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CHEMISTRY
OF
VITAMINS AND HORMONES

BY

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PREFACE

The chemistry of vitamins and hormones is largely a growth of the past fifteen years and its development during this period has been spectacular. As the result almost all of them have now been prepared in a pure condition and their chemical constitutions established beyond doubt and confirmed by synthesis. In several cases synthesis is carried out in commercial quantities

The present book is intended to give a direct and clear account of the subject in a small volume. In view of its limited scope extensive bibliography is not provided. References are given only to the most important chemical contributions. Further, to mention the names of all the workers, who are particularly numerous in this field, would have made the description cumbersome, and hence any failing in this direction should be pardoned. Owing to the world war there has been serious difficulty in obtaining literature relating to very recent advances and the printing of the book has also taken a considerable length of time. However, all efforts have been made to bring the account up to date.

For convenience of treatment the fat-soluble vitamins are placed together first and the water-soluble vitamins follow. Where there are several ways of isolating a vitamin or hormone, of proving its constitution or of synthesising it, the most direct ones are generally chosen, and some others which may involve important chemical technique are also mentioned. Information about certain groups of compounds like the sterols and carotenes and also about certain methods and phenomena will be useful for understanding the chemistry of vitamins and hormones. This could not be conveniently introduced in the main part of the book without affecting continuity of discussion or description. Hence such information is provided separately at the end of the book under notes.

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CHAPTER I

VITAMINS AND THEIR GENERAL CHARACTERISTICS

The discovery and the subsequent investigation of vitamins have marked an epoch in the progress of our knowledge of disease and nutrition. In the history of civilization various ideas have been current at different times regarding the cause and cure of diseases. Some of them are even now noticed simultaneously in the different strata of society. Probably the oldest and most primitive idea was to attribute them to evil spirits whose anger results in plague and pestilence. Sacrifices and other ceremonies were performed in order to appease them. A definitely higher stage of development was the theory of poisons or toxic substances as the cause of ailments, and treatment therefore consisted in removing these poisons. According to the humoral theory associated with some of the ancient civilizations, in a state of health the humors are present in equal concentration in the body. When one of them predominates there is disease and treatment consists in restoring the equilibrium between the humors. With the entry of the microscope as one of the tools of the scientist and the discovery of micro-organisms such as bacteria and fungi, the bacterial theory of diseases held sway from the beginning of the nineteenth century and it was considered an almost inevitable necessity to find a micro-organism as the cause of every known disease. Viruses were added later on. All the above represent positive causative agents and were easy to understand. The existence of deficiency diseases and their causation by the lack of certain essential substances were more difficult to realise and were understood only very recently. One such deficiency is that of vitamins which are normally present in food-stuffs.

A parallel development in our ideas of food may also be traced. Primitive man slowly accumulated knowledge

regarding harmful and harmless food, digestible and indigestible; but his first criterion would have been the feeling of satisfaction arising out of a full stomach. More and more information was collected later on regarding the detailed properties of different kinds of food. But marked progress was not possible till chemical knowledge came to be applied to this important subject. Food was then recognised as supplying the energy required for work and maintaining body temperature and also as providing the necessary material for body growth and replenishment. Proteins, carbohydrates and fats were found to be the important components; their capacity to supply energy and promote growth was carefully studied. Later came the recognition of the vital role of minerals, particularly of calcium and phosphates. The latest addition to our knowledge in this field is that certain accessory food factors or adjuvants, present only in very small amounts in our foods, play a very important part in maintaining the health and growth of animals. These are known as vitamins.

The name "vitamine" is due to Funk, one of the early workers in this subject. It implies that all the vitamins are nitrogenous and basic in nature. Though it is correct as far as the anti-beri-beri vitamin on which he was working and many other members of the B group are concerned, it is not satisfactory in regard to the large number of other vitamins which are neither nitrogenous nor basic. However, by long usage the name has got established and is now used with the omission of the final 'e.'

The vitamins have a number of common properties which justify their being grouped together. They are required only in small quantities and even in such amounts they bring about marked physiological effects. Specificity of function is another characteristic. Each vitamin is related to a definite disease. The lack of the vitamin causes the disease and the supply of it is the remedy. In general they are complex compounds; but the complexity of the molecules varies very much. The pellagra-preventive factor is one of the simplest, being identical with nicotinic acid; some others, however,

possess very complex molecular structures. They are usually divided into two groups, (*a*) fat-soluble and (*b*) water-soluble. Vitamins A, D, E and K belong to the first category. They consist of large molecules rich in carbon accounting for their solubility in fats and fat solvents. Further, entire molecules or large portions are based on isoprene units. Members of the B group and vitamin C are water-soluble. The water-soluble properties are due to diverse structural features such as salt form and presence of lyophilic groups of various types. Some of the vitamins contain nitrogen as a component element and they belong to the B group. The rest are non-nitrogenous. Regarding their supply, animals seem to depend almost completely on plants. It is true that some of these, among them vitamin A, do not occur as such in plants and are produced in the animal organisms by certain transformations. But even in such cases the main complex structures of the vitamin molecules are evolved in plants. All animals are not alike in their requirements of the various vitamins. There are animals which can get on without a supply of certain vitamins. This may be due to their capacity to make them or to get on without them. This point has to be particularly noted in translating the results of experiments carried out with lower animals to the case of human beings. Sometimes groups of allied compounds have the same property and function as one vitamin. Several antirachitic principles (*e.g.* D_2, D_3 and D_4) are definitely known; further there are three tocopherols (vitamin E) and two K vitamins. Among the related compounds, however, there are differences in physiological potency.

In the chemical study of vitamins the chief difficulty lay in their isolation. They occur in very small amounts associated with large quantities of other organic substances. Concentration of the vitamins and their purification involved special technique. With a view to following up the degree of concentration suitable methods of assay had to be evolved. They had to be rapid and reliable. Though biological methods satisfied the second requirement, they suffered from being slow.

Hence chemical and physical methods were discovered and used. Once a pure sample of the vitamin was made, things moved forward with great speed. Besides the establishment of the constitution, the subsequent step, synthesis, has also been achieved very fast. It is now correct to say that not only are the details of the chemistry of these once elusive bodies definitely known, but several of them are articles of commercial supply. Indeed in most cases synthetic products have displaced compounds of natural origin.

The vitamins form a large and growing group of compounds. In the early days when very little was known about them, the letters of the alphabet such as A, B C, D etc. were used for denoting them. Later on these have been supplemented by names indicating their physiological properties and their chemical characteristics. As an example may be mentioned vitamin C which is also called ascorbic acid (antiscorbutic acid). Similarly vitamin B_1 is now called aneurin indicating that it is anti-neuritic, and thiamin since it contains sulphur. Some letters like K and P have been employed for designating the newer vitamins, omitting the intervening letters of the alphabet. This is due to the fact that these are the first letters of the names of the vitamins Koagulations vitamin, Permeability vitamin.

Though vitamins are required only in very small quantities and have very powerful physiological properties, no cases of harmful effects of over-dosage are commonly met with. The margin between the therapeutic and the toxic dose is very large. This may be due to the ease with which the body mechanism can deal with excesses and dispose of them. Earlier reports about the adverse effects of injudicious administration of synthetic vitamin D (calciferol) to infants and children seem to be due to the presence of toxic impurities. Heavy doses of nicotinic acid may produce flushing and irritation of the skin and occasionally gastro-intestinal symptoms. They seem to depend upon individual variations in response and the effects are transitory. Similarly excess of thiamin (vitamin B_1) is reported to

the most easily tolerated. Even this, however, in doses of 1–2 grams daily for several weeks is said to affect the urine leading to the formation of urinary calculi. These unusually large doses have to be avoided not so much for the possible toxic effects as in the interest of economy of these vital food factors.

As has already been mentioned the vitamins are active physiologically in remarkably small doses. In the earlier days of work on vitamins, particularly before they were isolated in a pure condition and characterised, their preparations could be assayed and quantitatively evaluated only in terms of their physiological potency and this was the basis of the international and other units. The standards were originally arbitrary but now they are fixed in relation to pure substances. In the case of the simpler vitamins like nicotinic acid, inositol, choline etc. which were already known pure long before their vitamin properties were discovered, the tendency is to assay them in terms of the pure substances as so many milligrams.

The relationship of new compounds occurring in nature to well known raw materials like carbohydrates, fats and proteins, and their evolution from them have always formed a fascinating field of chemical enquiry. A great deal of valuable chemical work has been done particularly in connection with the alkaloids, terpenes, anthocyanins and anthoxanthins. Some amount of thought has been bestowed on the subject of vitamins also from this point of view. These ideas are presented and amplified where necessary and fresh suggestions are offered in the appropriate parts of this book.

CHAPTER II

AXEROPHTHOL: VITAMIN A

The very first characteristic to be noticed in connection with vitamins was their capacity to promote body growth. Later on, when different members of the vitamin group were recognised, the one primarily responsible for the maintenance of growth was designated vitamin A or the growth factor. It was further realised that it is intimately connected with the maintenance of the epithelial tissues in proper condition and the resistance of the body to infection from various diseases. The term 'anti-infective principle' has also therefore been applied to it. Lack of this vitamin is closely associated with certain diseased conditions of the eye such as xerophthalmia and night blindness. In giving this vitamin a distinctive name the property of curing xerophthalmia has been chosen and it is called 'Axerophthol'. Utilising the above discovery further, a property known as dark adaptation has been employed for studying the vitamin A levels of individuals in various communities. The principle involved is that small quantities of vitamin A are continually being supplied in the form of visual purple to the retina of the eye and upon this supply depends the capacity for vision If a person is suffering from low vitamin A level, his capacity to supply sufficient amount of it quickly when placed in the dark becomes less and his vision is defective. Even apart from these considerations an adequate supply of this vitamin is absolutely essential since it has far-reaching effects on several normal physiological processes.

Occurrence:—Vitamin A, as such, is present exclusively in animal sources. Of these, eggs, milk, cream and butter may be said to be highly important for nutrition. Though the amount of this vitamin present in these articles of diet is very small (*e.g.* about 3 I.U. per gram of milk) they are the most handy and easily available food-sources containing it. It must, however, be remarked

that the quality of milk and butter in regard to this vitamin is dependent on the fodder of the cow. Those obtained from grass-fed animals are rich, whereas those obtained from stall-fed animals are poor. The reason for this has been traced to the high amount of carotene present in green fodder, which undergoes transformation in the animal into vitamin A. Sun-drying of grass for hay-making leads to considerable loss of carotene. Consequently, methods have been developed for preserving grass and other similar green fodder without this loss. One of the earlier methods to be devised was conversion into silage. In this process green fodder is cut into bits and stacked in pits out of contact with air and allowed to undergo fermentation. Certain nitrogenous and phosphatic mineral compounds are also added with a view to increase the food value. A more recent method of preservation is to dry the grass, immediately after cutting, in special grass-driers which make use of a current of hot air for rapid evaporation of the moisture in the material. When employed with care, they are capable of yielding dry green grass in which there is practically no deterioration of the carotene, proteins or minerals or their digestibility. Therefore grass dried in this manner is equal in nutritive value to concentrated foods and in addition contains almost all the carotene originally present in the fresh material.

The liver fats of various animals and particularly those of grass-fed animals are very rich sources of vitamin A. However, from the point of view of commercial supply, cod-liver oil occupies the most important place. It has been valued for a long time owing to the easy digestibility of the fat. Now it has been found to be a convenient source for the supply of the fat-soluble vitamins A and D. The quality of this oil also varies with the locality and the season, being largely dependent upon the food available to the fish. It has been stated that eventually, the life of these fish depends upon the simpler marine organisms like algae, diatoms and small sea plants. The cod lives on smaller varieties of fish which in their turn derive their sustenance from marine plants. The variation in the quality of cod-liver oil should there-

fore be traced to the changing conditions of marine plant life. Further, methods of preparation and of storage have a profound effect since vitamin A is easily oxidized in the presence of light. Preservation in coloured bottles with the exclusion of air and moisture is quite necessary. The British and the United States Pharmacopoeias have prescribed a minimum of 600 international units per gram for the vitamin A potency of cod-liver oil of good quality.

Many other fish liver oils appear to be richer sources of vitamin A and some of them are remarkably potent containing about one per cent of the vitamin. Haddock, halibut, shark and tunny may be mentioned under this category. Halibut liver oil is sometimes found to be several hundred times as rich as the liver oil from the cod. Hence it has been employed as the most suitable starting point for the isolation of vitamin A and also for vitamin therapy where a very rich source enabling small dosage is desirable. In the warmer parts of the globe where the cod is scarce, shark-liver oil is a good substitute. It is generally 10—16 times as rich as cod-liver oil; but the quality is highly variable, some species of shark yielding oils even less potent than cod liver oil.

Though vitamin A does not occur as such in the plant kingdom, requisite amounts of it can be derived from vegetable foods. As already pointed out, carotene readily undergoes change in the animal system to yield vitamin A. Hence it is called 'Pro-Vitamin A' and the most important vegetable sources of this substance satisfactorily supply the needs of animals in regard to this vitamin. Under this category come all green and leafy vegetables, such as cabbage, lettuce, green peas and chillies, yellow and brown maize, red carrot, yellow and brown sweet potatoes and yellow and red onions. Among fruits, tomatoes and bananas, yellow oranges, mangoes and papayas may be mentioned. Red palm oil obtained from a palm growing in Africa and Malaya is employed as a very convenient source for the supply of carotene since it has an inoffensive odour and taste and is therefore easily administered. Annatto seeds which yield a dye known as bixin, a familiar colouring matter for

butter, also contain a high percentage of carotene. Greenness in leaves and yellow or orange colour in roots and seeds are rough but reliable indications of vitamin value. Thin green leaves are amongst the best sources, assaying well over 1000 international units per hundred grams, and are frequently found to be twenty times as good.

The discovery [1] of carotene as provitamin A emanated from the early observation of Steenbock that the vitaministic activity of plant food-stuffs appeared to be proportional to the amount of the carotenoid pigments and in particular carotene itself present in them. It was first thought that carotene might be the actual vitamin. The experiments of Karrer and von Euler showed definitely that the vitamin from cod-liver oil is different from carotene, but that carotene could replace vitamin A in dietaries. Actually the potency of animal foodstuffs e.g. milk and butter is the sum of the units due to vitamin A and carotene.

In spite of the large expansion of the fish oil industry in recent years, the majority of human beings continue to depend mainly on carotenoid provitamins rather than on preformed vitamin A. There are a number of compounds of the carotenoid group capable of functioning in this manner (see carotenes under 'notes'). The most important of these are the carotenes (α, β and γ) having the formula $C_{40}H_{56}$ and the related compound cryptoxanthin, $C_{40}H_{58}O$. Each of these possesses at least one β-ionone unit in its molecular structure. β-Carotene has the highest potency since it contains two such units.

Isolation: It was first established by McCollum and Davies in 1914 that vitamin A present in butter fat could be concentrated in the unsaponifiable fraction. This discovery was subsequently applied by other workers to the case of cod liver oil. Fractional distillation of the unsaponifiable matter of this oil gave highly potent preparations, but Drummond and Baker showed in 1929 that the active substance was present in these concentrates in such minute amounts that direct attempts to isolate it by ordinary chemical means were bound to be futile. Hence more potent sources of the vitamin such as halibut

liver oil were examined with more fruitful results. The unsaponifiable matter from this oil containing vitamins A and D and sterols was dissolved in methyl alcohol and the solution chilled to temperatures as low as $-60°$ whereupon most of the sterols crystallised out. The resulting concentrate contained at least 50% of vitamin A [2].

Further purification was effected in two ways. Karrer and coworkers [3] employed the chromatographic method. The sterol-free concentrate was dissolved in petroleum ether and passed down a column of Merck's Fasertonerde and subsequently washed down the column with the same solvent. Vitamin A was adsorbed mainly on the middle third of the column and elution with petroleum ether-methyl alcohol mixture gave a concentrate which could be purified further by a repetition of the process. The product was an extremely viscous golden yellow oil and was regarded as almost homogeneous.

The second method of concentrating the vitamin employed molecular distillation. Using a specially constructed molecular still Heilbron and coworkers [4] succeeded in fractionating the sterol-free portion of the unsaponifiable matter of halibut liver oil without decomposition ; a fraction boiling at 137-138° and containing the major portion of the vitamin was thus obtained. This product was indistinguishable from that got by chromatography.

In the latter half of 1937, Holmes and Corbet [5] succeeded in getting a crystalline preparation melting at 7° by prolonged cooling at $-60°$ of an aqueous methyl alcoholic solution of rich concentrates. However, to judge from the properties of allied compounds its melting point was too low. By the crystallisation of rich concentrates obtained from various fish liver oils and halibut visceral oil, using a number of solvents such as methyl alcohol, ethyl formate, petroleum spirit and propylene oxide at temperatures as low as $-35°$ to $-70°$, Baxter and Robeson [6] succeeded in 1942 in obtaining yellow crystals of pure vitamin A melting at 64°. According to these authors the crystals previously prepared by Holmes and Corbet contained methyl alcohol.

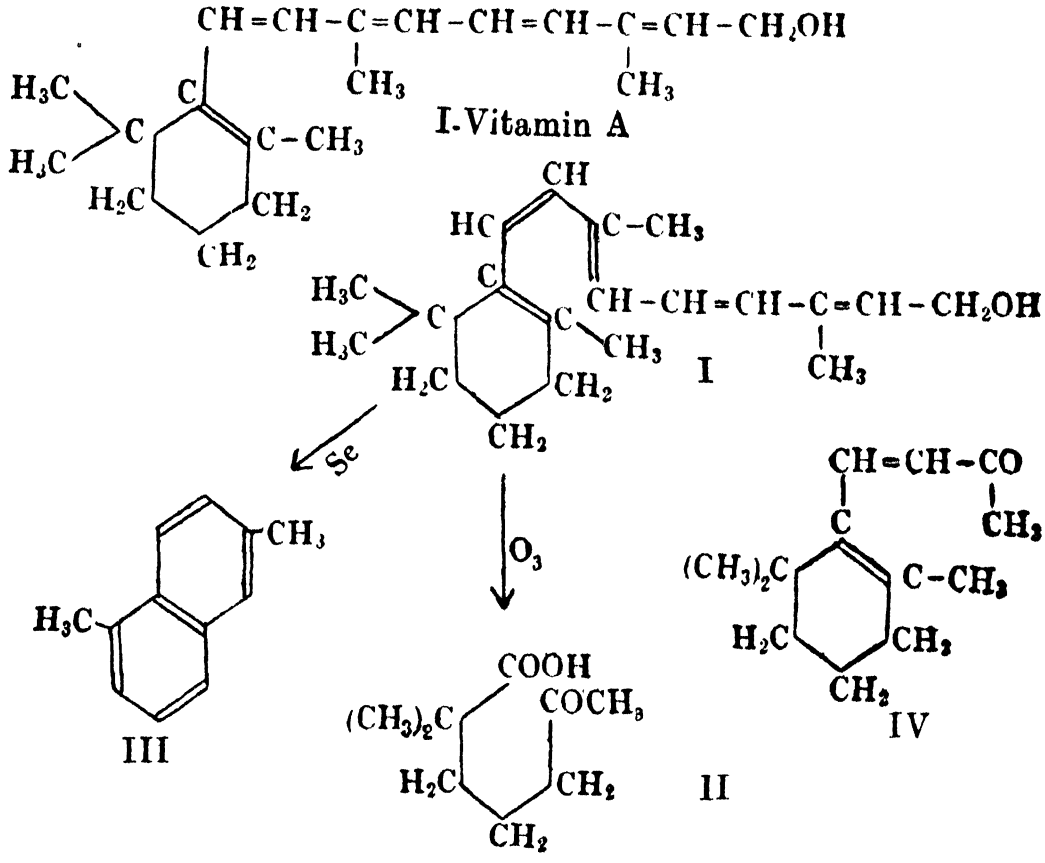
As already mentioned, vitamin A can be distilled in very high vacuum without appreciable loss. Hickman has used this procedure for separating vitamins A and D in the free condition or as esters with fatty acids. He has thus shown that in the oils these vitamins occur as esters. The methods outlined above, particularly in their improved forms, are being employed for obtaining concentrates of vitamin A in commercial quantities.

Properties :—As mentioned earlier, the purest form of crystalline vitamin A melts at 64°. It is insoluble in water but is readily soluble in organic solvents and in fats; hence it is grouped under 'fat-soluble' vitamins. It is optically inactive and isotropic. Its absorption spectrum exhibits a band with a maximum at 328 mμ. (extinction coefficient, $E^{1\%}_{1\ cm}$ in ethyl alcohol = 1700). In chloroform solution, it produces a bright blue colour with antimony trichloride and the absorption bands are at 583 mμ. and 620 mμ; the intensity of this colour offers a measure of the vitamin A content. With concentrated sulphuric acid it produces a violet colour. The vitamin is adsorbed by norite (charcoal) and by silica gel tenaciously. In the absence of air vitamin A is stable to heat but it is destroyed in air due to oxidation. Antioxidants protect it. Extreme caution has to be taken against the effect of light which is quite destructive.

In its properties and reactions vitamin A exhibits great similarity to carotenes, particularly β-carotene. They have similar growth-promoting property and the antimony trichloride reaction is also similar. There seems to be sufficient evidence for believing that carotenes are converted into vitamin A in the liver. These facts have been of great use in the determination of the constitution of vitamin A.

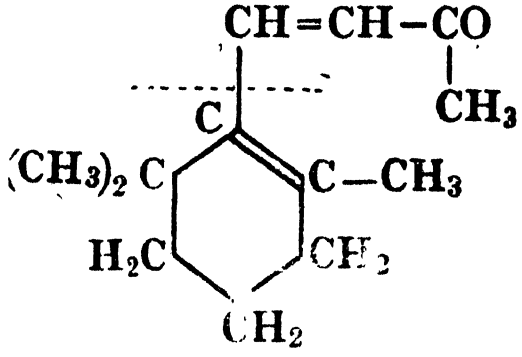
Constitution :—The molecule of vitamin A contains only carbon, hydrogen and oxygen and its molecular formula has been established as $C_{20}H_{30}O$. The molecular weight determined by various methods yielded values ranging between 300 and 320, the value corresponding to the above formula being 286. A comparison has been

effected with β-carotene using the diffusion method. From the relative rates of diffusion the molecular weight of vitamin A should be nearly half that of β-carotene. On reduction it absorbs 5 molecules of hydrogen and the decahydro-derivative is known as perhydrovitamin A. Further, the vitamin is a primary alcohol forming esters with acids. The presence of one β-ionone ring (IV) in the molecule was established by Karrer, Morf and Schöpp[7] who found that ozonolysis yielded one molecular proportion of geronic acid (II). This acid is well known as a normal oxidation product of β-ionone. On the basis of the above evidence, they[8] suggested formula (I) for the vitamin. This was corroborated by studies of absorption spectra which showed that the vitamin molecule contained 5 double bonds. Additional support was provided by the work of Heilbron, Morton and Webster[9]. By the action of hydrochloric acid on the vitamin they obtained a product which was originally considered to be cyclised vitamin A but has more recently been shown to be anhydro-vitamin A. When this product was subjected to selenium dehydrogenation it gave a good yield of 1:6—dimethyl-napthalene (III) which was also obtained by direct dehydrogenation of the vitamin itself. Its formation definitely established the terpenoid nature of the vitamin. The transformations can be represented as below:



Synthetic work:—Synthetic support for the above structure was provided by Karrer and Morf [10] in 1933, by the synthesis of perhydro-vitamin A (X). They started with β-ionone (IV), a molecule containing 13 carbon atoms and added to it suitably seven carbon atoms in four stages. Two pairs of C atoms were introduced by carrying out Reformatsky reaction twice using zinc and bromo-acetic ester; another pair was added by malonic ester synthesis and a methyl branch built up by the action of zinc methyl iodide. At appropriate stages reduction was carried out. The details are given below: β-Ionone (IV) was condensed with ethyl bromo-acetate (Reformatsky reaction) and the product dehydrated to give ethyl β-ionylidene acetate (V) which was hydrogenated catalytically to (VI). Reduction of this ester yielded the alcohol (VII) whose bromide was condensed with malonic ester and converted by standard reactions into the C_{17} carboxylic acid (VIII). Treatment of the acid chloride with zinc methyl iodide gave the C_{18} ketone (IX). By a repetition of the Reformatsky reaction, conversion of the hydroxy ester into the bromo ester and

stepwise reduction of the product using first zinc-copper and acetic acid and subsequently sodium and alcohol the saturated C_{20} alcohol (X) was obtained.



$\xrightarrow[Zn]{CH_2Br.\ COOEt}$

IV

$$C_9H_{15}-CH=CH-\underset{\underset{CH_3}{|}}{C}=CH-COOEt.$$

V

$\xrightarrow[\text{reduction}]{\text{catalytic}}$

$$C_9H_{17}-(CH_2)_2-\underset{\underset{CH_3}{|}}{CH}-CH_2-COOEt$$

VI

$\xrightarrow[EtOH]{Na}$

$$C_9H_{17}-(CH_2)_2-\underset{\underset{CH_3}{|}}{CH}-CH_2-\underset{(CH_2Br)}{CH_2OH}$$

VII

$\xrightarrow[\text{synthesis}]{\text{Malonic ester}}$

$$C_9H_{17}-(CH_2)_2-\underset{\underset{CH_3}{|}}{CH}-(CH_2)_3-\underset{(COCl)}{COOH}$$

VIII

$\xrightarrow{ZnMeI}$

$$C_9H_{17}-(CH_2)_2-\underset{\underset{CH_3}{|}}{CH}-(CH_2)_3-\underset{\underset{CH_3}{|}}{CO}$$

IX

$\xrightarrow[\text{reaction}]{\text{Reformatsky}}$

$$C_9H_{17}-(CH_2)_2-\underset{\underset{CH_3}{|}}{CH}-(CH_2)_3-\underset{\underset{CH_3}{|}}{\overset{\overset{OH(Br)}{|}}{C}}-CH_2-COOEt$$

$\xrightarrow[\text{reduction}]{\text{Stepwise}}$

$$C_9H_{17}-(CH_2)_2-\underset{\underset{CH_3}{|}}{CH}-(CH_2)_3-\underset{\underset{CH_3}{|}}{CH}-CH_2-CH_2OH$$

X

The synthetic alcohol and the product obtained by hydrogenation of a rich concentrate in the presence of platinum oxide were viscous oils possessing physical properties which agreed very closely. In order to make the proof of identity quite unambiguous Karrer and his co-workers prepared solid derivatives independently from each sample and compared them. The following series of reactions were carried out:

$$C_{19}H_{37}CH_2OH \xrightarrow{HBr} C_{19}H_{37}CH_2Br$$

X

$$\xrightarrow[\text{synthesis}]{\text{Malonic ester}} C_{19}H_{37}-CH_2-CH_2\cdot \underset{(COCl)}{COOH}$$

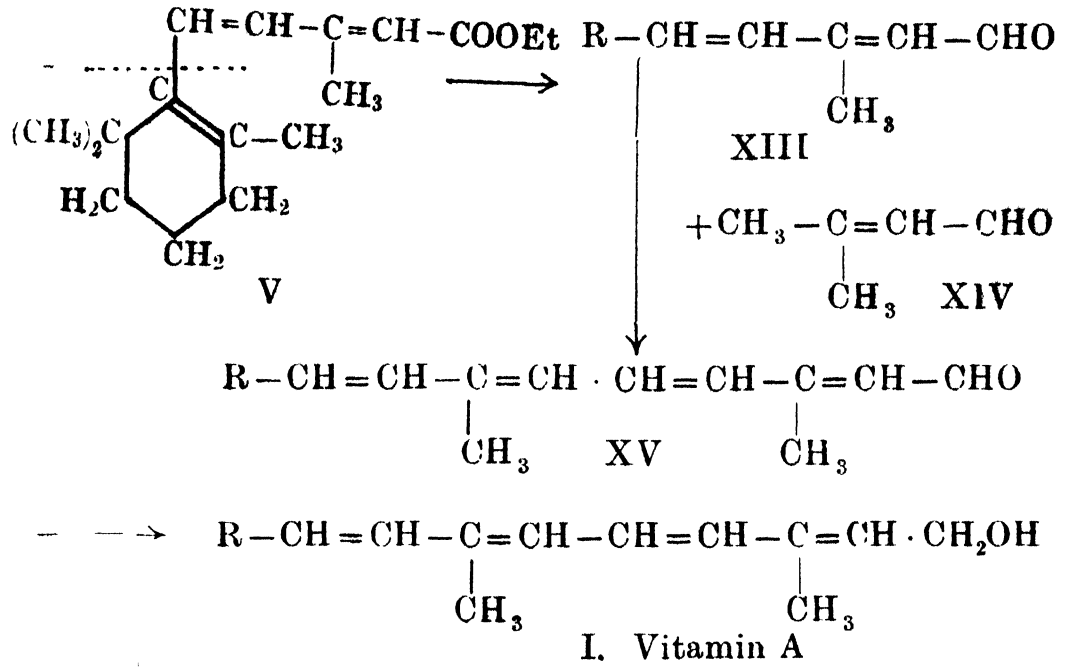
XI

$$\xrightarrow{ZnMeI} C_{19}H_{37}CH_2\cdot CH_2-\underset{\displaystyle CH_3}{\overset{\displaystyle |}{CO}}$$

XII

The melting point of the acid (XI) and that of the semi-carbazone of the ketone (XII) were the same in the two series and the mixed melting points were also the same.

The synthesis of vitamin A itself in an impure condition was claimed by Kuhn and Morris[11] in 1937 who also started with β-ionone (IV) and condensed it with zinc and iodoacetic ester to get the ethyl ester of β-ionylidene acetic acid (V). It was converted into the corresponding aldehyde (XIII) by a series of reactions and the product condensed with β-methylcrotonic aldehyde (XIV) using piperidine acetate as catalyst. The resulting compound was expected to be the aldehyde corresponding to the primary alcohol, vitamin A. It was carefully reduced using aluminium isopropoxide in isopropyl alcohol solution. The final product was considered to contain 7·5% of vitamin A as estimated by the antimony trichloride colour reaction and biological tests. But several workers including Karrer have reported unfavourably on this synthesis of the vitamin. They have failed to confirm it and it does not seem to be of much value.



I. Vitamin A

R represents cyclohexene ring part.

Some convenient derivatives:—For studying samples of vitamin A it is useful to have stable, crystalline derivatives which could be identified by melting points and other physical constants and by analysis. One such is obtained by the action of maleic anhydride on vitamin A acetate. Two molecules of the anhydride add on and form a crystalline product melting at 261°, and having the formula $C_{30}H_{36}O_8$. A crystalline addition product is produced even when fresh liver oils are directly treated with maleic anhydride. This however is found to be the dimaleic adduct of vitamin A palmitate and yields on saponification palmitic acid as one of the products. Thus there is definite proof that the vitamin occurs in these oils as the palmitate.

Crystalline derivatives of the above mentioned type are not physiologically active, nor can the vitamin be recovered from them. But the β-napthoate obtained by the action of β-napthoyl chloride and pyridine and melting at 76° exhibits vitamin A activity and can be saponified to give vitamin A. More recently Baxter and Robeson [12] (1942) have prepared the following aliphatic esters of vitamin A in crystalline condition:—the acetate melting at 57°, the palmitate melting at 27° and divitamin A succinate melting at 76°. They are all pale yellow in colour.

Assay:—The biological method involves the measurement of growth response of rats which have been originally deprived of vitamin A. It may be carried out as follows. About 30 newly-weaned rats are kept on a diet free from vitamin A until they cease to grow. They are then divided into different groups as uniformly as possible and each group is administered daily a certain known amount of the preparation under test or a standard preparation. The rats which resume growth are weighed once a week for three or four weeks and the average increases in weight of the rats in different groups are determined. Equal growth responses with particular doses of the unknown and of the standard preparation indicate equal physiological potency; from this the vitamin A potency of the unknown in international units per gram can be calculated.

The method can also be carried out as a prophylactic test. For this purpose groups of rats suitably arranged are given doses of the preparation being tested and of the standard preparation from the beginning of the experiment instead of only after the cessation of growth. Comparisons are then effected between the different groups and the vitamin A potency of the unknown is calculated as in the curative method. In spite of various improvements the biological method has not yet become as rapid as could be desired.

The spectrophotometric method measures the ultraviolet absorption at 328 mμ. which is a characteristic physical property of vitamin A. When applied to the determination of vitamin A in samples of cod-liver oil which conform in all other respects to the standard specifications, it gives a trustworthy measurement, but may be misleading in the presence of other substances which also show absorption in the same region but have no physiological potency (see 'compounds related to vitamin A' under notes). In the event of a discrepancy between the values obtained by this and the biological method, the latter alone is accepted as correct.

For the determination of vitamin A potency by this method, an oil is saponified and the unsaponifiable matter is extracted and dissolved in a suitable volume of

dehydrated alcohol or cyclohexane to produce a soluti of the concentration required for the instrument to used. The ultraviolet absorption at 328 mμ. is measu and the result calculated with reference to the origir oil. The vitamin A potency in I.U. per gram is obtain by multiplying the extinction coefficient by a suital factor ($E^{1\%}_{1\,cm} \times 1700$). Like the biological method, tl spectrophotometric method is also of limited applica lity, because of the need for expensive equipment a the lack of strict specificity. It may also be mention that the solvent employed in making up the fin solution has a great influence on the absorption; f example, using ethyl alcohol or isopropyl alcohol t results are about 12% higher than when cyclohexane employed. Further, the colour density does not seem be strictly a linear function of the concentration vitamin A.

A more handy method is the colorimetric one inv ving the use of the Lovibond tintometer according to Ca and Price. It utilizes the bright blue colour reacti of the vitamin with antimony trichloride. It is n claimed to be quite accurate but it agrees with tl biological method fairly satisfactorily and is qui reliable for comparative purposes with oils of know history. The accuracy can, however, be increas considerably by studying the intensity of the absorpti bands of this blue colour using the spectrophotomet (620 and 583 mμ. bands in concentrates and 606 a 572 mμ. bands in unconcentrated oils). Cholesterol a other sterols, squalene, carotenes and various carotenoi also yield blue products when treated with antimor trichloride. The position of the absorption band, ho ever, serves to identify the particular chromogen presen

In this method the vitamin A content of the samp is expressed in terms of "blue values". It can be co verted into international units by using an appropria factor (Potency in international units = Carr-Price bl value × 30). It has been found that the addition guaiacol in the Carr-Price reaction gradually chang the initial blue colour to a relatively permanent reddis

purple which is specific for vitamin A, and can therefore be better utilised for the estimation.

Standard, biogenesis, function and requirements:— A pure sample of β-carotene is now employed as the international standard for evaluating vitamin A activity. Small specimens of β-carotene dissolved in cocoanut oil along with a little hydroquinone as stabiliser, are supplied as reference standard to nutritional workers in different countries. As a subsidiary standard cod-liver oil (assayed) is available. The international unit of vitamin A activity is defined as the amount of activity contained in 0·6 microgram (0·6 γ or 0·0006 milligram) of the international standard of β-carotene (or 1 microgram of a mixture of α- and β-carotenes which was previously employed). Pure crystalline vitamin A has a potency of 3 to 3·3 million international units per gram.

In view of the symmetrical structure of β-carotene having two β-ionone units, it was originally assumed to give rise to two molecules of vitamin A. α- and γ-carotenes with one β-ionone unit per molecule have only half the potency of β-carotene. The vitamin was therefore considered to be produced by simple hydrolytic fission of the carotenes at the central double bond.

$$R - CH = CH - R + 2H_2O \rightarrow 2R{-}CH_2OH$$

$$R{-}CH = CH - R' + 2H_2O \rightarrow R - CH_2OH + R' - CH_2OH$$

But weight for weight vitamin A in alcohol has twice the activity of β-carotene and hence one molecule of β-carotene yields only one molecule of vitamin A in the animal body. A different type of fission leading to two different molecules should therefore be involved and the two halves should have equal chances of turning out to be active or inactive. A possible process involving hydration and reduction will be as follows:

$$R - CH = CH{-}R' \rightarrow R{-}CH_2OH + R'{-}CH_3$$

$$\text{or } R - CH_3 + R'{-}CH_2OH$$

Another possibility which is not excluded is the origin of the vitamin by oxidative destruction of one end of the molecule of carotene. Even then both halves should suffer equally in order to explain the relative potencies of β-carotene and other provitamins. The conversion is

considered to take place in the liver with the help of an enzyme called carotenase. At the same time nothing very definite can be said about the biochemical origin of the carotenes and their derivatives except that they are made up of isoprene units.

As already mentioned, vitamin A exists in fish liver oils and also in intestinal oils mostly in the combined state as esters of long chain fatty acids. These esters have a higher biological value than the free alcohol probably due to their greater stability to oxidation. The vitamin is considered to be a structural material. It is concerned with the structural integrity of the cell rather than with the chemical processes taking place in the cell contents. It seems to perform its biochemical function in chemical combination with other substances rather than in the free state. Since it has an aliphatic primary alcohol structure, it can combine with fatty acids, bile acids and proteins. It seems to be absorbed in the system as a bile acid compound, transported into the blood and lymph and also stored in the liver as fatty acid ester, and to play its part in the visual purple of the retina as a compound with protein.

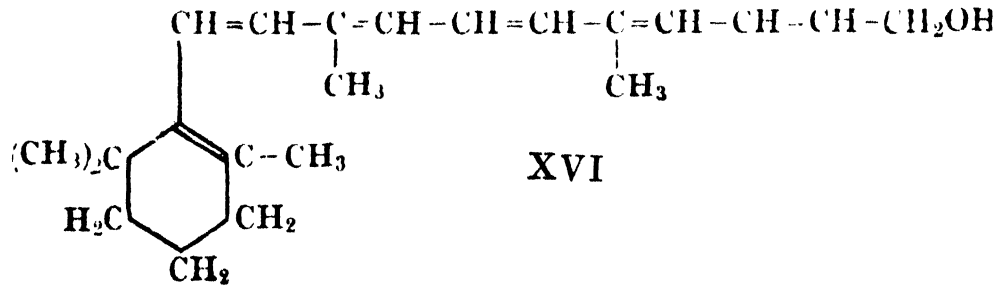
Vitamin A seems to exhibit complete specificity of action since even small modifications of the molecule destroy activity. Besides the β-ionone unit, the series of conjugated double bonds appears to be essential and reduction products are inactive.

There has been considerable change in opinion regarding the vitamin A requirements of different groups of individuals. The most recent opinion seems to be as follows: 5,000 international units per day for adult men and women, 6,000 units during pregnancy and 8,000 units during lactation. Children of varying ages require amounts ranging from 1,500 to 6,000 units; boys of 16 to 20 years are considered to require 1,000 units more than adults. Comparatively larger quantities are required when carotenes are used for supplying the vitamin since their conversion into the vitamin is in most cases incomplete.

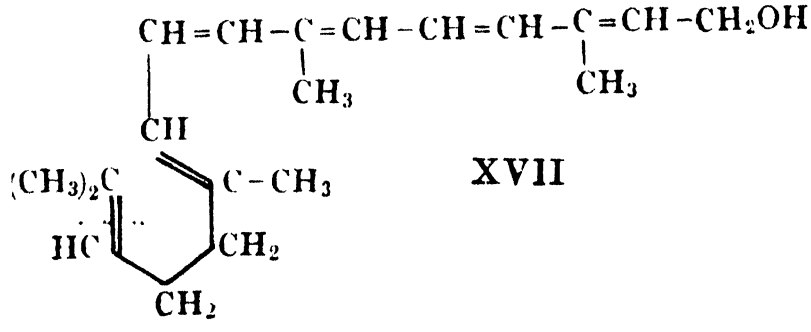
VITAMIN A

Recent careful examination[13] of liver oils from various sources has revealed the existence of another compound closely related to vitamin A and this is called vitamin A_2. With liver oils obtained from fresh water fish in particular the antimony trichloride reaction yields a new set of bands at 650 mμ. and 693 mμ. and these are attributed to vitamin A_2. The free compound itself shows absorption maxima at 280 and 355 mμ. Like vitamin A it is strongly adsorbed on alumina and has other similarities. It is the major component in fresh water fish liver oils whereas vitamin A is predominant in marine fish liver oils. It does not seem to occur in mammals, birds and reptiles.

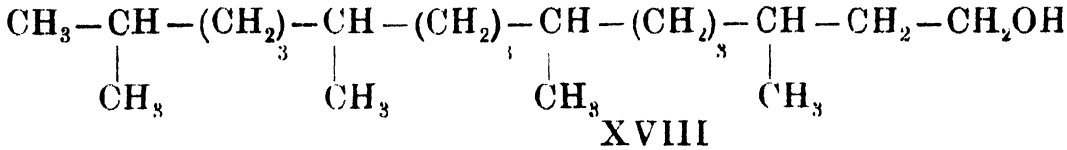
Originally there was great difficulty in separating it from vitamin A and the early experiments had to be conducted with samples which were more or less impure. From the fact that its ultraviolet absorption indicated the existence of a system of six conjugated double bonds and that ozonolysis gave geronic acid showing the presence of a β-ionone ring it was considered to be a C_{22} analogue of vitamin A having one more double bond (formula XVI). But its behaviour during molecular distillation indicated that it had the same number of carbon atoms as vitamin A. More recently (1943) Karrer and Bretscher[14] reported the isolation of almost pure samples of vitamin A_2 from winter-pike liver oil and winter-trout liver oil. Purification was effected first by chromatography using calcium hydroxide and subsequently by molecular distillation. They consider that the molecule of vitamin A_2 has the same number of carbon atoms as vitamin A, but an additional double bond arises from the opening of the ring (formula XVII). This agrees with the observation that ozonolysis of the substance gives rise to acetone, fission taking place at the dotted line. It has been further confirmed by hydrogenating the compound whereby dihydrophytol (XVIII) has been obtained and characterised as its allophanate melting at 73°. Consequently vitamin A_2 bears the same relationship to lycopene as vitamin A bears to β-carotene.



XVI



XVII



XVIII

According to the above authors though vitamin A_2 may be important for fishes it is not so for mammals. The purest samples of vitamin A_2 have only a tenth of the physiological activity of vitamin A on rats. Even this is considered to be due partly to contamination with vitamin A itself, and partly to the capacity of the rat to cyclise vitamin A_2 to vitamin A to a limited extent. This is in accordance with the known structural specificity for vitamin A activity; the absence of the β-ionone ring indicates that the compound should be inactive.

CHAPTER III

VITAMIN D

(Antirachitic Principle)

Rickets and caries are two diseases which arise from vitamin D deficiency. The former is common among children, affecting the bony skeleton particularly of the limbs, whereas dental caries affects not only children but also older people. A condition, known as osteomalacia, which is common among women particularly during pregnancy and lactation and affects the pelvic bones, is also due to vitamin D deficiency.

The evolution of our ideas regarding the cause and cure of rickets in the twenties of this century not only established the existence of this vitamin but also led to the synthetic production of vitamin D_2 (calciferol) on a commercial scale. It was observed that administration of cod-liver oil cured rickets and hence the claim was made that it was a deficiency disease and that the concerned vitamin was present in cod-liver oil. On the other hand, rickets was found to be prevalent among people living in crowded cities without access to sunlight, and removal of the patient to better surroundings with plenty of sunlight brought about a cure. It was therefore considered that the disease was due to the lack of hygienic conditions, air and light, and had nothing to do with vitamin deficiency. Later work, besides bringing about an interesting reconciliation of the two views, supplied important information about the chemical nature of the vitamin. Next to the study of the action of sunlight and ultraviolet light on the patient it was noticed that irradiated food materials had a curative effect. Careful examination of the components of food led to the observation that the fatty matter, particularly the unsaponifiable portion, was the material concerned. Since the unsaponifiable matter of fats and oils contains a high percentage of sterols, the effect of irradiation on this important group of organic compounds was next investigated resulting in fruitful discoveries. It was finally

realised that animals are capable of producing and supplying their own vitamin D requirements when exposed to sunlight, because the skin contains sterols (see notes) which after conversion into vitamin D by irradiation are absorbed by the system. The antirachitic action of cod liver oil is due to the presence of the vitamin in it.

The differentiation of vitamin D from vitamin A was first made by McCollum in 1922[1]. That the organic factor of cod-liver oil responsible for the cure of rickets was not vitamin A was clear from the fact that the oil which had been treated with a stream of air bubbles while hot, no longer contained vitamin A, but was still potent in the prevention or cure of rickets. This factor was called vitamin D. Ten different substances all of which are derivatives of sterols, have been claimed to have vitamin D properties. Only three of them are important. These three are considered to occur in different natural sources and have been obtained synthetically also.

Occurrence:—Though the raw materials for the formation of vitamin D are available plentifully in nature the vitamin itself is not widely distributed. Most of the animals seem to make their own vitamin with the help of sunlight. As external sources may be mentioned butter, cream and milk which are important items of food. Good summer butter has 80 I. U. per 100 grams which is about 1% of the value of cod-liver oil. Cow's milk in summer contains about 20 I. U. per litre. Since this concentration is too small for infant feeding for which purpose milk is otherwise admirably suited, fortification with calciferol or fish liver oil concentrates has been advocated. Adding irradiated yeast or cod-liver oil to the feed of cows also improves the quality of milk. The flesh of certain oily fish and eggs are richer in this vitamin. The quality of eggs varies very much with the feeding of the hens and with the season. All the vitamin D is present in the yolk and five eggs yield as much as one spoonful of cod-liver oil (about 4 c.c.). Thus the concentration of the vitamin is low even in them. For therapeutic purposes cod-liver oil is an important source (about 100 I.U. per gram) though certain other fish liver oils

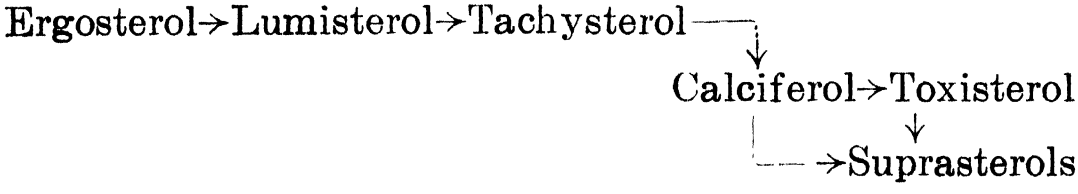
such as those of the halibut are many hundred times richer and are used where greater concentration is required. It will be clear from the above discussion that all natural foods containing vitamin D are of animal origin. Living plant tissues are generally considered to be devoid of it and they acquire it only on irradiation. Vitamin D is stable to heat and as already mentioned, it is much more stable to oxidation than vitamin A. Hence it is unaffected by the usual processes of cooking and preservation, but prolonged exposure to light may cause destruction.

Calciferol : Preparation :—Early experiments showed that irradiation of crude cholesterol gave an active preparation having antirachitic properties and hence cholesterol was considered to be the precursor of the vitamin. Later, a specially purified sample of the sterol failed to be activated and this led to a search for an explanation of the activability of crude cholesterol. The answer came mainly from physico-chemical considerations. If a compound could be activated by a particular wave length of light, it should first be absorbed by it. Hence crude cholesterol should absorb light in the region of 300 mμ., the activating radiation. As a matter of fact it was found to do so and further the degree of activability was proportional to the intensity of this band. Purified cholesterol which could not be activated showed no absorption in that region. Hence crude cholesterol should contain an impurity, possibly also of the nature of a sterol, which exhibits absorption at 300 mμ. Of the several possible impurities, ergosterol (7-dehydrocholesterol was not known then) was found to absorb light in this region. Therefore it might be the precursor of vitamin D. Actually when ergosterol was irradiated, the original absorption bands slowly underwent change and disappeared with further irradiation to give other bands. At an appropriate moment in the experiment it was found that the product of irradiation had a remarkably high antirachitic potency, some 20 to 50 thousand times (in later experiments some 250,000 times) that of ordinary cod-liver oil Thus it was established in 1927[2] that ergosterol is provitamin D and

this immediately led to the commercial synthesis of calciferol.

For producing a commercial vitamin preparation at a low price there were two essential requirements, (1) a cheap and abundant source of ergosterol and (2) a knowledge of the correct conditions for producing the maximum yields of calciferol. These problems have been successfully solved. An easily available and plentiful source of ergosterol has been found in yeast (about 2% of the dry weight) and as the result of careful investigation of the changes brought about by irradiation, the best conditions for producing a high yield of calciferol are now known. Careless and excessive irradiation may carry the chemical change beyond the calciferol stage producing toxic substances.

The photochemical transformation of ergosterol is quite complex. It proceeds in overlapping steps with the formation of a series of products of which calciferol is not the last. This intricate system has been studied extensively by Windaus and his associates at Goettingen and can be conveniently represented as below:



At no stage of the irradiation is any one of these substances present alone. If the operation is not unduly prolonged calciferol is the chief product. Under the best of conditions it forms only 50 to 60% of the total, tachysterol and lumisterol accounting for most of the remainder. The reaction is best carried out in solution in a suitable solvent with agitation and exclusion of air. Wave lengths longer than 270 mμ. are the most suitable and shorter ones cause the destruction of the vitamin.

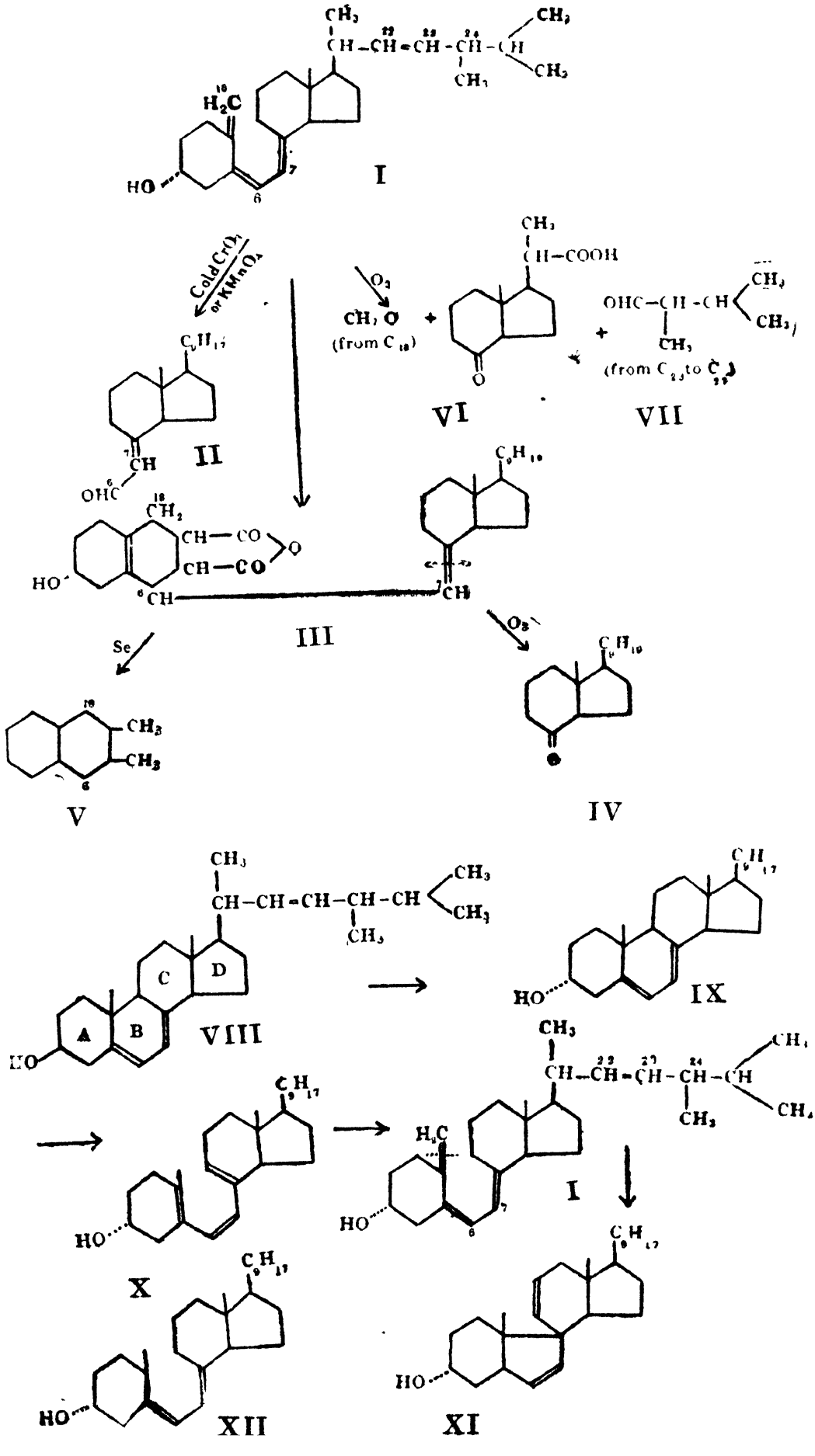
The preparation of calciferol[3] in a very pure condition was accomplished in 1932 and it involved the overcoming of a large number of difficulties. Since the product of irradiation was a mixture of isomeric compounds with very similar molecular structures their separation was not easy. The irradiated material was purified as follows. Unchanged ergosterol was first removed by cooling an

alcoholic solution and also by precipitation with digitonin. On treatment with maleic or citraconic anhydride the other isomers reacted more readily than calciferol and their adducts could be easily removed by means of alcoholic alkali. Calciferol could then be crystallised from acetone. A more convenient method as described in the B. P. Addendum, 1936 utilises the 3:5-dinitrobenzoic ester for purposes of purification. It is as follows. The product of irradiation, after removal of the solvent, is dissolved in 95% alcohol and strongly cooled. After removal of the ergosterol that separates out, the filtrate is concentrated in vacuo and the concentrate treated with 3:5-dinitrobenzoyl chloride in pyridine solution. On the subsequent addition of water, a mixture of 3:5-dinitrobenzoates separates out. It is recrystallised from acetone until the specific rotation of the crystals in benzene solution is + 57—60° using sodium light or + 68—72° using mercury light. The calciferyl dinitrobenzoate thus obtained is then hydrolysed with boiling alcoholic sodium hydroxide and the free vitamin recrystallised from methyl alcohol. Calciferol is also known as vitamin D_2 since by mistake the name vitamin D_1 was first given to a crystalline molecular mixture of calciferol and lumisterol.

Properties :—Calciferol is a secondary alcohol having the formula $C_{28}H_{44}O$ and is isomeric with ergosterol. It is soluble in fats and fat solvents and insoluble in water. The crytalline sample is colourless and odourless and melts at 117°. It is dextrorotatory, $[\alpha]_D$ + 125·5° using mercury light and + 105° using sodium light, in ethyl alcohol solution, and it has a strong absorption band in the ultraviolet with maximum at 265 mμ. With antimony trichloride in chloroform solution it produces an orange yellow colour. It does not get precipitated with digitonin. As an alcohol it forms esters and that obtained using 3:5-dinitrobenzoyl chloride melts at 147° and has been used for effecting the purification of calciferol. It reacts with citraconic anhydride and maleic anhydride rather slowly at room temperature, but more readily at higher temperatures, to form addition products. It is fairly stable to heat and can be distilled in high vacuo.

It is strongly active in the cure of rickets, active potency being 40 million I. U. per gram and thus it is 400,000 times as potent as average cod-liver oil.

Constitution[4]:—The constitution of calciferol (I) is based mainly on its relation to ergosterol (VIII). It contains four double bonds as against the three of the sterol. This is obviously due to the opening of a ring during irradiation and that it is the ring B that suffers this change could be understood from the products of various degradations of the calciferol molecule. The first evidence was obtained by oxidation with cold chromic anhydride or potassium permanganate when an $\alpha:\beta$-unsaturated aldehyde, $C_{21}H_{34}O$ (II) was isolated. Further, calciferol gave two isomeric adducts with maleic anhydride and these formed dihydro-derivatives on partial hydrogenation, the double bond in the long side chain getting saturated (III). Degradation of these with ozone gave a ketone, $C_{19}H_{34}O$ (IV) corresponding to the right half of the molecule, and selenium dehydrogenation produced 2:3-dimethyl-naphthalene (V) corresponding to the left half, the carboxyls being converted into methyl groups. These reactions are satisfactorily explained on the basis of structure (I) for calciferol. The production of 2:3-dimethylnaphthalene is of particular significance. It indicates definitely that the addition of maleic anhydride involves carbon atoms 6 and 18. The presence of an exocyclic methylene group in calciferol and of an ethylenic bond in its side chain has been demonstrated by direct ozonolysis which yields formaldehyde and methyl-isopropyl-acetaldehyde (VII) along with a keto-acid, $C_{13}H_{20}O_3$ (VI).


I
Cold CrO3 or KMnO4
O3
CH2O (from C18)
VI
VII
(from C23 to C27)
II
III
Se
V
IV
VIII
IX
X
XII
XI

The various products of irradiation of ergosterol (VIII) leading to the production of calciferol (I) are given in page 29. Ring B of ergosterol is unstable and is readily affected by irradiation since it is neither benzenoid nor completely saturated. The first change brought about by the activating rays is considered to be stereo-isomeric, the OH at C_3 becoming trans to the CH_3 at C_{10}. This is concluded from the fact that lumisterol (IX) and other irradiation products do not form a precipitate with digitonin. All natural sterols including ergosterol in which the arrangement is cis react with digitonin. The above isomeric change is considered to take place at C_{10}. The next stage involves a deeper alteration in the molecule. Tachysterol (X), like calciferol which follows it, has four double bonds instead of the three found in ergosterol and lumisterol. The fourth double bond arises from the rupture of ring B. The exact structural formula of tachysterol is not known with certainty but since it forms the same dihydro-derivative as calciferol, the two should be closely related isomers having the same carbon skeleton. Formula (X) is tentatively accepted. In the change from tachysterol to calciferol, the exocyclic double bond is formed and the molecule reaches a comparatively stable form.

Over-irradiation leads to the formation of toxisterol which is also called substance 248, since it has a characteristic single absorption band with maximum at 248 mμ. It is most readily formed when alcohol is the solvent used and subsequently passes over into suprasterols. When ether is the solvent it may not appear in detectable amounts, probably owing to its passing over into the next stage very fast. Its constitution is not yet definitely known.

Of the two suprasterols, suprasterol I has been better investigated. In it, the number of double bonds has again been reduced to three and this is considered to be achieved by the formation of a new 5 atom ring as shown in formula (XI).

Among the irradiation products of ergosterol, tachysterol (X) also has assumed considerable commercial importance during recent years, since its reduction pro-

duct, dihydrotachysterol (XII) has been found to be very useful in the treatment of tetany.

Vitamin D_3 : Isolation:—The spectacular success in the synthetic production of calciferol which has remarkable antirachitic potency, led to the assumption for some time that it was identical with the naturally occurring vitamin of fish-liver oils. But it was soon realised that the two are different. The most important difference was observed with regard to their potency with different animals. When samples standardised with rats as test animals were compared using chicks, calciferol was found to be much less potent. It was however noted that the D concentrates obtained from fats of various species of animals were identical. The isolation of a pure sample of this naturally occurring vitamin, now known as vitamin D_3, was effected in the years 1936 and 1937 from the unsaponifiable fraction of tuna-liver oil in the first instance and subsequently of halibut-liver oil somewhat in the following general manner [5].

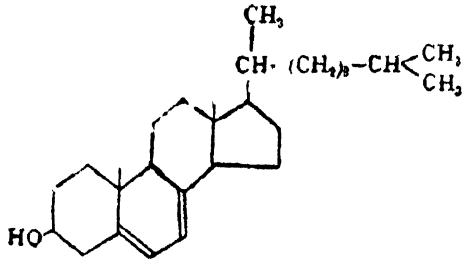
As has been already mentioned in connection with vitamin A, most of the sterols were removed by freezing a methyl alcoholic solution to −6°. The liver-oil concentrates were then rich in vitamins A and D. The former was removed as far as possible by (1) partition between immiscible solvents using benzene-ligroin mixture as one layer and 90 and 95% methanol as the other layers and (2) a preliminary adsorption over aluminium hydroxide. Vitamin D was present in the filtrate and was then purified by chromatography using granulated aluminium hydroxide and a red indicator (No. 33) for locating the position of the required zone. The red layer was extracted with benzene-ligroin mixture and the adsorption process was repeated again. Cholesterol was then precipitated by cooling the methanol solution and also by treatment with digitonin. Further purification was effected by conversion into the meta-dinitrobenzoate and subjecting the ester to chromatographic adsorption. From the pure ester the vitamin was regenerated and repeatedly recrystallised.

Properties and constitution :—The pure vitamin is a colourless solid melting at 83°. It exhibits positive

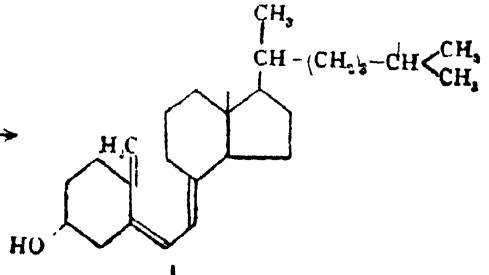
optical rotation, $[\alpha]_D$, +87·6° in acetone solution and has the same absorption maximum as calciferol. It resembles the latter closely in solubility and physiological action, in giving an orange yellow colour with antimony trichloride and in forming a 3:5-dinitrobenzoyl derivative (yellow needles melting at 132°). It also forms a crystalline allophanate melting at 173°.

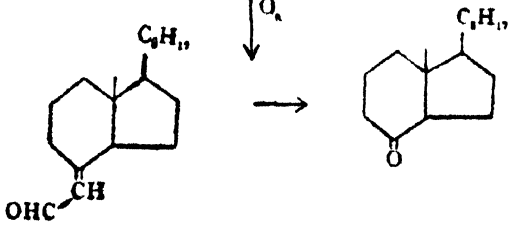
The vitamin has the formula $C_{27}H_{44}O$ and contains three double bonds. Its constitution had already been anticipated; from theoretical considerations 'the provitamin D' accompanying cholesterol in animals was expected to be 7-dehydrocholestrol (XIII). This was prepared by Windaus, Lettree and Schenck starting from cholesterol (see 'sterols' under notes) and was found to be highly activable[6]. The natural vitamin of fish-liver oils was shown to be identical with the irradiation product first by comparison of their derivatives and later by direct comparison of the crystalline samples themselves. Based on the striking parallelism between the two pairs, (1) ergosterol and calciferol and (2) 7-dehydrocholesterol and vitamin D_3, the constitution of D_3 was expressed as in (XIV). This was further supported by the degradation[7] of the compound with ozone whereby an aldehyde with the formula $C_{20}H_{34}O$ (XV) and a ketone with the formula $C_{18}H_{32}O$ (XVI) were produced.

XIII 7-Dehydro-cholesterol



XIV Vitamin D_3



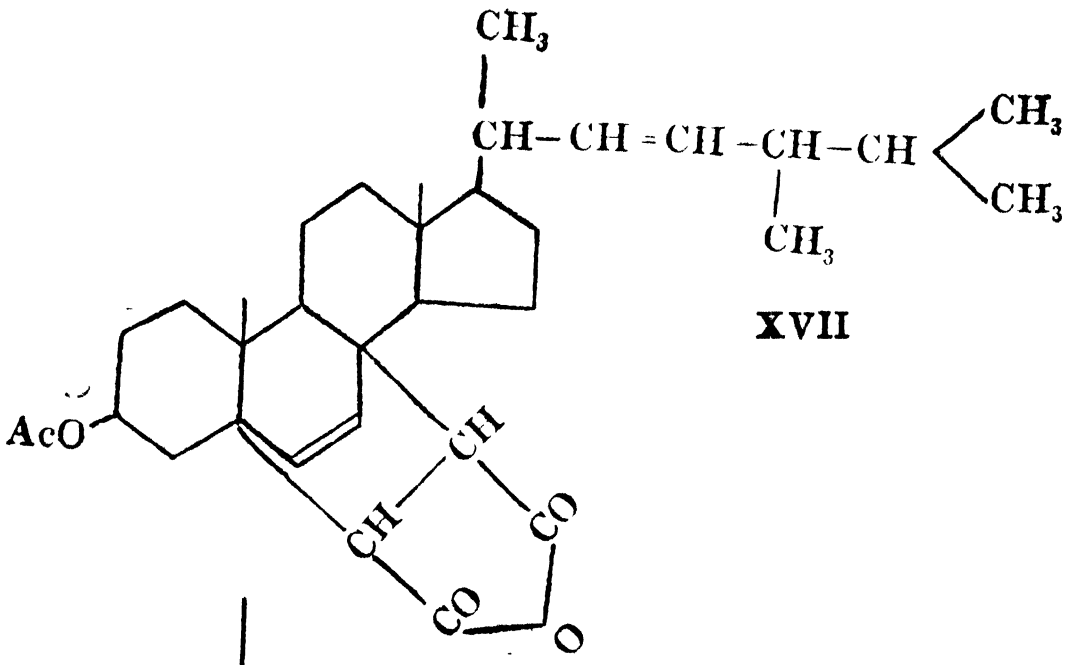


XV XVI

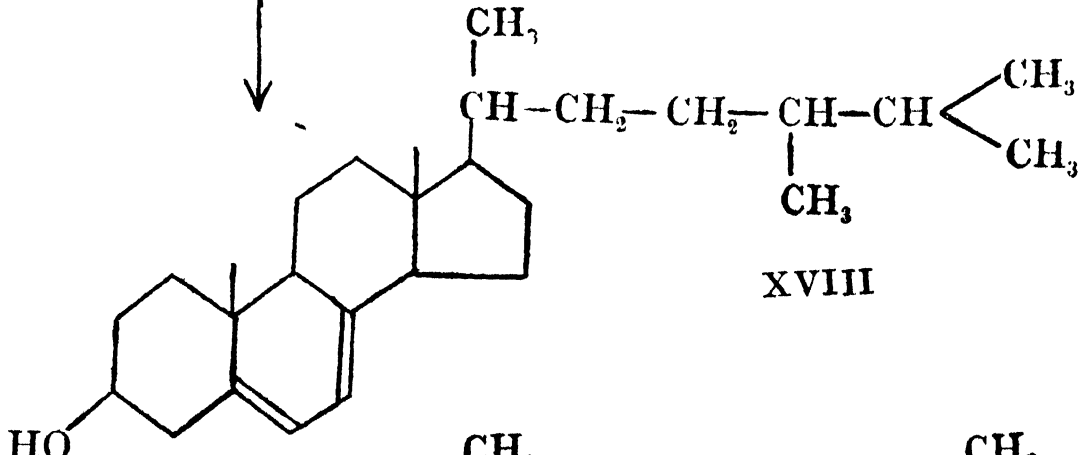
Biogenesis:—The infomation on the subject of the origin of vitamin D_3 in animals was rendered more complete when Windaus discovered, in the course of his examination of sterols from various sources, that skin fat contains 7-dehydrocholesterol and that the fat obtained from hog's skin is very rich in this substance (6%.)[8]. This confirmed the original conception regarding the formation of vitamin D when animals are exposed to sunlight or ultraviolet radiation. More recently 7-hydroxy-cholesterol has been found in ox-liver. This may be physiologically significant since it is an intermediate in the conversion of cholesterol into 7-dehydrocholesterol in the laboratory. A similar transformation is obviously taking place in the animal system also.

Cholesterol → 7-Hydroxy-cholesterol →
7-Dehydrocholesterol → Vitamin D_3.

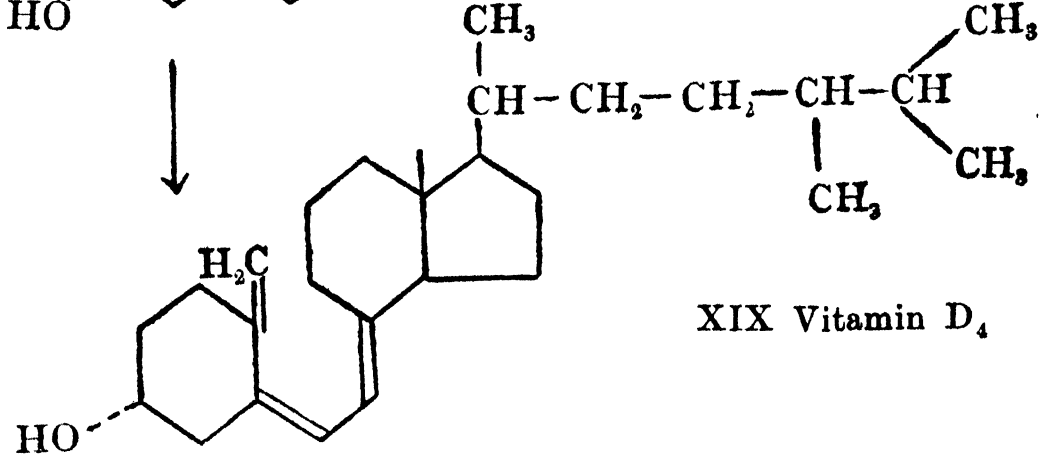
Vitamin D_4:—Based on analogy with 7-dehydrocholesterol which does not have an ethylenic double bond in the side chain, Windaus[9] prepared in 1937 a third member of the vitamin D group, viz. vitamin D_4 by the irradiation of 22-dihydroergosterol (XVIII). This new sterol had already been obtained by Windaus and Langer in 1933 by the careful reduction with hydrogen of the maleic anhydride adduct of ergosteryl acetate (XVII), subsequent removal of the added maleic anhydride by thermal decomposition and final saponification of the acetate. It melts at 152° and has a specific rotation of −109° in chloroform. When this dihydro-ergosterol was irradiated, vitamin D_4 (XIX) was obtained. The new vitamin melts at 107° and produces positive rotation, $[\alpha]_D^{18}$ +89·3° in acetone. It has the same absorption maximum as calciferol. It appears to be slightly less antirachitic for rats than the other two D vitamins, while for chickens its activity, per rat unit, is intermediate.



XVII



XVIII



XIX Vitamin D_4

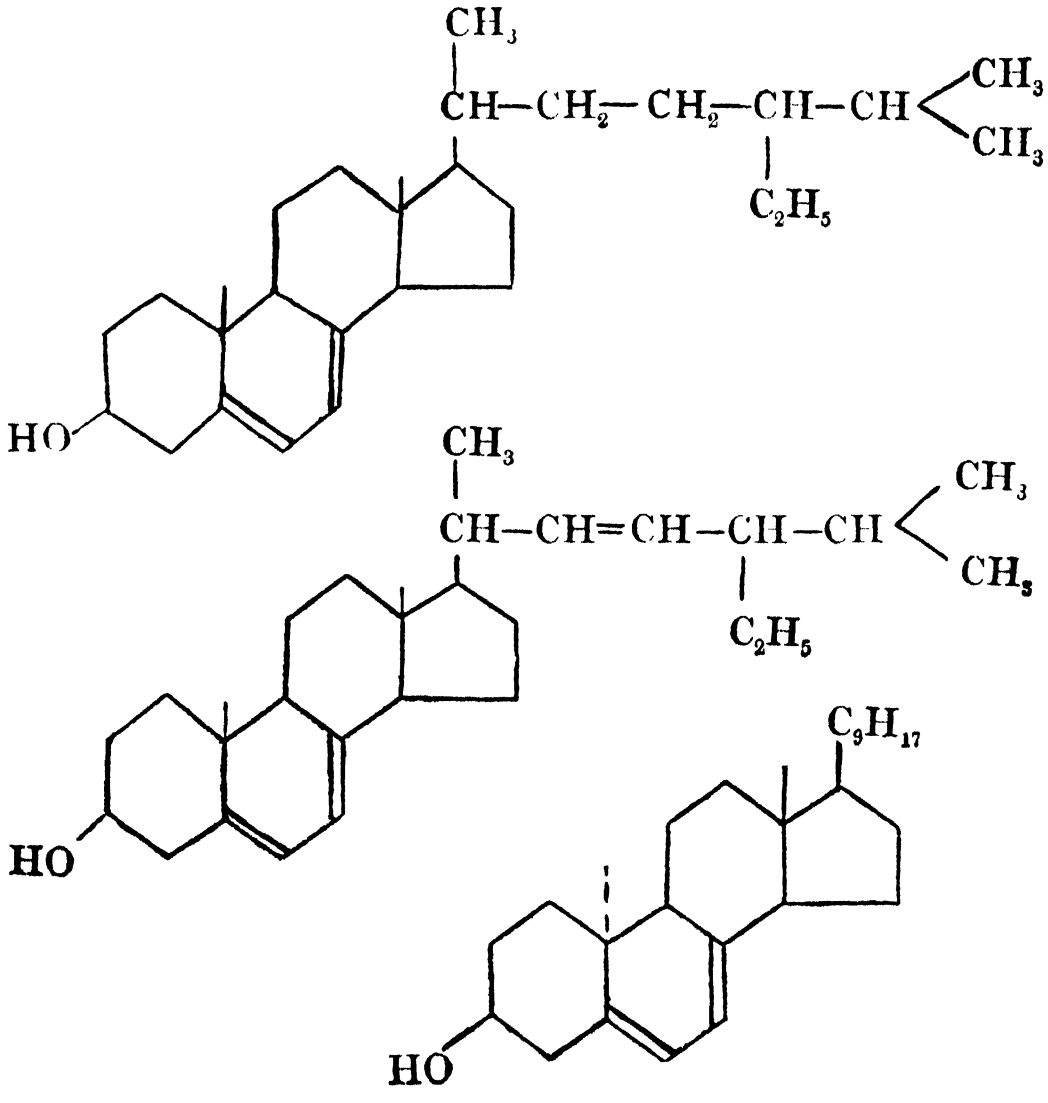
Structure and Antirachitic properties :—Three important members of the vitamin D group have been described above. Vitamin D_1 is a misnomer as already pointed out since it refers to a mixture of calciferol and lumisterol. Vitamin D_2 is pure calciferol obtained by

the irradiation of ergosterol. Though it is known mainly as a synthetic product, it is considered to be present in irradiated yeasts and fungi and to some extent in animal sources also. Vitamin D_3 is identical with the antirachitic principle of fish liver oils in which it is present mostly as fatty acid ester. It is present in all animal sources and it has also been obtained artificially from 7-dehydrocholesterol. Vitamin D_4 was also first known as a synthetic product from a synthetic sterol, 22-dihydroergosterol. It, however, appears to be the vitamin present in irradiated vegetable foods and is called 22-dihydrocalciferol.

From the foregoing account of vitamins D_2, D_3 and D_4, the only requisite for a sterol to be capable of functioning as provitamin D may seem to be the presence of conjugated double bonds in the 5 : 6 and 7 : 8 positions. This, however, is not correct, for the nature of the entire sterol molecule seems to have a large influence on the antirachitic potency of the irradiation product. Slight changes in the side chain are responsible for marked differences in activity when tested on rats and chicks. So far as rats are concerned vitamins D_2 and D_3 are of equal potency, but the former is barely half as active as the latter for chicks. Again D_4 has only half the activity of D_2 for rats and is more active than D_2 for chicks.

It has further been noticed that irradiation of 7-dehydrositosterol (XX) results in only a weakly antirachitic product while 7-dehydrostigmasterol (XXI) is entirely devoid of activity after irradiation. These compounds differ from 7-dehydrocholesterol only in the nature of the side chains. Even certain compounds like pyrocalciferol (XXII) which have not only the required unsaturated system in ring B but also suitable side chains, are unable to function as provitamin D. The disposition of the 3-hydroxyl too has no uniform effect. Whereas the important provitamins have the cis configuration, epi-lumisterol which not only has the cis configuration but resembles them in many other respects also, is not activable. Thus the structural requisites for provitamin-D property seem to be fairly specific and variations are possible only within narrow limits.

XX 7-Dehydro-sitosterol



XXI 7-Dehydro-stigmasterol XXII Pyrocalciferol

Assay :—The biological method employs calcification of bones in rats as the criterion. The estimation can be carried out either by the curative or by the prophylactic procedure. In the former, young rats are fed on a rachitogenic diet until they become severely ricketic. They are then divided into groups and each group is administered a definite dose of the preparation under test or a standard preparation until partial healing takes place. The extent of calcification of the bones is then estimated by an examination of the X-ray photographs of the bones (the radiographic method) or by the line test or by the bone ash method. In the line test the bones are examined visually after staining with

silver nitrate and in the bone ash method the percentage of minerals is determined on the dehydrated and defatted bones. From the degree of healing obtained with the unknown and the standard, the potency can be calculated. In the prophylactic method the rats are fed on diets containing known amounts of the standard and unknown preparations even from the outset. The general principles in this method are the same as in the curative method.

Of the chemical or colorimetric methods, the most selective is that depending on the production of a blue colour (absorption bands at 545-550 mμ and 590-600 mμ) by the action of bromine in chloroform (Tortelli-Jaffe reaction). This reaction is not given by vitamin A or carotenes or by the oxidation products and isomerides of vitamin D. It is claimed that by means of it vitamin D can be determined in cod-liver oil samples with such small quantities as 0·01—0·05 c.c.

