes where it has the specific function during mitosis of enabling the chromosome halves to part: that is, it is essential in the metaphase to anaphase step of mitosis. Folate deficiency prevents the synthesis of the two types of nucleic acid necessary for growth after division.

Folinic acid is the only member of the group which has an influence on blood formation, and in this function it is closely associated with



*Fig. 21. Some interactions of vitamins B and vitamin C in nucleic acid synthesis.*

vitamin B<sub>12</sub> and ascorbate. The deficiency interferes with development of the nucleus of the precursor cell in the bone marrow, with arrested maturation of red cells.

**Adrenal.** The rôle of folic acid derivatives in the synthesis of catecholamines is well established and is shown in diagrammatic form in Fig. 22.

In this sequence the tetrahydrofolate assisted conversion of tyrosine to dihydroxyphenylalanine appears to be the rate limiting step in noradrenalin and adrenalin synthesis. The end products inhibit the tyrosine to DOPA conversion, a typical example of the negative feedback mechanism in which the final product of a series of reactions inhibits the initial steps in production when concentration in the medium attains a sufficient level.

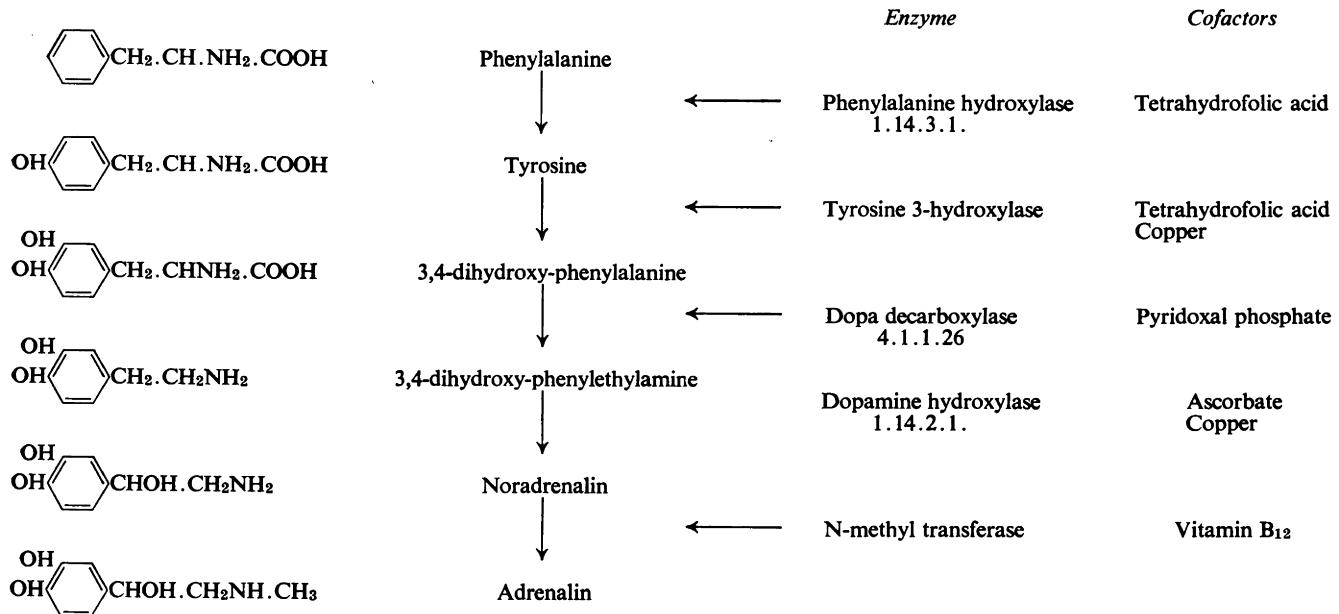


Fig. 22. Enzymes and cofactors in the synthesis of the catecholamines from phenylalanine.

In addition to their synthesis in the adrenal medulla, the catecholamines are produced in the sympathetic nerves and ganglia, and in the argentaffin cells of various organs and tissues. The main product in the nerves is noradrenalin, which acts as the chemical mediator for the transmission of nerve impulses in the sympathetic division of the autonomic nervous system. Insufficient catecholamine release in stress will obviously have widespread effects throughout the body.

Tetrahydrofolic acid has a co-enzymatic rôle in the hydroxylation of progesterone to 17  $\alpha$ -hydroxyprogesterone in the biosynthetic pathway of the adrenocortical steroids.

**Gonads.** The administration of folic acid increases the androgenic action of testosterone, but the mechanism of this action is not clear. Prolonged deficiency interferes with the sensitivity of the tissues to oestrogens, and the ovarian hormones are insufficient to maintain pregnancy.

**The prostaglandins.** Folic acid appears to function in the conversion of 8, 11, 14 eicosatrienoic acid to prostaglandin (see Chapter 9 on Essential Fatty Acids).

#### Vitamin B<sub>12</sub> or cyanocobalamin

Vitamin B<sub>12</sub> is a complex porphyrin derivative containing a cobalt atom, linked to a nucleotide (Fig. 23). The 'B<sub>12</sub> vitamins' are various metabolites which can be used by vertebrates. Animals are dependent for their dietary supply on the synthesis of B<sub>12</sub> by micro-organisms. Although the intestinal flora synthesise large amounts, this source is apparently not available, except in those animals which practise coprophagy. B<sub>12</sub> is present in adequate amount only in foods of animal origin, such as meat, eggs, cheese and milk. Vegetarians, as a group, therefore, are most likely to show signs of deficiency.

Absorption of the vitamin is almost entirely dependent on conjugation with a mucoprotein of low molecular weight (about 15,000) normally secreted by the stomach, and known as the 'intrinsic factor'. The compound is absorbed more easily through the intestinal wall than is the vitamin alone. Interference with absorption in man is caused by infestation with *Diphyllobothrium latum*, a tapeworm with an intermediate stage in fish, which often parasitises man in the Scandinavian countries and in other places where fish forms a large part of the diet. The tapeworm competes with the host for intestinal B<sub>12</sub>, thereby causing secondary megaloblastic anaemia. Pyridoxine deficiency also interferes with assimilation of B<sub>12</sub>.

The cobalt atom is an essential part of the vitamin molecule, and animals reared on cobalt deficient soil suffer a secondary B<sub>12</sub> deficiency. Ruminants are able to synthesise and use the B<sub>12</sub> molecule if cobalt is present in the diet.

Absorption appears to be conditioned by the endocrine activity of the host, as can be shown by the fact that thyroidectomy in rats prevents absorption; the situation can be remedied by feeding thyroxine or desiccated thyroid gland.

Most of the body stores are in co-enzymatic form. The B<sub>12</sub> group of co-enzymes forms a distinct group of corrinoid compounds. They are not as readily absorbed from the intestine as B<sub>12</sub> itself. Their metabolic

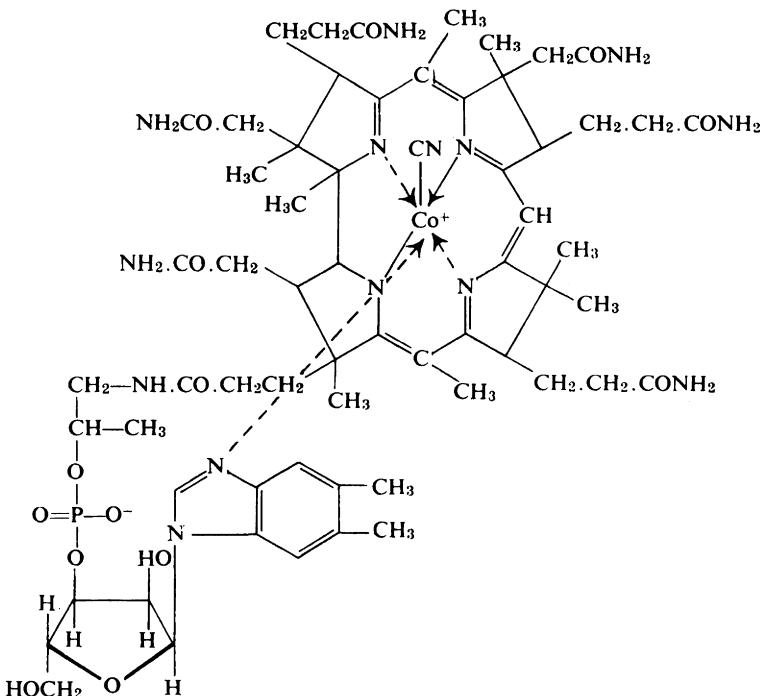


Fig. 23. The vitamin B<sub>12</sub> molecule or cyanocobalamin.

functions have not as yet been completely elucidated but it is known that they have no special function in pernicious anaemia.

B<sub>12</sub> has an important role in amino-acid metabolism, and its absence leads to inhibition of methionine synthesis, with resulting decrease in essential amino-acid and protein synthesis. Absence also leads to a block in RNA synthesis (Fig. 21). In this latter reaction and in trans-methylation reactions in choline and adrenalin metabolism, the vitamin functions in close association with folic acid.

The administration of cortisone to normal rats causes a rise in serum B<sub>12</sub> levels, possibly by releasing the vitamin from its tissue stores. The administration of either ACTH or cortisone causes increased urinary

excretion of B<sub>12</sub>, which may result from a cleavage of the link binding protein and vitamin in the tissues, or destruction of the carrier protein.

In B<sub>12</sub> deficiency there is failure of reproduction in both man and animals. The condition in man is associated with failure of maturation of spermatozoa. Low levels of cyanocobalamin may lead to sterility in women, or to foetal abnormality.

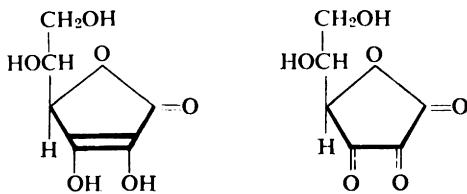
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## *Chapter 6*

### **Vitamin C**

Vitamin C is widely distributed in nature in both plant and animal tissues. It is a water soluble monosaccharide, occurring in the reduced and oxidised forms known as ascorbic acid and dehydroascorbic acid respectively (Fig. 24). Both forms are biologically active and both have



*Fig. 24. L-ascorbic acid and L-dehydroascorbic acid.*

the ability to prevent or cure scurvy. Of the two forms, dehydroascorbic is relatively more soluble in lipid. Within the animal body the distribution is uneven. Tissues with a high vitamin C content include the pituitary, adrenals, the lens and aqueous and vitreous humours of the eye, the intestines and the white blood cells. Tissues with a relatively low vitamin C content are the brain, muscles and red blood cells.

The vitamin is synthesised within the animal body from glucuronic acid, which is first converted to L-gulonic acid in a reaction which requires reduced NAD as cofactor. The gulonic acid is then converted via L-gulonic acid lactone and 2-keto-L-gulonolactone to ascorbic acid. The last step in the conversion cannot be undertaken by animals which lack the required enzyme. These include man, other primates, the Indian fruit bat and the guineapig. Most other animals possess the necessary enzyme for vitamin C synthesis and for them ascorbic acid, although essential, is not a vitamin. It is interesting that the agouti, which is closely related to the guineapig is able to synthesise its own vitamin C. Amphibians, reptiles and most birds are likewise independent of dietary supplies. Conditioned deficiency may arise in the C-independent animals during other deficiency states. It is known for example that in A deficiency in rats, the synthesis of ascorbate by the liver microsomes is greatly reduced.

Biochemically, the main task of ascorbic acid and dehydroascorbic acid is to function as intermediates in hydrogen transfer systems.

Requirements are high in physical stress, in pregnancy and in infectious disease. In infections accompanied by fever, the oxidised form tends to accumulate in the blood; return to normal health reverses the balance with a rise in ascorbic acid level. C-avitaminosis, manifested as scurvy, occurs in old people who lack the ability or the desire to consume balanced diets, and symptoms of the disease may be well advanced before the condition is diagnosed. Artificially fed infants, too, are susceptible to the condition in early post-natal life; this is because cow's milk is relatively low in vitamin C, and the small amount present is readily oxidised, even when the milk is fresh. C-deficient infants and guineapigs excrete in their urine  $\beta$ -hydroxyphenylpyruvic acid and homogentisic acid. The latter substance, on oxidation, gives a dark colour to the urine, as in the condition known as alkapturia which is said to be an inherited metabolic defect. In the C-deficient infant and guineapig, the activity of the oxidase (1.14.2.2) catalysing the conversion of  $\beta$ -hydroxyphenylpyruvate to homogentisic acid, and the conversion of the latter to maleyl acetoacetate (1.13.1.5) are dependent on the presence of ascorbate. The activity of the enzymes can be restored *in vitro* by the addition of ascorbic acid to the catalytic systems.

When vitamin C is lacking in the diet, depletion of the tissues is slow in man, but relatively rapid in the guineapig, in which scurvy can be induced in three to four weeks. The guineapig has the highest requirement for vitamin C, relative to its weight, of any of the mammals so far investigated.

In some vitamin C dependent reactions, ascorbic acid is oxidised to dehydroascorbic acid in a reaction catalysed by ascorbate oxidase, a copper containing enzyme (1.10.3.3). Regeneration takes place through monodehydroascorbic acid, a partially oxidised form. Electron transfer is mediated by a flavin transhydrogenase (Fig. 25). In many reactions

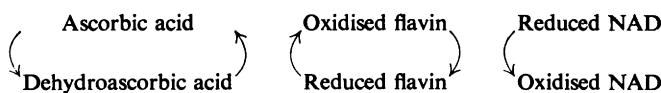
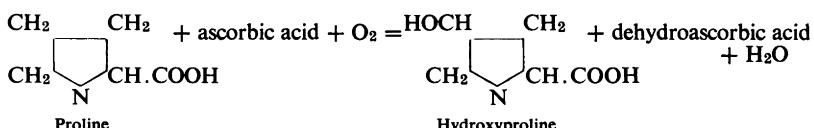


Fig. 25. Regeneration of ascorbic acid in electron transport mechanism.

*in vitro*, the ascorbic acid can be replaced by other electron donors, but these have no antiscorbutic action *in vivo*.

In scurvy, the primary lesion is the development of abnormal connective tissue. Vitamin C is required for the synthesis of collagen. Normally, the turnover of collagen is low, but in prenatal life and early post-natal life, synthesis is required for the supporting connective tissue of most organs, including the endocrine glands, and for their vasculature. The essential process mediated by ascorbic acid is the formation of hydroxyproline from proline (Fig. 26) and of hydroxylysine

from lysine. The basic collagen molecule consists of three linked polypeptide chains of aminoacid residues. Hydroxyl groups attached to a proportion of the proline units give strength and rigidity to the molecule. These hydroxylations occur after the aminoacids are linked together into the peptide chain, and require for their action, oxygen, ferric iron,  $\alpha$ -ketoglutarate and ascorbate. The enzyme concerned in proline hydroxylation is known as collagen proline hydroxylase. Defective hydroxylation within the synthesizing fibroblast gives rise to



*Fig. 26. Hydroxylation of proline, with ascorbic acid as the cofactor for the hydroxylating enzyme.*

the formation of abnormal collagen precursor, called procollagen. This substance may not be extruded from the cell as is the normal precursor, tropocollagen; if it is extruded then it polymerises into an abnormal collagen which lacks the tensile strength of the genuine article. In addition to its requirement for collagen synthesis, it is believed that ascorbate is also an essential cofactor in forming the intracellular cement substance which binds the lining cells of blood vessels to their basement membrane and to each other.

**Pituitary.** The amount of vitamin C in the pituitary is relatively high, but its function there is not known. Experimentally, the content of vitamin C can be depleted by injections of hypothalamic extracts in the rat. In this animal, there is a sex difference in tissue ascorbate content, and hypophysectomy in the male rat reduces tissue levels to those characteristic of the female. It is assumed that this sex variation is dependent on differences in liver biosynthetic enzyme activities, themselves under the control of pituitary-stimulated testicular androgens.

**Thyroid.** Experimental hyperthyroidism reduces tissue concentrations and increases requirements for ascorbic acid. In experimental deficiency in guineapigs there is haemorrhagic infiltration of the gland (or bleeding from capillaries in the gland), particularly in acute scurvy. In chronic scurvy the gland becomes hyperplastic and hypersecretory. All three changes are reversible by restoration of dietary ascorbate to an adequate level.

**The Adrenal Cortex.** The adrenal cortex is rich in vitamin C where its presence can be demonstrated by chemical, histochemical or biological

methods. In the rat the ascorbate level may reach 400–450 mg/100 gms as compared with 1·5 mg in plasma, 35 mg in the lung, or 60 mg in the spleen. The adrenal ascorbate content is greater in animals able to synthesise it, than in those animals which require exogenous supplies of preformed vitamin. It is present chiefly in the zona fasciculata and the zona reticularis, in association with the mitochondria, mainly as ascorbic acid—there is very little detectable dehydroascorbic acid present.

In spite of the great amount of work being done on corticosteroids there is as yet no definite knowledge of the role of vitamin C in the adrenal cortex, and at the moment one can only assume that the two forms of the acid are involved in oxidation and reduction reactions as hydrogen acceptors and donors.

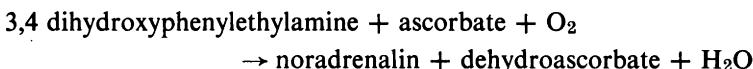
Stimulation of the adrenal cortex by ACTH or by adrenalin leads to depletion of both ascorbic acid and cholesterol. Depletion of the former has been used in countless experiments as an index of the effect of various stressful stimuli, such as infectious diseases, physical trauma, and reaction to shock of various kinds. The autonomic nervous system does not appear to play any direct rôle in depleting the hormonal or ascorbate content of the cortex.

ACTH exerts its secretory effect on the two inner zones of the cortex, the outer zone, or zona glomerulosa being largely independent of hypophyseal control. With the electron microscope, cells can be seen to respond to ACTH stimulation by increase in the number and size of mitochondria and by a decrease in the number and size of lipid vacuoles in which it is assumed that formed cortical hormones are discharged from the cell. A sustained increase in the level of ACTH stimulation induces a sustained increase in the rate of hormonal production and a rise in the level of the pyridine nucleotides which have a coenzymatic rôle in the hydroxylation of steroid molecules. There is a simultaneous rise in the content of enzymes and co-enzymes required for the energy-yielding systems of the cells. However, if the stressful stimulus is unduly prolonged or excessive, a state of adrenal exhaustion supervenes, with complete depletion of ascorbate. Mitochondria become reduced in size and number. This is associated, in prolonged depletion, with haemorrhage into the gland due to capillary fragility, when the supporting framework of the adrenal blood vessels is no longer able to contain the pressure of the circulating blood. This is assumed to be due to inability to synthesize collagen to maintain the structural integrity of the capillaries. Although the turnover of new collagen is relatively low in the adult, it is an important and continuing process in infants. In the aged too, there is a requirement in blood vessels for new collagen to reinforce walls weakened by the depolymerization of old collagen, which is an inevitable aging process.