The orange yellow colour (absorption maximum at 500 mμ) obtained when vitamin D is treated with antimony trichloride in chloroform solution has also been employed for the determination of this vitamin. The sensitivity of the reaction is considerably enhanced by the presence of 2% of acetyl chloride and this permits the determination of vitamin D in quantities as small as 2 γ. Fair agreement with the results of bio-assays is obtainable with fish-liver oils containing more than 10,000 I.U. per gram, if corrections are applied for the presence of vitamin A and sterols or if they are destroyed by suitable treatment.

Standard, function and requirements:—The international standard for vitamin D is a 0·01% solution in olive oil of ergosterol irradiated in accordance with a standard procedure internationally agreed upon, and it is kept at the National Institute for Medical Research, London. One international unit corresponds to 1 mg. of this preparation and is equal to 0·025 γ of crystalline calciferol.

Vitamin D plays its physiological role by controlling the metabolism of calcium and phosphorus. Vitamins D_2 and D_3 are of equal potency for human beings just as

for rats. The requirements of individuals of the vitamin vary considerably depending on various factors. Through exposure to the sun considerable quantities are produced on the skin normally. The optimal amount for an adult, however, seems to be 300 I.U. or less per day. It is quite desirable to give the vitamin during pregnancy and lactation (about 800 units per day). Babies, children and adolescents seem to require 300-400 units a day besides what they get in milk and other items of normal food. This is what is normally contained in about a tea-spoonful of cod-liver oil of good quality and corresponds approximately to 0·01 mg. of the pure vitamin. Among vitamins, vitamin D is therefore, the one required in the smallest quantity. For therapeutic purposes, besides the highly potent liver oils of the halibut, tunny and similar fish, their concentrates and irradiated ergosterol are prepared on a large scale and are available in various forms.

CHAPTER IV

TOCOPHEROLS : VITAMIN E

This vitamin, first recognised about the year 1924, is also known as the anti-sterility vitamin because its absence brings about sterility in animals. Its physiological role differs in the two sexes. For a considerable length of time it was not considered to be important, especially for human beings. Later, however, it was found that besides bringing about sterility, vitamin E deficiency is responsible for various other ailments, and hence adequate supply of it is necessary for normal health. Great importance attaches to this vitamin in connection with the raising of stock where fertility is a matter of primary concern.

Occurrence:—Leafy vegetables such as spinach and lettuce are good sources of this vitamin and so also are grains like wheat, rice and corn. Certain kinds of wheat contain one mg. per 100 g, and about 55 % of this is present in the embryo. Wheat-germ oil is one of the most potent sources. It has therefore been employed for the treatment of vitamin E deficiency and also for the isolation of this vitamin. Cotton-seed oil also contains an appreciable quantity of it. The occurrence of anti-oxidants or inhibitols is an important factor in the potency of these oils since in their absence rancidity sets in early and the vitamin is decomposed.

Isolation:—The isolation of vitamin E could be accomplished only with difficulty. A large amount of work had to be done by different chemists and a number of new methods tried before successful isolation of the pure vitamin was possible. Wheat-germs when cold-pressed gave 10 % of oil which was then worked up. The first stage in the process consisted in the separation of the unsaponifiable portion of the oil which contained a large quantity of sterols besides a small amount of vitamin E. It was estimated that the proportion was 9 : 1. After the removal of sterols using digitonin, an oil was

obtained which was still quite impure. Further concentration was effected by partition using various immiscible solvents. High vacuum distillation was not successful. Finally success was achieved by the use of cyanic acid, which reacts in general with alcohols and phenols to form solid derivatives called allophanates. Using this technique Evans, Emerson and Emerson[1] (1936) were able to prepare a crystalline allophanate melting at 158, which on hydrolysis yielded a light yellow oil. This was named α-tocopherol. It exhibited marked vitamin E activity, 2-3 milligrams being sufficient to cure sterility in rats. The mother liquor left after the removal of α-tocopheryl allophanate yielded another crystalline compound melting at 144°. When this was collected and hydrolysed a new tocopherol was obtained; it was called β-tocopherol. Its vitamin activity was nearly a half of that of the α-compound.

There was considerable loss of vitamin E in the above process during the saponification. This was avoided by Moss and Drummond[2] who used, instead, adsorption by alumina from a solution of the oil in light petroleum. By this method great improvement in yield was effected; two kilograms of wheat-germ oil yielded one gram of α-tocopheryl allophanate and 0·75 gram of β-tocopheryl allophanate.

Working with cotton-seed oil, besides the α-allophanate, a new compound melting at 138° was obtained. It was the allophanate of γ-tocopherol. The free γ-compound has only about a third of the activity of α-tocopherol.

Vegetable sources differ markedly in the composition of tocopherols. As explained above wheat-germ oil contains both α- and β-tocopherols whereas cotton-seed oil has α- and γ-tocopherols. Only α-tocopherol is present in lettuce, and palm and corn oils are similar to cotton-seed oil in containing no β-tocopherol. Corn-embryo oil is particularly suited for the isolation of γ-tocopherol since it is predominantly the major component, α- being present only in minor amounts. There exists some difference between the composition of the germ oils from American and European wheats. The former contains

α-tocopherol as the major component whereas in the latter the β-compound predominates. Karrer's experiments on the estimation of tocopherols indicate that they occur free and not as esters.

In the following table are set forth the total tocopherol (mixture) content of some materials examined by Karrer[3]:—

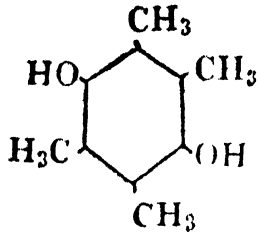
Unsaponifiable fraction from wheat-germ oil	...	13·4%
Wheat-germ oil	...	0·52%
Wheat-germs	...	0·026%
Unsaponifiable fraction from corn-germ oil	...	10·2%
Corn-germs	...	0·016%
Unsaponifiable fraction from lettuce	...	4·3%
Lettuce (dry)	...	0·055%
Unsaponifiable fraction from linseed oil	...	2·34%
Unsaponifiable fraction from olive oil	...	0·935%
Unsaponifiable fraction from sesame oil	...	0·63%

Properties:—The three tocopherols are very similar in their properties. They are fat-soluble and they dissolve easily in the common organic solvents also. They are stable to heat and reagents provided oxygen is excluded. They are particularly susceptible to oxidation. Rancid oils and fats do not possess any vitamin E activity since the tocopherols are destroyed by oxidation characteristic of rancidity. They are ordinarily viscous oily liquids with a pale yellow colour, but at low temperatures they can crystallise. The melting points are round about 0°. Their absorption spectra are characteristic. There has been some difference in the observations of different workers; but the maxima are round about 294 mμ.

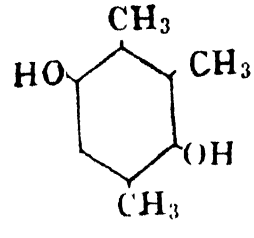
Constitution:—α-Tocopherol has the formula $C_{29}H_{50}O_2$ and β-, $C_{28}H_{48}O_2$; the γ-compound is isomeric with the β-. Obviously the difference consists in the existence of one more methyl group in the α-compound. Otherwise their reactions are quite similar. The presence of a hydroxyl group capable of forming esters such as the

acetate and the allophanate was recognised early. The tocopherols were first considered to be related to sterols mainly in view of their formulae (C_{29} and C_{28}) but a study of the absorption spectra soon revealed that the idea was erroneous. When they were converted into their allophanates the absorption maximum was shifted towards shorter wave-lengths by about 20 mμ. and the absorption itself became less intense. Such a behaviour is characteristic of phenols when converted into their esters. Further the colour reactions and the ready oxidisability of tocopherols also indicated that they are phenolic in nature; that they are monohydric phenols was evident from their derivatives.

The function of the second oxygen atom was not so obvious. By the distillation of α-tocopherol in high vacuum Fernholz[4] (1937) obtained durohydroquinone (I). John[5], and Todd and coworkers[6] repeated this experiment with β-tocopherol whereby ψ-cumoquinol (II) was produced. The difference between these two products of decomposition rests in the existence of an extra methyl group in durohydroquinone, thus again supporting the original idea that the tocopherols are homologues.



I

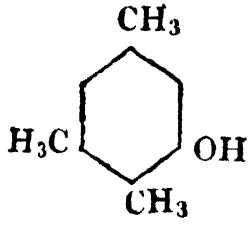


II

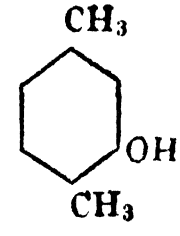
At this stage the suggestion was made that the vitamins were simple ethers of the above hydroquinones involving an alkene group $C_{19}H_{37}$. This was supported by the fact that many alkyl ethers of phenols were known to undergo cleavage by pyrolysis into the phenols and unsaturated hydrocarbons. Studies of the absorption spectra again showed that this was not correct. Meanwhile by the action of hydriodic acid on α- tocopherol (John[7], 1938), 2, 3, 5-trimethylphenol (III) was obtained, whereas with β-tocopherol 2, 5-dimethylphenol (IV) was produced. It may be noted that these two degradation

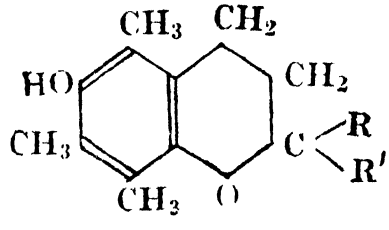
products not only lack a hydroxyl group when compared with the corresponding products of decomposition by heat, but there is loss of a methyl group also. The disappearance of the hydroxyl may be due to reduction by the hydrogen iodide, but the loss of the methyl group cannot be explained on the basis of the ether formula. It was therefore concluded that the tocopherols contained a pyran or a furan ring fused on to the benzene ring so that their structures should be represented as in (V) or (VI). This was in accordance with expectations based on the study of absorption spectra.

III

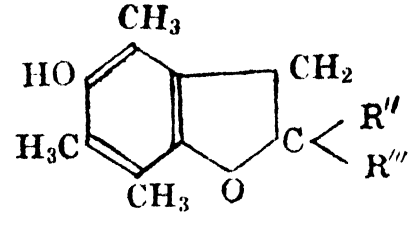


IV





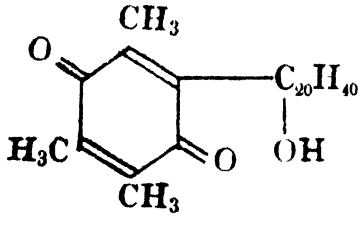
V—Chroman



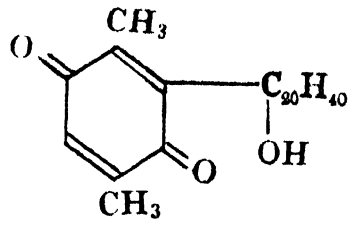
VI - Coumaran

The question was finally settled by oxidising the tocopherols by means of a mild oxidising agent[8]. The one employed in this case was silver nitrate. Karrer later showed that gold chloride is better. The products were quinones with the whole carbon skeleton intact and an extra oxygen atom as in (VII) and (VIII). By means of reductive acetylation, acetyl derivatives of the corresponding quinols were produced. Careful hydrolysis of the acetates gave the quinols (IX) and (X). Thus the existence of a second (oxide) ring was established and that it was a pyran and not a furan ring was settled in 1938 by Fernholz[9] who degraded α-tocopherol by means of chromic acid. The more important of the products were (1) a C_{21} lactone (XIII), (2) a C_{18} ketone (XIV) and (3) a C_{16} acid (XV). The lactone

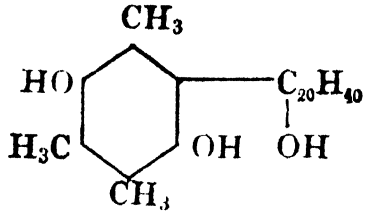
VII



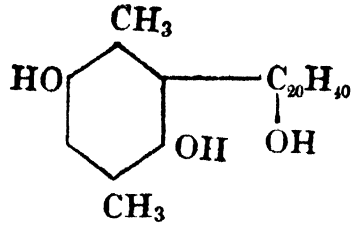
VIII



IX

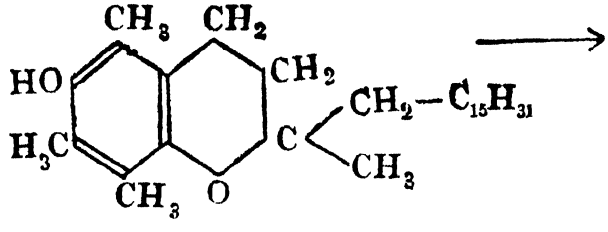


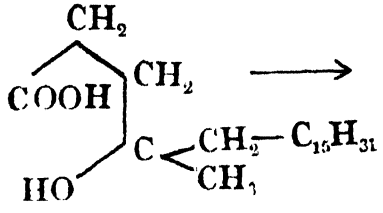
X



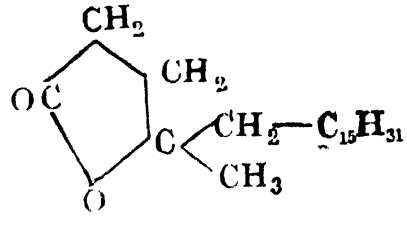
(XIII) was readily formed and was derived from a γ-hydroxy acid (XII). The hydroxyl group of this new acid could be esterified only with difficulty and could not be oxidised to a carbonyl group. It belonged therefore to a tertiary alcoholic group. All the above facts could be explained only on the basis of the following chroman structure (XI) for *α*-tocopherol.

XI

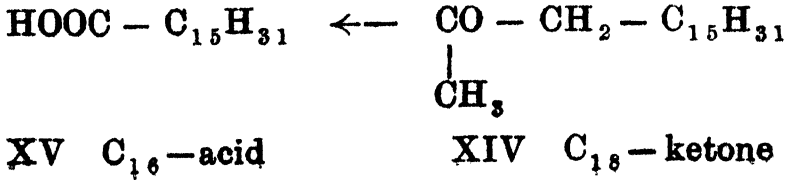




XII



XIII C_{21}-lactone

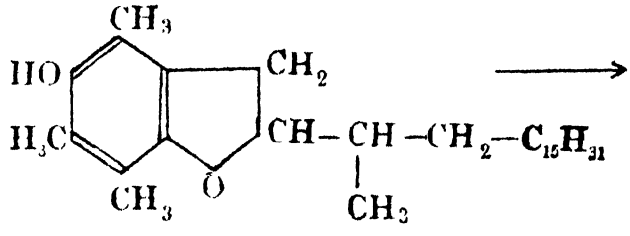


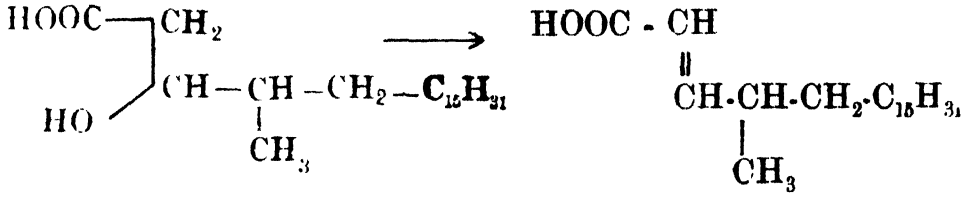
XV C_{16}—acid

XIV C_{18}—ketone

The alternative coumaran structure (XVI) fo α-tocopherol would on oxidation have given rise to th β-hydroxy acid (XVII) having a secondary alcoholi group. Such an acid would have been easily transforme on heating into the unsaturated acid (XVIII) an further would be capable of easy oxidation to th corresponding ketonic acid.

XVI

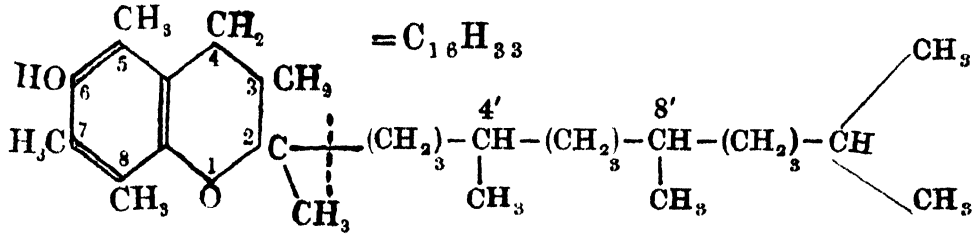


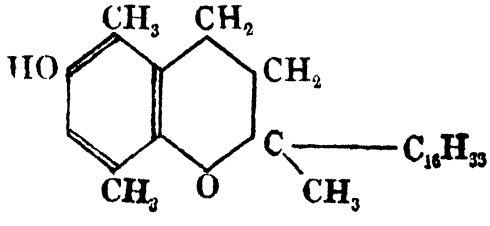


XVII XVIII

That the alkyl side chain $C_{15}H_{31}$ was derived fron phytol was deduced mainly from experience gained i other fields of natural products and from C-methyl deter minations. Hence α- and β-tocopherols have been re presented as in formulae (XIX) and (XX) respectively.

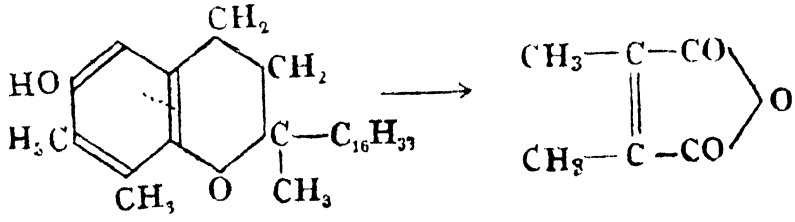
XIX. α-Tocopherol (5 : 7 : 8—Trimethyl-tocol)





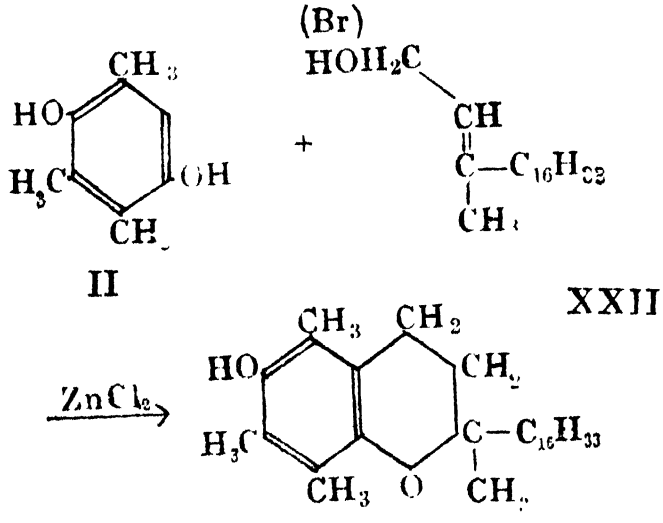
XX. β-Tocopherol (p-xylotocopherol or 5 : 8-dimethyl-tocol).

γ-Tocopherol [10] is isomeric with β-tocopherol and also yields ψ-cumoquinol (II) on pyrolysis. On oxidation it forms the same lactone (XIII) as given by α-tocopherol indicating identity of side chains in the two compounds. Another common oxidation product is dimethyl-maleic anhydride (XXIa) showing that two methyl groups are ortho to each other in the aromatic part of the molecule. Further, the reaction of γ-tocopherol with allyl bromide is characteristic of a free nuclear position ortho to the phenolic hydroxyl. These data strongly indicate that γ-tocopherol is o-xylotocopherol (XXI) and this conclusion is corroborated by its synthesis from 2:3-xyloquinol (XXIV).



XXI. γ-Tocopherol (o-xylotocopherol or 7 : 8-dimethyl-tocol.) XXI a

Synthesis [11]:—The synthesis of the tocopherols could be accomplished easily and it confirmed the constitutions proposed for them in a definite manner. Karrer (1938) sythesised α-tocopherol by heating ψ-cumoquinol (II) and phytyl bromide (XXII) in benzene solution in the presence of a catalyst (zinc chloride) which was later found to be unnecessary. Todd and coworkers simplified the procedure considerably and they obtained the com-



II XXII

XIX. α-Tocopherol

pound by heating ψ-cumoquinol and phytol (XXII) in the presence of zinc chloride. In either case the reaction is free from any complications and very high yields of dl-tocopherol are obtained, especially if the condensation is carried out in a nitrogen atmosphere.

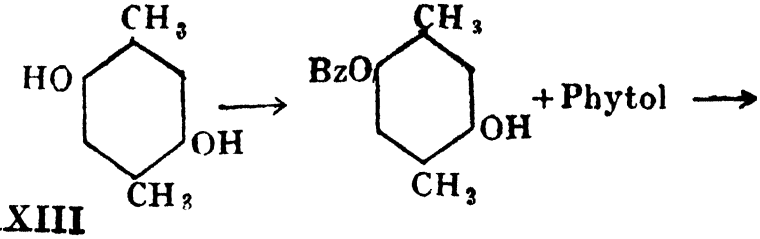
Recently the technique of preparing pure synthetic α-tocopherol has been improved. The crude synthetic compound is oxidised to the quinone (VII) by means of ferric or auric chloride or by means of silver oxide in a medium of dry ether. The quinone is reduced to the quinol (IX) with sodium bisulphite. This compound is readily purified since it is sparingly soluble. It is finally cyclised to α-tocopherol in dioxane solution in the presence of stannous chloride and hydrogen chloride.

The synthetic substance has the full vitamin potency of natural α-tocopherol but the derivatives have somewhat different melting points. By fractionating the 3-bromocamphor-sulphonate of the synthetic dl-compound from alcohol, Karrer obtained a crystalline fraction melting at 48° and having the specific rotation of +32°. This was found to be identical with the bromocamphor-sulphonate from natural α-tocopherol.

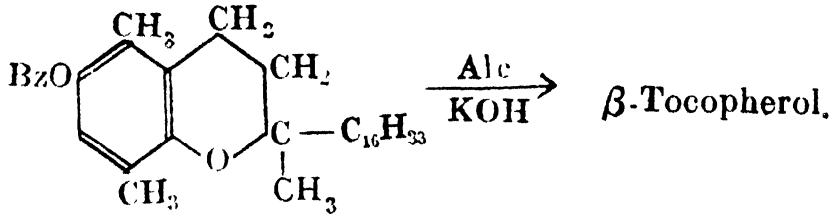
Several isomerides [12] of α-tocopherol are possible and the isomerism is complex due to the existence of three asymmetric centres (2, 4′ and 8′) in the molecule. Besides the natural α-tocopherol, synthetic samples have been obtained using trimethylquinol on the one hand and natural d-phytol, l-phytol and synthetic dl-phytol. They seem to be all racemic for one reason or other and do not exhibit optical activity. They have identical physiological property and the sole difference seems to rest in the melting points of the allophanates which range between 161° and 192°.

The condensation of phytyl bromide or phytol with 2 : 5- and 2 : 3-xyloquinols (XXIII and XXIV) to form β- and γ-tocopherols respectively is somewhat complex. Due to the existence of two reactive nuclear positions in these quinols, two phytyl residues react with one of quinol. This difficulty was overcome by Todd and coworkers [13] by using the monobenzoyl derivatives which form the benzoates of β- and γ-tocopherols (XXV and

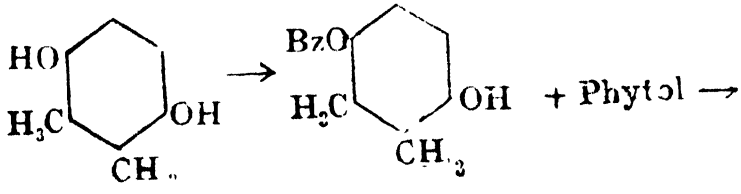
XXVI). These are finally debenzoylated by heating with alcoholic potash.



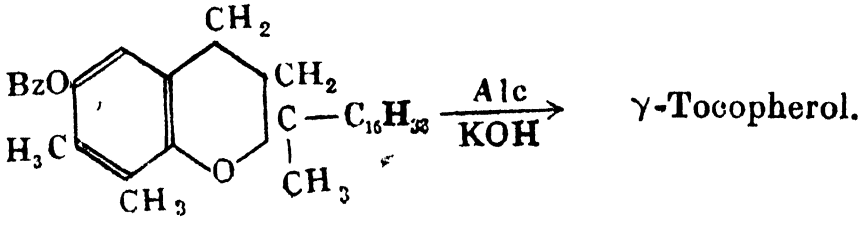
XXIII



XXV



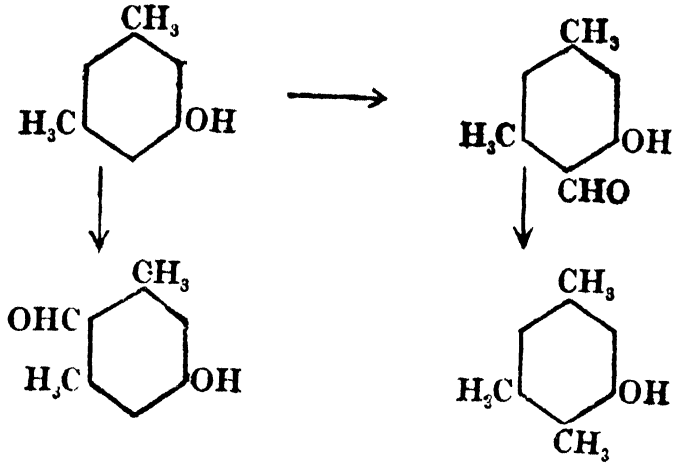
XXIV



XXVI

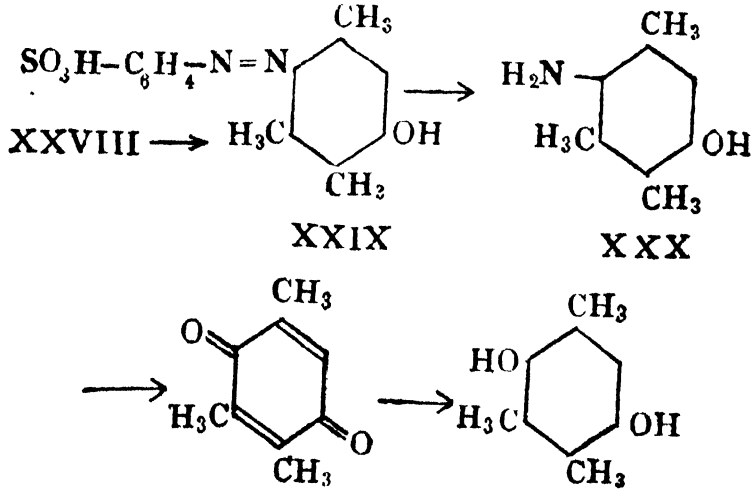
A difficult part of the synthesis of the tocopherols is the preparation of the concerned quinols[14], ψ-cumoquinol in the case of α-tocopherol and 2 : 5- and 2 : 3-xyloquinols in the case of β- and γ-tocopherols respectively. The first stage in the preparation of ψ-cumoquinol is to get 2 : 3 : 5-trimethyl phenol (XXVIII). This is made from sym-xylenol (XXVII) as follows. The xylenol is subjected to the Gattermann condensation using hydrogen cyanide and hydrogen chloride in the presence of aluminium chloride. Both ortho and para aldehydes are produced. The former distils with steam and is thus easily isolated. Clemmensen's reduction converts the aldehyde into the required trimethylphenol (XXVIII).

XXVII



XXVIII

The above trimethylphenol can also be obtained from the phenolic fractions of coal tar. It is the main component of the fraction boiling at 230°. For getting the quinol, the phenol is coupled with diazotised sulphanilic acid and the azo-dye (XXIX) is subjected to reductive cleavage. The amino-phenol (XXX) thus produced is oxidised to the quinone (XXXI) which can be isolated by steam distillation. Subsequent reduction gives ψ-cumoquinol in very high yields.

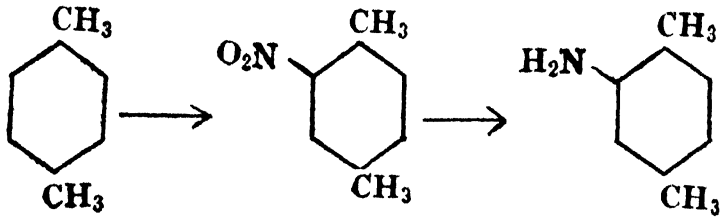


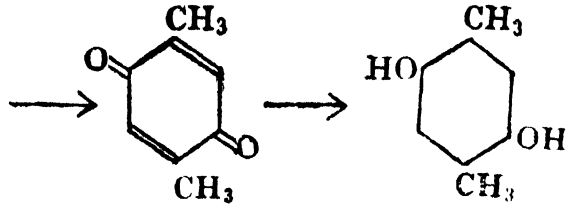
XXIX XXX

XXXI (II) ψ cumoquinol.

Para- and ortho-xyloquinols (XXIII) and (XXIV) are made from the corresponding xylenes. p-Xylene (XXXII) is nitrated and the mono-nitro compound (XXXIII) reduced to the amine (XXXIV). Oxidation with chromic acid yields the p-quinone (XXXV). Final reduction with zinc dust and acetic acid produces p-xyloquinol (XXIII) in high yields.

XXXII XXXIII XXXIV

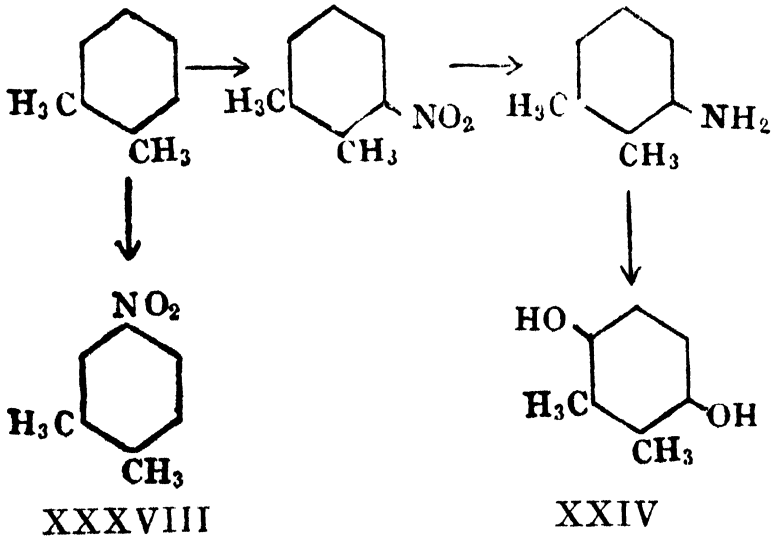




XXXV XXIII

In the case of o-xylene (XXXVI) two mono-nitro compounds are possible. The required 3-nitro-1 : 2-xylene (XXXVII) is best obtained by the action of sulphuric and fuming nitric acids, the isomer (XXXVIII) being obtained as the main product if a mixture of nitric and acetic acids is used. The subsequent stages are similar to those described above for p-xyloquinol.

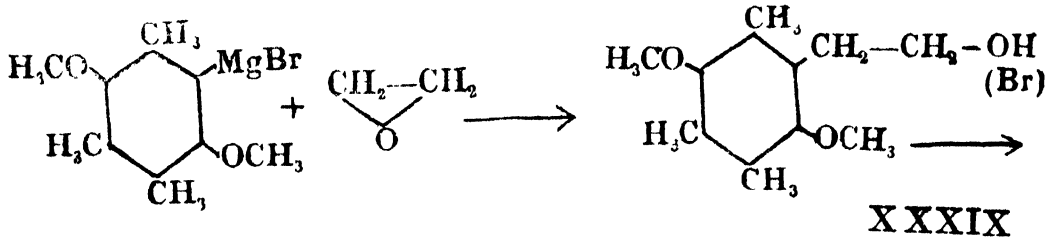
XXXVI XXXVII



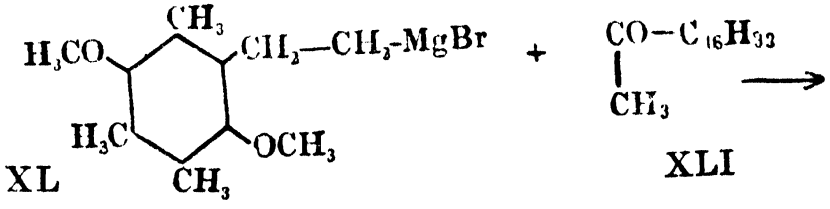
XXXVIII XXIV

The methods of tocopherol synthesis so far described involve the use of natural or synthetic phytol and suffer from two draw-backs. They fail to provide unequivocal proof of the chroman structure for the vitamin and phytol is limited in availability and is costly. The more

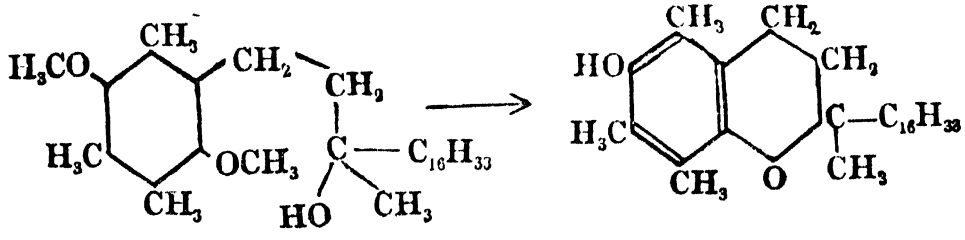
recent work of Smith and coworkers[15] eliminates these defects. One of their methods utilises the carbinol (XXXIX) which is transformed into the bromide and subsequently into the Grignard compound (XL). Condensation of this with 'phytol ketone' (XLI) yields the carbinol (XLII) which smoothly undergoes demethylation and cyclisation to yield α-tocopherol under the influence of hydrogen bromide in acetic acid.



XXXIX

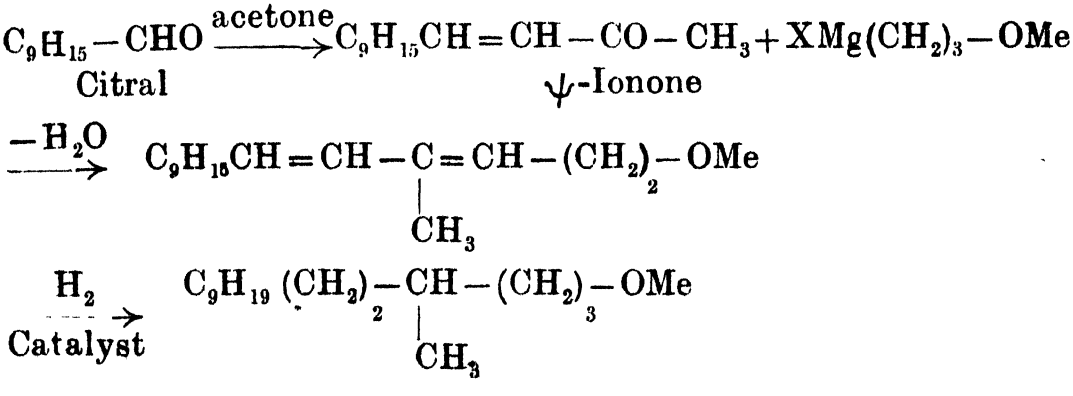


XL XLI



XLII α-Tocopherol

'Phytol ketone' (XLI) required for the above synthesis was originally obtained from phytol by the action of ozone or chromic acid; it can now be made from citral, which is a more readily available source, by the series of reactions indicated below.



$$\xrightarrow{HBr} C_9H_{19}-(CH_2)_2-\underset{\underset{CH_3}{|}}{CH}-(CH_2)_3-Br \text{ (XLIII)} + Mg \text{ and then } CH_3CHO$$

$$\longrightarrow C_9H_{19}-(CH_2)_2-\underset{\underset{CH_3}{|}}{CH}-(CH_2)_3-CHOH-CH_3$$

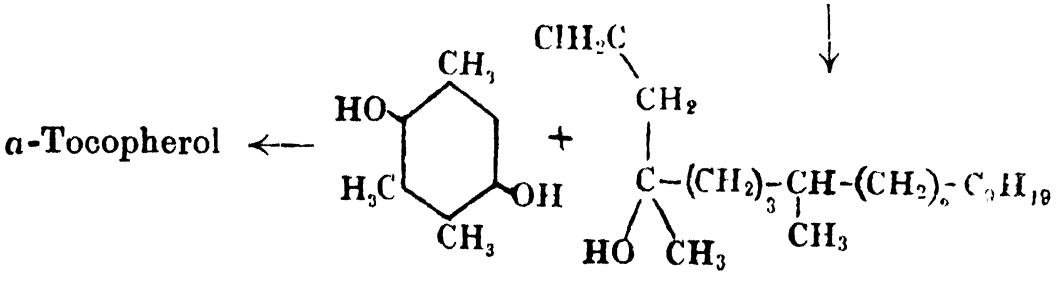
$$\xrightarrow[AcOH]{Na_2Cr_2O_7} C_9H_{19}-(CH_2)_2-\underset{\underset{CH_3}{|}}{CH}-(CH_2)_3-\underset{\underset{CH_3}{|}}{CO}$$

XLI Phytol ketone

Another method employed by the above workers involves the condensation of trimethylquinol with the halogenated alcohol (XLIV) which is prepared from the bromide (XLIII) in the following manner.

$$XLIII + Mg \longrightarrow C_9H_{19}-(CH_2)_2-\underset{\underset{CH_3}{|}}{CH}-(CH_2)_3-MgBr +$$

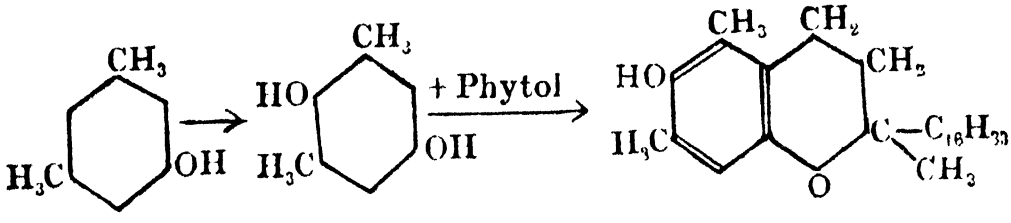
$$CH_3-CO-CH_2-CH_2Cl$$



XLIV

Vitamin E activity and structure of tocopherols :—In the study of structural requirements for vitamin E activity, the size of the phytol part as found in the tocopherols seems to be the minimum. A decrease in the length of the side chain by one isoprene unit leads to inactivity, but with one isoprene unit more the product is still active. Regarding the aromatic nucleus the presence of three methyl groups as in α-tocopherol was originally considered to be essential for maximum vitamin E activity. With only two methyl groups (β- and γ-tocopherols) the activity is only half that of α-tocopherol, while a single methyl group alone present in the nucleus is not enough for any appreciable activity. Later it was found that in contrast to β- and γ-tocopherols, m-xylotocopherol [16] (5 : 7-dimethyl-tocol) (XLVII) is quite as good as α-tocopherol, though it contains only

two methyl groups in the aromatic nucleus. Two methyl groups are therefore enough, but they should be present in the correct positions. This discovery opens up possibilities of the simpler m-xylotocopherol being substituted for α-tocopherol. The preparation of the new tocopherol starts from m-xylenol (XLV). Oxidation with alkaline persulphate yields the corresponding quinol (XLVI) which is condensed with phytol according to methods already described.



XLV XLVI XLVII. m-Xylotocopherol (5 : 7-dimethyl-tocol)

The hydroxyl group para to the bridge oxygen is quite essential for vitamin activity, though it can be modified by esterification with simple carboxylic acids like acetic and succinic acids or with phosphoric acid without reducing the activity appreciably. Probably the esters undergo hydrolysis readily in the animal and the free tocopherol functions. But conversion into ethers or into the allophanate results in complete loss of activity. The optical state of the asymmetric carbon atoms in the pyran ring and in the side chain have no effect since, as already stated, a number of stereo-isomers have identical physiological activity.

From the results of a large volume of work it has been found that many compounds which do not have any near relationship to the tocopherols exhibit some vitamin E activity. Consequently it may be stated that there is no rigid specificity of structure for this physiological property. But these substances are required in comparatively very large doses and are not of any value as substitutes for the tocopherols.

Assay:—The biological method employs rats as the experimental animals. It is based upon the ability of vitamin E-deficient females to bear living young, following oral administration of the test substance. Besides

this, there exist one physical and three chemical methods for the assay of materials containing tocopherols.

It was found, even at an early stage of the work on vitamin E, that a close parallelism existed between the biological activity of vitamin E preparations and the intensity of the absorption at 294 mμ. The latter proved to be a reliable guide in subsequent work in following the process of concentration of the vitamin present in naturally occurring materials. But for purposes of assay this method suffers from the same defect as the chemical methods described below.

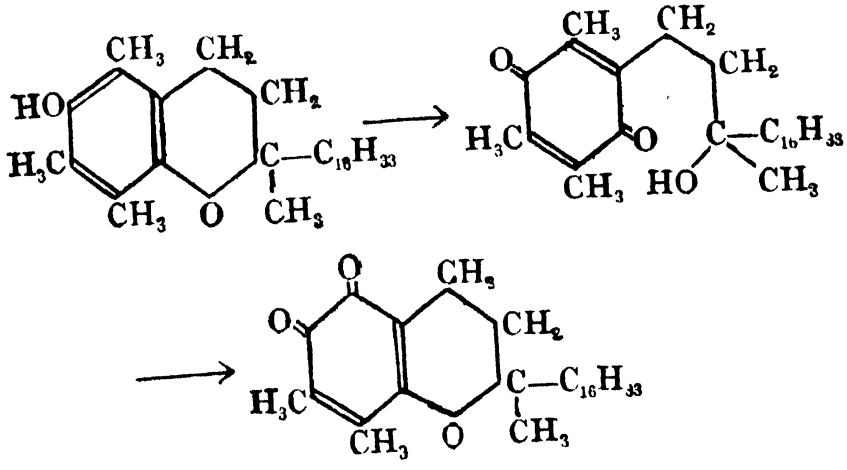
The chemical methods of assaying tocopherols make use of their easy oxidisability. Since the three naturally occurring tocopherols belong to the same chemical type, the chemical methods do not distinguish between them but give the sum of all the three. As, however, the biological activities of the three tocopherols differ, the chemical methods cannot be used to replace the bio-assay although they agree fairly satisfactorily. They are briefly given here.

Potentiometric titration with gold chloride has been employed by Karrer very successfully. It involves the oxidation of the vitamin by the gold salt as already explained. Powerful reducing substances like glutathione and ascorbic acid are removed from the sphere of influence by partition between water and petroleum ether or ether, these compounds being soluble in water but not in the organic solvents in which the tocopherols are soluble. Carotenoids also react with gold chloride and must be either eliminated first or determined independently.

The second method involves the oxidation of the tocopherols with ferric chloride. The ferrous ions formed are converted into a red complex by the addition of $\alpha : \alpha'$-dipyridyl and the intensity of colour is measured. In this method, which is more satisfactory than the previous one, a correction factor is applied for carotenoids.

On the addition of concentrated nitric acid to tocopherols in absolute alcohol and heating for a short while, the colourless or yellow solution becomes orange

red and in a few minutes turns intense cinnabar red. The colour is said to be due to the formation of o-quinones as shown below; the change involves elimination of a methyl group from position 5.



The cooled solution is then used for the determination of the extinction coefficient by means of a Pulfrich photometer. Carotenoids do not interfere in this method.

Standard, function and uses :—The acetate of dl-*α*-tocopherol is easily produced by the action of acetic anhydride and it is more stable and useful than the free tocopherol. It was originally known as a bright yellow, highly viscous oil; but it has recently (1943) been obtained as a crystalline solid as the result of repeated crystallisation from methyl alcohol and ethyl formate at −30°. It melts at 27° and has an absorption band at 286 mμ., $E^{1\%}_{1\ cm}$ =41·2. It has the formula $C_{31}H_{52}O_3$ and is used as the standard for vitamin E. The international unit is 1·0 mg. of the standard preparation. When orally administered it has the specific activity of preventing resorption gestation in female rats deprived of vitamin E. It is used in the form of a 1% solution in olive oil and the standard is supplied by the National Institute for Medical Research, London. The 'rat unit', also called the 'fertility dose' is the smallest amount of the vitamin which, when given by mouth daily to vitamin-deficient female rats during the whole period of gestation (21 days), will just cure sterility and bring about in 50 per cent of the animals the birth of living young ones. This dose is equal to 2 - 3 mg. of *α*-tocopherol.

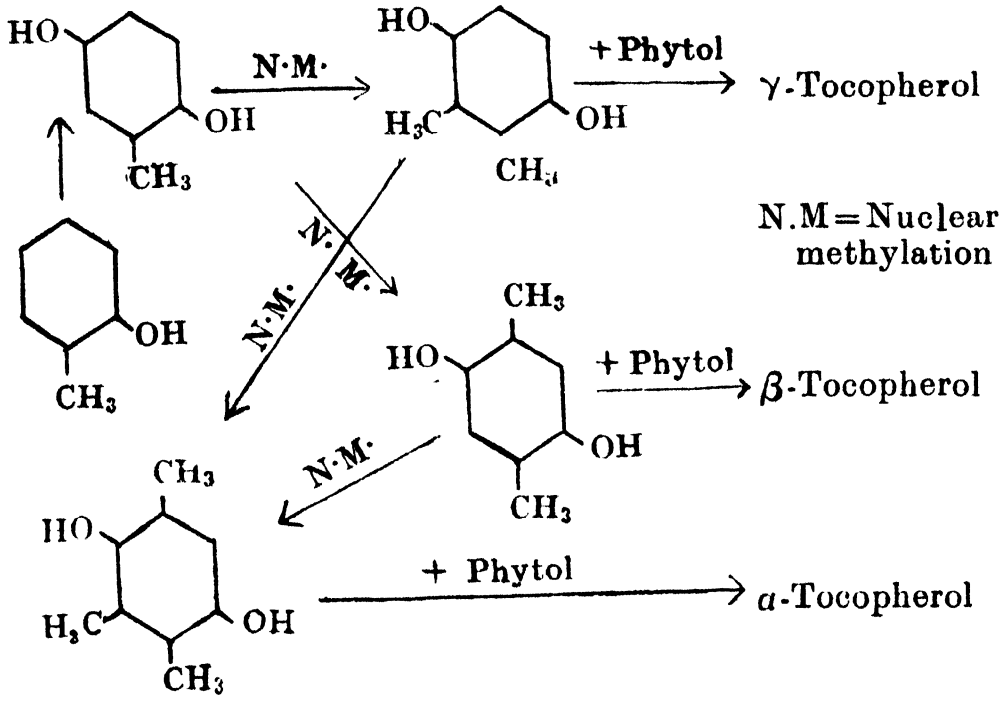
It is double this amount for the β- and about three times for the γ-compound.

Vitamin E is considered to be necessary for normal and efficient utilisation of chemical substances of the phenanthrene group such as sterols, vitamin D and sex hormones. It has also been found to play an important part in the utilisation of vitamin A. It is commonly used for stabilising vitamin A in fats and it may play a similar part in the animal. There is evidence to show that it is the major anti-oxidant in certain fish liver oils.

The use of vitamin E has been advocated for the treatment of habitual abortion in women and for a similar purpose in the veterinary field. Wheat-germ oil and its concentrates have been generally employed and highly successful results have been reported. The synthetic dl-α-tocopherol is sold in the form of the acetate which is at least as active as the free phenol and is at the same time more stable to oxidation.

Biogenesis :—The tocopherols could be considered to consist of two parts, the phytol part and the polymethyl-quinol part. Phytol occurs in abundance in the vegetable kingdom and forms part of the green pigment chlorophyll. It is built up of isoprene units. It will be interesting to note in this connection that plants lacking in chlorophyll, particularly fungi, do not contain vitamin E.

The construction and origin of the second part are not yet clear The poly-alkylated phenol nucleus is unique. It may, however, be noted that somewhat similar types occur in lichen acids and mould products. In connection with the biogenesis of the former[17] it has been suggested that the primary aromatic skeleton has already a methyl group in it and others are introduced by nuclear methylation. A similar procedure could be adopted in working out a tentative scheme for the evolution of the tocopherols. In this connection it should be noted that the isomeric β- and γ-tocopherols lack a methyl group each in different positions. Ortho-cresol forms therefore a suitable starting point for the biogenetic scheme.



Compounds related to o-cresol such as salicin and helicin occur in nature and may well be the source of the cresol in the plant. Nuclear methylation can take place by means of formaldehyde; somewhat similar reactions have been carried out even under laboratory conditions.

The tocopherols are produced entirely in the plant kingdom. The animal system does not seem to be capable of synthesising them to any extent. When a mixture of phytol and trimethyl-hydroquinone was administered orally no growth-promoting effect was noticed. The combination of the two which is so easy in the laboratory does not seem to take place in the animal.

CHAPTER V

VITAMIN K

(Koagulations Vitamin)

The discovery of vitamin K is associated with the study (1929-1934) of a deficiency disease known as chicken scurvy which could not be prevented by ascorbic acid. It was shown by Dam and his collaborators in Copenhagen, that when young chicks were fed on a diet deficient in certain fat-soluble substances, but otherwise adequate in every respect, they developed haemorrhages in the skin, mucous membrane and other parts of the body with prolongation of blood-clotting time. The bleeding was discovered to be due to a fall in the concentration of prothrombin in the blood and could be cured by the administration of vitamin K. Though this condition is very rare in human beings, the investigations connected with this vitamin have led to new uses for it in connection with many diseases such as anaemia and obstructive jaundice which require the improvement of blood-clotting time for cure.

Occurrence:—The presence of this fat-soluble, thermostable vitamin was first noted in hog-liver fat which contains 30-60 parts per million. Later it was found that vegetables are better, particularly the portions in active growth. Green leaves and growing stems such as alfalfa, cabbage, cauliflower, spinach and grass are rich (about 240 parts per million), and are suitable for the extraction of the vitamin. Chestnut leaves (480 parts per million) form the most potent source. Flowers, fruits, roots, seeds and nuts are generally poor in it; cod-liver oil contains no vitamin K.

Two members of this group (K_1 and K_2) are known and they were isolated from different sources during the years 1936-39. For obtaining vitamin K_1 alfalfa hay has been found to be the most suitable, whereas for vitamin K_2 putrefied sardine meal (550 parts per million) has been employed. When sardine meal, casein and certain other food-stuffs are allowed to stand in a moist condition

they develop anti-haemorrhagic properties due to bacterial action. It has therefore been suggested that this vitamin is produced by bacterial metabolism in the lower portions of the intestines of animals (mammals) and absorbed, and hence the deficiency of it is not so common. But in chicks absorption from the lower intestines does not take place and consequently deficiency arises.

Isolation[1] of Vitamin K_1 (α-Phylloquinone):—Artificially dried alfalfa meal was extracted by percolation with petroleum ether and the vitamin purified using a modified chromatographic adsorption method. In this case 'decalso' and 'permutit' (two synthetic zeolites) were employed and a solution having approximately a concentration of 2–5% of the vitamin was allowed to pass down the column. The first layer of the chromatogram contained all the chlorophyll which was thus easily removed. Four or five adsorptions were necessary to obtain a product essentially pure. The vitamin was eluted by successively washing the adsorbate with petroleum ether, benzene-petroleum ether mixture and acetone.

The vitamin is extremely sensitive to both light and alkali and hence purification has to be effected very carefully. Repeated crystallisation at low temperatures and distillation at low pressures give the pure vitamin as a lemon yellow oil; but it has been found better to convert it into the dihydro-diacetate by reductive acetylation using acetic anhydride, sodium acetate and zinc dust and then regenerate the vitamin. The dihydro-diacetate is a crystalline solid melting at 62-63° and having about half the potency of the vitamin. The regeneration of the vitamin from this compound is effected by means of an ingenious technique, employing magnesium methyl iodide and dilute hydrochloric acid for deacetylation and atmospheric oxygen for oxidation.

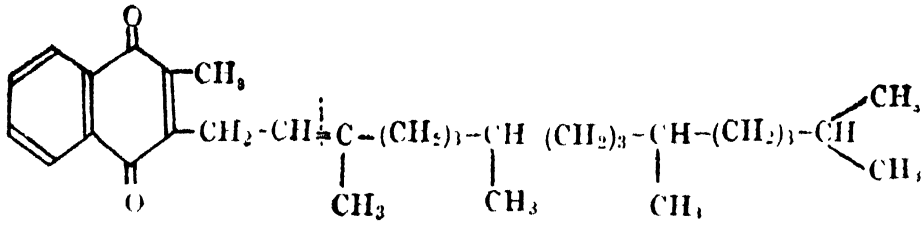
Properties:—In the pure state the vitamin is a lemon yellow oil distilling between 115 and 145° at 2×10^{-4} mm. pressure. When crystallised from acetone or ether at −70°, the crystals melt at −20° approximately. It is soluble in fat solvents, insoluble in water and only

sparingly in methyl alcohol. It exhibits a characteristic fluorescence when exposed to the light from an argon lamp. This is similar to that exhibited by pure phytol and is obviously due to the phytol part of the molecule. Compounds which do not possess a phytol moiety but which are otherwise closely analogous to vitamin K do not show this fluorescence.

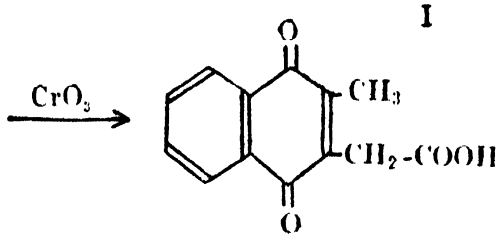
Constitution[2] of Vitamin K_1: This vitamin has the molecular formula $C_{31}H_{46}O_2$ and is optically inactive. It is very sensitive to alkali and undergoes reduction easily. One of the earliest observations which gave an important clue to its constitution was that sodium ethylate reacts with it to give a transient blue colour which fades to reddish brown. This reaction was shown by Fieser to be a general property possessed by all 2-allyl-*a*-naphthoquinones. The idea that vitamin K_1 is a derivative of 1 : 4-naphthoquinone was supported by its easy reduction to the corresponding hydroquinone. As has already been mentioned, the procedure using reductive acetylation gave rise to a convenient derivative for purposes of characterisation and purification. By a careful study of the ultraviolet absorption spectrum and colour reactions, it was then surmised that the vitamin is a 2 : 3-disubstituted 1 : 4-naphthoquinone. Further, when subjected to catalytic hydrogenation the vitamin took up 8 atoms of hydrogen, *i.e.*, 2 atoms in excess of what was necessary for the formation of the tetrahydro derivative of the dihydroquinone; this showed definitely the presence of one ethylenic linkage in a side chain of the molecule. Oxidation of the vitamin using chromic acid gave more detailed information. It yielded a mixture of products from which two acids were isolated. One of these was phthalic acid, showing the presence of an unsubstituted benzene ring. The other was 2-methyl-1 : 4-naphthoquinone-3-acetic acid (II). When the diacetate of dihydrovitamin-K_1 (III) was similarly oxidised, 1 : 4-diacetoxy-2-methyl-naphthalene-3-acetic acid (IV) and 2 : 6 : 10-trimethyl-pentadecan-14-one (phytol ketone) (V) were obtained. The ketone was produced also by the ozonolysis of the diacetate. These reactions gave definite evidence

regarding the location of the ethylenic double bond. The constitution of vitamin K_1 is therefore represented as 2-methyl-3-phytyl-1 : 4-naphthoquinone (I). The degradations are indicated in the following formulae :

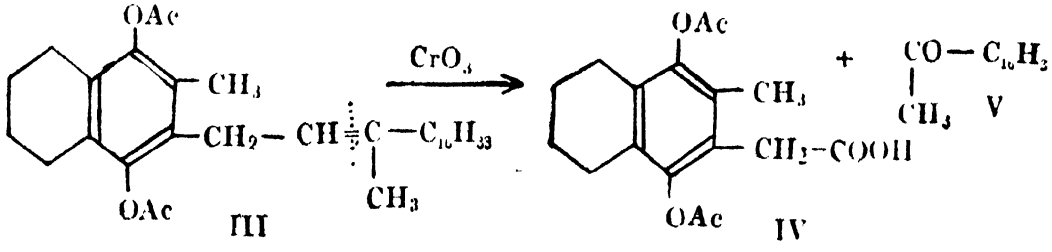
I Vitamin-K_1 (2-methyl-3 phytyl-1 : 4-naphthoquinone)



I



II



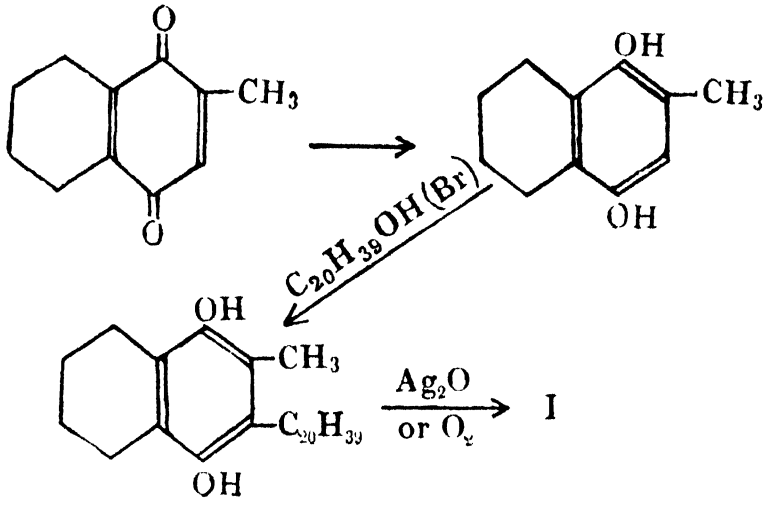
III IV V

Synthesis[3] **of Vitamin K_1 :**—The synthesis of the vitamin was effected in the year 1939 almost simultaneously by three different groups of workers. The following is the method employed by Fieser. 2-methyl-1 : 4-naphthoquinone (VI) obtained by the oxidation of 2-methylnaphthalene with chromic acid in acetic acid solution, was reduced to the hydroquinone (VII) with sodium hydro-sulphite and the product condensed with phytol in dioxane solution using oxalic acid as the condensing agent. The condensation product was rendered free from the unchanged hydroquinone by extracting an ether solution of the mixture with dilute alkali. The purified ether solution was distilled to remove the solvent, the residue was taken up in petroleum ether and the solution chilled. By this process the dihydrovitamin (VIII) was obtained as a solid. It was separated

and converted by oxidation with silver oxide into vitamin K_1.

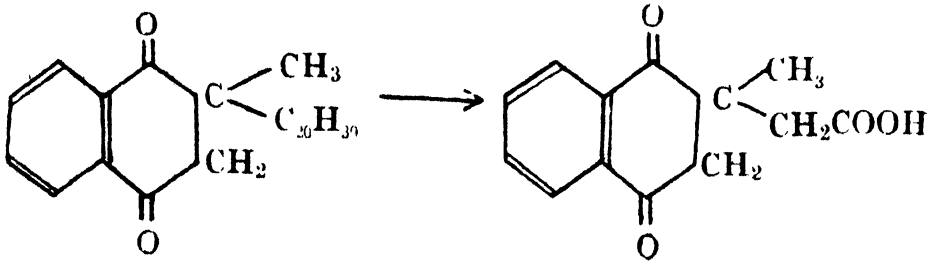
A modification of this process was adopted by Doisy and collaborators. They employed phytyl bromide and made it react with a benzene suspension of the monosodium salt of 2-methyl-1 : 4-naphtha-hydroquinone. Subsequent oxidation using atmospheric oxygen yielded vitamin K_1 (I).

VI VII



VIII

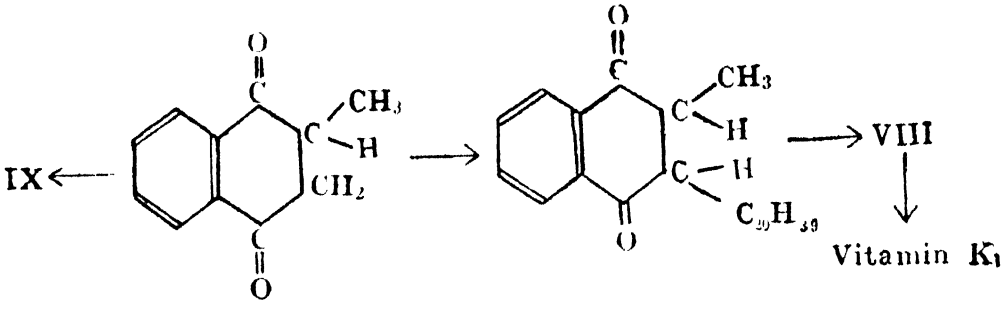
The yields in the synthesis of vitamin K_1 are only moderate. A considerable amount of a liquid by-product is also formed. It gives on oxidation with chromic acid 2-methyl-2:3-dihydro-1:4-naphthoquinone-2-acetic acid (X), possesses antihaemorrhagic activity and can be converted into vitamin K_1 to some extent by pyrolysis. The constitution of this substance is therefore represented as 2-methyl-2-phytyl-2:3-dihydro-1:4-naphthoquinone (IX).



IX X

It appears that in the above synthesis 2-methyl-1 : 4-naphtha-hydroquinone (VII) reacts in the ketonic form

(VII a). If the phytyl group enters the 3-position, the product (VIII a) can undergo isomeric change into the hydroquinone (VIII) which can be subsequently oxidised to vitamin K_1. On the other hand, if the substitution should take place in the 2-position, compound (IX) results directly and no further change is possible.



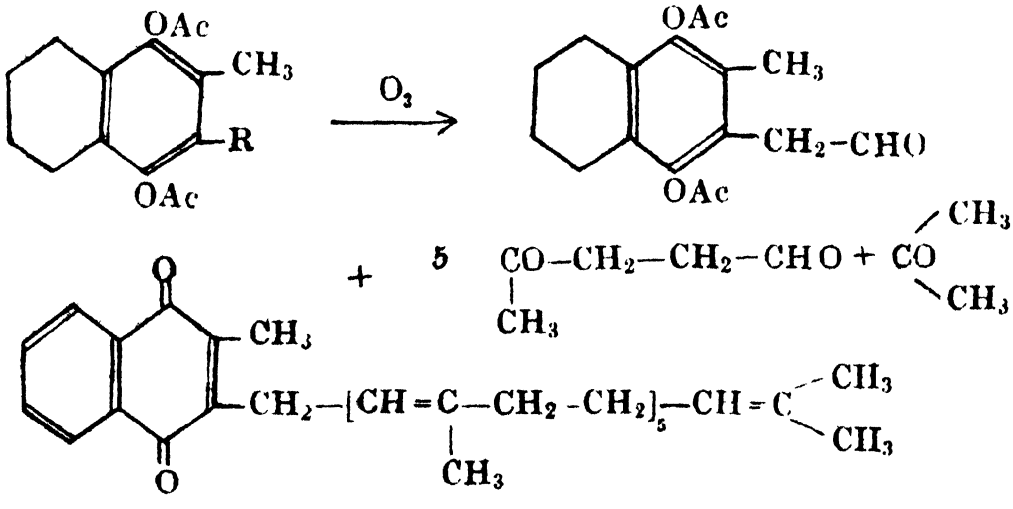
VIIa VIIIa

Isolation of vitamin K_2 ;—It is obtained from fish meal which is first extracted with petroleum ether in order to remove fatty matter that is likely to interfere in the subsequent stages of the process. The defatted meal is then allowed to undergo putrefaction, usually for about a fortnight. The vitamin which is produced during this process is subsequently isolated by a method very similar to that employed for vitamin K_1. It is a light yellow crystalline solid melting at 54°. Its potency is 60 per cent of that of vitamin K_1 and it is generally less soluble. It is also optically inactive.

Constitution[4] of vitamin K_2 :—The molecular formula of this substance was established by Doisy and coworkers (1940) as $C_{41}H_{56}O_2$. It exhibits more or less the same properties as vitamin K_1, particularly in its instability towards light and alkali and its capacity to undergo reversible reduction. By reductive acetylation, it gives rise to a crystalline derivative, diacetate of dihydro-vitamin K_2 which melts at 60°. The ultra-violet absorption curves of the free vitamin and of this derivative are very similar to those of vitamin K_1 and its dihydro-diacetate respectively. Bromination and reduction reactions indicate that it is a derivative of 2-methyl-1 : 4-naphthoquinone with 6 double bonds in the side chain. Oxidation experiments were not so successful as with vitamin K_1, since only phthalic acid could be detected. However, by

the action of ozone on the diacetate of dihydro-vitamin K_2 (XI) an ozonide was formed, which when decomposed with zinc in ether, gave a good yield of 1 : 4-diacetoxy-2-methyl-naphthalene-3-acetaldehyde (XII). This was found to be identical with the aldehyde obtained from vitamin K_1 by a similar reaction. As other products of ozonisation, were obtained levulinic aldehyde (XIII) and acetone in the form of the bis-2 : 4-dinitrophenyl-hydrazone and 2:4-dinitrophenyl-hydrazone respectively. The quantities of these (approximately 5 and 1 molecular proportions) suggested that the side-chain of vitamin K_2 is derived from farnesol. From all these considerations the constitution of vitamin K_2 is represented as 2-methyl-3-difarnesyl-1:4-naphthoquinone (XIV).

XI XII



XIV Vitamin K_2 (2-methyl-3-difarnesyl-1 : 4-naphthoquinone).

Assay:—The biological assay is based on the prolongation of blood-clotting time produced by a deficient diet. It may be carried out as follows. A large group of chicks from the same hatch are maintained on a diet deficient in the K-vitamins. After about two weeks they show a blood-clotting time of about an hour or more (as against 1 to 5 minutes in normal chicks) and are then ready for the assay. After administration of the vitamin-containing preparation, they are bled and the clotting time determined either on the whole blood or on the plasma. Control experiments are carried out

simultaneously with a standard antihaemorrhagic preparation. This method is the best and has been rendered fairly speedy.

A physical method involving measurement of the extinction co-efficient of the absorption band at 248 mμ. was used by Karrer as a guide in the purification of preparations containing this vitamin. It can also be determined polarographically in a solution of potassium chloride in aqueous isopropyl alcohol.

Two colorimetric methods are known. When sodium ethylate reacts with vitamin K_1 it gives a transient blue colour fading to reddish brown. Almquist used this reaction and found that it gave results agreeing with bioassays. Another utilises the reaction with dinitrophenyl-hydrazine and ammonia. The vitamin or a related compound is treated with 2 : 4-dinitrophenyl-hydrazine in hydrochloric acid. Later ammonia is added and the mixture diluted with water. A stable green colour appears and it can be extracted with amyl alcohol. The intensity of the colour is proportional to the quantity of the quinone originally present.

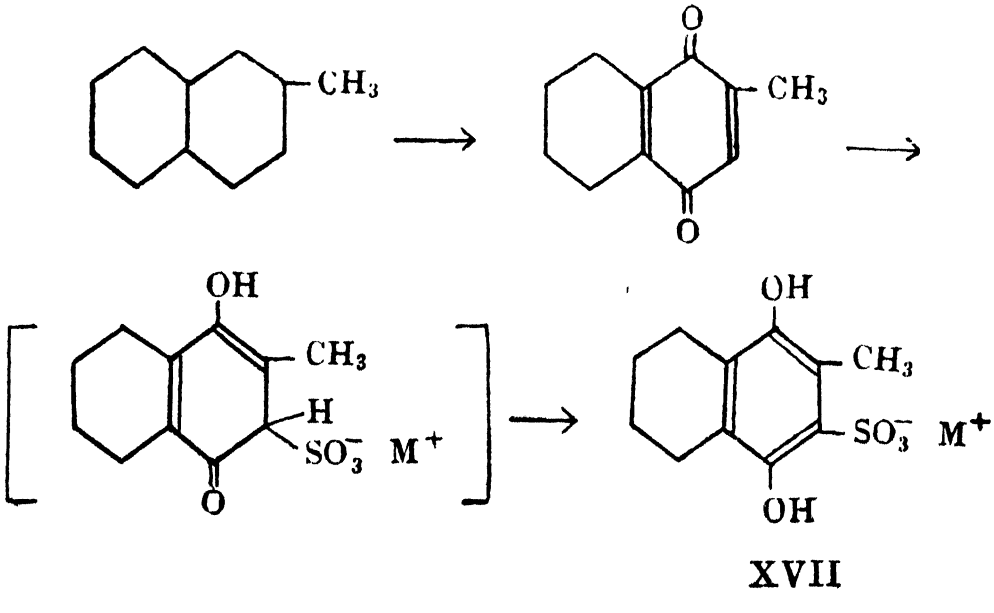
A reduction method is also employed. Vitamin K_1 and related quinones are reduced with Raney nickel in n-butyl alcohol solution and the product subsequently titrated with 2 : 6-dichlorophenol-indophenol (see under ascorbic acid).

Many different units are in use. One of these employs a standard alfalfa extract. It is the specific anti-haemorrhagic activity of 0·8 mg. of the standard extract. It is interesting that, based on this unit, the potency of vitamin K_1 is 1,000 units per milligram (Thayer-Doisy).

Structural features and Vitamin K properties: A number of naphthoquinone derivatives have been found to have the characteristic vitamin K properties. A methyl group in the 2-position seems to be essential for marked potency. Vitamin K_1 has this and in addition a phytyl side chain in the 3-position. Further lengthening of the unsaturated side-chain in position 3 lowers the activity slightly since vitamin K_2 has only 60% of the activity of vitamin K_1. 2 : 3-Dimethyl-naphthoquinone

and 2-methyl-3-hydroxynapthoquinone (phthiocol) which occurs as a pigment in the tubercle bacilli, also have some activity though considerably much less than the above vitamins. It appears that disubstitution is not an advantage since the simpler 2-methyl-1 : 4-naphthoquinone (XVI) is the most active substance known. It is at least twice as active as vitamin K_1. It is quite stable and easily handled and has therefore been used as a standard for the estimation of vitamin K. It is now prepared on a large scale by the chromic acid oxidation of 2-methyl-naphthalene which is found in low temperature coal-tar. In view of its high potency (2,000 alfalfa units per mg.) and the simplicity of its preparation it is now extensively used in therapy as vitamin K substitute under the name 'menadione'; it is freely soluble in oils and sparingly in water. The corresponding hydroquinone and 2-methyl-4-amino-1-naphthol are practically as good, probably due to their ready conversion in the animal system into the quinone. A number of water-soluble derivatives of these simple compounds have also been introduced. The most important of them are the sulphate and the phosphate of the hydroquinone or the hydrochloride of the amino compound. The following compound (XVII) is an example of another type. The substance obtained by the treatment of 2-methyl-1 : 4-naphthoquinone with bisulphite has been found to be highly water-

XV XVI

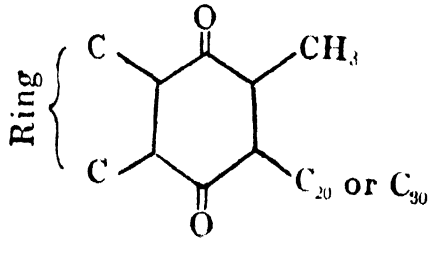


XVII

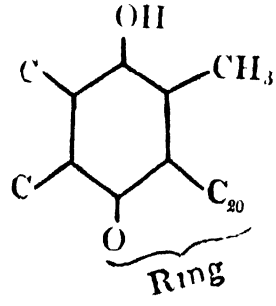
soluble and to possess vitamin K activity equivalent to that of the quinone contained in it.

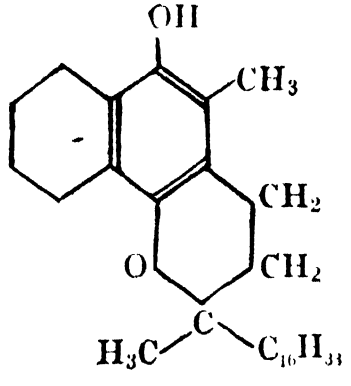
There are several points of resemblance between the K vitamins and the tocopherols. They are derivatives of p-quinones or quinols, have nuclear methyl groups and have long hydrocarbon portions derived from phytol or farnesol. The resemblance becomes striking if their skeletons are compared in the manner shown in formulæ (XVIII) and (XIX). Naphthotocopherol (XX) has been recently prepared by refluxing vitamin K_1 in acetic acid with stannous chloride. Structurally it differs from α-tocopherol (XXI) in having a condensed benzene ring in the place of two methyl groups. However it exhibits appreciable vitamin E as well as K activity. Such correspondence between two ortho-methyl substituents and a condensed benzene ring has been noted in carcinogenic-hydrocarbons also.

XVIII Vitamin K.

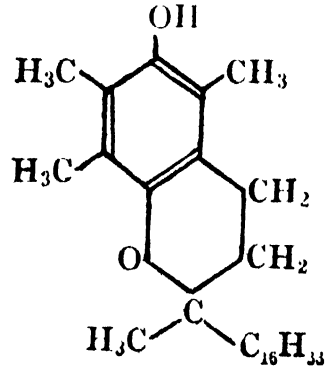


XIX α-Tocopherol





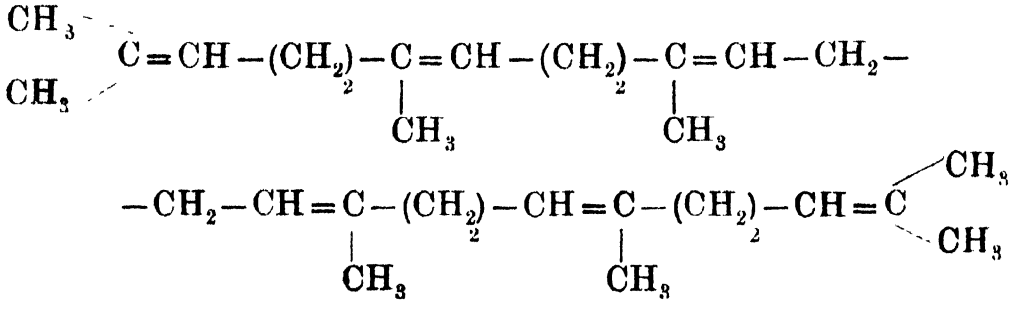
XX Naphtho-tocopherol.



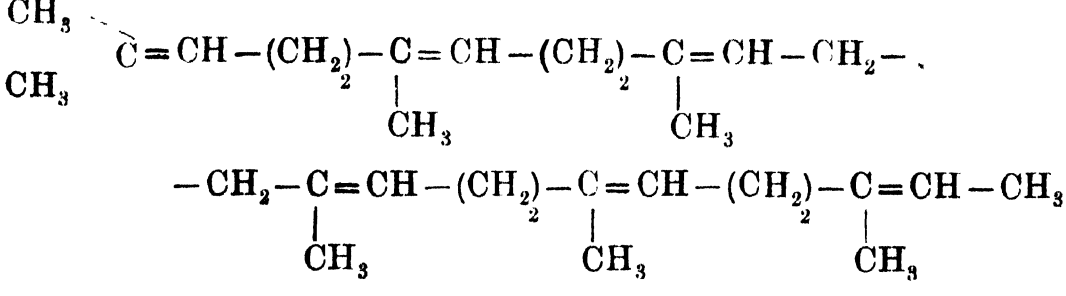
XXI α-Tocopherol.

The structural make up of the K vitamins[5] could be analysed into (1) the long unsaturated side chain and (2) the naphthoquinone part. The former, $C_{20}H_{39}$ or $C_{30}H_{49}$ is based on the isoprene model. It is significant

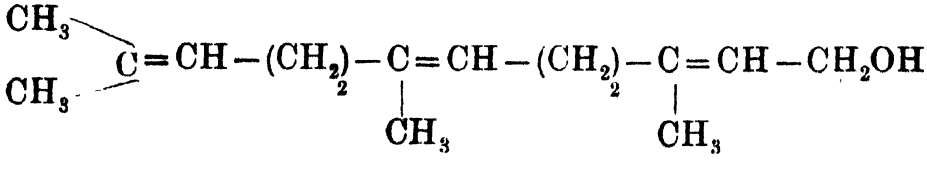
that vitamin K_1, present in green leaves, has the phytyl unit derived from phytol which is so abundant in this plant material. Vitamin K_2 is prepared from fish meal and it has the $C_{30}H_{49}$ unit (difarnesyl) resembling closely the triterpene squalene, $C_{30}H_{50}$ (XXII) which is characteristic of fish oils. The difference between the isomers lies in the fact that the squalene molecule is symmetrical whereas the difarnesene molecule is not. Difarnesene (XXIII) itself does not seem to occur in nature but the sesquiterpene unit common to both is found abundantly in the sweet-smelling alcohol farnesol (XXIV) and its isomer nerolidol (XXV).



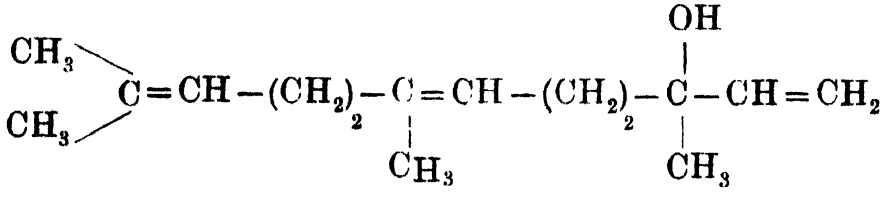
XXII Squalene



XXIII Difarnesene



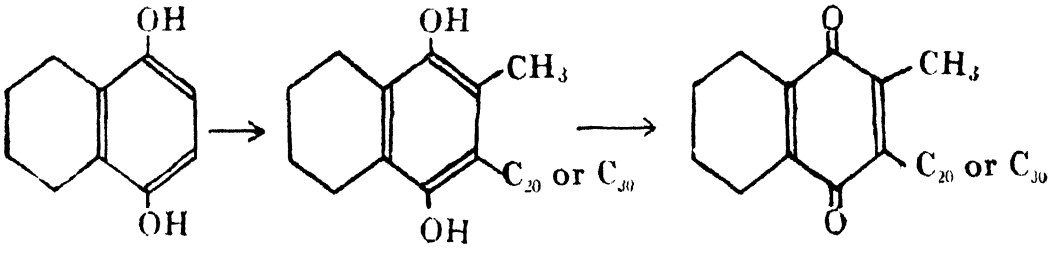
XXIV Farnesol



XXV Nerolidol

As regards the naphthoquinone part it is well known that several hydroxy-derivatives of 1 : 4-naphthoquinone

are found in plants and animals (see naphthoquinones under notes). The naphthalene nucleus does not seem to occur in any other form. The simplest of these are lawsone (3-hydroxy-naphthoquinone) and juglone (5-hydroxy-naphthoquinone) and the others seem to be evolved from the above two by the introduction of the required groups. It may therefore be reasonable to suggest that 1 : 4-naphthoquinone or-hydroquinone is produced as an important stage in the production of the vitamins and nuclear methylation and phytylation (or introduction of difarnesyl) take place subsequently. Just as in laboratory synthesis, the hydroquinone, being more reactive, may be employed in the life processes also and subsequently oxidation to the quinone effected.



VITAMINS OF THE B GROUP

A number of water-soluble vitamins belong to this group; usually they occur together in the same source. They exhibit general resemblance in a number of properties; almost all of them contain nitrogen, are basic in nature and seem to be capable of functioning as co-enzymes At present ten different entities are definitely known and their chemical and physiological properties have been studied in detail Excepting folic acid the constitutions of the others are well settled and their synthesis has been effected. They are (1) thiamin or aneurin, originally called vitamin B_1, (2) riboflavin, also known as vitamin B_2 or G, (3) nicotinic acid (amide), (4) pyridoxin or adermin or vitamin B_6, (5) pantothenic acid, (6) biotin, (7) inositol, (8) p-aminobenzoic acid, (9) choline and (10) folic acid.

With regard to group characteristics the B group vitamins cannot be considered to be in the same category as the D, E or K groups. The several members present in any of the latter groups have the same physiological property, though their potencies may differ. Further, their chemical structures are fundamentally the same, the differences being confined to certain minor details such as the number of nuclear methyl groups or the length and nature of side chains. On the other hand the individual members of the B group exhibit large differences. They differ markedly in their physiological properties and chemically they belong to different structural types. Each is therefore an individual and has to be studied separately.

The vitamins of the B group occupy a unique position in biology. They appear to be universally required by all organisms, from the single-celled protozoa, bacteria and yeast up the scale to the most highly developed plants and animals. Presumably they are constituents of fundamental enzyme systems essential for life processes and they have considerable degree of uniformity in their function wherever they may be found.

CHAPTER VI

THIAMIN OR ANEURIN: VITAMIN B_1

Deficiency of this vitamin in man leads to a disease known as beri-beri in which shortage of breath, paralysis, dropsical swellings and numbness, particularly in the hands and feet, are the prominent symptoms. The corresponding condition in other animals is known as polyneuritis and is characterised by loss of muscular coordination, spastic movements, retraction of the head and paralysis. Loss of appetite is one of the striking accompaniments of this deficiency disease and this leads to progressive decline in weight even in the early stages. Beri-beri is very common among rice-eating populations in countries like Japan, China, India, Malaya, Dutch Indies and the Philippine Islands. It is also prevalent in Newfoundland and Labrador, where people live mainly on flesh and fat. It has been known to make its appearance as an epidemic among soldiers in times of war when preserved foods form the staple diet. Polished rice is responsible for the occurrence of the disease among rice-eating populations, for the vitamin is lost in the rice-polishings. To rectify the defect, hand-pounded and par-boiled rice have been advocated. Hand-pounding leaves behind a good portion of the outer layer of the rice containing the vitamin intact. Par-boiling is better because during this treatment the vitamin diffuses into the body of the grain and consequently is not lost in the course of the subsequent milling and polishing.

Occurrence:—This vitamin is found widely distributed in nature though in small quantities (1—4 parts per 10 million parts). The richest sources are rice-polishings (20 parts per million), yeast, eggs and liver. Green leafy vegetables come next, while milk, fish and meat also contain small quantities of it; nuts and legumes are also fairly good sources. Yeast extract which is sold under the name 'marmite' is a good preparation containing thiamin. Preparations from

rice-polishings have also been made. It is claimed that molasses form a very good source of this vitamin. This is true if the carbonation method is adopted for sugar manufacture. When, however, the sulphitation method is employed, the resulting molasses are devoid of the vitamin, since it is decomposed by sulphites. The vitamin occurs free and also in combination with protein. In most cases it is easily liberated by treatment with dilute acids.

Isolation :—Of all the raw materials suitable for the preparation of thiamin, rice-polishings have been found to be the most satisfactory. The earliest successful experiment was made by Jansen and Donath in the East Indies in 1926. They, however, got only a very small yield. In 1932 Windaus was also successful in isolating it. But the first satisfactory method for preparing large quantities of the vitamin was worked out in 1934 by R. R. Williams[1] in America. This involves several stages of operation, during which careful watch is kept over losses of the vitamin. The most important steps are mentioned below :

Rice-polishings are first extracted with water acidified with sulphuric acid so as to bring the p_H to 4·5 ; a little toluene is also added to prevent fermentation. Thiamin which is easily soluble, is extracted and the solution drawn off. The extract is next treated with Fuller's earth which has the capacity of adsorbing thiamin rapidly. The next stage is the recovery of the vitamin from the adsorbate. This is effected very satisfactorily by employing an ingenious technique.[2] The Fuller's earth adsorbate is extracted with a solution of quinine sulphate. Quinine displaces the vitamin which goes into the solution completely. The solution is then filtered from the Fuller's earth. Excess of quinine sulphate separates out on neutralising the solution. Addition of baryta and subsequently helianthin precipitates impurities. The vitamin is then precipitated with silver nitrate at p_H 7·5 and regenerated from the silver salt precipitate by means of hydrochloric acid. Subsequent stages consist in the removal of alcohol-insoluble impurities and precipitation of thiamin as the phosphotungstate.

As a further step in the purification, the vitamin is converted into the gold chloride double salt which is again decomposed with molecular silver. Thiamin in the form of its chloride-hydrochloride is finally recrystallised from alcohol. By this procedure Williams was able to obtain 5 grams of the vitamin salt for every ton of rice polishings ; the yield is about 25 per cent of the total quantity present (2 mg. per 100 g.). The method has been worked on a factory scale, giving rise to several pounds of the pure compound.

Properties :—The free base of thiamin has not been isolated owing to its extreme instability. The chloride-hydrochloride is reported to exhibit dimorphism, one form melting at 249° and the other at 232°. The bromide-hydrobromide behaves similarly, the two forms melting at 227° and 219° respectively. They contain water of crystallisation. The first compound is a colourless crystalline solid and has the formula $C_{12}H_{17}ON_4SCl$, HCl, $\frac{1}{2}H_2O$. It is easily soluble in water and alcohol and fairly stable to the action of heat. From this it could be inferred that the process of cooking of foods does not seriously destroy thiamin present in them, but the water with which grains or vegetables are cooked should not be thrown off, since it is an extract of the vitamin. It should, however, be noted that pressure cooking, wherein temperatures over 100° are involved, and high p_H are quite injurious. Thiamin is readily adsorbed by Fuller's earth. By the action of potassium ferricyanide in alkaline medium, it changes into a fluorescent compound known as thiochrome. It also combines with diazonium salts to form coloured azo-dyes.

Constitution :—The successful preparation of the vitamin in large quantities enabled the problem of its constitution to be solved rapidly. There are two ways in which the molecule could be split into two halves. Windaus in 1934 carried out careful oxidation with nitric acid, and obtained two products, having the formulae $C_7H_{11}O_5N_3$ (I) and $C_5H_5O_2NS$ (II) respectively. But the details of the reaction and the nature of the fission products were not well understood. A more im-

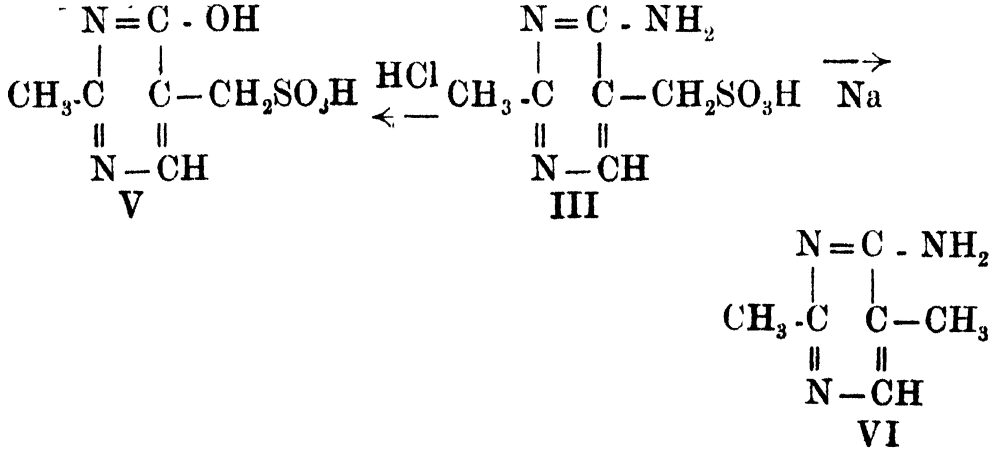
portant observation was made by Williams[2] in 1935. He noticed that thiamin breaks down into two simpler substances (III) and (IV) by treatment with sodium sulphite in a weak acid solution ($p_H = 5$) at room temperature.

$$C_{12}H_{18}N_4OSCl_2 + Na_2SO_3 \rightarrow \underset{\text{III}}{C_6H_9N_3O_3S} + \underset{\text{IV}}{C_6H_9NOS} + 2\,NaCl$$

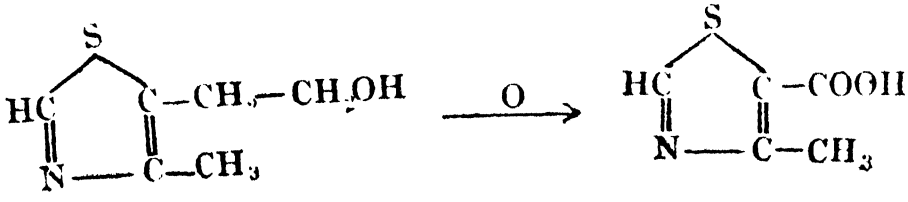
The constitution of the vitamin could be deduced if the nature of (III) and (IV) could be understood. (III) could be hydrolysed by heating with water under pressure when sulphuric acid was produced or by fusing with alkali when a sulphite was obtained. These indicated that it had a sulphonic acid grouping. By treatment with aqueous hydrochloric acid, (III) gave rise to another crystalline compound (V) whose reactions indicated that it was also a sulphonic acid and that it contained a hydroxyl group. A comparison of the formulae of (III) and (V) showed that the latter was derived by the substitution of a hydroxyl group in the place of an amino group present in the former.

$$\underset{\text{III}}{C_6H_9O_3N_3S} + H_2O + HCl \rightarrow \underset{\text{V}}{C_6H_8O_4N_2S} + NH_4Cl$$

Thus the existence of a sulphonic and an amino group in (III) was established. Studies of the ultraviolet absorption spectrum indicated that compound (III) has a pyrimidine structure and this idea was confirmed by its reduction by means of sodium in liquid ammonia to 2 : 5-dimethyl-6-amino-pyrimidine (VI). Subsequently compound (V) was shown to be identical with 2-methyl-6-hydroxypyrimidine-5-methylsulphonic acid obtained synthetically and hence it followed that (III) is 2-methyl-6-aminopyrimidine-5-methylsulphonic acid[3]. The relation between (III), (V) and (VI) may be represented as below :

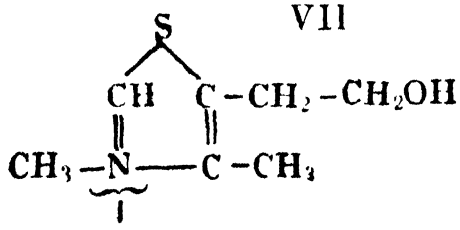


Compound (IV) proved to be a primary alcohol with a thiazole nucleus and on oxidation gave compound (II) originally obtained by Windaus. The latter was found to be identical with 4-methyl-thiazole-5-carboxylic acid which had already been prepared by Wohmen in 1890. Consequently the constitution[4] of the former was arrived at as the corresponding ethyl alcohol. The thiazole nucleus had never before been found in nature and its recognition in thiamin was first based on the peculiar stability of the sulphur linkage in it and also on a study of the ultra-violet absorption spectrum.

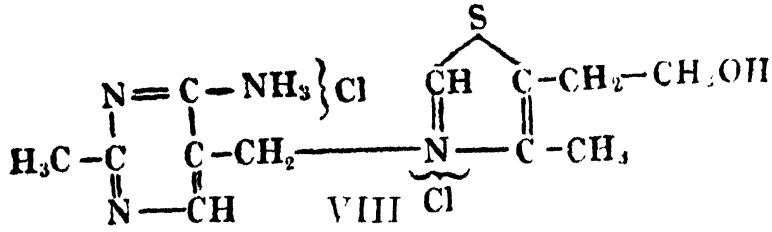


IV II

Finally the mode of linkage of the two nuclei to each other was disclosed by potentiometric titration of thiamin with alkali[5]. The point of attachment of the pyrimidine ring was indicated by the position of the sulphonic acid group in the cleavage product (III); but the position in the thiazole ring was not so obvious. It was inferred from the peculiar behaviour of thiamin chloride-hydrochloride when titrated with alkali. After one equivalent of alkali had been consumed, further additions did not produce any steady rise in alkalinity. There was only a temporary rise after each addition and it slowly fell in a few minutes. This was indicative of the production of a comparatively slow rearrangement. Altogether three equivalents of alkali were used up before any permanent rise in alkalinity could take place. An analogous behaviour was exhibited by the methiodide of 4-methyl-5-hydroxyethyl-thiazole (VII). The constitution of thiamin (chloride-hydrochloride) was therefore represented as shown in (VIII)[6] and it is in conformity with all its properties and reactions.



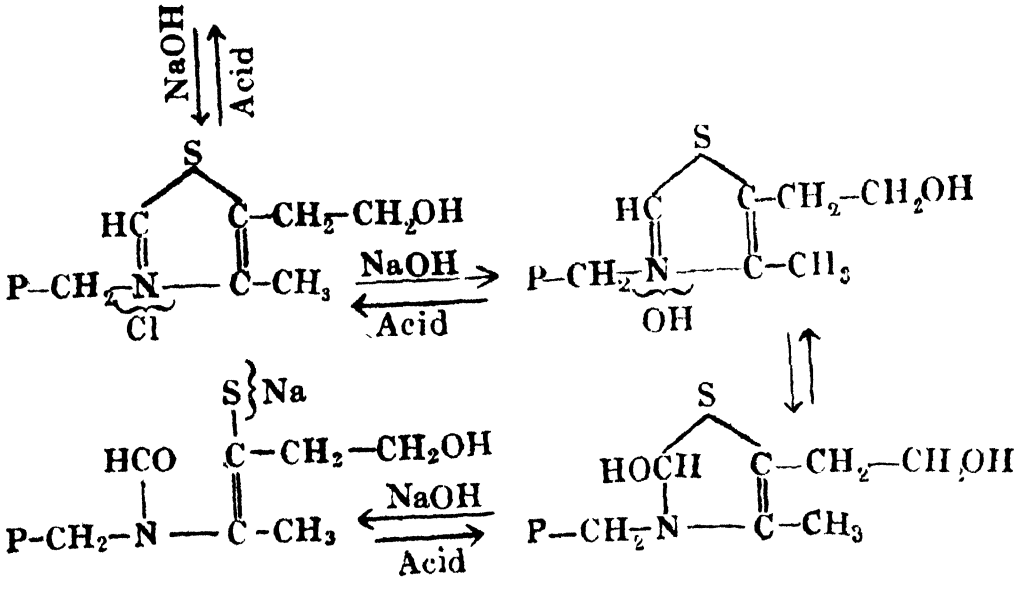
VII



VIII

The action of alkali involves two successive rearrangements in the molecule and the changes are represented below.

Thiamin chloride-hydrochloride



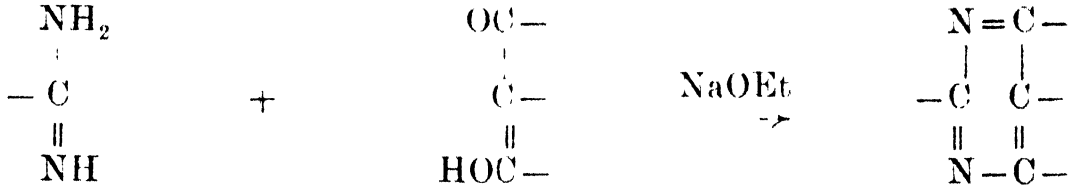
P = Substituted pyrimidine.

The whole series of steps is reversed on the addition of acid and the original thiamin can be completely recovered.

Synthesis:—The synthesis of the two halves of the vitamin molecule not only established their respective constitutions but also enabled the synthesis of thiamin itself to be effected easily. The methods given below are those of R. R. Williams and his collaborators[7] described in 1937.

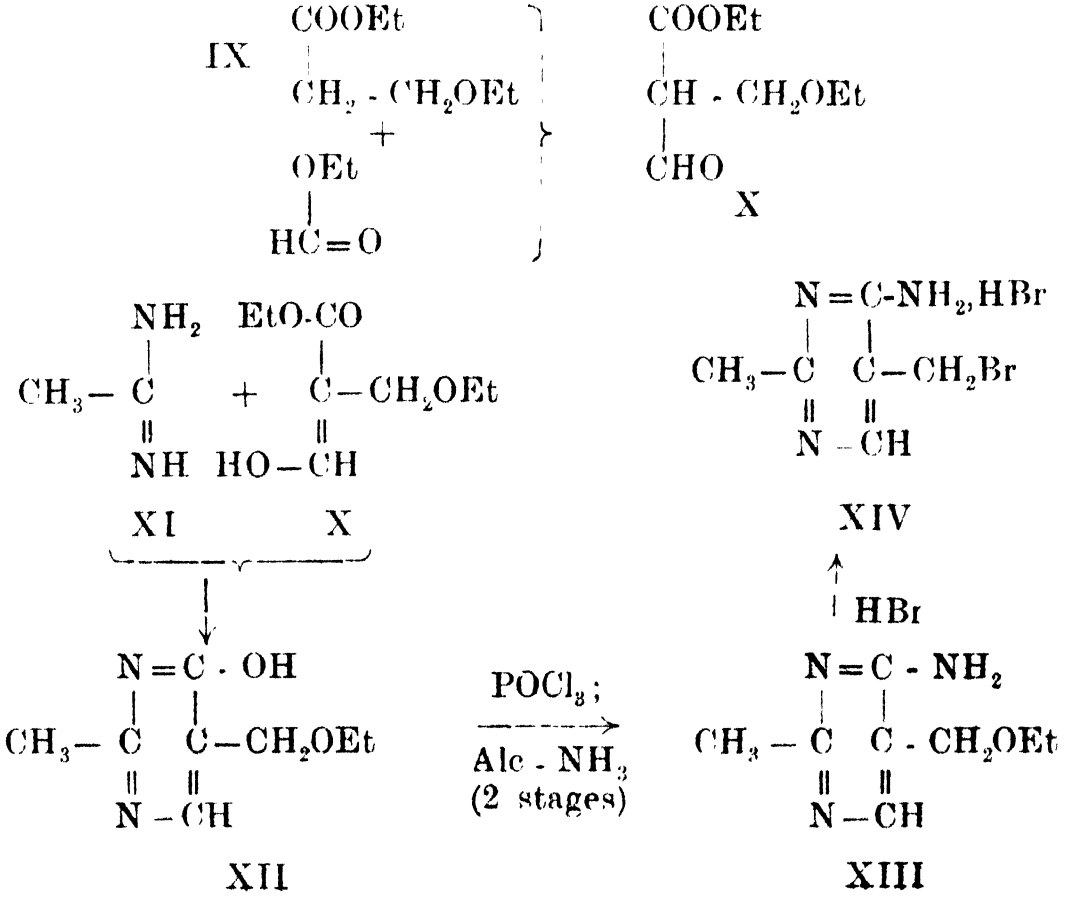
The Pyrimidine part:—One of the general methods for the synthesis of pyrimidines involves the condensation of amidines of carboxylic acids with β-diketones or

ketonic esters, the latter reacting in the enolic form as given below:

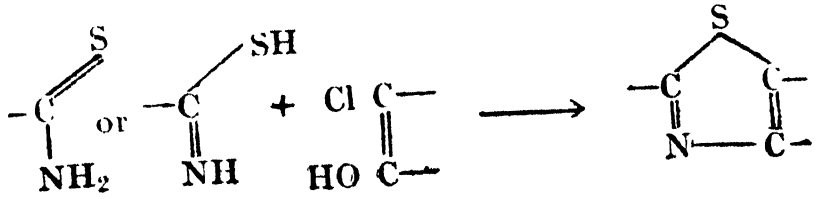


Amidine β-Diketone (enolic form) Pyrimidine

For the synthesis of the pyrimidine part of thiamin the required diketone was obtained by the action of ethyl formate on ethyl β-ethoxy-propionate (IX). The formyl derivative (X) when condensed with acetamidine (XI) gave 2-methyl-5-ethoxymethyl-6 - hydroxy - pyrimidine (XII). The hydroxyl group of this compound was replaced successively by chlorine and amino group by treatment with phosphorus oxychloride and alcoholic ammonia leading to compound (XIII). Subsequent treatment with concentrated hydrobromic acid involved the replacement of the ethoxyl group by bromine, and 2-methyl-6-amino-pyrimidine-5-methyl bromide (XIV) was obtained in the form of its hydrobromide.



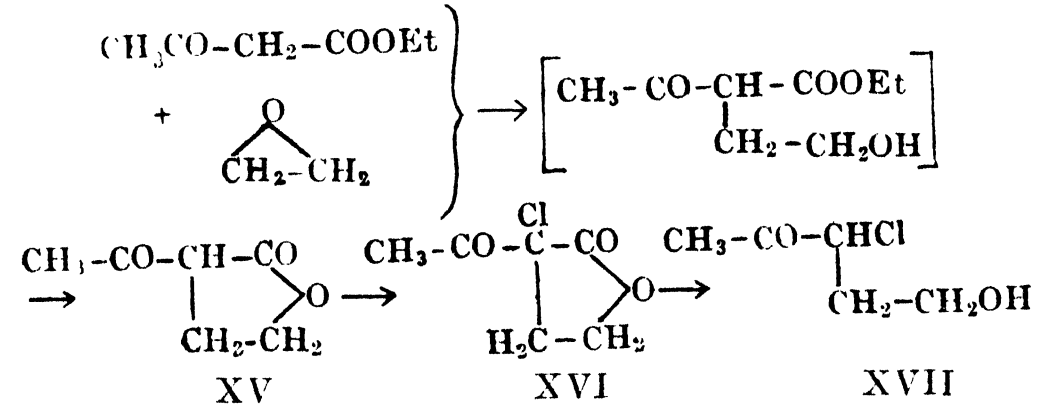
The Thiazole part :—Thiazoles are formed by the interaction of thioamides and *α*-chloroketones or *α*-chloroaldehydes. The general reaction may be represented as follows :



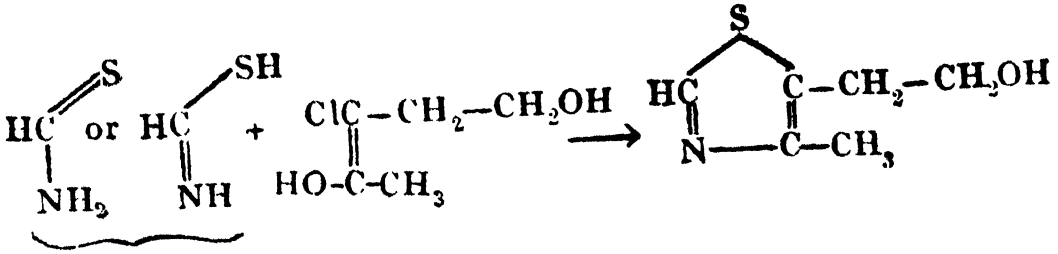
Thioamide Ketone (enolic form) Thiazole

For the thiazole part of thiamin the components required are thioformamide (XVIII) and 3-chloro-4-ketopentanol (XVII). The latter was synthesised from acetoacetic ester as follows :

Ethylene oxide underwent condensation with ethyl acetoacetate in the presence of sodium ethoxide to form a lactone (XV) which yielded the corresponding chloro-compound (XVI) when treated with sulphuryl chloride. Subsequent hydrolysis with dilute hydrochloric acid involved decarboxylation also leading to the formation of 3-chloro-4-ketopentanol (XVII). Reaction of (XVII) with thioformamide (XVIII) yielded 4-methyl-5-*β*-hydroxyethyl-thiazole (IV).

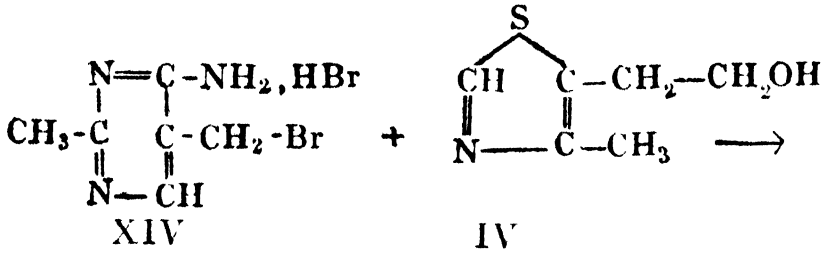


XV XVI XVII

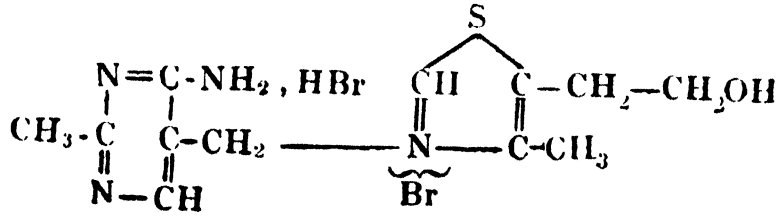


XVIII XVII IV

When the two components (XIV and IV) were heated together condensation took place readily to produce thiamin bromide-hydrobromide (VIII). This was found to be identical with the product obtained from natural sources. From the bromide, the corresponding chloride could be obtained by boiling an alcoholic solution with silver chloride.



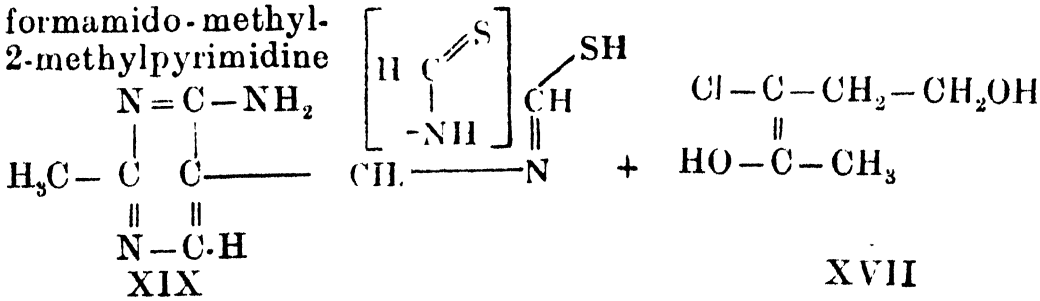
XIV IV



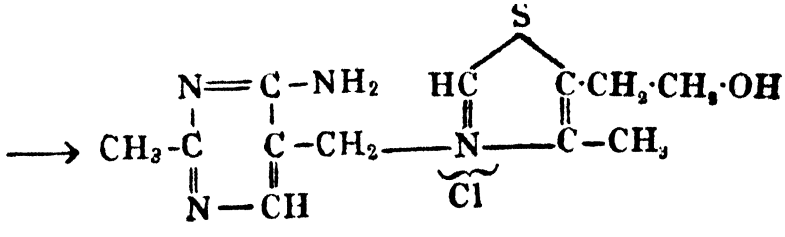
VIII

Another synthesis of thiamin was described by Todd and Bergel[8] (1937); it involved the formation of the thiazole ring upon the appropriate thioformamide containing the pyrimidine unit (XIX) as shown below:

6-amino-5-thioformamido-methyl-2-methylpyrimidine



XIX XVII



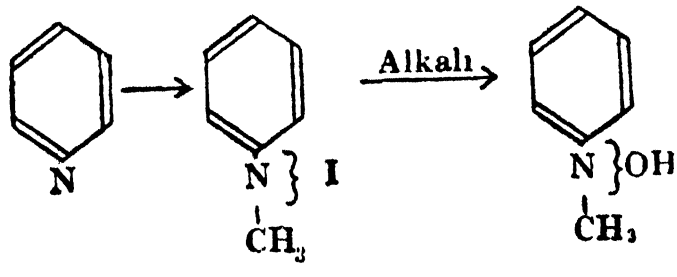
Thiamin chloride

The various stages in the synthesis of thiamin have been so perfected that at the present time the synthetic method is much cheaper than isolation from rice-polishings. Not only are large quantities of this sub-

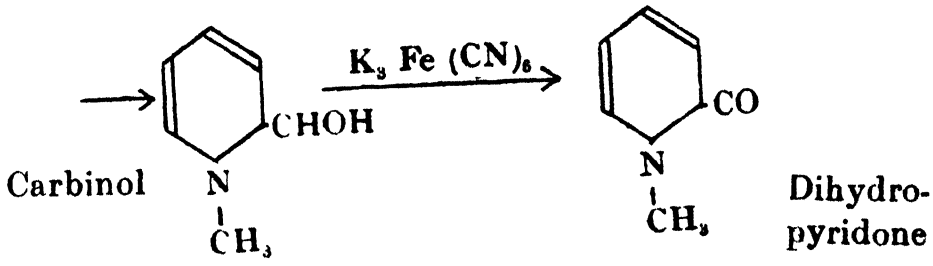
stance needed for the making of vitamin preparations and medicinal foods, but considerably greater quantities are used in America and Britain for the enrichment of flour. Each pound of flour thus fortified contains not less than 1·66 mg. of thiamin. The large part played by chemical research in this line is indicated by the fall in price of thiamin during the past decade. In 1935 the bulk price was 300 dollars per gram; in 1940 it was 1 dollar and in 1942, 0·53 dollar per gram.

A number of analogues of thiamin have been synthesised and tested for vitamin activity. Small changes such as the replacement of the methyl groups in the pyrimidine and thiazole parts with ethyl or propyl groups do not seriously affect the potency and highly active products are obtained. Nearly all other changes, e.g., absence of the hydroxyethyl group or substitution of the free nuclear position in the thiazole ring and replacement of the amino group in the pyrimidine ring with a hydroxyl group result in considerable or total loss of activity. Thus structural characteristics are highly specific for thiamin activity.

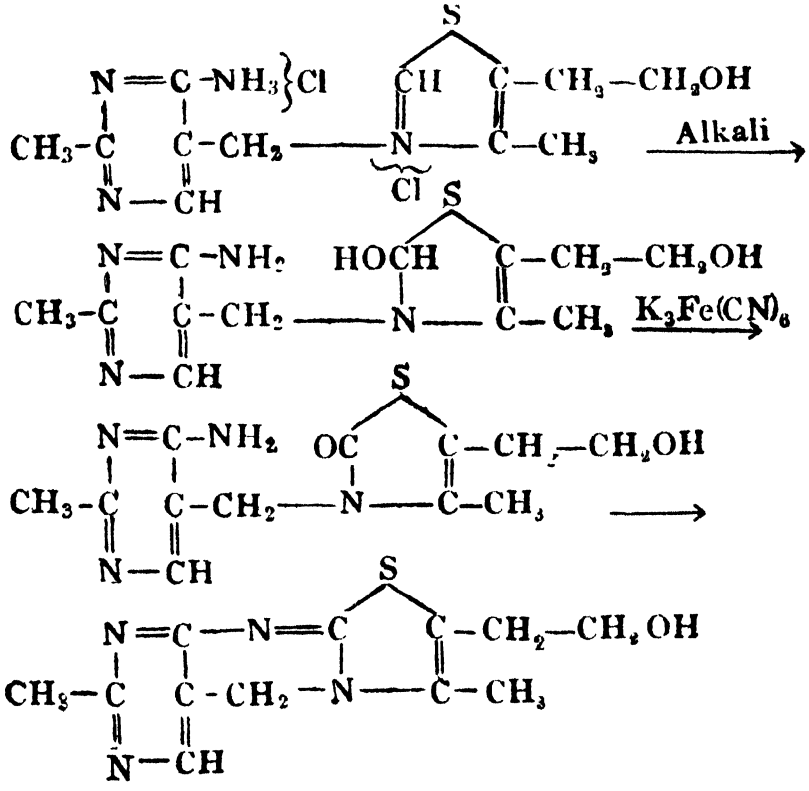
Thiochrome:—The constitution of thiamin given above is supported by the formation of thiochrome (XX), when the vitamin is oxidised with alkaline potassium ferricyanide. This compound was first isolated by Kuhn from yeast in 1933 and was particularly noted for its intense blue fluorescence in neutral or alkaline solution. Barger, Todd and Bergel [9] (1935) were the first to identify it as an oxidation product of thiamin. The behaviour of the vitamin is analogous to that of pyridinium salts under the same conditions. The latter yield dihydropyridones, the pyridinium hydroxide and the carbinol being the possible intermediate stages.



Methyl pyridinium hydroxide



In a similar manner the formation of thiochrome (XX) is explained as given below :



XX Thiochrome

Assay :—As in most other cases the earlier methods of assay were biological. The British Pharmacopoeia prescribes a curative rat growth method in which young rats, freshly weaned, are kept on thiamin deficient diet until they cease to grow. Some of the rats are then fed on the unknown preparation under test while others are given a standard preparation of known potency. Here again, as in the case of other vitamins, equal growth response indicates equal thiamin content. The cure of polyneuritis in birds, particularly pigeons and the rice-birds of Java, has also been taken as a criterion for purposes of assay. But the pigeon test is considered to be the least accurate of the standard methods. A third

method makes use of the changes in the normal rhythm of the heart which result from thiamin deficiency and which can be measured by means of an electrocardiograph. This method known as the bradycardia method is fairly specific and accurate and is most conveniently carried out with rats as test animals. The heart rate falls from a normal of about 500 per minute to 250 as the result of vitamin deficiency.

Chemical methods for the assay of the vitamin are much quicker than the biological methods and in addition are quite reliable. They are based on the estimation of substances produced from the vitamin by well known reactions.

The thiochrome method has been found to be the easiest and at the same time most satisfactory. The chemistry of thiochrome has already been discussed. The thiamin solution is treated with 0·1% solution of potassium ferricyanide in the presence of aqueous sodium hydroxide. The thiochrome that is formed is extracted with isobutyl alcohol, the extract made up to a definite volume and the depth of fluorescence estimated using a fluorimeter. The strong blue violet fluorescence is proportional to the concentration of thiochrome and the use of isobutanol not only increases the intensity of the fluorescence, but helps the separation of the thiochrome from interfering impurities. For biological materials in which the vitamin exists in a combined form, extraction with (0·1N) sulphuric acid is generally sufficient to liberate it. In some cases enzymes (diastatic type) have to be employed. Purification by passing the impure extract through a column of synthetic zeolite (decalso) has also been found to be advantageous. The vitamin is adsorbed and later on eluted using potassium chloride solution. The thiochrome method has been found to be very satisfactory even for materials of low potency.

A simpler method involves the formation of a red azo-dye when the vitamin solution is mixed with diazotised p-amino-acetophenone. Coupling is said to take place in the free nuclear position of the thiazole part. The dye is extracted with isobutyl alcohol and the solution made up to a definite volume and compared

with a standard obtained from known quantities of thiamin. Sulphanilic acid can also be employed for the diazotisation and coupling. But p-amino-acetophenone (Prebluda-McCollum reagent) has been found to be the best. This method is said to be reliable only for materials of fairly high concentrations.

A microbiological method has also been suggested for the assay of thiamin. It depends upon the fact that the vitamin is essential for the growth of yeast. The rate of yeast growth is measured by the evolution of carbon dioxide when it is grown on a suitable medium.

Unit, requirement and function:—During the earlier days of the study of thiamin, Fuller's earth adsorbates were used in the treatment of beri-beri and the international unit of vitamin B_1 was taken as the activity of 10 milligrams of a standard Fuller's earth adsorbate, the quantity required for curing polyneurites in a rat. It has now been found that this unit is equivalent to three micrograms (millionths of a gram) of thiamin. Consequently 1 milligram of the pure vitamin chloride has a potency of 333 I.U.

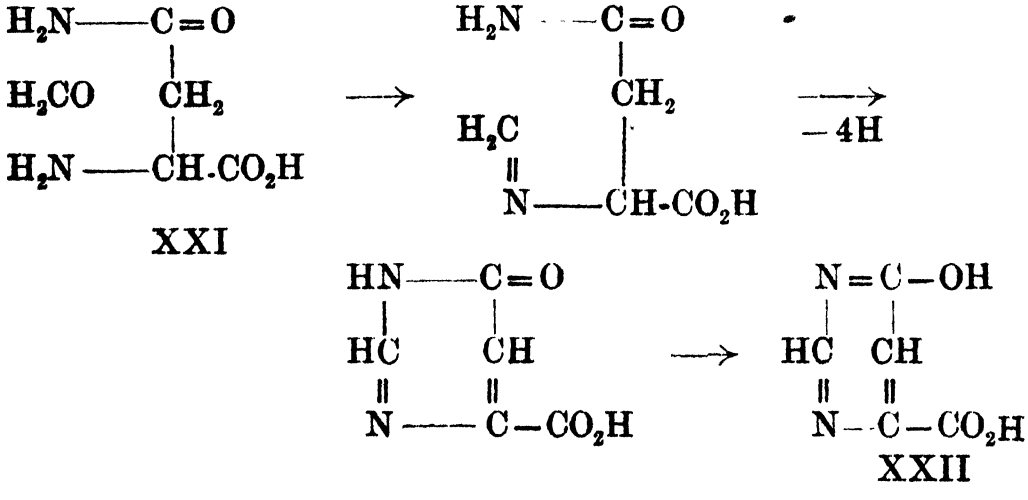
Human requirements of thiamin vary depending upon food and other conditions of life. Carbohydrates increase and fats decrease the need whereas proteins have no effect. The minimum on which man can subsist is 0·6 milligram of thiamin per day. To function efficiently the daily dose should be about twice this amount and for safety about 2 mg. The nutritional status of an individual in regard to this vitamin can be assessed from its excretion in urine. The average normal adult excretion is considered to be 20 I.U. per day; a smaller output will indicate deficiency. The vitamin is not a dietary essential for ruminants such as the cow, which seem to be capable of synthesising it in their rumen.

Thiamin functions in animal tissues in the form of its pyrophosphoric ester, cocarboxylase, as was first indicated by Lohmann and Schuster (1937). Its precise role in carbohydrate metabolism lies in the disposal of pyruvic acid. There seems to be sufficient evidence to warrant the belief that it influences fat metabolism also in an indirect way. It also plays an important part in

the growth of a wide variety of micro-organisms and of lower and higher plants.

Biogenesis:—In tracing the biological origin of thiamin it is simplest to consider it as arising from two parts just as in the laboratory synthesis. There is some experimental support for this. Plants and to some extent animals are found to have the capacity for synthesising thiamin from the two components. It may be presumed that this represents the last step in the biogenesis of the vitamin.

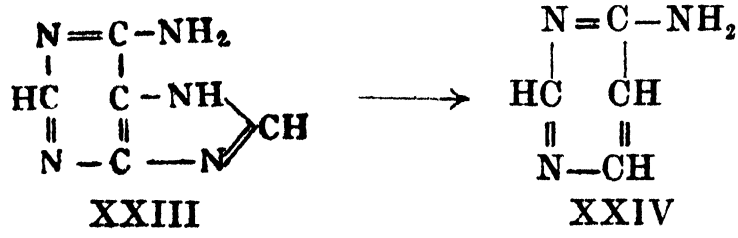
Pyrimidines occur fairly widely in nature as for example in nucleic acids. It is possible that they are produced either from simpler substances or are derived from the degradation of the more complex purines. In favour of the first alternative may be mentioned the formation of a pyrimidine compound from asparagine (XXI). It reacts with formaldehyde to give a methylene derivative which when oxidised undergoes transformation into 6-hydroxypyrimidine-4-carboxylic-acid (XXII).



XXI

XXII

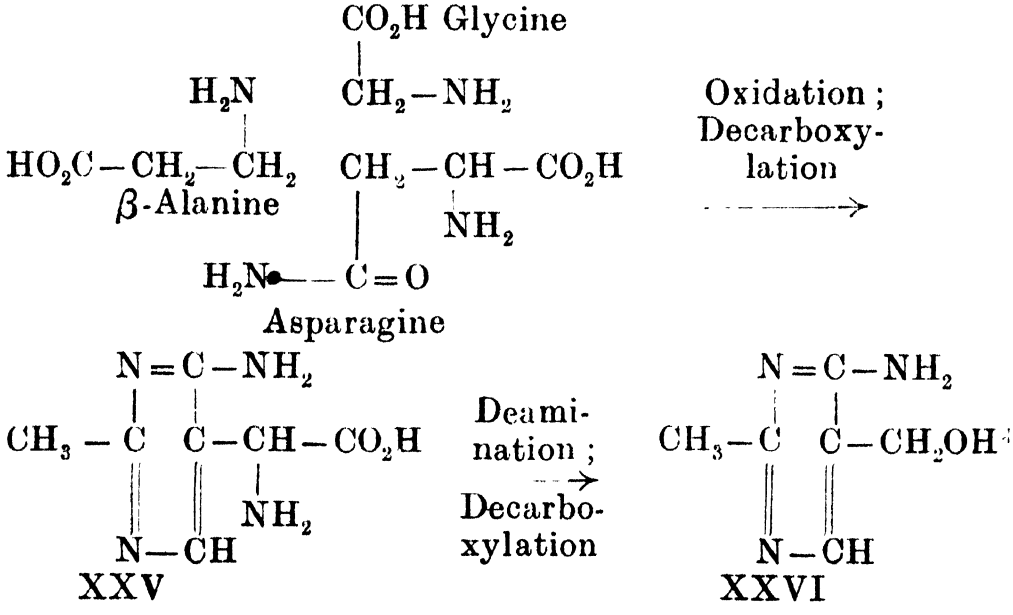
Their possible origin from the purines may be illustrated by the degradation of adenine (XXIII) which yields 6-aminopyrimidine (XXIV) by hydrolysis.



XXIII XXIV

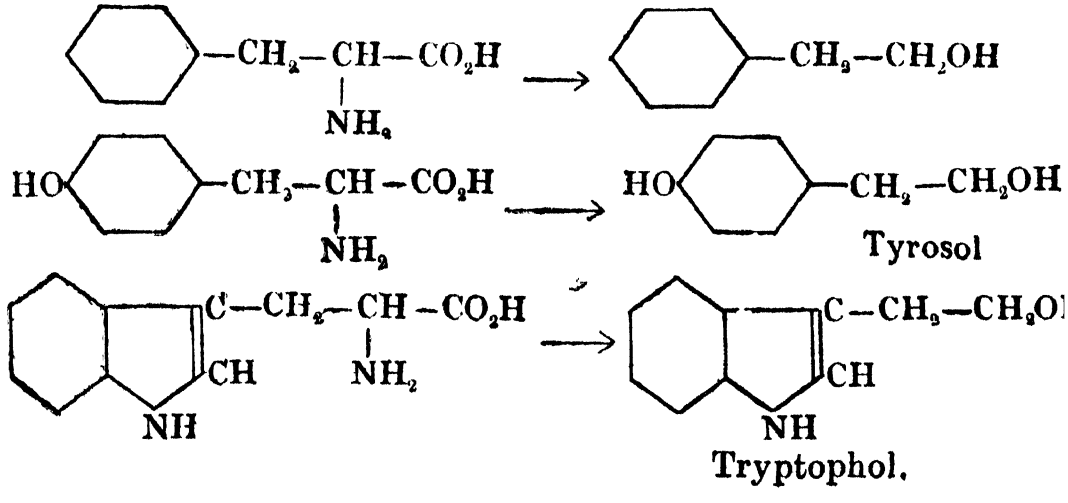
The position seems to be far more complex with regard to the origin of the pyrimidine half of the thiamin

molecule which has a methyl substituent in the 2-position, a hydroxymethyl group (free or as chloride or similar ester) in the 5-position and an amino group in the 6-position (XXVI). No experimental data useful for the purpose seem to be available. However, without entering into details, the following plausible suggestion could be made regarding the amino-acid units that may be involved in its biological synthesis.

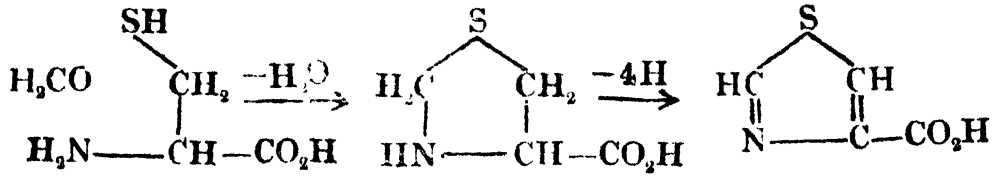


* The alcohol may be esterified and may exist either as a chloride or as any other ester.

Of the processes involved, decarboxylation can take place readily in living tissues, and so also the conversion of the amino-acid unit into the hydroxymethyl side chain. The latter reaction is analogous to the formation of phenylethyl-alcohol from phenylalanine, tyrosol from tyrosine and tryptophol from tryptophane by the action of fermenting yeast.

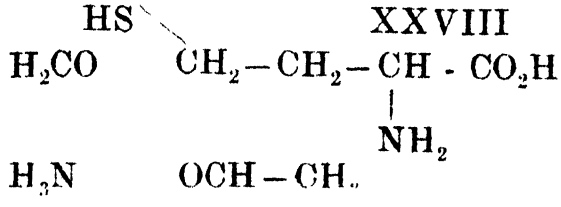


The thiazole nucleus is peculiar to this vitamin and does not occur elsewhere in nature. A simple derivation of this nucleus seems to be possible from cysteine (XXVII) by condensation with formaldehyde and subsequent oxidation.

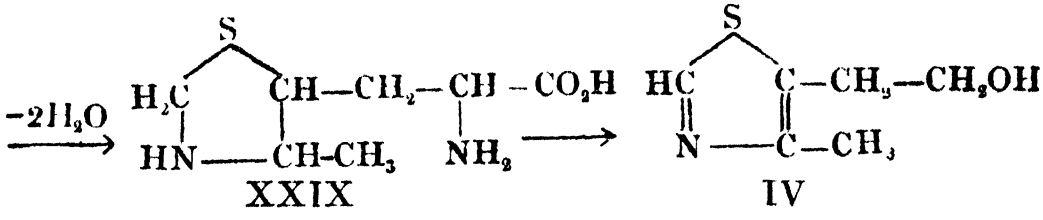


XXVII

The particular thiazole (IV) present in thiamin has ethanol and methyl side chains in the 5- and 4-positions respectively. Harington and Moggridge [10] made the interesting suggestion that this structure possibly arises from the condensation of methionine, acetaldehyde and ammonia and subsequent degradation of the resulting amino acid (XXIX) to the alcohol (IV). In the following representation instead of methionine, the corresponding thiol (XXVIII) and formaldehyde are used as being more convenient. They have experimentally shown that the amino-acid (XXIX) is in fact decomposed by yeast to give a good yield of the thiazole.

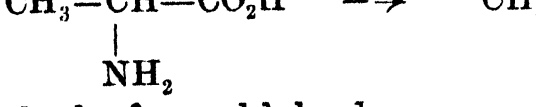


XXVIII



XXIX IV

The acetaldehyde unit required for the building up of the thiazole part may arise from the oxidation of alanine. This point has been established in the synthesis of harman under almost biogenetic conditions.



Similarly formaldehyde may arise from glycine though a simpler origin is possible. Then thiamin may be said to be formed entirely from amino acids.

CHAPTER VII

RIBOFLAVIN

The isolation of riboflavin was made about the year 1933 from several sources. The name flavin was given because of the yellow colour. It is water-soluble and basic in nature and has the characteristic physiological property of promoting growth (water-soluble growth factor). Originally different names were given for samples obtained from different sources, such as ovoflavin from eggs, lactoflavin from milk and hepaflavin from liver. The pure substances were later shown to be identical and the name riboflavin was finally adopted. It was also known as vitamin B_2 and vitamin G.

Riboflavin deficiency in man is marked by inflammation and scaliness of the lips and cracks at the angle of the mouth. It stunts the growth of the young and in adults brings about a condition of premature ageing. These symptoms respond rapidly to treatment. The vitamin is said to be of importance in the rearing of pigs and poultry.

Occurrence: Its occurrence is more wide-spread in food materials than that of the fat-soluble growth factor, vitamin A. The most important dietary sources are eggs, milk, green leafy vegetables and meat. They contain about 2 parts per million. Liver and yeast are much richer yielding about 20 parts per million. Though it is also found in grains it is less abundant in them than thiamin. It appears to be formed primarily in the green leaves of actively growing plants and it is found there in higher concentration than elsewhere. As the leaves mature it seems to diminish in amount.

Preparation:—For the preparation of pure riboflavin egg albumen was first used and it was reported by Kuhn and his collaborators[1] that 30 mg. of the pure flavin were obtained from 30 kg. of dry egg-white corresponding to 10,000 eggs. As a more convenient source they used whey. It was mixed with about a twelfth of its volume of concentrated hydrochloric acid, Fuller's earth added

and the mixture stirred for an hour and a half. The adsorbate was then removed and washed free of all acid. It was subsequently eluted with a mixture of pyridine, methanol and water (1 : 1 : 4). The extract was concentrated in vacuo to remove methanol, and impurities were removed by extracting the solution with ether and then adding excess of acetone. After concentrating this solution to small bulk the vitamin was adsorbed again, this time using Frankonit. The second eluate was concentrated and treated with excess of acetone and some acetic acid. As the solution was concentrated in vacuo colourless impurities separated and they were filtered off at various stages. Creatine was removed as the picrate by the addition of saturated picric acid solution. Further concentration in vacuo led to the formation of crystals of riboflavin and it was finally re-crystallised from acetic acid. It was later found that the precipitation of the vitamin as the sparingly soluble thallium salt as one of the stages of purification greatly improved the yield. Kuhn and his collaborators reported a yield of 1·0 gram of crystalline riboflavin from 5,400 litres of whey, which amounts to about 20% of the total quantity present. In general the above procedure has been adopted with minor modifications in the case of other materials also.

Properties :—The pure substance is obtained in the form of orange-yellow, needle-shaped crystals decomposing at about 285°. It is bitter to the taste and is slightly soluble in water and alcohol. Its solution in water gives an intense yellowish green fluorescence which is maximum when the solution is neutral. It exhibits marked optical activity in alkaline solution, the specific rotation in 0·1N sodium hydroxide being −114°, and has absorption maxima at 446, 366, 267 and 220 mμ. It is fairly stable to heat and to the action of mineral acids and oxidising agents but is very sensitive to the presence of alkalies. One characteristic property is that it can be reduced to a colourless leuco-compound and the change can be reversed in the presence of air. For the reduction sodium hydrogen sulphide, zinc and acetic acid, and hydrogen in the presence of platinum have been

employed. Sun light and ultraviolet light cause decomposition of the vitamin into different products depending on other conditions.

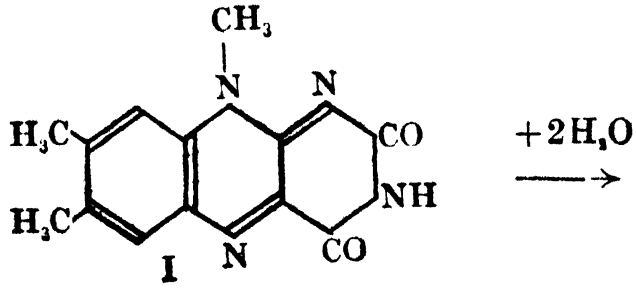
Constitution:—Certain amount of useful early work on Warburg's yellow enzyme rendered the study of riboflavin easier. As will be mentioned later on, riboflavin forms part of this enzyme. The vitamin has the molecular formula $C_{17}H_{20}O_6N_4$. It forms a tetra-acetate due to the presence of four hydroxyl groups. In the elucidation of the constitution of the molecule the study of two important degradation products, (1) lumiflavin and (2) lumichrome has played the most important role.

Lumiflavin:—This product was investigated by Kuhn and his coworkers[2]. When riboflavin is exposed to sunlight or ultra-violet light in an alkaline medium it undergoes decomposition giving rise to a yellow compound, lumiflavin (I) having the formula $C_{13}H_{12}O_2N_4$. Unlike riboflavin it is insoluble in water and soluble in chloroform but it resembles riboflavin in exhibiting the same yellowish-green fluorescence in solution. The decomposition involves the splitting of a side chain rich in hydroxyl groups ($C_4H_8O_4$) which is responsible for the solubility of the original vitamin in water.

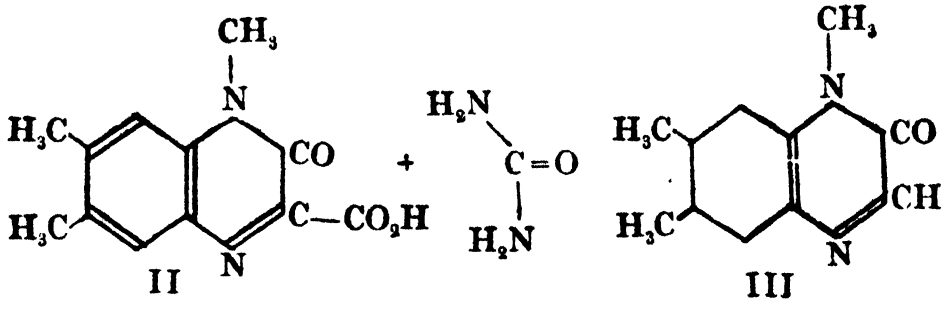
Lumiflavin (I) has a methylimino group and does not undergo acetylation or deamination. It therefore contains neither hydroxyl nor amino groups. It, however, undergoes decomposition with aqueous alkali giving rise to a molecular proportion of urea and a keto-carboxylic acid having the composition $C_{12}H_{12}O_3N_2$ (II). Since two molecules of water are taken up for this change, it is concluded that the decomposition involves a ring containing two carbonyl groups, one of which goes off in urea while the other hydrolyses to a carboxyl. The acid loses carbon dioxide on heating, leaving a compound (III) with the formula $C_{11}H_{12}ON_2$ and with the properties of a dihydroquinoxalone.

From the general similarity in properties and reactions exhibited by lumiflavin with the class of compounds known as alloxazines (IV) which had been prepared and studied by Kuhling as early as 1891, the constitution (I) was assigned for the flavin. It is there-

fore 6 : 7 : 9-trimethyl-isoalloxazine. The decomposition with alkali takes place as indicated below :

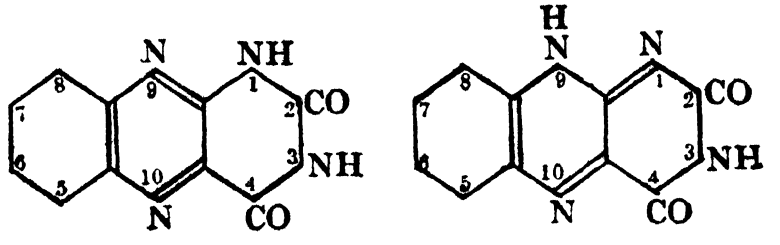


I



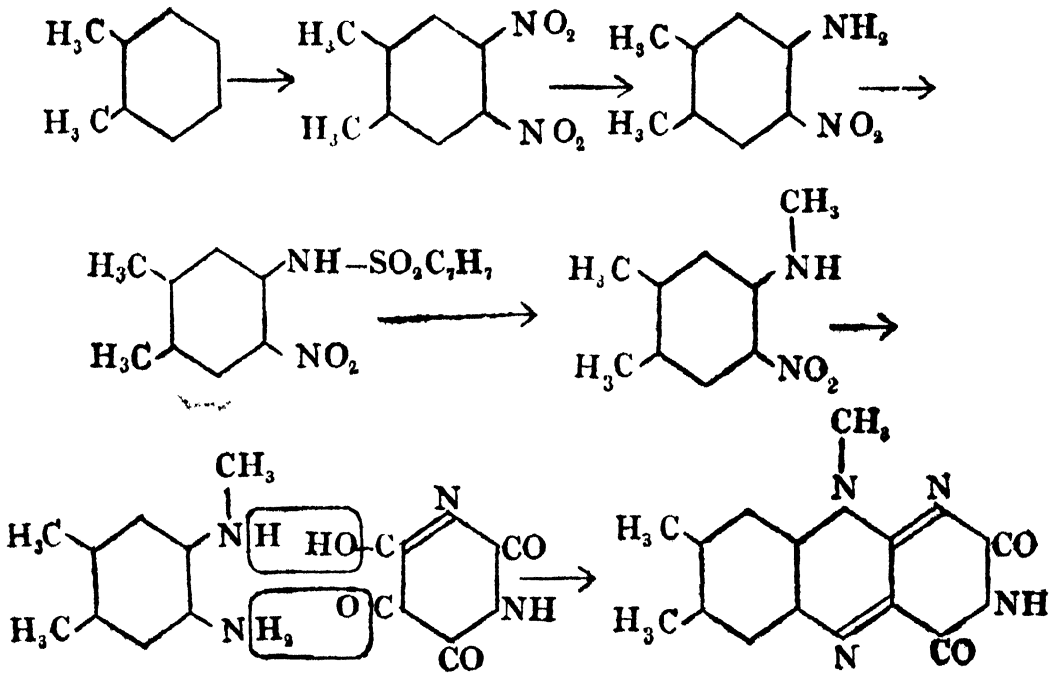
II

III



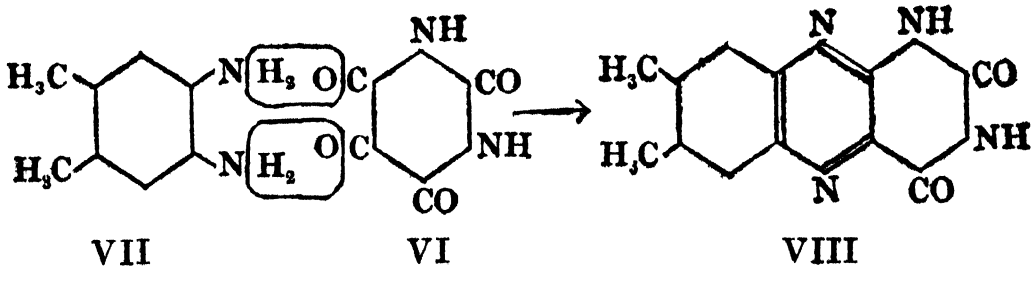
Alloxazine IV Iso-alloxazine

The above structure of lumiflavin was confirmed by Kuhn's synthesis starting from o-xylene. On nitration it yields 4 : 5-dinitro-orthoxylene which forms on partial reduction 1 : 2-dimethyl-4-amino-5-nitrobenzene. The next stage is its convertion into the monomethyl amine. For this purpose the p-toluene-sulphonyl derivative is made, methylated with dimethyl sulphate and hydrolysed with sulphuric acid. The second nitro group is then reduced with stannous chloride in hydrochloric acid. The resulting compound, N-methyl-4 : 5-diamino-ortho-xylene (V) in the form of its hydrochloride condenses readily with alloxan (VI) in warm aqueous solution to give a good yield (75%) of lumiflavin (I).



V VI (enol form) I

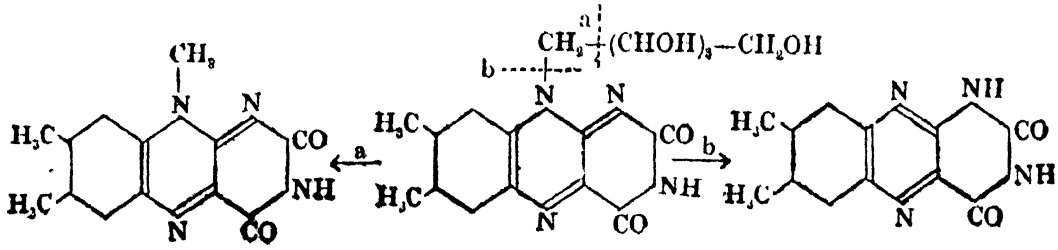
Lumichrome:—Karrer[3] discovered that when riboflavin is irradiated in dilute methyl alcoholic solution it undergoes degradation in a different way to yield lumichrome, $C_{12}H_{10}O_2N_4$ which gives an intense sky-blue fluorescence in solution. It is very similar to lumiflavin in properties and has an analogous constitution, the only difference being that it is devoid of an N-methyl group. Its structure as 6 : 7-dimethyl-alloxazine (VIII) was established by comparison with a synthetic sample obtained by the condensation of 4 : 5-diamino-orthoxylene (VII) with alloxan (VI).



VII VI VIII

From the established constitutions of lumiflavin and lumichrome, that of riboflavin could be derived as represented in (IX). The formation of these two degrada-

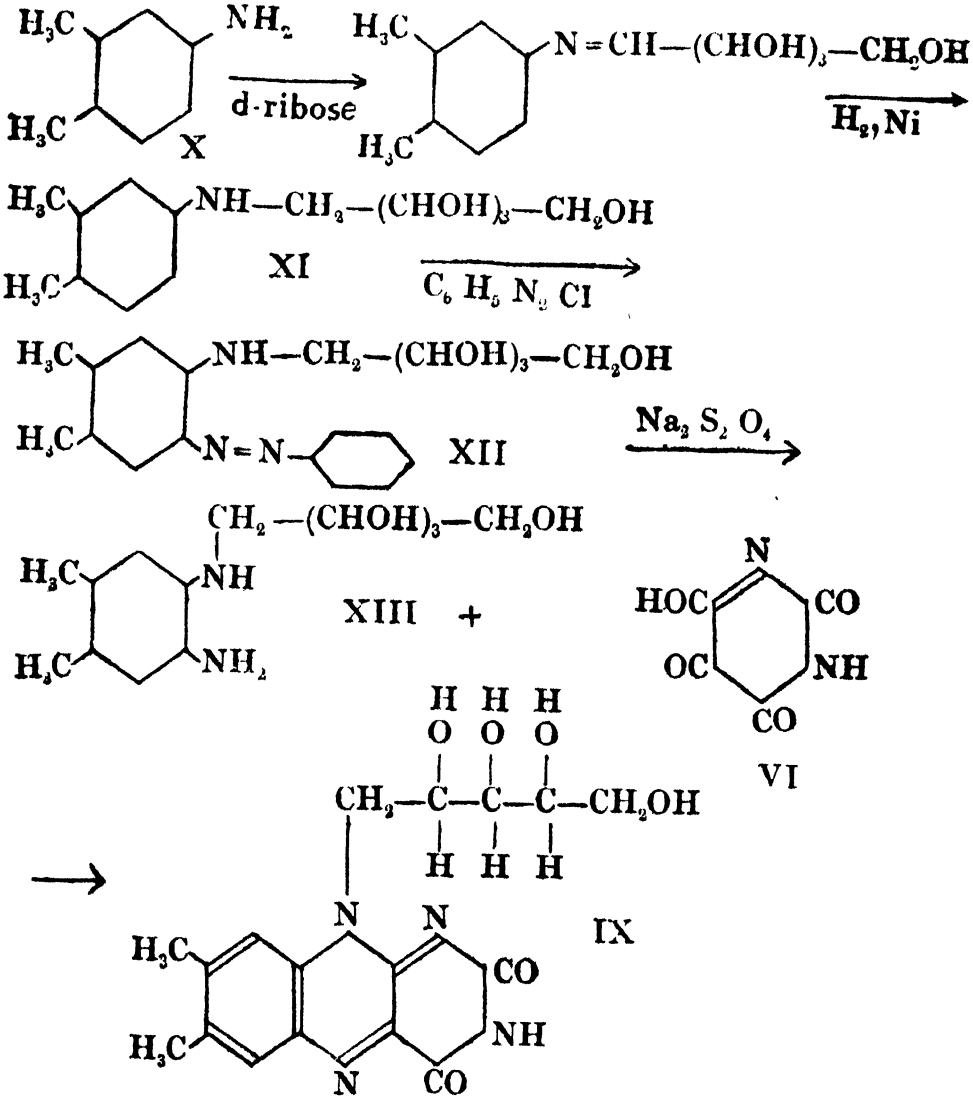
tion products (I and VIII) is due to rupture at the positions (a) and (b) as shown below :



I IX VIII

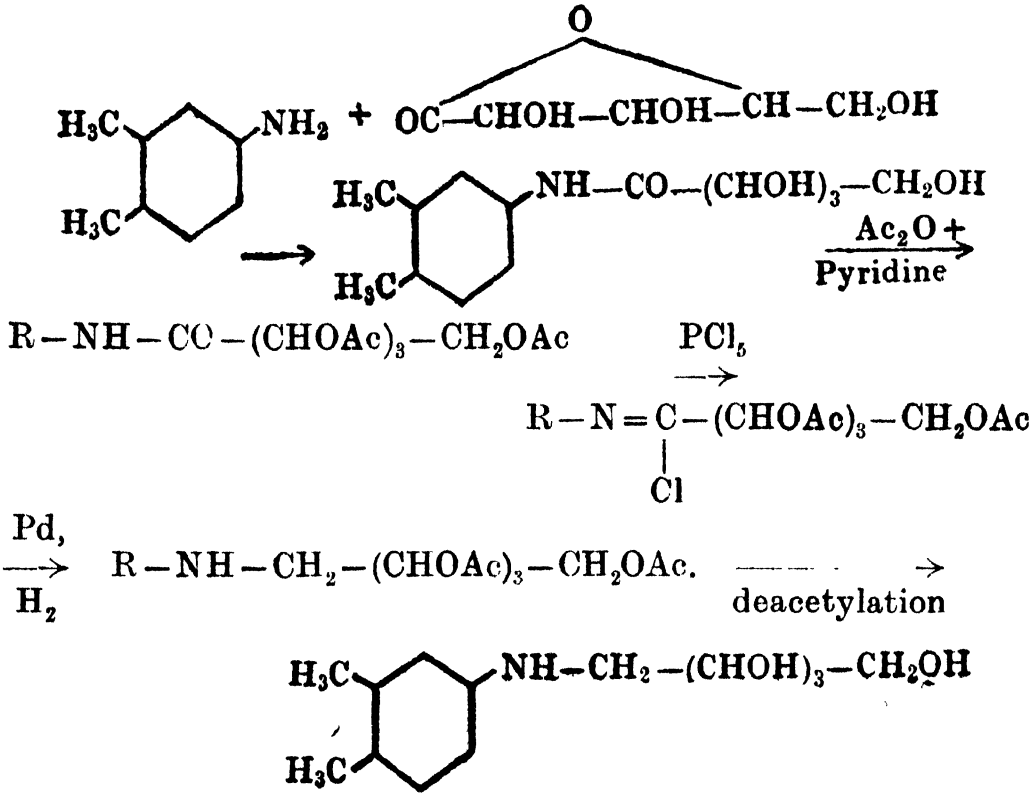
It is obvious that the oxygen-rich side chain is derived from pentitol. There are eight possibilities for the structure of this chain since it has three asymmetric carbon atoms. Of the possible isomers the l-arabityl derivative has a small vitamin activity; the d-ribityl derivative, however, is found to be identical with the naturally occurring flavin in all respects including physiological potency. Hence the derivation of the name riboflavin, though it should be more correctly ribitylflavin, and the full name of the compound is 9-d-1'-ribityl-6 : 7-dimethyl-isoalloxazine. It may be mentioned here that the side chain is related to d-ribose which is a pentose widely present in nucleic acids.

Synthesis[4] :—The various stages in the synthesis of riboflavin were worked out during 1934 by two groups of chemists, one headed by Kuhn and the other by Karrer. The method finally perfected by Karrer in 1936 is given here. 4-Amino-o-xylene (X) is condensed with d-ribose and subjected to the action of hydrogen in the presence of nickel or palladium. This is known as reductive condensation. The product is the ribityl derivative of the amine (XI). It is then coupled with phenyldiazonium chloride which attacks it in the 5-position to give an azo dye (XII). When this dye is reduced with sodium hypo-sulphite it yields the ortho-diamino-derivative (XIII). The final condensation with alloxan (VI) is effected in acetic acid solution in the presence of boric acid which considerably enhances the yield. The reported yield is 38% calculated on the ribose employed, this sugar being the most costly of the chemicals involved.



The above synthetic method has been adopted on a manufacturing scale. The main difficulty lies in the availability of ribose which is a rare sugar. Though it is widely distributed in nature as part of nucleosides and nucleotides it occurs only in small quantities, the yield from yeast, a relatively rich source, being only about 0·05—0·1%. Kuhn and also Karrer have described improved methods for obtaining it synthetically from d-glucose (see notes). Still in view of its high cost attempts have been made recently to avoid it and use more easily accessible compounds. One such method[5] employs d-ribonic acid. The essential stages of this synthesis are indicated below :

d-Arabonic acid $\xrightarrow[\text{change}]{\text{epimeric}}$ d-ribonic acid $\longrightarrow$ d-ribonolactone



Further stages are as already described

A rival to the synthetic process is the recovery of riboflavin as a by-product in the dairy industry in countries where this industry is highly developed. Besides the use of the substance in various vitamin preparations and medicinal foods, the idea of enriching flour with it is becoming more and more popular. For this purpose about 1·2 mg. are required per pound of flour. It is also used in the poultry industry to stimulate egg-production. The estimated world production for 1945 is roughly 90 tons. The interest in this vitamin and the large improvements made in its manufacture will be evident from the considerable fall in price during recent years. In 1938 the bulk price was 17·5 dollars per gram and in 1942 it was only 1·25 dollars.

Structure and vitamin properties:—A large number of synthetic flavins have been prepared and examined for vitamin activity. From the results it is found that the possibility of deviation from the riboflavin structure without affecting the vitamin activity is very small. The pentitol side chain is essential as lumiflavin which has not got it, is inert. Of the stereoisomers only l-arabityl-flavin has some activity (one third); the

others are inactive. Even the closely related ribosidoflavin exhibits no activity. As for the substituents in the nucleus, the two apparently inactive methyl groups in the 6 and 7 positions are necessary for maximum activity, though one alone in either of these positions (6 or 7) gives some activity. With no methyl substituent activity disappears and definite toxicity is found to appear. Methyl groups in the other positions of the benzene ring definitely inhibit activity.

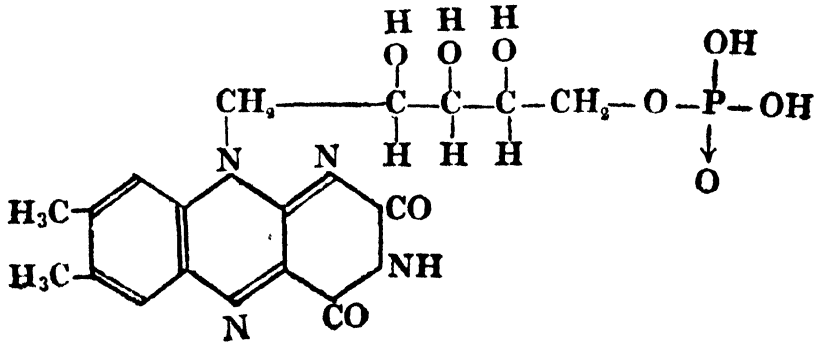
Assay:—One of the characteristic properties of riboflavin is its capacity to promote growth. This has been utilised in the biological method of Sherman and Borquin using rats. Though accurate and reliable, this procedure takes at least 28 days thereby greatly restricting the usefulness of the process besides adding to its cost. The following rapid methods have been found to be satisfactory.

The fluorescence method employs the intensity of the fluorescence given by the flavin solution in violet light. It has already been stated that aqueous solutions of the flavin give an intense yellowish green fluorescence. In the case of biological materials they are usually subjected to a preliminary treatment with papain and standard taka-diastase at 37-40° for two hours in order to liberate the vitamin from combination. Proteins are then coagulated by heating at 80° and removed either by filtration or by centrifuging, and the clear solution is subsequently employed for the measurement of the fluorescence.

The microbiological method is based upon the fact that riboflavin promotes the growth of micro-organisms. The organism employed is a lactic acid-producing bacillus, Lactobacillus helveticus. It is grown on a synthetic medium containing a known amount of the preparation under test but otherwise free from the vitamin. The rate of growth is estimated either from a measurement of the turbidity of the culture after incubation for 24 hours or from the amount of acid produced after incubation for 72 hours. Control experiments are conducted at the same time using standard preparations of the vitamin.

Unit, requirement and function:—The international unit for riboflavin has not yet been defined. For practical purposes the Sherman-Borquin unit is adopted. This represents the activity of 2·5 micrograms of riboflavin. The daily requirement of the vitamin for an adult is about 2—3 milligrams. A larger amount is required by women during pregnancy; children under ten can manage with less. The deficiency of this vitamin can be found out by a study of its excretion in urine.

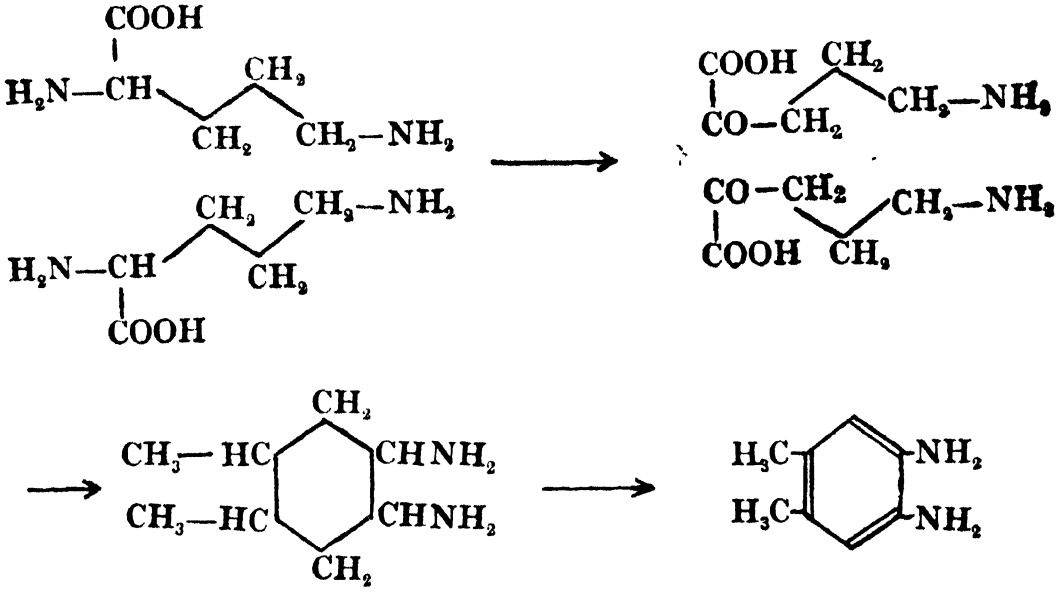
Riboflavin exists in plants and animals to a large extent in the combined form. Its phosphoric ester (XIV) plays an important part in the activity of Warburg's respiratory enzyme which is responsible for biological oxidations. When this ester is combined with the specific protein or enzyme the active enzyme itself is generated. This explains the important role of the vitamin in the living organism. Further, the isolation of this enzyme by Warburg and Christian in 1932 and their preliminary study of the non-protein part were of considerable help in the subsequent development of the chemistry of riboflavin.