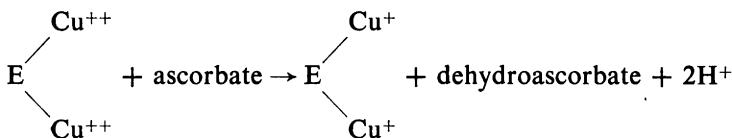
In old age there is decreased cortical response to the ACTH stimulus.

This can be restored to a certain extent by administration of ascorbic acid, which causes a rise in blood 17-hydroxysteroids and in excreted urinary 17-ketosteroids. The connection between ascorbic acid and steroid hormone synthesis is therefore marked, but not as yet understood.

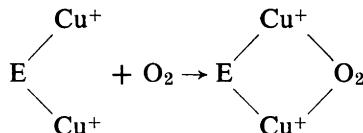
**Adrenal Medulla.** A high concentration of vitamin C is found in the medulla of mammals. It is present in the form of dehydroascorbic acid, and is one of the cofactors required in the enzymatic catalysis of dihydroxyphenylethylamine to noradrenalin, thus:



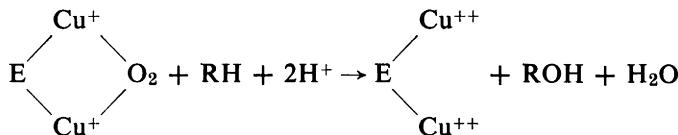
The enzyme concerned, Dopamine hydroxylase (1.14.2.1) has been isolated from bovine adrenal glands, and has been shown to be a copper containing protein. During the reaction part of the protein-bound copper undergoes cyclic reduction and oxidation. The enzyme (E) is first reduced by ascorbate.



The reduced form then reacts with oxygen



and the substrate (RH)



to give the hydroxylated product; in this case, dihydroxyphenylethylamine (RH) is converted to noradrenalin. The protein-bound copper is also reoxidised to the Cu<sup>++</sup> state during this reaction. The function of ascorbate therefore in catecholamine synthesis is to reduce the essential metal on the enzyme so that the reduced enzyme may mediate in the hydroxylation reaction (Friedman and Kaufman, 1966).

**The Gonads.** Vitamin C is present in high concentration in the interstitial cells of the testis and in the ovary.

There is a sex difference in the activities of the liver enzymes responsible for ascorbate synthesis in the rat, manifested as a much greater activity in the male, with concomitant higher level of ascorbate in the tissues. One of the enzymes, gulonolactone hydrolase, which is activated by growth hormone, is much reduced in activity in the hypophysectomized animal, with resulting decrease in tissue ascorbate levels in both sexes.

Hypophysectomy in the male rat decreases the activities of gulonolactone hydrolase, gulonate NADP reductase, and gulonolactone oxygen oxidoreductase to the levels characteristic of female rats. Castration of male rats leads to a reduction in activity of all three enzymes to the female level. It seems therefore that the higher tissue levels of ascorbic acid are dependent on the rate of hepatic biosynthesis, which in turn is kept at a high level in the male rat by the stimulatory effect of testicular androgens (Stubbs, McKernan and Haufrect, 1967).

Stimulation of the rabbit ovary by gonadotropin causes a rapid and marked decrease in ovarian interstitial cell ascorbate content, suggesting a correlation between ascorbate level and oestrogenic hormone production. The decrease is followed by a moderate compensatory increase in ascorbate content.

Gonadotropic stimulation of the corpus luteum of pregnancy likewise causes a rapid decrease in ascorbate content, followed by a pronounced rise. This also suggests an association between vitamin C and steroid production (Hökfelt, 1950).

However, increased production of hormone in non-steroid producing glands, such as the anterior lobe of the pituitary, is also associated with decreased ascorbate content, which may perhaps reflect increased activity of the gland. Much work remains to be done on this problem.

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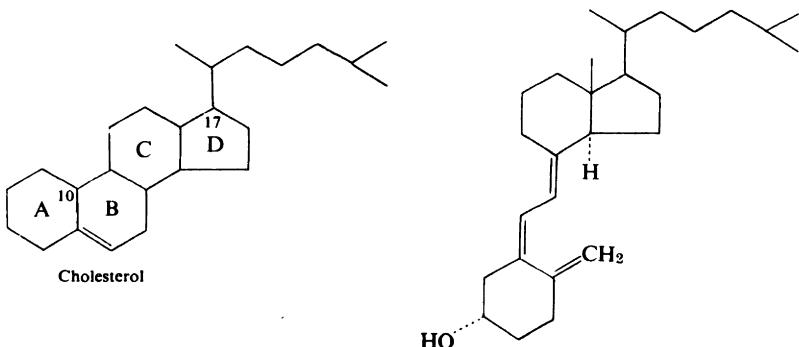
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## *Chapter 7*

### **Vitamin D**

The antirachitic vitamin  $D_3$  or cholecalciferol, occurs naturally in the animal body, where it is formed in the skin by irradiation of its provitamin, 7-dehydrocholesterol; all the higher animals have the ability to synthesize the provitamin. In foodstuffs, cholecalciferol is found in high quantity in fish oils, and in moderate amount in egg yolks. It is also present in cow's milk, normally at a level less than half of that in human milk. In cows' milk it is associated with calcium and phosphorus levels 3–5 times greater than the human levels. Recent work has suggested that preformed  $D_3$  occurs in palm oil and in graminaceous leaves, stalks and roots.

Artificially produced steroid alcohols, closely resembling vitamin  $D_3$ , include  $D_2$  (calciferol),  $D_4$ ,  $D_5$  and  $D_6$ . These antirachitic substances as a group are characterised by the opening of the B ring of the cholesterol molecule, and by the presence of a methylene group at position 10 in place of the methyl group (Fig. 27). The synthetic



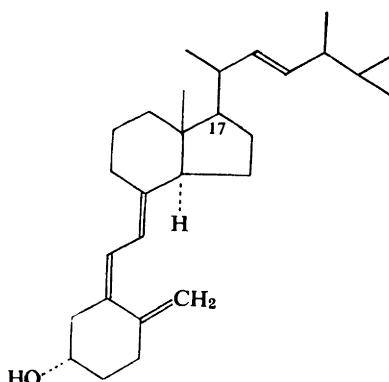
*Fig. 27.* The structure of vitamin  $D_3$  or cholecalciferol. The side chain on carbon 17 is the same as that of cholesterol.

vitamin  $D_2$  differs structurally from  $D_3$  in having an unsaturated side-chain. It is prepared by irradiation of ergosterol, a vegetable sterol present in ergot and in yeasts.  $D_2$  varies in its antirachitic potency in various animal species, and is rather more toxic than the naturally occurring animal vitamin (Fig. 28).

Vitamin  $D_3$  can be assimilated as such from the diet. Its absorption

depends on the presence of bile in the intestinal canal, so that any liver disease interfering with bile secretion, or chronic intestinal disease interfering with absorption may lead eventually to vitamin D depletion.

The vitamin, whether ingested or endogenously formed is stored mainly in the liver, but is also present in the bones, skeletal muscle, and in the blood plasma in which it is associated with the  $\alpha_2$  globulin and albumin fractions. It is found too in the intestinal walls, the adrenals and the kidneys, in conjunction with the microsomal fractions of the cells. Some of the ingested vitamin is apparently esterified in the intestinal wall and a small proportion may be esterified in the blood plasma by a cholesterol esterifying enzyme. Fraser and Kodicek (1968)



*Fig. 28. The structure of vitamin D<sub>2</sub> or ergocalciferol. The side chain on carbon 17 is unsaturated.*

have shown that in the rat lymph stream, vitamin D is esterified with palmitate, stearate, oleate and linoleate. These esters are apparently the storage forms of the vitamin.

In contrast to vitamin A, vitamin D is stored in relatively small amounts in the animal body, and therefore is more readily depleted. Excessive dietary cholecalciferol may be excreted in the bile, conjugated with glycine and taurine. Very little is excreted in the urine.

Vitamin D functions mainly as a regulator of the calcium-phosphorus balance of the body. This is achieved (1) by increasing the intestinal absorption of calcium and phosphorus and (2) by the mobilization of phosphate from the tissues and (3) by the control of the transfer of calcium and phosphorus between blood and bone. Vitamin D in adequate amount raises the calcium and phosphate to such a level in the tissue fluid that calcium salts become precipitated in the bone matrix when there is need for growth or remodelling of bone.

The normal integrity of bone is a function of the osteocytes, which

are concerned with the metabolism and composition of both organic and inorganic phases. The osteoblasts (bone forming cells) and the osteoclasts (bone dissolving cells) are closely related cells and it is likely that osteocytes may be differentiated into one or the other in response to hormonal stimuli.

The numerous factors controlling change in bone are far from being understood as yet, but it is known that in prenatal life vitamin A is concerned in the balance between osteoblastic and osteoclastic activity. In early postnatal life the pituitary growth hormone is concerned, and at all stages the parathyroid hormone in close association with vitamin D exercises a regulatory influence on the turnover of bony metabolites.

The conversion of organic phosphorus in tissue fluid to inorganic phosphate probably takes place in the bone itself, the change being mediated by an alkaline phosphatase in the osteoblasts. Histochemically, it can be shown that when bone is being formed the osteoblasts give a strongly positive reaction for alkaline phosphatase, and it is thought that this enzyme is activated by vitamin D. The enzyme is present in ossifying cartilage, bone, kidneys, adrenals, intestinal wall and liver and operates at an optimal pH of about 9. Normally, blood plasma is slightly supersaturated with calcium phosphate and deposition in the tissues is prevented by the inhibitory action of inorganic pyrophosphate. Alkaline phosphatase has the ability to destroy this inhibitor and thus allow local deposition of calcium salts.

Solution of bone with the liberation of calcium is dependent on osteoclastic activity. The lysosomes in these multinucleate cells can be shown histochemically to contain acid phosphatases, zinc-containing enzymes, which exert their lytic effect at a pH of 7 or less. Suitable environment for the activity of these enzymes may be provided by the increased release of citric acid and lactic acid induced in bone under the influence of parathyroid hormone and vitamin D. Calcium is mobilised in the form of soluble calcium citrate complexes for transfer to circulating blood. The osteoclastic lysosomes also contain proteases which have the ability to dissolve the collagen which forms a very large part of the organic matrix of bone.

The above-mentioned presence of alkaline phosphatase in the soft tissues suggests that a similar activation of the enzyme by combined vitamin-hormone stimulus may exercise control over the intestinal absorption of dietary phosphorus, in which organic phosphate must be converted to inorganic phosphate before it can be utilized by the body. Whether the vitamin and hormone act in conjunction in phosphate uptake and retention by the renal tubule appears to be still the subject of debate, but there are grounds for thinking that the passage of Ca and other divalent cations including Mg and Zn across membranes is under the combined control of parathormone and cholecalciferol.

Perhaps the most important function of vitamin D is the hormonally

assisted transfer of calcium, magnesium and zinc from the intestinal canal to the plasma. Research has been concentrated on calcium metabolism but a growing interest in the importance of magnesium as a structural part of bone and as an enzyme activator should restore the balance in the near future. Ionized calcium, which is the physiologically active fraction of the plasma calcium, forms about 60% of the total, and its level is kept constant within narrow limits by the combined action of vitamin D, parathyroid hormone and calcitonin.

### The Parathyroid and Vitamin D

The parathyroid secretes, in its chief cells, a polypeptide hormone. For obvious reasons, nearly all the basic research on this hormone has been carried out with the bovine product, which is a polypeptide of molecular weight about 10,000. Release of the hormone occurs in response to a fall in the circulating blood calcium level, as when the diet is low in calcium or vitamin D. Changes in blood phosphate levels have no direct effect on hormone release but increased plasma phosphate may cause precipitation of calcium phosphate, thus reducing the circulating ionized calcium and causing a secondary release of parathyroid hormone. It can be shown experimentally that magnesium ions exert an effect on hormone release similar to that of calcium, but quantitatively much less. Excessive levels of both cations cause a drop in hormone production. The main function of the parathyroid hormone is the maintenance of the balance between the intake and the excretion of calcium, with the object of maintaining calcium homeostasis in the blood. Although the basic mechanism of its action is not known, vitamin D is known to be essential for most of the reactions activated by the hormone. The known target organs for parathyroid hormone include the small intestinal wall, the kidneys and the bones. Parathyroid hormone, in the presence of adequate vitamin D promotes the absorption of calcium from the small intestine. There is as yet no evidence that its ability to stimulate phosphate absorption is vitamin dependent. In the kidneys parathyroid hormone stimulates the urinary excretion of phosphate by preventing tubular reabsorption. Simultaneously there is decreased excretion of magnesium and calcium. Vitamin D is thought not to be greatly concerned in these processes, as the transport across the renal cellular membranes can take place to a certain extent in the absence of the vitamin. Normal urinary excretion of calcium is dependent on the plasma calcium level, which in turn is dependent on both vitamin D and parathyroid hormone activities. Excessive amounts of the hormone, whether endogenous as in hyperparathyroidism, or given by injection, cause a decrease in blood phosphate and an increase in the blood calcium.

Parathormone is known to increase the production in the bone of lactate and citrate, the calcium salts of which are readily soluble, and

may contribute to the rise in total blood calcium. Vitamin D likewise can be shown to increase blood citrate when given to rats on a normal or D deficient diet and it is likely that the vitamin and the hormone are synergistic in this respect.

The hormone mediates its effect on bone by stimulating the activity of osteoclasts, with resulting solution of bone salts and breakdown of the collagenous matrix. The mechanism of the differentiation of mesenchymal cells into osteoclasts is not understood. The transition in function probably arises when the hormone induces the synthesis of increased quantities of lysosomal enzymes, or activates the enzymes already present in these subcellular organelles.

Osteoclasts function locally as a group, and the stimulus to clumping may also be hormonally induced, although this question remains to be resolved. A further debatable point is whether lysosomal enzymes must be released from their limiting membrane before they are activated, or whether the enzyme substrate is ingested by the cell in a pinocytotic process and fused with the lysosomes before the enzymatic reaction takes place. Electron microscopical evidence indicates that the pinocytosis-fusion method occurs under certain conditions, but it is difficult to visualise such a process in the case of bone salts. It may be that the potentially devastating effect of the release of lytic enzymes is kept under control by the aggregation of osteoclasts around a crystal of bone salts so that lytic changes may proceed in the centre of the giant cell thus created, without damage to surrounding tissues. Prolonged excessive doses of parathyroid hormone in the experimental animal stimulate osteoclastic activity to such an extent that rarefaction of bone occurs. The collagenous matrix is attacked by proteolytic enzymes giving rise to a condition known as osteoporosis. This condition also occurs naturally, in hyperparathyroidism.

Adequate supplies of vitamin D are essential for the activation of osteoclasts by parathyroid hormone, and in D deficiency the tissues may be quite unresponsive to hormonal effect. To a certain, but limited extent the inhibitory effect of avitaminosis D may be overcome by the provision of excess hormone, but in no sense does one compensate for the other. The hormone and the vitamin act synergistically, and must be present in the tissue fluids in optimal proportions for calcium homeostasis. Deficiency of either parathyroid hormone or vitamin D leads to hypocalcaemia and tetany. The latter, although usually associated with a drop in blood calcium may in some cases be due to the drop in blood magnesium which is a feature of both hypoparathyroidism and avitaminosis D.

**Calcitonin** (or thyrocalcitonin) is a protein hormone elaborated in the parafollicular or C-cells of the thyroid gland in domestic animals. In man it is found in the thyroid, parathyroid and thymus. It has the

ability to oppose the action of parathyroid hormone by lowering the blood calcium and inhibiting solution of bone crystals. The hormone has only recently been discovered and work is still in progress to determine its mechanism of action, and functions. A comparative study of the secreting cells has been undertaken by Moseley and his associates (1968), and their list of references should be consulted for details of the discovery, isolation, chemical characterisation and functions of this new endocrine system.

Calcitonin is stored in the microsomal fraction of the secreting cells. It is a lipophilic polypeptide with a molecular weight of about 3,600. There are marked species variations in its composition. The stimulus to secretion is hypercalcaemia in the blood passing through the thyroid gland, and in prolonged hypercalcaemia the gland may be completely depleted of hormone. During periods of hypocalcaemia the hormone tends to accumulate in the parafollicular cells.

In the experimental animal thyrocalcitonin causes a drop in Ca and phosphate levels but magnesium level remains unaltered. *In vitro* it inhibits the action of parathyroid hormone on bone resorption. This action of calcitonin appears to take place in the bone itself, where it either inhibits the mobilization of bone calcium and phosphorus into the extracellular fluid, or promotes the deposition of calcium.

Normally the calcium ion concentration of the blood is restricted within very narrow margins by the balance between the hypercalcaemic action of parathormone acting as a coarse adjustment and the hypocalcaemic action of calcitonin acting more rapidly, and for a short term only, as a fine adjustment. Any disturbance in calcium homeostasis which causes a rise in the circulating level of one hormone causes a fall in the level of the other. The mechanism of action of calcitonin is not apparently a direct inhibition of the parathyroid hormone calcium mobilizing system, as calcitonin is able to exert its effect in parathyroidectomised animals. In addition, thyrocalcitonin is able to exert its effect in D deficiency, a condition in which parathormone is unable to mobilise calcium.

**D deficiency.** The deficiency syndrome varies with the species concerned, and varies in degree in members of a given species. It is, for example, difficult to induce D deficiency rickets in rodents unless the calcium/phosphorus ratio of the diet is unbalanced. The deficiency state, manifested as rickets is likely to arise in artificially fed human infants. In adults the condition of osteomalacia may occur during periods of physiological stress such as pregnancy and lactation, or following gastrectomy or low intake. Low levels of vitamin D may be prevalent in people deprived of facilities for skin irradiation, including miners and the inhabitants of high latitudes during the long winter months. Dark-skinned immigrant children in relatively sunless Britain are

particularly prone to D deficiency, as their skin sterols are screened from the activating effect of solar irradiation by their natural melanin pigmentation, which normally protects them from excessive irradiation in their own countries.

Compensatory hyperparathyroidism occurs in both D deficient and calcium deficient subjects in an attempt to adjust calcium homeostasis. This it may well do, in the case of calcium deficiency, but only at the expense of further demineralization of bone. In D deficiency, as already mentioned, the bony tissues are unresponsive to parathormone. Intestinal absorption of calcium in D deficiency is greatly reduced and this too is apparently a parathormone dependent reaction. It is not as yet clear whether the hormonal effect on phosphate metabolism is independent of vitamin D, or whether the reactions concerned may be induced by very low concentration of the vitamin, which, though sufficient for phosphate turnover, are quite insufficient to activate calcium translocations.

Although in infantile rickets, absorption of both calcium and phosphorus is reduced, the blood calcium level is not usually much diminished, but the blood phosphorus is markedly low. Serum alkaline phosphatase is generally high, suggesting that there is some failure in the activation of the enzyme, with consequent interference with phosphate metabolism in the target organs. A further complication is the low availability of the divalent cations, which are essential cofactors in phosphatase reactions, as a consequence of failure in the intestinal mechanism for their absorption in D deficiency.

Renal rickets arises when a renal tubular or intertubular defect, inherited or acquired, interferes with the reabsorption of phosphorus in the tubules. Phosphate is also lost through the bowel wall and excreted as insoluble calcium phosphate. The condition is associated with secondary hyperparathyroidism, causing defective calcification of bones, and metastatic calcification of arteries. Skeletal deformities and dwarfism occur in the young, and osteomalacia develops in adults suffering from chronic renal disease.

**Vitamin D excess.** Vitamin D resembles the other fat soluble vitamin A in being highly toxic when given in excess. It has been calculated that the lethal dose is about two thousand times that of the physiological dose, but prolonged pathological alterations can be induced with doses much lower than this. High blood calcium levels can be expected in children fed vitamin D supplements together with cow's milk, which has an unnaturally high calcium level for the human species although its calcium content is admirably suited to the requirements of the calf. It is said that vitamin D excess may occur as a result of prolonged exposure to ultra-violet light.

D excess increases the rate and quantity of absorption of calcium

and phosphorus from the food. Blood calcium and phosphorus levels then are raised to the point where calcium is deposited in the soft tissues, particularly in the kidney, either in the cortex or the medulla depending on the animal species involved. The blood vessel walls are subject to metastatic calcification, but all organs, including the parathyroid, may be affected in prolonged excess. Interstitial deposits of calcium in the lungs may prove fatal in both man and animals. The deposited salts are identical with those normally found in bone.

There must be few fields of hormonal research in which results obtained by various groups of workers show so many irreconcilable discrepancies as the subject of parathyroid hormone, vitamin D, and calcium and magnesium interrelations. A small part of the confusion can be attributed to species variations in reactivity to given hormonal or vitamin preparations. For example in some animals there is withdrawal of calcium from bones in hypervitaminosis D. The rat and the rabbit are two such creatures. In cattle, however, excessive doses of vitamin D seem to favour mineralization of bone, which becomes heavy and dense.

Another cause for discrepancy may arise from the fact that much of the hormonal work in the field is pursued with parathormone of bovine origin. Although there seems to be a certain amount of homogeneity in parathormones of different origin, slight variations in molecular structure may be detected by the antibody forming apparatus of the host body, so that in time immunity develops to the alien hormone. This immunity may be directed both against the variant part of the molecule, and against the homogenous part of the molecule in its rôle as hapten.

The complex interactions of parathormone, calcitonin, vitamin D, the phosphatases and the divalent cations concerned in their chemical reactions are still not clearly understood. Elucidation of the many problems concerned with calcium and magnesium homeostasis must await further information on the nature of the alterations in cell membranes caused by variations in vitamin and hormone content of tissue fluids. As a general premise it can be said that vitamin D and parathormone act synergistically in the transport of calcium across membranes, and that the hormone is not dependent on the vitamin, or is only very slightly dependent on the vitamin in its effects on phosphate transport. To state however that the hormone-vitamin complex activates cell membranes to allow passage of the extremely important cations simply underlines our ignorance of the true nature of the mechanism involved.

**Vitamin D and the Thyroid.** Vitamin D, according to some workers has the effect of increasing the basic metabolic rate, when given to normal animals, and it is suggested that the effect is mediated by the thyroid.

In experimental hyperthyroidism, vitamin D counteracts the increased calcium excretion, and returns the calcium balance to normal. D deficiency alone apparently has no direct effect on thyroid function.

**Vitamin D and the Adrenal.** Adrenalectomy of itself does not interfere with the known activities of vitamin D.

Cortisone has been used to mitigate the effects of hypervitaminosis D in man. From experimental work, it would appear that any beneficial effect obtained could be attributed to the inhibition by cortisone of calcium transport across the intestinal wall. Presumably a much more lasting effect could be obtained by dietary control. Cortisone has no beneficial effect on hypervitaminosis D in the rat.

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As this book was about to go to press DeLuca (Fed. Proc., **28**, 1678, 1969) reported the strong possibility that the metabolically active form of vitamin D<sub>3</sub> is 25-hydroxycholecalciferol (See figures 7 and 27 for the position of the hydroxyl group on the sidechain of the cholecalciferol molecule.)

## Chapter 8

### Vitamin E

A group of at least eight substances, occurring in plant and animal tissues, some of which are endowed with vitamin E activity, has been given the generic name of tocopherols. They are all derivatives of a hypothetical compound known, in abbreviated form, as tocol (Fig. 29).

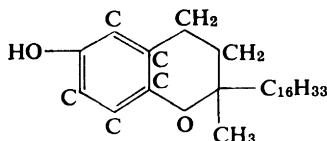


Fig. 29. Tocol.

This is composed of a 6-hydroxyl chromane nucleus, with a methyl group and an isoprenoid side chain at the two position (Fig. 30).

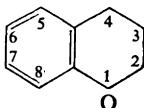


Fig. 30. The chromane nucleus.

The tocopherols differ only in the number and position of the methyl groups on the benzene ring of the chromane nucleus, which is numbered as in the figure above. Biologically the most active of the E group is  $\alpha$ -tocopherol, or 5,7,8 trimethyltocol, which has the constitutional formula given in Fig. 31.

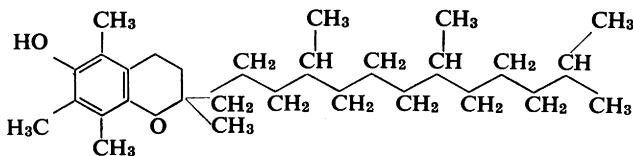


Fig. 31.  $\alpha$ -tocopherol or 5, 7, 8 trimethyltocol.

$\beta$ -tocopherol, which has about one third of the activity of  $\alpha$ -tocopherol, has methyl groups at the 5 and 8 positions, and is therefore known as 5,8 dimethyl tocol.  $\gamma$ -tocopherol, or 7,8 dimethyl tocol has

about one fifth of the potency of  $\alpha$ -tocopherol, and has methyl groups at positions 7 and 8. Several monomethyl tocopherols are known, but these are apparently not biologically active. The tocopherols are widely distributed in nature, in green plants and in cereals. The  $\alpha$  and  $\beta$  forms occur in barley and wheat; the less active  $\gamma$  form is found in soya beans and ground nuts.

Unesterified tocopherols are rather unstable and tend to lose their activity under the influence of ultra-violet light or oxidising agents. Oxidative rancidity of fats or oils containing tocopherols entails loss of potency of the vitamins. The tocopheryl esters are in general more stable, and retain their biological activity for longer periods.

Vitamin E, which belongs to the fat-soluble group of vitamins, is easily absorbed from the intestine, in the presence of adequate amounts of bile and pancreatic juice. Unlike the water-soluble vitamins, it is neither synthesized nor broken down in the intestine. It is absorbed mainly *via* the lymphatics, the acetate being absorbed more slowly than the free alcohol form. Once through the intestinal barrier, it is distributed throughout the tissues of the animal body, the main storage depots being the liver and adipose tissue. About 90% of tissue tocopherol is in the  $\alpha$  form. Distribution is uneven within the animal body. There is a remarkably high level in the pituitary. The adrenal, ovary, testes and uterus have fairly high contents in comparison with such tissues as lung, kidney, spleen, pancreas and muscle. Quaife *et al.* (1949), give values of 90 mg. per 100 gm. for the pituitary, 34 mg. per 100 gm. for the adrenal, with all the other tissues having a content of 6 mg. per 100 gm. or less calculated on a fat weight basis. Discrepancies between the figures given by various authors arise not only because vitamin E decomposes easily, but also because some authors calculate E content as total tocopherols, others as  $\alpha$ -tocopherol only. The latter estimates are the more meaningful with regard to biological activity.

Variations in tissue E levels occur in hypercholesterolaemia and in pregnancy, when levels rise above normal. Levels decline following prolonged physical or mental stress, anoxia and malnutrition. The presence of large amounts of unsaturated fatty acids in the diet, or deficiency in protein, vitamins A<sub>1</sub>, B<sub>6</sub> or the essential fatty acids, tend to deplete stores of vitamin E. Malabsorption occurs in cases of biliary obstruction, and diarrhoea associated with achlorhydria; in sprue, coeliac disease and fibrocystic disease of the pancreas, or in any prolonged impairment of fat absorption.

Human infants and young animals are born with low reserves—placental transfer is minimal—but requirements are high in neonatal life. The more rapidly the infant grows, the greater are the requirements, and in animal populations it is the more rapidly growing individuals which succumb to the pathological effects of E deficiency. High requirements in infancy mean that several months may elapse before adequate

reserves are built up by the growing infant. The artificially fed human infant is at a particular disadvantage. If he is given cow's milk he can expect to get only 60 mg. tocopherol per 100 ml., compared with the 240 mg. per 100 ml. present in human milk. Human colostrum has the exceedingly high value of 1,480 mg. per 100 ml. and it is fair to assume that the human infant needs this amount in the first few days of life. Occasionally children are born with an inherited defect in the ability to metabolise  $\alpha$ -tocopherol.

There is as yet no agreement as to the exact rôle of vitamin E in cellular metabolism. The intracellular locus is in the mitochondrial membranes and in the membranes of the microsomes. In the former it occurs within the phospholipid layers, in close proximity to co-enzyme Q, the cytochromes of the electron transfer chain, the carotenoids and cholesterol. These spatial associations naturally suggest functions which have been the subject of much detailed and painstaking work.

$\alpha$ -tocopherol seems to share with retinol the function of maintaining the integrity of the membranes of intracellular organelles. As an indicator of this function one may consider the leakage of lysosomal enzymes with resultant severe cellular damage, associated with deficiency of either vitamin. Excess of either vitamin has a similar effect and membrane integrity would seem to depend on optimal proportions of the two metabolites.

Each intracellular lipoprotein membrane has its own characteristic fatty acid pattern. The lipids of these membranes are prone to oxidative damage, with inactivation or acceleration of transmembrane movement, destruction of the cytochromes of electron transport and uncoupling of oxidative phosphorylation in the case of the mitochondria.  $\alpha$ -tocopherol, as a strong antioxidant, may be expected to protect against such damage by inhibiting peroxidation of the lipid components of cell membranes. By protecting vitamin A from oxidation, it may prevent changes in cell membranes attributable to failure of sulphation of the mucopolysaccharides associated with these membranes. Membrane damage can be seen in electron micrographs of biopsy specimens of human jejunal mucosa in cases of E deficiency. The mitochondria and the endoplasmic reticulum lose their normal osmophilia and their outlines become indistinct. When the patients are given adequate vitamin E the membranes regain their affinity for osmium tetroxide, and the organelles are clearly outlined as in the normal healthy cell. The plasma membrane and the Golgi apparatus are less vulnerable to E deficiency in this respect and manage to retain their tintorial ability. Histological sections viewed under the light microscope fail to show any defect (Molenaar, I., Hommes, F. A., Braams, W. G., & Polman, H. A. 1968).

Another rôle suggested for vitamin E in its mitochondrial location is a purely mechanical one—that of maintaining cytochromes b and c

in correct spatial configuration for electron transport. However it seems more likely that the vitamin has a more active rôle to play, and indeed it can be shown *in vitro* that the activity of cytochrome c reductase in striated mammalian muscle is greatly enhanced by the presence of the vitamin. Slater *et al.*, (1961) suggest a catalytic rôle for  $\alpha$ -tocopherol, rather than a structural rôle.

Deficiency of tocopherol and selenium causes a decrease in the concentration of coenzyme Q in the tissues of the rabbit and the rat—a condition which can be remedied by the administration of  $\alpha$ -tocopherol. It has been suggested therefore that  $\alpha$ -tocopherol or a metabolite thereof may be concerned in the biosynthesis of coenzyme Q, one of the electron carriers in terminal electron transport. It seems to be fairly definitely established that vitamin E has a rôle to play in the electron transfer chain, but there is as yet no unanimity on the exact position or function of the vitamin in the chain of reactions.

*In vitro* experiments have shown that decreased oxidation of  $\alpha$ -ketoglutarate in liver cell homogenates can be restored to normal by the addition of  $\alpha$ -tocopherol. Therefore it has been suggested that  $\alpha$ -tocopherol, or a quinonoid metabolite thereof, has a cofactoral rôle in the action of  $\alpha$ -ketoglutarate dehydrogenase ( $\alpha$ -ketoglutarate to succinyl CoA, Fig. 13), which is essential for the correct functioning of the Krebs cycle of aerobic respiration, which takes place in the mitochondrial matrix.

Until the exact biochemical rôle of  $\alpha$ -tocopherol is determined, it will be necessary to define the function of the vitamin in terms of the pathological changes elicited by its absence from, or excess in, the body. The signs of E deficiency in the mammalian body vary widely with the age, sex and species of the animal. The most marked result of deficiency is dystrophy of smooth, cardiac, and skeletal muscle—a condition which affects *inter alia* the smooth muscle cells of the endocrine gland blood vessels. Liver necrosis too is seen—the final stage of liver cell damage, which in the early stages must interfere with normal protein and steroid turnover. Liver necrosis and muscular dystrophy together suggest a major and specific metabolic rôle for  $\alpha$ -tocopherol, and lend substance to the view that it has a coenzymatic rôle in the basic mechanisms of cellular respiration.

The vitamin is a potent anti-coagulant and in its absence thrombi form readily in the blood vascular system. The cerebellar encephalomalacia noted in E deficient chicks, and also seen in the human infant, appears to develop on a basis of vascular thrombosis.

The well-known antioxidant activity of  $\alpha$ -tocopherol has been the subject of a vast amount of experimentation. The fact that for example, synthetic antioxidants such as methylene blue substitute for vitamin E in the prevention of foetal resorption is only one example of this facet of its activity. It would appear that in this context, the synthetic

antioxidant spares residual stores of vitamin E for the more essential metabolic processes. In the metabolism of unsaturated lipids, vitamin E functions non-specifically, as an antioxidant, but is depleted in the process, so that diets containing such lipids require larger amounts of the vitamin, in the active  $\alpha$ -form, than do those with more saturated lipids. In the field of animal nutrition, it is important to realise that the leaf fats of herbage are highly unsaturated and are demanding in their requirements for  $\alpha$ -tocopherol. In the past, tocopherol values of foods have been estimated as total tocopherol, that is  $\alpha$ ,  $\beta$  and  $\gamma$  tocopherols, and the estimations have given rather deceptive results, in view of the varying potency of the three forms. Cod liver oil is an important anti-metabolite for vitamin E. Its protection from oxidation uses up stores of the vitamin, and no doubt provides one more stress factor for the artificially fed infant to cope with. Lipid peroxides are highly toxic for several enzyme systems, and it is believed that the organic peroxides induced by ionizing radiations, and by lack of vitamin E are responsible for major interference with cellular enzymatic reactions.

Vitamin E in the diet protects vitamin A from oxidative destruction, and it has even been suggested by some authors that this is its sole function. However, the great difference in the deficiency syndromes caused by these two essential food factors renders such a theory untenable.

In some of the deficiency states associated with low levels of E, selenium is of vital importance. It may function as an essential cofactor, in some, if not all of the enzymatic reactions concerned. Selenite deficiency is apparently not concerned in the causation of nutritional encephalomalacia, but it is known to be low in animals affected with nutritional muscular dystrophy, and liver necrosis. This point becomes of great importance when comparing results of experiments carried out on animals reared on food from selenium deficient soils, as for example, in some areas of Sweden and Finland, with results from animals reared on selenium adequate soils. Cobalt and magnesium ions may substitute for selenium to a certain extent, for they have some protective effect in dietary liver necrosis. Geographical areas of selenium excess are known, in which the balance is upset in the opposite direction and the syndrome of selenium toxicity is seen in domestic animals. This is thought to be due to the incorporation of selenium, instead of sulphur, into cysteine, and is in effect, a cysteine deficiency disease.

The importance of vitamin E in maintaining membrane integrity is reflected in the cases of red blood cell fragility seen in the newly born human infant, which respond to treatment with  $\alpha$ -tocopherol. Haemolysis in the E-deficient infant can be prevented by oral administration of the vitamin, but not by giving the vitamin to the mother in the last three months of pregnancy. Human placental transfer is inefficient

in comparison with transfer in the rat, in which red cell fragility in the new-born can be avoided by giving the mother vitamin supplementation.

Macrocytic anaemia arising in E-deficient experimental animals has its human counterpart in infants suffering from extreme protein-calorie malnutrition. In these children, the macrocytic anaemia, described as a maturation arrest, does not respond to  $B_{12}$ , folates, ascorbic acid or iron, but  $\alpha$ -tocopheryl phosphate injections, supplemented with  $\alpha$ -tocopheryl acetate given orally for several days produces an encouraging haematologic response and a diminution in the associated creatinuria.

Capillary fragility is another feature of the E deficient state. Exudative diathesis in chicks, and sclerema neonatorum in human infants, both oedematous conditions, and the latter with defective fat metabolism, are thought to arise by exudation of plasma through defective capillary walls. Both are apparently reversible by  $\alpha$ -tocopherol therapy.

$\alpha$ -tocopherol is essential for the development, function and regeneration of the endocrine glands. The exceedingly high concentration of the vitamin in the pituitary gland has yet to be explained, although it is fairly certain that the vitamin is essential for gonadotropin production or release. Changes in the gland in E deficiency are so late as to suggest that the pituitary holds on to its supply until all other bodily sources are depleted. Changes found in this gland are therefore probably secondary to tocopherol mediated changes in target organs.

E deficiency causes degeneration of testicular tubular cells with secondary hyperplasia of the pituitary basophil cells responsible for the production of follicle stimulating hormone. These cells are generally peripherally situated in the pituitary gland and located near the large portal vessels. In the E deficient rat these glycoprotein-producing cells can be stained by the periodic acid Schiff method and shown to have grossly swollen cytoplasm. Increase in size, number and secretory activity presumably indicates an attempt to stimulate activity of the seminiferous tubules by over-production of the relevant hormone. Associated, but much less marked hyperplasia of the luteinising hormone producing cells reflects the need for small amounts of luteinising hormone for the re-establishment of spermatogenesis. The male accessory glands are left intact in E deficiency and there may in fact be some hyperplasia of testicular interstitial cells. Such hyperplasia may in certain circumstances progress to neoplasia.

In the female there seems to be an order of precedence for the production of pituitary hormones. In partially E deficient rats, the production of thyrotropic and lactogenic hormones appears to be switched off before gonadotrophic hormone production ceases. Animals ovulate and go through the oestrus cycle normally; mating and implantation are normal, but there is inability to rear the young. In more advanced deficiency, the luteinising activity is depressed and there is resorption of foetuses or production of 'cretinous' young. The stunted

growth of these animals and changes in their pituitary and thyroid glands suggest the development of pituitary dwarfism with secondary cretinism. The presence of small degenerated acidophilic cells, with pyknotic nuclei and no distinct cytoplasmic granules in the E depleted experimental animal can be correlated with depletion of somatotropin and prolactin and the associated retardation of growth in the young and inability of the female to rear her litter.