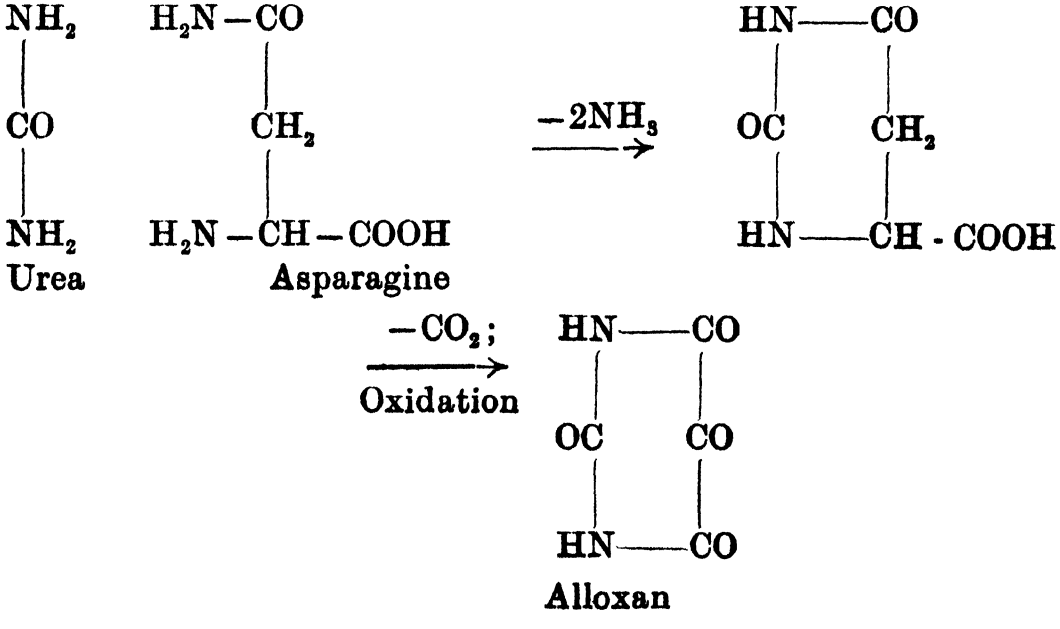
XIV

Biogenesis:—Riboflavin has a unique type of structure. In studying its construction an easily recognised part is the side chain which is obviously derived from d-ribose. This sugar is fairly widely distributed in plants and animals and its formation in nature is discussed later on under ascorbic acid. Just as in the laboratory synthesis, the isoalloxazine portion may be considered to consist of (1) the diamino-o-xylene unit and (2) the alloxan unit. Based on the general consideration that the nitrogenous vitamins are derived from

amino acids the first part could be built up from two units of ornithine as indicated below :



Alloxan is an important tetrahydroxy-pyrimidine. It is well known as an oxidation product of uric acid and could possibly arise from this source. An alternative scheme would be to consider it as derived from asparagine like other pyrimidine compounds in general. The details may be represented as below :



CHAPTER VIII

NICOTINIC ACID OR NIACIN

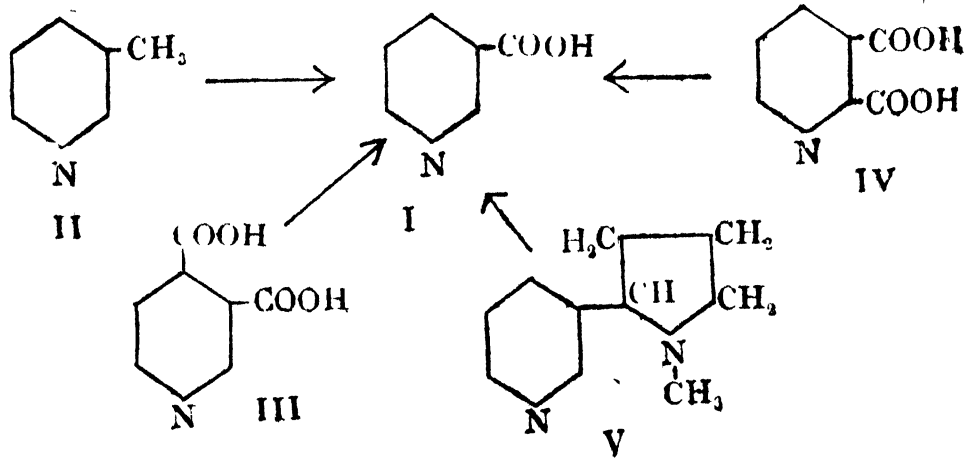
(The Pellagra-Preventive Factor).

Pellagra is a disease which is endemic in places where the population subsists mainly on maize, as in certain parts of America, Italy and the Balkans. The early symptoms are ulceration of the mouth and symmetrical reddening of the skin particularly after exposure to sunlight. The advanced stages are characterised by enteritis, dermatitis and degenerative changes in the nerves and the spinal cord. Untreated pellagrins usually develop mental symptoms and the disease is often fatal. After a great deal of change in our ideas regarding the cause and cure of pellagra, it was established in 1937 that nicotinic acid or its amide is the substance involved and hence it is known as the pellagra-preventive factor. The acid is remarkably effective in curing pellagra in human beings and also the condition known as black tongue in dogs. It is one of the simplest of compounds that possess vitamin properties and was known as early as 1870 as an oxidation product of nicotine. Its occurrence in nature was first noted by Funk and by Suzuki about the year 1912 in their study of yeast and rice-bran with reference to polyneuritis. Its importance in nutrition was however realised only much later.

Occurrence:—Nicotinic acid and its amide seem to occur widely in plant and animal tissues though in small amounts. One of the richest sources is yeast, which contains about 60 milligrams per 100 grams dry weight. Among cereals, wheat and soya-bean have the highest nicotinic acid content, oats and maize the lowest and barley, rye and rice intermediate amounts. It is present in the liver in good concentration (15 mg. per 100 grams of fresh tissue). Cooking and other forms of commercial processing cause only minor loss of this vitamin.

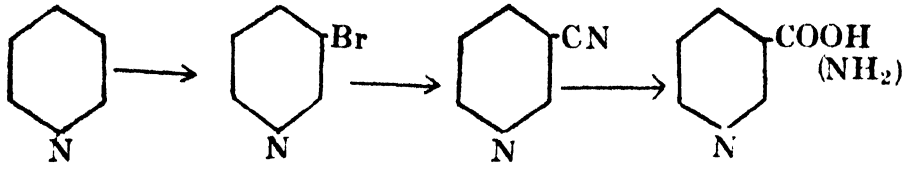
Properties and Constitution:—Nicotinic acid is a colourless crystalline solid melting at 234°. It is soluble in

water and faintly acidic to taste. The amide melting at 126° is far more soluble in water and has a slightly bitter taste. Nicotinic acid is a monobasic acid having the molecular formula $C_6H_5O_2N$ (I). Since it yields pyridine on decarboxylation it is a carboxylic acid of that base. The position of the carboxylic group is established from the formation of this acid when β-picoline (II) is oxidised with chromic acid. Further, it is produced when cinchomeronic (III) and quinolinic (IV) acids are decomposed by heating; these two dicarboxylic acids have each a carboxyl group in the 3-position and this is left behind. It should be noted that nicotinic acid is a stable carboxylic acid whereas its isomers picolinic and isonicotinic acids undergo decomposition on heating.



Commercial Preparation:—The easiest method of preparing nicotinic acid for therapeutic purposes is by the oxidation of nicotine (V). Waste tobacco leaves are used for the purpose and crude nicotine prepared from them is oxidised using either potassium permanganate, or chromic acid or nitric acid. The last reagent seems to be the most economical. It has been recently claimed that oxidation with 27% nitric acid at 98° (3 hours) gives a 70% yield of nicotinic acid[1]. Thus large quantities are manufactured and are readily available for use. A convenient method of preparation starting from pyridine[2] has also been worked out. Direct bromination of this substance gives rise to β-bromopyridine (VI). When the bromo compound is heated with cuprous cyanide at 160-70° for one hour β-cyanopyridine (VII) is formed. It can be purified by vacuum distillation and hydrolysed

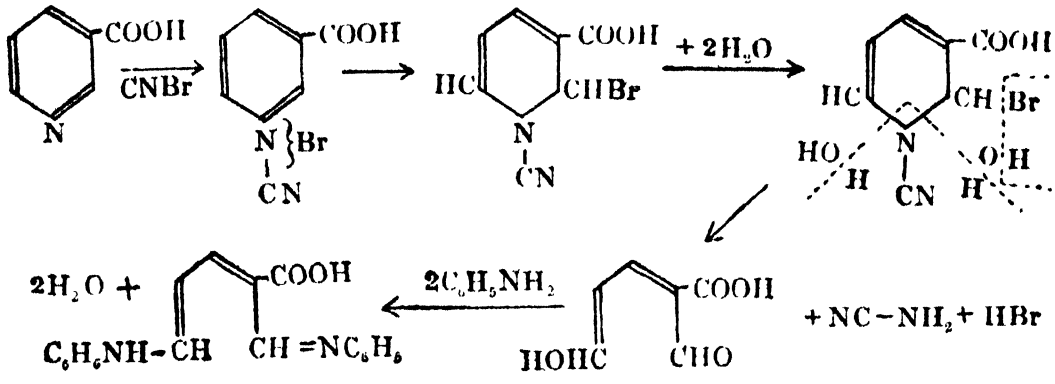
by means of alcoholic sodium hydroxide to yield nicotinic acid. Addition of hydrogen peroxide is claimed to be favourable for high yields. If the hydrolysis is effected by means of concentrated aqueous ammonia at 107-109° a very good yield of the amide (VIII) is obtained.



VI VII VIII

Nicotinic acid is available in the market in various pharmaceutical forms, sometimes in combination with other vitamins and sometimes alone. It is present in medicinal foods and is also used in the preparation of enriched flour each pound of which should contain not less than 6 mg. of nicotinic acid. The production for 1943 has been given as 200 tons. Since this acid is an old and well known compound the decrease in its price as a result of improvements in manufacture is not so marked as with some other vitamins. Still it is not negligible; in 1937 the bulk price of this substance was 35 dollars per pound and in 1942 it came down to 6·5 dollars in spite of war time rise in prices.

Assay:—The estimation of nicotinic acid in plant and animal materials is not of any value in regard to its preparation in bulk because it is not isolated conveniently from them. However, in connection with the dietary survey or clinical study of deficiency of this factor in nutrition, its assay in these materials becomes important. The biological method employs the cure of black tongue in dogs, which is caused by lack of this vitamin. It is time-consuming and expensive. A rapid chemical method utilises the golden yellow colour produced by the action of cyanogen bromide and aniline (Konig's reaction). It depends upon the break-down of the pyridine ring with the removal of nitrogen and the subsequent combination of the product with two molecules of the aromatic amine.



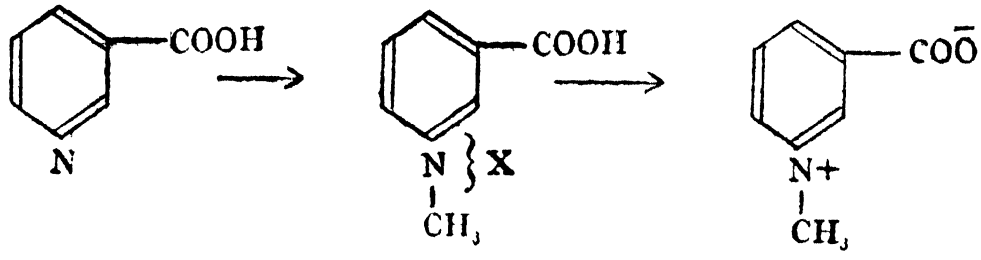
p-Amino-acetophenone gives a more intense colour than aniline. In order to obtain reproducible results the reaction is conducted in a slightly acid medium (p_H 6·0) and the solutions are protected from light as far as possible. Since in biological materials the vitamin occurs to a large extent in the combined form, a preliminary hydrolysis is necessary to get the total nicotinic acid, and adsorption on charcoal is helpful in eliminating interfering substances. Though this method is fairly good, errors arise due to the reaction being given by other pyridine compounds having no antipellagra activity. The errors become very high with grains and grain products. It is claimed, however, that under suitable conditions it can be reliable.

Bacterial methods are said to be superior to the chemical methods in sensitivity and suitability for batch analysis. They are based upon the fact that the vitamin is essential for the growth of the micro-organism, Bacillus proteus. The bacterial tubes are incubated for several hours at 37° and the turbidity is read with a photo-electric photometer. This procedure is not suitable for coloured or turbid extracts. In such cases it is convenient to use Lactobacillus arabinosus. The lactic acid produced is estimated by titration with alkali using bromothymol blue as indicator. In this estimation the incubation is usually for 72 hours.

For the assay of a mixture of nicotinic acid and the amide, the microbiological analysis is made at first for the total quantity; the amide is then converted into β-aminopyridine (inactive) by the action of bromine and potash and the remaining nicotinic acid estimated.

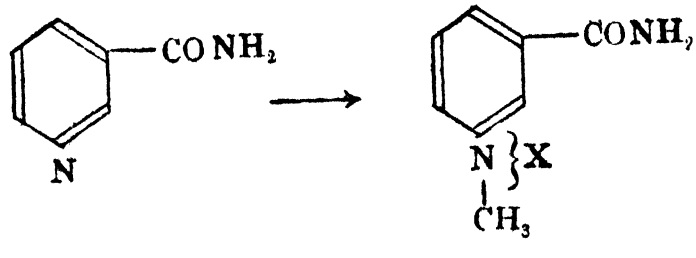
Unit, metabolism and structural specificity:—Since nicotinic acid had been known in a pure form long before its importance as a vitamin was recognised, there was no need for biological units for purposes of assay. Quantities are expressed only in milligrams. Like other members of the B group of vitamins nicotinic acid exists largely in combination as a component of coenzymes. In these the amide of the acid is present in combination with ribose, phosphoric acid and adenine.

The chief end product of nicotinic acid metabolism is considered to be trigonelline and in this form it is excreted in the urine. The change is brought about by the methylation of the tertiary N atom as shown below:



Trigonelline

Along with trigonelline there occurs in urine a fluorescent compound which has been identified as the analogue derived from nicotinic amide. This substance has been isolated as the halide or picrate and has been called "N'-methyl-nicotinamide" and "trigonellamide"; but the correct name would be "amide of N-methyl-pyridiniumhydroxide-3-carboxylic acid". The estimation of this fluorescent compound in urine is considered to be useful for the assessment of the nutritional status of individuals regarding nicotinamide.



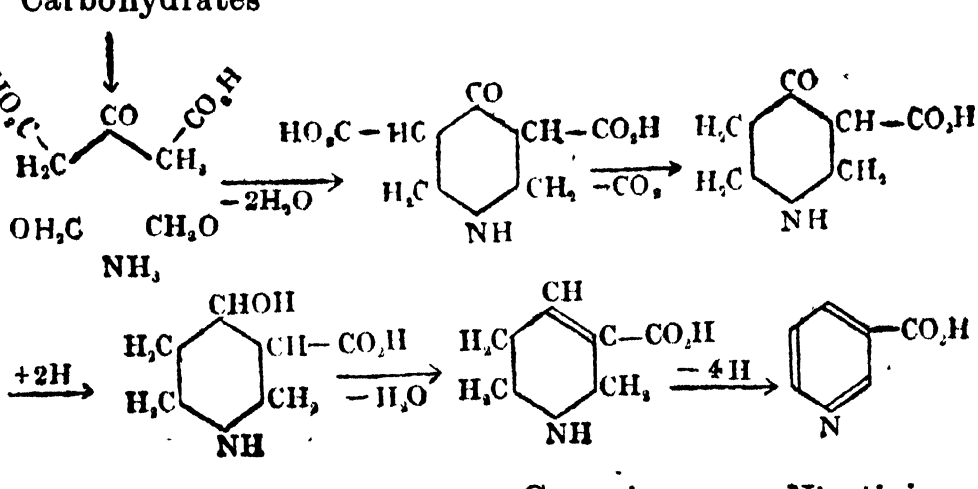
N'-Methyl-nicotinamide

Besides nicotinic acid and its amide, the simple esters and substituted amides are also physiologically

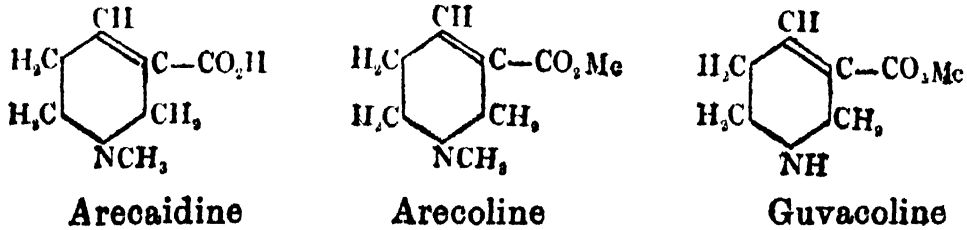
active. This is considered to be due to their capacity to yield nicotinic acid in the living organism. Even quinolinic acid has curative properties for this reason. But other variations in molecular structure lead to inactive products. It is particularly the case if the ring nitrogen is substituted, as for example trigonelline is inactive.

Alkyl-amides of nicotinic acid have attracted some attention because of their use in the treatment of shock, collapse and cardiac defects. The most useful of these is N-diethylnicotinamide also called coramine.

Biogenesis:—Several compounds related to nicotinic acid occur in nature. The areca nut alkaloids, arecaidine, arecoline, guvacine and guvacoline and the betaine-like compound trigonelline come under this category. The evolution of these molecules from acetone-dicarboxylic acid, formaldehyde and ammonia was suggested by Robinson[3] in 1917 in connection with his theory of phyto-chemical synthesis of alkaloids. Following his scheme the biogenesis of nicotinic acid may be represented as below:



Guvacine Nicotinic acid

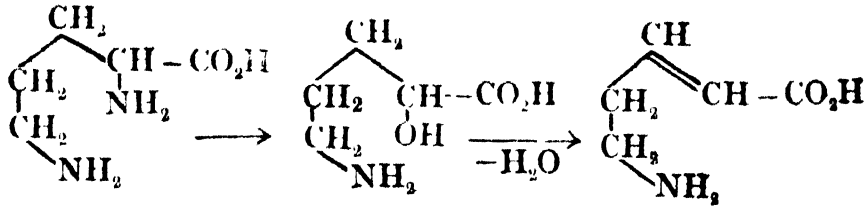


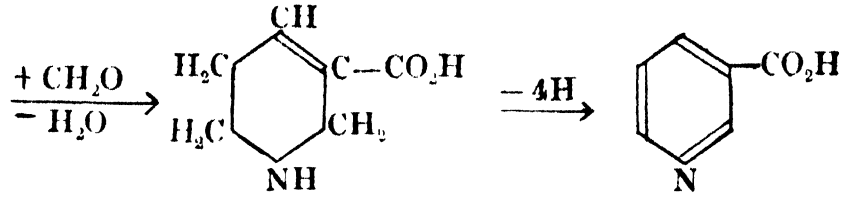
Arecaidine Arecoline Guvacoline

The possible origin of nicotinic acid from the naturally occurring amino acid ornithine has been suggested by

Guggenheim[4]. The following represents a modification of his scheme:

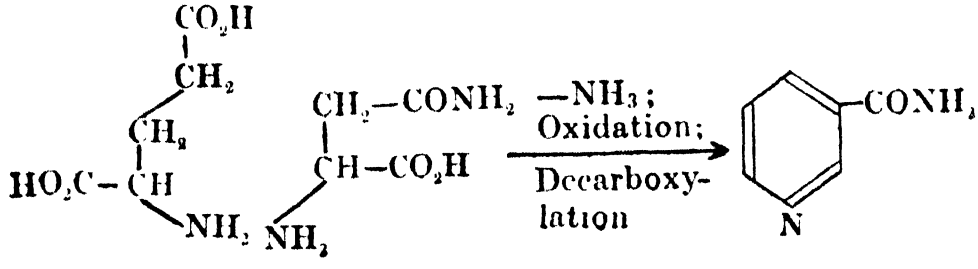
Ornithine





Guvacine Nicotinic acid

A third possible mechanism for the biogenesis of the vitamin is suggested by the observation that the amide of nicotinic acid is formed[5] by heating a solution containing asparagine and glutamic acid. By extracting the resulting mixture with ether the substance could be isolated. The following indicates the possible union of the two amino acid molecules:



Glutamic acid Asparagine

CHAPTER IX

PYRIDOXIN OR ADERMIN (VITAMIN B_6)

Pyridoxin is also known as the rat dermatitis (acrodynia) factor since its absence causes the above mentioned disease in the rat. Its relation to human nutrition is not yet quite clear. It has, however, been suggested that the physiological function of the vitamin is connected with the utilisation of unsaturated fatty acids. There seems to be also an intimate relation between this substance and the formation of hæmoglobin. It is therefore considered to be essential in human nutrition. It is always used in conjunction with thiamin and riboflavin. Like nicotinic acid it is also a pyridine derivative and the name is derived from this characteristic.

Occurrence and Isolation :—This vitamin is widely distributed in plants and animals. Whole grains and fresh meat contain about 5-10 parts per million. Molasses also form a good source. It is however most abundant in yeast (about 50 parts per million), wheat germ (50 parts) and rice polishings (15 parts). Its isolation in a crystalline form was reported in 1938 independently by P. Gyorgyi and a number of workers in America and by Kuhn in Germany, rice polishings being generally used. Later Greene[1] described a simplified procedure giving greater yields. He found that ether was particularly useful for concentrating the vitamin and impurities were best removed by means of silver nitrate. He employed the colour reaction with ferric chloride (intense reddish-brown) for following the stages in the concentration.

An aqueous rice-bran extract was employed and it was extracted with ether at p_H 7·5. This effected a fifty-fold increase in the concentration of the vitamin. It was then transferred to dilute sulphuric acid and the solution concentrated to small bulk. After adjusting the p_H again to 7·5, impurities were precipitated with silver nitrate. Almost complete adsorption then took place at p_H 2 on the addition of Fuller's earth. The vitamin was eluted

from the adsorbate with baryta, and by the judicious use of sulphuric acid and barium chloride, inorganic salts were removed almost completely and a solution of the hydrochloride of the vitamin was obtained. After concentrating the solution the residue was purified using alcohol, acetone and ether. Finally pyridoxin hydrochloride was crystallised from absolute alcohol. Yields of 10-15 per cent of the vitamin could thus be obtained.

Properties and constitution[2] :– Pyridoxin melts at 159° and has the molecular formula $C_8H_{11}O_3N$. Its hydrochloride melts at 209° with decomposition and is readily soluble in water. In dilute hydrochloric acid solution it gives a strong absorption band with maximum at 291 mμ. The vitamin is stable to acids and alkalies and is optically inactive. It contains no methoxyl or N-methyl groups, but gives evidence for the presence of one C-methyl residue (Kuhn and Roth's method). With aqueous ferric chloride it gives a deep reddish-brown colour similar to that produced by β-hydroxy-pyridine.

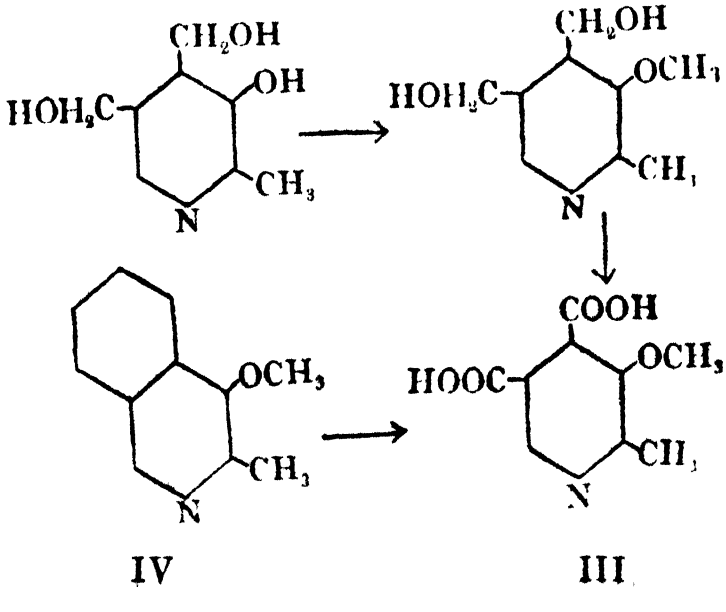
Since pyridoxin does not react with nitrous acid, the basic nitrogen should be tertiary in character. In view of the absence of an N-methyl group, the molecular formula, stability and the reactions of the molecule, this nitrogen atom should be involved in an aromatic ring like pyridine.

Estimation of active hydrogen atoms by means of magnesium methyl-iodide (Zerewitinov's method) indicates the presence of three. The vitamin forms a triacetate which can be distilled in vacuo and which is biologically active. That one of the active hydrogen atoms is present in a phenolic hydroxyl is proved not only by the ferric chloride colour, but also by methylation using diazomethane. Selective methylation takes place leading to the formation of a monomethyl ether (II). The other two active hydrogen atoms should be present in two primary alcoholic hydroxyls because they can be oxidised to carboxyl groups. Thus by the oxidation of the monomethyl ether (II) using barium permanganate, is obtained a dicarboxylic acid (III) whose properties reveal the final constitution of pyridoxin. At this stage it will be clear that a pyridine ring exists in the

molecule, since 3 out of the 8 C atoms of the vitamin have been accounted for in the alcoholic groups and in the C-methyl group and 5 more remain to form the 6-atom ring along with the nitrogen.

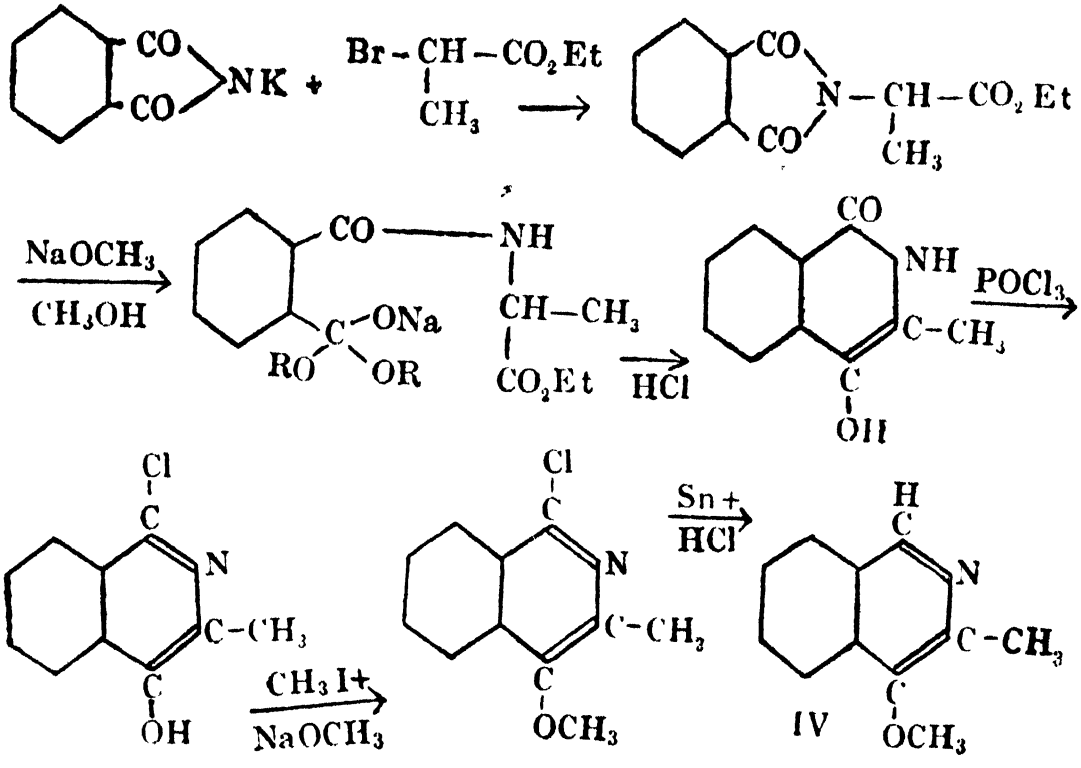
The dicarboxylic acid does not give any colour with aqueous ferrous sulphate which is taken as indication that no carboxyl group is in the α-position of the pyridine ring. On fusion with resorcinol it gives a fluorescent dye showing that the two carboxyl groups are vicinal. They should therefore be in the β : γ positions and it follows that in the vitamin the primary alcoholic groups are in these positions. From the ferric chloride colour it could be inferred that the phenolic hydroxyl is in the second β-position. Finally the location of the CH_3 has now to be fixed in one of the two α-positions. Since the vitamin gives the specific test with dichloroquinone-chlorimide for a free nuclear position para to the phenolic hydroxyl, the methyl group should be in the α-position which is ortho to the phenolic hydroxyl group. The constitution of pyridoxin is therefore 2-methyl-3-hydroxy-4 : 5-dihydroxymethyl-pyridine (I). It explains correctly all its properties and reactions. The action of diazomethane gives rise to the 3-methyl ether (II) and subsequent oxidation yields the dicarboxylic acid, 2-methyl-3-methoxypyridine-4 : 5-dicarboxylic acid (III). These formulations have been confirmed by the preparation of the dicarboxylic acid (III) from 3-methyl-4 methoxy-isoquinoline (IV).

I II

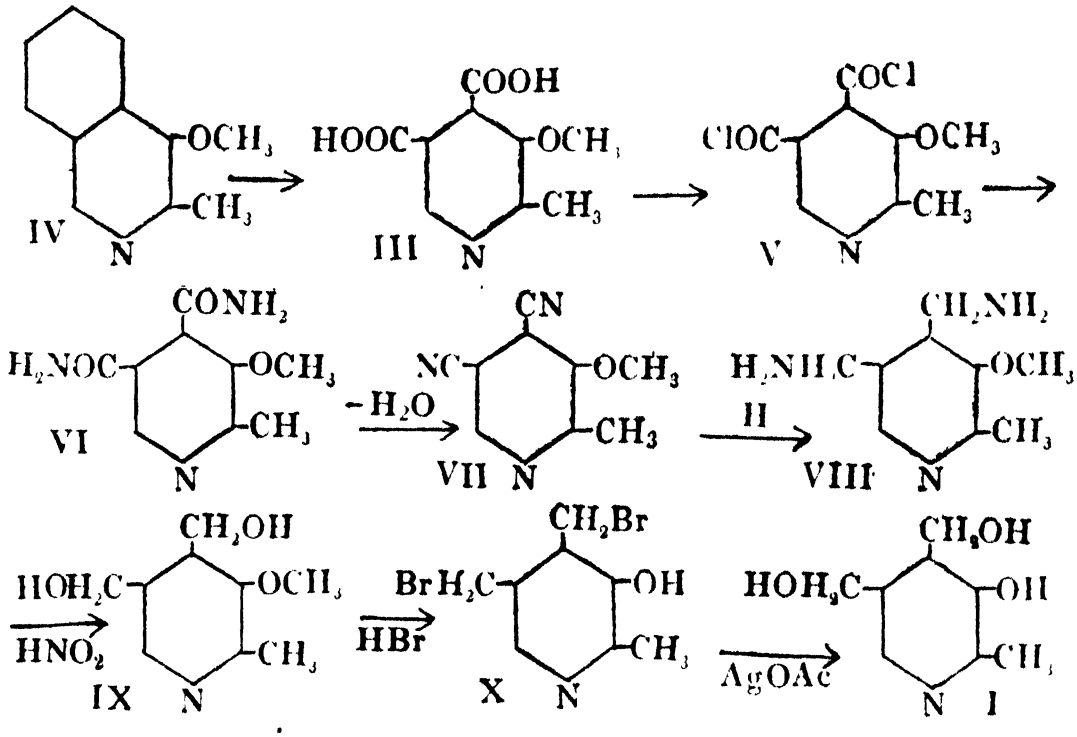


IV III

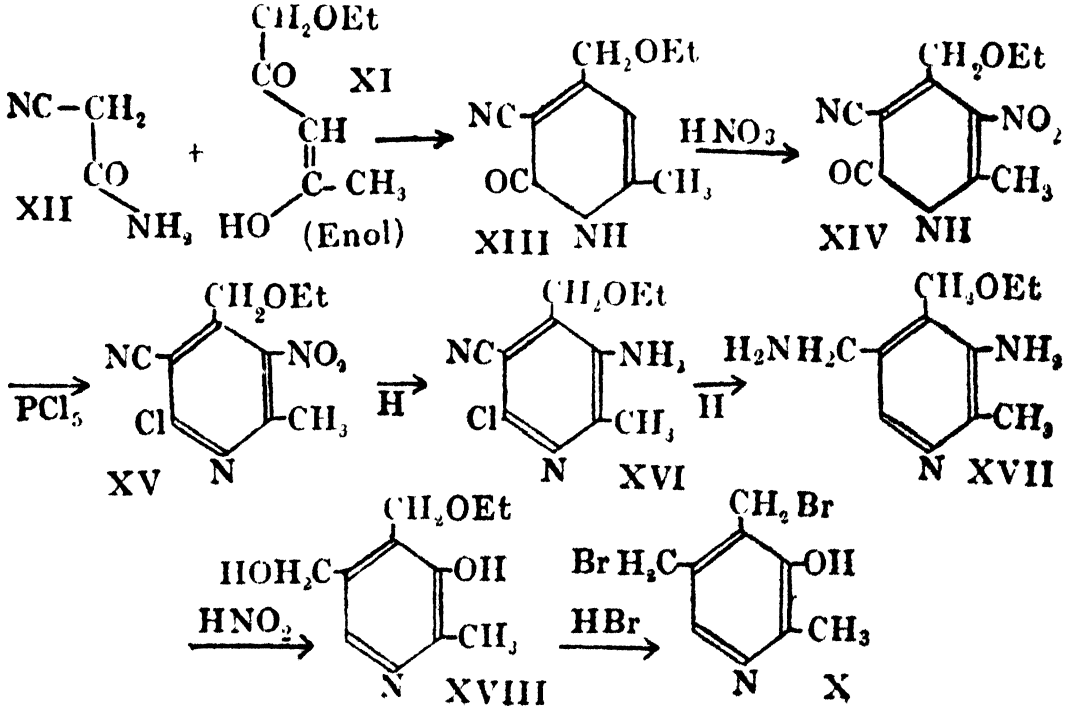
The synthesis of the above mentioned isoquinoline is based on a method discovered by Gabriel and Colman for building up the isoquinoline skeleton. It starts with phthalimide and bromopropionic ester; the various stages are indicated below:



Synthesis[3]:—Amongst the various methods adopted for the synthesis of pyridoxin, the one described by Kuhn (1939) is as follows. The starting material is the isoquinoline (IV) mentioned above. It is nitrated whereby a nitro group enters the benzene ring and it is subsequently reduced. Oxidation of the amino compound with potassium permanganate gives rise to the dicarboxylic acid (III). The diamide (VI) is obtained through the acid chloride (V) by the action of ammonia. On dehydration the dinitrile (VII) is obtained which when submitted to catalytic reduction forms a diamino compound (VIII). Action of nitrous acid on the amino body yields the monomethyl ether (IX) of pyridoxin. Demethylation with hydrobromic acid changes also the carbinol groups into bromides (X). Reconversion into carbinol groups is finally brought about by the action of silver acetate.

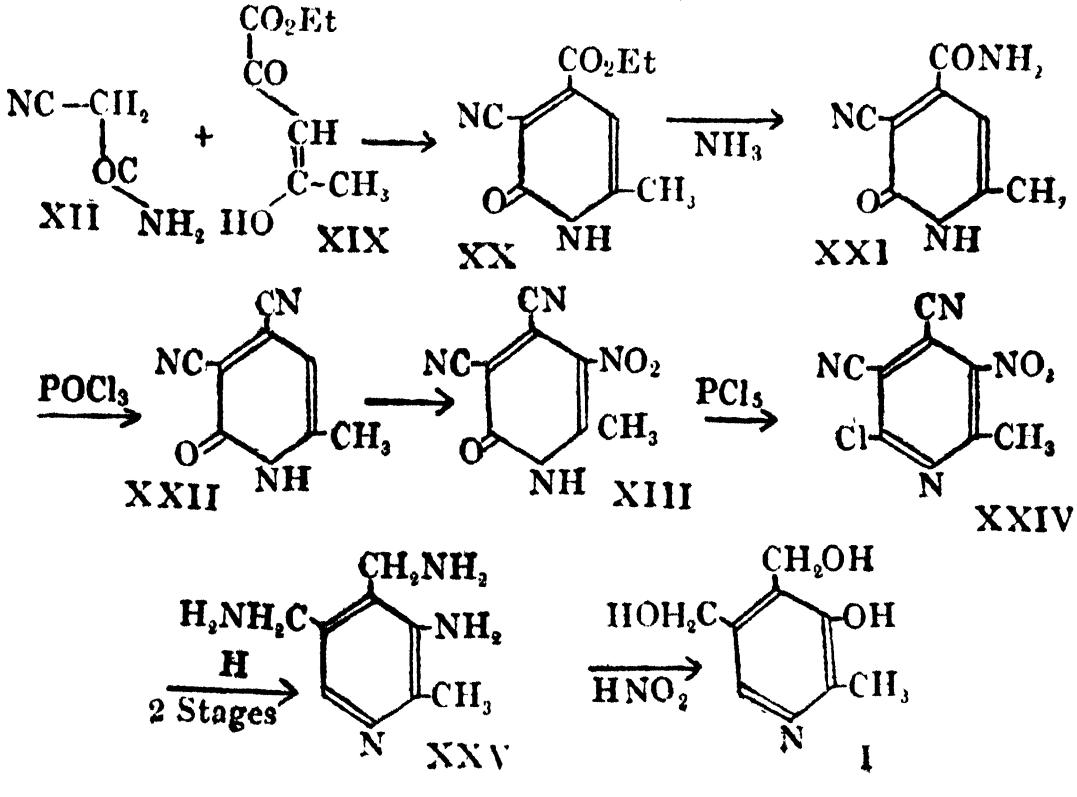


Another method which is capable of adoption on a large scale is that of Harris and Folkers (1939). By the condensation of ethoxy-acetylacetone (XI) with cyano-acetamide (XII) they obtained 2-methyl-4-ethoxymethyl-5-cyano-6-pyridone (XIII). Nitration yielded the 3-nitro derivative (XIV) which when treated with phosphorus pentachloride in chlorobenzene solution formed 2-methyl-3-nitro-4-ethoxymethyl-5-cyano-6-chloro- pyri-



dine (XV). By regulated catalytic reduction the nitro group was first reduced to amino (XVI) and subsequently the chlorine atom was removed and the CN reduced to CH_2NH_2. The resulting amino compound (XVII) was converted by nitrous acid into the corresponding hydroxy compound (XVIII) which when treated with 48% hydrobromic acid gave the dibromo compound (X) obtained also by Kuhn's method. A modification of this method in which the last stages have been simplified has been patented in 1942.

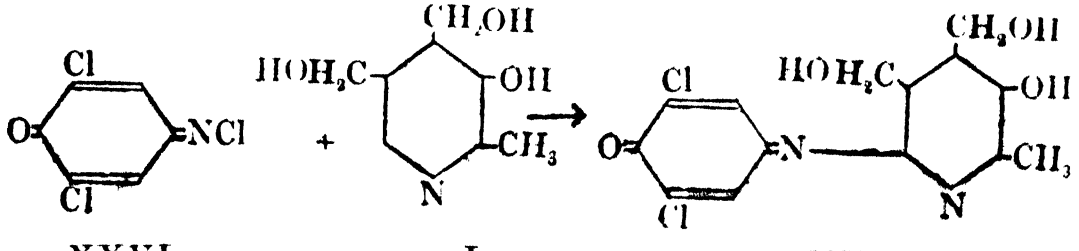
A new route to pyridoxin developed by Mowat, Pilgrim and Carson starts with 4-carbethoxy-5-cyano-2-methyl-6-pyridone (XX) which is obtained by the condensation of ethyl acetyl-pyruvate (XIX) with cyanoacetamide (XII). The carbethoxy compound (XX) is converted into the nitrile (XXII) via the amide (XXI). Subsequent nitration yields the compound (XXIII) which is converted by phosphorus pentachloride into the 2-chloro-pyridine (XXIV). Stepwise catalytic reduction of this substance results in the formation of the triamine (XXV) which is converted into the vitamin (I) by the action of nitrous acid.



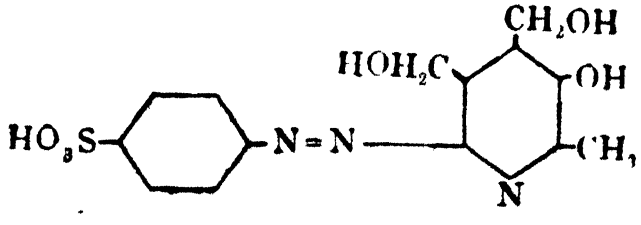
The ethyl homologue has also been prepared. It is found to have less than 2% of the activity of pyridoxin. Related compounds without free hydroxyl groups have feeble or no activity. But the acetates are fully active probably due to ready hydrolysis in the organism.

Ever since its synthesis was first reported in 1939 there has been considerable interest in this vitamin and it is now produced on a commercial scale. There has also been appreciable reduction in price on account of improvements in technique. It was sold at 12 dollars per gram in 1939 while in 1941 the price was less than 3 dollars per gram.

Assay :—The biological method employs the capacity of the vitamin to cure dermatitis in young rats. Among the more rapid chemical methods the most satisfactory procedure utilises a modification of the phenol test. 2 : 6-Dichloroquinone-chlorimide (XXVI) reacts with phenols forming blue coloured compounds and the position attacked by the reagent is para to that of the phenolic hydroxyl. In the case of pyridoxin the reaction produces the compound represented by (XXVII). It is carried out at p_H 7·6 and a butanol solution of the reagent is added to an aqueous solution of the vitamin. The alcohol layer is separated and the blue colour read after 40 minutes. The method is considered to be very sensitive. Diazotised sulphanilic acid has also been used for the estimation. It couples with the vitamin to form a dye which may be represented as in (XXVIII.)



XXVI I XXVII

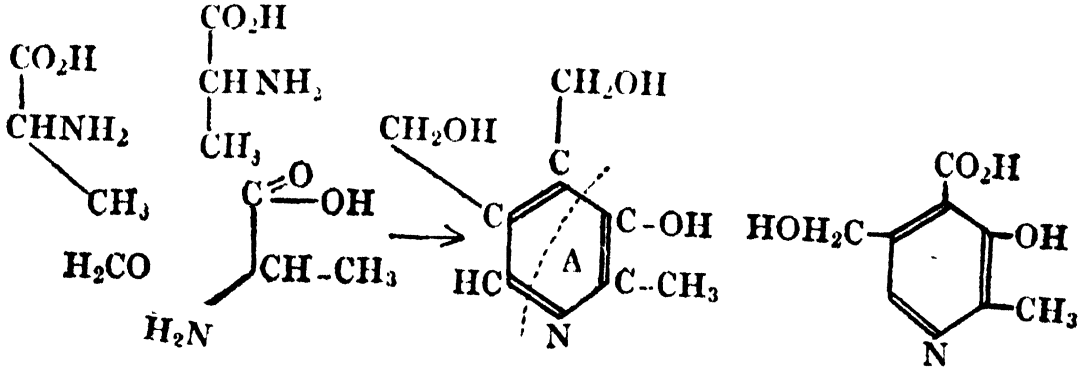


XXVIII

In the examination of biological materials the vitamin has to be liberated from combination with protein or starch, and interfering substances have to be removed. Pepsin digestion followed by tungstic acid precipitation is employed for the removal of proteins. Silver nitrate precipitates purines, pyrimidines and related compounds. Final purification employing adsorption with alkaline earths is also employed.

The international unit is not defined. One rat unit is equal to 10γ of pyridoxin and is defined as that amount necessary per day to cure or prevent typical symptoms of avitaminosis in a rat. The daily adult requirement is considered to be 2 mg.

Biogenesis :—Though pyridoxin is only a small molecule and is a pyridine derivative, the origin of its structure is not clear. It has however been shown that alanine is capable of replacing pyridoxin effectively for the growth of micro-organisms[4]. This may indicate the possible biosynthesis of the vitamin in plants from this amino acid. The existence of this unit in the molecule will be clear from the portion marked (A) in formula (XXIX). The origin of the remaining parts and particularly the two orthocarbinol groups is not so obvious. It is now suggested that they may arise from two amino acid units somewhat in the manner described in the case of thiamin. Alanine units can again be employed for this purpose as indicated below.



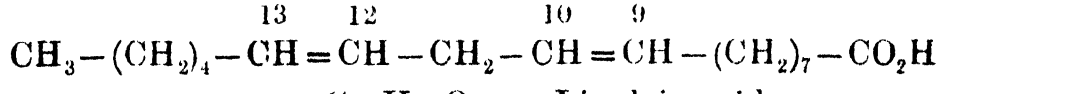
XXIX XXX

4-Pyridoxic acid[5] is the name given to a product of oxidative metabolism of pyridoxin present in urine. It is 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine (XXX) resulting from the oxidation of the carbinol

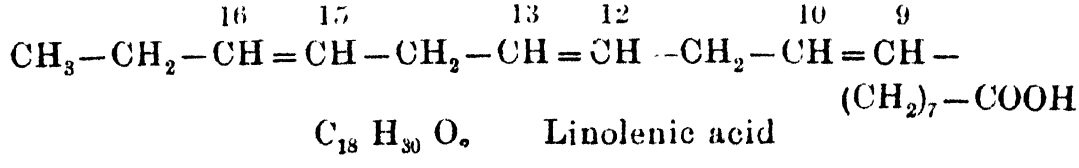
group of the vitamin in the 4-position. It is a fluorescent compound melting at 247°. Its lactone is readily formed in the presence of hydrochloric acid. This acid can be obtained by the oxidation of pyridoxin with potassium permanganate.

Some other substances having high biological activity on micro-organisms seem to accompany pyridoxin in natural materials. They have been referred to as "Pseudo-pyridoxin". They are considered to be closely related to pyridoxin and to be formed from it by processes involving amination and partial oxidation, possibly leading to the formation of an amino-methyl or an aldehyde group in the 4 position.

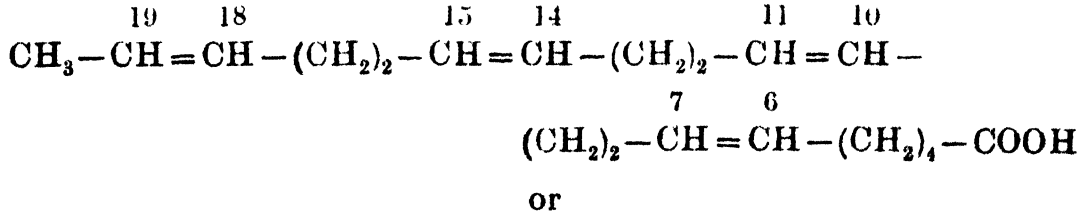
Vitamin F:—Certain symptoms of acrodynia in rats usually associated with adermin deficiency could be cured by the administration of what are known as nutritionally essential fatty acids (vitamin F). These are unsaturated acids like linoleic, linolenic and arachidonic acids. In cases of eczema in children there is found a decrease of unsaturated fatty acids in the serum. Administration of vitamin F-containing fractions cures the eczema and raises the unsaturated fatty acid level of the serum.



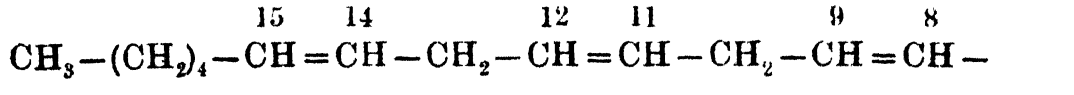
$C_{18}H_{32}O_2$ Linoleic acid



$C_{18}H_{30}O_2$ Linolenic acid



or



$C_{20}H_{32}O_2$ Arachidonic acid

Pyridoxin and these fatty acids seem to be interrelated in their functions and it has been claimed that either factor can independently cure acrodynia. It is possible, however, that the symptoms of the two deficiencies are similar and not identical. The unsaturated acids also differ amongst themselves appreciably in their effects on growth and on the skin lesions. A more recent observation which promises to throw light on the mode of action of vitamin F is that in its deficiency there is an impaired absorption of ordinary fats.

CHAPTER X

PANTOTHENIC ACID

This was first recognised as early as 1933 by R. J. Williams and his co-workers as a factor capable of promoting the growth of yeast and bacteria. A study of the physical and chemical characteristics of extracts from different sources such as liver and rice-bran which could stimulate yeast growth revealed that the activity is due to a single water-soluble, acid substance. The name pantothenic acid was chosen to indicate its universal occurrence. Several years later (1938) it was found that liver extract contains a factor which is necessary for the prevention of typical dermatitis in chicken and which is different from the other known members of the vitamin B group. Consequently it was named the chick dermatitis factor. A critical examination of its properties by several workers revealed that it was identical with pantothenic acid. It has also been shown to be necessary for rat growth. Thus pantothenic acid is a vitamin of importance in animal nutrition.

Occurrence :—Though pantothenic acid is widely distributed, it occurs only in very small quantities. However, it is in general more abundant than riboflavin. Yeast, egg-yolk, dried whey, dried butter-milk, sweet potato and bran of grains (rice bran) are good sources. By far the richest is liver in which it is present to the extent of forty parts per million. Liver extracts have therefore been used as the starting point for the isolation of this vitamin. It is interesting to note that royal jelly of the honey bee contains six times as much pantothenic acid as is present in liver.

Isolation :— There were several difficulties in the isolation of pantothenic acid in a pure condition. Since it occurs only in minute amounts, considerable quantities of liver tissue had to be employed. In an experiment 250 kilograms of liver, after an extended process of extraction, yielded only about 3 grams of the crude

vitamin which was approximately 40 per cent pure. From this the remaining impurities could be removed only with great difficulty. Pantothenic acid and its derivatives are highly hydrophilic and show very little tendency to crystallise even after purification. Further it is unstable and can be handled only in the form of a neutral salt. The earlier work was again handicapped by the lack of quick chemical and physical methods of assay, only a biological method being available.

The first successful preparation of calcium pantothenate in an amorphous condition was made by R. J. Williams et al.[1] (1938) starting from sheep's liver. The process was exceedingly laborious. The essential feature of their work was the separation of the vitamin from a variety of sugars and other neutral and basic water-soluble substances by means of the brucine salt. This was extracted with chloroform which left behind sugars and similar compounds. A preliminary adsorption on charcoal was found to give better results.

The aqueous extract of autolysed liver was first treated with Fuller's earth and filtered in order to remove basic and other impurities. Sulphuric acid was then added to the filtrate to bring the p_H to 3·6, and pantothenic acid adsorbed on norite in the cold. The adsorbate was quickly eluted using ammonia and the p_H of the solution brought to 6·5 by means of oxalic acid. Addition of excess of brucine yielded the brucine salt which was isolated by evaporating the solution and extracting the residue with chloroform. It was purified by partition between water and chloroform repeatedly and thus a thirty-fold concentration of the vitamin was effected. The next stage was conversion into the calcium salt by the use of lime water. Purification of calcium pantothenate consisted in (1) precipitating it from concentrated aqueous solution with absolute alcohol, (2) treatment with mercuric chloride in aqueous or alcoholic solution to remove basic impurities, (3) fractional precipitation from an aqueous alcoholic solution with isopropyl ether, and (4) a final fractional precipitation from pyridine using acetone. The product was a colourless powder and was found to have a potency of 11,100 as

compared with a standard rice bran extract. Further fractionation resulted in no increase in potency and hence it was taken to be substantially pure.

A later improved method[2] employs a concentrate of the alcohol-soluble fraction of liver extracts. It is brought into aqueous solution and subjected to a preliminary adsorption with norite at p_H 9·5 whereby impurities are effectively removed. Instead of using brucine, the purification of pantothenic acid is effected by repeated extraction with absolute ethyl alcohol and precipitation of impurities using acetone or ether. Finally the barium salt is prepared from aqueous solution using baryta and purified.

The preparation of pure samples of pantothenic acid or of its salts could not be made till after its synthesis.

Properties and Constitution[3]:—The substance is distinctly acid and exhibits no amphoteric behaviour. It is highly soluble in water and obviously contains a number of hydrophilic groups in the molecule. It is inactivated by alkali. The products of esterification and acetylation are also inactive, but hydrolysis regenerates the original compound with its activity. It stimulates the growth of yeasts and other micro-organisms and is essential to them. It is fairly stable and cooking of foods does not entail any extensive destruction of this principle.

The constitution of pantothenic acid was established mainly by the work of Williams and his co-workers in America during the years 1939 and 1940. The difficulties were really great. The quantity of the substance available for investigation was very small and neither the vitamin nor its derivatives had been obtained pure enough for correct analysis and determination of the formula. Experiments had to be done with impure specimens only. Even in 1939 the calcium salt analysis gave a formula which is now known to be wrong. Pantothenic acid is therefore one of those special cases where the molecular structure was elucidated mainly from a study of the products of decomposition even before the substance itself could be isolated in a pure state.

Pantothenic acid was found to be a mono-carboxylic acid forming salts and esters. It was aliphatic in nature

as evidenced by its ultra-violet absorption spectrum and it contained no double bonds. The molecular weight was found to be between 150 and 200. There was one nitrogen atom in the molecule but it was non-basic in its function. The compound was not an amide since ammonia was not liberated by hydrolysis, nor did nitrous acid react with it. In all probability it seemed to be a peptide. The observation that minute amounts of β-alanine (I) are effective in stimulating yeast growth led Williams to suggest a relationship between this amino-acid and the vitamin. This idea was confirmed by the isolation of β-alanine hydrochloride as one of the products of hydrolysis using hydrochloric acid. Thus it was clear that the vitamin is a peptide (II) with β-alanine as one part and another acid as the second part.

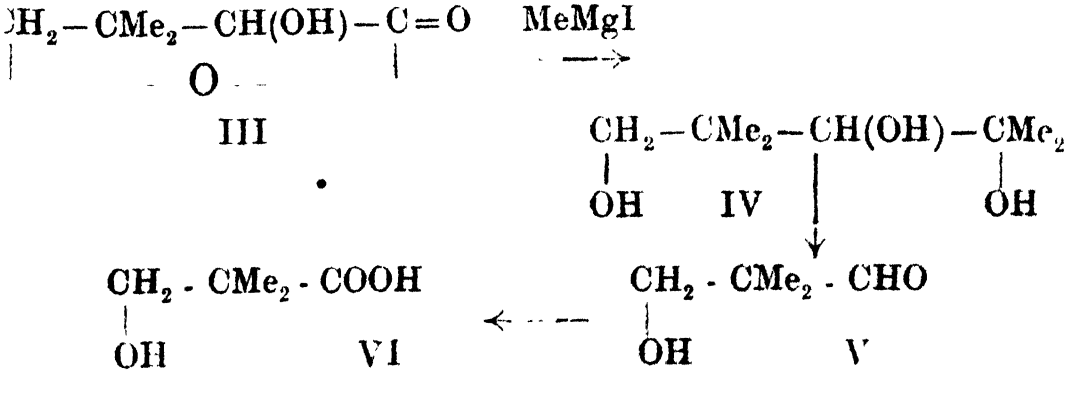
$NH_2.CH_2.CH_2.COOH$ (I) $\qquad$ $R.CO.NH.CH_2.CH_2.COOH$ (II)

Regarding the constitution of the second half certain inferences could be drawn from the properties of the vitamin itself. The latter was known to contain no methoxyl groups; its non-volatility and hydrophilic nature suggested that the acid half should consist of a polyhydroxy acid, and this was supported by the action of acetone which combined with it in the same manner as with sugars forming acetone compounds.

Earlier work carried out with very small quantities indicated that the non-β-alanine fraction consisted of a dihydroxy- acid capable of forming a lactone readily. Hence a hydroxyl group should be located in the γ or δ position. The other hydroxyl should be in the α-position, for after pantothenic acid had been hydrolysed with alkali the solution gave a ferric chloride test (clear yellow) characteristic of α-hydroxy acids. In confirmation of this a micro-method of determining α-hydroxy acids was applied to the product of alkaline hydrolysis; the carbon monoxide evolved in the reaction, $R\,CH(OH)\,CO_2H \xrightarrow{H_2SO_4} R\,CHO + CO + H_2O$, was measured and the volume found to agree very well with expectations. Quantitative determinations of β-hydroxyl group based on the formation of an unsaturated compound indicated its absence.

At this stage due to the co-operation of Williams and his co-workers with the Merck laboratories (U.S.A.) it was possible to devise a rapid method of extraction of pantothenic acid and to obtain sufficient quantities of the second half in a crystalline condition for detailed investigation. It melted at 91° and was optically active, $[\alpha]_D^{26°}$, - 49·8°. Analysis and cryoscopic molecular weight determination indicated the formula $C_6H_{10}O_3$. Titration showed the absence of a free carboxyl group, but on heating with alkali one equivalent was consumed. The stability of the lactone and the rate of lactonisation showed that it was a γ- and not a δ-lactone. The presence of a free hydroxyl group in the lactone was confirmed by the preparation of a mono-acetate and a 3:5-dinitrobenzoate. Determination of C-methyl by Kuhn and Roth's method gave indications for the presence of a gem-dimethyl group.

The above facts suggested that the constitution of the compound is 1-α-hydroxy-β:β-dimethyl-γ-butyrolactone (III). It was confirmed as follows. When treated with magnesium methyl iodide (Grignard reagent) the compound gave rise to a trihydric alcohol (IV). Oxidation of the new alcohol with lead tetra-acetate resulted in the loss of three carbon atoms and the formation of a hydroxy-aldehyde (V). Alkaline silver oxide converted the aldehyde into a hydroxy acid (VI) which was found to be identical with hydroxy-pivalic acid (β-hydroxy-α:α-dimethyl-propionic acid). These reactions and the nature of the products obtained at the several stages are given below. They agree very satisfactorily with the constitution (III) proposed above for the lactone.



Consequently the constitution of pantothenic ac could be represented as in (VII) (i.e.) d-α : γ-dihydrox; β : β-dimethyl-butyryl-alanine.

$$CH_2-CMe_2-CH(OH)-CO-NH-CH_2-CH_2-COOH$$
$$\quad\quad\quad

$$HCMe_2 \cdot CHO + HCHO, Aq. \xrightarrow{K_2CO_3} CH_2(OH) \cdot CMe_2 \cdot CHO$$
VIII IX

$$\xrightarrow[KCN]{NaHSO_3,} CH_2(OH) \cdot CMe_2 \cdot CH\langle^{CN}_{OH} \quad (X) \xrightarrow[\text{boil}]{\text{Conc-HCl,}}$$

$$CH_2 \cdot CMe_2 \cdot CH(OH) \cdot C{=}O + NH_2 \cdot CH_2 \cdot CH_2 \cdot COOEt$$
(III: the lactone ring O bridges the CH(OH) and C=O) I

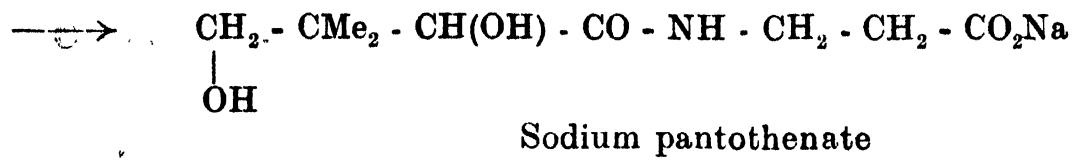
$$\xrightarrow{\text{2 stages}} CH_2 \cdot CMe_2 \cdot CH(OH) \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot COOH$$
(OH on the CH(OH)) VII

An interesting point to note in connection with the reactions outlined above is that the l-lactone gives rise to d-pantothenic acid and vice-versa.

The preparation of optically active forms of pantothenic acid has been reported by other workers also. Kuhn and Wieland[5] obtained racemic pantothenic acid by heating a mixture of the benzyl ester of β-alanine and the racemic lactone at 100°. It was resolved by using the quinine salt into the d- and l-acids. The d-acid had a specific rotation of + 27° and was physiologically active; the l-form showed no activity. Reichstein and Grussner[6] again reported the preparation of methyl dl-pantothenate from the racemic lactone and methyl d-pantothenate from the l-lactone. The latter was biologically active in doses of 10γ.

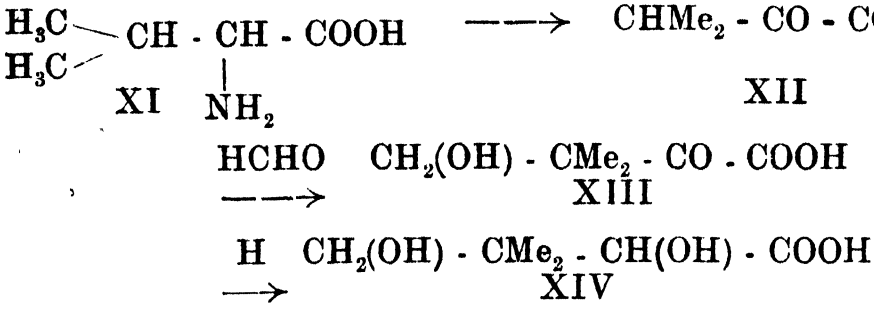
The crystalline sodium salt[7] of pantothenic acid can be easily obtained by a number of alternative synthetic methods. The most convenient of these involves the heating of the dry lactone with the sodium salt of β-alanine and very good yields are obtained. The condensation takes place as given below. The product crystallises from isopropanol as clustered aggregates of parallel fibres and melts at 122°. It is the most suitable salt and is used as the standard for the vitamin.

$$CH_2{-}CMe_2{-}CH(OH){-}C{=}O + NH{-}CH_2{-}CH_2{-}CO_2Na$$
(III: the lactone ring O bridges the CH(OH) and C=O) (H on the NH)

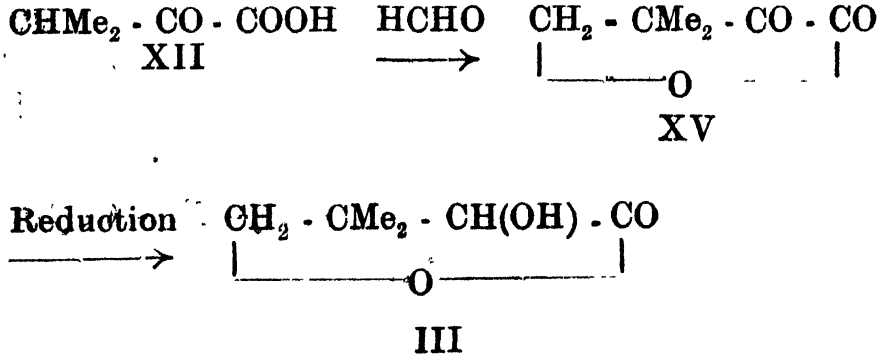


Sodium pantothenate

Biogenesis :—Kuhn and Wieland [8] have suggested a scheme for the biogenesis of pantothenic acid. Starting from valine (XI) oxidative deamination would lead to the formation of α-ketoisovaleric acid (XII). The next stage involves the action of formaldehyde which introduces a carbinol group forming the hydroxy-ketonic acid (XIII). Subsequent reduction should yield the required dihydroxy acid (XIV) which can produce pantothenic acid by condensation with β-alanine.



In support of this theory Kuhn and Wieland have experimentally shown that the keto-acid (XII) condenses with formaldehyde in the presence of potassium carbonate to form α-keto-β:β-dimethyl-γ-butyrolactone (XV) which is the lactone of the acid (XIII). When it is reduced catalytically the corresponding hydroxylactone (III) is obtained in the racemic form. If, on the other hand, biochemical reducing agencies are employed the l-form of (III) is produced. β-Alanine (I) required for the final stage of the biogenesis may be produced by the decarboxylation of aspartic acid (XVI) as shown below:



$$\underset{\text{XVI}}{COOH \cdot CH(NH_2) \cdot CH_2 \cdot COOH} \xrightarrow{-CO_2} \underset{\text{I}}{CH_2(NH_2) \cdot CH_2 \cdot COOH}$$

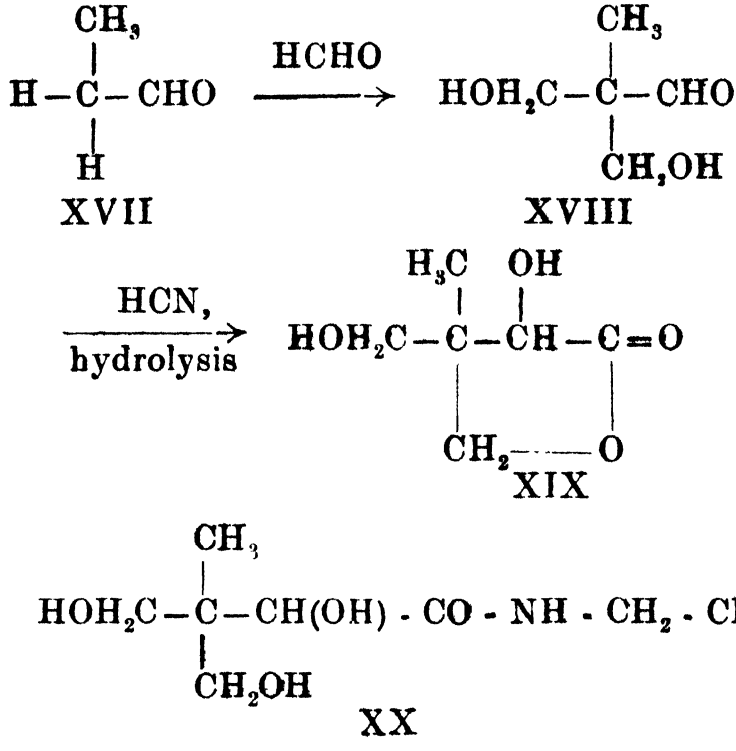
Assay:—The biological assay utilises chickens and is based on the capacity of the vitamin to prevent or cure dermatitis in them. Rats are also used for this assay. The response of micro-organisms to pantothenic acid is highly sensitive and specific. Very small quantities such as 0·0002γ of calcium pantothenate per c.c. can be easily detected. It has been found most convenient to use the lactic acid bacterium, Lactobacillus helveticus. The production of lactic acid during 24 hours is estimated by direct titration. The method is very similar to the estimation of riboflavin ; both these vitamins are essential for the organism.

The international unit of pantothenic acid has not been defined ; the sodium salt has been recommended as the standard. One chick unit is defined as one-tenth of the amount which will just provide for maximal growth, when fed daily to a chick three weeks old, in conjunction with a diet free from the vitamin. This unit corresponds to 14γ of pantothenic acid.

Structural specificity and uses:—Naturally-occurring pantothenic acid is dextro-rotatory. The lævo-form is inactive biologically and the dl-acid is half as active as the dextro-. It has been noted that the esters of pantothenic acid are active in rats and chicks but are practically inactive with regard to micro-organisms. This difference may be due to the conditions prevailing in animals being favourable for hydrolysis. The precise function of pantothenic acid is not yet known. But it is believed that it is a constituent of an enzyme system like most of the members of the B group of vitamins.

A number of closely related analogues of pantothenic acid have been prepared using amino-acids like aspartic acid, alanine, lysine and β-aminobutyric acid in the place of β-alanine. None of these is active and hence the high structural specificity of pantothenic acid is established. Variation in the non-nitrogenous part has also been explored. Amongst the products the only analogous compound showing activity is hydroxy-

pantothenic acid[9] (XX). Its synthesis is effected from propaldehyde (XVII) by the same series of reactions as employed in the synthesis of pantothenic acid itself. The only difference in the course of the reactions arises when formaldehyde acts on (XVII); two carbinol groups are introduced on the α-carbon atom of the aldehyde and this ultimately leads to the formation of hydroxy-pantothenic acid (XX).



XVII XVIII XIX XX

The use of pantothenic acid is said to be quite popular in America and it is added to a large proportion of the milk consumed. The calcium salt is also sold in various pharmaceutical forms. With increasing use of this substance considerable reduction in price has resulted, though it was first made available on a commercial scale only in 1940. In that year its bulk price was 2·5 dollars per gram whereas in 1942 it was just one-fifth of the above figure.

CHAPTER XI

BIOTIN (VITAMIN H, BIOS II B)

Biotin is a growth factor needed by yeast and other micro-organisms. The substance which stimulates the growth of the micro-organisms in the nodules of the roots of certain leguminous plants and called 'coenzyme R' has been found to be identical with it. Similar identity has been established with vitamin H, a factor needed by rats and chicks for protection against nutritional injury which arises when the diet contains much raw egg-white. Under similar conditions human beings also seem to require it. The action of this food in producing deficiency has been traced to a component in the crude protein called avidin, which unites with vitamin H (biotin) and thus renders it unavailable to the organism. Avidin has been recently obtained in a crystalline form and has been shown to be a protein-carbohydrate complex.

Occurrence:—Biotin seems to be universally present in minute concentrations. Animal sources are markedly better than vegetable materials. Yeast, egg-yolk, liver, kidney, pancreas and milk are among the richest sources. Considerable amounts are present in casein, rice- and wheat-brans and in cattle manure (particularly urine). The vitamin is invariably present in a form which is insoluble both in water and in fats and is rendered water-soluble by a process of autolysis in the case of yeast and by a suitable process of digestion in the case of liver. It is probably one of the most physiologically potent substances known, having marked effects on yeast growth even in a dilution of 1 in 4×10^{11} and being curative for rats in daily doses of the order of 0·04 γ.

Isolation:—The isolation of biotin from natural sources is extremely difficult, since even in liver which represents one of the richest sources, it is present in a concentration of less than 0·0001%. Some idea of the

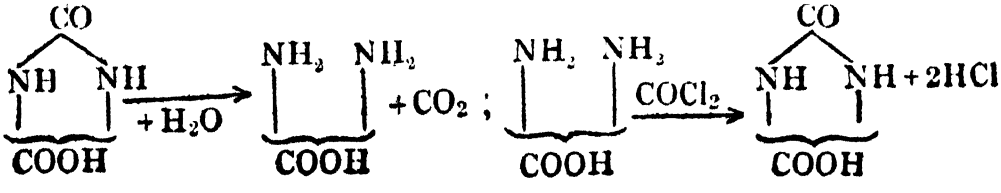
very low yields and the magnitude of the work involved could be realised from the fact that for one gram of biotin 360 tons of yeast or one and a half million eggs had to be employed. Starting from egg-yolk and adopting a very laborious process Kögl[1] succeeded in isolating small quantities of biotin methyl ester melting at 161·5°. Its preparation from liver is less difficult and this source was employed by Du Vigneaud et al[2]. The alcohol-insoluble fraction of beef-liver was subjected to high pressure hydrolysis and a preliminary purification was effected by precipitating inert material from the aqueous solution with alcohol and acetone. Biotin was then precipitated as the phosphotungstate and recovered by decomposition of the salt with baryta. This crude product containing about 0·1% biotin was subjected to esterification conditions with methanol and hydrogen chloride. The ethyl acetate-soluble portion was dissolved in chloroform and subjected to chromatographic adsorption using Brockman's aluminium oxide, and the active material was eluted with methanol-acetone mixture. After a second adsorption and elution the active material was dissolved in chloroform and the solution extracted with dilute hydrochloric acid. The acid extract which contained most of the active material was re-esterified. Subsequent extraction with ethyl acetate and concentration of the ethyl acetate solution gave crystals of the methyl ester of biotin. Recrystallisation from methanol-ether mixtures followed by sublimation in high vacuum gave the ester as fine needles melting at 166 °. It had a vitamin H activity of 28,000 units per mg.

A more convenient source for the preparation of biotin was later found to be commercial milk concentrates[3] containing 0·1 to 0·2% of biotin. The procedure was a slight modification of the one used for the isolation from liver. The biotin present in the crude concentrates was converted into the methyl ester and the ester was purified by adsorption from chloroform solution first on decalso and subsequently on activated alumina. It was eluted with methanol-acetone mixture and from this solution the methyl ester was obtained in a cry-

stalline form. Subsequent vacuum sublimation and recrystallisation yielded it in a pure condition melting at 166°.

Biotin could be readily obtained from the ester by saponification with cold alkali and subsequent acidification. It crystallises in the form of long thin needles and melts at 232° with decomposition. It is dextro-rotatory, $[\alpha]_D^{22°}$ + 91° in 0·1N sodium hydroxide.

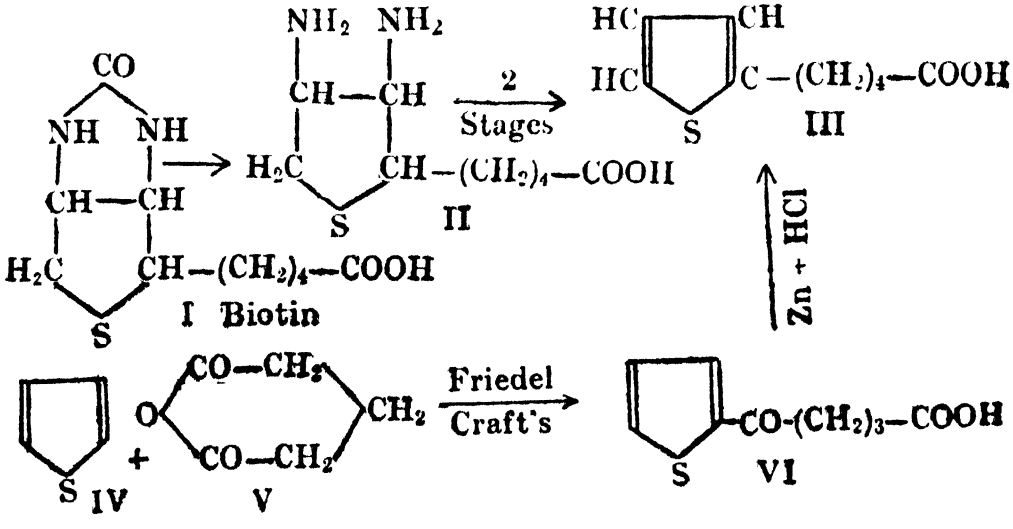
Constitution[4]:—Biotin like thiamin contains sulphur. The empirical formula ascribed to it is $C_{10}H_{16}O_3N_2S$. Titration with alkali indicated that the compound is a monocarboxylic acid. The well-known methyl ester has the composition $C_{11}H_{18}O_3N_2S$. In its spectrum there is no specific absorption in the ultraviolet thereby showing the absence of aromatic rings. Experiments relating to the stability of the substance towards a variety of reagents and treatments, employing yeast growth as a criterion of activity, yielded valuable preliminary information and these were followed up by chemical study. Acylating, alkylating and carbonyl reagents did not react. No nitrogen was produced when biotin was treated with nitrous acid by the van Slyke method and the ninhydrin reaction was also negative, both indicating that it is not an amino-acid. Treatment with baryta at 140° for 20 hours led to the formation of an acid having the formula $C_9H_{18}O_2N_2S$. This contained two free amino groups and gave a dibenzoyl derivative. Its formula showed that it should have been derived from the parent biotin with the loss of one carbon and one oxygen atoms. The most plausible explanation of the change involves the cleavage of a cyclic urea derivative as indicated by the partial formulæ given below. This assumption has been confirmed by the partial synthesis of biotin from the diamino-carboxylic acid by the action of phosgene.



The sulphur atom in biotin exists in a stable com-

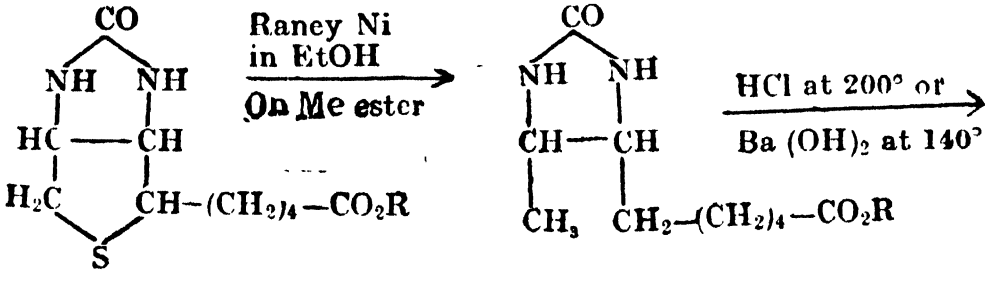
bination thus indicating that it is involved in a thioether structure. This is supported by the fact that on treatment with hydrogen peroxide and glacial acetic acid a crystalline sulphone is obtained in 90% yield. In this change there is no loss of carbon or hydrogen atoms but two oxygen atoms are added.