**The Thyroid.** In E deficiency in rabbits there is marked hyperplasia of the thyroid gland. This is probably secondary to deficiency of thyrotropic hormone, since degranulation of the associated pituitary cells can be demonstrated in advanced depletion. Thyroid hyperplasia is seen too in E deficient rats, and the thyroid glands of the litters born to these animals are also hyperplastic, showing enlarged vesicles filled with colloid. There is often some fibrous tissue between the vesicles. Such animals generally fail to survive.

Hyperthyroidism increases the rate of utilization of  $\alpha$ -tocopherol and rapidly depletes the organism, while hypothyroidism has the reverse effect.

**The Pancreatic Islets.**  $\alpha$ -tocopherol is said to reduce the insulin requirements of diabetics as a result of improved glycogen storage in muscle cells, rather than as a result of potentiation of insulin action.

**The Adrenal.** Although it has been known for many years that there is a peculiarly high concentration of vitamin E in the adrenal, its function there remains a mystery. In an organ devoted to steroid production, it is difficult not to assume that the vitamin is intimately concerned in steroidogenesis. The high adrenal concentration is many times that found necessary in other organs for the prevention of oxidation of tissue fats. In prolonged deficiency, lipofuscin pigment accumulates in the cells of the zona reticularis, suggesting that at least part of the function of the vitamin there is to prevent the autoxidation of the esters of highly unsaturated fatty acids, which in combination with protein derived from the host cells form the substance known as lipofuscin, or 'wear and tear' pigment.

As previously noted, the adrenal has a very high content of vitamin C, and in those animals which are able to synthesize ascorbate, vitamin E helps to maintain and restore the enzyme gulonolactone oxidase (1.1.3.8), necessary for the final stage of ascorbic acid synthesis.

Unless experimental rats are kept depleted very early in life, before they have time to build up stores of  $\alpha$ -tocopherol, they may take several months to deplete. At the end of this time there is lowered

adrenal lipid content, and progressive adreno-cortical degeneration, similar to that caused by hypophysectomy. Adrenal atrophy is exacerbated by simultaneous selenium deficiency.  $\alpha$ -tocopherol is one of the three vitamins known to be depleted in adrenal stress, the others being pantothenic acid and ascorbic acid.

E deficiency in young swine has been shown to lead to the formation of gastric petechial and ecchymotic haemorrhages and ulcers. The appearances are exactly those described as occurring in man by Addison, over a hundred years ago, in his paper on 'The Constitutional and Local Effects of Disease of the Suprarenal Capsules'. The disease, adrenal cortical failure, which came to be known by his name, has many causes, including tuberculosis and carcinoma, but the invariable association with gastric damage was a prominent feature of Addison's report. 'How far', he wrote, 'these gastric symptoms when present are referable to sympathy existing between diseased (adrenal) capsules and the stomach . . . a more extended observation will probably determine hereafter'. Addison can hardly have thought that even 100 years thereafter, we would still be ignorant of the true nature of the sympathy existing between the adrenal cortex and the stomach lining. We can only hazard a guess that defective corticosteroid synthesis in tocopherol or pantothenic acid depletion leads to loss of tone in the gastric blood vessels, so that there is insufficient blood supply to keep the more superficial mucosal cells alive.

In experimental E deficiency, microthrombi can be seen in the gastric mucosal capillaries, and exudation of plasma into adjacent tissue with the deposition of fibrin—a condition known as plasmatic vasculosis or rather more inelegantly as 'sludging of blood'. Nutritional myopathy in the smooth muscle cells of the larger branches of the gastric artery indicate the presence of a state of atony there.

There is evidence that adrenocortical hormone stimulates the activity of phosphatase in the duodenal epithelium and this aspect of an intriguing subject is one which in all probability would repay investigation.

If it were permissible to extrapolate the findings in young E deficient swine to conditions in the young human infant, one would expect to find artificially fed infants developing areas of gastritis, with bouts of vomiting, until stores of vitamin E become gradually and painfully built up over the course of months. Certainly healed ulcers can be seen in young piglets at a very early age if they have been subjected to nutritional stress.

**The Prostaglandins.** Tocopherols, in their role as antioxidants, protect the essential fatty acids, linoleic, linolenic and arachidonic acids, from peroxidation and hydroperoxidation. In view of the rôle of these polyunsaturated fatty acids as precursors of the prostaglandins, vitamin E has an essential role to play in the maintenance of these hormones at an adequate level.

**The Gonads.** Sterility was one of the first conditions to be noted in E deficiency. The significance of the normally high level of tocopherol in testes, ovaries and uterus still eludes us. In the male rat there is destruction of the germinal epithelium in tocopherol depletion, but the Leydig cells remain normal, and the accessory glands are unaffected, indicating that the hormonal functions of the organ are not impaired. It is thought that  $\alpha$ -tocopherol is concerned in nucleic acid synthesis and it may be that rapidly dividing cells, like the germinal epithelium are particularly at risk in the deficiency state.

In the female rat, sterility is due to foetal resorption, rather than to failure to conceive. Implantation is normal but disruption of the vascular relationship between the foetal and the maternal placentae results in death of the foetus from anoxia and starvation.

Oestrogen excretion in the E deficient female rat drops during pregnancy, and acceleration of the fall at about the 18th day coincides with the time of foetal death. Dietary supplementation of the dam with  $\alpha$ -tocopherol during the first week of gestation prevents death of the litter. Tocopherol deficiency acts, not directly on the ovary, but *via* the gonadotrophic hormone. Death and resorption of the foetuses is due to the marked drop in luteinising hormone.

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## *Chapter 9*

### **The Essential Fatty Acids**

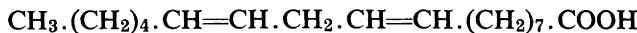
Fats in the animal body occur as simple or compound lipids. The more important of the simple lipids are the neutral fats or triglycerides, which are glyceryl esters of fatty acids, most commonly of palmitic, stearic and oleic acids. Thus a given triglyceride is composed of one molecule of glycerol and three molecules of fatty acids, which may possibly be three different radicals. The compound lipids are more complex and incorporate glycerol or some related substance, fatty acids, various nitrogen containing bases and often a phosphate group. The most important compound lipids are the phosphatides (or phospholipids) which contain glycerol, two molecules of fatty acid (usually unsaturated), phosphate, and nitrogenous base. Examples are the lecithins and the cephalins. Other compound lipids are the sphingomyelins and the cerebrosides, which also contain fatty acids. The compound lipids are integral parts of the limiting membranes of cells and their contained organelles. They also take part in lipid transport and are present in quantity in nervous tissue.

The fatty acids in both simple and compound lipids vary with the species of animal, the age, the physiological status and the diet consumed. In the naturally occurring lipids they are straight chained molecules, usually with an even number of carbon atoms. They may be saturated, that is without double bonds, or unsaturated, with one or more double bonds (monoenoic, dienoic, trienoic, tetraenoic etc, or simply polyenoic if there are more than two double bonds). The polyunsaturated fats of most animals are generally formed by elongation of exogenous unsaturated acids. Fairly non-specific enzymes catalyse the desaturation and elongation of the fatty acid chains. New double bonds are introduced by a polydesaturase. An acyl transferase elongates the fatty acid in two-carbon moieties (Its coenzyme, the acyl carrier protein, contains pantothenic acid).

There is an important group of fatty acids known collectively as the essential fatty acids (EFA's) or, in some countries, as the vitamin F group. These include linoleic acid, linolenic acid and arachidonic acid. They owe their vitamin status to the facts that they are vital for growth and health, and that they cannot be synthesized within the animal body, with the proviso that arachidonic acid may be synthesized *in vivo* from linoleic acid. Thus, arachidonate requirements are met by the provision of dietary linoleate.

The conversion of the dienoic linoleic acid to the tetraenoic arachidonic acid requires reduced NAD and reduced NADP. Pyridoxal phosphate may play some rôle in the conversion, possibly cofactoral, possibly less specific.

Linoleic acid has the formula—



It is thus an eighteen carbon chain, with two double bonds, and is known as 9,12 octadecadienoic acid (octadeca = eighteen, diene = two double bonds). The double bonds occur at the ninth and twelfth carbon atoms, counting in the conventional method from the carboxyl (COOH) group.

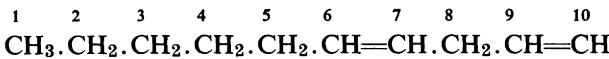
Linolenic, or 9,12,15 octadecatrienoic acid has the formula—



Arachidonic acid is 5,8,11,14 eicosatetraenoic acid, with 20 carbon atoms and four double bonds, or in chemical shorthand it is a 20:4 fatty acid.



It can be seen from the formulae above that if the carbon atoms are numbered from the terminal methyl ( $\text{CH}_3$ ) group, instead of the more usual method from the carboxyl group, that all three fatty acids have double bonds at the six and nine positions, and that the double bonds are linked by methylene bridges, thus:



These are apparently the two features which distinguish the essential fatty acids from other polyunsaturated fatty acids in the series.

The presence of the double bonds in the polyunsaturated lipids implies that the fatty acids are more reactive, and combine more easily with atmospheric oxygen, and therefore undergo *oxidative* rancidity more easily than the saturated fats. The more highly unsaturated a fat, the more readily it will oxidise. (*Hydrolytic* rancidity involves the liberation of free fatty acid from fat by lipase action, in the presence of moisture.) The rate of oxidation of natural fats depends on their content of antioxidants, such as vitamin E, which is the main physiological antioxidant. Oxidation and peroxidation destroy the essential fatty acids, carotene and vitamin A and deplete the  $\alpha$ -tocopherol content. In the modern methods of hardening food fats to prolong storage life, much of the essential fatty acid activity is lost in the hydrogenation process. Unnatural isomers of the polyenoic acids are formed, which do not have the biological activity of the naturally occurring EFA's.

The essential fatty acids are widely distributed in natural organic material. They occur most abundantly in vegetable oils and to a much lesser extent in animal fats. Seed oils are the usual source of linoleic and linolenic acids. Cottonseed, sunflower seeds and groundnuts give a good yield of linoleic acid, while linolenic acid is present in linseed. However, quantities vary extensively in seeds from different geographical sources. Arachidonic acid can be recovered in high yield from bovine adrenal glands.

Within the animal body, the various phosphatides tend to have their own preferred fatty acid pattern, and if this should include one or two of the essential fatty acids, substitution by a more readily available fatty acid seems to entail impairment of biological activity of that phosphatide to a greater or lesser extent. Within the cell, the EFA's are present in the microsomes, and in mitochondria and outer cell membranes. They occur as esters of cholesterol, as the lipid fraction of some lipoproteins and in various phosphatides. The EFA's are apparently preserved by their incorporation into structural lipids, as for example the arachidonate present in the  $\beta$  position of a phospholipid. Otherwise they undergo oxidative degradation in the same way as do the inessential long-chain fatty acids. A typical phosphatide, for example lecithin (phosphatidylcholine) contains varying amounts of essential fatty acids, depending on its source. The lecithin from eggs contains about 10% of linoleic acid, while soya-bean lecithin contains about 80% of linoleic acid. This acid is present too in the phosphatidylethanolamine of blood platelets, and in various phosphatides of the arterial wall, where arachidonic acid is also found. As may be imagined, interference with the integrity of cell membranes in such situations can have far-reaching effects.

Generally speaking, the distribution of the polyenoic acids, whether as part of phosphatides or triglycerides or cholesterol esters varies from one organ to another in the body. In adipose tissue alone, the polyenoic acid content may vary according to the anatomical situation. Perirenal fat may be quite different in composition from subcutaneous fat in the same body.

Analysis of human milk shows that the essential fatty acid content is much higher than that of cow's milk, but in both cases the type of fat present is influenced by the dietary fat intake. The optimal level of EFA intake for the human infant has been shown experimentally, and not surprisingly, to be close to the level present in human milk, although less than half that level will cure the eczematous skin condition which arises in the EFA deficient infant; bottle-fed babies are about seven times as prone to eczema as naturally fed babies. The figure suggested as suitable for infants is about 2-4% of the daily dietary calories given as linoleic acid. In the adult a reasonably varied diet is unlikely to be deficient in EFA's. Only in conditions of fat malabsorption or when

artificially treated foods are used to excess, is EFA deficiency likely to occur.

Of necessity, most of the work on EFA deficiency has been done in experimental animals, although there are a few records of unfortunate human infants being deprived of the vitamins for lengthy periods. Young animals are particularly susceptible to deficiency; increasing age brings with it increasing difficulty in inducing the symptoms. The young male is even more susceptible to the deficiency than the young female. This latter difference does not seem to be entirely related to increased calorie consumption in the male, or higher basal metabolic rate. It is thought that the mobilization and transport of cholesterol is dependent on EFA's, and since male animals need more EFA than do females, for various purposes, there is less available for cholesterol transport. This is possibly one of the many reasons why cholesterol accumulates to a greater extent in the masculine arterial wall, in atherosclerosis.

In the deficient rat, the first symptom noticed is increased scaliness of the skin of the paws and tail. The skin of the entire body becomes increasingly permeable to water. Affected male rats become sterile, with degenerative testicular changes, and atrophy of accessory sex organs. Female rats ovulate irregularly, and become infertile or even sterile. If litters are born, then the neonatal mortality is high.

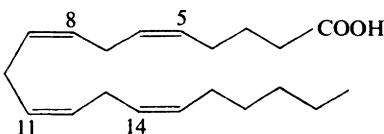
Defective phospholipid synthesis in EFA deficiency, as already noted, leads to both structural and functional defects in mitochondrial and outer cell membranes. The abnormal accumulation of fat in the liver of experimental rats is accompanied by increased fragility of liver mitochondria and interference with oxidative phosphorylation. Cell membrane damage in EFA deficiency is associated with increased capillary fragility and permeability.

A fairly constant feature of the deficiency state is the presence of kidney damage, including accumulation of lipid in tubular epithelium, proteinaceous tubular casts, intertubular extravasation of blood and necrosis of pelvic epithelium. Deficient animals are highly susceptible to bacterial invasion. Epithelial damage and reticulo-endothelial blockade with accumulated fat might be considered as contributing to the increased susceptibility to infection.

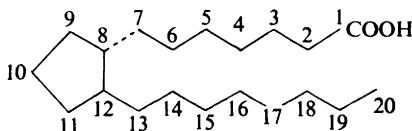
Within recent years, Bergström and his colleagues (1966) at the Karolinska Institute in Stockholm have identified and categorised a new series of hormones known as the prostaglandins. This brilliant work led to the elucidation, by Van Dorp and his associates (1966) of what must surely be the most important function of the essential fatty acids, namely their rôle as precursors of the prostaglandins. These hormones are derivatives of a parent C-20 acid, prostanoic acid, and have smooth muscle stimulating and vasodepressor activity. The prostaglandins are not, as might be thought, solely the prerogative of

those endowed by nature with a prostate gland. Their original isolation from seminal fluid determined their name, in accordance with a presumed, though mistaken, prostatic origin. Subsequently it has been shown that they are present in many organs, in both males and females. A very high concentration occurs in the seminal vesicles of sheep. These organs are not bladder-like sacs as in man, the horse and the rat, but are rather compact glandular organs, with a lobulated surface closely resembling the human prostate histologically.

The presence of prostaglandins in the seminal fluid of primates, sheep and goats in high titre, and their effects on the musculature of the female reproductive tract are thought to assist the process of fertilization by



*Fig. 32.* The structural configuration of a prostaglandin precursor. This is arachidonic, or 5, 8, 11, 14 eicosatetraenoic (20:4) acid. The essential fatty acids, being in the all-cis forms, are already folded over at the appropriate point for conversion to the prostaglandins.



*Fig. 33.* The numbering of the carbon atoms in the prostaglandin molecules.

aiding the migration of spermatozoa. They appear to be readily absorbed by the vaginal wall, and therefore provide one example of an exogenous hormone. As with the adrenal steroids, simple changes in the molecule lead to marked changes in biological activity. The six primary prostaglandins are PGE<sub>1</sub> and PGF<sub>1α</sub>, derived from homo-γ-linolenic acid (20:3); PGE<sub>2</sub> and PGF<sub>2α</sub>, derived from arachidonic acid (20:4); and PGE<sub>3</sub> and PGF<sub>3α</sub>, derived from 5,8,11,14,17 eicosapentaenoic acid (20:5). The last mentioned acid is synthesized from dietary linolenic acid. Cofactoral requirements for the enzymatic reactions concerned include folic acid derivatives.

In the formation of the prostaglandins the long-chained precursor fatty acid molecules are folded over, with carbons 8 and 12 joining to form a five-membered (cyclopentane) ring. The ring becomes oxidized with a hydroxyl and a keto group. A hydroxyl group is introduced on the sidechain, and there is shifting of the double bonds as in the diagrams shown (Figs. 32, 33, 34).

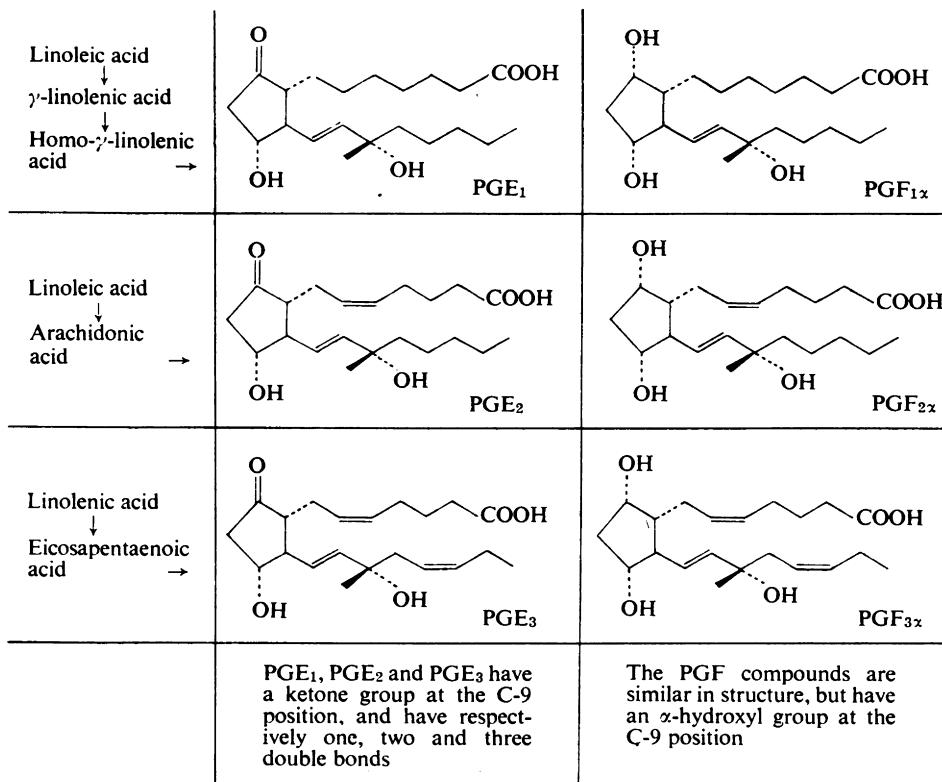


Fig. 34. The six primary prostaglandins.  $\alpha$ -substituents, indicated by interrupted line bonds, are located on the same side of the cyclopentane ring as the carboxyl side chain attached to C-8.  $\beta$ -substituents are oriented on the opposite side, indicated on the diagram by a heavy type bond.

A secondary group of prostaglandins formed by dehydration of the primary members has an ultra-violet absorption band at 217 m $\mu$  (nanometers). These were at first known as PGE<sub>1</sub>-217 etc., but have now been renamed according to the following table, which is included because both forms of identification are still used in the literature.

(Members of the 217 or A group have a double bond between C10 and C11.)

<i>Old nomenclature</i>	<i>New nomenclature</i>
PGE <sub>1</sub> -217	PGA <sub>1</sub>
PGE <sub>2</sub> -217	PGA <sub>2</sub>
19-OH PGE <sub>1</sub> -217	19-OH PGA <sub>1</sub>

Similarly derivatives with an absorption band at 278 nanometers are known as follows. (Members of this 278 or B group have a double bond between C8 and C12.)

<i>Old nomenclature</i>	<i>New nomenclature</i>
PGE <sub>1</sub> -278	PGB <sub>1</sub>
PGE <sub>2</sub> -278	PGB <sub>2</sub>
19-OH PGE <sub>1</sub> -278	19-OH PGB <sub>1</sub>

Although the prostaglandins have been found in greatest quantity in seminal fluid, they are also present in varying mixtures in endometrium, adrenal glands, pancreas, thymus, ovary, lungs, nervous tissue, iris and kidney. In fact, most tissues examined so far have been able to undertake the biosynthesis of these substances, which may therefore be called tissue hormones.

The functions of the different PGs vary with the species of animal, the tissue, and even the physiological state of the tissue. Knowledge of their mode of action is still at the speculative stage. At present it is believed that they act as modulators of the cyclase reactions mediated by such hormones as ACTH, glucagon, vasopressin, the catecholamines, and histamine. These hormones influence the release and activity of enzymes in target tissues, thus determining the specific metabolic functions of those tissues. A typical effect of the prostaglandin compounds is peripheral vasodilation of small arteries.

Of the secondary group of prostaglandins, PGA<sub>1</sub> appears to be identical with 'medullin', a smooth muscle-stimulating lipid, with blood pressure lowering activity, and found so far in the renal medullae of man, rabbit, cattle and swine. PGE<sub>2</sub> may also be a component of medullin, which is really a collective term covering antihypertensive lipids in the renal medulla. The constitution of these lipids varies between species, but as an example the main vasodepressor lipid in the rabbit renal medulla is PGE<sub>2</sub>—a fact which is in accord with the presence of arachidonic acid as the main essential fatty acid in that situation,

the PGE<sub>1</sub> precursor, homo- $\gamma$ -linolenic acid being present in much lower quantity.

There is a surprisingly large amount of PGE<sub>1</sub> in the calf thymus—apparently one of the two smooth muscle stimulating factors present in that gland. A third thymic hormone has already been demonstrated by Levy *et al.*, (1963) which has the ability to restore immunologic competence to the lymphocytes of newly born thymectomised mice, and to prevent the wasting syndrome which normally follows early thymectomy. In comparison with the great volume of work done on thymic lymphocytes the paucity of information on thymic hormones is striking, particularly in view of the present interest in transplant rejection.

**Pituitary.** Pituitary changes in EFA deficiency may possibly be primary. There is more evidence for thinking however that the changes are secondary to degeneration in target organs. In the early stages of deficiency there is hypertrophy of the cells which produce tropic hormones for gonads and adrenals. In rats, increases in pituitary acidophils have been seen after only two months on a fat-free diet, suggesting increased demand for ACTH and the gonadotrophic hormones. Increases in pituitary basophils have also been noticed by some authors, denoting an attempt to remedy the agalactia and stunting of growth, which are features of the disease, by the provision of prolactin and growth hormone.

**Thyroid.** Although the basal metabolic rate is undoubtedly increased in EFA deficiency, there seems to be some doubt as to the rôle of the thyroid in initiating this rise. Hyperthyroidism of itself, is known to stimulate the metabolism of the EFA's.

In the deficient rat, the thyroid is reported to be hyperactive, with small follicles containing reduced stores of colloid. Some follicles are collapsed and contain colloid in their epithelial cytoplasm (Alfin-Slater and Bernick, 1958). Such features are not constantly present and one must attribute the variable reports in the literature to varying success in depletion of the experimental animals.

**Adrenal Glands.** Opinions differ on the effect of EFA deficiency on the adrenal gland. Alfin-Slater and Bernick (*loc. cit.*) report atrophy, with decreased width of *zona fasciculata* and *reticularis* in the rat. Other authors note hypertrophy of adrenal cortex in the male rat but not in the female.

Chemically the character of the adrenal fatty acids is profoundly modified and the output of adrenal steroids is reduced in EFA deficiency. The clear cells of the *zona fasciculata* normally contain free cholesterol, cholesterol esterified with fatty acids, and possibly also cholestryol

sulphates. Grant (1968) has shown that cholesterol arachidonate is depleted (in the human adrenal) to a greater extent than saturated fatty acid esters of cholesterol, following ACTH stimulation. Within the cell, transfer of these esters to the mitochondria for conversion to pregnenolone is under ACTH control. It is possible that the mitochondrial enzymes have a high specificity for cholesterol arachidonate in the adrenal cortex, but this remains to be investigated.

Although the mechanism of prostaglandin action in the adrenal is not known, it may be associated with the recognized ability of these hormones to activate lipolytic enzymes. The knowledge that nervous stimulation releases prostaglandins from tissues led Ramwell *et al.*, (1966) to investigate the release of PG's into the adrenal venous effluent after acetylcholine stimulation. Their results showed that PGF<sub>1 $\alpha$</sub>  appeared in the venous outflow from perfused feline adrenal, when catecholamine secretion was evoked by acetylcholine stimulation. Among various interesting speculations they suggest that acetylcholine activates endogenous phospholipase A to split off prostaglandin from membranes and thus allow catecholamine to escape. They acknowledge of course that there is as yet no firm evidence that prostaglandins are incorporated in structural phospholipids, but conjecture that prostaglandin release may be an accompaniment of secretory activity in other organs and tissues, including nervous tissue.

**The Pancreatic Islets.** There seems to be a well defined relationship between insulin and the essential fatty acids. Depletion of EFA's occurs

Linoleic acid	CH <sub>3</sub> .(CH <sub>2</sub> ) <sub>4</sub> —(CH=CH—CH <sub>2</sub> ) <sub>2</sub> —(CH <sub>2</sub> ) <sub>6</sub> .COOH
$\gamma$ -linolenic acid	CH <sub>3</sub> .(CH <sub>2</sub> ) <sub>4</sub> —(CH=CH—CH <sub>2</sub> ) <sub>3</sub> —(CH <sub>2</sub> ) <sub>3</sub> .COOH
*Homo- $\gamma$ -linolenic acid	CH <sub>3</sub> .(CH <sub>2</sub> ) <sub>4</sub> —(CH=CH—CH <sub>2</sub> ) <sub>3</sub> —(CH <sub>2</sub> ) <sub>5</sub> .COOH
Arachidonic acid	CH <sub>3</sub> .(CH <sub>2</sub> ) <sub>4</sub> —(CH=CH—CH <sub>2</sub> ) <sub>4</sub> —(CH <sub>2</sub> ) <sub>2</sub> .COOH

Fig. 35. Synthesis of arachidonic acid from linoleic acid. The first step is inhibited in insulin deficient animals.

\* Also known as dihomo- $\gamma$ -linolenic acid.

more rapidly in diabetic rats fed on EFA deficient diets than in normal control animals on the same diet. Depletion of polyunsaturated fatty acids may be an accompaniment of the increased mobilisation and metabolism of fats usually found in diabetes.

It is suggested that in the diabetic animal there is impaired conversion of linoleic to arachidonic acid, and indeed Mercuri *et al.*, (1966) have shown that a defect occurs in the desaturation of linoleic acid to  $\gamma$ -linolenic acid in the liver microsomes of alloxan diabetic rats. The defect is prevented by pretreatment with insulin. The postulated route of synthesis of arachidonic acid from linoleic acid is seen in Fig. 35. It

would seem reasonable therefore to provide dietary supplementation with arachidonic acid for the diabetic patient.

In view of the skin defects associated with EFA deficiency in the rat, it is interesting to note that susceptibility to skin infections in such animals has its counterpart in a similar propensity to skin infection in diabetic human patients.

**The Gonads.** The gonads are adversely affected in EFA deficiency. Deficient male rats become sterile and unwilling to mate. Their testes become small and hypoplastic and there is reduction in prostatic and seminal vesicle weight. Sterility is associated with arrest of spermatogenesis at the secondary spermatocyte stage. Some seminal tubules are denuded of germinal epithelium although the Sertoli cells seem to survive unharmed. Giant cells may be found in tubular lumina, a fairly non-specific result of dietary deficiency.

Injection of either linoleate or gonadotropin restores the situation to normal. Prostate and seminal vesicles return to their correct weight following the injection of interstitial cell stimulating hormone—a possible indication of pituitary inadequacy in EFA deficiency. Testosterone injections have a similar effect. The main defect seems to be in testosterone production, although this is rather a hen-and-egg situation in that it is difficult to know whether pituitary or Leydig cell insufficiency came first.

It has been shown experimentally that pituitary follicle stimulating hormone assists the incorporation of linoleic acid into testicular lipids, possibly as one facet of the general stimulation of testicular growth by this hormone. The effect is more pronounced in the immature animal than in the mature animal. Linoleic acid continues to be incorporated into spermatozoa at all stages of spermatogenesis. Interstitial cell hormone and testosterone have a similar effect, and in mild deficiency, testosterone alone may restore germinal epithelium to a functional state.

Testosterone is the main hormone elaborated by the Leydig cell and is presumed to be derived from the cholesterol esters present in those cells. Whether the cholesterol is esterified with linoleate or some other fatty acid is not known; nor is the complete biosynthetic pathway for testosterone production established, although it is assumed that the hormone is derived from its precursor in much the same enzymatic way as the adrenal steroids are produced.

There are some grounds for thinking that pituitary changes are secondary to degeneration of the sex organs. These changes vary with the duration of the deficiency. After about two months of EFA deficient diet, hypertrophy of gonadotropin producing cells may be seen in rats. After about six months, there may be decrease in number and shrinkage of the relevant cells. The latter changes indicate exhaustion, following

excessive stimulation consequent on low circulating levels of secretion from target gonadal cells.

Linoleate and arachidonate, which are abundant in testicular tissue are the EFA's which have a curative effect in male infertility. Linolenate, which is present in much lower concentration normally, is not curative. A similar situation exists in the female rat, in which linolenate is not an essential fatty acid for reproduction. Linoleate or arachidonate supplementation of the diet will reverse the changes of EFA deficiency in this animal. These include irregular ovulation, or complete failure to ovulate. If mating occurs, foetuses may be resorbed, or litters may be produced which fail to survive the first few days of life. Prolonged gestation, difficult parturition and agalactia are all attributable to EFA deficiency. The litters born to deficient animals are themselves very deficient, and show the gross signs of scaly skin and tail necrosis. This suggests that polyunsaturated fatty acids are able normally to cross the placental barrier.

The complicated metabolic effects of the polyunsaturated fatty acids and the various prostaglandins in different animals and tissues are summarised in Polyunsaturated Acids, Part Two, volume 9 of *Progress in the Chemistry of Fats and Other Lipids*, edited by Ralph T. Holman and published by Pergamon Press, 1968. This issue also contains a review of our knowledge to date of Essential Fatty Acid Deficiency.

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## *Chapter 10*

### **Vitamins and Hormones in Carcinogenesis**

In the field of cancer research it is recognised that malignant new growth may be caused by many different factors. Estimates of the relative importance of these factors vary according to the interests of the investigator concerned. It is probable that the majority of human tumours are initiated by chemical carcinogens. A relatively high proportion of new growths in animals and birds can be attributed to viral stimulation, but the recognised viral tumours of man form a very small proportion of the total. Tumours resulting from irradiation are more commonly seen in man than in animals. In both, the status of hormones as inducers of malignant transformation, and the function of hormones in controlling the growth and character of tumours are now well established. The effects of varying levels of vitamin excess and deficiency in hormonally induced and controlled tumours form the basis of much experimental work in laboratory animals. It is however difficult to collect such information in man, and reliable results can be gained only from detailed case reports of abnormalities of endocrine metabolism, correlated with the subsequent establishment of cancerous growths.

Information on the mechanism of carcinogenesis is accumulating rapidly, and an insight into the relationship between vitamins and hormones is useful in the understanding of the etiology and growth of the endocrine dependent tumours. As we have seen, vitamins are required as precursors of hormones, as activators of steps in hormonal synthesis, and as potentiators of the action of hormones in their influence on cell membranes. It follows then that excess or deficit of specific vitamins must induce some modification in the quality of the hormones which they help to synthesise, or some change in the effect of those hormones on their target cells. In addition, defective vitamin status may modify the capacity of the target cell to react to normal hormonal stimulus.

The endocrine dependent tumours may be defined as those which result from some abnormality of endocrine activity, causing interference with the action of one or more growth controlling mechanisms. As a result, a genetic change arises in the target cell, which allows its progeny to multiply indefinitely, given suitable nutritional conditions. The change in the target cell is often associated with loss of ability of its descendants to differentiate along their normal pathway, and to

carry out their predestined functions. The change to malignancy may be at least partially reversible, in that some neoplasms caused by excessive endocrine activity may regress when the initiating stimulus is removed.

The general state of nutrition of the host is known to exert an influence on the onset and growth of tumours. For example, obesity in man is associated with a higher than normal incidence of cancer. Laboratory rats on high fat diets suffer a high incidence of spontaneous tumours, and excessive dietary fat favours the development of artificially induced tumours. Malnutrition, in the sense of undernutrition, seems to render animals more susceptible to carcinogenic agents. Tumours in undernourished animals tend to grow relatively more rapidly than those in well nourished animals, and seem to be able to utilize available nourishment more efficiently than do the host cells.

The change in status of the hormonally dependent cell to the autonomous state is induced by several different factors which may act singly, but more often act synergistically, in which case they are known as cocarcinogens. These factors may be grouped broadly into:

1. Chemical agents
2. Viral agents
3. Ionizing radiations
4. Genetic factors
5. Hormonal imbalances.

Knowledge of the synergistic action of carcinogens stems largely from the painstaking work of Bittner (1957), spread over many years, on the development of mouse mammary carcinoma. Bittner, by prolonged inbreeding of mice, produced strains which were highly susceptible to mammary carcinoma, and strains which were relatively unsusceptible, thus demonstrating the influence of genetically inherited susceptibility in the development of a neoplasm. By removing newly born mice from their high cancer or low cancer strain mothers and allowing them to suckle foster-mothers, he was able to show that an infective agent was present in the maternal milk of high cancer strains. The infective agent (milk factor or Bittner factor) was carcinogenic usually only in pregnant or lactating mice, a fact which suggested the operation of some hormonal influence. In pursuance of this line of thought, it was found that removal of both ovaries at an early age prevented the development of mammary carcinoma in susceptible mice. According to the author, 'Of the inherited hormonal patterns, one was found which induced mammary cancer in virgin females having the other inciting factors; another hormonal mechanism apparently either inhibits or delays the development of the disease in susceptible breeders.'

This demonstration of the interaction of hormonal, genetic and viral factors did much to establish on a sound basis the principles of co-carcinogenesis upon which much subsequent work was founded.

Bittner's selective breeding for high and low cancer incidence has proved a remarkably useful procedure in experimental animals. The occurrence of genetic susceptibility to cancer in man however is rather limited. There are few authentic cases of high cancer families in the human race, due no doubt to widespread antipathy to inbreeding. The known cancer families in man have usually inherited a predisposing factor for cancer, such as intestinal polyposis, rather than a direct predisposition to develop malignancy. On the other hand, the tendency in man to develop multiple tumours indicates some degree of individual susceptibility.

The concept of initiation and promotion of tumours as a two-stage mechanism in neoplasia has gained many adherents. Although it is not possible to state categorically that a given agent, physical, chemical or biological, is either an initiator or a promoter of tumour growth, the terms are useful to specify respectively the first and second agents in the progression from normality to neoplasia. The initiator is generally thought of as a factor which produces a latent, or dormant, change in a cell which sensitises that cell to the action of the promoting factor. An initiating factor may be for example a single dose of radiation. It is usually rapidly acting, and the change it produces may be irreversible. The promoters of neoplasia, on the other hand, act more slowly, and prolonged action on their part may be necessary before a new growth becomes manifest.

In view of the well established existence of cocarcinogenesis, a brief reference to some aspects of non-hormonal carcinogenesis may not be out of place, before we consider hormonal carcinogenesis.

**1. Exogenous chemical carcinogens.** These are now numbered in many thousands. The first chemical carcinogens to be studied were polycyclic hydrocarbons, and, as a group, these remain perhaps the most important today. Members of the group are produced as a result of incomplete combustion of organic material in, for example, cigarette smoke and engine oils. In general, these substances produce cancer locally, that is, they produce skin cancer if applied directly to the skin, or lung cancer if they are inhaled. The nature of their action is not yet established.

An important point about the hydrocarbons is that they are metabolised in different ways by different species; for example some substances which are carcinogenic for rats are not carcinogenic for primates. Within species, too, there are strain differences in reaction to their effects, and even individuals vary widely in their response. Individual variation may depend on the comparative efficiency of detoxifying systems within the host, or more particularly on the nutritional status of the host with respect to vitamin levels. Ingested carcinogenic hydrocarbons are detoxified more easily by a liver provided with an

adequate supply of vitamins of the B complex. Of these, possibly riboflavin is the most important. It seems to be a constituent of a coenzyme in the system required to break down some chemical carcinogens, and therefore its presence in adequate amount modifies the effective dose of a carcinogen. On the other hand, excess riboflavin is known to favour the development of experimental mammary tumours.