The other important reactions of biotin and its derivatives are given below. When the diamino-carboxylic acid (II) was methylated using methyl sulphate and potash (modified Hofmann's exhaustive methylation) and the product was subsequently refluxed with hydrochloric acid ε-2-thiophenylvaleric acid (III) was obtained. The identity of this compound was established by synthesis starting from thiophene (IV) which was subjected to the Friedel and Craft's reaction with glutaric anhydride (V) and subsequently reduced with zinc and hydrochloric acid. The formation of a thiophene derivative in the above reaction pointed to the presence of this ring system in (II) and consequently in biotin also. The nature and position of the side chain were also clear from the constitution of (III).

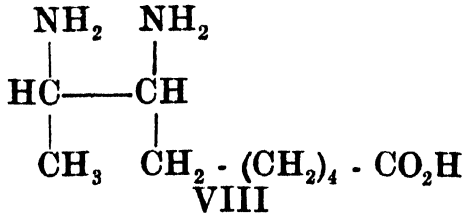


Further information particularly with regard to the position of the amino groups in (II) was obtained from the study of dethio-biotin. Dethiobiotin (VII) is a compound which results when the methyl ester of biotin is reduced with Raney-nickel in boiling ethyl alcohol. It is free from sulphur but it contains the same number of C, N and O atoms as biotin and it could be converted into

a diamino-carboxylic acid (VIII) in the same manner as biotin itself. The structure of this diamino compound as 7 : 8-diamino-nonoic acid was definitely established by synthesis starting from ε-bromo hexoic acid (ester) (X) and the monosodium derivative of acetoacetic ester (IX) through a series of reactions as indicated below :

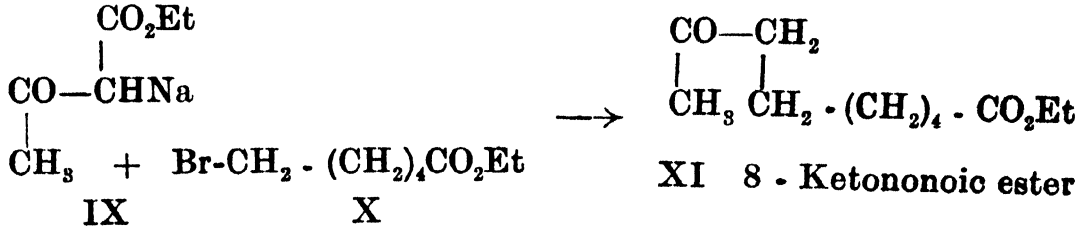


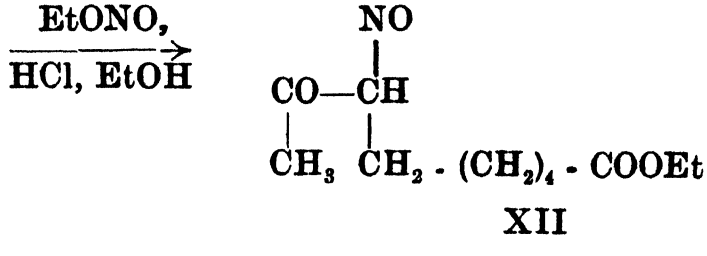
I VII Dethiobiotin

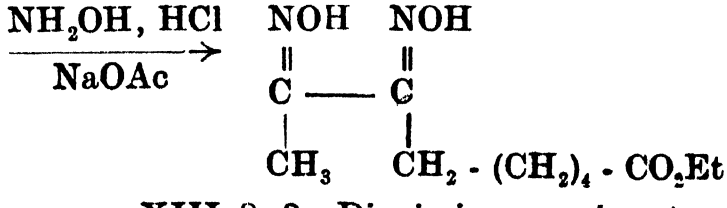


VIII

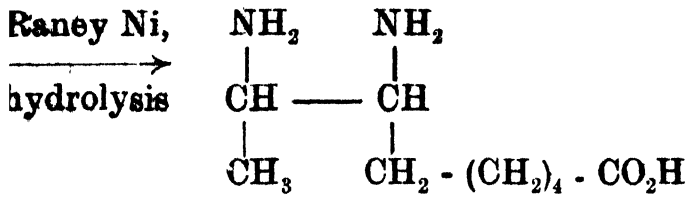
In formulae I and VII, R = H in biotin and dethiobiotin and Me in their methyl esters.





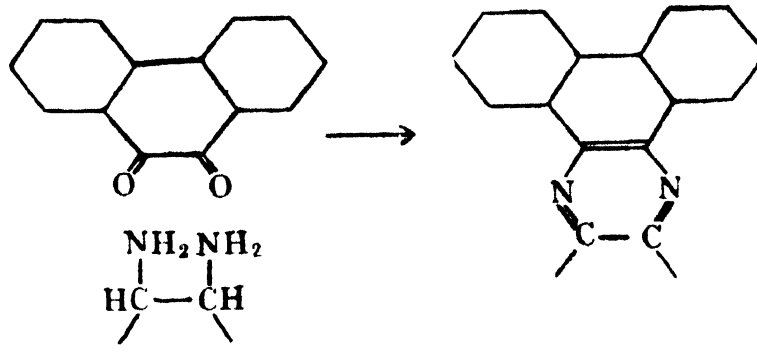


XIII 8 : 9 · Dioximino-nonoic ester



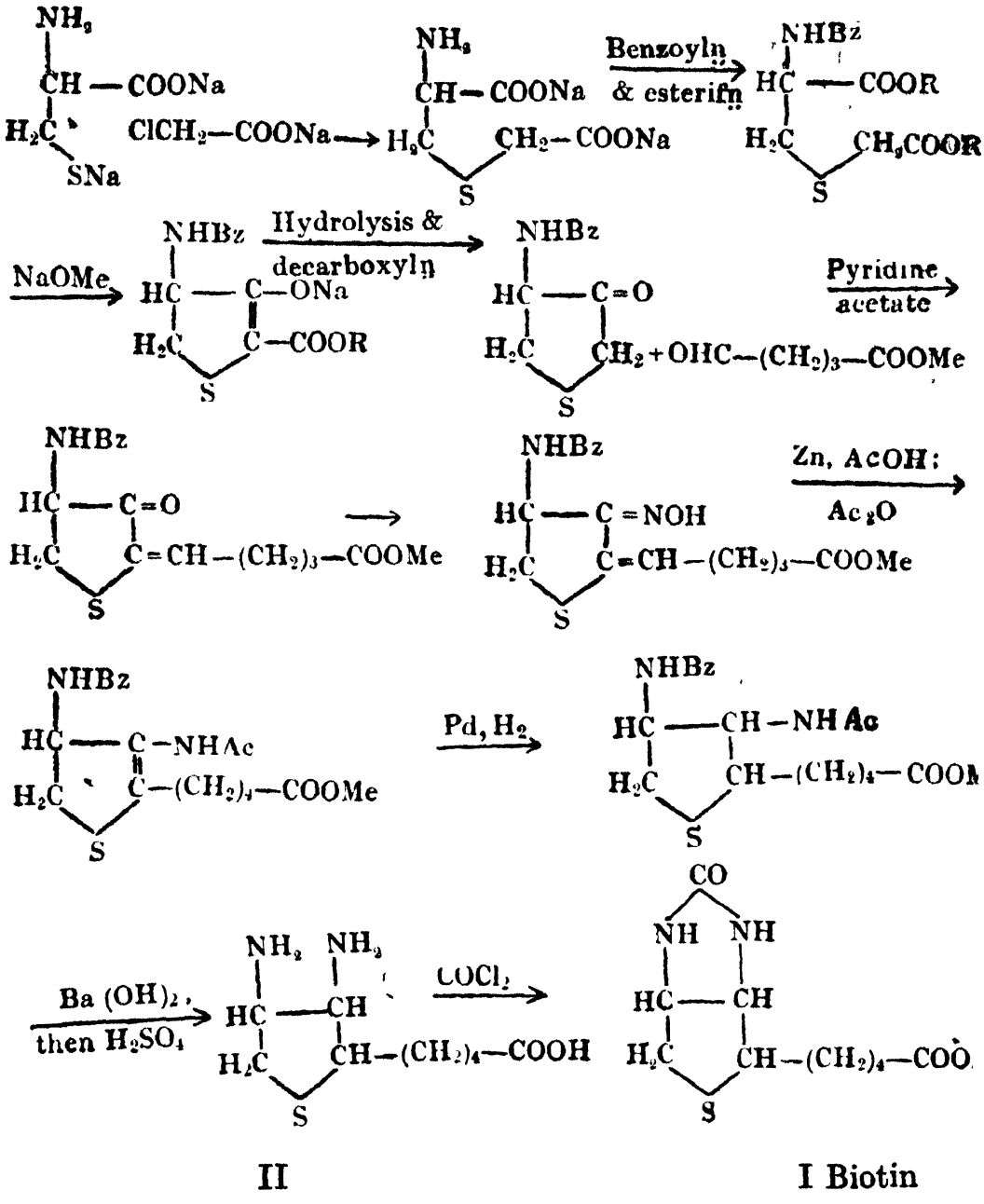
VIII 7 : 8 - Diamino-nonoic acid

An observation which indicated that the urea unit in biotin is present in a 5-atom ring was that the diamino-monocarboxylic acid (II) could smoothly condense with phenanthrene-quinone to give a quinoxaline derivative. Such condensation is characteristic of 1 : 2-diamines and not of 1 : 3-diamines.



From all the above data it could be concluded that biotin has the constitution represented by (I). It may be interesting to note that the structure of biotin was correctly predicted in 1942 from the nature of the functional groups and the ratio of hydrogen to carbon, even before the presence of the reduced thiophene ring was independently established and the reactions of dethiobiotin were investigated.

Synthesis[5]:—For the complete synthesis of biotin which was reported in 1944, l-cysteine, chloracetic and glutaric acids are the starting materials. The stages in the synthesis are indicated below:

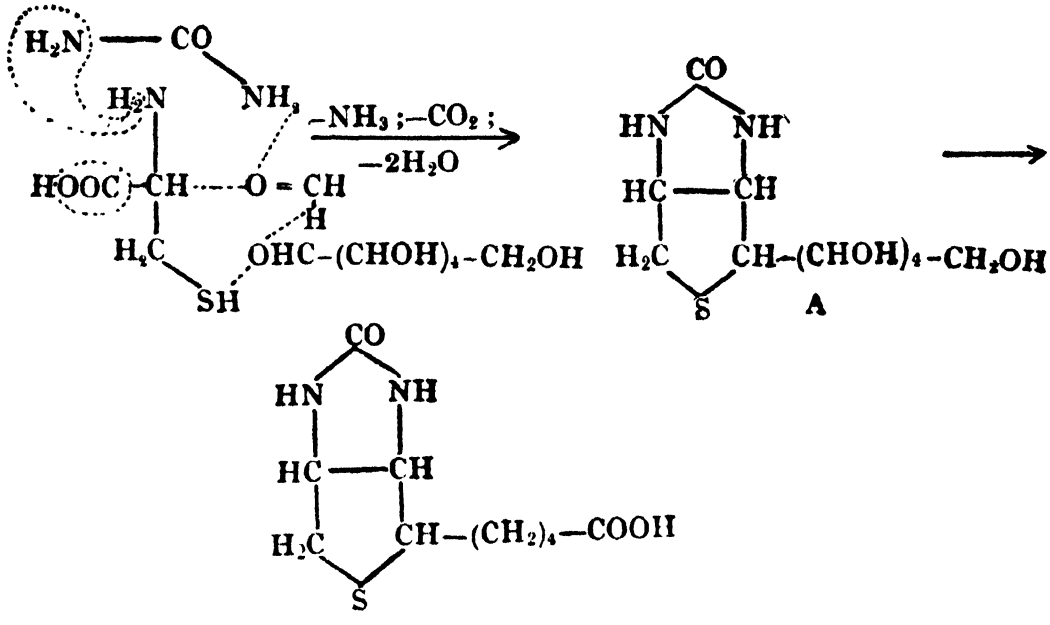


II I Biotin

Since there are three asymmetric centres in the biotin molecule the product of the above synthesis is a mixture of isomers. From this dl-biotin could be separated and resolved through its ester with l-mandelic acid, yielding the naturally occurring d-biotin.

The molecular structure of biotin seems to be highly specific with regard to its physiological activity and all the functional groups seem to be essential. It has been found that the diamino-carboxylic acid (II) has about a tenth of the activity of biotin; yet this compound differs from biotin in not combining with avidin.

Biogenesis:—It seems to be almost certain that the biochemical synthesis of biotin, like the laboratory synthesis, also starts with the naturally occurring thiol-amino acid, cysteine. Some amount of information is available regarding the units required by micro-organisms for the synthesis of biotin. It has been found that pimelic acid[6] could replace biotin for certain bacilli, and the amount of the vitamin formed increases with increasing amounts of pimelic acid and cysteine supplied. Pimelic acid thus appears to be a possible precursor. However, this acid with 7 carbon atoms does not seem to occur in nature. Using materials more easily available in plants and animals, viz., glucose, formaldehyde and urea, the following scheme may be suggested.



B. Biotin

The formation of the skeleton (A) involves elimination of ammonia, carbon dioxide and water during the condensation of the units. The modification of (A) to (B) resembles the conversion of sugars into fatty acids in which reduction should play a predominant part though there is oxidation of the end carbon atom. The details of the transformation are not yet quite clear.

Assay and Standards:—The method of assay for biotin utilises its capacity to promote yeast growth, an unknown sample being compared with a known standard. The earlier rat method is based on a secondary

deficiency disease brought about by egg-white injury and is not convenient. The methyl ester is commercially prepared for therapeutic purposes and is supplied in 1 c.c. ampoules containing quantities of 25γ. Biotin is 3-5 times more effective when given parenterally than by mouth.

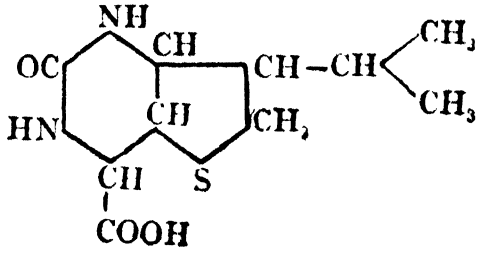
One rat unit is the daily dose of a given preparation or food-stuff which in four weeks brings about complete cure of egg-white injury in rats. This corresponds to an activity of 27 million 'rat units' per gram of the methyl ester of biotin. One 'saccharomycetes unit' (S.U.) is the amount of biotin which produces growth of a special strain of yeast under defined conditions; 1 g. of biotin = 25,000 million S. U.

α-Biotin:—The most recent work of Kögl and his collaborators[7] at Utrecht seems to indicate that biotin of the egg is different from that of liver and milk though they are isomeric. The component of egg-yolk has since been designated α-biotin and that of liver and milk β-biotin. They differ appreciably in their physical properties and physiological potency as indicated in the following table.

	M.P.		Optical rotation, $[\alpha]_D$		Potency of the methyl ester in units per mg.
	of methyl ester	of free acid	of methyl ester	of free acid	
α-Biotin	161—162°	220°	+47° in $CHCl_3$ +82° in MeOH	+51° in 0·1N NaOH	10,000
β-Biotin	163—164°	232—233°	+39° in $CHCl_3$	+91° in 0·1N NaOH	28,000

Kögl has reported that a mixture of the α- and β-esters showed a melting point depression of 20-30° and that a mixture of the free acids melted at 197-202°. Some of the decomposition products also are found to differ. As the result of detailed investigation of α-biotin, the following structure (XIV) has been suggested for it. It will be noticed that though this structure also has a reduced thiophene ring, the size of the nitrogen ring

and the points of fusion between the two are different from β-biotin. The carbon atoms too are not in an unbranched chain. If this should be eventually confirmed, the biotins will be unique examples of much larger differences in structure than known in other vitamins.



XIV. α-Biotin

CHAPTER XII.

MINOR VITAMINS OF THE B GROUP

The term vitamin B_2 complex has been employed to designate a large number of water-soluble entities. Riboflavin, nicotinamide, pyridoxin, pantothenic acid, biotin, meso-inositol, choline, p-amino-benzoic acid and folic acid come under this category. The term 'bios' was proposed long ago to denote certain factors capable of promoting yeast growth. Three different entities have since been shown to be present in 'bios', viz., bios I identified as meso-inositol, bios II A identified as pantothenic acid and bios II B identified as biotin. As already stated these also form part of the vitamin B_2 complex.

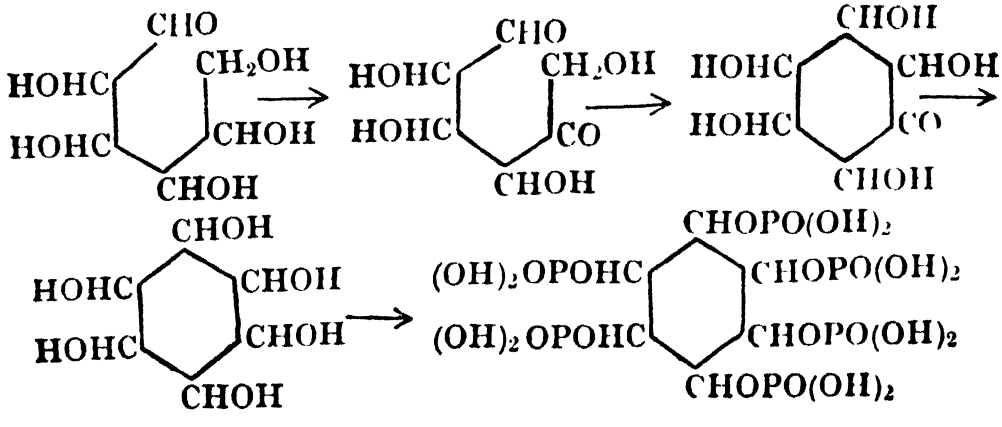
All the above mentioned substances have been shown to be nutritional factors for animals, though in some cases their significance in human nutrition is not yet clearly known. A brief account of those members of the B_2 complex that have not already been dealt with in detail is given in the following pages.

Meso-inositol is a hexahydric alcohol related to cyclohexane and is widely distributed in animals and plants. It occurs in the heart, lungs, liver, muscle and brain, the concentration being about 150-175 mg. per 100 grams of fresh tissue. Among plant sources it is found in beans, in the leaves of the oak, ash and asparagus and in all parts of the grape vine. Some plants yielding milky latex, unripe peas and the leaves of the walnut and the mistletoe are convenient sources for the preparation of the compound.

On a large scale it is prepared[1] by the hydrolysis of phytin which is obtained either from wheat-bran or as a by-product in the starch industry. It occurs free to the extent of 0·4% in the roots of Decalepis Hamiltonii[2] and can be readily extracted by means of 90% methyl alcohol. It melts at 225° and has the formula $C_6H_{12}O_6$ (I). It is soluble in water and has a sweet taste.

A disease called alopecia characterised by hairlessness of the trunk and cessation of growth has been found in mice fed on a restricted diet. Cure could be effected by the addition of meso-inositol. Consequently it is also called anti-alopecia factor for mice. Certain derivatives like inositol hexa-acetate and phytic acid (inositol hexaphosphate, II) have also been found to have some activity. It should be noted however that, whereas in small amounts phytic acid acts as a vitamin for mice or as a growth stimulant for yeast, in larger amounts it becomes an anti-vitamin by combining with calcium and making it unavailable to the animal body. Meso-inositol can be estimated in plant and animal materials by its effect on yeast growth. A number of closely related alcohols including d- and l-inositols do not possess physiological activity.

Inositol is considered to arise in nature from glucose though the change may not be direct and may involve intermediate stages of oxidation, condensation and reduction [3].

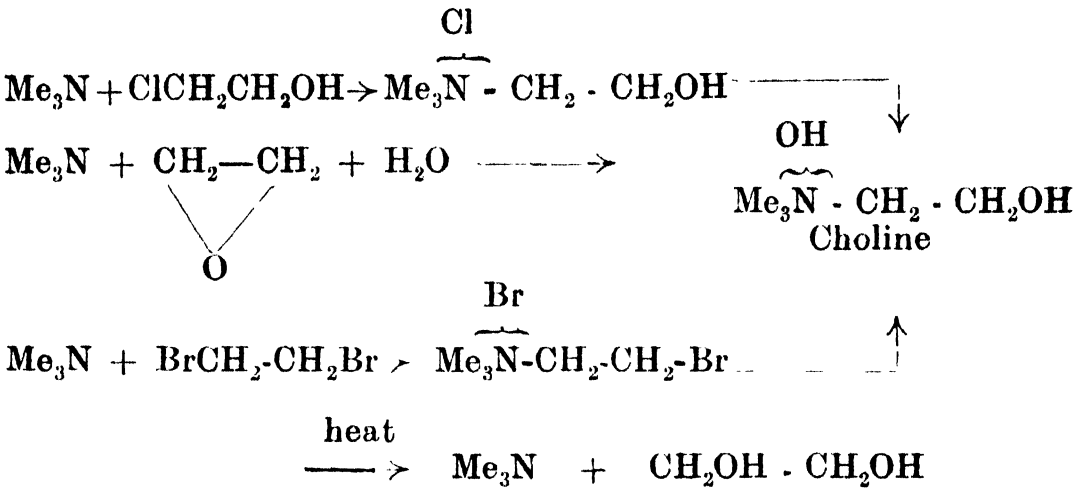


I Inositol

II Phytic acid

Choline occurs widely in plants and animals. Liver, pancreas and yeast seem to be rich sources yielding about 250 mg. per 100 grams of fresh material whereas wheat flour contains only 140 mg. Its isolation which is rather difficult, was first made from hog-bile and subsequently from egg-yolk and white mustard seed. Its synthesis [4] is comparatively easy and can be effected in a number of ways, such as heating ethylene chlorhydrin with trimethylamine and subsequent conversion of

the chloride into the hydroxide, or more directly by heating ethylene oxide with trimethylamine and water. But the best method is to start with ethylene dibromide, convert it into trimethyl-bromo-ethyl-ammonium bromide and then heat it with potash.



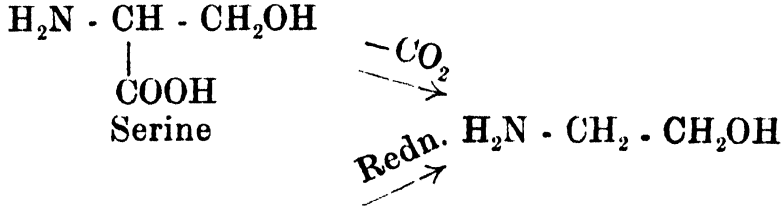
Choline is extremely hygroscopic. Hence it is generally obtained in the form of a syrup and special conditions are required for getting it in a crystalline condition. On heating, it does not melt or distil but decomposes to give trimethylamine and glycol. It is very soluble in water and alcohol and sparingly in anhydrous organic solvents and is a strong base. Most of its salts are soluble in water but a few like the periodide are sparingly soluble and are useful in the detection and estimation of choline. The characteristic odour of trimethylamine which is given off when choline is heated with concentrated sodium or potassium hydroxide can also be employed for its detection. This reaction, however, lacks specificity since betaine behaves similarly.

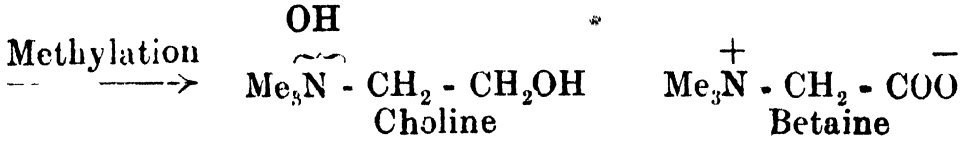
Under certain conditions choline is a dietary essential for rats; it is also needed by poultry. Its physiological role lies in the regulation of fat metabolism. It encourages phospholipid formation in which both fats and sterols are used up. Absence of it causes an excessive deposition of fat in the liver and haemorrhages in the kidney. It also functions in the organism as a biological methylating agent, though for this purpose it can be

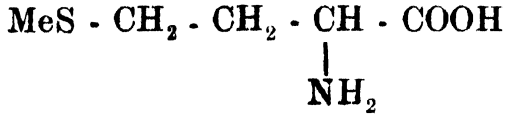
replaced by methionine or betaine. It is not yet definite whether choline or its biological equivalents are needed by man; but they seem to be needed by other animals. It may be added here that acetylcholine plays an important role in the physiology of the parasympathetic nervous system.

There are important points of similarity between thiamin and choline. Both are quarternary bases and have the ethanol unit. There is also a physiological association of the two with reference to the deposition of fat in the liver.

Some work has been done on the biogenesis[5] of choline in the rat. It has been shown that ethanolamine is the immediate precursor and this undergoes methylation, the methyl groups being derived from methionine. The amine itself could be produced either by the decarboxylation of serine or by the reduction of glycine.







Methionine

p-Aminobenzoic acid (PABA):—Loss of pigmentation in the fur of rats kept on deficient diets was noted by a number of investigators. Silver foxes, dogs and guinea-pigs have also been shown to be susceptible to this deficiency. A new vitamin identified by Ansbacher as p-aminobenzoic acid is involved. Its presence encourages colouring of the skin and hair. It has also been recognised as a growth-promoting factor for plants and

bacteria and the view is held that sulphanilamide acts as a bacteriostatic by making this compound unavailable to bacteria. Though clinical applications have already been put forward there seems to be some doubt whether alternative factors, biotin or pantothenic acid, do not cure grey hair in rats.

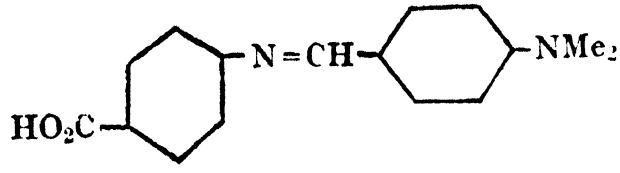
p-Aminobenzoic acid occurs in small quantities in almost all plant and animal foods. Among vegetables cabbage seems to be the richest, containing 14 parts per million of the dried material. Yeast, rice-bran and rice-polishings are also good, containing more than 10 parts per million. Yeast extract is therefore highly rich in this vitamin.

p-Aminobenzoic acid was first isolated from yeast but it is more easily obtained by synthesis. It was first made by Fischer in 1863 by the reduction of p-nitrobenzoic acid with ammonium sulphide. The pure substance crystallises in the form of colourless needles but the crystals acquire a yellow colour on ageing. It is unstable in the presence of oxidising agents particularly ferric salts. An interesting point about this acid is that though by itself it has no effect on the sense of touch or pain, several important local anaesthetics are esters derived from it or from similar compounds.

p-Aminobenzoic acid can be readily detected and estimated by virtue of its being a primary aromatic amine. It can be diazotised and made to couple with a suitable component, for instance dimethyl-α-naphthylamine, in acid solution to form a red pigment and this can be utilised for distinguishing it from other naturally occurring compounds, particularly vitamins. This method may not, however, be suitable when synthetic substances like sulphanilamide are also present.

For the assay of PABA when present in vegetable sources or in biological materials, either chemical or microbiological methods have been employed. The reaction of the vitamin with p-dimethylamino-benzaldehyde to form a Schiff's base having a bright yellow colour has been the basis of a convenient procedure for chemical assay. The specific growth-promoting effect of the vitamin for such organisms as Lactobacillus

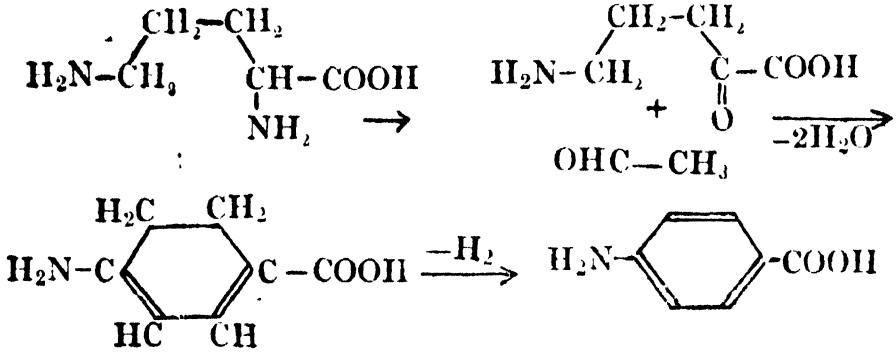
arabinosus has also been employed by different workers for the assay of the vitamin.



Schiff's base

As in the case of several of the members of the B group of vitamins, the biogenesis of PABA seems to be based on a naturally occurring amino acid. The following scheme starting from ornithine can be suggested.

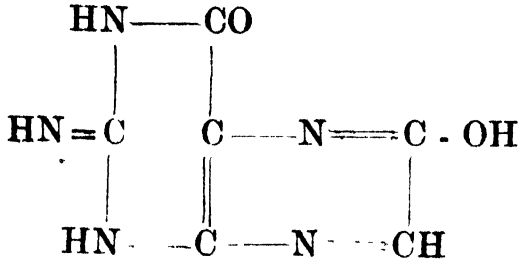
Ornithine



p - Aminobenzoic acid

Folic acid:—An acid substance necessary for the nutrition of yeast and Streptococcus lactis was discovered by R. J. Williams in 1941. It was named folic acid since the original concentrate was obtained from leaves. It has subsequently been shown to be present in liver, kidney and yeast and it seems to be essential for the growth of all micro-organisms. From 1,000 lbs. of spinach Williams et al.[6] have obtained 1·2 mg. of amorphous folic acid. It is sparingly soluble in organic solvents and has a molecular weight of about 400. Analysis indicates an approximate empirical formula $C_{15}H_{15}O_8N_5$. It is readily inactivated by most chemical and physical operations. The interesting observation has been made that it has anti-anaemic properties similar to xanthopterin,[7] the pigment of the wing of the butterfly, and its absorption spectrum indicates that like

xanthopterin it is also a pyrimidine derivative. The acid is possibly related to another nutritional factor known by the name "grass juice factor" which is needed for guinea-pigs.



Xanthopterin

CHAPTER XIII

ASCORBIC ACID (VITAMIN C)

This vitamin is related to a well-known disease called scurvy and hence the name ascorbic acid. Scurvy, particularly associated with sailors and hence called "mariners' disease", has been known for hundreds of years and the curative value of lemons has also been appreciated for a considerable length of time. The early symptoms of the disease are weakness in the joints and spongy gums. In more advanced stages subcutaneous haemorrhages, loose teeth, fragility of bones and often œdema result. The condition responds very quickly to treatment with the vitamin. It has been found that cows and rats are not susceptible whereas monkeys and guinea-pigs resemble human beings in being readily affected by the lack of this vitamin.

Ascorbic acid is one of the most reactive substances known and it is quite sensitive to the action of air and heat and hence considerable difficulty was experienced in its isolation. The achievement goes mainly to the credit of Szent-Györgyi who first isolated several grams of it from adrenal cortex in the year 1928. This was done in the course of his search for the factor responsible for oxidation and reduction in animal and plant systems. Later (1932) when King and his collaborators in America succeeded in isolating a small quantity of it from lemons also, its identity with the antiscorbutic principle was established. Szent-Györgyi then made a vigorous search for a rich plant source of this vitamin and hit upon the Hungarian chillies (Capsicum annuum) as the most satisfactory. From this he made about 4 kilograms of ascorbic acid and this paved the way for the subsequent rapid development of our knowledge regarding the chemical constitution of this important vitamin.

Occurrence:—Ascorbic acid is the most abundant of the vitamins. It is associated with those parts of plants

in which active metabolic processes are in progress. In the resting parts such as seeds it is absent, but as they germinate it again makes its appearance.

The most important sources of this vitamin in edible food-stuffs are fresh vegetables like amaranth, spinach, cauliflower, cabbage and acid fruits such as the citrus fruits, pine-apple and tomato. They contain 30-70 mg. of the vitamin per 100 grams of the material. Germinating seeds such as gram are also important sources of supply. Among the richer sources may be mentioned drumstick, parsley, capsicum, guava and cashew-apple. They contain several hundred milligrams of ascorbic acid per 100 grams. Though potatoes are relatively poor (10-15 mg. per 100 grams) they should be considered to be a valuable source on account of their cheapness and large consumption. For intensive vitamin administration the Indian goose-berry and rose-hips are very useful. It has been found that in the former the vitamin is far more stable to oxidation than in many other natural sources and the material can therefore be dried or dehydrated without marked deterioration in quality. Rose-hips have always been favoured both in Eastern and Western medicine and confection of rose-hips is a very popular preparation. Fully ripe hips of many common species grown in Britain contain over 1% of the vitamin and cases with much higher percentages have been reported. They seem to be liable to considerable variation depending on species and habitat. As a recent addition to the list of rich sources of ascorbic acid may be mentioned the tender shoots of Vitis quadrangularis, a creeper which grows wild in waste lands in several parts of India and which finds occasional use as a food and as a drug.

In all the above cases drying or cooking destroys part of the vitamin, the extent of the loss depending on the conditions employed; æration, enzyme action and catalytic effect of copper ions are quite harmful. Canned fruits are believed to retain-much of their original potency unimpaired and spray-drying of fruit juices has been a very successful method of preserving the anti-scorbutic potency of juicy fruits.

Isolation:—The preparation of ascorbic acid on a large scale from plant sources has ceased to be of technical importance since it can be more easily obtained by synthetic methods. But in the evolution of our knowledge of the substance its large scale production from Hungarian paprika by Szent-Gyorgyi [1] was a great chemical achievement. It is therefore described here. The fully ripe capsicum fruits were pulped and pressed and the juice was treated with lead acetate and some formic acid. The precipitate consisted of impurities and ascorbic acid was present in the solution. After separation, the solution was again treated with lead acetate along with enough ammonia to make it just alkaline to phenolphthalein. This time the precipitate contained the lead salt of the vitamin and it was isolated by centrifuging. It was decomposed by the addition of sufficient amount of hydrochloric acid, and the lead chloride was filtered off and washed in order to recover all the vitamin. The filtrate was concentrated rapidly under reduced pressure to a syrup containing not more than 20% water and this was extracted repeatedly with excess of acetone. The acetone solution in which most of the vitamin was present was evaporated to a sticky syrup under reduced pressure and again extracted with dry acetone. When this extract was concentrated to small bulk, treated with butyl alcohol and the remaining acetone removed under reduced pressure, the vitamin slowly crystallised from the butyl alcohol solution kept at 0° for several days. The crystals were separated by means of the centrifuge, washed with acetone-methanol mixture and recrystallised from a mixture of methanol and dioxane (4 : 1). The total recovery was 25% of the vitamin present in the fruits. Some variation in the above method was made under certain conditions; the preparation of the sodium salt was sometimes a useful stage in the purification.

Properties:—The pure substance is a colourless crystalline solid, slightly acid to taste and very readily soluble in water. It is insoluble in oils, chloroform, light petroleum and ether. When adequately protected from oxidation it can be crystallised from water, the

ower alcohols and acetone. It melts at 196° and has the pecific rotation of +23° in water, +50° in methyl alcoıol and +112° in dilute sodium hydroxide solution. 'he large increase in rotation in alkaline solution is ıoteworthy. This is obviously due to the ionic state of he acid molecule. An aqueous solution of ascorbic acid, lightly acidified, exhibits strong absorption at 245 mμ. ndicating the presence of an α:β-unsaturated carbonyl roup in the molecule. It gives an intense violet colour vith aqueous ferric chloride. The most characteristic roperty is its strong reducing power. It is consequenty unstable in the presence of air, particularly at high $_{H}$, and readily reduces solutions of iodine, potassium ermanganate, ammoniacal silver nitrate and other imilar reagents.

Constitution[2]:—Using material supplied by Prof. zent-Gyorgyi, the problem was solved in 1933 by several roups of workers, the Birmingham group led by Professor W. N. Haworth being the fore-most. Though he vitamin has a comparatively small molecular size, here was considerable difficulty in studying it on ccount of its high reactivity and the new type of structure that it represented.

Ascorbic acid has the molecular formula $C_6H_8O_6$. An aqueous solution is acid to litmus and it reacts with arbonates with the evolution of carbon dioxide. It is a veak monobasic acid forming salts of the general formula $C_6H_7O_6$ (M). Its composition and solubility give trong indications that it is closely related to the simple exoses. Treatment with boiling hydrochloric acid ives a quantitative yield of furfural thus showing that here are at least five carbon atoms in an unbranched hain as in ordinary pentoses. In this reaction there is esemblance to uronic acids. Acetone condenses with scorbic acid as it does with sugars and forms a monoisopropylidene derivative.

The presence of enolic hydroxyl groups is indicated y the bright colour given with ferric chloride. Since a imethyl ether is readily formed by the action of diazoıethane two such groups should be present in the scorbic acid molecule. The strong reducing and the

distinctly acidic properties of the vitamin are related, and they depend on the existence of these enolic hydroxyl groups. This was established by the study of the product of gentle oxidation. When ascorbic acid is oxidised in neutral or acid solution by mild oxidising agents the product is dehydro-ascorbic acid, also called oxy-ascorbic acid, having the formula $C_6H_6O_6$. The change is reversible and ascorbic acid can be recovered by the action of hydrogen sulphide.

$$\text{Ascorbic acid } C_6H_8O_6 \underset{H_2S}{\overset{I_2}{\rightleftharpoons}} \text{Dehydroascorbic acid } C_6H_6O_6$$

Dehydroascorbic acid is also antiscorbutic due to the readiness with which it could be reduced in the living system. But it is neutral in reaction, behaves as a lactone, and does not form furfural on heating with hydrochloric acid. It has no enolic groups and gives no colour with ferric chloride. Thus the acid and reducing properties disappear with the enolic hydroxyl groups. The lactone ring of dehydroascorbic acid may be considered to exist in ascorbic acid also. Further, the composition of the salts of the latter show that salt-formation takes place without the lactone ring opening and hence the acid properties are due to other structural features.

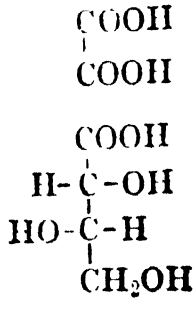
A $-\overset{OH}{\overset{|}{C}} = \overset{OH}{\overset{|}{C}}-$ B $HO_2C-\overset{OH}{\overset{|}{C}} = \overset{OH}{\overset{|}{C}}-CO_2H$

That a structure like (A) having two enolic hydroxyl groups is responsible for the special properties of ascorbic acid was confirmed by a comparison with dihydroxy-maleic acid (B). Both exhibit the same oxidation-reduction reactions and they show the same change in the absorption spectra when they are subjected to oxidation. Both are capable of being methylated with diazomethane to yield dimethyl ethers whose absorption spectra stand in similar close relationship with those of the parent substances.

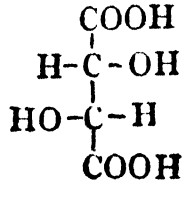
A more direct proof of the absence of a carboxyl in ascorbic acid was provided by Hirst. He showed that

the dimethyl derivative obtained by the action of diazomethane dissolved in alkali without splitting off a methyl group. It thus behaved like a lactone; a free carboxyl group, if present, would have got esterified by diazomethane and the resulting compound should not dissolve in alkali without the initial hydrolysis of the ester involving the loss of a methyl group. This directly led to the discarding of the earlier formulæ embodying a carboxyl group and to the formulation of the correct one now accepted.

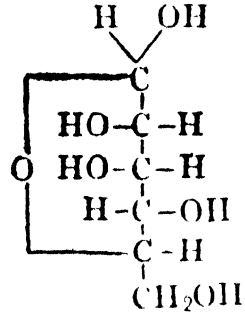
Further insight into the constitution of ascorbic acid was obtained by its oxidation in alkaline medium using sodium hypoiodite. Oxy-ascorbic acid which is first produced undergoes degradation to yield oxalic acid and l-threonic acid (I). The formation of the last acid gives definite evidence for the configuration of the three bottom carbon atoms of ascorbic acid. This is confirmed by further oxidation of the threonic acid whereby dextrotartaric acid (II) is obtained. These observations definitely prove that ascorbic acid is a derivative of l-gulose (III).



I. l-Threonic acid



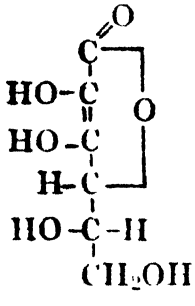
II. l (+) Tartaric acid



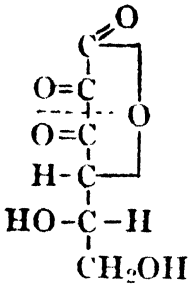
III. l-Gulose

Based on the above properties and reactions the constitution of ascorbic acid has been deduced as a derivative of l-gulofuranolactone (IV). This satisfies all requirements and embodies in it the enol structure, the lactone ring and the l-gulose skeleton. Crystallographic and X-ray measurements made by E. G. Cox are also in agreement with this formula. Oxy-ascorbic acid can then be represented by the formula (V) for the anhydrous and (Va) for the hydrated form. It will be clear that the oxidation of ascorbic acid could also be considered to

be due to the addition of two hydroxyl groups at the double bond. The oxidative break down of the oxy-acid is indicated by the dotted line. Experiments on the methylation of ascorbic acid using restricted amounts of diazomethane show that the 3-hydroxyl is the most reactive and is the one that ionises most. This is in agreement with expectation from structural considerations since the electromeric effect initiated by the carbonyl group will affect this hydroxyl most as shown in formula (VI).

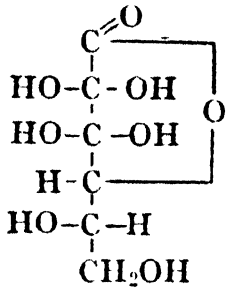


IV. l-Ascorbic acid

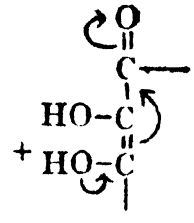


V

Oxyascorbic acid

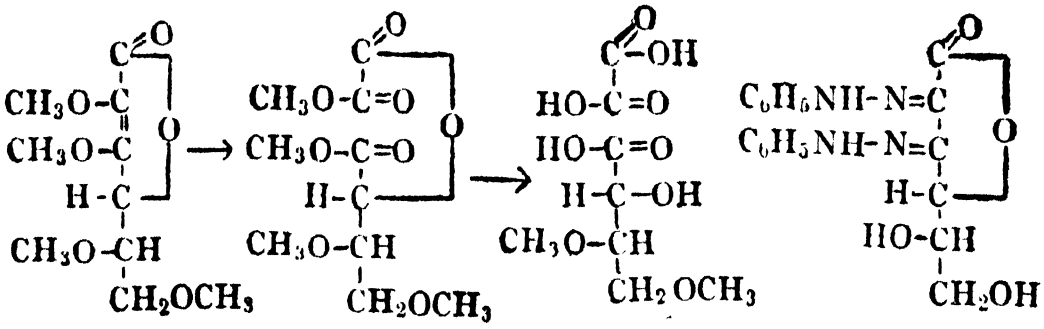


Va



VI

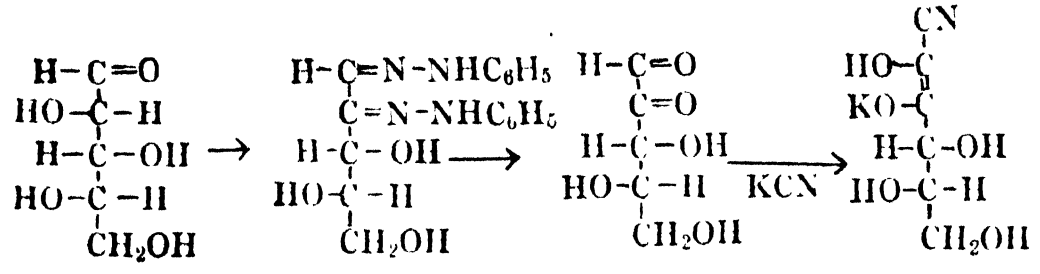
The furano-lactone structure given above locates the ring on the right hand side of the carbon skeleton and this agrees with the dextro-rotatory properties of l-ascorbic acid. The size of this ring was established by a study of its fully methylated derivative which was prepared by methylation in two stages, (1) with diazomethane yielding the dimethyl ether and (2) with methyl iodide and silver oxide yielding the tetramethyl ether (VII). When subjected to decomposition with ozone it broke down at the double bond forming an intermediate compound (VIII), which produced on hydrolysis with alkali, 3 : 4-dimethyl-l-threonic acid (IX) and oxalic acid. Treatment of compound (VIII) with ammonia yielded the amides of the above two acids. The presence of a 5-atom ring was thus proved. Any other ring structure would have given rise to a differently substituted threonic acid.



VII VIII IX X

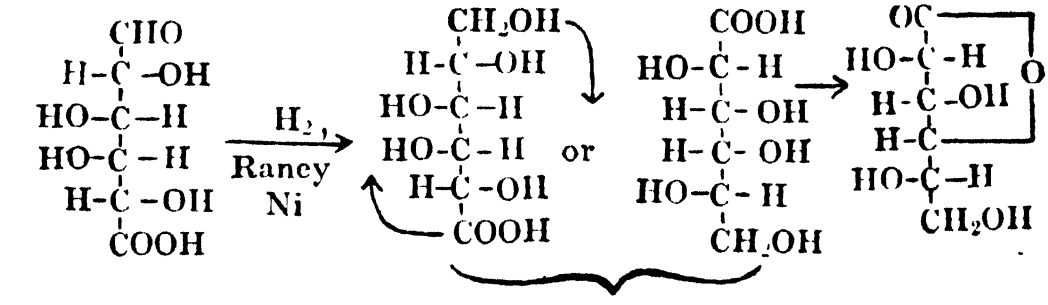
The most easily accessible and characteristic derivative of l-ascorbic acid is the yellow diphenylhydrazone (X) melting at 210°. It is best obtained after preliminary conversion of the vitamin into its immediate oxidation product by means of iodine.

Synthesis:—The first synthesis[3] was effected in 1933 by the method of Reichstein. Even before the correct constitution of the vitamin was elucidated this worker obtained d-ascorbic acid starting from d-xylose. Later when the molecular structure of l-ascorbic acid was established by the Birmingham school, Haworth and subsequently Reichstein reported the synthesis of this substance. l-Xylose (XI) was converted into xylosone (XII) by the well known method passing through the osazone. The osone when treated with potassium cyanide in aqueous solution yielded the cyanhydrin (XIII) which was found to have the imide ring structure (XIV). This substance was capable of reducing iodine just like ascorbic acid and it underwent quantitative conversion into l-ascorbic acid by treatment with 8% hydrochloric acid at 40-50°.

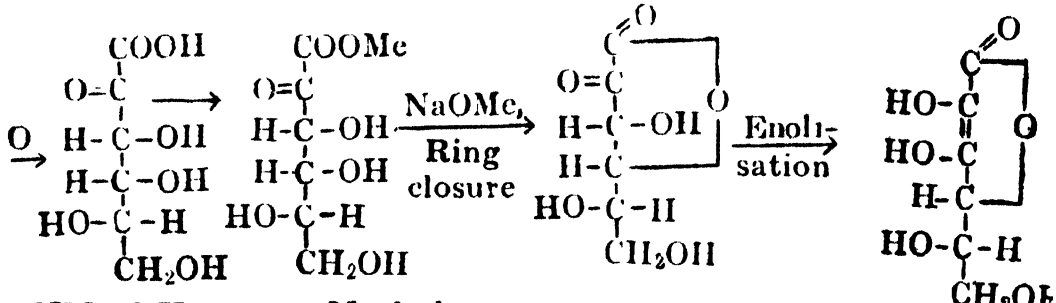


XI. l-Xylose XII XIII

which is then oxidised by means of sodium chlorate in the presence of vanadium pentoxide to yield 2-keto-l-galactonic acid (XXIII.) The subsequent stages involving esterification, ring closure and enolisation are the same as already described in connection with 2-keto l-gulonic acid (XVIII). The steric difference about the 3rd carbon atom between these two keto-aldonic acids is of little consequence in the synthesis of l-ascorbic acid, since the asymmetry about this carbon atom disappears in the final dienol structure of the vitamin. The most difficult part of the new method is the oxidation where the yield amounts only to 25%, but the raw materials are extremely cheap, being by-products of the beet sugar and the fruit industries. A yield of fifty pounds of ascorbic acid per ton of pulp is claimed.



XXI. d-Galacturonic acid — XXII. l-Galactonic acid — γ-Lactone of XXII



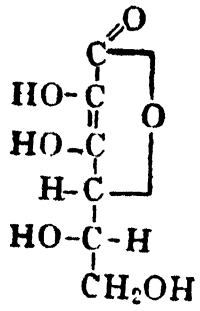
XXIII. 2-Keto-l-galactonic acid — Methyl ester of XXIII — l-Ascorbic acid

Ascorbic acid has gained the special distinction of being the first of the synthetic vitamins to be introduced commercially. It is available in the market in various pharmaceutical forms and is added to beverages, confectionery and to certain special foods. In spite of the large occurrence of vitamin C in nature it is stated that

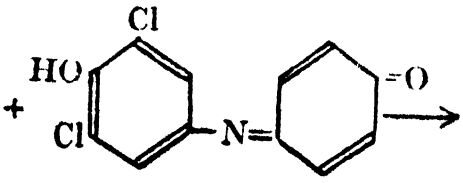
In 1940 about 17 tons of synthetic vitamin C were made in the U.S.A. and the figure reached nearly 100 tons in 1942. The estimated world production for 1945 is 1,200 tons. This has produced a tremendous effect on the bulk price of the vitamin. In 1934 when natural ascorbic acid first became available commercially the cost was something like 213 dollars per ounce. There was a big drop to 3.25 dollars when the synthetic material came into the market in 1937 and in 1942 it further went down to half this value, i.e., 1.65 dollars.

Assay:—The biological method employs guinea-pigs as test animals. It has already been mentioned that like human beings these animals are susceptible to scurvy. The degree of scurvy is estimated by an examination either of sections of the incisor teeth or of macroscopic scorbutic lesions. Both prophylactic and curative tests are employed. These have largely been replaced by chemical methods which are more rapid and accurate.

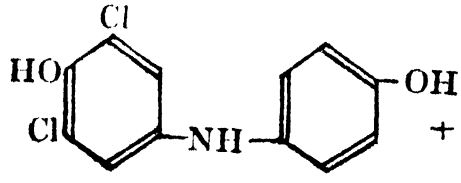
From the beginning of the studies on ascorbic acid, its characteristic reducing property has been used for its estimation. Szent-Györgyi originally employed iodine solution for estimating the reducing power of plant extracts and this gave him valuable information regarding their ascorbic acid content. But this method has now been abandoned owing to the fact that reducing sugars and certain amino acids such as cysteine and glutathione also reduce iodine. Tillmann's discovery that the oxidation-reduction indicator, dichlorophenolindophenol (XXIV) or the corresponding bromo-compound is preferentially reduced by ascorbic acid and that it is comparatively unaffected by other compounds present in tissues, has been widely used in evolving methods of estimation. Chloracetic acid is commonly employed for preparing the tissue extracts. Owing to the high cost of the above dye alternatives have been sought. Methylene blue is reported to be satisfactory when employed under specific conditions. It is however defective in being rather slow.



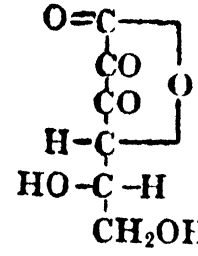
IV



XXIV 2 : 6 - dichloro-phenolindophenol
(Blue in alkali, red in acid.)



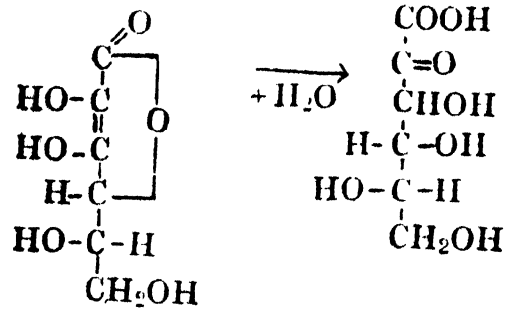
Reduced indicator (colourless.)



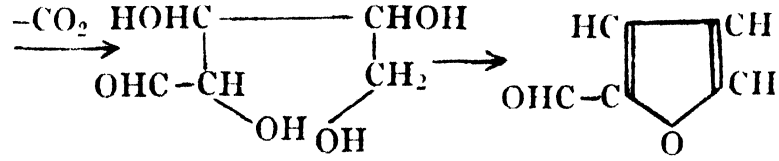
V

The method of assay has to be particularly rapi(since ascorbic acid changes rapidly in solution. Th(chief sources of error are (1) oxidation during the prepa ration of the extract and (2) existence of other reducin(agents. The first is mainly due to the action of coppe and iron ions and enzymes containing them and it i prevented or minimised by the use of phosphoric acid The second is not so important with most natural pro ducts, particularly if the titration is carried out rapidl and if the solution is acid (p_H less than 5, usually 1 t(3.5).

It is probably better to have an independent metho(and in this respect the conversion of ascorbic acid int(furfural by the action of boiling hydrochloric acid ha(been investigated. The reaction may be considered t(proceed as follows:



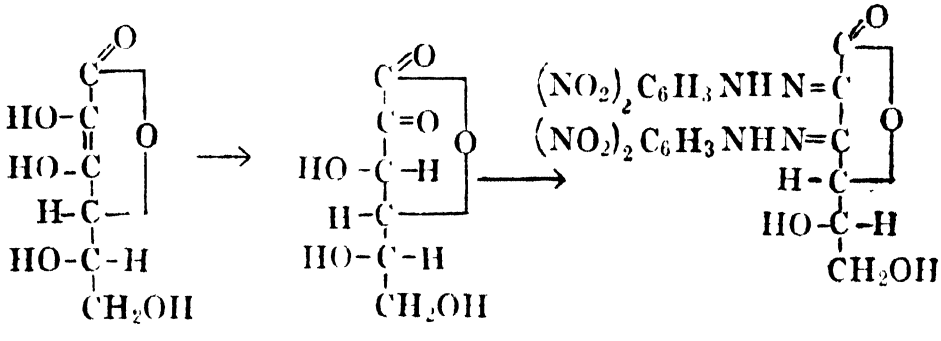
IV



Furfural

The furfural evolved is estimated colorimetrically after treatment with a large excess of aniline acetate. A very weak solution of freshly distilled furfural is employed as the standard. Though the method is good, it is time-consuming and is subject to interference from uronic acids and pentoses.

Estimation of the vitamin by means of its sparingly soluble dinitrophenyl-osazone (XXV) has also been suggested. The compound melts at 271° and its formation may be represented as below :



IV XX XXV

Function, requirements and structural specificity :—The international unit is equivalent to 0·05 mg. of ascorbic acid. Some information is available regarding the physiological function of the vitamin. It obviously acts as a hydrogen-carrier in cellular metabolism since it is capable of reversible oxidation, but the exact reactions involved are still unknown. It is a general cell-stimulant and is responsible for its healthy condition and it increases the resistance of the body to infection. The daily intake of an adult should not be below 25 mg. Of this nearly half is lost in urine and urine analysis gives an indication of the deficiency or otherwise of the patient. An average intake of 60-70 mg. per day has been advocated for good health and about 100 mg. per day make provision for safety. Even children and infants

require roughly the above quantities in view of the large demands made by rapid growth.

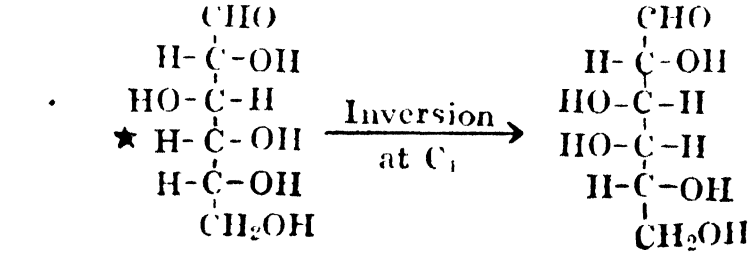
A number of sugar derivatives having the typical structure of ascorbic acid (2:3-dienol-4-lactone) have been prepared. They have similar chemical properties, but they differ markedly in their physiological activity. Very few of them have any antiscorbutic property and particularly d-ascorbic acid has none. The most active of these is l-rhamno-ascorbic acid having about a fifth of the potency of l-ascorbic acid.

Biogenesis:—Ascorbic acid is remarkable among the vitamins in several important respects. While having a small size it represents a novel chemical type having special chemical properties. Extraordinarily large amounts of it are required by human beings and other organisms for maintenance, and its availability in nature is also comparatively large. There is no clear experimental evidence regarding its origin in plants and animals. As already mentioned it occurs in largest quantities in organs where active metabolism is in progress. In resting parts such as seeds it is totally absent, but it reappears as soon as germination takes place and life processes begin. Based on these characteristics and the structure of the substance it could be generally stated that it is derived from carbohydrates.

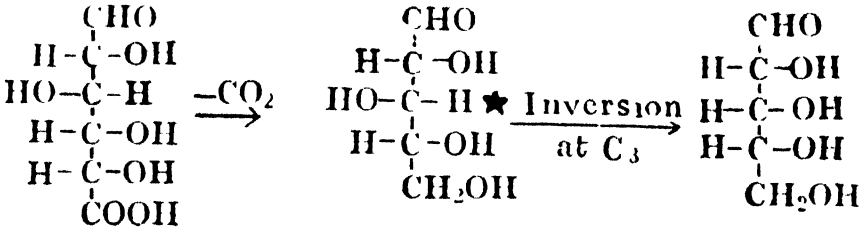
Though a number of biological experiments have been done in regard to the biogenesis of ascorbic acid no definite results could be recorded. It may be simple to regard it as an oxidation product of sorbitol just as in the commercial synthesis, the alcohol being first produced by the reduction of glucose. But this alcohol is not so abundant or widely distributed in nature. In view of the wide distribution of ascorbic acid and the readiness with which it is formed it seems to be quite possible that Nature adopts a mechanism which is simpler and more direct than what is used in the commercial process.

Among monosaccharides the most primary and abundant are the isomers glucose (XV) and fructose (XXXII). In solution under mild basic conditions they can undergo inter-conversion. It looks quite possible that ascorbic

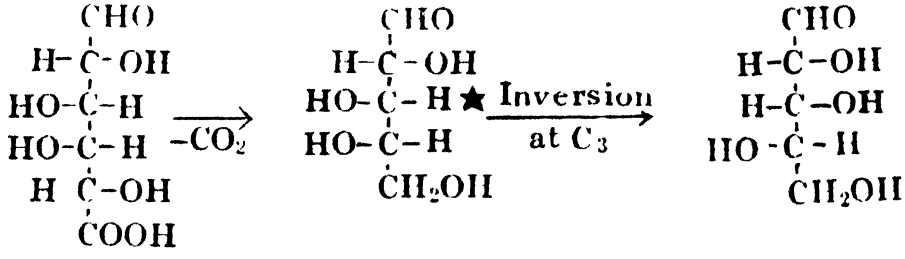
cid is produced from the ketose, the first stage consisting in the conversion of d-fructose into l-sorbose (XVII). he transformation of fructose to sorbose involves Walen inversion on the 5th carbon atom. This type of iechanism was originally suggested by R. Robinson[5] or the formation of d-galactose (XXVI) in nature from -glucose. Similar inversion seems to be involved in he formation of some of the naturally occurring pentoes, particularly d-ribose (XXIX) and l-lyxose (XXXI). 'he following formulae explain the reactions :



XV d-Glucose XXVI d Galactose



XXVII -Glucuronic acid XXVIII d Xylose XXIX d-Ribose

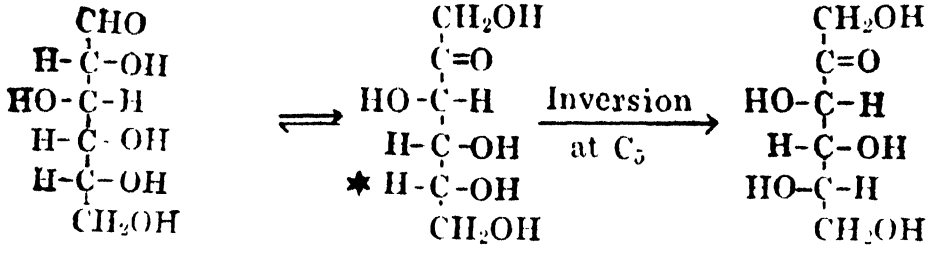


:XI d-Galacturonic acid XXX l-Arabinose XXXI l-Lyxose

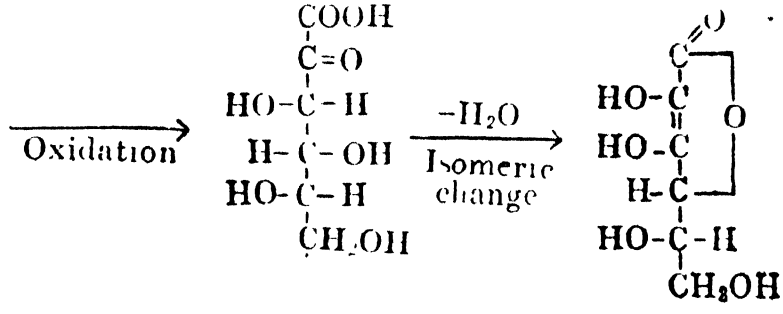
'he inversion is considered to be brought about in the ourse of phosphate formation involving the particular arbinol grouping and subsequent hydrolysis of the ester.

The later stages in the biogenesis of ascorbic acid rom sorbose may be analogous to those employed in the ommercial synthesis. In representing these changes

the open chain forms of the sugars are used below for the sake of simplicity. It may, however, be pointed out that the pyranose ring in d-fructose does not involve the hydroxyl group on carbon atom 5 and hence facility for inversion exists.



XV d Glucose XXXII d-Fructose XVII l-Sorbose

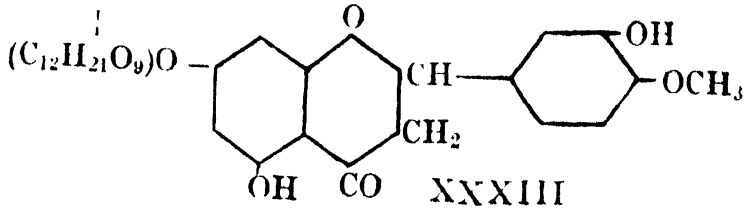


XVIII 2-Keto-l-gulonic acid IV l-Ascorbic acid

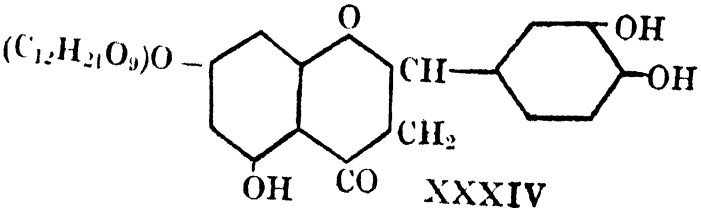
Vitamin P (Permeability Vitamin):—Under this term are included certain mixtures of flavanone and flavone glycosides which are found widely distributed in fruits. Szent-Györgyi noticed that there are certain symptoms of scurvy which are not cured by vitamin C. These are associated with pathological fragility of the capillary walls and their permeability to plasma proteins and are attributed to the lack of vitamin P. The flavanone and flavone glycosides having curative properties were first isolated from Hungarian paprika and later from lemon juice and peels, and more recently from rose hips. These are also well known rich sources of vitamin C. The term 'citrin' was first used for a crystalline product obtained from lemons and this was shown by Brückner to consist of a mixture of the glycosides, hesperidin (XXXIII) and eriodictyn (XXXIV). Later some quercitrin (XXXV) was also found to be present. The possibility that the active principle is the glycoside of the chalkone (XXXVI)

ɔrresponding to hesperidin has also been suggested. The ɛistence of this vitamin, however, does not seem to be ılly established.

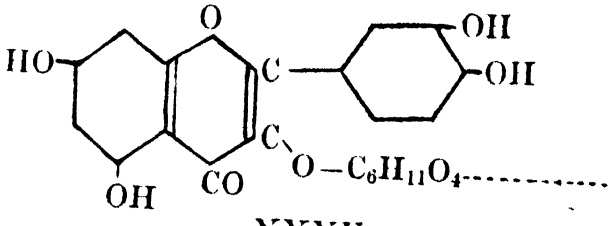
Rhamnoglucose residue



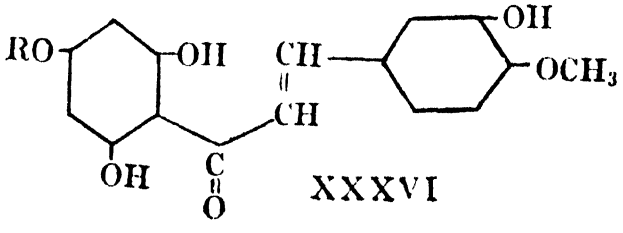
XXXIII Hesperidin



XXXIV Eriodictyn



XXXV Quercitrin — Rhamnose residue.



XXXVI

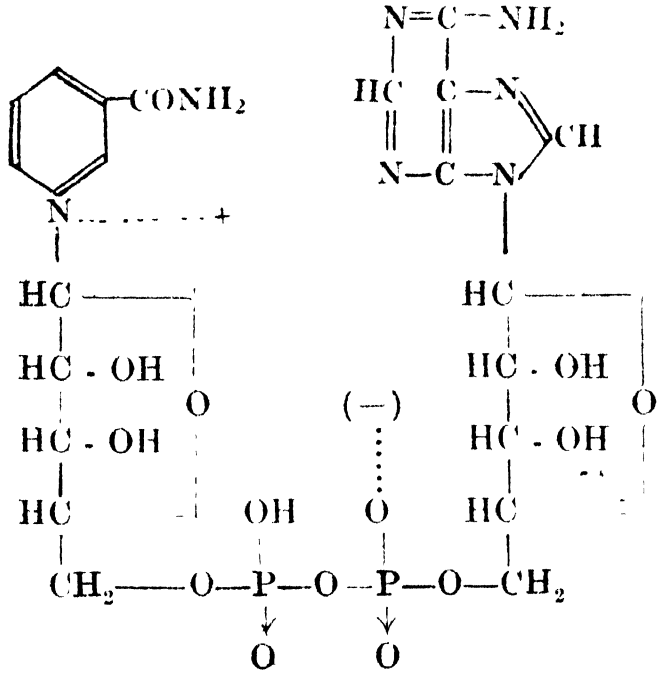
CHAPTER XIV

VITAMINS AND ENZYMES

Though the importance of vitamins in animal nutrition has been known for a long time, the details of their biological functions are not clear even now in the case of a large number of them. Since they are required only in very small quantities as compared with other items of food such as carbohydrates, proteins and fats, they have been considered in general to play their part as biological catalysts. Though the main functions of vitamins A, D, E, and K are understood, they cannot be expressed in chemical terms. The position is slightly better with reference to ascorbic acid. It appears to be an oxidation - reduction catalyst and can act as a reversible hydrogen acceptor and donor, but the actual processes catalysed by it are not yet known. In regard to some members of the B group of vitamins, however, detailed information has been obtained during the past ten years.

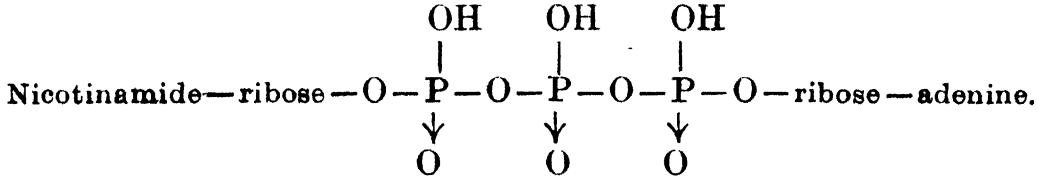
The fundamental discovery of the 'yellow oxidation enzyme' by Warburg (1932) and the subsequent proof that riboflavin is a component of this enzyme, created a great deal of interest and stimulated active work in the study of the relation of vitamins to enzymes. As the result, nicotinic acid amide, riboflavin and thiamin have been shown to be the chief components of a number of coenzymes. These coenzymes are stable, dialysable organic compounds essential for the activity of enzymes. Owing to the pioneering work of Warburg and his colleagues and of Euler and his coworkers, the mode of action of certain coenzymes in enzymic processes is fairly clear. After combination of the coenzyme with a specific protein or enzyme, it is capable of being alternately reduced and oxidised and in this way transfer of hydrogen from a substrate to the final oxidising agent is accomplished. There is, therefore, no doubt that the nutritional effects of the above mentioned vitamins are directly related to their known roles in enzymic behaviour.

Coenzyme I, also called cozymase[1], is a compound of nicotinic acid amide with two molecules of ribose, two of phosphoric acid and one of adenine. Its constitution is expressed as follows:



Coenzyme I: Cozymase.
(Diphosphopyridine Nucleotide).

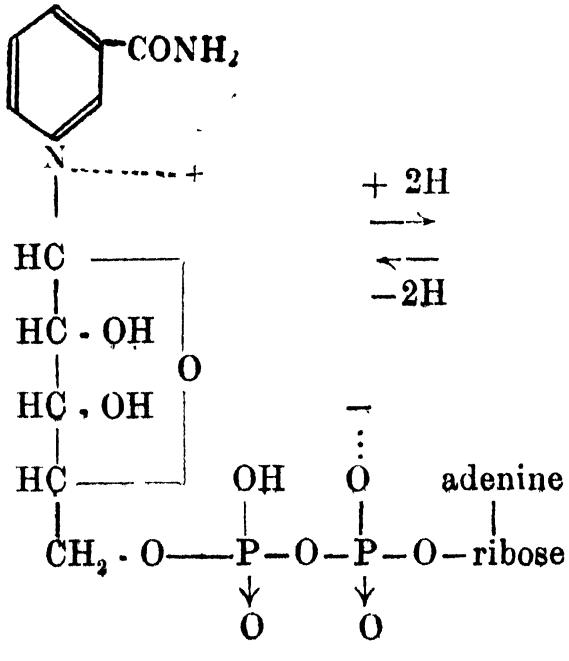
It is usually referred to as diphosphopyridine nucleotide and is considered to be necessary for the activity of the specific enzymes oxidising alcohol, lactate, malate, triose-phosphate, α-glycerophosphate and glucose. Coenzyme II is built in a similar manner but with three phosphoric acid groups linked together instead of two and is therefore referred to as triphosphopyridine nucleotide.



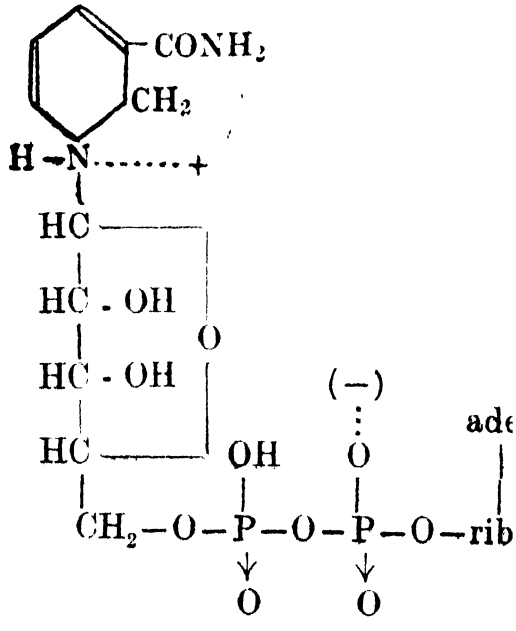
Coenzyme II.
(Triphosphopyridine Nucleotide).

This coenzyme is considered to be necessary for the activity of the enzymes oxidising hexosemonophosphate, glucose and 6-phospho-gluconate.

The coenzymes combine reversibly with their appropriate specific proteins which are the enzymes. For example cozymase combines with alcohol dehydrogenase to form a highly dissociated compound. The substrate, in this case alcohol, after combination with and activation by the enzyme, reduces cozymase. In this reaction a double bond in the nicotinic acid portion of the nucleotide becomes saturated. The reduced cozymase is then oxidised by another enzyme system, and thus by a chain of reductions and oxidations, the hydrogen of the substrate (alcohol) is finally conveyed to oxygen. Lactate, in the presence of lactic dehydrogenase, will not reduce sodium ferricyanide without the addition of cozymase. At present no known substance can replace it in such a system.



Coenzyme I



Reduced coenzyme I

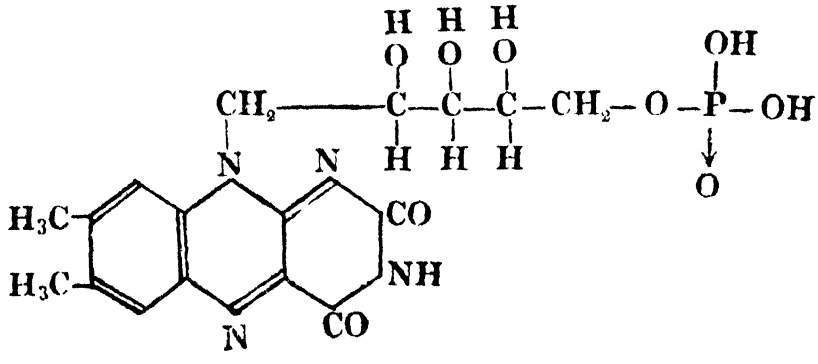
$$CH_3 \cdot CH(OH) \cdot COO^- \xrightarrow{-2H} CH_3 \cdot CO \cdot COO^-$$

$$Na_3Fe(CN)_6 \xrightarrow[H]{NaOH} Na_4Fe(CN)_6 + H_2O$$

Coenzyme II behaves similarly and the mechanism of its oxidation and reduction is the same. But each coenzyme is specific for a particular type of dehydrogenase. The two coenzymes are widely distributed in the

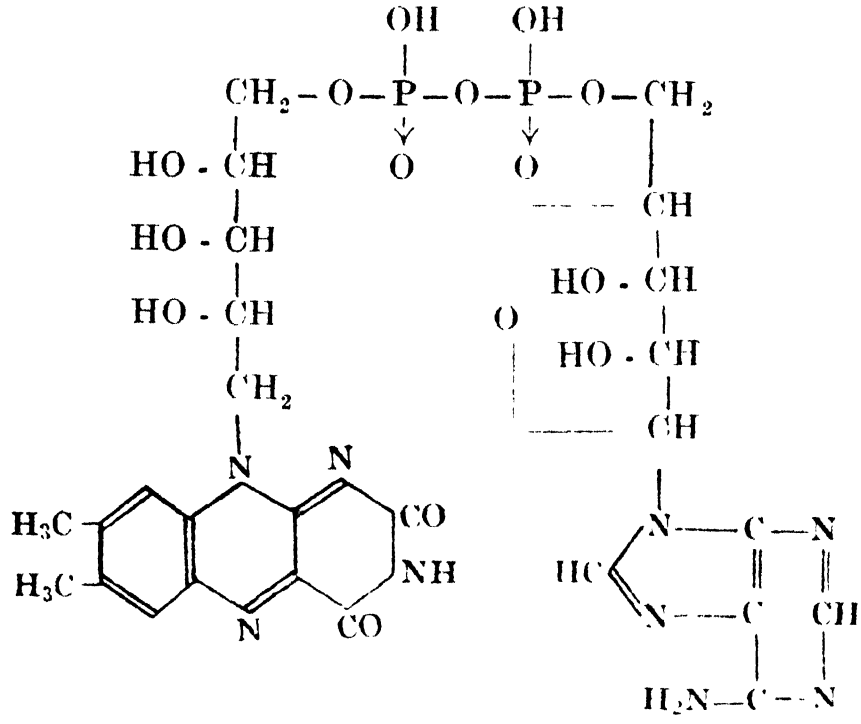
animal body and it is stated that nicotinic acid deficiency leads to a definite decrease of the coenzyme contents of the liver and muscle.

As has already been mentioned, the first discovery of the relationship existing between vitamins and enzymes was the existence of riboflavin phosphate as a component of Warburg's respiratory or oxidising enzyme (also known as the yellow enzyme)[2] which was isolated from yeast. The enzyme could be separated into two parts, (1) riboflavin phosphate and (2) the specific protein. Neither of these could act independently as a carrier of oxygen; they function only in combination. The vitamin phosphate is therefore considered to be a coenzyme.