Much work has been done on the modifying effect of vitamin A on cancer induced by polycyclic hydrocarbons. One example of this line of research is the work done by Saffiotti *et al.*, (1967) on the induction of bronchogenic carcinoma in hamsters by the intratracheal instillation of benzpyrene and hematite. These authors found a marked inhibition of the induction of squamous cell tumours in the animals fed adequate quantities of vitamin A palmitate. This suggests a systemic effect of the vitamin on cancer induction. Other authors have found that hypovitaminosis A enhances the growth of dimethylbenzanthracene (DMBA) induced tumours in hamsters. Whether these opposing effects are concerned with the known activity of vitamin A in assisting the synthesis of adrenal steroids is not yet known, but defective hormonal synthesis is recognised as having a cocarcinogenic effect in many situations. In the case of established spontaneous mammary carcinomata in mice, there is generally no response to treatment by steroid hormones. However, DMBA induced mammary carcinoma in rats is known to be hormone dependent. Such tumours regress following ablation of the ovaries or the pituitary. Induction of the tumours also is hormone dependent, as is shown by the fact that they cannot be induced in the hypophysectomized rat. Induction in such animals is dependent on providing exogenous supplies of growth hormone, oestrogen and progesterone.

The topical application of vitamin A along with DMBA potentiates the action of the carcinogen, and it is believed that the effect here is the result of increased permeability of cellular and subcellular membranes induced by vitamin A, facilitating the entry of the carcinogen into the cells. The combined effect of the vitamin and the carcinogen is to produce larger and more anaplastic tumours than does the carcinogen alone (Levij and Polliack 1968).

An interesting hypothesis worthy of further consideration is that the flat molecules of some carcinogenic hydrocarbons (this does not include DMBA which is non-planar) or their metabolized derivatives, show some structural similarity to the steroid hormones. They may therefore substitute in some way for the polycyclic skeleton of the steroid molecule, or compete with the hormones or their derivatives, giving rise to hormone imbalance. Other planar carcinogenic molecules may substitute for the purine and pyrimidine bases in the DNA helix, such as the adenine-thymine pair, causing errors in transcription.

Actinomycin D, already mentioned as an inhibitor of RNA synthesis,

is a chemical carcinogen in rats, producing sarcomata after repeated subcutaneous injections. Its ability to bind to chromosomal DNA allows it to interfere with cellular division and differentiation. As a result it causes not only tumour formation, but also dysmorphogenesis in the foetal rat.

Changes induced by chemical carcinogens may so alter the metabolism of the target cells, that their response to hormones is profoundly modified. For instance, chemically induced prostate tumours in mice are endocrine dependent while they remain glandular in type. When they undergo squamous metaplasia, as for example after serial transplantation, they become independent of hormonal control.

**2. Viral carcinogens.** Oncogenic viruses belong to both major viral groups. Examples of DNA viruses include the infective agent of the human wart, adenoviruses, the Shope rabbit papilloma, and the polyoma virus originally isolated from mice. RNA viruses include the Rous Sarcoma of chickens, the mouse mammary carcinoma virus, the agents of leukaemia in mice and leucosis in fowls, and the recently isolated monkey sarcoma virus. Malignant transformation of cells by viral agents is to some extent a reflection of failure on the part of the virus to establish itself (although the immunologist might consider this failure to be the result of a good host defence mechanism). A well established virus grows rapidly within a cell, and kills it, thus releasing large numbers of viral particles. The carcinogenic virus is able to infect the host cell, but does not replicate rapidly enough to kill it. It appears that in the case of DNA viruses, the viral chromosome merges with the host cellular chromosomes. In some way as yet unknown, it interferes with the normal regulatory processes of the cells, which then escape the control mechanism responsible for maintaining the equilibrium between cell proliferation and cell destruction. As a result of the chromosomal merger, when the host cell divides, the daughter cells contain replicas of the virus and are themselves cancerous.

In the case of the carcinogenic RNA viruses, viral particles may modify messenger RNA, or substitute for a part of, or the whole molecule. In this way, new information for the synthesis of protein may be introduced, leading to changes in cellular metabolism, as well as viral replication. However, at the present state of our knowledge, this theory fails to explain how new genetic information is passed on to the daughter cells in a fast growing tumour. There are indications that *in vitro* at least, virally infected cells may occasionally revert to the untransformed state, but this appears to be an uncommon happening *in vivo*, where perhaps the conditions for multiplication of transformed cells are more favourable.

The problem of isolating virus from cancerous cells is an exceedingly difficult one. Recently, however, it has been shown that when virally

infected cancer cells are fused with normal cells, the latent virus becomes unmasked and can be recovered from the hybrid cell, in which it is able to multiply. The importance of the technique for the detection of the viral origin of tumours can hardly be overestimated (Watkins and Dulbecco, 1967).

The carcinogenic effects of viruses may be reinforced by cocarcinogenic factors. For example in the induction of the Shope papilloma, painting the skin with tar shortens the incubation period of the virally induced tumour and increases its virulence. Cater (1951) has shown that the growth and spread of Rous sarcoma in chicks is favoured by pre-existing vitamin E deficiency. The cocarcinogenic effect of hormones on viral tumour induction has already been mentioned in connection with the Bittner mouse mammary carcinoma.

**3. Ionizing radiations.** The carcinogenic effects of ionizing radiations have formed the basis of a prodigious amount of scientific research since the early recognition that prolonged exposure to X-rays gave rise to dermatitis and eventually to carcinoma of the skin of the hands and forearms of radiologists. The mutagenic effects of ionizing radiations were first studied in lower forms of life, such as bacteria and fruit flies, and this work has now been extended to studies of mutations of mammalian cells in culture.

The majority of cancers produced by radioactive substances are sarcomatous in type, both in man and animals. Radium and plutonium, for example, stimulate the formation of bone sarcomas. Plutonium too will often give rise to the formation of fibrosarcomas at the site of inoculation. Non-penetrating radiations cause superficial damage, resulting in the later appearance of skin carcinomas. Single whole body exposures to penetrating ionizing radiations have been known occasionally to cause tumours in experimental animals, even at a dose of less than 1,000 roentgens. Tumours are produced with more regularity however by successive doses of irradiation which cause accelerated proliferation of exposed cells.

Exposure to ionising radiations may be an occupational hazard—there are many examples of this among miners working with radioactive ores. Therapeutic exposure to irradiation has claimed its victims among patients as well as radiologists. Hypopharyngeal carcinoma, for example, has been seen in patients given excessive X-ray treatment for thyrotoxicosis. The malignant condition takes many years to develop following exposure to irradiation. In the much discussed bronchial carcinoma of cigarette smokers, radioactive polonium in the inhaled cigarette smoke has been suggested as the initiator of the malignant change in bronchial epithelium.

Ultra-violet light is a carcinogen to which we are all exposed. Moderate doses cause reddening of the skin and stimulate an increase

in the amount of protective melanin pigment. Higher doses often cause blistering and death of the superficial layers of the skin. Prolonged high doses give rise to cancer in man and animals if they are not protected by an adequate layer of melanin pigment. Thus, white Australians have a high rate of basal cell carcinoma. The morbidity rate increases steadily from South to North Australia. To put it more succinctly, morbidity varies inversely with the latitude and directly with the intensity of actinic radiation. The dark-skinned races have a very low incidence of skin carcinoma, with the unfortunate exception of the albino individuals of those races. In the animal kingdom, white faced cattle have a predisposition to corneal and conjunctival carcinoma.

X-rays and  $\gamma$  rays damage cells by means of their high energy photons, which collide with and displace electrons from atoms within the cell. The deprived atoms become highly reactive and immediately strive to combine with other atoms within the cell. The most important intracellular targets are the nucleic acids. If an ionized atom should form part of the DNA strand, its alteration will give rise to defective genes when the cell replicates. The daughter cells will then have undergone a genetic mutation. If mutation should occur in the genes which control cell division, then uncontrolled cell division may follow.

Proteins within the cell, particularly the all important enzymes, are readily damaged by ionizing radiations. Irradiation of cells gives rise to reducing and oxidising radicals, as well as to organic peroxides within the cell. Reducing radicals can be shown to denature proteins, as for example ribonuclease, by splitting the disulphide bridges between the sulphur-containing amino-acids of the molecule, thus causing a breakdown of the tertiary structure which is necessary for full catalytic activity. There is some evidence too that the DNA polymerase is sensitive to irradiation in this manner. Hydroxyl radicals may react peripherally with macromolecules, by removing hydrogen (to form water) leaving a free surface radical. It could happen that the affected part of the molecule was the catalytically active site of an enzyme, so that defective function would result. The organic peroxides too are highly reactive, and it may be that vitamin E, the physiological anti-oxidant, has some rôle to play in protecting cells from small amounts of radiation damage.

In its function as a cocarcinogen, irradiation damage may inactivate quiescent oncogenic viral particles by damaging their protein coat, and thus releasing viral DNA. Irradiation as an initiating factor for malignant transformation can be demonstrated *in vitro*. The irradiation of hamster cells considerably increases the sensitivity of the survivors to subsequent malignant transformation by polyoma virus.

Irradiation has a cocarcinogenic effect in hormonal imbalance. Kaplan and his colleagues (1951) have shown a striking increase in mortality and a significant augmentation of lymphoid tumour after

total body irradiation in adrenalectomized mice. These authors showed too that the injection of cortisone, either along with, or six weeks after total body irradiation significantly inhibited the development of lymphoid tumours.

**4. The Genetic Factor.** It cannot be doubted that in animals at least, there is a genetically inherited susceptibility to cancer. Strains of mice highly susceptible to the mouse leukaemia virus have been identified, as have strains with low susceptibility to the virus. The validity of the cancer statistics in these strains may be questioned, as mice are known to carry, and be susceptible to several oncogenic viruses which may be activated by various stimuli. More acceptable are the statistics on strains of mice which are of high or low susceptibility to hormonally induced adrenal, gonadal, pituitary or lymphoid tissue malignancy. It is known that spontaneous tumours of mice are readily transplantable to members of the same inbred strain, but not to members of unrelated strains. Genetical homogeneity appears to be a prerequisite for success in transplanting these tumours. The situation in man is more difficult to elucidate. Genetic susceptibility may be thought to account for the fact that the very rare retinoblastoma is often found in several members of the same family. It is of interest too that when identical twins suffer from cancer, the tumours are often similar; when fraternal twins get cancer the tumours are more often dissimilar in nature.

In animals the genetic predisposition to hormonally induced tumours may be transmitted by either the male or the female parent. The alteration in the genes which determines the predisposition may exert its effect on the gland producing a stimulatory hormone, or on the receptor glands or tissues. A third possibility is that the inherited enzyme pattern of the organs responsible for inactivation or excretion of hormones may be such as to predispose to malignancy.

The interaction of hormonal and genetic factors has been demonstrated in mice of three different strains, the CE, DBA and C57 black strains. Ovariectomy in young CE mice results in the formation of carcinoma of the adrenal cortex, but in DBA mice nodular hyperplasia is the only visible change in the adrenal. The adrenals of C57 mice, on the other hand, are apparently little affected by ovariectomy. If the missing hormones are replaced in the first two strains of mice, then neither carcinoma nor nodular hyperplasia develop (Woolley, Dickie and Little, 1952).

**5. Hormonal Factors.** Tumours responsive to changes in hormonal environment are those which arise in a target organ as a result of excessive hormonal stimulation. Under certain conditions, such tumours may regress when the stimulus is removed. The principles can be

illustrated in the experimental animal by removal of the thyroid gland. The disappearance of the thyroid hormone from the circulating blood activates the negative feedback mechanism which induces the pituitary to secrete thyrotropin. Prolonged stimulation of the relevant pituitary cells leads eventually to tumour formation there. The growth of such pituitary tumours is inhibited, if thyroid hormone is given to the host animal, since the tumour cells are well differentiated physiologically and able to respond to target cell hormonal stimulus. If however, these tumours are transplanted serially, they become independent of hormonal control eventually. At this late stage, the tumour cells are dedifferentiated and have lost their ability to secrete thyrotropic hormone. If, instead of ablation, the thyroid gland is rendered atrophic by radioactive iodine, or its activity depressed by thiouracil, it becomes subject to prolonged bombardment by pituitary thyrotropic hormone, and eventually thyroid cancer develops. In the same way, defective thyroxine formation in man, whether from lack of iodine or of any other factor necessary for thyroxine synthesis, leads to tumour formation in a certain percentage of the population at risk.

Cyclic proliferation in a non-endocrine target tissue sometimes becomes excessive, so that target cells undergo neoplastic transformation. Such a condition arises in the mammary gland following excessive stimulation by oestrone from the ovary. The growth of established mammary gland tumours, and also of malignant melanoma and carcinoma of the uterine cervix is accelerated during pregnancy. It is generally accepted that only about half of the cases of mammary carcinoma in women are endocrine dependent. Many are promoted by prolactin as well as oestrogen and progesterone, and therefore growth is accelerated during lactation as well as pregnancy. Some few cases seem to be influenced by pituitary growth hormone. In the human male, mammary carcinoma is androgen dependent, and its growth may be retarded to a certain extent by the administration of oestrogens.

Experimentally, the injection of oestrogens into mice produces mammary tumours, and large doses give rise to lymphatic leukaemia. This latter condition rarely arises in man as a result of excessive oestrogen stimulation. Large doses of oestrogen in mice also, on occasion, stimulate the formation of endometrial carcinoma. The analogous condition is seen in women suffering from granulosa-theca cell tumours of the ovary. Such tumours produce excess oestrogen which promotes endometrial hyperplasia and eventually carcinoma. There is probably too a direct connection between granulosa and thecal cell tumours and the initiation of breast cancers.

Defective oestrogen production by the ovary, which could as we have seen, arise by lack of the necessary vitamins for its synthesis, calls forth prolonged gonadotropic hormone stimulation from the pituitary. The resulting prolonged stimulus on the ovarian cells brings about

tumour formation in that gland—another example of the failure of a feedback mechanism.

Tumours are fairly readily induced in experimental animals by excessive hormones or by hormone deprival, but the type of tumour elicited varies with the species of experimental animal used and with the route of administration of any hormone given in excess. For example, oestrogens, given subcutaneously to mice and rats produce spindle cell and round cell sarcomas; gonadectomy in mice and goats stimulates the formation of tumours in the adrenal cortex. The prolonged administration of antifertility steroids to animals, involving as it does serious endocrine disturbances, inevitably brings the risk of carcinogenesis. Whether the same situation will arise in the human population following the widespread present-day use of such steroids, will no doubt become apparent in ten or twenty years when experience of the use of such drugs will have covered a proportion of the human life span equal to that proportion necessary for tumour induction in animals.

**The Precancerous State.** In cases where it is possible to follow the natural history of the development of malignant tumours, it is often found that a histologically recognizable precancerous state exists. The sequence of events appears to be that first a group of normal cells becomes hyperplastic. This stage may or may not be followed by a metaplastic stage, and then a morphologically malignant but non-invasive stage, before the final irreversible change to the neoplastic state takes place. We have already seen how hyperplasia precedes neoplasia in certain thyroid tumours. Metaplastic changes are known to precede neoplasia in tumours of the human lung, gall bladder, urinary bladder and renal pelvis. Oestrogens promote keratinization in specific target organs, but vitamin A deficiency has a more generalized effect in promoting keratinizing metaplasia in epithelial tissues. Histologically, the epithelial overgrowth induced by vitamin A deficiency is indistinguishable from the hyperplasia and metaplasia which precede the establishment of bronchial carcinoma in man as a result of prolonged exposure to the carcinogenic effects of cigarette smoke or other air pollutants. It is worth considering whether the unfortunate *non-smoking* victims of lung cancer may have been deficient in vitamin A, or in some way unable fully to metabolize it. On a theoretical basis, it is possible that in the case of both smokers and non-smokers with lung cancer, A deficiency may have played a cocarcinogenic role.

Experimentally it has been demonstrated that the supply of adequate vitamin A has an inhibitory effect on tumours of the forestomach of hamsters induced by chemical carcinogens.

The natural history of the development of gastric ulcers has been the subject of much speculation in the past. It seems to be fairly well established that stress is an initiating factor. As we have seen, defective

adrenal steroid synthesis in animals is one stress factor leading to gastric ulceration. In man, the only animal who lives for prolonged periods with active ulcers, about 10% of gastric ulcers eventually become malignant. The contribution of several vitamins, including vitamin A, to normal adrenal steroid synthesis has already been pointed out, and needs no further emphasis.

The problem of altered steroid biosynthesis as a carcinogenic factor has attracted some attention. As an example, rats deprived of both vitamin E and pantothenic acid, both of which are necessary for normal steroid synthesis, develop testicular interstitial tumours. Cholesterol itself, the steroid precursor, in either the crude commercial form or the purified form, promotes the development of sarcomata when injected into mice, the morbidity rate depending on the strain of mice used. Oxidative derivatives of cholesterol are carcinogenic in the same animals—a particularly potent derivative is 6  $\beta$ -hydroperoxycholestene-3-one (Dunn, 1965). The possibility arises that defective steroids may substitute for the correct compounds but lack their normal biological activity—in particular they may lack the power to switch off the production of pituitary tropic hormones.

The concept of altered steroid metabolism has been used to explain the genesis of skin cancer in white skinned subjects exposed to excessive sunlight. The hypothesis is that abnormally large amounts of vitamin D may be produced by irradiation of the unprotected skin. The vitamin may act locally as a cytotoxic and oncogenic agent in either its normal, or in an oxidised state. Leakage of enzymes from lysosomes damaged by irradiation may play a part in the degradation of the steroid molecules.

Having considered a selection of the factors responsible for initiating and promoting neoplastic growth, it now seems obvious that basically, such growth follows structural changes in the genetic apparatus of the cell, or interference with the expression of the information encoded in the genes. Gross evidence of chromosomal damage is provided by the karyotypes of tumour cells. Most malignant cell populations are heterogeneous, that is, there are variations in their number of chromosomes and also sometimes in the form of individual chromosomes. It is true however, that some tumours have normal chromosome patterns, and that aneuploidy occurs in non-malignant cell populations. (Aneuploidy refers to a chromosome count, greater or less than the diploid number which is 46 in man—but not a multiple of the haploid number, which of course is 23.) Nevertheless there are grounds for considering that aneuploidy predisposes to malignancy. It is known for instance that persons affected with mongolism are much more prone to acute lymphoid leukaemia than are normal persons. Each body cell in the mongol contains one chromosome in triplicate, all the other chromosomes being in the usual duplicate state.

The major problem to be solved now is how hormones effect the permanent alteration in cell behaviour which is expressed as uncontrolled cell growth. Do those hormones which stimulate mitosis, such as the oestrogens, androgens and growth hormone activate DNA polymerase, and so stimulate reproduction in generation after generation of cells? How does lack of a hormone give rise to uncontrolled growth? Do some hormones perhaps act as repressor molecules or as corepressors, so that in their absence the production of new cellular protein is uncontrolled? Or do hormones control cell growth by interfering with the expression of genetic information at the ribosomal level? The answers to all these questions lie in the future.

Most somatic cells contain all the genetic information necessary for the organism, but at any given time probably 99% of this information is normally in a state of repression. Tumour cells may differ from normal cells in that certain repressors are absent. It is not yet known whether these repressors are hormones, or the histones which are normally associated with DNA, probably lying in the grooves of the DNA helix and preventing copying of the greater part of the genetic information. Whatever the nature of the repressor molecules, their absence in certain tumour cells not only allows uncontrolled growth, but also unmasks the ability to secrete peptide, hydroxy fatty acid and steroid hormones. Thus bronchial carcinoma cells have been shown to secrete material with gonadotropin-like activity. The presence of high levels of prostaglandins (mainly E<sub>2</sub> and F<sub>2α</sub>), has been detected by Sandler *et al.*, (1968) in bronchial carcinomas, an islet cell tumour of the pancreas and in phaeochromocytomas. Medullary carcinoma of the thyroid was associated with an overproduction of prostaglandins as well as with the secretion of calcitonin. It has been found too that human breast carcinoma cells have the ability *in vitro* to convert steroids to physiologically active hormones (Adams & Wong, 1968). The enzymes necessary for such a transformation are presumably derepressed in the tumour cell, either by deletion of the regulator genes, or inactivation of the repressors for their synthesis.

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## *Chapter 11*

### **Hormones and Vitamins in Prenatal Life**

Hormonal and vitamin imbalances which cause malaise or even pass unnoticed in post-natal life, may have a disastrous effect on the developing foetus. The rapid rate of growth of embryonic cells, which far surpasses that of most tumour cells, is the basic factor which determines the exaggerated foetal response to injurious stimuli. Interference with growth or metabolism of developing cells at an early stage is reflected ultimately in alterations at the stage of differentiation, with resulting malformations or impaired function of developing organs and tissues. The embryonic cell has much in common with the malignant cell, including high mitotic rate, and lack of differentiation in the early stages. It is therefore not surprising that most of the agents discussed in the previous chapter as being responsible for malignant transformation of cells are equally capable of causing dysmorphogenesis in the developing foetus.

Early foetal development depends to a certain extent on hormones supplied by the maternal endocrine organs and the placenta, and on vitamins and other food factors derived from the maternal blood supply. Eventually, however, each foetal endocrine organ grows, differentiates and matures, and in those animals which are born at a relatively well developed state, produce their own hormones, many of which are essential for further normal development of their receptor organs and tissues. As each organ develops, it influences the growth and maturation of other organs, and eventually the functional relationships between endocrine glands characteristic of post-natal life become established. Thus, normally, the pituitary-adrenal and the pituitary-gonadal axes are fully functional before birth. Receptor organs respond to hormonal stimulation when they acquire sufficient maturity to do so. The earliest growth and differentiation of endocrine glands is independent of stimulation by other foetal glands, but prenatal maturation requires the presence of a fully functional hormonal network.

The difficulties in working with foetal tissues are so great that we have little direct knowledge of the biosynthetic pathways by which the foetal endocrine glands synthesize their hormones, but there is no reason to believe that they are very different from the modes of hormonal synthesis in post-natal glands. Assuming this metabolic similarity, one must accept that vitamins are as essential for the synthesis and operation

of prenatal hormones as they are for post-natal hormones. Such an assumption is supported by the abnormalities of endocrine development and function found in animals partly or completely deprived of vitamins during the course of foetal life. The absence of the vitamin-containing coenzymes required for biochemical sequences in foetal organogenesis and metabolism has far-reaching effects. Many of the experimentally induced vitamin deficiencies cause malformations identical with those reputed to be hereditary in origin. As a rule, the demands of the mother for vitamins take priority over foetal demands, so that in many cases there may be no sign of maternal deficiency in essential food factors, while at the same time the foetus may be suffering marked deficiency. This means that a marginal maternal deficiency, which is very difficult to detect, may have grave consequences for the foetus.

Not all vitamin deficiencies cause recognizable defects in foetal development. Some cause foetal death, others provoke malformation. Many simply cause retarded growth, by interfering with foetal metabolic processes in a minor degree. Individual requirements for vitamins vary over a wide range, so that deficiency which proves teratogenic in one strain of animal, or in one individual, may not necessarily cause developmental defects in another strain, or individual. It seems therefore that genetic factors are important in determining susceptibility to vitamin deficiencies.

The subject of foetal nutrition is a very complicated matter, since each step in morphogenesis, as for example the formation of the cardiovascular system or the central nervous system, has its own individual requirements, both qualitative and quantitative, which must be satisfied if ontogenesis is to proceed normally. Knowledge of such requirements can only be attained by observing the results of the relevant deficiencies in experimental animals and in the very few cases in man in which a single conditioned deficiency results from therapeutic treatment. Such a condition arises for example when aminopterin, a folic acid analogue, is used to induce therapeutic abortion, and fails to achieve this object. The conditioned deficiency of folic acid which is thus induced gives rise to multiple congenital defects.

There is a marked resemblance between the pattern of congenital defects produced by hormonal deficiency, and that produced by vitamin deficiencies. The same pattern is induced too by chemical and viral teratogens and by irradiation, and it is the specific period of pregnancy during which the noxious stimulus acts which determines the pathological effect, rather than the teratogen itself. The whole wide range of defects in human morphogenesis can be duplicated in most cases in experimental animals by dietary manipulations. These defects may be grouped into:

(a) Agenesis—failure of localized portions of the foetus to grow. This term covers ectromelia (absence of limbs), ectrodactylyia (absence

of fingers or toes), absence of kidneys, or lungs, or of muscle groups, ribs, vertebrae or skin.

(b) Failure of tissue to unite normally as in anencephaly, spina bifida, harelip and cleft palate.

(c) Failure of tissues to divide normally, as in syndactyly and synophthalmia.

(d) Failure to take up a correct anatomical position as in renal, cardiac or testicular ectopia, and the various forms of talipes (club-foot).

(e) Persistence of foetal anatomical patterns into post-natal life, as in persistent foramen ovale and persistent ductus arteriosus.

A further category of congenital defect could be included, to cover overgrowth of tissues, but these might be more accurately classified as congenital tumours.

Carter (1968) lists the most common malformations of new born children in England and Wales as anencephaly, spina bifida, Down's syndrome, pyloric stenosis, cleft lip (with or without cleft palate), talipes equinovarus, congenital hip dislocation and congenital heart malformation. All these conditions, with the exception of Down's syndrome (mongolism) he attributes to the effect of a polygenicetic genetic predisposition, triggered off by some unknown intrauterine environmental factor. 'If' as Carter says 'the additional factors are, as they may well prove to be, nutritional, then it is reasonable to hope that we can protect the mother, and through her the foetus.'

It is known of course that virus infections, irradiation and chemical teratogens such as thalidomide cause a variety of congenital defects, but such cases form a small fraction of the total of foetal abnormalities, and it seems that much more could be done in the field of preventive medicine to cut down the large numbers of preventable congenital defects. In human foetal development, most abnormalities are established by the eighth to the tenth week of gestation. This means of course that the most important period for nutritional care occurs in the few weeks before and immediately after conception. This is not usually the time when diet is considered important. Developmental defects following maternal rubella infection, and treatment with thalidomide have spot-lighted dramatically the vulnerability of the foetus at an early stage of development. Nevertheless, most of our advances in knowledge of the interaction of the foetus with its environment must come from work with experimental animals. Although there are obvious dangers in extrapolation of the results gained from animal experiments, there is much to be learned from a comparative study. The animals most in use are rabbits, rats and mice, and of course the developing hen's egg provides the most easily accessible experimental model.

It should be realized that there is much variation in the state of maturity of animals at the end of their period of gestation. The newly

born rat and mouse are at a retarded state of maturity when compared with ruminants, some of which are able to rise to their feet and run with the herd within half an hour of birth. Comparison, then, with stages of human development requires a detailed knowledge of the timetable of morphogenetic events in the animal concerned, as compared with that of man. A valuable contribution in this field has been provided by Otis and Brent (1954) who have drawn up a graphic comparison of the times of appearance of 147 recognizable stages in development in mice and men. There is close comparison between the times of appearance of structures in the first seventeen days of mouse foetal life, and the first fifteen weeks of human foetal life. The total gestation period in the mouse is 19 days, therefore in comparison with man, the newly born mouse is distinctly underdeveloped.

The rat has a gestation period of 22 days. At the end of this time, the rat thyroid follicles are just starting to show a small amount of colloid, whereas in man, a similar stage is reached at 8–10 weeks. In the human foetus the adrenal medulla begins to function at 12 weeks and the adrenal cortex shows signs of secretory ability at or before 9 weeks. In the rat, migration of adrenal medullary cells into the primitive adrenal cortex does not begin until the 16th day of foetal life. Even at birth, cortical tissue is still undifferentiated. In man, the pancreatic islets become differentiated at 12–14 weeks compared with differentiation at the 19th or 20th day in the rat, whose beta-cells start producing insulin on the 22nd day. The alpha cells do not differentiate until after birth. The human anterior pituitary is well differentiated by the 12th week, that is, the end of the first trimester, a stage of development only reached by the rat towards the end of the gestation period. Similarly, sexual differentiation starts at 6 or 7 weeks in man, and at 13 or 14 days in the rat.

Keeping these marked differences in stages of maturity constantly in mind, it is possible to draw some comparisons between anomalies in development in human and animal foetuses caused by various factors acting at comparable critical periods in organogenesis. Such critical periods may occur just before the rudiments of an organ are detectable, or when the cells in the developing organ are most actively dividing. The formation of the anlagen (primitive masses of cells forming the rudiments of organs) and successive steps in differentiation of human endocrine glands related to length and age of embryos are described by Tonutti and Fetzer (1956) as also are the postnatal changes in maturation of endocrine glands up to the age of puberty. This work forms a useful reference for the minuter details of endocrine development.

As we have seen, the whole spectrum of human foetal abnormalities can be simulated experimentally in properly chosen animals, by dietary adjustments, at strictly defined periods of gestational time. This provides a valuable means of investigating the faults in the basic biochemical

mechanisms which underlie the pathological phenomena so produced. Foetal anomalies may arise as a result of faulty implantation of the ovum, or as a result of placental disease, but we are concerned here only with those defects which arise as a result of faulty hormonal balance or dietary deficiency. Deformities may arise directly in the cells of the deformed structure. Or they may be caused indirectly by faulty nutrition arising from insufficient maternal blood supply. Or again the foetal cardiovascular or hepatic output may be at fault, or there may be lack of control by the foetal endocrine system.

Careful control of the experimental model is essential. It is necessary to initiate a borderline deficiency or imbalance, since excessive interference with maternal nutrition will result in sterility, or death and resorption of the foetus. The same situation applies in the field of human nutrition where both high infant mortality and high incidence of foetal anomalies are seen in the underdeveloped countries, following maternal malnutrition.

#### **Pathogenesis of single vitamin deficiencies**

*Vitamin A.* The list of congenital abnormalities produced in experimental animals by maternal A deficiency is a long one. The details vary with the species of animal used, the intensity, and in particular the timing of the deficiency in pregnancy. The large number of malformations indicate a primary effect on the process of organogenesis. Piglets are born without eyes or with microphthalmos. Calves become blind from constriction of the optic nerve by relative overgrowth of osseous tissue. Rabbits develop hydrocephalus. It is the rat however, which shows the widest range of foetal abnormalities, including malformations of the eye, cardiovascular anomalies, resembling those seen in man, urogenital anomalies, diaphragmatic hernia, hypospadias and cryptorchidism. The last anomaly, failure of the testes to descend, is said to be due to the failure of the testicular Leydig cell to produce sufficient hormone to induce the descent. In view of the necessity of vitamin A for the synthesis of corticosteroids and testosterone, this explanation would appear to be satisfactory.

Excess of vitamin A given to pregnant females is also teratogenic. Deformities include cranial anomalies, cleft palate, harelip and eye defects, hydrocephalus, spina bifida and exencephalos. The teratogenic action of vitamin A excess in rats is potentiated by cortisone, although cortisone is not by itself teratogenic. The combined action is difficult to explain, as cortisone normally stabilizes the membranes which are said to be damaged by vitamin A excess. As has already been mentioned, excess vitamin A damages lipoprotein membranes. The organs distorted in vitamin A excess are derived from the neural tube, the lens primordia, and the oral cavity, and it is presumed that embryopathic changes are the result of direct attack by vitamin A on these ectodermal structures,

with alteration of the molecular arrangement of their cell membranes. Protection against the brain anomalies is given by the injection of protamine zinc insulin between the ninth and twelfth days of pregnancy.

Vitamin A is said to be able to pass through the human placenta, although carotene is retained. Premature babies are known to be deficient in vitamin A, perhaps because they have not had time to build up sufficient stores of the vitamin for the stressful first few days of life. Their diet should be supplemented with the preformed vitamin until their bodily stores become adequate.

The children of women with diabetes mellitus have a higher incidence of malformations than the children of non-diabetic women. Microcephaly, hydrocephalus, cardiac defects and cleft palate are common. Similarly the offspring of diabetic rabbits show gross deformities of the brain. It is reasonable to suggest that vitamin A deficiency is the basic cause, or one of the basic causes of such changes, since diabetic subjects are unable to convert carotene to vitamin A. If this fact is not recognised, and the subject given preformed vitamin A then such abnormalities might be expected in the children of a certain proportion of women at risk. It is essential too that at each level of embryonic development, the foetal blood glucose level should be in correct coordination with requirements; marked deviations from normal result in foetal malformation.

It was thought at one time that mongolism was the result of defective transfer of vitamin A from the mother to the foetus. Although mongolism is now thought to be a hereditary defect associated with trisomy in chromosome 21, the two theories are not contradictory, in view of recent investigations in the induction of chromosome anomalies by conditioned vitamin deficiency (*vide infra*).

*The vitamin B complex.* The necessity of members of the vitamin B complex for correct operation of the glycolytic pathway, the citric acid cycle and the electron transfer mechanism is emphasized by the marked disturbance of foetal metabolism caused by their absence. Often, a deficiency of a single metabolite lasting no more than a day can be shown to produce foetal abnormalities in rats. It will be recalled of course that these water soluble vitamins are not stored in the animal body. This means that animals are more readily depleted of B vitamins than they are of the fat soluble A vitamin. Foetal avitaminosis A is not readily achieved unless the dam is deprived of the vitamin from the time of weaning.

As might be expected, *riboflavin* deficiency causes, in the foetal liver, reduced oxygen consumption and reduced activity of such enzymes as succinic dehydrogenase and cytochrome oxidase. Severe deficiency of course causes sterility in rats, but borderline deficiency may allow the development of small misshapen foetuses, which often die *in utero* and become resorbed. Although there are differences in susceptibility to the

deficiency in different strains, the general syndrome is one of skeletal malformation and blood disorder. Micromelia (abnormally small limbs), short mandibles, cleft palate, and syndactylism with fused cartilaginous anlagen are all seen; foetuses are oedematous and anaemic. Degeneration of Wolffian bodies can often be detected. Deficiency induced by the antivitamin has been known to cause anophthalmia and microphthalmia.

*Thiamine* deficiency is responsible for anoestrus with sterility, or relative infertility in rats, depending on the degree of deprivation. Marginally deficient rats may become pregnant and give birth to undersized progeny, but the perinatal mortality rate is high. Thiamine is apparently required most urgently in the late stages of pregnancy, and foetal morphological anomalies are therefore not associated with deficiency of this vitamin, either in experimental animals or in human beri-beri patients. Embryopathy in thiamine deficient young is probably associated with pituitary or ovarian malfunction, since, as Nelson and Evans (1956) have shown, foetal death and resorption can be prevented by daily injections of oestrone and progesterone during pregnancy.

*Nicotinic acid.* There are apparently no foetal abnormalities in man associated with pellagra, but in experimental animals, development abnormalities can be induced by short term use of nicotinic acid antagonists. Pinsky and Fraser (1960) by giving 6-aminonicotinamide to mice on the ninth day of gestation were able to produce cleft lip and palate, and hind limb defects. The inhibition of nicotinamide adenine dinucleotide dependent reactions was suggested as the cause of the anomalies. The timing was critical. Inhibition on the tenth day failed to cause the anomalies. Even as short an inhibition as two hours (determined by giving nicotinamide two hours after the antagonist) was sufficient to cause lasting damage to the foetus. Similar malformations may be induced in foetal rabbits by the use of a nicotinic acid antagonist at a critical period in morphogenesis.

*Biotin.* Low levels of dietary biotin are not apparently teratogenic in the rat, but the progeny of deficient pregnant females may be resorbed *in utero* or fail to survive more than a few days of post-natal life. Degenerative changes may be found in the heart and blood vessels, and in the liver.

*Pantothenic acid* deficiency gives rise to a wide variety of foetal abnormalities mainly affecting the nervous system. The timing of the deficiency during gestation is of critical importance. The period of greatest need appears to be just before birth, in animals born mature. The need for coenzyme A, a pantothenic acid containing cofactor, for phospholipid synthesis, may be one of the basic factors in determining integrity of the nervous system. The vulnerable periods for myelination in the brain in the dog, man, rat and pig have been discussed by Davison and Dobbing (1966). These authors state that myelination starts at

different times in various areas of the nervous system, and the onset of myelination varies for each species. Myelin of course has a high content of phospholipid and defective synthesis may be expected to impair function to a certain extent prenatally, as it does post-natally in the demyelinating disease which lack of pantothenic acid causes.

Female rats treated with pantothenic acid antagonist at the relevant critical growth period give birth to young with interventricular septal defects and anomalies of the aortic arch. Cleft palate and club foot are also seen, as are hydronephrosis and hydroureter, and ectopic gonads. The pantothenic acid deficient pregnant rat has a tendency to resorb foetuses or to give birth to undersized young. The reproductive failure in this case cannot be cured by the provision of oestrogen or progesterone throughout pregnancy.

*Folic acid* is required for nucleic acid synthesis and as might be expected deficiency during pregnancy interferes with the normal development of organs and tissues. The effects produced in rats are similar to those induced by giving purine analogues (Kury *et al.*, 1968), which likewise are antagonists of nucleic acid synthesis. Mercaptoperine is an example of the latter. This drug has been used extensively for the treatment of acute leukaemia in man. If mercaptoperine, or aminopterin (a folic acid antagonist) is given to pregnant rats, the foetuses develop with cleft palate and harelip, deformed limbs, and malformations of the heart, diaphragm, urogenital system and adrenals.

The folic acid antagonist is known to cause the formation of spina bifida and to affect the development of the eye, and the closure of the body wall. There is a very broad spectrum of foetal abnormalities associated with folic acid deficiency. The incidence, and type of change vary with the degree and the duration of the deficiency. Even as short a period as 48 hours' deficiency causes a high incidence of abnormalities, the nature of which is determined by the gestational stage during which the deficiency is established (Nelson *et al.*, 1955). Foetal death and resorption in folic acid deficient rats are not prevented by the administration of the sex hormones.

It is interesting that the human foetus is able to concentrate folic acid from the maternal circulation. Foetal requirements for the vitamin are presumably much higher than maternal requirements, in order to keep pace with the tremendous mitotic activity occasioned by intra-uterine growth.

Variations in the maternal level of *vitamin B<sub>12</sub>* during pregnancy do not seem to affect the embryo. Vitamin B<sub>12</sub> is one of the water soluble vitamins which the foetus is able to accumulate and retain at a higher level than that found in the maternal circulation.