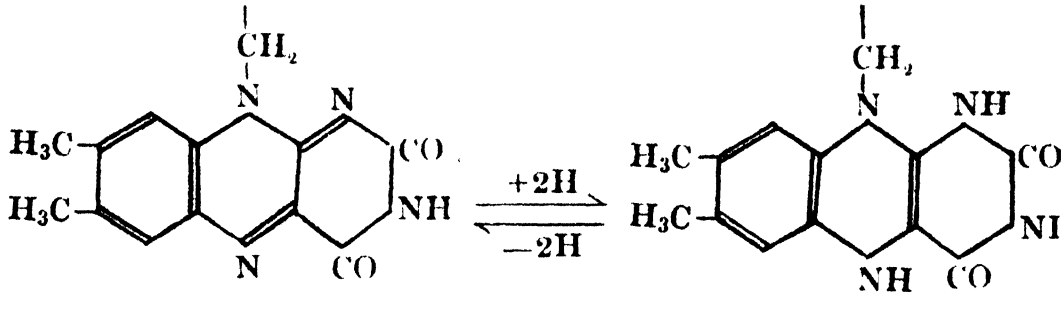
Riboflavin phosphate (coenzyme)

The above mentioned respiratory enzyme is also called a flavoprotein. A number of other flavoproteins are also known. They contain flavin-adenine nucleotide as the coenzyme, combined with specific proteins or enzymes. This coenzyme has the constitution represented below:



Flavin - adenine nucleotide (Coenzyme).

There is a striking similarity of structure betwee flavin nucleotide and cozymase, the only difference bein in the nature of the reducible groups ; in the former it i the isoalloxazine ring and in the latter the pyridin ring. The flavin nucleotides, however, enter into muc firmer union with their specific proteins; the chemica nature of these unions, however, is still unknown.



Coenzyme Reduced coenzyme

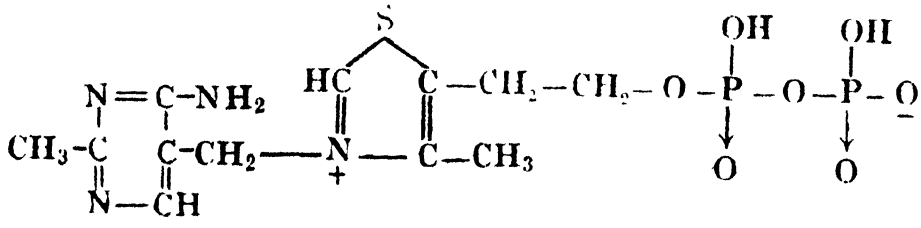
The combined action of cozymase and flavoprotein o the type mentioned above is necessary for the reductio of methylene blue or oxygen. Reduced cozymase com bines with the flavoprotein and transference of hydroge takes place. Cozymase is reformed and the flavi nucleotide is reduced in the isoalloxazine ring. Th

reduced flavoprotein is capable of reducing methylene blue or oxygen.

Reduced cozymase + flavoprotein ⟶ cozymase + reduced flavoprotein $\xrightarrow{\text{methylene blue or } O_2}$ flavoprotein + leucomethylene blue.

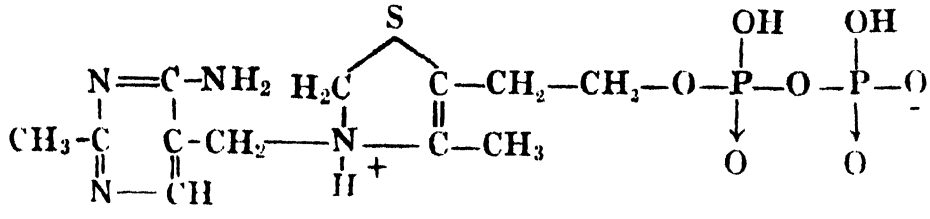
As already mentioned a number of flavoproteins are known to exist, each having a different catalytic behaviour. But all of them have the same coenzyme part i.e., flavin-adenine nucleotide. They differ only in the specific protein or enzyme part and this leads to differences in function as found in d-aminoacid oxidase, xanthine oxidase and the flavoprotein of milk or of the heart muscle.

Another coenzyme of considerable importance is cocarboxylase[3]. It is the pyrophosphoric ester of thiamin and its structure is as given below:



Thiamin Pyrophosphate (Cocarboxylase).

$\xrightarrow[\longleftarrow]{+\,2H}$
$-\,2H$



Reduced Cocarboxylase.

The discovery of this coenzyme was made by Auhagen in 1932. He found that carboxylase of yeast, the enzyme which breaks down keto-acids like pyruvic acid into the corresponding aldehyde and carbon dioxide, can be separated into two fractions, one consisting of a protein (enzyme proper) and the other of a thermostable factor termed cocarboxylase. The fractions individually

have no catalytic activity; together they possess the activity of the original enzyme.

$$CH_3 \cdot CO \cdot COOH \xrightarrow{\text{Carboxylase}} CH_3 \cdot CHO + CO_2$$

Lohmann and Schuster in 1937 obtained the coenzyme in a crystalline condition and established its constitution as thiamin pyrophosphate. It has subsequently been synthesised by chemical and enzymic methods starting from thiamin. This coenzyme is now known to be quite essential also for the oxidation of pyruvic acid in animal tissues.

$$CH_3 \cdot CO \cdot COOH \xrightarrow{O} CH_3 \cdot COOH + CO_2$$

Thus pyruvate undergoes two different degradation processes in vivo. In yeast, carboxylase in the presence of thiamin pyrophosphate (cocarboxylase), forms acetaldehyde and carbon dioxide from it. In animal tissues and in certain bacteria, it is oxidised by pyruvic acid oxidase to acetate and carbon dioxide, thiamin pyrophosphate again being necessary. Cocarboxylase therefore, plays an important part in the metabolism of carbohydrates.

$$\text{Glucose} \rightarrow CH_3 \cdot CH(OH) \cdot COOH \xrightarrow[\text{Dehydrogenase}]{\text{Lactic}} CH_3 \cdot CO \cdot COOH \begin{cases} \rightarrow CH_3 \cdot CHO + CO_2 \\ \xrightarrow{O} CH_3 \cdot COOH + CO_2. \end{cases}$$

It is also considered to catalyse the carboxylation of pyruvic acid in animal metabolism; this leads to the utilisation of the acid probably in the following manner:

$$CH_3 \cdot CO \cdot COOH + CO_2 \longrightarrow COOH \cdot CH_2 \cdot CO \cdot COOH.$$

The coenzymes are so important in the mechanism of enzyme action that they have been sometimes spoken of as the prosthetic groups of enzymes, the proteins being the carriers. It seems to be, however, more correct to consider them as specially important biocatalysts the special proteins being the enzymes proper. The active enzymes are then enzyme · coenzyme complexes. A

umber of biological oxidations can take place through hree essentially similar stages.

;) Substrate + Coenzyme I or II	Specific dehydrogenase	Oxidised substrate + Dihydrocoenzyme I or II
) Dihydrocoenzyme I or II + 2 Fe^{III} cytochrome	Dihydrocoenzyme dehydrogenase	Coenzyme I or II + 2 Fe^{II} cytochrome
) 2 Fe^{II} cytochrome + O	Cytochrome Oxidase	2 Fe^{III} cytochrome + H_2O.

a), (*b*) and (*c*) consist in the transfer of 2 hydrogen atoms or their equivalents) from a hydrogen donator to a ydrogen acceptor. Reactions (*a*) and (*b*) are anærobic whereas (*c*) is ærobic. According to modern nomencla- ure the term 'oxidase' is reserved for enzymes directly atalysing the reduction of molecular oxygen and hence ne enzyme taking part in reaction (*c*) is given that ame.

The above mentioned series of changes taking place n easy stages and leading finally to the formation of ater from hydrogen derived from the substrate and xygen from air is characteristic of biological processes nd is essential for the safety of the organism. In the aboratory it is possible to bring about this change in a ngle stage. But then, due to rapid evolution of energy iolent changes in temperature and other conditions ake place. These will be seriously injurious to the elicately balanced system of the living cell. If the aboratory process could be compared to a gigantic row up to the top of a hill or a precipitous fall from bove, the life process employing enzyme action resem- les a gentle climb up by means of easy steps or an asy climb down.

The vitamins of the B group seem to be invariably equired whenever energy transformations take place in ving tissues and their functions also seem to be inter- lated. It is highly significant that they are not only ound in organs concerned with active metabolism, but

even in the resting parts such as seeds, wherein t most important of this group of vitamins are present adequate amounts readily available for starting acti life processes again.

CHAPTER XV

HORMONES AND THEIR GENERAL CHARACTERISTICS

Even before the beginning of the twentieth century the influence of hormones and the effects of their deficiency were known in a general way, though their importance was not fully realised. The pressor action of the extracts of the suprarenal glands had been observed by Oliver and Schäfer towards the end of the last century and attempts were being made to obtain the hormone in a crystalline condition. The oral administration of the thyroid gland as a remedy for thyroid deficiency was an older practice and the effects of castration were known from ancient days. But the full significance of hormones and their functioning was clearly understood only in 1902 after the investigations of Bayliss and Starling on secretin. They studied the factors that bring about secretion of the pancreatic juice and established that it was not due to a nerve stimulus just as in the case of the secretion of the saliva but was the effect of a chemical stimulus. The term 'hormone' for substances of this class was first suggested by them and it has been commonly adopted. Ordinarily it is restricted to the specific secretions of certain glands which are called hormonal glands. They are ductless and they secrete the hormones inwards into the blood stream. Consequently they are also known as endocrine glands.

It has already been pointed out in connection with the vitamins that, in the evolution of our knowledge of diseases, the category of deficiency diseases was the last to be recognised. If the endocrine glands do not perform their functions adequately or not at all, hormonal deficiencies arise and in each case definite pathological conditions follow. For example, Addison's disease is linked up with the suprarenal glands; cretinism is related to the thyroid and lack of sex maturity to the pituitary

glands. These distinct conditions are quickly rectified by the supply of the concerned hormones from external sources.

It is therefore clear that hormones are quite specific in their physiological functions. They are required only in small amounts and even in low concentrations they produce large effects. The chief difficulty in their chemical study has been their isolation in adequate quantities. The required glands are usually small in size and special arrangements have to be made to collect large quantities of them and store them without deterioration. The yields of the hormones are invariably very small. For instance, even the improved method of Harington yields only 0·027 % of thyroxine from the thyroid gland. There are other cases where the yield is smaller. Considerable difficulty exists in separating them from the large amount of other chemical compounds accompanying them in the glands. Further, they are generally unstable and easily susceptible to oxidation and hydrolysis either by chemical reagents such as acids and alkalies or by the action of enzymes. In most cases special chemical properties had to be discovered enabling their isolation in a state of purity. Once the hormones were isolated in a pure condition the subsequent study leading to the establishment of their constitution may be said to have been comparatively easier. Synthesis soon followed in several cases.

Some of the hormones are fairly simple in their molecular structure, such as for instance adrenaline; some others are more complex, as represented by the sex hormones, while insulin may be taken as an example of the most complex molecules to study. Chemically they belong to various groups. Adrenaline is a base, thyroxine is an amino-acid and insulin a complex protein. The sex hormones and cortex hormones contain no nitrogen and are derived from sterols; they are therefore called steroid hormones. Some of them are phenolic and a large number are ketonic in nature. The plant hormones too are non-nitrogenous and are acids.

As a consequence of the advances in the chemistry of the hormones great improvements have been made in the treatment of hormonal deficiencies. Historically, the

administration of the fresh or preserved glands was the earliest practice. The next stage was the use of concentrated and stabilised extracts. The administration of the pure hormones was a later stage and meant considerable advance in the effectiveness of the treatment of diseases. After the constitutions of the hormone molecules were definitely understood, commercial production by synthetic methods was attempted and in certain cases this has been remarkably successful. Probably the last stage of progress in this line is the discovery of synthetic substitutes having smaller molecular size and capable of being manufactured easily. Some of them have been found to be more useful than the natural hormones from the point of view of stability and physiological potency.

The discovery of satisfactory and at the same time easy methods of assay played a very important role in the concentration and isolation of hormones from biological sources. The earlier methods had per force to be physiological and these continue to be the only available and correct ones to the present day in certain cases. After gaining some knowledge of the chemical nature of the hormones it was possible to evolve chemical and physico-chemical methods which are far more rapid.

In all that has been said above regarding the hormones, the existence of close parallelism between them and the vitamins is obvious. They are both connected with deficiency diseases, have powerful physiological action and the development of their chemistry and the treatment of deficiency diseases have progressed on similar lines. The only difference that could be noted as far as animals are concerned, is that vitamins are normally supplied as components of food-stuffs whereas hormones are normally produced by the endocrine glands.

CHAPTER XVI

ADRENALINE

This is the hormone of the adrenal gland. In 1849 Addison called attention to the importance of this tiny organ (weighing about five grams in man) and showed that a disease, now called after him, is associated with lesions of the gland. The adrenal gland (also called the suprarenal gland as it is situated above the kidney) consists of two histologically distinct portions, the medulla and the cortex. The former secretes adrenaline and the latter 'cortin' (cortex hormones). Addison's disease is now known to be due to pathological changes in the cortex evidently leading to the deficiency of cortin. Soon after Addison, Brown-Sequard showed in 1856 that the removal of both suprarenals in animals is fatal, and Vulpian found that a substance giving a green colour with ferric chloride and a red colour with iodine is present in the adrenal medulla. But the most interesting and important discovery was that of Oliver and Schäfer[1] (1894) who found that when extracts of the adrenals were injected intravenously into animals there was a marked rise in the blood pressure. Subsequently vigorous attempts were made in various laboratories to isolate the active principle in a crystalline state. Success was achieved simultaneously in 1901 by Takamine and by Aldrich in America. The active principle which was named adrenaline, was the first hormone to be obtained in a pure condition and this was accomplished even before the significance of hormones was fully understood.

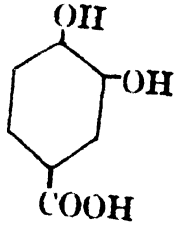
Isolation[2]:—The preparation of adrenaline from suprarenal glands is quite simple and it utilises the marked basic character of the substance. For production on a commercial scale the adrenal glands of oxen are most commonly employed. The minced glands are extracted with hot acidulated water and the extract is heated in order to coagulate most of the proteins. It is then filtered

and the clear filtrate concentrated in vacuo. The concentrated solution is treated with 2-3 volumes of alcohol whereby more impurities including the remaining proteins are precipitated After filtration, the alcoholic filtrate is concentrated in order to remove the alcohol. When ammonia is added to the residual aqueous solution, adrenaline is liberated and it slowly separates as sphæro crystals. It is finally purified by dissolution in alcohol containing oxalic acid (15%) whereby inorganic impurities are left behind. After filtration and dilution with water ammonia is added to precipitate the base. Throughout the process oxidation must be guarded against by using a vacuum or a carbon dioxide atmosphere. Frequently a layer of light petroleum serves the purpose

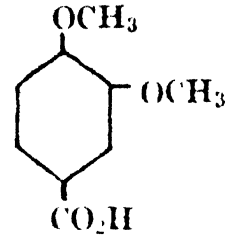
Properties: Adrenaline is also called epinephrine and suprarenin. It is a colourless crystalline substance melting at 211°. It is practically insoluble in water and most organic solvents but is soluble in acetic acid, mineral acids and caustic alkalies. Its solution in water is feebly alkaline to litmus. The hormone is comparatively stable in acid medium whereas alkaline conditions are highly detrimental. It imparts a green colour to ferric chloride solution and it is a strong reducing agent capable of reducing Fehling's solution and of being used as a developer in photography. The gland product is lævo-rotatory, having a specific rotation of $-53°$ in hydrochloric acid solution. The racemic form decomposes at about 230° and is much less active physiologically. This is due to the fact that d-adrenaline possesses only one-twelfth to one-sixteenth the physiological activity of the lævo isomer.

Constitution[3]:—The results of analysis and molecular weight determination agree with the formula $C_9H_{13}O_3N$. The first important item of information regarding its constitution was the existence of a catechol nucleus. This was indicated by the intense green colour produced with ferric chloride and the ease with which adrenaline was oxidised. More definite proof for the existence of this nucleus was furnished by the degradation experiments of Takamine and of Jowett. The former

obtained protocatechuic acid (I) by the fusion of adrenaline with potash. The latter first methylated adrenaline completely in order to protect the nucleus from disruption in the second stage which consisted in oxidising the methyl ether with potassium permanganate. The fission products were veratric acid (II) and methylamine. The isolation of protocatechuic and veratric acids not only furnished proof for the existence of the catechol nucleus but also showed the position of the side chain relative to the two phenolic groups. This arrangement is very commonly met with in a large number of vegetable substances.

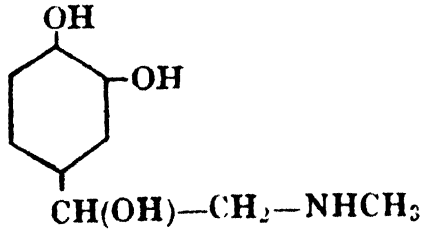


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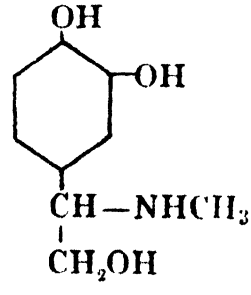


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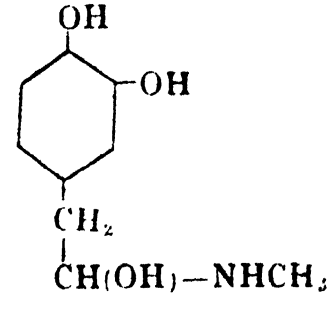
Von Fürth established the presence of a methylamino group and of an alcoholic hydroxyl group, both in the side chain. Boiling adrenaline with hydrochloric acid for a long time produced methylamine. These findings led to the following alternative formulæ for adrenaline.



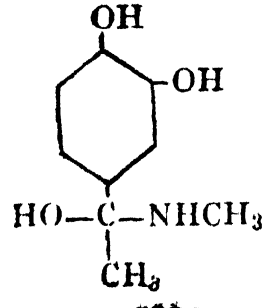
III



IIIA

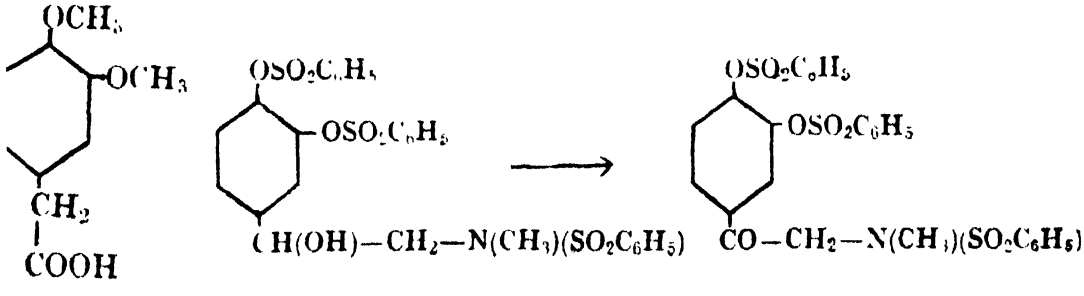


IIIB



IIIC

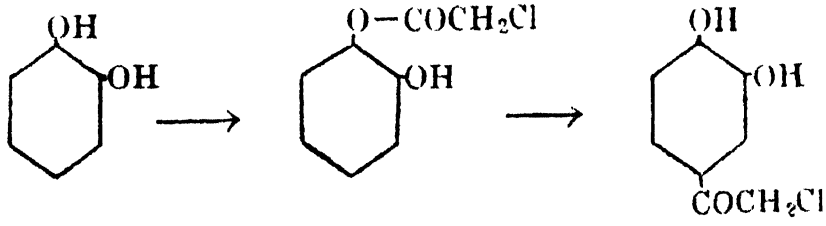
Of these, formula (III-B) would give rise to homoveratric acid (IV) on methylation and subsequent oxidation, while formulæ (III-A) and (III-C) would not explain the formation of pyrrole and skatole derivatives observed by certain workers when adrenaline was fused with potash. Hence the choice was in favour of formula (III) for adrenaline. This structure explains all its properties especially optical activity and its degradation to pyrrole, skatole and catechol derivatives. It further indicates that the compound is a secondary alcohol and not a primary alcohol as in (III-A) or a tertiary alcohol as in (III-C). This point was first proved by the synthesis of adrenaline itself but the usual proof of the existence of such a structure was given later by Friedmann who prepared the tribenzenesulphonyl derivative (V) of adrenaline thus protecting the catechol nucleus. This underwent oxidation to a ketone (VI) and not to an aldehyde or an acid.



IV V VI

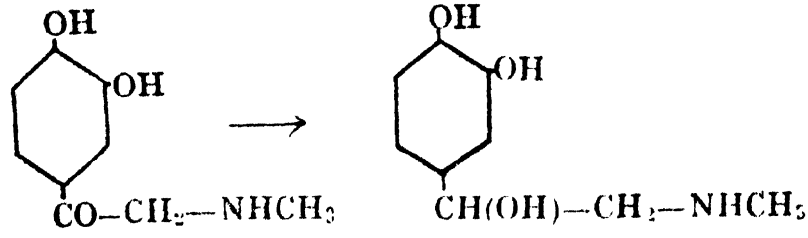
Synthesis [1]:—The synthesis of adrenaline was carried out almost simultaneously by Stolz in Germany and by Dakin in England. The starting point was catechol which condenses with chloracetic acid when heated in the presence of phosphorus oxychloride as the condensing agent. The product was chloracetocatechol (VIII). Due to the formation of much tar the yield was bad. Ott later investigated the mechanism of this reaction and showed that it takes place in stages, the primary product being the chloracetate of catechol (VII). He found that when equimolecular proportions of catechol and chloracetyl chloride were heated together in boiling benzene solution the chloracetate of catechol was produced without any resinification. When this ester

was subsequently warmed with 5-10% of its weight of phosphorus oxychloride, it smoothly underwent isomerisation giving chloracetocatechol (VIII).



Catechol (VII) (VIII)

The next stage in the synthesis was the replacement of the chlorine atom by methylamine. This was accomplished by using a large excess of concentrated methylamine solution in the cold. The resulting methylamino ketone (IX) known as adrenalone exhibits the pharmacological properties of adrenaline, though in a much smaller degree. Its reduction to adrenaline (III) presented considerable difficulty. It was originally carried out electrolytically or by means of aluminium amalgam, but a more satisfactory method due to Hoshino is catalytic reduction using hydrogen and palladium. The resulting product was dl-adrenaline, as it happens invariably in synthesis. It had only half the physiological activity of the natural (laevo) variety and this has been explained as being due to the very poor activity of the d-variety present in the mixture.

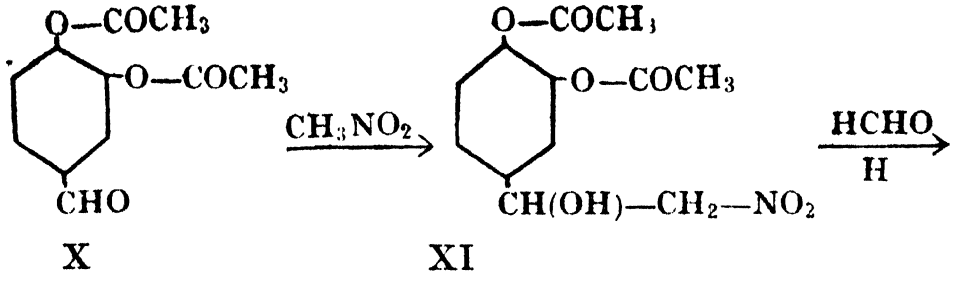


(IX) (III)

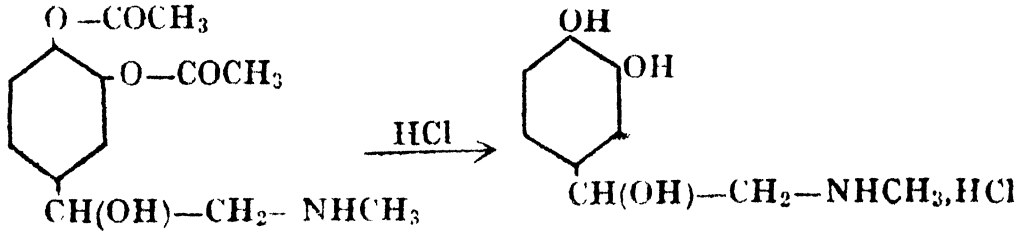
The first manufacture of the synthetic product was carried out in Germany. The German manufacturers could have placed on the market the synthetic racemic compound employing double the concentration normally used in the case of the natural laevo variety. But this was obviously not economical in a competitive market.

They therefore resolved it by means of its bitartrate utilising a discovery made by Flächer. When dl-adrenaline d-tartrate is heated with methyl alcohol, d-adrenaline d-tartrate dissolves and l-adrenaline d-tartrate remains behind. The latter is obtained optically pure by recrystallising from 95% methyl alcohol and subsequently decomposed to give the free base, l-adrenaline. The crude d-adrenaline obtained from the soluble portion of the tartrates is not wasted but racemised by heating with hydrochloric acid. On resolution, a further quantity of the l-variety is obtained and by repeating the process nearly the whole of the material could be converted into the desired physiologically potent l-form. The synthetic compound thus obtained is identical with the natural one both in its physiological and chemical properties.

Besides the original synthesis of adrenaline by Stolz and its modifications mentioned above, a number of other processes have been described in the literature. The following method is due to Nagai:—Diacetyl-protocatechuic aldehyde (X) is condensed with nitromethane and the condensation product (XI) is reduced with zinc dust and acetic acid in the presence of an equivalent amount of 35% formaldehyde to form (XII). After the removal of the zinc, enough hydrochloric acid is added to hydrolyse the acetyl groups and form a salt of the base. When the solution is evaporated in vacuo at a low temperature adrenaline hydrochloride (III) crystallises out. The reactions involved in the synthesis may be represented as below :—



X XI



XII III

In spite of the large improvements in the synthetic production of adrenaline, the natural product still continues to be used in considerable quantities. Adrenaline is one of the few cases in which the natural and the synthetic products have both been in use over a large number of years. This is due to the simplicity of the process of extraction, the yield being almost quantitative. It is 0·1 to 0·24% of the fresh beeves glands or nearly 1·5% of their dry weight, which is the amount actually present, as determined by blood pressure experiments. It has been estimated that a ton of adrenaline is the product from something like twenty million animals. Several million glands are used annually in the manufacture and it is considered that l-adrenaline can be produced more cheaply from the products of the highly organised American packing houses than by synthesis in chemical works.

War time research has indicated that India has vast resources of cattle wealth and that the glands are also of very good quality. The chief difficulty seems to be the lack of organisation for collecting and preserving them.

Uses:—The therapeutic uses of adrenaline are numerous. It is used as an injection before surgical operations in conjunction with cocaine or novocaine to produce localised anaemia and anaesthesia. By constricting the arterioles adrenaline checks undue bleeding and minimises the absorption of the local anaesthetic into the general circulation. As the result the local effect of the anaesthetic is more pronounced and its general toxic effect considerably diminished. Adrenaline is also used in the treatment of allergic conditions like spasmodic asthma and hay-fever. In the former rapid relief is obtained due to dilatation of the bronchial tubes

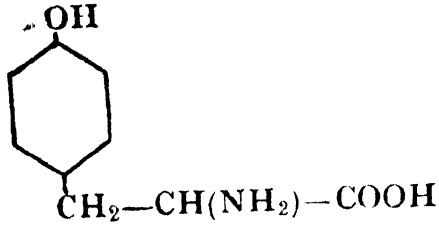
and the spasms cease and normal breathing is restored. It is sold under various trade names like adrenine, epinephrine, suprarenalin and vasoconstrictine. A 1 in 1000 solution of the hydrochloride in water, containing a little acid to prevent decomposition, is very commonly employed by injection. A stronger solution, 1 in 100 is also sometimes employed by spraying in the throat and nose from the mucous membranes of which it is readily absorbed.

Assay:—As there are a large number of adrenaline preparations, natural as well as synthetic, on the market and as the possibility of deterioration during storage is also great, there is need for their control using suitable methods of assay. Amongst them the physiological method is universally applicable and is carried out by measuring the rise in blood pressure; a specially prepared cat (spinal cat) is used for this purpose. The error in this case does not exceed 5%. A number of more rapid chemical methods have been described. They are of three types. The first employs the green colouration obtained with ferric chloride due to the catechol nucleus in adrenaline. Under carefully controlled conditions this is said to yield fairly reliable results. The red colouration produced from adrenaline by the action of a variety of oxidising agents is the basis of the second method. A number of reagents such as iodine, iodic acid and mercuric chloride have been used. Of these iodic acid seems to be the most satisfactory. A third method depends upon the reduction of tungstic acid by adrenaline to deep blue lower oxides of tungsten. The phosphotungstic acid reagent of Folin and Denis is employed for this purpose, the limit of sensitiveness being 1 in 3,000,000.

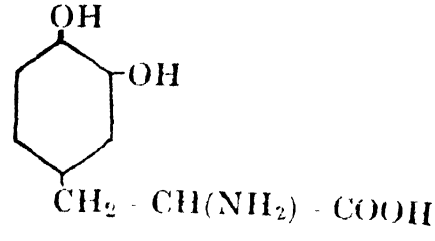
The drawback of the above colorimetric methods is that they are incapable of distinguishing between the various forms of adrenaline, l-, d- and racemic. Certain decomposition products of adrenaline also seem to give similar colour reactions. But this can be checked by the determination of the optical rotation using a polarimeter. The polarimetric study can serve as a reliable

preliminary examination for finding out if a sample of adrenaline is up to the standard or not.

Biochemical origin:—Besides the suprarenal gland adrenaline occurs in the venom of the Central America toad, Bufo agua. It has been estimated that as much as 5% of the dried venom consists of adrenaline. Many other toads have the same kind of venom. The suggestion has been made that tyrosine (XIII) is the ultimate origin of adrenaline in animals, and the first step in the transformation is the introduction of a second hydroxyl group leading to the formation of 3 : 4-dihydroxyphenylalanine (XIV).



XIII

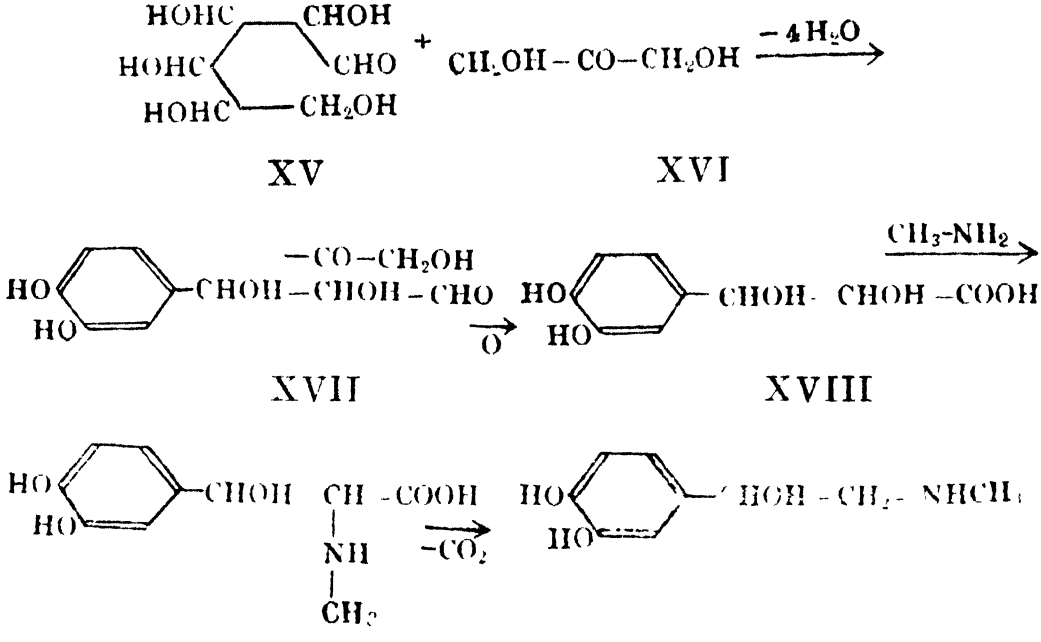


XIV

In support of this idea have been mentioned the occurrence of this dihydroxy compound in certain plants and lower animals and its formation in the course of the conversion of tyrosine into melanin, the pigment of the skin, by means of the meal worm oxidase or of dopase. The fact that an injection of it raises the sugar of the blood is explained as being due to the production of adrenaline from it. Isomeric 2 : 4- and 3 : 6- dihydroxy derivatives of phenylalanine do not possess this property. But the final stages of this scheme relating to the transformation of (XIV) into adrenaline have not been explained.

A better alternative scheme not involving tyrosine as an intermediate may also be considered. It is well established that tyramine, tryptamine, histamine and similar bases are produced in nature by the decarboxylation of tyrosine, tryptophane, histidine and other corresponding amino-acids. On this analogy the immediate precursor of adrenaline should be the amino-acid (XI). According to the theory advocated by (Sir) R. Robinson the 9-C system made up of a benzene ring and a si

chain of 3-C atoms arises from one molecule of hexose and one of triose. Compounds having two hydroxyl groups in the ring are more important and appear to represent earlier stages in evolution. Based on these ideas the stages in the formation of the required amino-acid (XIX) could be represented as below :



Among the reactions involved, the initial condensation and the subsequent oxidation and decarboxylation are known to take place easily in the living organism. The conversion of (XVIII) into (XIX) requires amination of an α-hydroxy acid. This reaction is not so well established as the reverse viz. deamination of an α-amino acid to an α-hydroxy acid. However, there seems to be sufficient support for it from feeding experiments in which certain essential amino acids could be substituted by the corresponding α-hydroxy acids ; the conversion of the latter into the former should therefore be taking place in the animal. Further it has been shown that α-keto acids (which are closely related to α-hydroxy acids) can be converted into the corresponding α-amino compounds under the influence of enzyme systems by the so-called transamination reaction, in which glutamic and aspartic acids are considered to play an important role.

Heavy adrenaline in which all the six hydrogen oms of the catechol nucleus of adrenaline have been placed by deuterium has recently been prepared by the tion of deuterium oxide on adrenaline in alkaline lution. The physiological action of 'heavy' dl- lrenaline is almost indistinguishable from that of dinary dl-adrenaline.

Physiological role:—As has already been mention- , when adrenaline is injected intravenously in minute iantities, it causes a marked rise in the blood pressure. nother important effect is the appearance of hyper- ycaemia (high concentration of blood surgar) leading glycosuria (appearance of sugar in the urine). This due to a disturbance of the normal equilibrium bet- een glycogen and glucose in liver-tissue and its being ifted in favour of glucose. According to Cannon, one the most important functions of the suprarenals is to epare the organism for defence in an emergency. olent emotional states like fear, rage or pain lead to marked discharge of adrenaline from the adrenal ands into the blood, with all its concomitant effects ce increase of blood pressure, quickening of the heart at and mobilisation of sugar reserves. Thus the uscle cells are equipped with sufficient amount of itritive materials for a supreme effort in the act of self- fence. Owing to the constriction of the perepheral ssels liability to external haemorrhage is reduced d thus the animal is safeguarded against any pos- ble injury.

It has been established that minute quantities of renaline are constantly present in the blood. That is must be continually supplied from the suprarenal ands follows from the fact that injected adrenaline ry rapidly disappears from the blood, being destroyed otherwise disposed of. Whether or not the small iounts, normally present in the blood-stream, actually fluence the tone of the vascular system and help to aintain normal blood pressure is yet to be settled. But e marked effect of adrenaline in raising the low blood essure in Addison's disease seems to indicate a close ationship between the functional activity of the

adrenal glands and the maintenance of normal blood pressure.

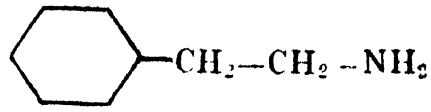
Analogues of Adrenaline:—The molecule of adrenaline (XX) is fairly simple. In view of its remarkable physiological properties a series of analogous substances having various possible combinations of the substituent groups have been made. The simplest of them is phenyl-ethylamine (XXI) and the others are derived from it by substitution in different ways. All of them have the characteristic property of adrenaline and cause a rise in blood pressure by constricting the arterioles. They have been described by Barger and Dale as "sympathomimetics".

As the result of the study of a large number of compounds certain characteristic influences of structural features on pharmacological action have been observed. The absence of hydroxyl groups in the benzene nucleus reduces the intensity but increases the duration of pressor action. Further, the stability of the compound is considerably enhanced. As an example neo-synephrin (XXII) could be mentioned. Though its pressor activity is small (1/25) compared with adrenaline, the duration of action is 5 times as much. Further, solutions of neo-synephrin can be sterilised by boiling whereas adrenaline solutions are unstable to heat.

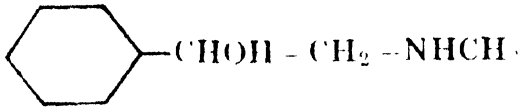
The presence of a methyl substituent in the α-position exerts a remarkable effect. It not only enhances the duration of pressor action, but also confers on the molecule stability to the action of body enzymes. Ephedrine (XXIII) and benzedrine (XXIV) are important compounds coming under this category. They are active when administered orally whereas adrenaline is not. Further, unlike adrenaline, they have a marked stimulant action on the central nervous system and therefore have the characteristic property of banishing sleep. The presence of an isopropylamine side chain and absence of substituents in the benzene ring seem to be factors favourable for this property.



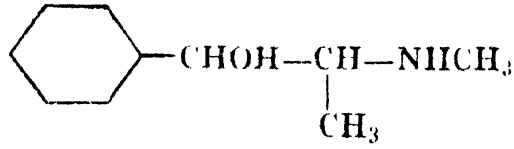
XX



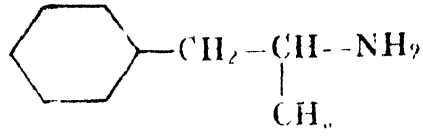
XXI



XXII



XXIII

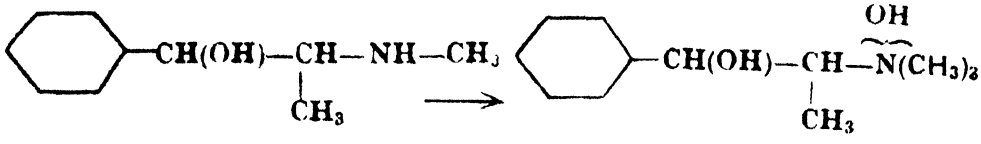


XXIV

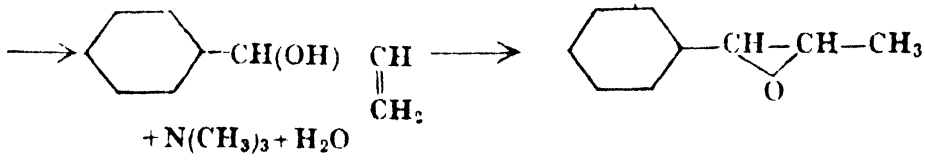
Ephedrine:—Ephedrine is an alkaloidal drug commonly prepared from the dried Chinese herb, Ephedra Sinica and related species. It has recently been found that Indian species of Ephedra are also quite suitable for the isolation of the alkaloid. l-Ephedrine which is the active principle, occurs in conjunction with other related bases, the most important of which is d-ψ-ephedrine. These are isomeric compounds and could be separated by the fractional crystallisation of their oxalates from water. l-Ephedrine oxalate is sparingly soluble whereas the salt of the isomeric compound is quite soluble.

In the determination of the constitution[5] of ephedrine much of the early work related to ψ-ephedrine and the methods were subsequently applied to the case of ephedrine also. It has the molecular formula $C_{10}H_{15}ON$. The existence of a secondary amino group and an alcoholic hydroxyl group is indicated by the formation of a nitroso derivative and a dibenzoyl compound. Degradation of the base with hydrochloric acid yields methylamine and further, during the course of the oxidation of the compound with potassium permanganate the same degradation product could also be obtained. These prove the existence of the grouping $-NHCH_3$. Oxidation with permanganate gives rise to benzoic acid and benzaldehyde, suggesting the presence of a hydroxyl

in the carbon atom of the side chain adjacent to the benzene ring. The constitution could therefore be inferred to be (XXIII). This is supported by exhaustive methylation and subsequent decomposition of the ammonium base (XXV). The products are trimethylamine and α-phenylpropylene-α : β-oxide (XXVI).

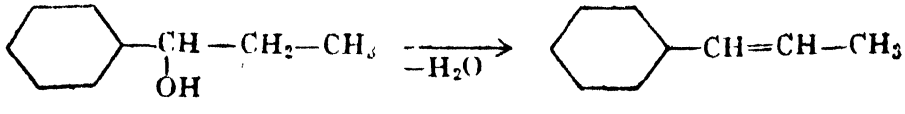


XXIII XXV

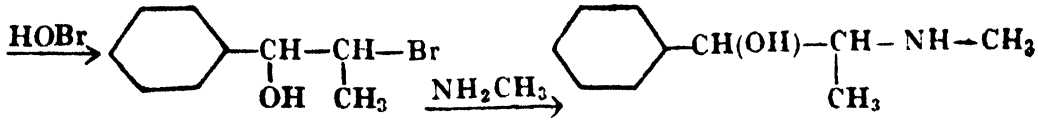


XXVI

A number of methods have been worked out for the synthesis of this useful substance. A convenient procedure due to Forneau[6] starts with phenyl-ethyl carbinol (XXVII) which on dehydration yields propenylbenzene (XXVIII). On treatment with hypobromous acid it yields the corresponding bromhydrin (XXIX) which on condensation with methylamine by heating in a sealed tube gives rise to inactive or racemic ephedrine. Final resolution gives rise to the l-form which is the natural variety.



XXVII XXVIII



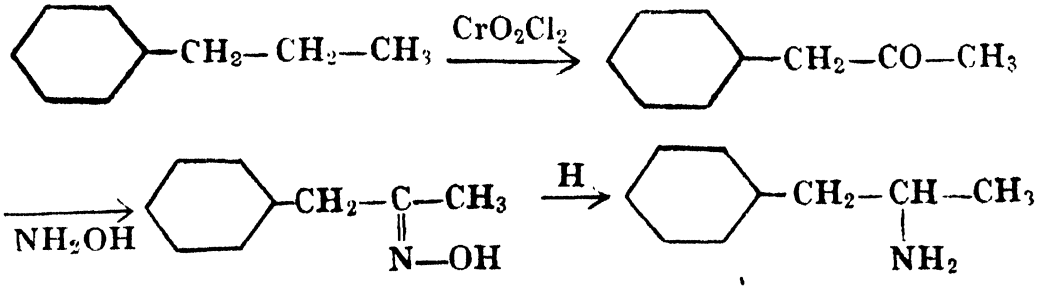
XXIX XXIII Ephedrine

Ephedrine is of low toxicity and does not lead to cumulative poisoning or habit formation. It is largely used in the treatment of asthma, hay fever and other allergic conditions and also to relieve spasmodic cough,

whooping cough and vomiting. Its action is not so powerful as that of adrenaline but it persists for a much longer time, and further it is effective even when given by the mouth. As already mentioned these characteristics of ephedrine are attributed to the absence of phenolic hydroxyl groups and the presence of an isopropylamine side chain.

Benzedrine:—This substance was originally used as an ephedrine substitute but is not suitable for this purpose on account of its inordinate stimulation of the central nervous system. It is, however, finding other uses in virtue of its capacity to banish sleep. It is also employed as an inhalant to remove congestion from the nose in the case of cold.

Benzedrine is prepared[7] from propylbenzene which yields on oxidation with chromyl chloride, benzyl-methyl ketone and not phenyl propionic aldehyde. The oxime of the ketone is reduced by means of sodium amalgam in dilute acetic acid medium and the racemic base is obtained. It is a colourless oil boiling at 205° and is a strong base readily combining with carbon dioxide. The hydrochloride melts at 145° and is extremely hygroscopic.



Benzedrine

CHAPTER XVII

THYROXINE

The thyroid gland which is situated in the throat plays an important part in the maintenance of basal metabolism and growth and also in the development of faculties both mental and physical. It is the largest of the endocrine glands and weighs about 30 grams in man. Defective thyroid secretion leads to exophthalmic goitre, myxoedema and cretinism. The former is due to excess of activity and the latter to subnormal activity. The story of the discovery of the thyroid hormone began with the efforts to counteract thyroid deficiency which marked the first great triumph of gland therapy. In 1882 Kocher recognised that the operative removal of the thyroid in goitre produced symptoms similar to those of myxoedema, namely, thickening of the skin, loss of hair and failure of mental activity. This led successively to experiments on the trasplantation of normal thyroid tissue into the myxoedematous patient, to the injection of extracts and to the oral administration of the gland. These modes of treatment were found to be quite effective in myxoedema and also in cretinism. The favourable effect of iodine therapy and the subsequent discovery that iodine is a normal constituent of the thyroid gland indicated that the active principle concerned is associated with iodine.

Isolation[1]:—After a great deal of valuable exploratory work carried out by a number of workers on the isolation of the thyroid hormone success was first achieved by Kendall in America (1919). The hormone is now known to exist in the thyroid gland in peptide combination as a constituent amino acid of the characteristic thyroid protein, iodothyroglobulin. Hydrolysis is therefore necessary for liberating it. Kendall conducted the operation in two stages, first with aqueous sodium hydroxide and subsequently with baryta. He utilised the acidic nature of thyroxine and was guided by iodine estimations of the crude products at various stages of the

extraction and purification. Thus he obtained a pure crystalline substance containing 65% of iodine and having the physiological activity of the thyroid gland. He called it thyroxine. His attempts to elucidate the constitution of this hormone were unsuccessful mainly due to the very small yield of the compound that he got.

Harington, whose name is intimately associated with the development of the chemistry of this hormone, had therefore first to solve this initial difficulty of the yield in the isolation of the substance. In this he was highly successful (1926). He employed good quality dried gland and greatly improved Kendall's method of isolation resulting in about 25 times as much yield. He thus obtained enough material to determine the correct constitution of thyroxine (1926) which subsequently enabled Barger and Harington to synthesise it (1927). The essence of Harington's method is the use of hot baryta for hydrolytic extraction of thyroxine, first using a 10% solution and later a 40% solution. With glands rich in iodine a considerable proportion of the hormone separates even after the first hydrolysis in the form of the crude barium salt. After final hydrolysis with 40% baryta almost the whole quantity of the hormone comes out as this salt. It is decomposed by suspending it in hot 1% sodium hydroxide and adding a slight excess of sodium sulphate solution. Barium sulphate thereby gets precipitated and the sodium salt of thyroxine is left in solution. From this, crude thyroxine could be obtained in a granular condition by acidifying when hot. It is subsequently purified by dissolving in alcoholic alkali and acidifying the clear solution with acetic acid. A better method of purification is to make use of the sparing solubility of the sodium salt. The crude material is dissolved in boiling 0·5% sodium carbonate, cooled and allowed to stand in the ice-chest. The sodium salt is collected, dissolved in alcoholic alkali and decomposed with acetic acid.

The sample of thyroxine obtained by the above method is racemic and melts at 231° and decomposes above 250°. By employing the method of enzymic hydrolysis with trypsin Harington succeeded later (1930) in

obtaining a small sample of the optically active (l-) hormone from the thyroid gland. This sample melted at 235° and had an optical rotation, $[\alpha]_{5461}$, of $-3{\cdot}8°$ as the sodium salt in 60% alcohol solution.

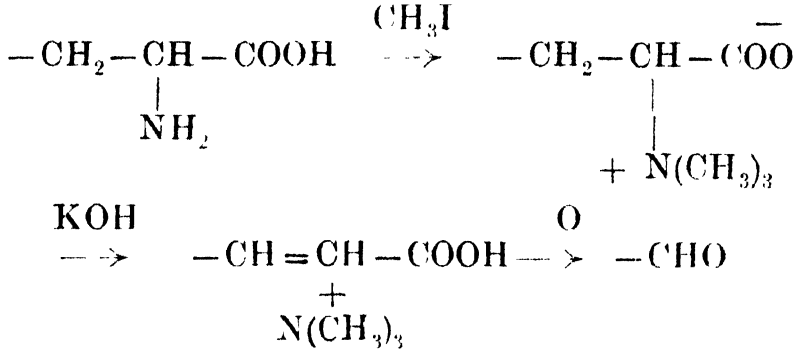
Properties:—Thyroxine crystallises as colourless rosettes and sheaves of fine needles. It is very sparingly soluble in water and insoluble in organic solvents but it dissolves fairly easily in ammonia and alcoholic alkalies. Its characteristic physiological property is its capacity to increase the rate of basal metabolism.

Constitution[2]: Since 65% of the molecule of thyroxine is iodine there was serious difficulty in interpreting analytical data and determining the exact number of the different atoms present. Further due to the sparing solubility of thyroxine, its molecular weight could not be found out. There was also difficulty in studying the degradation of thyroxine itself, since the products which would contain iodine, could not be easily recognised owing to the lack of necessary reference compounds. It was therefore necessary to replace the iodine atoms by hydrogen without changing the rest of the molecular structure. An elegant method adopted by Harington for this purpose involved the shaking of an alkaline solution of thyroxine with colloidal palladium in a hydrogen atmosphere. This removed the iodine quantitatively as iodide and the amount of hydrogen used up was found to correspond exactly to the amount of iodine split off, so that no extra addition of hydrogen had taken place. The product which was free from iodine was originally referred to as des-iodothyroxine but was subsequently named thyronine. Its analysis could be interpreted correctly, and since it was fairly soluble its molecular weight could be determined. It was found to have the composition $C_{15}H_{15}O_4N$ and from the iodine content of thyroxine it followed that thyroxine has four atoms of iodine in it and that it has the molecular formula $C_{15}H_{11}O_4NI_4$.

The first step in the determination of the constitution of thyronine was the recognition that it was an α-amino acid. This was made out from the fact that on treating the substance with nitrous acid (Van Slyke's method)

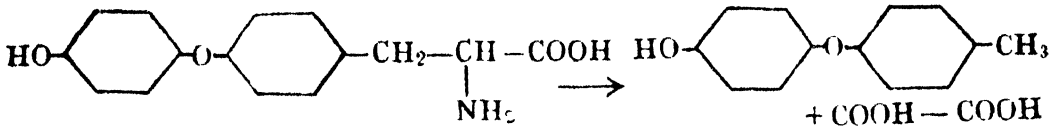
the whole of the nitrogen was eliminated and that an intense violet colouration was produced on boiling it with a solution of triketohydrindene hydrate. The second reaction is a characteristic of α-amino acids. The substance further gave the Millon's reaction which is specific for compounds containing a hydroxy-phenyl unit. Thus of the four oxygen atoms two are in the carboxyl and a third in the phenolic hydroxyl group. The fourth oxygen atom was rather unreactive and was probably involved in an ether linkage.

The next step was to break down the amino acid side chain by known stages. For this purpose the substance was fully methylated by heating with methyl iodide in alkaline alcoholic solution. The product was a betaine containing four methyl groups, one having entered the phenolic hydroxyl and three being linked with the nitrogen. On heating it with concentrated potassium hydroxide nitrogen was lost as trimethylamine and an unsaturated acid was left behind. On oxidation, this product yielded an aldehyde. These reactions are characteristic of compounds having an alanine side chain. The various stages can be represented as below :

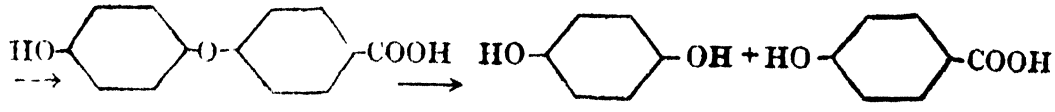


Thyronine could then be represented by the partial formula $HO.C_6H_4(C_6H_4O).CH_2.CHNH_2.CO_2H$. The nature and the mode of linking of the unknown residue was rendered clear by a study of the products obtained by the gentle fusion of the compound with potash (180-222°). Four substances were recognised of which the following three, quinol, p-hydroxybenzoic acid and oxalic acid were present only in small quantities. The main product was a phenol having the formula $C_{13}H_{12}O_2$. It was evidently the primary product of the potash fusion. From its reactions it was identified as

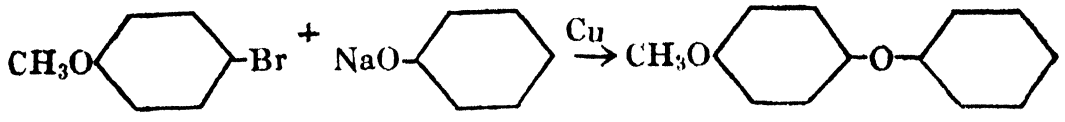
4-hydroxy-4′-methyl-diphenyl ether (II) and the identity was confirmed by synthesis. The degradation of thyronine (I) by means of alkali may then be represented as given below. Oxalic acid is derived from the side chain of the molecule and the others are obtained from the diphenyl ether by further change. The break down of the diphenyl ether system takes place after initial oxidation of the methyl group.



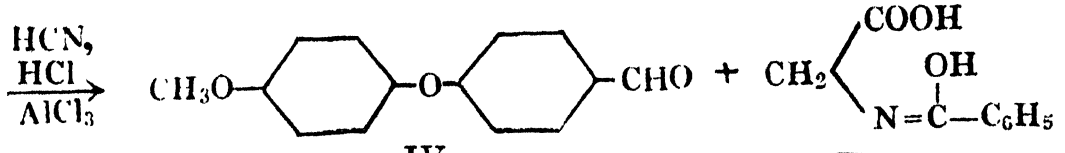
I Thyronine II



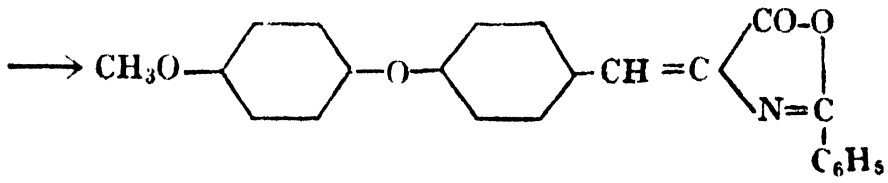
The constitution of thyronine was thus found to be as represented in (I) and this was confirmed by synthesis. 4-Methoxy-diphenyl ether (III) was made by the condensation of sodium phenate with p-bromoanisole in the presence of copper powder (Ullmann's reaction). An aldehyde group was then introduced in the 4′ position (IV) by the method of Gattermann using anhydrous hydrocyanic acid under the influence of hydrogen chloride and a condensing agent, zinc or aluminium chloride. The alanine side chain was then built up by condensation with hippuric acid to form an azlactone (V) and subsequent hydrolysis and reduction. Concentrated hydriodic acid and phosphorus brought about the final reactions very satisfactorily including reduction, debenzoylation and demethylation. The product (I) was identical with thyronine.



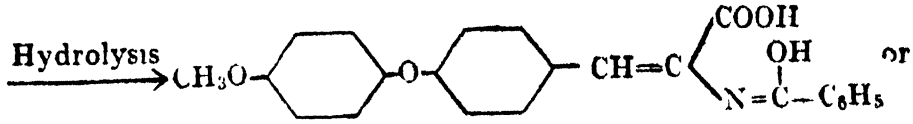
III

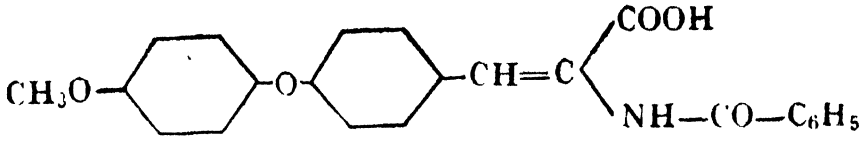


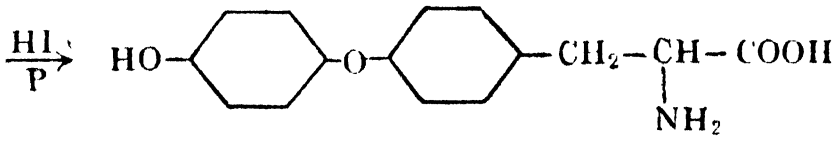
IV Hippuric acid



V



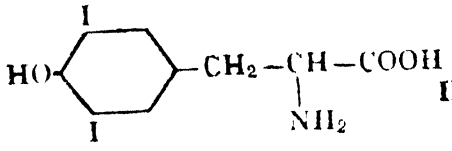




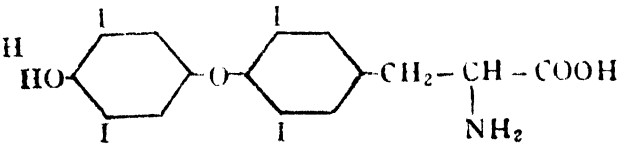
I

After the constitution of the iodine-free compound had thus been established the next step was to ascertain the position of the four iodine atoms in thyroxine itself. This was done partly by analogy and partly from a consideration of the reactions of thyroxine. The relationship between thyronine and tyrosine is very close. One of the very few naturally occurring organic iodine compounds of known constitution is 3 : 5-diiodotyrosine (VI) which is present in the skeletal portions of corals and which can also be obtained by the hydrolysis of the common bath sponge. It seemed therefore plausible that thyroxine is derived from two molecules of diiodotyrosine, one molecule having lost its side chain (VII). The experimental support for this was a colour reaction given by thyroxine. When it was treated with nitrous acid a yellow colour was produced which deepened on boiling and changed to red when the liquid was cooled and rendered alkaline with ammonia. This reaction is characteristic of phenols iodinated in both ortho positions. Further when thyroxine was fused with potash at a high temperature it gave products which showed pyrogallol reactions. From this it was clear that the

molecule passed into 3 : 4 : 5-trihydroxy derivatives. This reaction is in conformity with the structure (VII) for the hormone. But the most definite evidence came from the synthesis of the compound itself as described below.



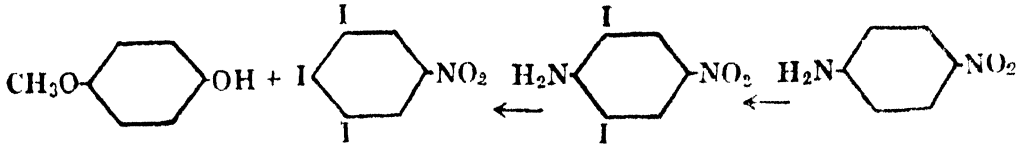
VI



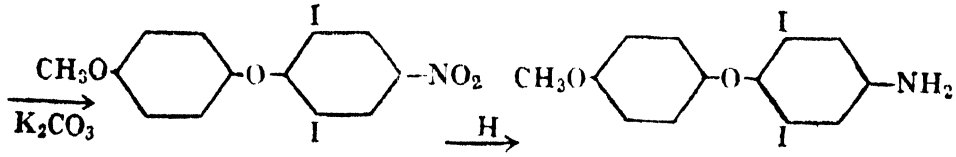
VII Thyroxine

Synthesis :—The main difficulty in the synthesis of thyroxine was due to the fact that the iodine atoms, particularly of the inner phenyl ring, could not be introduced after the main structure had been completed. Consequently a benzene derivative with the iodine atoms already in the proper positions had to be employed. The starting point was the commercial substance p-nitraniline (VIII) which was iodinated to the diiodo compound (IX) and the amino group subsequently replaced by an iodine atom according to the method of Sandmeyer. The immediate product was 3 : 4 : 5-triiodonitrobenzene (X). This compound was condensed with hydroquinone mono-methyl ether (XI). The reaction took place with facility due to the high reactivity conferred on the iodine atom in position 4 by the nitro group. It took place at a low temperature in methyl ethyl ketone solution in the presence of finely powdered potassium carbonate as the condensing agent. In the resulting diphenyl ether (XII) the nitro group was reduced (XIII) and the amino group replaced by a cyanogen group according to the method of Sandmeyer thus yielding the cyanide (XIV). It could be converted into the corresponding aldehyde (XV) by reduction with anhydrous stannous chloride. The condensation of the aldehyde with hippuric acid was carried out as already described under thyronine, and the subsequent reduction, demethylation and debenzoylation proceeded smoothly with hydriodic acid in the presence of phosphorus when some quantity of acetic anhydride was also used in order to improve the solubility. The final introduction of the required pair of iodine atoms was successfully accom-

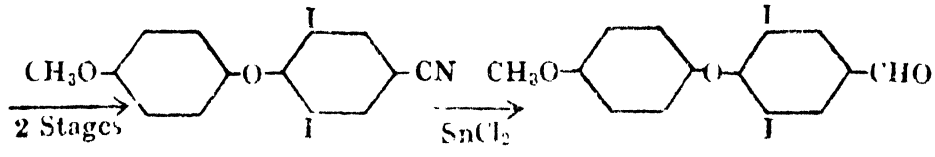
plished by dissolving the diiodoamino acid (XVI) in concentrated ammonia and adding the calculated quantity of potassium triiodide solution. When the free acid was liberated it was found to be identical with dl-thyroxine from the glands.



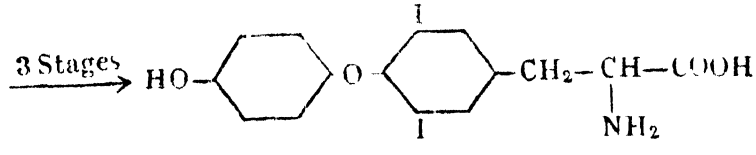
XI X IX VIII



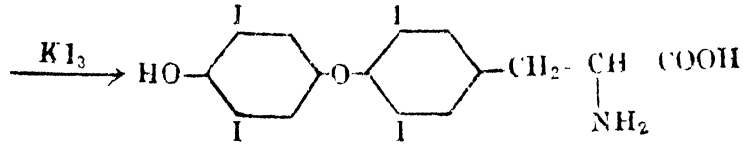
XII XIII



XIV XV



XVI



VII Thyroxine

As could be seen there are eleven stages in the synthesis of thyroxine and the final yield will very much depend upon the efficiency of the transformations at each stage. All the reactions have now been made to yield well over 50%; still due to the large number of steps involved the final yield of thyroxine is about 5% of the p-nitraniline used. Even then it can be easily made at about half the price of the natural product obtained by the improved method of Harington.

The resolution of the synthetic dl-compound into its optical antipodes was not feasible on account of its sparing solubility. Harington (1928) overcame the difficulty by resolving dl-3 : 5-diiodothyronine (XVI) into the d- and l-forms and introducing two additional iodine atoms into the 3′ and 5′ positions of each separately in the same manner as with the racemic compound (XVI) itself. The sample of l-thyroxine obtained by this method melted at 235° with decomposition and had an optical activity, $[\alpha]^{21°}_{5461}$, of −3·20° in alcoholic alkali, while the sample of d-thyroxine melted at 237° with decomposition and had the specific rotation of + 2·97° in alcoholic alkali. A mixture of the synthetic d- and l-specimens sintered at 228° and melted at 231°.

A comparison of the physiological activity of the synthetic d- and l-forms of thyroxine employing the growth of tadpoles showed that both the forms were active in small concentrations, the l-form in slightly smaller concentrations than the d-. However, employing the method of oxygen consumption of rats, both preparations were found to produce definite effects in doses of 4 mg. per kilogram body weight and the l-form appeared to be three times as potent as d-thyroxine.

Two isomerides of thyroxine with modifications in the left half of the molecule have been prepared, (1) 2′ : 4′-diiodo-3′-hydroxy compound and (2) 3′ : 5′-diiodo-2′-hydroxy compound. The former is physiologically inactive whereas the latter has about one twenty-fifth of the activity of thyroxine.

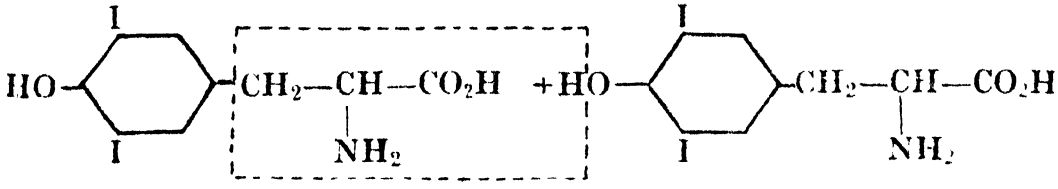
There are two important official preparations containing thyroxine. They are (1) desiccated thyroid (fat-free) which should contain 0·1% of iodine in combination as thyroxine and (2) thyroxine-sodium which is the sodium salt of thyroxine and contains 61-65% of iodine.

Assay :—As mentioned in an earlier para the biological method involves the study of the growth of tadpoles or of oxygen consumption of rats. The metamorphosis of tadpoles is hastened by feeding them the gland or thyroxine. The shortening of the tail has been used for quantitative studies of thyroid activity. The effect

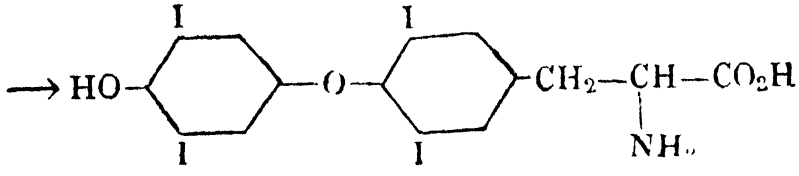
on basal metabolism is perhaps more important. The injection of 1 mg. of thyroxine into a patient suffering from myxoedema will increase the basal metabolism by 2·8%. But the pharmacopoeial method is chemical and is based on the estimation of iodine in the liberated thyroxine sample.

Thyroid is assayed by first boiling it with aqueous sodium hydroxide to hydrolyse the iodo-thyroglobulin, precipitating crude thyroxine from the hydrolytic extract by acidification and subsequently estimating the iodine in the precipitate in the same manner as in the case of thyroxine-sodium. The iodine in the latter compound is determined by fusing a known weight of it with excess of solid sodium carbonate thus converting the organic iodine into the inorganic form as sodium iodide. The excess of sodium carbonate is decomposed and the sodium iodide is quantitatively oxidised to iodate by treatment with excess of bromine. After the removal of the unused excess of bromine by means of a little phenol, excess of potassium iodide is added when free iodine proportionate to the amount of the iodate is liberated and is estimated by titration with a standard solution of sodium thiosulphate.

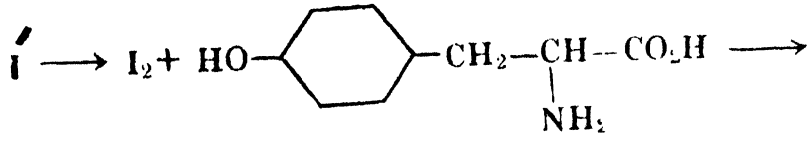
Biogenesis of Thyroxine and iodination of proteins: — Speculation regarding the biochemical origin of thyroxine actually preceded the final proof of its constitution. The immediate precursor was considered to be 3:5-di-iodotyrosine and the change was represented as in (XVII) given below. As already mentioned this hypothesis was helpful in arriving at the final constitution of thyroxine which was later confirmed by synthesis. Subsequent investigations have not only proved the correctness of the idea, but have further provided valuable information relating to the mechanism of the reaction and opened out possibilities of preparing the hormone by the iodination of proteins.



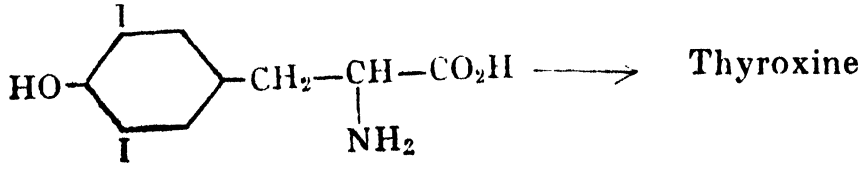
XVII



When thyroxine was discovered, 3:5 - di-iodotyrosine was the only other naturally occurring amino acid containing iodine It was first known to occur in corals and sponge Careful analysis of the thyroid gland showed that it is also an important source; as a matter of fact the total iodine of the gland is distributed between thyroxine and di-iodotyrosine, the latter being slightly in excess This association definitely indicated that the former can arise from the latter. Such a relationship was also supported by the fact that natural tyrosine, its diiodo-derivative and natural thyroxine have the same l-configuration. But the most interesting results were obtained by tracing the movement of iodine in the animal body using radioactive iodine. Most of the iodine administered as iodide, moved to the thyroid and was soon found in organic combination first as the di-iodotyrosine and subsequently as the thyroxine component of the characteristic protein of the gland, thyroglobulin.



XVIII



The whole process is considered to take place in the thyroid and the preliminary conversion of the iodide

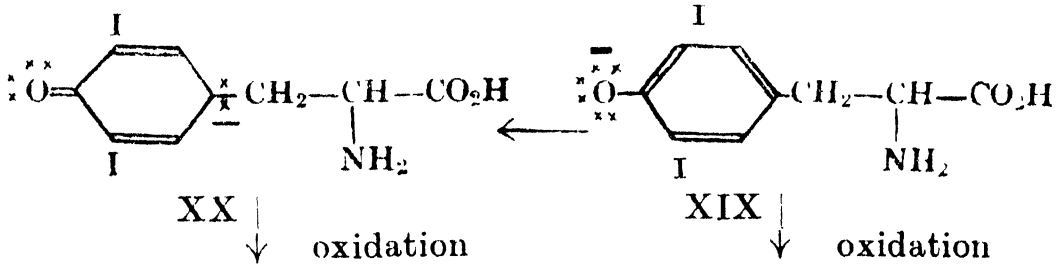
into iodine involves an oxidative system. Regarding the second stage in the above scheme (XVIII), earlier work on the iodination of proteins has revealed that the tyrosine unit is a remarkably efficient iodine-binding constituent of the protein molecule. In fact it is possible by choosing proper conditions to titrate the tyrosine in a protein with iodine to form di-iodotyrosine almost quantitatively.

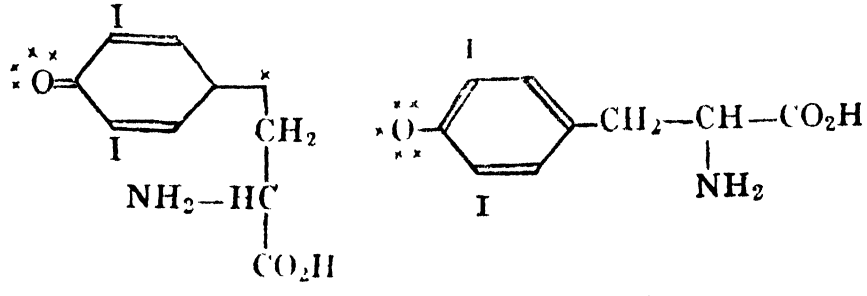
Our knowledge of the final stage of the biogenesis began with the publication of the important work of Ludwig and Mutzenbecher [4] in 1939. They described a method of iodination of casein and other proteins yielding physiologically active products from which it was actually possible to isolate thyroxine. It was comparatively drastic employing an alkaline medium and an elevated temperature and appeared to be favourable for oxidative side reactions probably involving sodium hypoiodite. For the successful preparation of thyroxine by this process, the conditions have to be carefully controlled. The reaction is quite complex and seems to depend in an unknown way on the detailed structure of the protein. Simple considerations of the presence of tyrosine units in the protein and of the addition of the requisite amount of iodine are not enough. However, the discovery is very important as it enables the development of alternative sources for thyroxine. According to the experiments of Harington, [5] commercial casein was treated with 20% of its weight of iodine in the presence of sodium bicarbonate (p_H 8·5) at 37°. The product contained 7·7 to 8·7% of organic iodine and possessed thyroid activity equal to $\frac{1}{300}$ of that of thyroxine. Hydrolysis with barium hydroxide yielded an active fraction containing 37-45% of iodine; from this pure thyroxine could be obtained readily. The yield of the crystalline hormone was about 100 mg. from 100 grams of iodinated casein. Better yields (400 mg.) are claimed by Reinecke and Turner [6] in their more recent work.

Von Mutzenbecher [7] later reported that thyroxine is formed in small quantities (0.23%) during prolonged incubation of di-iodotyrosine in a slightly alkaline medium. The conditions were considerably modified by Harington

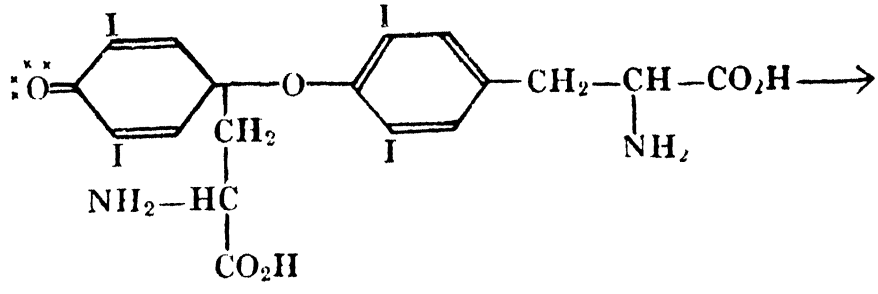
leading to a yield of 3·4%. Di-iodotyrosine was dissolved in enough sodium hydroxide to have the p_H at 10 and oxidised by means of 20% hydrogen peroxide. In order to save thyroxine from oxidative destruction it was removed, as it was being formed, from further contact with the oxidising agent by shaking with butyl alcohol.

Harington[8] has given an explanation of this reaction. Since it takes place in an alkaline medium anions of di-iodotyrosine are involved. The two electromeric forms (XIX) and (XX) suffer oxidation by losing one electron each and give rise to reactive radicals (XXI) and (XXII) which combine together (XXIII) with the elimination of the side chain yielding thyroxine. The presence of iodine atoms in the positions ortho to the hydroxyl group inhibits reaction in these positions and makes it go in the desired manner involving the para position.

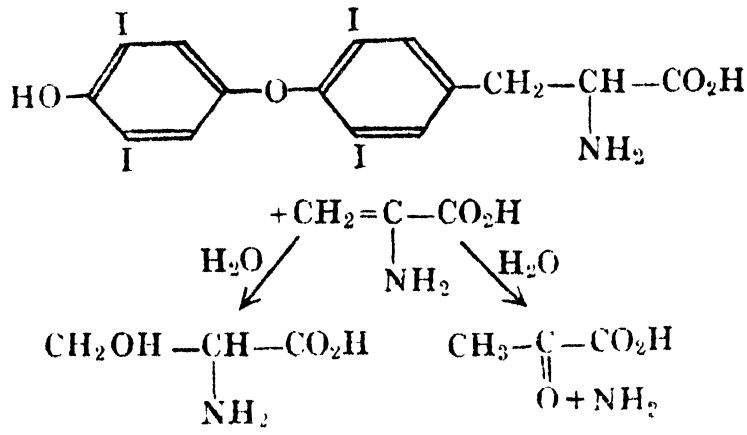




XXII XXI



XXIII

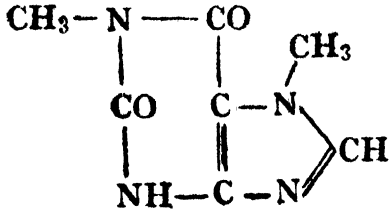


Important support for the above mechanism could be provided if the lost side chain could be detected in the form of serine or pyruvic acid. Johnson and Tewkesbury[9] claimed that they were able to identify pyruvic acid and ammonia among the oxidation products. But this does not seem to be conclusive.

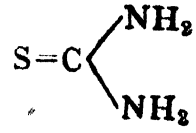
Summing up the results so far obtained the following stages in the biogenesis of thyroxine are now clear. Iodine taken in the food is absorbed as iodide; by the action of oxidising systems in the body tissues free iodine is liberated. This has two functions, (1) iodinating tyrosine and (2) oxidising the resulting di-iodotyrosine to thyroxine. Owing to the special capacity of the thyroid gland to concentrate iodine, the process of synthesis of thyroxine and its subsequent storage takes place almost entirely in this gland.

Antithyroids:—In the animal body the activity of thyroxine seems to be in a way controlled by the presence of an antithyroid substance known as paraxanthine.[10] Its chemical constitution is 1 : 7 dimethylxanthine (XXIV) and it has been found to be present in the liver and in urine. It is considered to control the metabolic rate by interaction with thyroxine. A similar reduction of thyroid activity is also brought about by the administration of thiourea and related compounds. They have been shown to be capable of rectifying hyperthyroidism known as Graves' disease. Experiments indicate that they function by inhibiting the synthesis of thyroxine by the gland rather than by neutralising its effect by chemical interaction. Thiourea (XXV) has

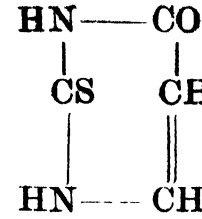
the clinical disadvantage of producing nausea; thiouracil (XXVI) is claimed to be free from this defect. Sulphanilamide has also been shown to possess this inhibiting property of thiourea.