The production of skeletal deformities in the young born to rachitic females is associated with maternal deficiency of *vitamin D*.

Prolonged severe *E deficiency* in rats causes sterility; in less severe

deficiency implantation may take place, but the foetuses usually become resorbed and there is regression of the corpora lutea. Marginally deficient rats given alpha-tocopherol supplement before the ninth day of gestation are able to produce normal young. If the treatment is delayed until the twelfth day, then the young animal may develop exencephaly, hydrocephalus, syndactyly and an oedematous condition of the tissues. Abnormalities in the E deficient young rat are thought to be due to degenerative changes in, and haemorrhages from, the placental vessels. The resulting drop in blood supply causes foetal malnutrition and asphyxiation.

Using the developing chick embryo as an experimental model Adamstone (1931) was able to demonstrate in E deficiency the presence of a ring of intensive cell proliferation in the blastoderm. The vitelline blood vessels were choked by the development of this lethal ring, and became degenerate. Many chick embryos suffered massive haemorrhage into the exocoel, usually from a ruptured cardiac atrium.

The occurrence of abnormalities in marginally deficient rats is mitigated to a certain extent by oestrone or progesterone injections, but in the more severe deficiency characterised by foetal resorption, hormones appear to have no curative effect.

In *essential fatty acid* deficiency embryos fail to maintain their normal rate of growth, and there is a low rate of neonatal survival. The blood vessels appear to undergo degenerative change and there may be haemorrhages into the tissues of the limbs and tail in the rat.

Turning now from the problems created by failure to provide exogenous supplies of essential food factors to the developing foetus, we must next consider the problems arising from failure of the foetal glands to develop the harmonious relationships which are essential for normal growth. It is known that abnormal foetal hormone production is responsible for both metabolic disorders and gross morphological errors in development. Illustrative of these are the vascular derangements caused by excess adrenalin, and the defective closure of the palate caused by excess ACTH. Defects in the receptor tissues are the cause of dysharmony in the endocrine system.

In studying the principles which govern hormonal action, we have already seen that:

1. A drop in production of metabolites by receptor tissue induces overproduction of hormone from the stimulatory organ. Thus, adrenal failure induces excessive pituitary activity, sometimes even to the point of exhaustion. Similarly, excessive target tissue function cuts off the production of stimulatory hormone.

2. Excessive amounts of circulating hormone, whether of exogenous or endogenous origin, cause reduced activity and atrophy of the producing gland. Thus, if oestrogens are injected into an animal, if they are produced in excess, as by a tumour, or if they are not broken down

by reason of the liver being diseased, then the normal oestrogenic cells cease production (Oestrogenic tumour cells seem to escape this control mechanism).

These two principles apply in prenatal life, just as they do in postnatal life.

The study of the rôle of hormones in prenatal development is a difficult one, but various experimental procedures are being developed which allow investigation to proceed, and have already contributed much to our knowledge of the subject. Foetal endocrine glands can be removed surgically, or inactivated by irradiation or by chemical procedures. Much information can be acquired too from Nature's own 'experiments', such as the anencephalic monsters which provide information on the role of the foetal hypothalamo/pituitary axis, by a study of the abnormalities which arise in its absence. *In vitro* cultivation of gland rudiments is another source of information about the interactions of the developing endocrine glands and their secretions.

**The Pituitary Gland.** The effects of extreme deficiency or complete lack of pituitary hormones have been studied in experimental rats and rabbits decapitated *in utero*, and in human anencephalics by Bearn (1968). In the experimental animals, decapitation three days before Caesarean section at full term in the rats, or seven days before full term in the rabbits, did not interfere with the growth rate or with the bony development of the foetuses, even though at this stage in development the pituitary is normally differentiated and growth hormone can be detected in it. These experimental findings are in accord with the known fact that skeletal growth in human anencephalics is not retarded. In their case, the hypothalamus is definitely absent, but careful search often reveals the presence of the anterior hypophysis, usually small and underdeveloped. It has been said that these hypophyses contain substantial amounts of ACTH. As it is the function of the pituitary to form hormones, and the function of the hypothalamus to modulate their release, it is possible that the undoubtedly hypoplastic changes in target organs in anencephalics are due to failure in the hypothalamic release mechanism, or to extreme shortage of ACTH in the circulating blood.

Experimental hypophysectomy causes a marked drop in foetal liver glycogen, possibly due indirectly to lack of glucocorticoid stimulus. The situation can be remedied by injections of ACTH.

The foetal pituitary-thyroid axis has been the subject of much study. Several investigators have found that the embryonic thyroid gland is able to differentiate independently of the pituitary, but that in the absence of the pituitary stimulus, excretion of thyroxine is much reduced.

The pituitary-adrenal axis is well developed in the prenatal period, although after birth in the rat there is a temporary state of refractoriness

to adrenalin stimulation. Hypophysectomized embryos have very underdeveloped adrenal cortices. If however, these animals are given adrenocorticotropin the adrenals develop normally. Bearn (*loc. cit.*) found that the adrenal weights of rats decapitated four days before term were less than half those of their litter mates and much less well vascularised. An interesting finding in this study was that the thymus weights in decapitated foetuses were much increased. They could be kept down to normal weight by injections of ACTH. The doses of ACTH used in the Bearn's experimental rats were admittedly excessive and produced overgrowth of the adrenals; in these circumstances the thymuses were reduced in size in comparison with normal thymuses. The conclusions therefore were that the adrenals were under pituitary control, and also that the adrenals provide an inhibitory influence on thymus growth. Reduction in size of the foetal thymus also follows the injection of corticosteroid into the mother. The effects of hormones on the immunological apparatus of the body might be considered as by-products of endocrine research. The toxic effects of cortisone on lymphocytes, the inhibitory effects of stress on the production of antibody, and the stimulation of phagocytosis by oestrone and progesterone are fascinating by-ways which invite exploration.

To return now to the adrenal, the foetal zone which normally comprises four-fifths of the cortex, is almost absent in anencephalics. The fact that adrenal hypoplasia should occur in both man and experimental animals in these circumstances suggests that maternal ACTH is not able to cross the placental barrier.

Studies on the hypophyseal-gonadal axis show that gonadotropic stimulation is not essential for the differentiation of the genital tracts, but at a certain late stage in pregnancy (22–24 days in the rabbit) embryonic hypophyseal gonadotropin is essential for the normal functioning of the testis. At a later stage in development, the hypophysis again becomes inessential. Histologically the gonadotropin producing cells can be shown to be in a high state of activity in the few days preceding their requirement for testicular development. Thereafter, they become relatively inactive.

**The Thyroid Gland.** The development of the foetal thyroid takes place between the eighth and fourteenth week in man. There is some evidence that the thyroid can abstract labelled iodine from the blood before follicles are visible histologically. The time of development of the thyroid in animals varies with the species, being earlier in those animals and birds born mature than in those born immature. In the sheep it is well differentiated at the 50th day out of 150 days total gestation, in the rat at the 18th day out of 21. Elimination of foetal thyroid function has a profound effect on the embryo, which is perhaps even more obvious in birds than in mammals. The yolk sac remains outside the body cavity,

instead of being drawn within, as is usual. There is delay in the maturation of ossification centres and the growth of the embryonic plumage is much retarded. Likewise, in mammals there is delayed development. Lascelles and Setchell (1959) demonstrated the delay in bony development and maturity in lambs born to sheep treated with methylthiouracil, a drug which lowers thyroid function. The treatment seemed to reduce the protein bound iodine in the foetus much more than in the dam. As in other experimental animals, thyroid depletion caused an increase in the lipid content of the animals. Fat accumulated within the foetuses and the foetal plasma showed increases in all lipid fractions. Injection of thyroxin into affected animals restored the situation to normal by limiting fat accumulation.

A more important result of foetal thyroid inactivity however, is the effect on the central nervous system. The congenital cretinism arising in the children of hypothyroid mothers can be duplicated experimentally in rabbits and rats. Changes in the cerebral cortex in the hypothyroid foetus include decreased size of neurones and defective myelination. If the subjects are not treated in the very early days of post-natal life, then the brain damage becomes irreversible. Endemic and enzootic cretinism is associated with skeletal malformations as well as with congenital goitre.

Morphological changes have been noted in experimentally hypothyroid animals such as persistent foramen ovale in piglets. Rats develop the eye abnormalities which are associated with A deficiency, namely cataract, coloboma and occasionally anophthalmia. Cleft lip and cleft palate are common. Low succinic dehydrogenase activity, an indication of enzyme inhibition in hypothyroidism, can be restored to normal if the missing hormone is replaced early in life, but not later than three weeks after birth.

The injection of excessive pituitary thyroid-stimulating hormone into certain strains of rats causes hydronephrosis and hydroureters resembling that produced by giving excess vitamin A. The similarity between the eye lesions of hypothyroidism and of A deficiency recall the fact that several investigators have stated that in hypothyroidism the absorption of carotene and its conversion to vitamin A are decreased.

**The Pancreatic Islets.** Insulin and the associated blood glucose levels are among the most important requirements for maintaining a steady and normal rate of development in the foetus. In man, the pancreatic islets start to differentiate about the 7th week and by the 20th week both alpha and beta cells are present, in association with the capillaries necessary for circulating their secretion. Developmental abnormalities arising in diabetes have already been mentioned in connection with variations in maternal vitamin A status. The injection of insulin into experimental animals also causes foetal deformities in rabbits and in chicks. Following the injection of insulin on the fourth day of incubation,

chick embryos become hypoglycaemic. When they hatch, they show various skeletal abnormalities including rumplessness and a syrenoid condition (fusion of the lower limbs).

**The Adrenal Gland.** Both the adrenal cortex and the medulla produce their secretions in intrauterine life and are important for normal foetal development. In man the differentiated foetal cortices are already showing lipid accumulation by the tenth or twelfth week; the medullary chromaffin reaction becomes apparent at about 15 weeks. At midterm, the adrenal cortex is able to synthesize a variety of steroids, as is the foetal liver, which at this time has some claim to the status of an endocrine organ in this respect. The placenta too is able to convert pregnenolone to progesterone in the preparatory stage of steroid production (Solomon *et al.*, 1967). The chick embryo adrenal gland becomes visible by the 11th or 12th day of incubation. Differentiation in both man and birds is not under pituitary control until a fairly late stage, when the pituitary stimulus accelerates the growth of the gland and stimulates the accumulation of steroid precursors in the zona fasciculata. In the absence of the pituitary further growth is retarded.

The functional ability of the foetal adrenal is well demonstrated by the fact that removal of one adrenal stimulates overgrowth of the other. Just as in post-natal life, the compensatory hypertrophy is prevented if the animal is supplied with cortisone. Prolonged supply of exogenous cortisone retards adrenal development by inhibiting the production of ACTH and thus interfering with the negative feedback mechanism.

A common test used to detect adrenal activity is the increase in duodenal alkaline phosphatase in embryos. This enzyme increases markedly in the mouse duodenum from the 15th intrauterine day. The increase can be prevented by foetal adrenalectomy at the 12th intrauterine day. This suggests that the adrenal cortical secretion is important in the differentiation of the duodenal epithelium. Adrenal control of duodenal phosphatase is also exerted post-natally and it may be that the adrenal exhaustion following stress of various types has a contributory effect in impairing the function of the mucosa in duodenal ulceration. In the rat, cortisone stimulates the synthesis of alkaline phosphatase both in the duodenal microvilli and in the brush borders of the renal tubules.

Cleft palates can be induced by treating pregnant mice with cortisone or hydrocortisone from about the 11th to the 14th gestational day. The incidence varies with the strain of mouse used and may attain 100%. That the production of cleft palate by cortisone is not purely of experimental interest is demonstrated by the fact that 1% of the babies born to pregnant women treated with cortisone have cleft palates—a significant increase over the incidence in women not so treated.

In animals the adrenal medulla is known to have the ability to secrete catecholamines before birth. Extra-adrenal chromaffin bodies secrete

only noradrenalin. The intra-adrenal chromaffin tissue is able to convert noradrenalin to adrenalin before birth in both man and rats. The enzyme which catalyses the conversion, phenylethanolamine N-methyl transferase is under the control of the pituitary gland. (It will be remembered from Chapter 5, that vitamin B<sub>12</sub> is a coenzyme for the reaction.) In rats deprived of the pituitary by decapitation on the 17th day of gestation, the activity of this enzyme drops by about 80%. Not only ACTH, but also hydrocortisone acetate, has the ability to restore the activity towards normal. It seems therefore, that both the pituitary and the adrenal cortex have a modifying effect on the adrenalin/noradrenalin ratio of the medulla. The dramatic fall in enzymatic activity following foetal pituitary ablation suggests that maternal ACTH is not able to cross the placenta to substitute for the lost foetal hormone.

If adrenalin is given in excess to rat or rabbit foetuses, it causes degenerative changes in the foetal vascular system. As a result, some tissues become necrotic, and there may even be prenatal loss of limbs or tails.

**The Gonads.** Disturbances in the development of the gonads are known to occur as a result of the therapeutic use of sex hormones during pregnancy, or as a result of pathological processes occurring in the maternal gonads. In the male rat, the testicular interstitial cells are histologically demonstrable about the 15th day of gestation, in man about the 16th week. In the female the analogous periods for ovarian development are the first day of post-natal life in the rat and the 30th week in the female human foetus. Secretion by testicular and ovarian endocrine cells is stimulated by pituitary gonadotropin, and synthesis requires nicotinamide coenzymes, vitamin A and possibly other vitamins. Hypophysectomy leads to a reduction in the number of interstitial cells present in the testis and arrested development of the male genital tract.

Genetic sex is determined at the moment of conception. Gonadal sex is not determined until a later period. Somatic sex depends on the development, or the absence of the testicular androgens in mammals. The sequence of development in the rabbit provides an illustration of the process. The embryonic gonads are recognisable by about the 15th day of gestation, and they are identical in both sexes. In the presence of the functioning pituitary, the gonad in a genetic male then begins to secrete androgen. This is followed by regression of the Müllerian duct and the development of prostatic rudiments. Full development of somatic masculinity occurs when the internal and external genital apparatus and the secondary sex characters appear. Loss of testicular activity just before gonadal sex differentiation, by castration or by chemical means, inhibits the process, and the animal develops along the female line of differentiation. In such a case, a genetic male acquires

feminine characteristics and is therefore pseudo-hermaphrodite. The gonadectomised genetic female suffers no such disability, and continues to develop along the female pattern. We must conclude therefore, that foetal testicular hormones are essential for the development of the portion of the Wolffian duct which forms the vas deferens, and for the formation of prostatic anlagen from the urogenital sinus. The interstitial cells of the embryonic testis are thus the major factor in deciding somatic sex differentiation in mammals. In the rat, it is known that the ability of foetal testicular tissue to make testosterone from progesterone is at its height at about 18½ days gestation. The vitamin-containing co-enzymes necessary for steroid biosynthesis will therefore be required for several days prior to this time.

Sexual development in birds differs from that in mammals in that the ovarian hormones have the controlling influence in most cases investigated. This is possibly related to the fact that in mammals the males are the heterozygous sex (possessing an X and a Y chrosomome), and in birds the males are homozygous (possessing two X chromosomes).

Human cases of bilateral ovarian agenesis (Turner's syndrome), at one time thought to involve chromosomal females only, are now known to include both genetic males and genetic females. The lack of testicular hormone in the genetic males apparently allows the formation of the infantile female type of genitalia and secondary sex characters, as in the experimental rabbit. Turner's syndrome in man is associated with anomalies of the central nervous system and the cardiovascular and skeletal systems.

### **Chromosomal abnormalities**

Most of those agents which were mentioned in the previous chapter as causing chromosomal abnormalities and carcinogenesis are able, in the foetus, to produce teratogenesis; ionizing radiations, viruses and antimetabolites are all teratogenic. Genetically determined foetal abnormalities are by now well recognised. In some cases the chromosomal damage is visible to the electronically aided eye, in other cases the genetic defect can be traced only by an examination of family history. Some genetic abnormalities can be duplicated exactly by environmental changes *in utero*, and these are known as phenocopies, that is, they are non-hereditary defects mimicking the defects induced by mutant genes. Examples are the syndactyly, cleft palate and coloboma induced by A deficiency, riboflavin deficiency or folic acid deficiency.

The purines and pyrimidines, which require folic acid for their synthesis, form part of the prosthetic groups of enzymes, the absence of which may cause foetal abnormality. Mercaptopurine, for example, which is used in the treatment of human leukaemia, if given to pregnant

rats, induces foetal abnormalities, including cleft lip and palate, deformed limbs, malformation of the urogenital and cardiovascular systems and of the adrenals (Kury *et al.*, 1968).

It is important to distinguish between phenocopies and true genetic defects. Ingalls, Ingenito and Curley (1964), while investigating the teratogenic effects of a nicotinamide antagonist in mice, were able to show that the malformed foetuses had a high proportion of chromosomal abnormalities as compared with normal foetuses. Polyploid cells and cells with fragmented chromosomes were found not only close to the palatal defect which occurred in 95% of the test animals, but also at some distance from the lesions. Examination of the maternal bone marrow cells also revealed a rise in chromosomal abnormalities as compared with the normal control animals. Six days after the injection of the teratogen 56% of the maternal bone marrow cells showed fragmentation of the chromosomes and 6% were polyploid. Had the previous history of the animals not been known accurately, the chromosomal anomalies and congenital malformations of the foetuses would have been interpreted as true hereditary defects. Here we have proof that the genetic substance itself is susceptible to environmental injury. It remains to be seen how far-reaching the effects of such damage may be—whether they may be passed on to succeeding generations in a latent or an overt form.

As in neoplasia, so in teratogenesis, karyotypes may not always be visibly abnormal, but the possibility remains that mutations not yet visible by present microscopic methods nevertheless do exist. Such alterations may provide the molecular lesion which causes eventually the production of tumours or malformations. Down's syndrome, or mongolism, provides the obvious link-up between teratogenesis and neoplasia. The affected persons have an incidence of leukaemia grossly in excess of that of other people.

Genetic defects are not always transmitted by the maternal chromosomes. The paternal contribution, although contained in one cell only, nevertheless provides half the programme for the fashioning of the new organism, and must be held responsible for the defects as well as the good qualities of the progeny. This can be illustrated experimentally by treating rabbit sperm with colchicine. The foetuses of rabbits impregnated with this sperm show various anomalies (Chang, 1944). Similarly a paternal chromosomal defect has been suspected as being the cause of multiple anomalies in a human infant. In a case reported by Day *et al.*, (1967) the mother had a normal karyotype, but the father and child had part of the 18th chromosome missing. Physically, the father was apparently normal; the infant had various abnormalities including microcephaly, cryptorchidism, hypospadias and talipes. Damage to paternal chromosomes as a cause of foetal anomaly is a fairly new concept in human medicine and one which has been slow

to gain acceptance. In the field of animal medicine, economic considerations have ensured that the nutritional requirements of both the sire and the dam are held of equal importance for the development of healthy progeny.

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