XXIV



XXV



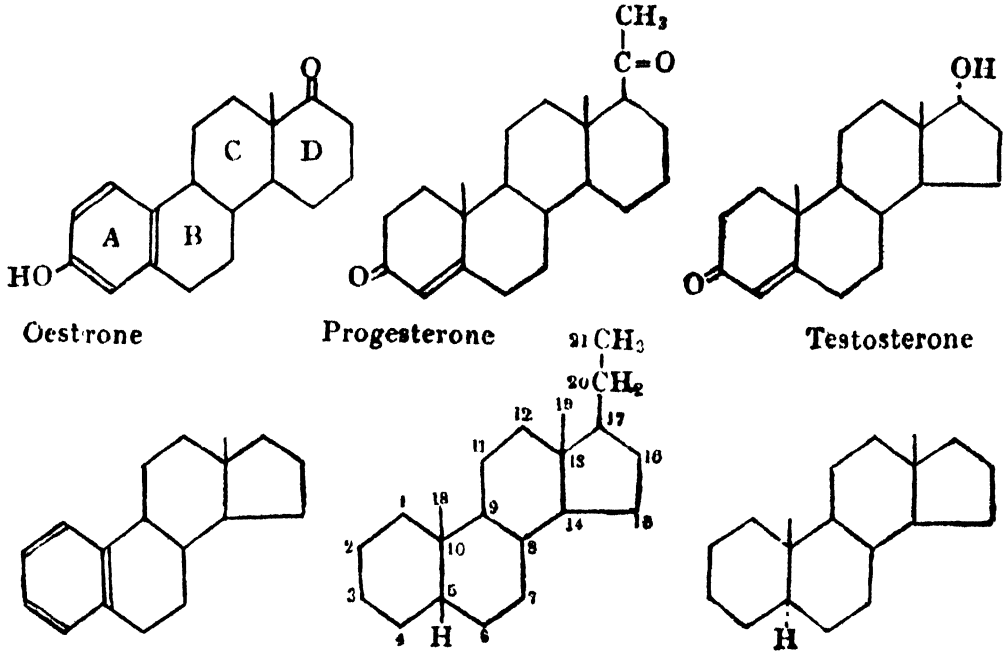
XXVI

CHAPTER XVIII

SEX HORMONES

Though the physiological effects of castration were known from ancient times, close investigation of the gonads as organs of internal secretion really started about the middle of the last century. By studying the results of castration and of implantation of the sex glands, physiologists were able to determine some of the effects due to their secretion. The effects of the gland extracts on castrated animals were later investigated. As the result, definite methods of bioassay were developed for the evaluation of these hormones.

Active study of the chemistry of these substances really started somewhere about 1929 and its progress ever since has been remarkable. Three important types have been isolated and their complicated molecular structures completely elucidated. Further, methods have been developed for obtaining them synthetically and at the same time some simpler synthetic analogues of great value have also been made. The sex hormones are structurally related to the sterols and are probably derived from them. Consequently they are known also as the steroid hormones. The three types are : (1) female hormones or oestrogenic hormones, (2) pregnancy hormone or corpus luteum hormone and (3) male hormones or androgenic hormones. Typical members of these groups are given below along with their structural formulae. Their systematic names are derived with reference to the fundamental hydrocarbons, oestrane (C_{18}), pregnane (C_{21}) and androstane (C_{19}). The numbering of the carbon atoms that has been generally adopted in this group of compounds is also indicated :



The pituitary :—The production of these hormones by the gonads is regulated by the secretions of the anterior lobe of the pituitary. The pituitary which is just the size of a large pea in adult man, is one of the most important of the hormonal glands. It is situated at the base of the brain and consists of an anterior lobe, a posterior lobe and an intermediate part. Each seems to be having definite functions. Extracts of the posterior lobe contain at least two active substances, one of which has the characteristic property of bringing about contraction of the uterus and the other of causing constriction of the capillaries and contraction of the intestines. These extracts also have an antidiuretic effect and are useful in the treatment of a disease called diabetes insipidus. The intermediate part of the gland contains a principle which makes frogs black. This effect on the colour is due to the expansion of special black cells in the skin. Its precise function in the human organism is not known. The anterior lobe is by far the most important and has received much attention. It has been called the 'dictator-gland' and also as the 'conductor of the gland orchestra.' Extracts of the anterior pituitary contain a large number of active principles (six have been so far definitely recognised) which produce marked

effects of various kinds. These effects can be divided into two types, general and specific.

The general effects involve the metabolism of the whole body. An animal deprived of its pituitary does not grow but remains a dwarf. The condition can be corrected by the injection of extracts of the anterior lobe of the pituitary. Over-activity of the gland produces giants. It has also an effect on sugar, protein and fat metabolism. A glycotropic substance generated by this lobe tends to increase blood sugar and thus has an effect opposed to insulin.

The specific effects are mostly on other hormonal glands. Removal of the pituitary causes atrophy of the suprarenals, the thyroid and the sex glands. The injection of suitable extracts stimulates them to activity again. The stimulating effect of the pituitary on the other glands seems to be to some extent mutual. Extracts of the anterior pituitary contain two gonadotropic substances which are capable of stimulating the sex glands of both the male and the female. Another active substance of the pituitary is the lactogenic hormone, prolactin. It is a protein and has been obtained in a crystalline condition.

Very little is known about the chemistry of these active principles but the gland extracts find large use. Commercial preparations are made from the pituitary glands of cattle and they are standardised biologically.

CHAPTER XIX

OESTROGENIC HORMONES

Occurrence : —These hormones were the first among the sex hormones to be obtained in a pure and crystalline condition. The ovaries and follicles of animals were the earlier materials to be employed but working with these was found to be extremely difficult. Their isolation and study were considerably simplified due to the discovery by Aschheim and Zondek in 1927 that they are present in human pregnancy urine. But far more potent sources were later discovered in the urine of pregnant mares and finally in the urine of the stallion. The former yields 100,000 mouse units per litre and 1,000,000 M.U. per day whereas the stallion urine is richer yielding 170,000 M.U. per litre. As compared with these human pregnancy urine is much poorer, having only about 21,000 M.U. per litre. The extraordinary occurrence of such large quantities of female hormones in the urine of male animals is characteristic of the horse group (equidae): male zebra yields 36,000 M.U. per litre of urine. However, it should be noted that smaller quantities are present in all urines including those of the males of all species. A more interesting point is that the stallion urine is a more convenient source since the hormone oestrone is very readily obtained pure from it. A similar observation relates to the existence of considerable amounts of oestrogenic hormones in the male sex gland. Obviously at some stage or other female and male sex hormones have common ground. It is therefore to be emphasised that the term 'female sex hormone' refers to the activity of the material and not to its source. A similar statement holds good for male hormones also.

Oestrogenic hormones also occur in the vegetable kingdom as well as in certain lower animals. Pure oestrone has been isolated from palm-kernel extract and oestriol from female willow flowers. What part these

hormones play in plant physiology still remains obscure. Their distribution in nature seems to be still a biochemical puzzle.

Isolation[1]:—The procedure employed for the isolation and purification of the oestrogenic hormones is rather complex, since it has to effect concentration of about a million-fold and separation of substances that are very similar. Pregnancy urine of women and of mares or the urine of the stallion is employed as a convenient source. The hormones are present largely as glucuronides or sulphates and have therefore to be liberated by heating after the addition of concentrated hydrochloric acid. They are then extracted with organic solvents, the extracts are rendered free of acid, and the material is purified to a considerable extent by partition between immiscible solvents. Since the oestrogens are phenolic compounds and stable under alkaline conditions they are separated as a group from others by means of alkali. Among themselves there are differences in solubility in different solvents and this could be used for further separation. For example oestrone and oestriol are major components of oestrogen mixtures and they could be separated by using the immiscible solvents, benzene and aqueous alcohol. Oestrone is found in the benzene layer and oestriol in the aqueous alcohol layer. The introduction of a new reagent by Girard (Girard's reagent T: $(CH_3)_3\underbrace{N}_{Cl}—CH_2—CO—NH—NH_2$) renders the isolation of ketonic members of the hormone group easier. It reacts readily with ketones to form water-soluble derivatives. Fatty matter and non-ketonic hormones can then be removed by extraction with ether. The ketonic substances are subsequently liberated by warming with excess of dilute acid. The final purification of the hormones is effected by distillation in high vacuum followed by recrystallisation.

Eight compounds of the oestrogenic group have so far been isolated from urine and tissues of the genital organs. Of these three are more important.

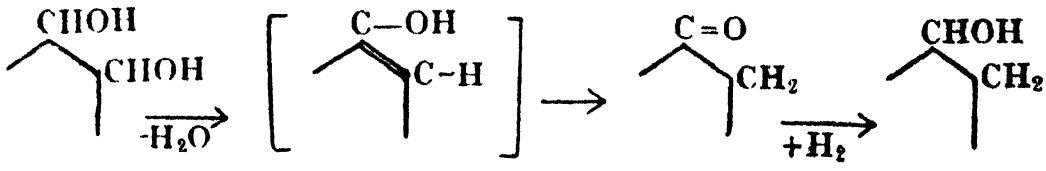
(1) **Oestrone** also called oestrin and theelin, is easily soluble in alcohol and can be crystallised from it. Its

melting point has been given as 254, 256 and 259°. It has a specific rotation of + 156° in dioxane solution. It contains a phenolic and a ketonic group and has the formula $C_{18}H_{22}O_2$.

(2) **Oestriol** is a trihydroxy compound; one of the hydroxyl groups is phenolic and the others are alcoholic. It is sparingly soluble in water and ether and readily soluble in aqueous alkali. It melts at 283° and has a specific rotation of + 30° in dioxane. It has the formula $C_{18}H_{24}O_3$. Its close relationship to oestrone is indicated by the fact that when dehydrated with potassium bisulphate it yields oestrone. Two hydroxyl groups should therefore be contiguous. It contains no ketonic group. Compared with oestrone it has only feeble activity.

(3) **Oestradiols**[2]:—By the reduction of oestrone in alkaline media two oestradiols, α- and β- are formed and they can be separated by fractional crystallisation from acetone. They are found in the urine of pregnant mares and are stereoisomeric. The α-compound melts at 178° and has a specific rotation of + 80° in dioxane solution. It has the trans configuration (OH at C_{17} is trans to CH_3 at C_{13}) and is the most potent of the naturally occurring oestrogens. It is considered to be the true ovarian hormone since it has been found in the ovaries of pigs. On account of its high activity it is preferred to oestrone and hence is commercially prepared from it. Its benzoyl derivative (C_3-benzoyl) has a prolonged and better action and it has not only been used widely for therapeutic purposes but has also been adopted as a standard for the assay of oestrogenic preparations. β-Oestradiol melts at 223° and has a specific rotation of + 54° in dioxane. It has the cis configuration and is less active than oestrone. α- and β-Oestradiols have the molecular formula $C_{18}H_{24}O_2$. They have no ketonic properties, but have two hydroxyl groups, one of which is phenolic and the other alcoholic (secondary).

Constitution:—The structural investigations of these compounds are mainly due to Butenandt, Doisy and Marrian. From the above description of the three hormones it is clear that they are closely related. The relationship can be represented as below:



Oestriol Oestrone Oestradiol

Physical studies such as surface film and X-ray measurements suggested even at an early stage that the oestrogens are structurally related to sterols. Experiments on catalytic hydrogenation indicated the existence of three double bonds belonging to an aromatic ring. A hydroxyl is situated in it and this accounts for the phenolic properties of the compounds. All of them exhibit selective absorption in the ultra-violet at 280-85 mμ. and this is closely similar to that of phenolic bodies. The other three rings of the main structure are saturated.

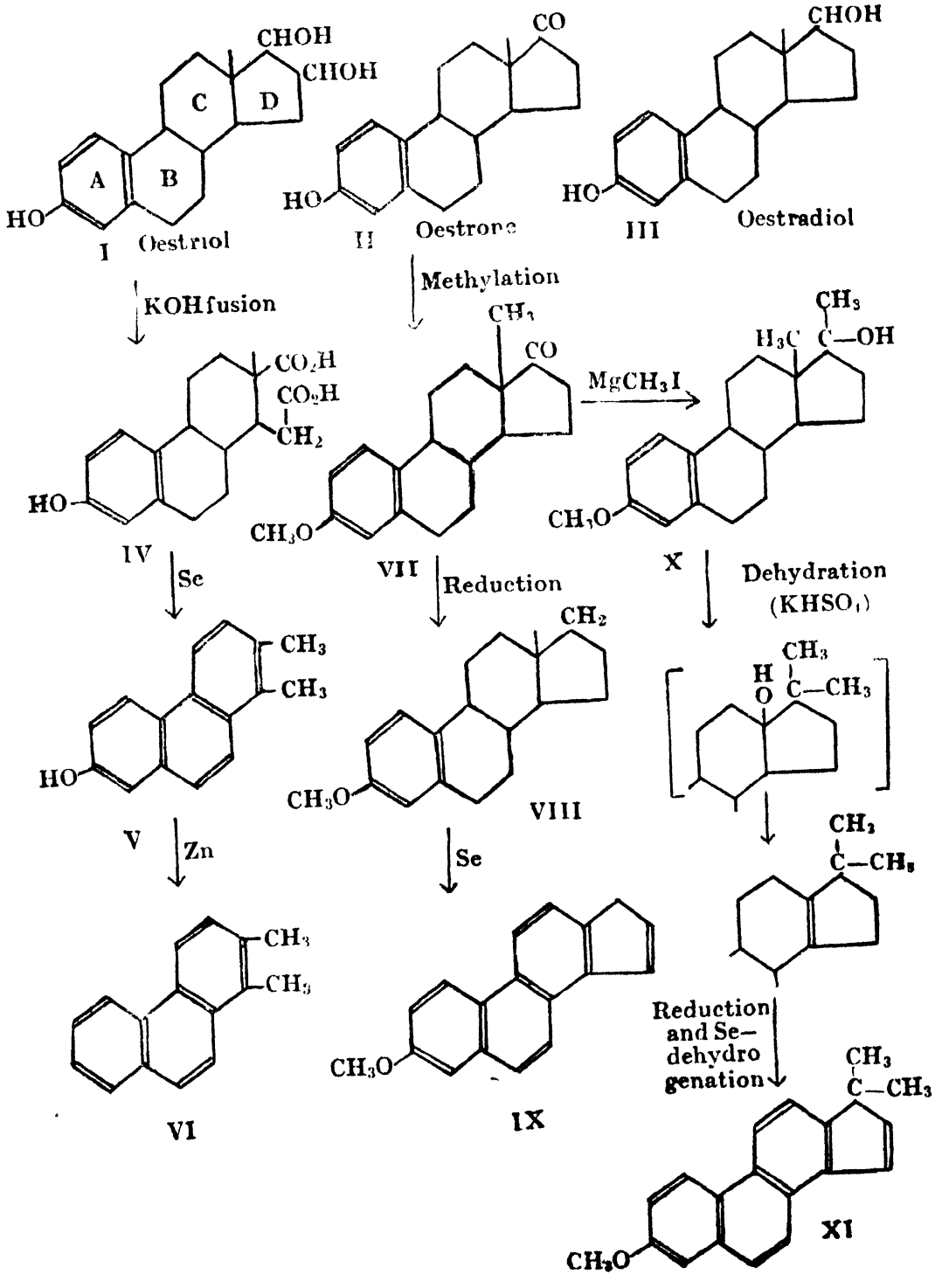
In view of the above mentioned close structural relationship between the three compounds, degradations effected with any one of them yielded information applicable to the whole group. The following transformations are important.

(1) On subjecting oestriol (I) to potash fusion it gave rise to oestric acid (IV), a phenol-dicarboxylic acid having the same number of carbon atoms. It readily formed an anhydride but gave no cyclic ketone indicating that it is obtained by the splitting of a five atom ring existing in oestriol. Selenium dehydrogenation of the acid yielded a dimethyl-phenanthrol (V) which underwent change by zinc dust distillation to 1 : 2-dimethylphenanthrene (VI). These reactions established the presence of a phenanthrene ring system [3] in the molecules of the oestrogens.

(2) The position of the phenolic hydroxyl group and the nature of the fourth ring were ascertained by a degradation [4] starting from oestrone (II). It was methylated (VII) and the ketonic group reduced to CH_2. Dehydrogenation of the product, deoxy-oestrone methyl ether (VIII) with selenium yielded 7-methoxy-1 : 2-cyclopentenophenanthrene (IX). The exact constitution of this substance was established by synthesis. These experiments proved conclusively that the hormones contain the ring system of the sterols and that the hydroxyl is

located in the same position. Since, however, it is phenolic in nature ring A of these hormones should be aromatic.

(3) The point that had still to be settled was the position of the ketonic group. The methyl ether of oestrone was again employed for this purpose. It gave on interaction with methyl magnesium iodide a tertiary



alcohol (X) which was subjected to dehydration, reduction and selenium dehydrogenation in succession. The final product was 7-methoxy-3' : 3'-dimethyl-1 : 2-cyclopentenophenanthrene (XI) which was also obtained by synthesis. Thus the carbonyl group of oestrone was shown to be in position 17.

The complete structures of oestriol (I), oestrone (II) and oestradiol (III) could then be given. Based on them the transformations described above are satisfactorily explained (see p. 209).

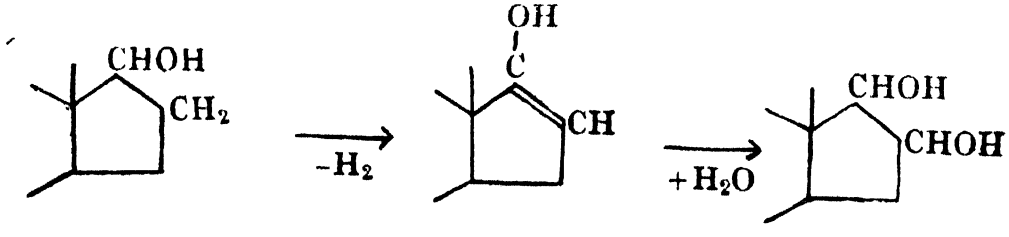
As already mentioned, α-oestradiol is considered to be the true ovarian hormone. In urine however the two stereoisomers of oestradiol are present along with oestrone and oestriol. As the result of a detailed study of oestrogen metabolism involving administration of adequately large doses of the hormones, it is concluded that the following reactions can occur in the mammalian organs.

α - Oestradiol $\rightleftharpoons$ Oestrone $\longrightarrow$ Oestriol

$\uparrow\downarrow$

β- Oestradiol

Direct change from oestradiol to oestriol is not excluded; it may take place as given below :

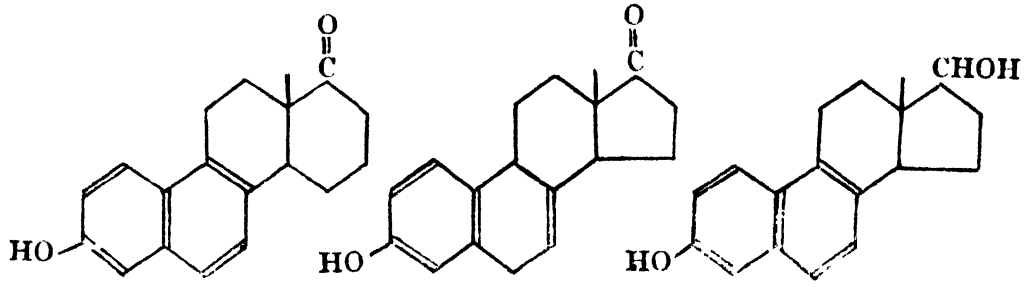


Oestradiol Enol of oestrone Oestriol

Even the conversion of oestrone to oestriol has been considered to involve the above enol form as the intermediate stage.

The Equilenin Group :—A number of closely related and more highly unsaturated oestrogenic hormones were isolated in small yields by Girard[6] from the urine of pregnant mares. They are characteristic of this source and have not been found in human pregnancy urine. They occur along with oestrone and are related to it.

Of these the compound known as equilenin is present in the largest quantity. The others which are present in much smaller amounts are hippulin, equilin and 17-dihydroequilenin. Equilenin has been investigated most thoroughly. It is a hydroxyketone and has two double bonds more than oestrone. Its properties and reactions indicate that in it ring B has also become aromatic and the constitution may be represented as given below. It has only a tenth of the activity of oestrone.



Equilenin Equilin 17-Dihydroequilenin

Equilin and hippulin contain only one double bond more than oestrone. They are isomeric and seem to differ in regard to the position of this fourth double bond. Equilin has been shown to have it between the 7 and 8 positions. A physiologically interesting observation of Girard emphasises further the close relationship between oestrone and these hormones. He found that the urine of pregnant mares contains at the beginning of pregnancy mainly oestrone; later equilin with another double bond in ring B predominates and at the end of pregnancy equilenin with ring B completely aromatic becomes the major component.

Assay and uses:—The oestrogenic hormones are responsible for the development of sex characters in the female. This is indicated by a condition known as oestrus. In castrated animals an injection of these substances re-establishes the oestrus cycle. Based upon this Allen and Doisy have worked out a well known method of biological assay, called the "oestrus test", using castrated female rats and mice. A second method studies the effect of the oestrogenic preparation on the weight of the uterus of young and ovariectomised animals. The results are generally expressed in mouse units. By international agreement one mouse unit is defined as the

effect produced by 0·1 γ of a standard oestrone preparation. As a subsidiary standard the 3-benzoyl ester of α-oestradiol is used. This unit, also called the benzoate unit, is represented by the specific activity produced by 0·1 γ of the ester.

There is considerable variation in the estimated biological activity of the natural oestrogens. The values depend largely on the exact technique of the assay. The following gives an idea of the large range in the results obtained: employing different techniques the activity of α-oestradiol was found to vary from 2·5 to 12 times that of oestrone; similarly β-oestradiol gave values varying from 1/25 to 1/10 that of œstrone.

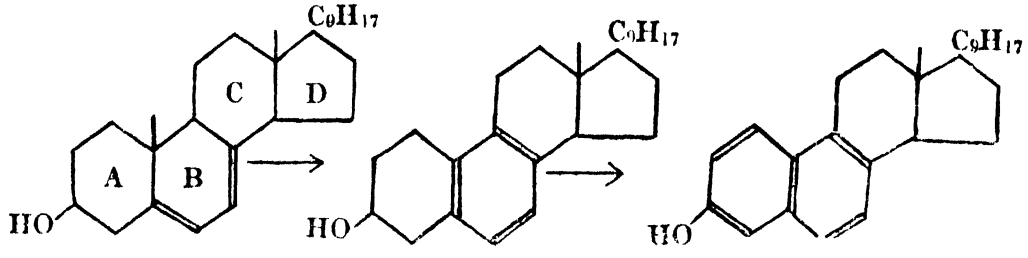
More recently chemical methods of assay have also come into frequent use. The Kober reaction is specific for the oestrogens and has been employed for this purpose. When the solid material is heated at 100° with a mixture of phenol-sulphonic acid and concentrated sulphuric acid a yellow colour is obtained. On adding water and heating again a highly specific pink colour develops and its intensity is estimated. β-naphthol-sulphonic and guaiacol-sulphonic acids are claimed to be better. In the study of urine, preliminary extraction of the oestrogens and separation from other chromogens is necessary for accurate results.

Another sensitive reaction for natural oestrogens is based on their ability to combine with phthalic anhydride in the presence of anhydrous stannic chloride. The products have a deep pink colour with a greenish yellow fluorescence. The method is said to be highly delicate and capable of detecting as little as 0·25 γ of oestrone.

The most obvious use of the oestrogens is in the treatment of disorders that arise from the malfunctioning of the ovaries. They have also been employed to remove the unpleasant effects that sometimes occur during menopause. An altogether new use that has been found for them is in the treatment of vaginitis in children.

The hormones are usually administered in the form of intra-muscular injections. Implantation of crystalline tablets under the skin is a novel procedure which has been adopted where action is desired over long periods.

Attempts at Partial Synthesis:—Attempts were made by several workers to obtain oestrone by the degradation of ergosterol. They were based on the important work of Windaus on the oxidising action of certain dyes (*e.g.*, eosin) on this sterol in the presence of light. The immediate product was a complex compound which yielded on pyrolysis neo-ergosterol having the ring (B) aromatic. When dehydrogenated in the presence of platinum black it gave rise to dehydro-neoergosterol



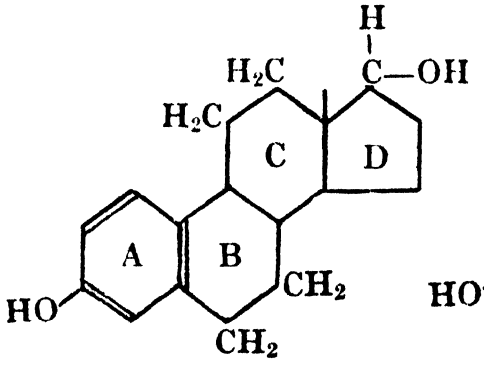
Ergosterol Neo ergosterol Dehydro neo-ergosterol

Starting from the last compound reduction of ring B and oxidation of the side chain were expected to yield oestrone. This could not be achieved,. Reduction seems to affect ring A in preference to ring B. In view of these results and the physiological observation of Girard regarding the equilenin group that ring B becomes aromatic only at a later stage, it seems obvious that Nature does not adopt the above procedure to evolve the oestrogens from sterols. It appears more probable that some intermediate involving partial dehydrogenation of ring A is first produced and that this intermediate is one of the androgenic substances as embodied in the general scheme of biogenesis of the sex hormones drawn up by Ruzicka (see Chapter XXII).

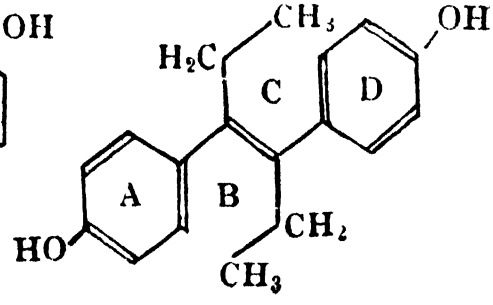
Synthetic Oestrogenic compounds:—The availability of natural oestrogens is limited and their isolation is costly. For a long time their synthesis was not achieved. Though some valuable results have been obtained in this direction recently, the synthesis of the complex skeleton is not economical In view of the great importance of oestrogens in medicine, a large amount of work has been done in attempts to make simple synthetic substitutes, and some of the most spectacular chemical achievements of recent years have been accomplished in this field. The

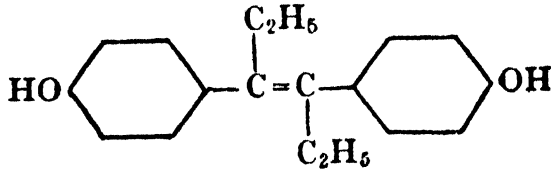
earlier preparations were complex compounds having the phenanthrene nucleus. Subsequently it was realised that such a complex nucleus is not absolutely essential and simpler phenolic compounds were examined. Certain diphenyl derivatives were found to be useful. An important discovery was made by Dodds and Lawson[8] that the demethylation product of anethole had an activity equal to that of oestrone. Though their original conclusion that anol or para-hydroxy-propenylbenzene was the active product, was later shown to be wrong, it encouraged hope in the discovery of simple and useful synthetic oestrogens. But the most successful compounds were discovered by Dodds, Golberg, Lawson and Robinson[9] as the result of speculation regarding the possible spatial relationship between natural and synthetic oestrogens (see below). They are derivatives of stilbene and diphenylethane and their names are (1) stilboestrol, (2) hexoestrol and (3) dienoestrol.

Oestradiol

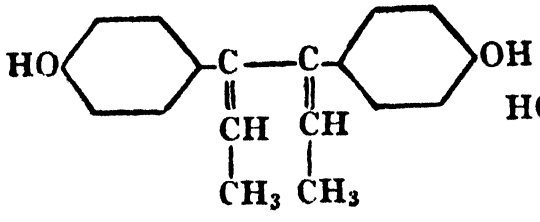


Stilboestrol

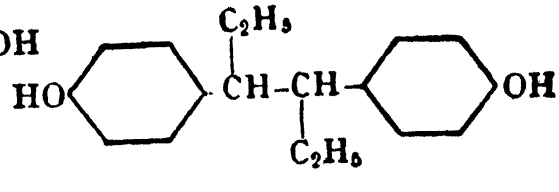




Stilboestrol



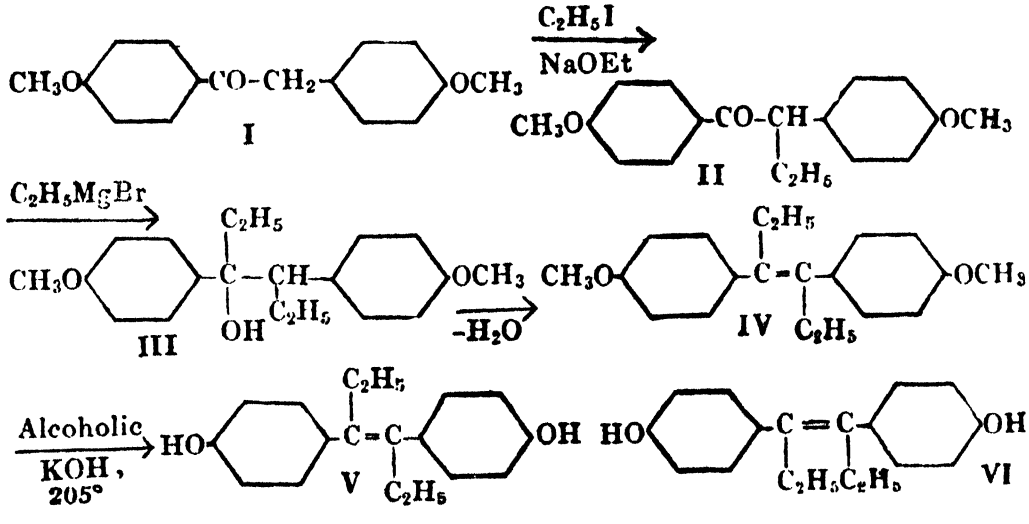
Dienoestrol



Hexoestrol

These compounds are made easily and are now commercially available. They are more powerful than even α-oestradiol and have the additional great advantage of being effective by oral administration. Their esters are somewhat weaker in potency, but their effect is prolonged and hence they are also used.

Stilboestrol is 4 : 4'-dihydroxy-α : β-diethylstilbene (V). It is prepared[9] starting from deoxyanisoin (I) which on reaction with alcoholic sodium ethoxide and ethyl iodide gives α-ethyl-deoxyanisoin (II). When treated with ethyl magnesium bromide the latter forms 3 : 4-dianisylhexan-3-ol (III) which undergoes dehydration under the influence of acids, acetic anhydride or phosphorus tribromide to yield 4 : 4'-dimethoxy-α : β-diethylstilbene (IV). Stilboestrol (V) is obtained from the dimethoxy compound by heating it with alcoholic potash in a sealed tube at 205° and it has the trans structure.

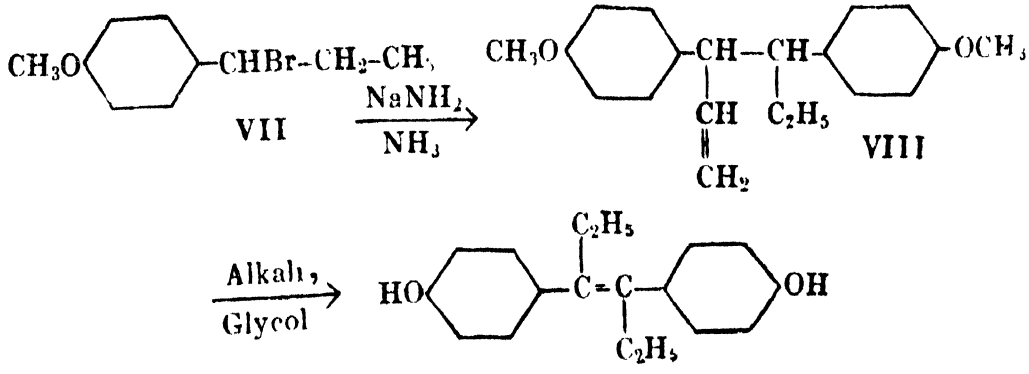


Stilboestrol ψ -Stilboestrol

During the dehydration of the carbinol (III), besides the ether of stilboestrol, a considerable amount of an isomeric by-product is obtained. This consists of the methyl ether of ψ-stilboestrol (VI); it undergoes isomerisation into stilboestrol ether in the presence of sunlight.

A more direct method of synthesis seems to be that of Kharash and Kleiman[10]. They treated anethole hydrobromide (VII) with sodamide in liquid ammonia and obtained 40% yield of a substance believed to be

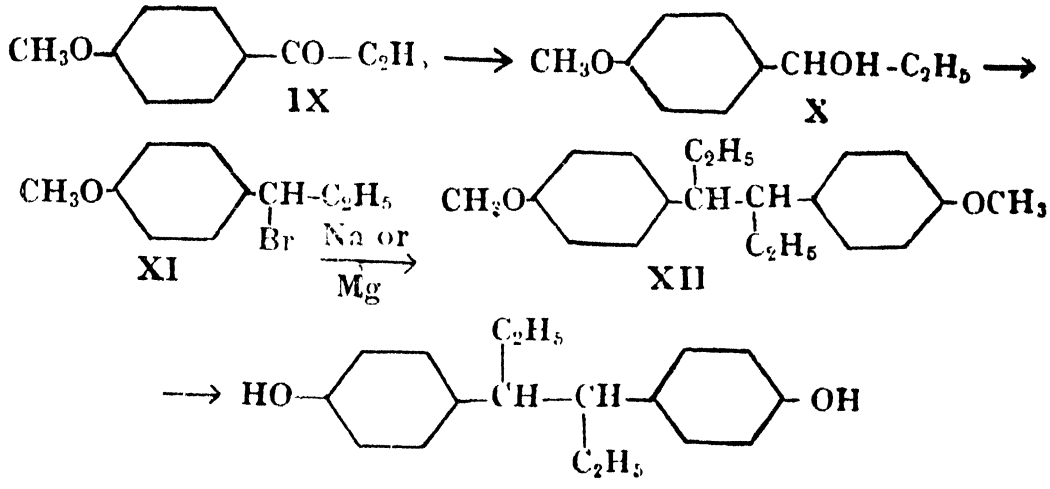
(VIII). It underwent isomerisation on demethylation and yielded 55% of stilboestrol (V).



V Stilboestrol

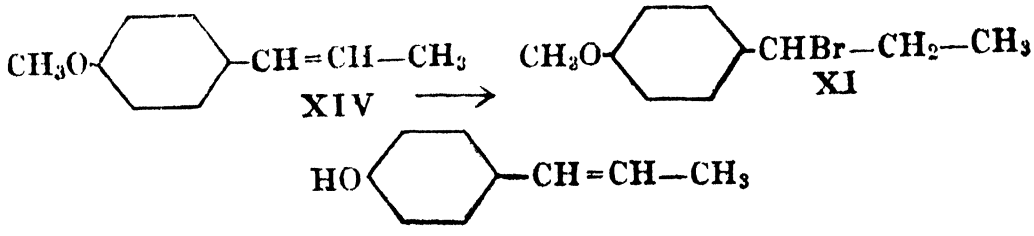
Hexoestrol[9]:—Stilboestrol has three times the activity of the natural ovarian hormone α-oestradiol. Hexoestrol has a still higher potency and is less toxic. It is the meso-form of dihydrostilboestrol and is the most potent oestrogen known. The dl-form called isohexoestrol is much less active physiologically. Hexoestrol is obtained by the hydrogenation of ψ-stilboestrol (VI) or dienoestrol (XVIII) in the presence of palladised charcoal as catalyst, whereas stilboestrol itself gives isohexoestrol under these conditions. An economic procedure[11] for the preparation of hexoestrol, therefore, starts with the by-product of stilboestrol synthesis which as already mentioned consists essentially of ψ-stilboestrol-dimethyl ether; it is hydrogenated and demethylated by heating with magnesium ethyl iodide.

A number of independent methods have also been worked out for the preparation of hexoestrol. One of these due to Bernstein and Wallis[12] (1940) starts with p-methoxy-propiophenone (IX). This is first reduced to the carbinol (X) and converted into the bromide (XI) by treatment with dry hydrogen bromide at 0°. On allowing an ethereal solution of this substance to remain in contact with sodium wire or magnesium the dimethyl ether (XII) of hexoestrol is produced and is subsequently demethylated by boiling with hydriodic acid.



XIII Hexoestrol

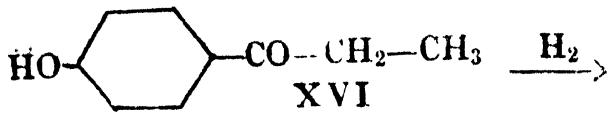
Another method due to Docken and Spielman[13] (1940) starts with anethole (XIV). It is treated with hydrogen bromide and the product (XI) subjected to further treatment as in the synthesis given above.



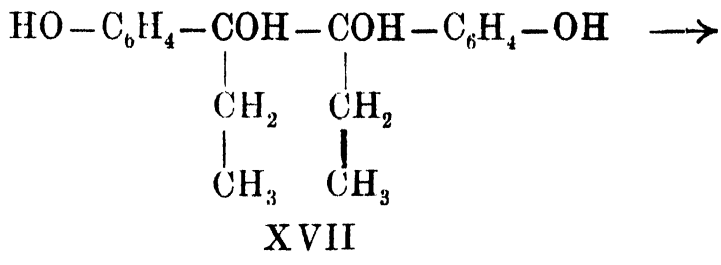
XV Anol

It may be recalled here that Dodds and Lawson originally reported that anol (XV) obtained by the demethylation of anethole with alcoholic potash at 200° was as active as oestrone. Subsequent careful examination has revealed that this activity is due to the presence of a persistent impurity identified as hexoestrol. It is now known that this demethylation yields a complex mixture of products.

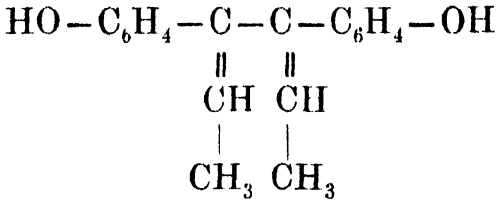
Dienoestrol[9] (XVIII) is another highly active oestrogen and its preparation starts from p-hydroxypropiophenone (XVI). Reduction of the ketone with aluminium amalgam in moist ether or electrolytically yields the corresponding pinacol (XVII). Dehydration is then brought about by treatment with acetic anhydride or acetyl chloride, followed by hydrolysis of the acetoxy groups.



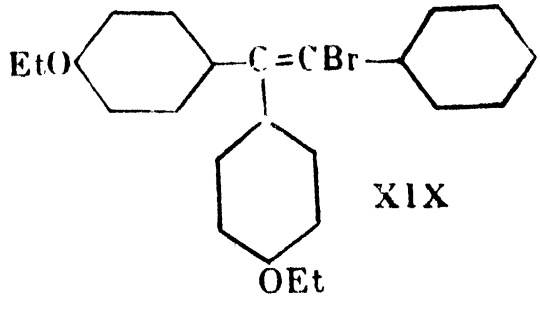
XVI



XVII



XVIII Dienoestrol



XIX

The foregoing compounds were made on the basis of configurational resemblance to oestradiol. But this does not seem to be an essential criterion as indicated by a number of oestrogens which are derivatives of triphenyl-ethylene. A diethoxy derivative of triphenyl-bromo-ethylene[14] (XIX) is noted for producing prolonged effects.

CHAPTER XX

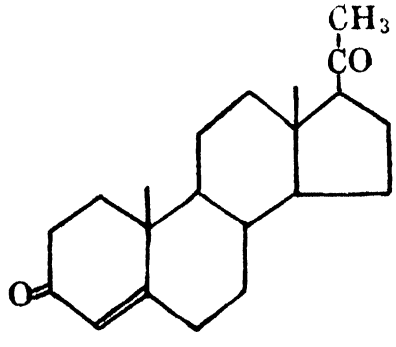
CORPUS LUTEUM HORMONE (PROGESTERONE)

The female hormones or oestrogens cause the development of female characteristics and prepare the animal for motherhood. But they do not function in support of pregnancy which is controlled by another hormone called progesterone. When the ovary has discharged its ovum, the corpus luteum, a small body in the ovary starts secreting progesterone. If pregnancy does not occur this secretion ceases after a few days.

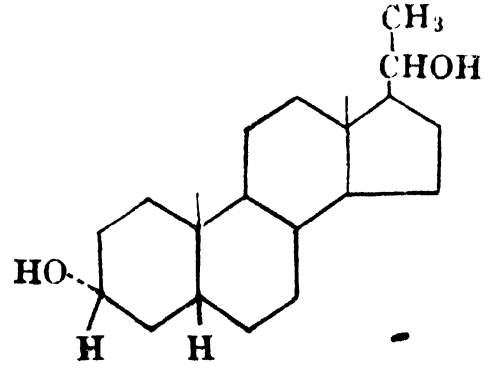
Isolation:—Progesterone was isolated in 1934 from the corpora lutea of pregnant sows by a number of workers at about the same time. An improved procedure was given by Allen and Goetsch [1] in 1936. Pigs' ovaries containing large pink corpora were minced and extracted with methyl alcohol. On diluting the extract with an equal volume of water fatty matter separated out. The solution was subsequently extracted with petroleum ether using a special counter-current extractor. 70 to 80% of the hormone originally present could thus be obtained in the petroleum ether solution. The solid left after distilling off the solvent, was taken up in 70% ethyl alcohol and extracted with small volumes of petroleum ether whereby impurities were removed. After diluting the alcoholic solution with water, it was again extracted with petroleum ether. This time progesterone was extracted and oestrone left behind in the aqueous alcohol. When the solid residue obtained from the petroleum ether solution was treated with ether and chilled to $-20°$ crude progesterone was obtained in a crystalline condition; it could be further crystallised from dilute alcohol or pyridine. The ether mother liquor gave an oil from which more progesterone could be obtained by conversion into the disemicarbazone and hydrolysis with mineral acid. Progesterone is considered to occur also in the placenta which exhibits marked activity; but it

has not been successfully isolated from this source. On the other hand, its presence in the adrenal gland is definitely established by its isolation.

Properties and Constitution[2]:—Progesterone exists in two crystalline modifications, the α-form (prisms) melting at 128·5° and the β-form (needles) melting at 121°. Both are identical physiologically. It has the formula $C_{21}H_{30}O_2$ and is a diketone capable of forming a dioxime. The existence of a close chemical relationship between progesterone and pregnanediol ($C_{21}H_{36}O_2$), an inactive alcohol isolated several years earlier (1929) from pregnancy urine, seemed to be possible. The constitution of the alcohol was already known as 3-(trans)-20-dihydroxypregnane and hence progesterone should be a related diketone with a double bond. Since its solutions exhibited strong absorption in the ultraviolet at 233 mμ., there was indication for the existence of an $\alpha:\beta$-unsaturated carbonyl group (C=C-C=O) in the molecule. A peculiar property of this substance is its instability to alkali. These characteristics are correctly explained by the following structure for progestrone.

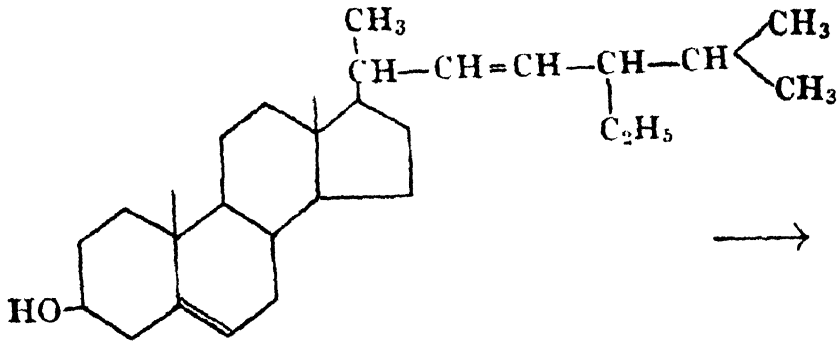


Progesterone

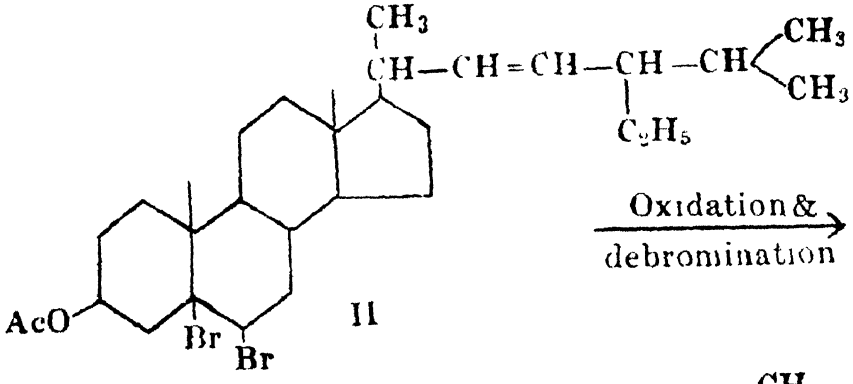


Pregnanediol

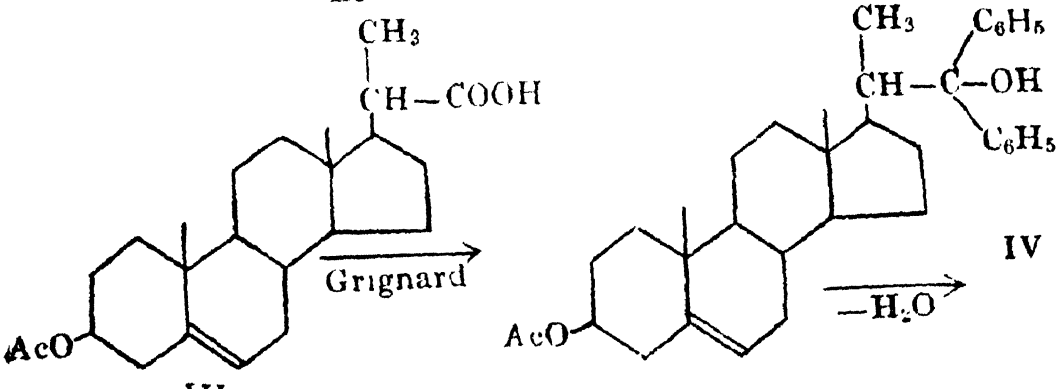
The above constitution was confirmed by the synthetic work of Butenandt and of Fernholz (1934) who were able to prepare it starting from stigmasterol (I). Stigmasterol forms a dibromide in which the double bond in the ring system is saturated. The acetate of this dibromide (II) is oxidised and then debrominated. This results in the production of 3-acetoxy-Δ^5-bis-nor-cholenic acid (III). This acid is subsequently converted into the ketone (VII) by Wieland degradation as given below. Its methyl ester is subjected to treatment with magne-



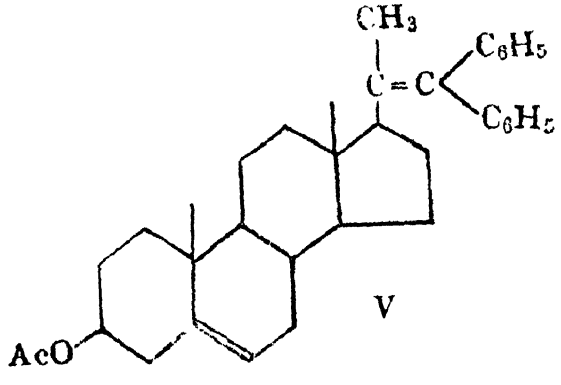
I Stigmasterol



II



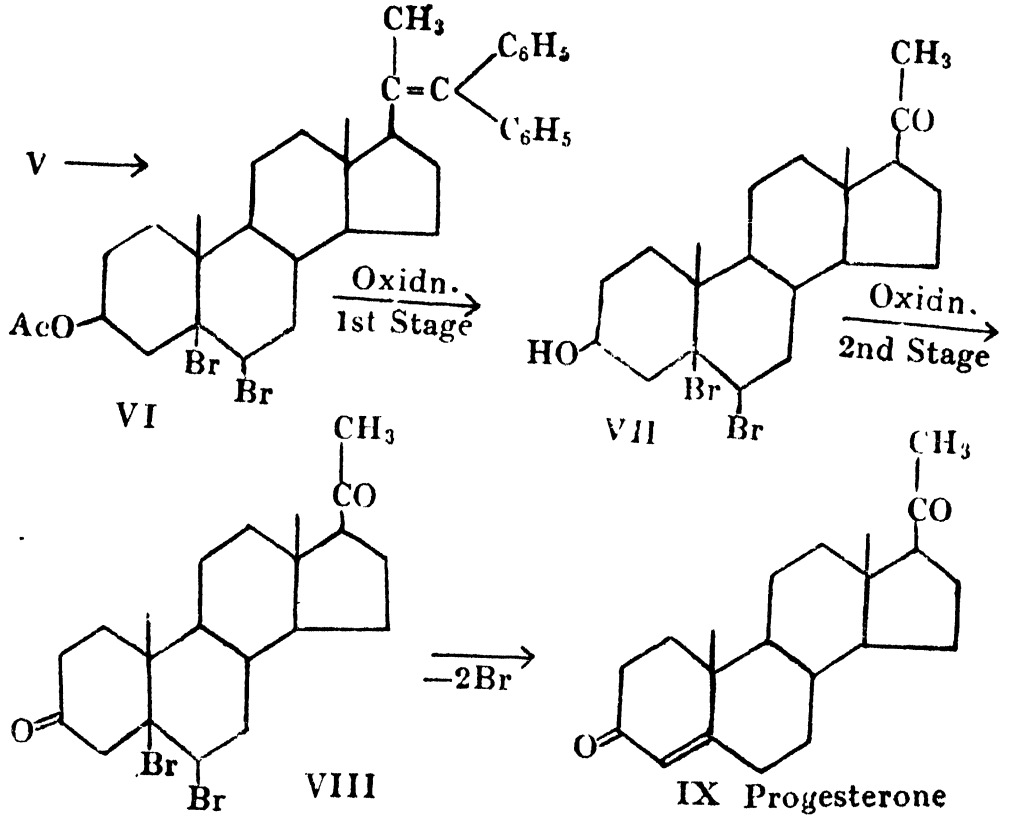
III IV



V

sium phenyl halide, when it first forms a tertiary alcoholic grouping (IV) which quickly undergoes dehydration giving rise to the acetate of the unsaturated alcohol (V). This compound is oxidised in two stages, the double bond of the ring being protected by the presence of bromine atoms. The first stage yields a hydroxy-ketone

(VII) involving oxidation in the side chain present in position 17. During the second stage, oxidation of the secondary alcoholic group in position 3 takes place forming a diketone (VIII). When the bromine atoms are finally removed by means of zinc, migration of the double bond also takes place giving rise to progesterone (IX).

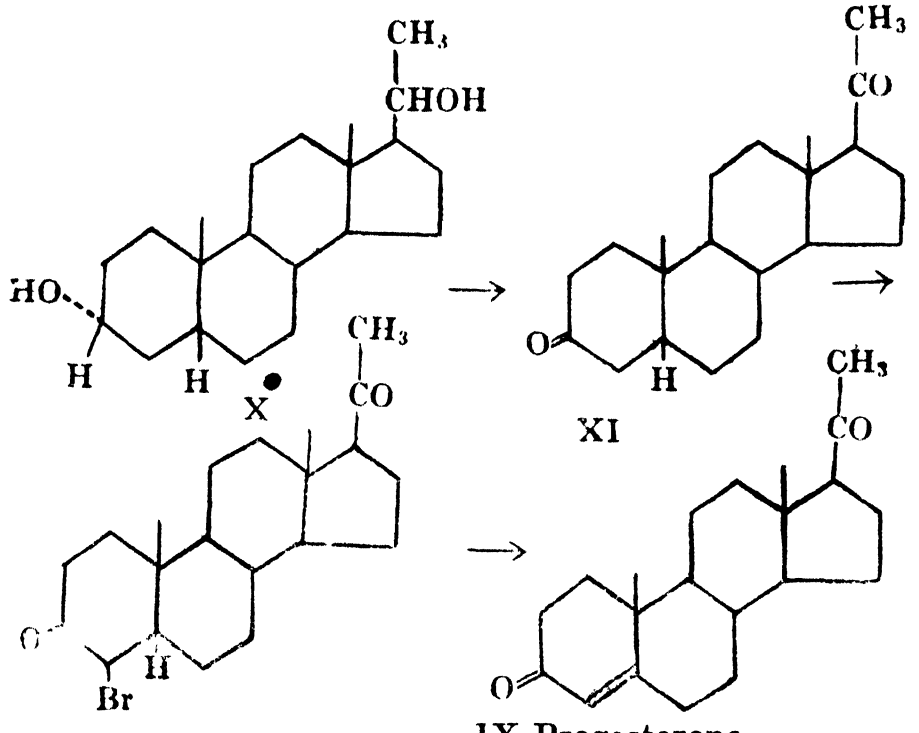


Several methods have been examined for the preparation of progesterone from the more easily available sterol, cholesterol. They involve cholesterol-dibromide or cholestenone as an intermediate stage and oxidation is effected using potassium permanganate, chromic acid or hydrogen peroxide. Final debromination is done if the bromo-compound is employed. But the yields seem to have been poor. Sapogenins, particularly diosgenin, are also considered to be suitable sources.

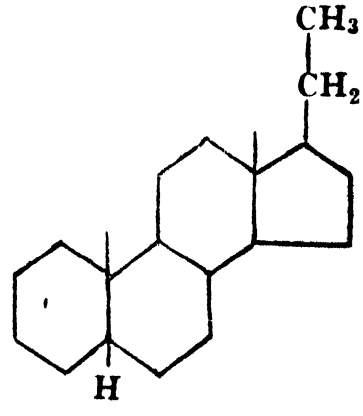
Pregnanediols[3]:—Unlike the oestrogens which tend to undergo oxidation during the course of elimination, progesterone undergoes reduction. It has not so far been found in urine probably due to rapid transformation. The most important of the reduction products are the isomeric dihydric alcohols, pregnanediol and allopregna-

nediol. They are excreted as glucuronides in considerable quantities in the urine in the later stages of pregnancy or after the administration of progesterone. Of the two, pregnanediol is the major component and is easily isolated from urine. It has been found in the highest concentration in bull's urine. It has the composition $C_{21}H_{36}O_2$ and the constitution represented by formula (X). It can be converted into progesterone in the following manner. On oxidation it yields pregnanedione (XI). An ethylenic double bond is then introduced by bromination in the 4 position and removal of hydrogen bromide by means of pyridine.

Pregnane-diol Pregnane-dione



IX Progesterone

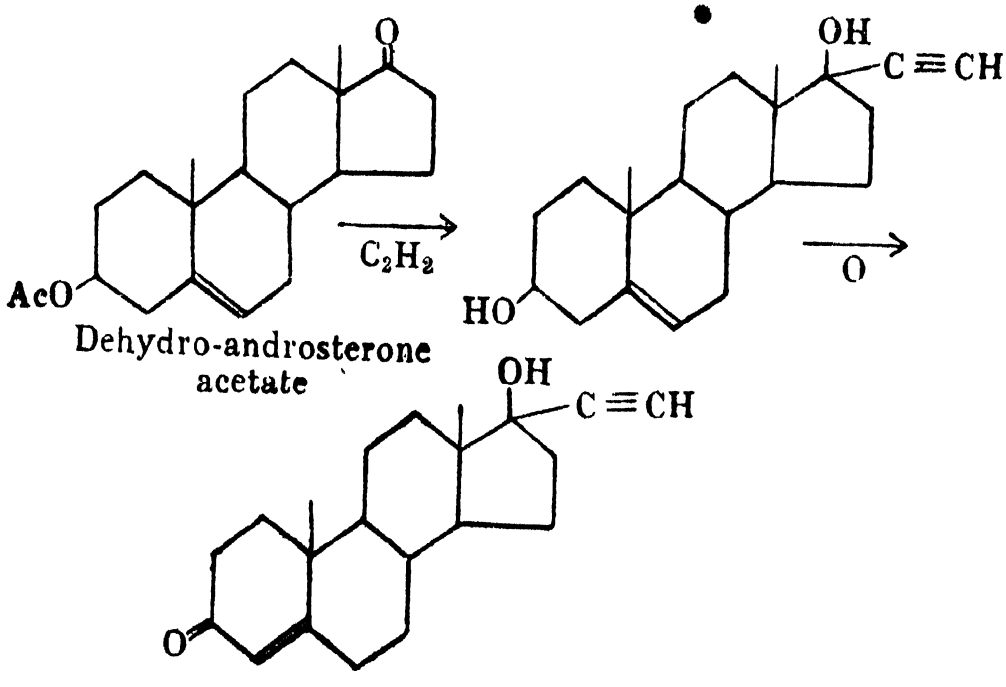


XII Pregnane

Progesterone and other compounds mentioned above are considered to be derivates of the hydrocarbon pregnane (XII) which can be obtained by the reduction of pregnanedione.

Assay and uses:—Progesterone is usually detected and assayed by its effect on the uterus, as revealed in histological section, of pregnant rabbits from which the ovaries have been removed after the onset of pregnancy. This is known as the Allen-Corner test. 1 mg. of pure progesterone is a rabbit unit. Though the hormone has been prepared artificially and a number of other substances with progestational activity have been discovered, none of these is easy to make. Treatment with progesterone is still very expensive. Its obvious use is to maintain pregnancy and it has been employed in cases of habitual abortion in the form of an oil solution injected intramuscularly.

An artificial compound having progesterone-like activity and active even by oral administration is pregneninolone[4] (XIII). It is synthesised from dehydroandrosterone acetate by reaction with acetylene and subsequent oxidation.



XIII Pregneninolone

CHAPTER XXI

ANDROGENIC HORMONES

From the urine of animals both male and female, and from testicular extracts a number of androgenic hormones have been isolated. When injected into castrate or immature males they bring about the restoration or development respectively of male characteristics. Four such compounds are known of which two, androsterone and testosterone, are more important.

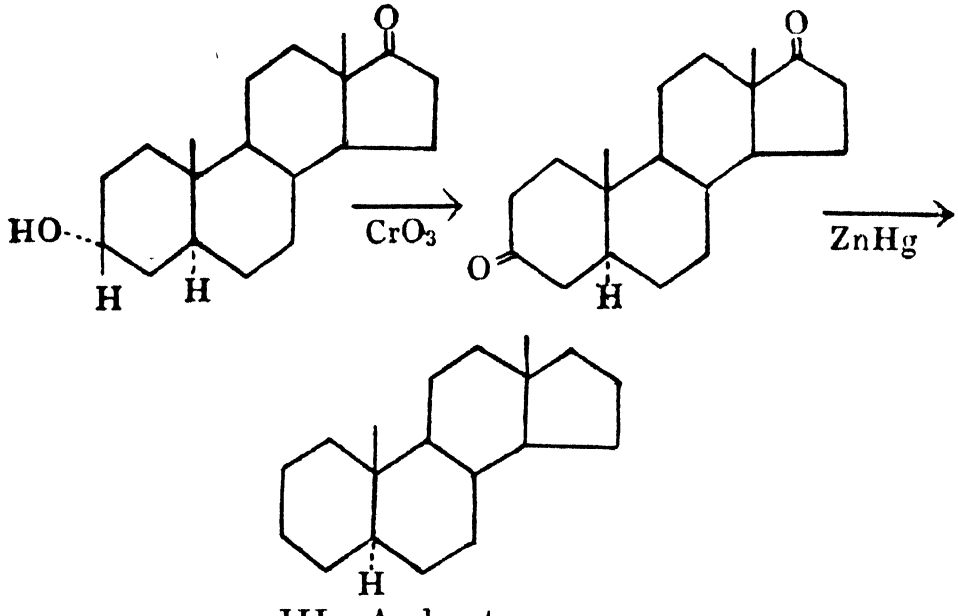
Androsterone: Isolation[1]:—Androsterone is the male hormone present in the largest quantity in urine and it occurs mostly in the combined form as sulphate. In the earlier method adopted by Butenandt, concentrated male urine was employed. It was treated with acid and extracted with chloroform. Acid and phenolic matter were removed by shaking the extract with aqueous potash. The neutral fraction was then subjected to steam distillation in order to remove volatile impurities, and boiled successively with alkali and acid. The unsaponifiable matter contained the hormone which was purified by partition between solvents. Androsterone was then converted into the oxime and recovered by decomposition with acid.

Considerable improvement in yield and ease of operation has been achieved by the use of Girard's reagent T and chromatography. When the neutral fraction mentioned above is treated with this reagent the ketonic components form water-soluble products and others are removed by extraction with ether. The ketones are then regenerated by hydrolysis, and subsequent purification and separation effected by chromatography using Brockmann's alumina as adsorbent and carbon tetrachloride as solvent. Yields ranging from 0·2–0·4 mg. of androsterone per litre of normal urine (male or female) have been obtained.

Constitution:—Androsterone melts at 184°, has a specific rotation of +96° in alcohol and has the formula

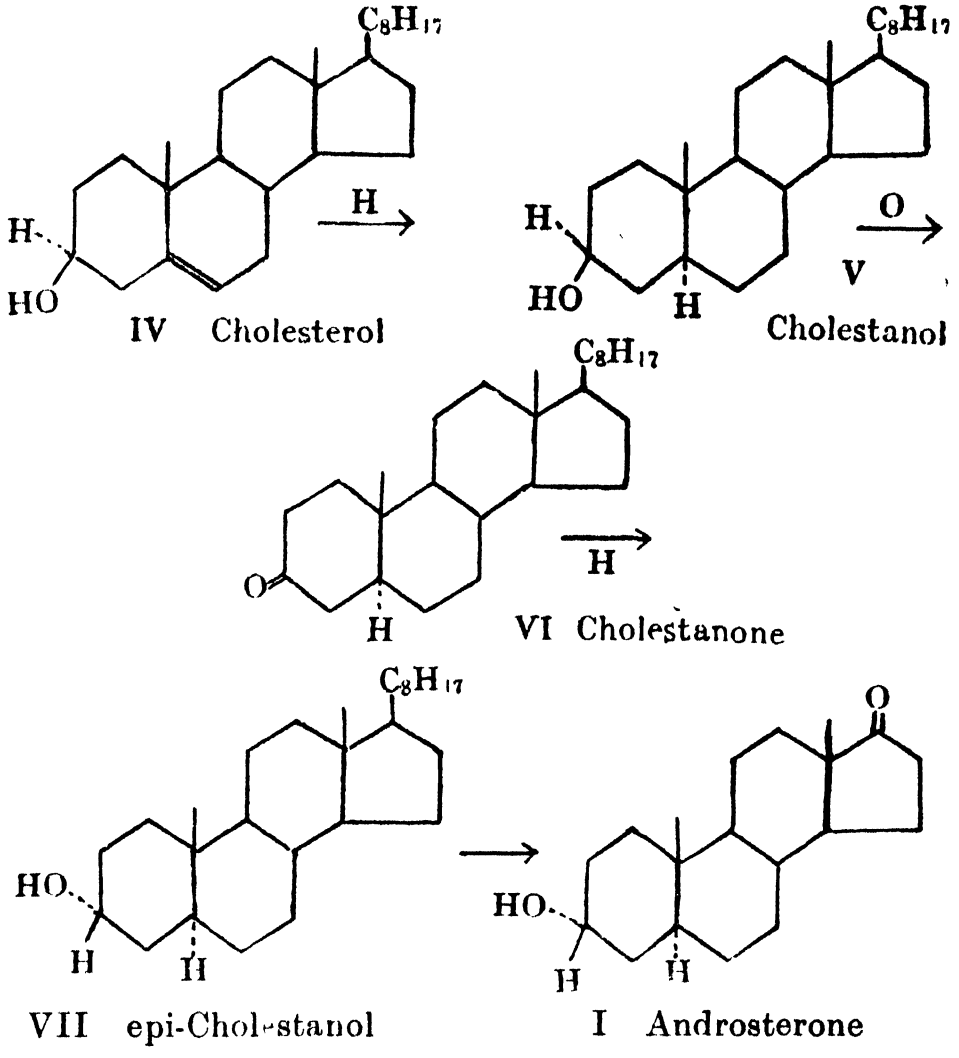
$C_{19}H_{30}O_2$. It has no ethylenic double bonds and it is a hydroxy ketone, one of the oxygen atoms thus being in a hydroxyl group and the other in a ketonic group. The formula shows that it contains eight hydrogen atoms less than a saturated aliphatic ketone and that the compound must therefore be tetracyclic. Assuming that it was a sterol derivative Butenandt [2] (1933) suggested the formula (I). He was able to oxidise it with chromic acid to a diketone, androstanedione (II) which when subjected to reduction with zinc amalgam (Clemmensen's method) yielded the hydrocarbon, androstane, $C_{19}H_{32}$ (III).

I Androsterone II Androstanedione

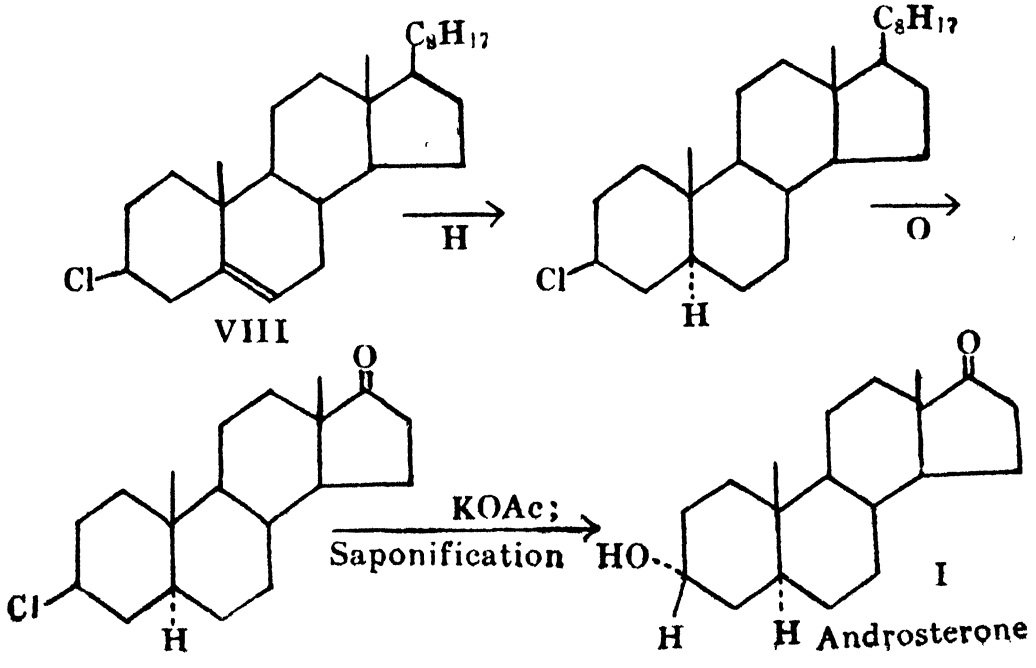


III Androstane

Partial synthesis :—Ruzicka and his collaborators [3] (1934) succeeded in proving the relation between androsterone and the sterols by preparing it from epi-cholestanol (VII). The starting material was cholesterol (IV) which, when subjected to catalytic hydrogenation in neutral solvents, formed dihydrocholesterol or cholestanol (V). On gentle oxidation this yielded cholestanone (VI). Reduction of this ketone with hydrogen in acid media gave epi-cholestanol (VII), the stereo-isomeric change taking place during the reduction. The acetate of this compound was oxidised by means of chromic acid, whereby the side chain in position 17 was removed resulting in androsterone acetate which on hydrolysis gave androsterone (I).

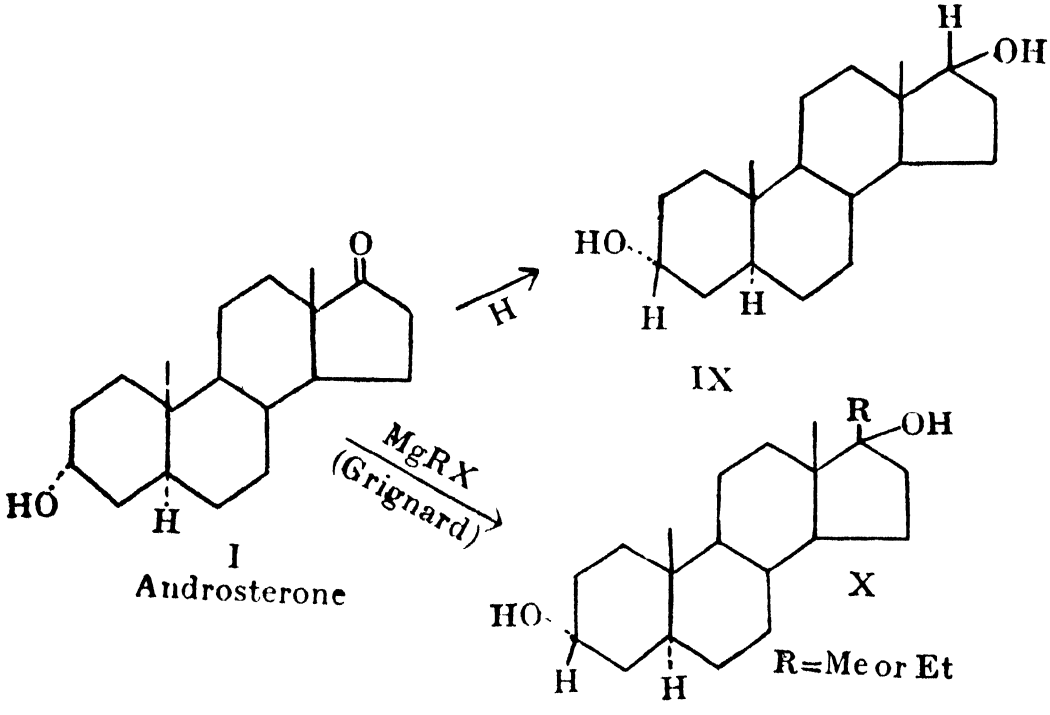


Though the above stages may appear to be easy, the yields were remarkably small, the technical difficulties



being mainly due to the limited solubility of the products. A simpler method has been described by Marker[4] using cholesteryl chloride (VIII). After hydrogenation of the double bond and oxidation of the side chain, chlorine is replaced by hydroxyl by treatment with potassium acetate and subsequent saponification. During this replacement Walden inversion takes place to give directly androsterone.

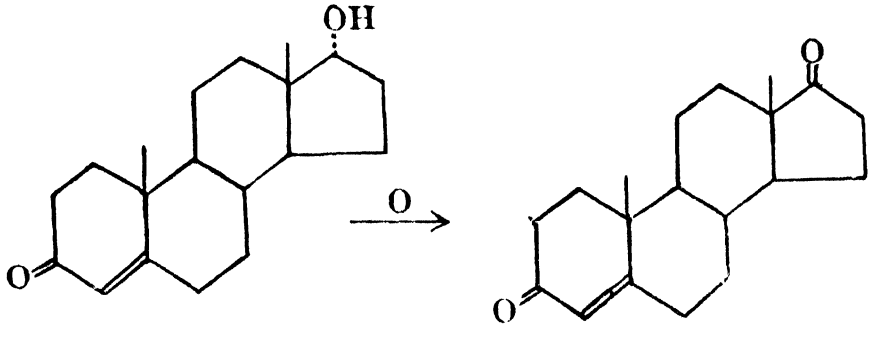
Reduction of androsterone (I) yields androstanediol (IX) and treatment with Grignard reagent, 17-methyl- and ethyl-androstanediols (X). These compounds are of interest because they are about three times as potent physiologically as androsterone.



Dehydroandrosterone:—Androsterone is accompanied in the urine by lesser quantities of Δ^5-dehydro-androsterone (XVI) having the composition $C_{19}H_{28}O_2$. It is physiologically less potent than androsterone and it can be made in good yield from cholesterol[5]. Cholesteryl acetate-dibromide (XIII) is oxidised and debrominated. The acetate of dehydroandrosterone (XV) thus obtained is subsequently saponified (see under synthesis of testosterone).

Testosterone[6]: Properties and Constitution:—Examination of testicular extracts indicated the presence of a

hormone far more powerful than androsterone (1935) and this compound was further characterised by instability in the presence of alkali. It was called testosterone. It crystallises as needles from acetone, melts at about 154° and has a specific rotation of +109° in ethyl alcohol. It has the molecular formula $C_{19}H_{28}O_2$ and contains an alcoholic and a ketonic group The presence in the spectrum of strong absorption at 240 mμ. showed that it is an $\alpha:\beta$-unsaturated ketone. These properties and its inactivation by alkali strongly indicated that it is structurally similar to progesterone. The structure of testosterone as given in formula (XI) was established by David (1935) who oxidised it to Δ^4-androstene-dione (XII), a substance whose constitution was already known and which had already been prepared by other methods.

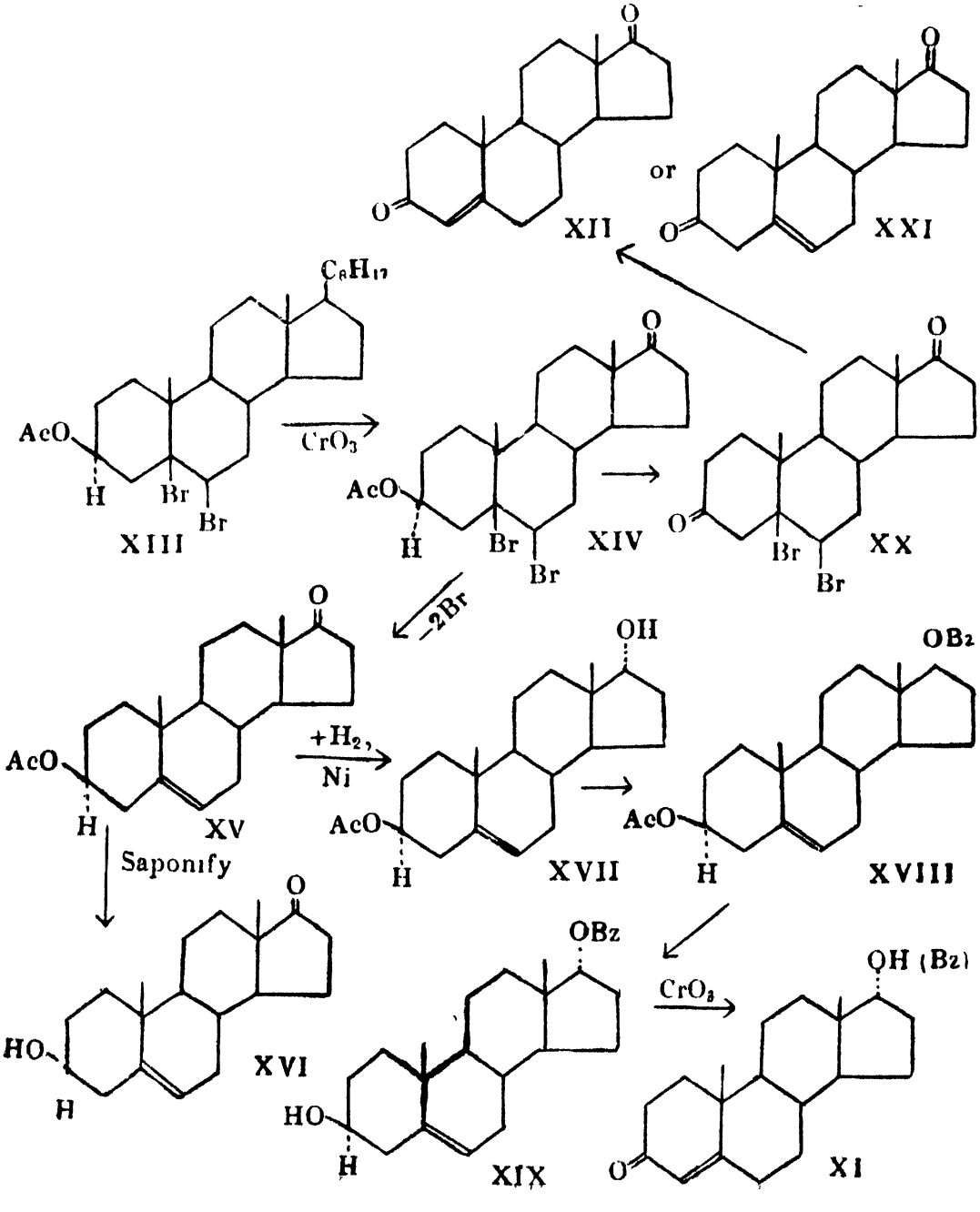


XI Testosterone XII Androstene-dione

By means of a novel method of reduction using fermenting yeast the reverse change, from androstenedione to testosterone, could be effected. In this reaction only the keto group at C_{17} is attacked; this partial reduction is more difficult to carry out by purely chemical methods (see page 231).

Partial Synthesis:—The constitution of testosterone was confirmed by the synthesis developed independently by Butenandt and by Ruzicka at about the same time (1935). Cholesterol was again the starting point. It was acetylated and treated with bromine whereby the dibromide of cholesteryl acetate (XIII) was obtained. Oxidation of this substance with chromic acid followed by debromination resulted in the formation of dehydroandrosterone acetate (XV). To get testosterone (XI) the keto group at C_{17} was first reduced and then the OH

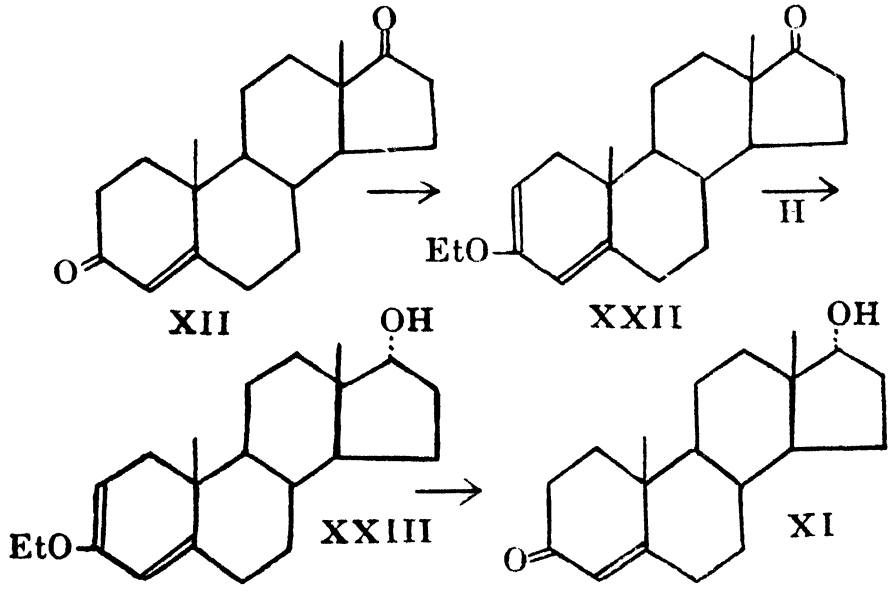
at C_3 oxidised in the following manner. The compound (XV) was reduced in the presence of nickel to form the acetate of Δ^5-androstene-diol (XVII). When it was benzoylated, the 3-acetoxy-17-benzoyloxy compound (XVIII) was obtained. On careful hydrolysis at a low temperature the acetyl group in position 3 alone underwent removal leaving a hydroxyl group in that position (XIX). Oxidation again with chromic acid of this hydroxylic compound produced testosterone benzoate. An improved method employs for this oxidation the 5 : 6-dibromide and later effects debromination with zinc.



Shifting of the position of the double bond takes place in either case. Final debenzoylation gives testosterone (XI).

The immediate product of oxidation of (XIII) is the acetate (XIV). After hydrolysis it can be oxidised to the diketone (XX) and then debrominated. Depending on the method of debromination either Δ^4-androstene-dione[7] (XII) (zinc and acetic acid) or its Δ^5-isomer (XXI) (zinc and weakly acid alcohol) can be obtained. The former is more potent physiologically and is more important otherwise also, being a product of oxidation of testosterone. It is also found in the adrenal cortex.

An alternative route[8] for the synthesis of testosterone employs Δ^4-androstene-dione (XII) which is converted into the 3-enol ether (XXII). The keto group at C_{17} is then reduced giving (XXIII) which on hydrolysis reverts to the keto form, testosterone (XI).

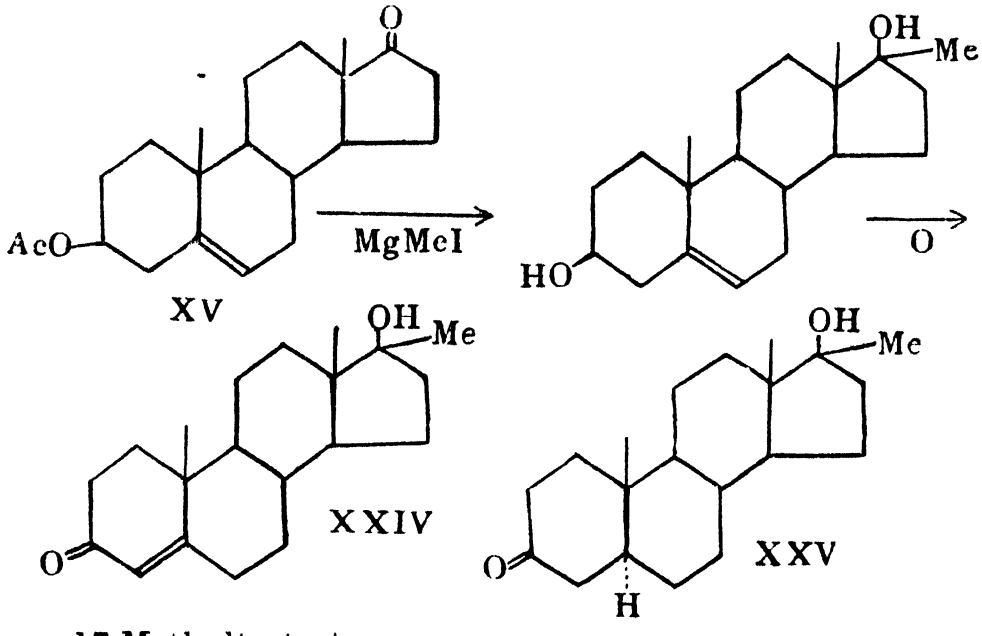


Testosterone

As has already been mentioned, the preferential reduction of the keto group at C_{17} in (XII) can be achieved more easily by biochemical methods. Since the yield of testosterone from sex glands is very poor (90-270 mg. from one ton of gland tissue) the synthetic methods mentioned above assume considerable importance.

Uses:—Testosterone is used in clinical practice for replacement therapy, particularly in the treatment of enlarged prostate. It is usually administered in the

form of the propionate, which is dissolved in oil and injected intramuscularly. The synthetic androgens, 17-methyl-testosterone (XXIV) and 17-methyl-dihydrotestosterone (XXV) have been found to be more useful since they could be given orally and have a more powerful action than the natural hormones. The former can be prepared[9] from dehydro-androsterone acetate (XV) by means of the Grignard reaction with methyl magnesium iodide and subsequent oxidation.



17-Methyltestosterone

The dihydro compound (XXV) is prepared in a similar way starting from androsterone. It is considered to be the most potent of all known androgens, natural and synthetic.

Assay of Androgenic Hormones : –The older method is biological and is very reliable. It is known as the Gallagher-Koch method and depends upon the increase in the comb surface of capons which have been treated with the male hormones. The growth of the comb can be correctly determined by means of a shadowgraph. The international comb unit is defined as 0·1 mg. (100γ) of the male hormone androsterone. The intramuscular injection of one I.U. daily for 5 days gives an easily measurable but not inconveniently large growth of the comb of the capon. Androgens can also be assayed by their action in increasing the weight of the prostates and seminal vesicles of castrate and immature rats.

There are differences in the potency of androgens when compared by the two methods. Though the former is more precise the latter may be considered to be more significant physiologically.

Zimmermann colour reaction provides a convenient chemical method for the rapid assay of the androgenic hormones. It is based on the observation that compounds with a reactive methylene group (-CO-CH_2-) react with m-dinitrobenzene in the presence of alcoholic potash to form red coloured products. The androgen sample is treated with the nitro compound and potash in alcoholic solution at 25°, diluted to a standard volume with alcohol and the colour intensity estimated. The results agree with those of the biological (comb growth) method satisfactorily. In the analysis of urine the neutral ketonic fraction is obtained as a dry solid and used; oestrone and equilenin which belong to the phenolic fraction interfere unless they are removed, since they also give the Zimmermann reaction.

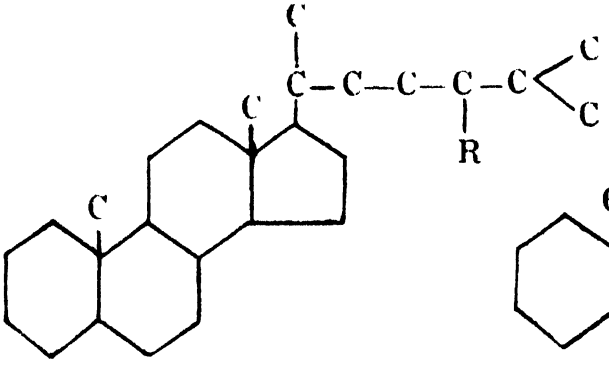
Testosterone is not eliminated in the urine as such, but only after transformation into androsterone and related compounds. This seems to account only for a part of the urinary androgens, the rest being derived from the hormones of the adrenal cortex.

CHAPTER XXII

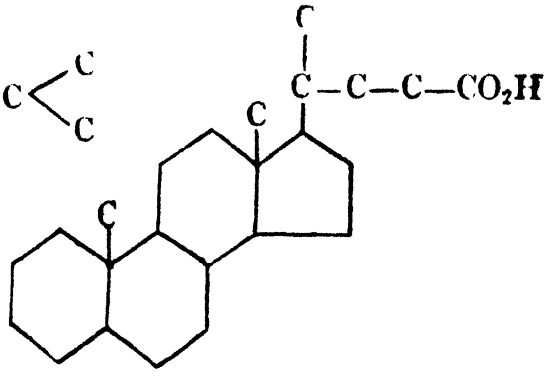
SEX HORMONES: THEIR BIOGENESIS AND TOTAL SYNTHESIS

Inter-relationship of the sex hormones and their biogenesis:—The sex hormones as a group have certain common characteristics. They occur together in the urine and in the sex glands and have the same ring skeleton which is well known to be present in the sterols.

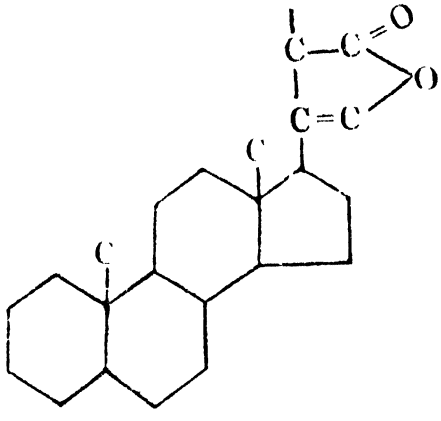
Amongst various groups of compounds related to the sterols the first important difference arises in the nature of the side chain attached to the 17 position. It is longest and devoid of any oxygen in the case of the sterols. In the bile acids and in the cardiac glycosides this chain is shortened and other modifications are caused by the presence of oxygen atoms. It is at its minimum or non-existent in the sex hormones. Progesterone retains most of the sterol characteristics and has two carbon atoms in the side chain. The four rings are non-aromatic and the two angular methyl groups exist. The androgens, androsterone and testosterone, come next. In them the chain in the 17 position has been removed. Otherwise the sterol structure is kept up. On the other hand, the oestrogenic hormones have undergone dehydrogenation rendering ring (A) aromatic. Thus the hydroxyl group in position 3 becomes phenolic in nature. This aromatisation is capable of proceeding further under certain conditions as are prevalent in mare's urine, to form equilenin in which even ring (B) has become aromatic. As a necessary preliminary to this structural change the angular methyl group in position 10 has been removed. It seems to be possible that the presence of these angular methyl groups renders the ring skeleton of sterols stable to dehydrogenation to a large extent. However they are not capable of preventing the change altogether. The main skeletons of the different groups of compounds referred to above are represented below:



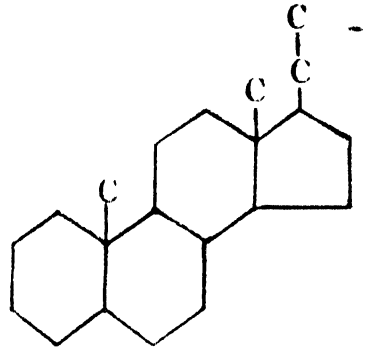
Sterols



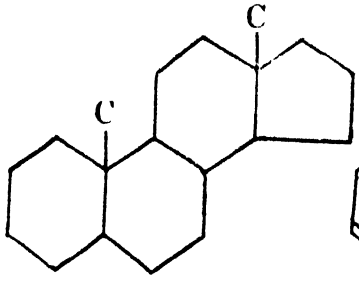
Bile acids



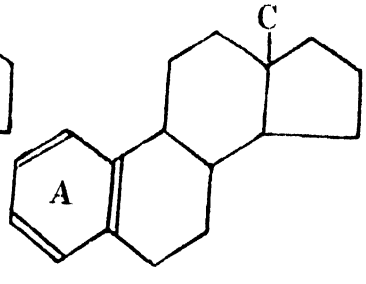
Cardiac aglucones



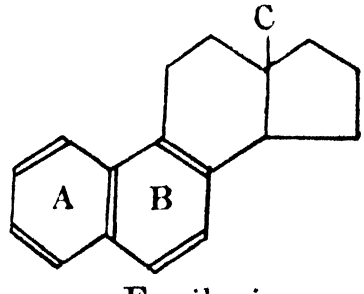
Progesterone



Androgens



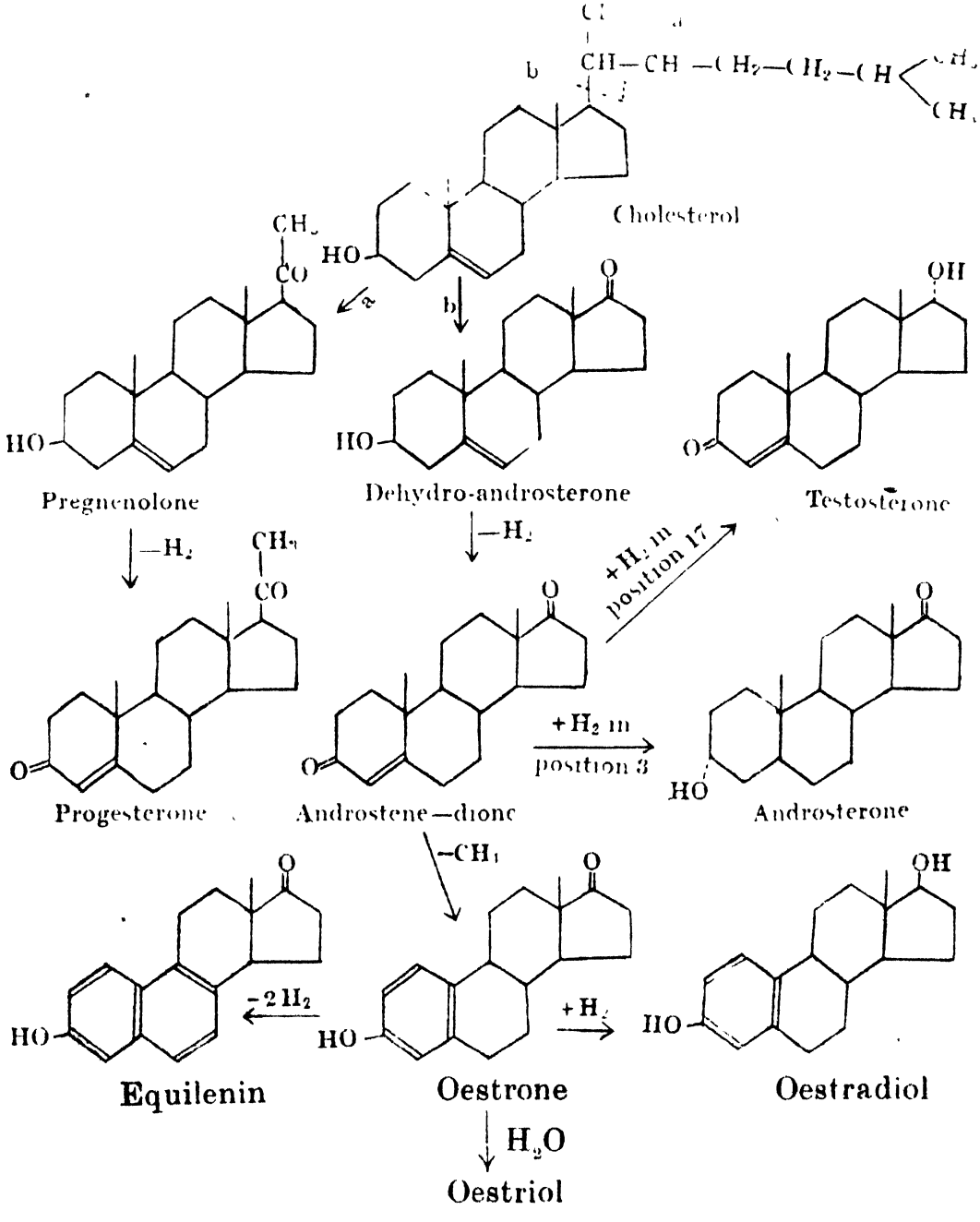
Oestrogens



Equilenin

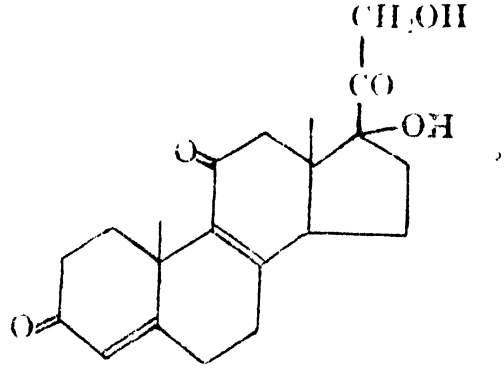
In regard to the biogenesis of the sex hormones their marked structural relationship with the sterols has been highly suggestive. They have been considered to be derived from this source by degradation. This is supported by the number of partial syntheses of the hormones starting from the sterols. Ruzicka[1] has drawn up an interesting scheme by which the sex hormones could all be obtained starting from cholesterol, the most important of the animal sterols. The essential features of the scheme are given below. It is in agreement with all known observations and particularly the later appearance of equilenin in mare's urine showing that it is derived from oestrone, and the remarkably

large occurrence of oestrone in the urine of male animals, indicating that urinary androgens and oestrogens have a common origin. It will be interesting to note that most of these hormones are bisexual in their action, some being predominantly androgenic and some predominantly oestrogenic. This bisexual property may be inherent in the molecules or may be due to secondary changes. It, however, emphasises the close relationship between them in regard to structure and origin.



In the production of the hormones the sterol side chain should be capable of fission in two ways, (1) as in

(*a*) to form progesterone and (2) as in (*b*) to form the others. There is adequate experimental analogy for this type of fission. By the chromic acid oxidation of cholesterol both dehydroandrosterone and pregnenolone have been obtained; but the yields are poor. The fundamental difficulty in these speculations arises out of our lack of correct knowledge regarding the genesis of the sterols themselves. There does not seem to be any reason to exclude the possibility of the hormones being built up independently of the sterols from some common source, though it may be along parallel lines. According to Marker[2] the steroid hormones including the sex and the cortex hormones are considered to be evolved from a hypothetical common precursor having the constitution (I) which is formed independently of the sterols. His theory is based on a detailed study of the steroid components of urine of various types.

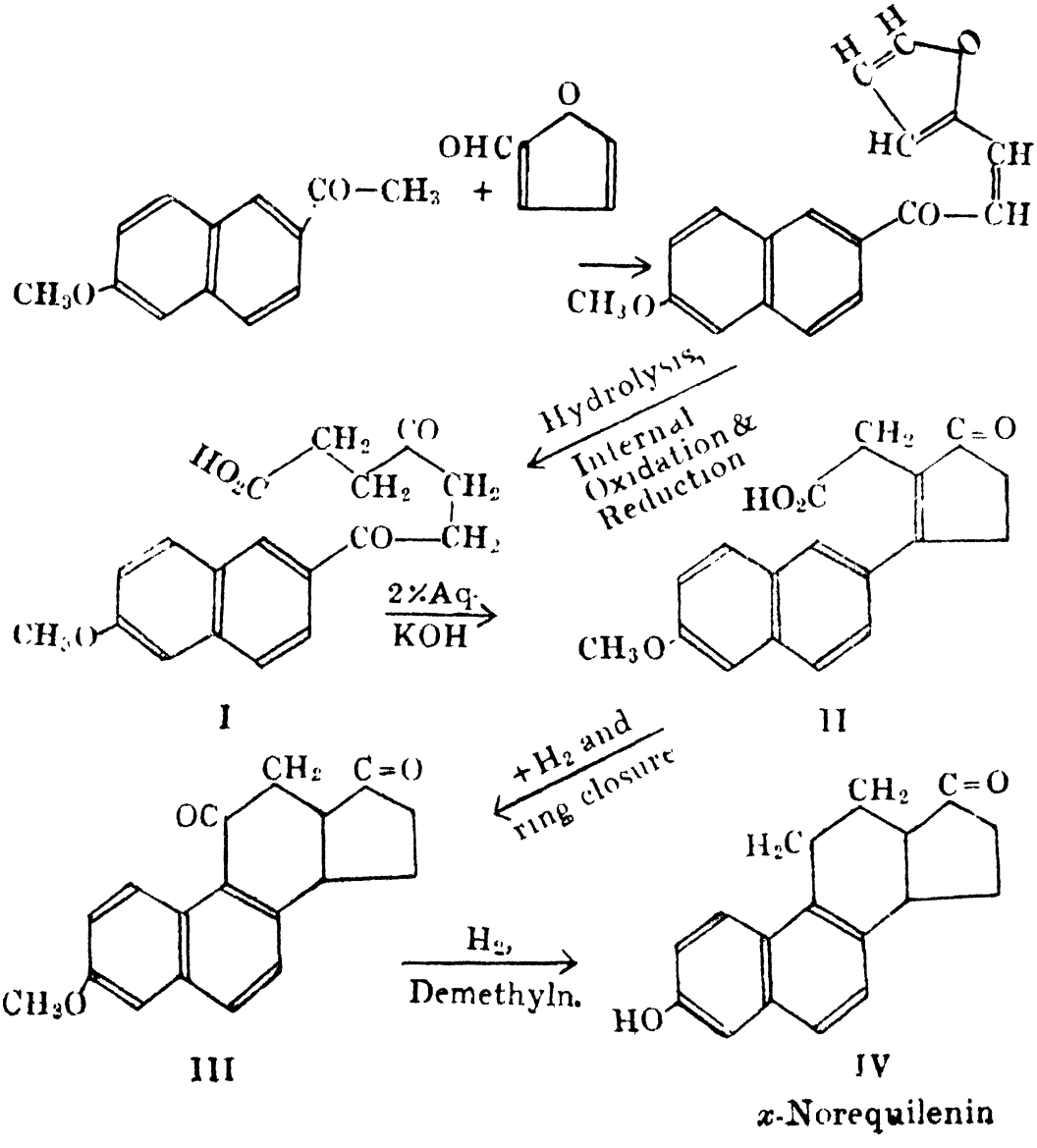


I $\Delta^{4\ 8}$-Pregnadiene-diol-17 : 21-trione-3 : 11 · 20

Total Synthesis of the Sex-hormones:—Though some of the sex hormones could be obtained by partial synthesis starting from naturally occurring sterols and their derivatives and introducing the necessary modifications of the skeletons, their preparation from more elementary substances, that is total synthesis is beset with great difficulties. One that is peculiar to the steroid group is due to the capacity of the tetracyclic structure to assume various stereoisomeric configurations, and synthetic methods invariably give rise to mixtures of a large number of isomerides. However, during the past several years a vast amount of synthetic work has been done with this object in view and some very remarkable

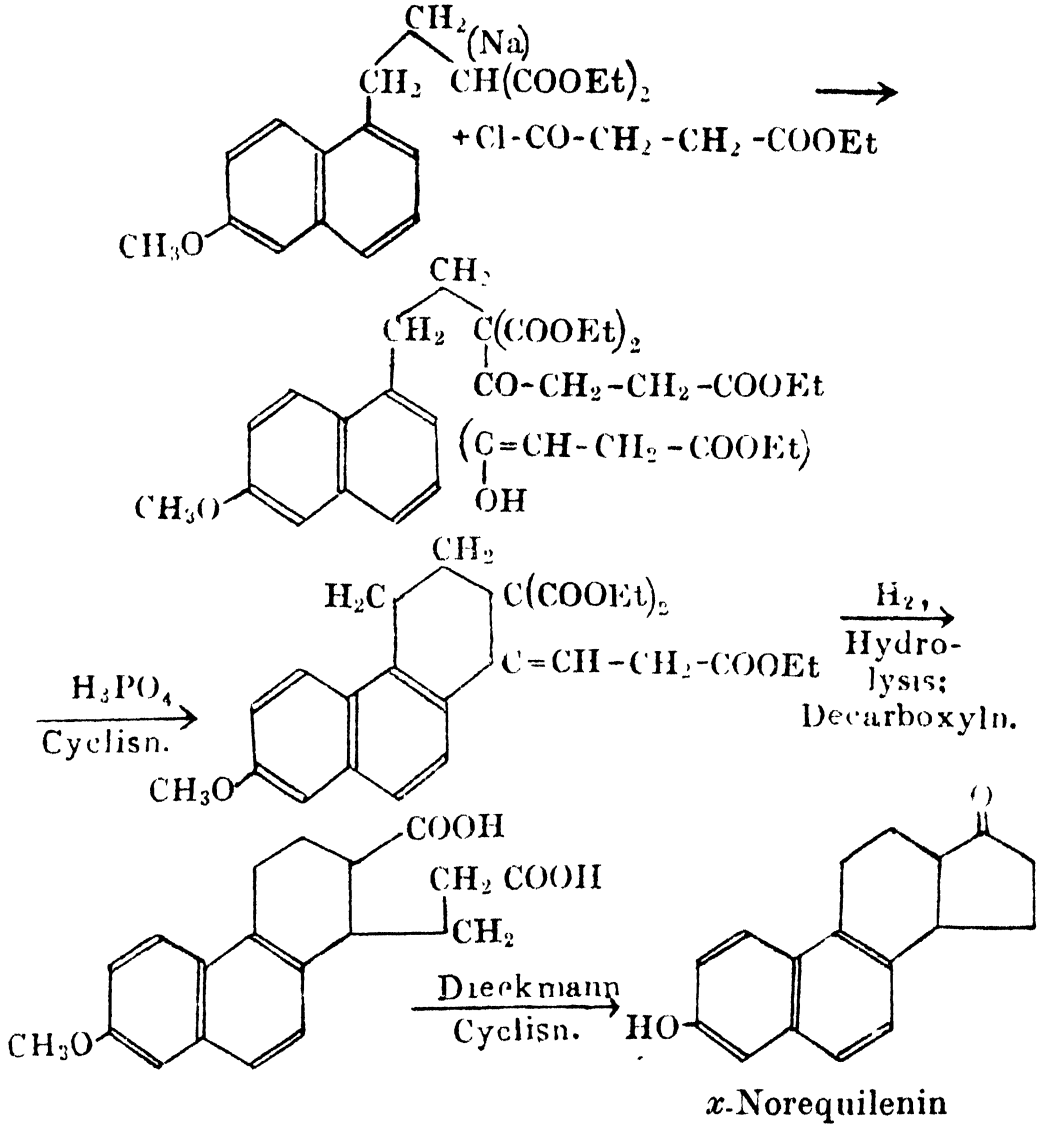
synthetic achievements have been recorded. Two schools of workers stand out most prominently in this connection, (1) (Sir) R. Robinson's school in Oxford and (2) W. E. Bachmann's school in Michigan. Already a number of important compounds of this group have been synthesised. They are *x*-norequilenin, *x*-noroestrone, d-equilenin, dl-oestrone and dl-androstene-dione. Some of the more important features of this work are described below.

***x*-Norequilenin**: This synthesis was effected by Koebner and Robinson[3] and it involved an ingenious double ring closure. The first important stage was 4 : 7-diketo-7-(6'methoxy-β-naphthyl)-heptoic acid (I). On treatment with alkali it underwent the first ring closure yielding a cyclopentenone derivative (II). It was hydro-



genated and then the second ring was closed by the action of phosphoric oxide in syrupy phosphoric acid solution to yield the diketone (III). Catalytic reduction of the CO group in the 11 position and subsequent demethylation produced x-norequilenin (IV). The prefix x is used to indicate that the stereochemical composition is not definite.

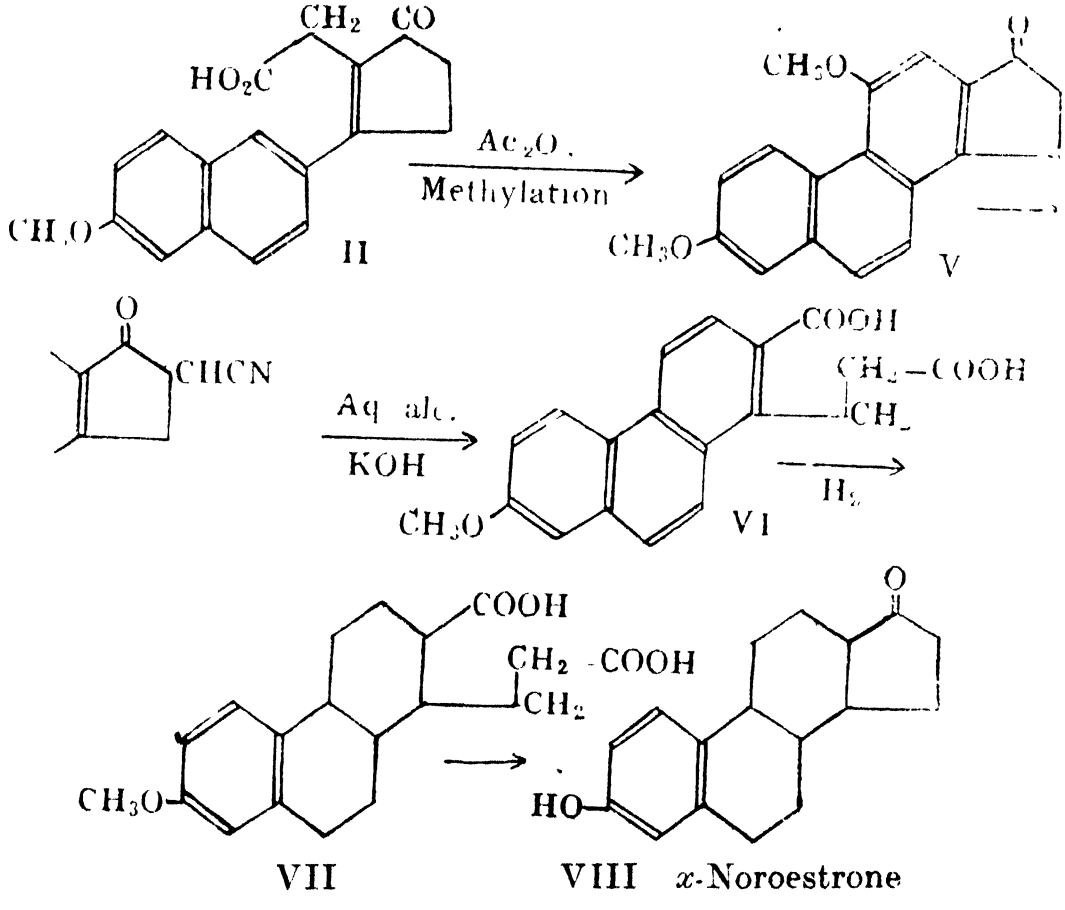
Later the same substance was prepared in a new way by Bachmann et al[4].



x-Norequilenin

Starting from the methyl ether of x-norequilenin and applying a new procedure of angular methylation, Robinson and coworkers[5] have more recently obtained in good yields dl-iso-equilenin (see formula XIV). This has the cis-configuration; the naturally occurring compound is d-equilenin having the trans- configuration.

x-**Noroestrone**: In this compound, ring B is in a reduced condition. To achieve this synthesis Robinson and Rydon[6] adopted another novel technique. By using a modification of the ring closure they converted compound (II) into 4 : 7-dimethoxy-3'-keto-1 : 2-cyclopentenophenanthrene (V). Since direct hydrogenation of this compound was not feasible they opened the five membered ring after the introduction of a nitrile group. Catalytic hydrogenation of the dicarboxylic acid (VI) to (VII) and pyrolysis of its lead salt and demethylation yielded *x*-noroestrone (VIII).

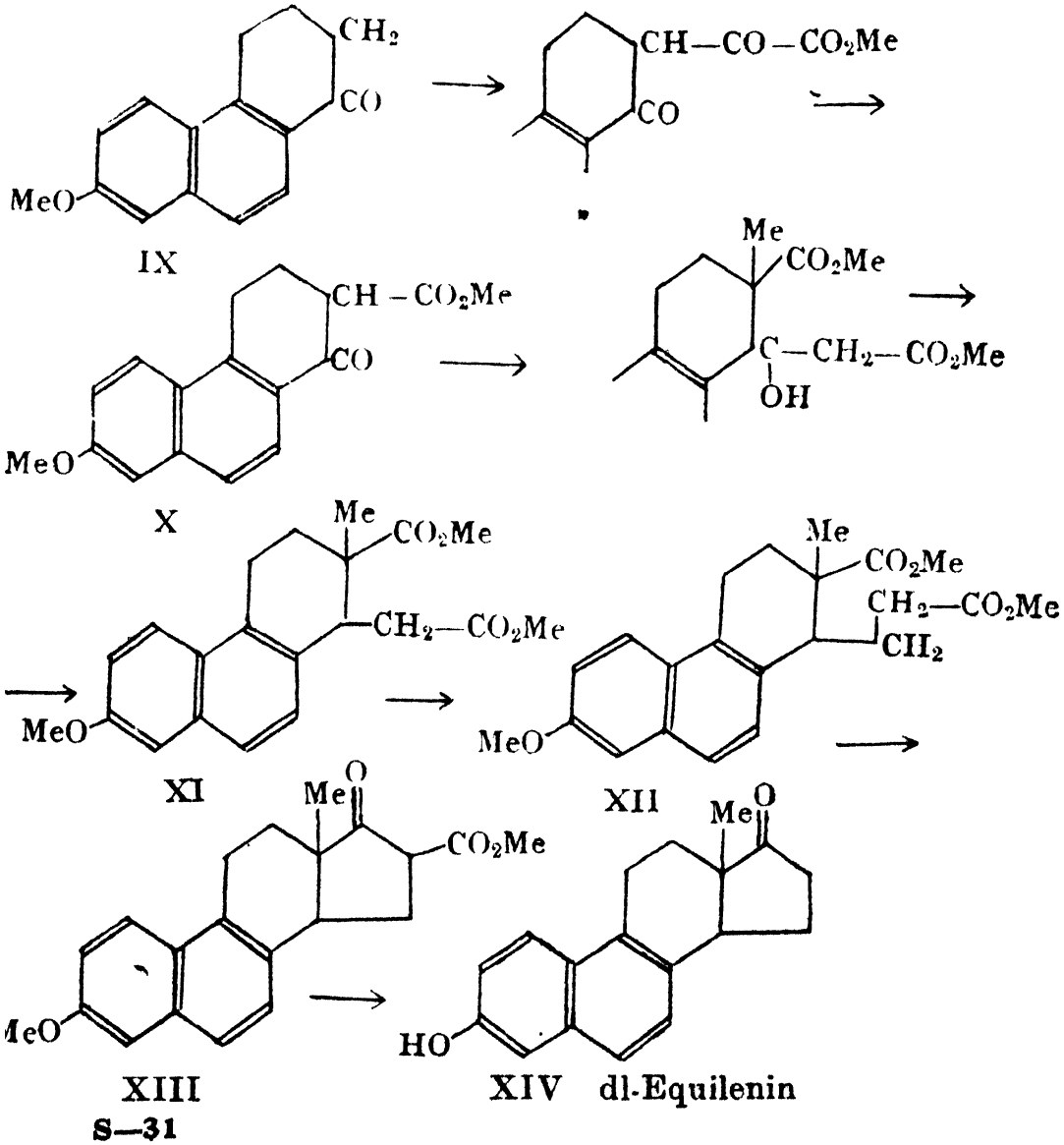


Equilenin and oestrone:—The synthesis of d-equilenin carried out by Bachmann, Cole and Wilds[7] illustrates the application of a new procedure also largely developed by the Robinson school and is an important achievement. It started with 1-keto-7-methoxy-1 : 2 : 3 : 4-tetrahydrophenanthrene (IX). This was condensed with methyl oxalate and when the product (glyoxylic ester) was heated, carbon monoxide was lost and the β-ketonic ester (X) was formed. C-Methylation in the α-position

using sodium and methyl iodide, Reformatsky's reaction, dehydration and reduction produced (XI) as a mixture of cis and trans forms. The free acids were separated and subjected to the Arndt-Eistert chain lengthening process giving the propionic ester (XII) in cis and trans forms. This new method utilises the action of diazomethane on an acid chloride. Decomposition of the diazoketone with water or alcohol in the presence of silver oxide gives the higher acid or its ester respectively and it involves molecular rearrangement.

$$RCO_2H \rightarrow R\text{-}COCl \xrightarrow{CH_2N_2} R\text{-}COCHN_2 \xrightarrow[EtOH,\ Ag_2O]{H_2O\ or} R\text{-}CH_2COOH\ (COOEt)$$

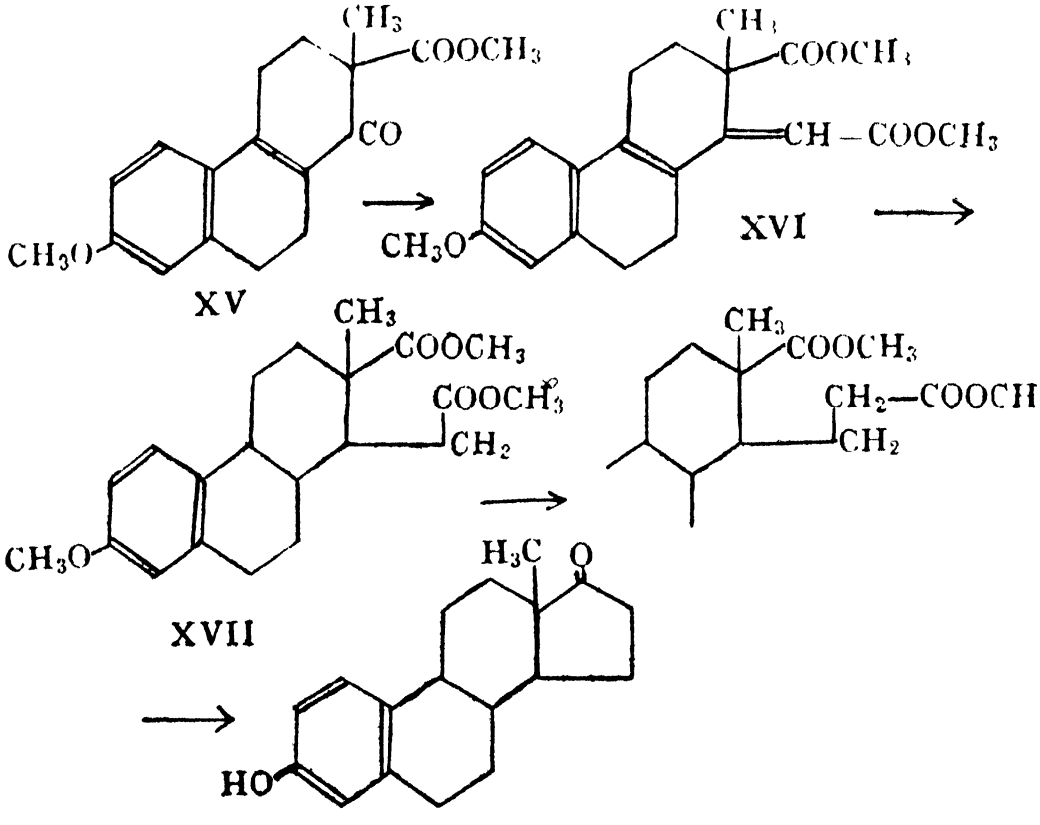
For equilenin synthesis the monoester, with the acetic acid group free, obtained by partial hydrolysis of (XI)



was employed and the diazoketone was decomposed with methyl alcohol yielding the dimethyl ester (XII).

From the trans di-methyl ester ring closure to (XIII) and subsequent hydrolysis, decarboxylation and demethylation yielded dl-equilenin (XIV). Resolution effected through the l-menthoxy-acetic ester gave the d-form identical with the natural hormone. This is thirteen times as active as the l-form. The above stages were worked out so efficiently that 90% yields were obtained in most of them with consequent very high yields of the final product.

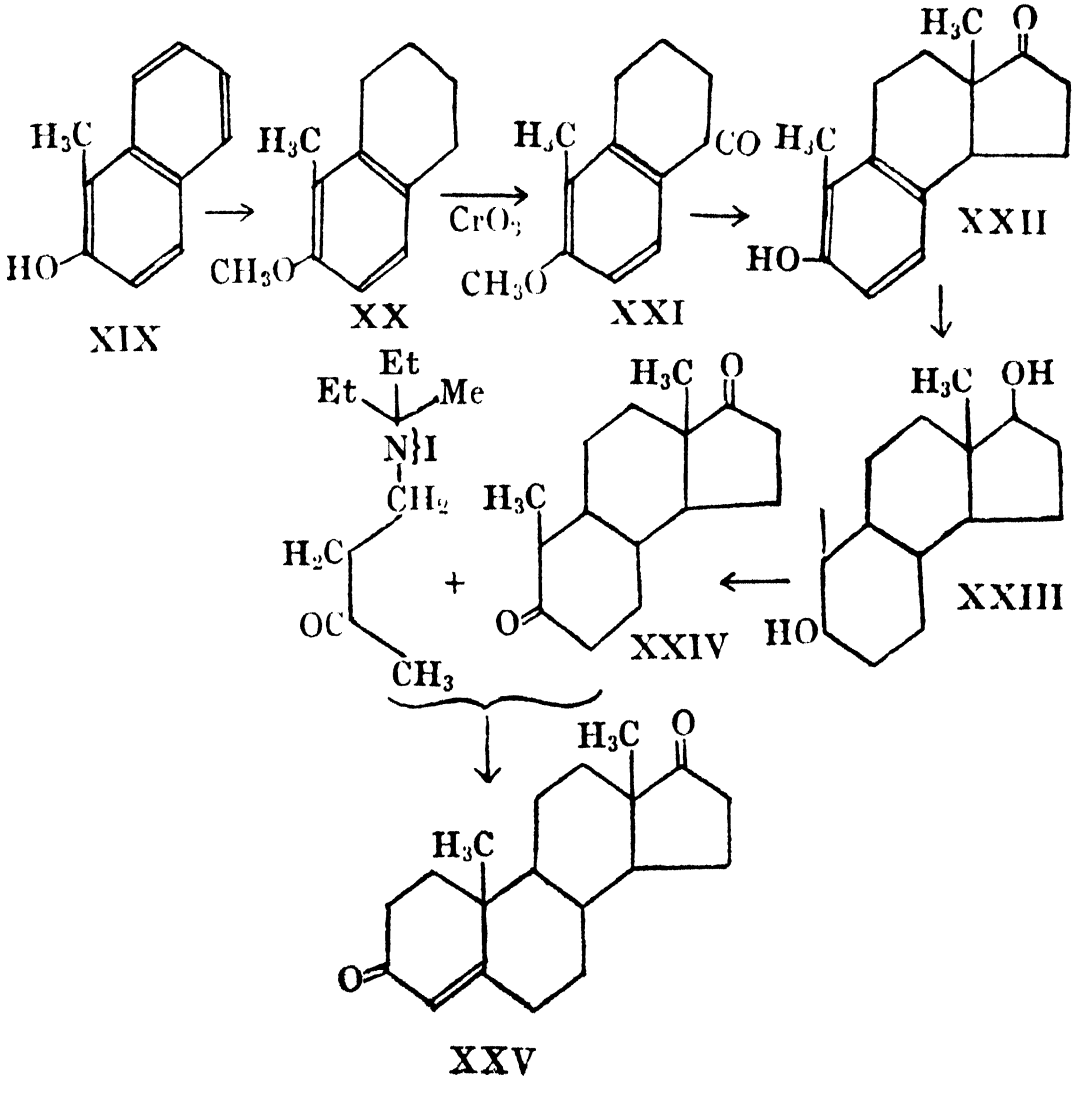
Employing the same basic procedure, Bachmann et al.[8], later effected the synthesis of a stereoisomer of oestrone. In this work more complicated stereoisomeric problems were involved. The starting material was the ketonic ester (XV) which was converted into (XVI) by Reformatsky's synthesis and dehydration using formic acid. Hydrogenation of the two ethylenic linkages was effected using palladium charcoal as catalyst (XVII). Subsequent stages were the same as given above for equilenin. The ultimate product (XVIII) obtained in



XVIII Oestrone and its stereoisomers

good yield was a mixture containing at the most eight dl-forms. It readily yielded a crystalline portion, dl-oestrone-*a*, which is an isomer of dl-oestrone. The uncrystallised residue was considerably more active physiologically and is expected to contain dl-oestrone itself.

Androstenedione:—The more recent synthesis of the androstenedione skeleton by Martin and Robinson[9] involves another interesting procedure known as Robinson-Mannich base method. It is very effective in building up a cyclohexenone ring. The synthesis started from 1-methyl-2-naphthol (XIX) which was catalytically reduced and subsequently methylated to yield 6-methoxy-5-methyltetraline (XX). Oxidation with chromic acid produced the tetralone (XXI) whose conversion into the phenolic ketone (XXII) was effected by methods indi-



cated in the synthesis of equilenin. During this stage two products were obtained, α-(cis) in larger amounts and β-(trans) in smaller amounts. The former was used for further stages. (XXII) could be reduced by high pressure hydrogenation using a special catalyst, palladium on strontium carbonate, to yield the saturated diol (XXIII). Subsequent oxidation led to the formation of the diketone (XXIV). When it was condensed with 4-diethylaminobutan-2-one-methiodide in the presence of sodamide, a mixture of compounds conforming to the formula (XXV) was obtained. The work has been completed with the α-(cis) series. The β-(trans) series is expected to yield the active hormone itself. Since androstenedione could be directly reduced to testosterone the above work, when completed, will mean the synthesis of testosterone also.

CHAPTER XXIII

CORTEX HORMONES

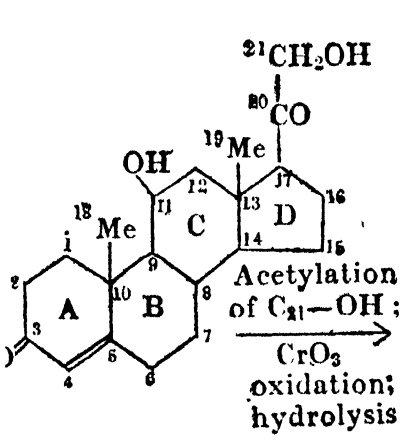
As has already been mentioned the adrenal glands consist of two parts, one being the medulla and the other the cortex. The former produces adrenaline and the latter the cortex hormones (once called cortin). The production of the cortex hormones which are essential for life, seems to be the more important function of the adrenals. A deficit of these hormones caused by injury to the glands, produces the typical symptoms of Addison's disease, bronzing of the skin, muscular weakness and an increase in the blood urea level. Other symptoms of deficiency are disturbance of salt and water balance, disturbance of carbohydrate metabolism and reduction of resistance to cold and shock of different kinds. Complete removal of the suprarenal cortex results in serious break down of the normal physiological functions and is accompanied by large changes in all the organs of the body. The circulation collapses as in the case of surgical shock or shock from burns. Invariably death ensues in a few days. On the other hand, over-development of the glands in children results in precocious sexual development. This observation gave early indication that these hormones should be closely related to the sex hormones and this conclusion was amply supported by a detailed study of their structures.

The systematic examination of extracts from the suprarenal cortex has resulted in the isolation and identification of nearly 30 different constituents which are closely related. Consequently there was considerable difficulty in separating them and confusion existed during the early investigations regarding the individuality of substances isolated by different workers.

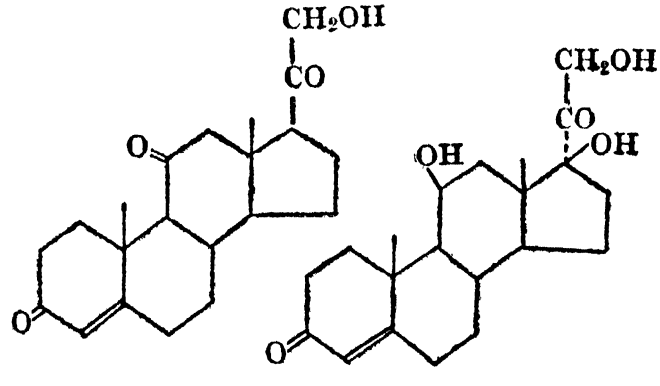
Isolation [1] :—The general procedure utilises their properties as steroid derivatives containing keto and hydroxyl groups. The most successful methods of Reichstein involve gentle treatments, large quantities of ox suprarenals being employed. In recent years whole

glands rather than the dissected cortices have been used as the starting material. Extraction is made with alcohol or acetone. Adrenaline that is also present, is easily separated by making use of its basic properties. Fats are removed by partition between hydrocarbon solvents and water or water-alcohol mixtures. The hydroxylated character of the hormone molecules leading to appreciable solubility in water is a factor involved in the above process. The resulting mixture of compounds is finally separated by fractional crystallisation, partition between solvents or by the application of Girard's reagent T. This ketonic reagent has been used for fractional formation of water-soluble derivatives. Separation has also been effected by fractional decomposition of these derivatives. Another process is chromatography of the hydroxy compounds as well as of their acetates. Since the compounds are in general sensitive to both acid and alkali mild conditions have to be employed.

Names and Properties:—Of the large number of constituents, six are cortical hormones; they have been found to be active according to various methods of assay, either by prolonging life in adrenalectomised animals or by curing or preventing individual deficiency symptoms. A mixture of a number of compounds seems to be responsible for cortical activity. Due particularly to the monumental work of T. Reichstein and his coworkers the constitutions of the hormones have been well established and the more important of them obtained by partial synthesis. They are thus also available more easily for physiological studies. Their names and formulae are given below:

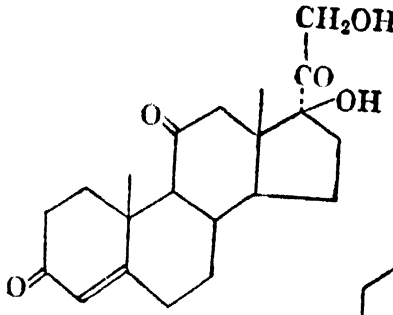


I Corticosterone $C_{21}H_{30}O_4$

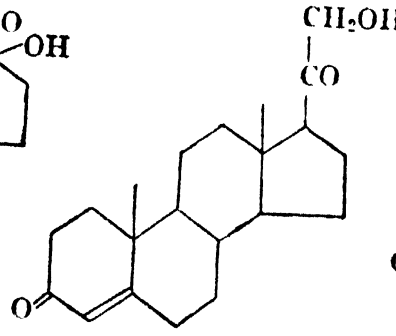


II Dehydrocorticosterone

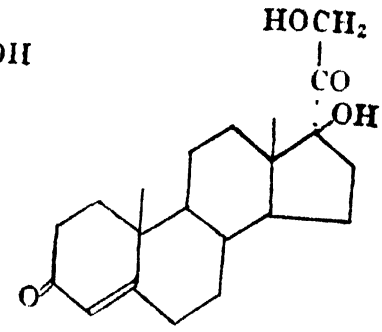
III 17-β-Hydroxy-corticosterone;



IV 17-β-Hydroxy-dehydrocorticosterone $C_{21}H_{28}O_5$



V Deoxycorticosterone $C_{21}H_{30}O_3$



VI 17-β Hydroxy-deoxycorticosterone. Substance S. $C_{21}H_{30}O_4$

[In III, IV and VI, the OH at C_{17} is considered to be above the plane of the ring and the CO · CH_2OH group below the plane of the ring].

Corticosterone (I):—It was the earliest of the hormones to be characterised and studied and is the most abundant in the gland. It crystallises from acetone in the form of plates melting at 180°; $[\alpha]_D^{18°}$, +223° in ethanol solution. Concentrated sulphuric acid gives an orange solution with an intense green fluorescence. By cautious oxidation of the acetate, the dehydro compound (II) can be obtained. It possesses most of the typical cortin properties.

Dehydrocorticosterone (II):—It crystallises as prisms from aqueous acetone and melts at 177°; $[\alpha]_{5461}^{25°}$, + 299° in ethanol. It can be obtained from compound (I) by careful oxidation[2] and it closely resembles it in most properties. A characteristic difference is the lack of fluorescence in sulphuric acid solution.

17-β-Hydroxycorticosterone (III):—It crystallises from ethanol in small prisms melting at 207°; $[\alpha]_D^{22°}$, + 167° in ethanol. Concentrated sulphuric acid yields an orange solution with green fluorescence. By careful oxidation it can be converted into the dehydro-compound (IV).

17-β-Hydroxy-dehydro-corticosterone (IV):—It crystallises from ethanol or acetone as rhombohedral crystals

melting with decomposition at about 215°; $[\alpha]_D^{25°}$, + 209° in ethanol. The pure substance does not exhibit fluorescence in sulphuric acid.

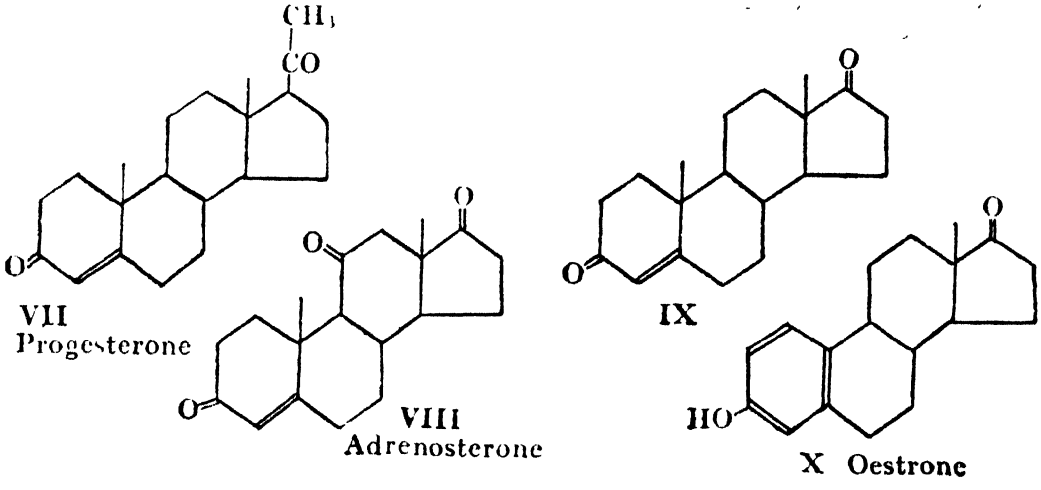
Deoxy-corticosterone (V):—It was first obtained synthetically and was later discovered in the gland extracts. It crystallises from ether as colourless plates melting at 141°; $[\alpha]_D^{22°}$, + 178° in ethanol.

Substance S (VI):—It crystallises from ethanol as flat spear-head shaped crystals melting at about 213° with decomposition. It gives a pure red colour with concentrated sulphuric acid.

All the six compounds are colourless substances exhibiting positive rotation. In virtue of the hydroxy-ketone unit in their structure they reduce alkaline silver solutions readily in the cold. Their absorption spectra exhibit a definite band at about 240 mμ. indicating the existence of $\alpha:\beta$-unsaturated carbonyl groups. The 21-mono-acetates are readily prepared and they form important derivatives particularly for biological tests. The members that have a hydroxyl group in the 11-position exhibit marked fluorescence in sulphuric acid solution.

The cortex hormones seem to fall into two definite groups not only chemically but also physiologically. The first four are more complex since they are oxygenated in ring C. The last two are simpler; on this account they are more easily obtained synthetically. As already mentioned deoxycorticosterone (V) was first known only as a synthetic analogue of corticosterone and was prepared with a view to support the constitution of corticosterone; its presence in adrenal cortex was detected later. This most easily available compound of the group appears to be the most potent physiologically. But the oxygenated compounds (I to IV) have certain distinctive features. They exhibit a remarkable effect on carbohydrate metabolism; they have a strong anti-insulin action whereas (V) and (VI) show little or no such activity. Hence the former are important for the clinical treatment of such conditions as Addison's disease and surgical shock.

Besides the typical cortex hormones described above, the glands contain amongst others the following:—(1) progesterone (VII) which has a small cortin activity; (2) adrenosterone having the constitution (VIII) and considered to be a product of degradation of compounds of the type (III) and (IV); this has male hormone activity equal to one-fifth of that of androsterone; (3) Δ^4-androstene-3 : 17-dione (IX) obtained in small amounts and also considered to be a degradation product; (4) oestrone (X).

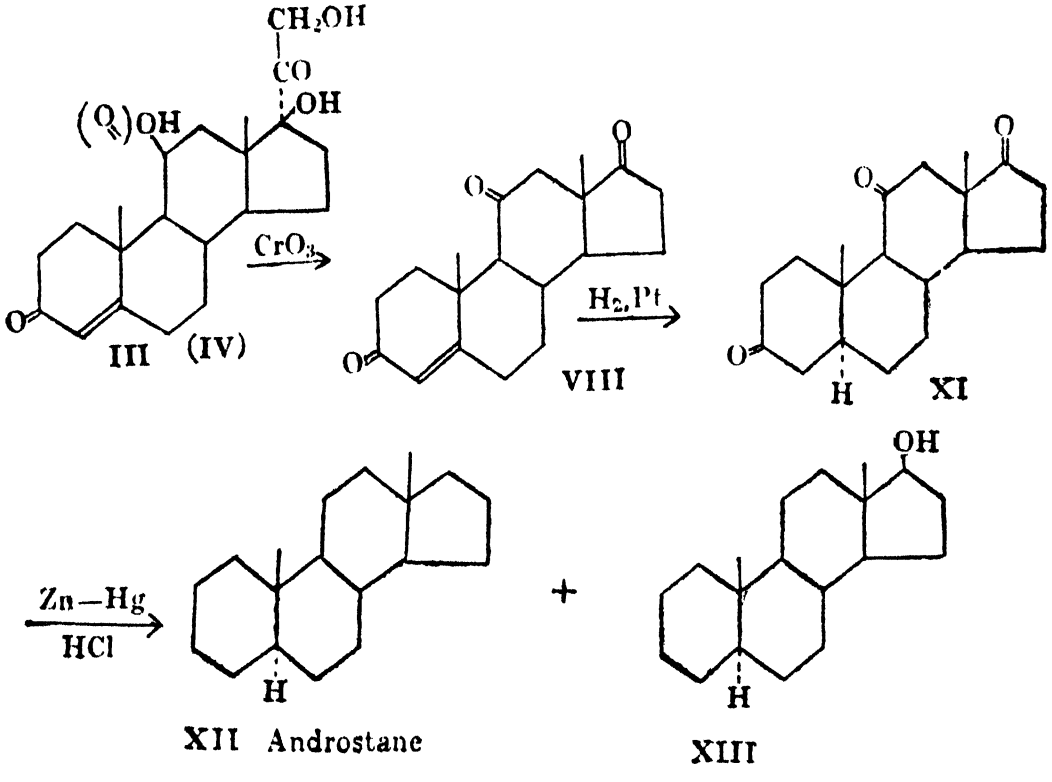


Constitution:—It was noticed early that these hormones possess male hormone activity and should therefore be related to the androgens. In regard to their instablility to alkali they resemble progesterone and testosterone. Further they exhibit strong absorption in the ultraviolet at 240 mμ., which is characteristic of a ketone group conjugated with an ethylenic double bond. They reduce alkaline silver solutions readily; this may be due to aldehyde or hydroxy-ketone groups. But oxidation with periodic acid[3] (a reagent specific for hydroxy-ketones) yields acids with one carbon less and this supports the latter alternative.

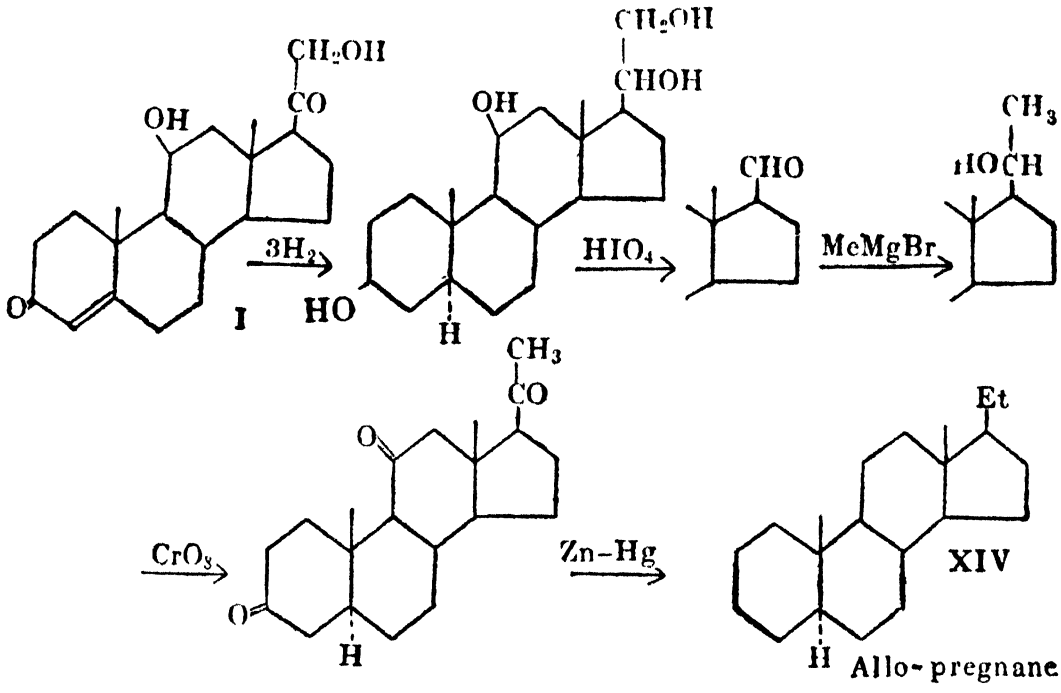
$$\underset{\text{Coticosterone}}{C_{19}H_{27}O_2-CO-CH_2OH} \xrightarrow{HIO_4} C_{19}H_{27}O_2-CO_2H + CH_2O$$

The presence of the steroid skeleton[4] was established by transformations starting from the 17-hydroxy-compounds (III) and (IV). As has already been mentioned, the former can be converted into the latter by the oxidation of the acetate with chromic acid and subse-

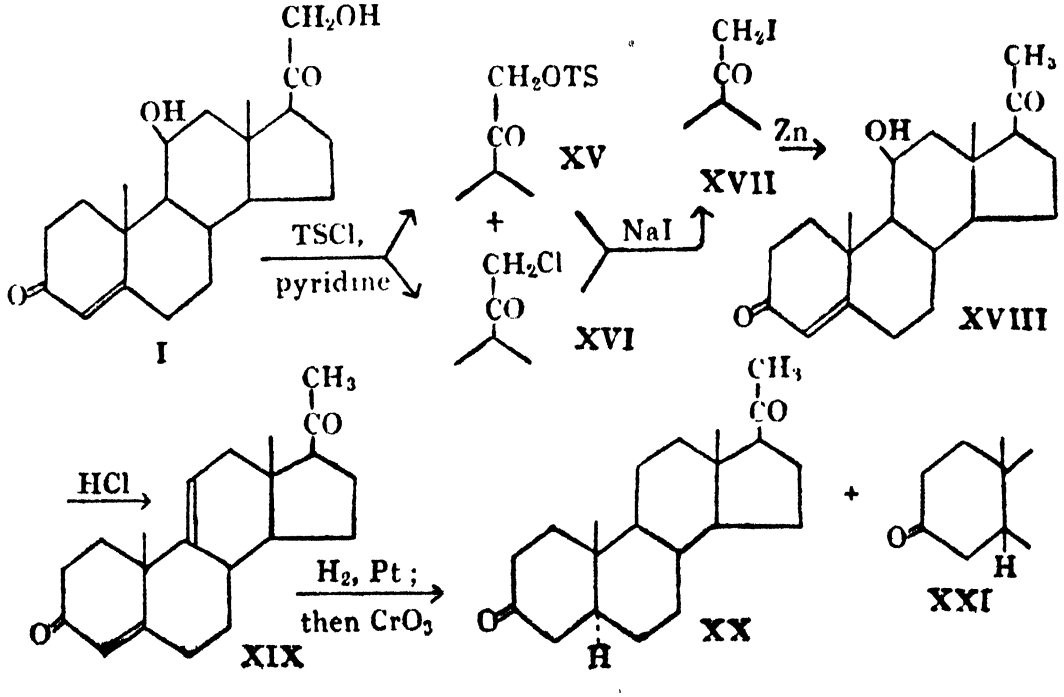
quent hydrolysis. Oxidation of either of them with chromium trioxide yielded the triketone, adrenosterone (VIII). The ethylenic double bond was reduced by catalytic hydrogenation in the presence of platinum and the dihydro compound (XI) reduced by means of zinc amalgam and hydrochloric acid (Clemmensen's reduction). The main product was found to be androstane (XII); a small amount of 17-hydroxy-androstane (XIII) was also formed. This result not only established the steroid skeleton but also located an oxygen atom in position 17 in adrenosterone and consequently in the hormones employed.



The presence of a hydroxy-ketone side chain was already established by periodic acid oxidation. Its location in the 17 position[5] was proved by the transformation of corticosterone (I) into allo-pregnane (XIV) by the following steps.

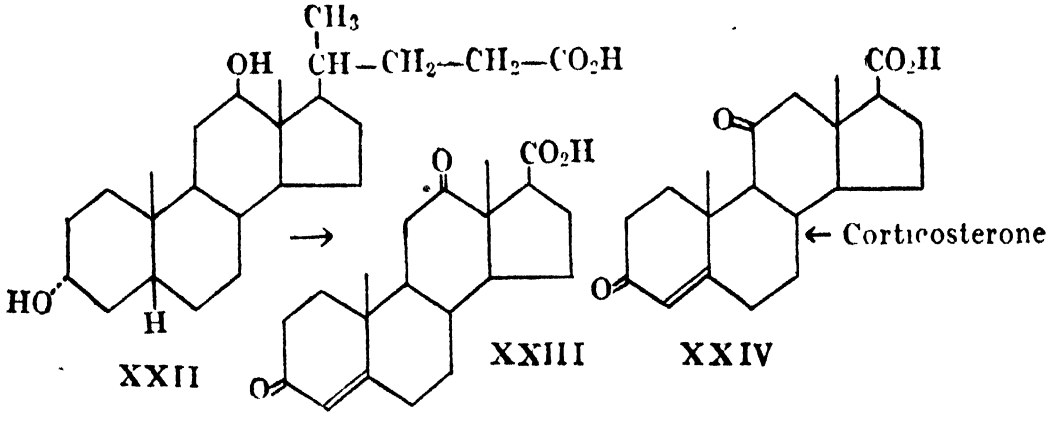


That two ketonic oxygen atoms are present in the 3 and 20 positions and that the double bond is present in the Δ^4-position was established by the following transformations starting again from corticosterone[6]. On treatment with p-toluene-sulphonyl-chloride and pyridine, corticosterone (I) gave a mixture of the ester (XV) and chloro-ketone (XVI). These readily yielded the iodo-ketone (XVII) by the action of sodium iodide in acetone and it was subsequently reduced with extreme ease to

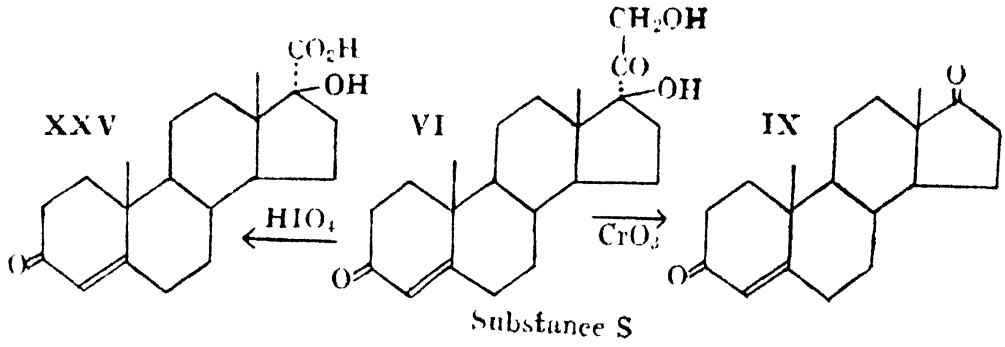


11-hydroxy-progesterone (XVIII). This compound was dehydrated with hydrochloric acid to give Δ^9-dehydro-progesterone (XIX) as the principal product. Hydrogenation of this and final oxidation of the product furnished allo-pregnane-3 : 20-dione (XX) together with a little of the stereo-isomer (XXI).

There were special difficulties in locating the oxygen atom in position 11[7]. This hydroxyl is extremely unreactive and can be acetylated only with great difficulty or not at all. The corresponding carbonyl group does not form any characteristic ketonic derivative. The position was originally fixed by elimination of other possibilities. It was shown that dihydro-adrenosterone (XI) which, as already mentioned, is a definite product of degradation cannot have the third oxygen atom in any other position. Its properties favoured the alternatives of 11 and 12 positions of which the former was preferred. The latter was ruled out definitely by the work of Hoehn and Mason who prepared (XXIII) by the degradation of desoxy-cholic acid (XXII) and showed it to be different from the corresponding acid (XXIV) obtained from corticosterone.

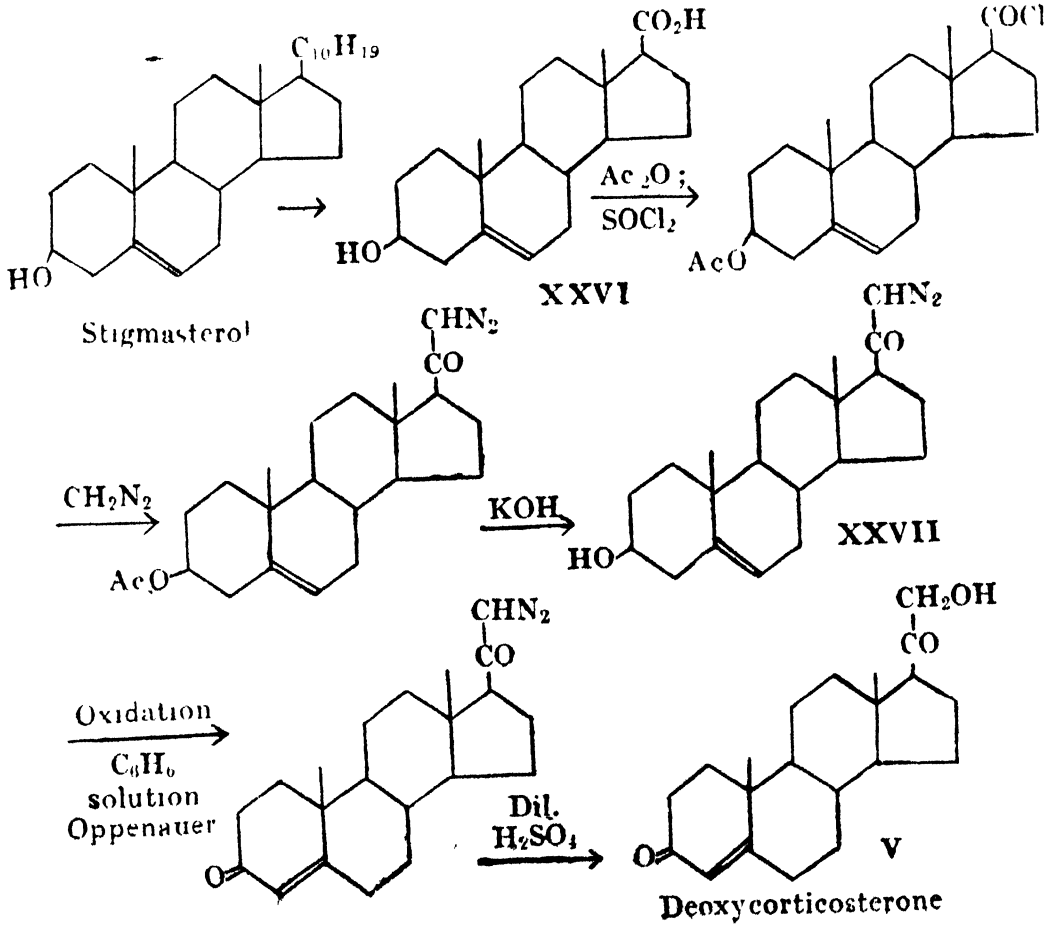


The determination of the constitution of the hormones without an oxygen atom in position 11 was much simpler, since they could be readily converted into known steroids. For expample substance S (VI) gave by oxidation[8] with periodic acid 17-(β)-hydroxy-3-keto-Δ^4-aetiocholenic acid (XXV) and with chromium trioxide furnished the known Δ^4-androstene-3 : 17-dione (IX).

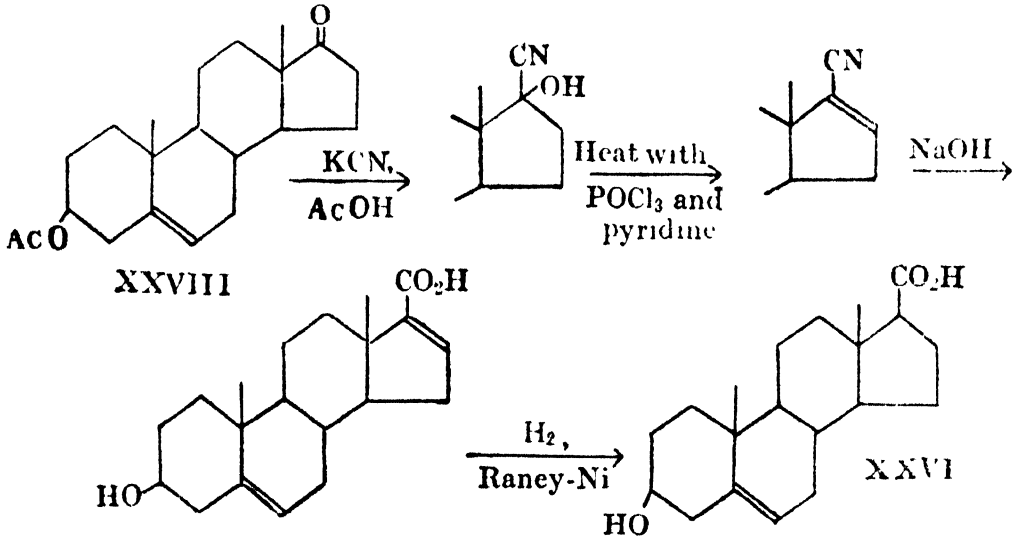


Partial synthesis:—Confirmation of the constitutions of the hormones has been obtained by the partial syntheses of three typical members. They are briefly described below.

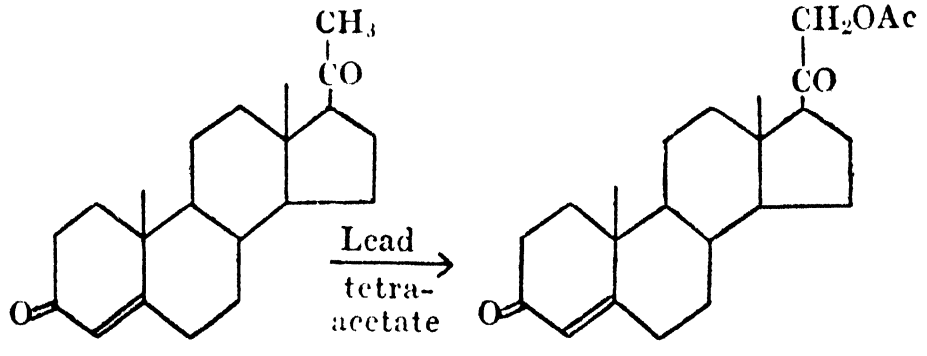
Deoxy-corticosterone:—This synthesis starts with stigmasterol which was first converted by Reichstein and Steiger[9] into 3-(β)-hydroxy-Δ^5-aetiocholenic acid (XXVI). The subsequent important stages are (1) the preparation of the diazoketone (XXVII), (2) oxidation of the hydroxyl group in the 3-position and (3) decomposition of the diazoketone group to yield deoxy-corticosterone.



The acid (XXVI) can also be obtained from sapogenins by degradation and from Δ^5-dehydro-androsterone acetate (XXVIII) by synthesis [10] as shown below:



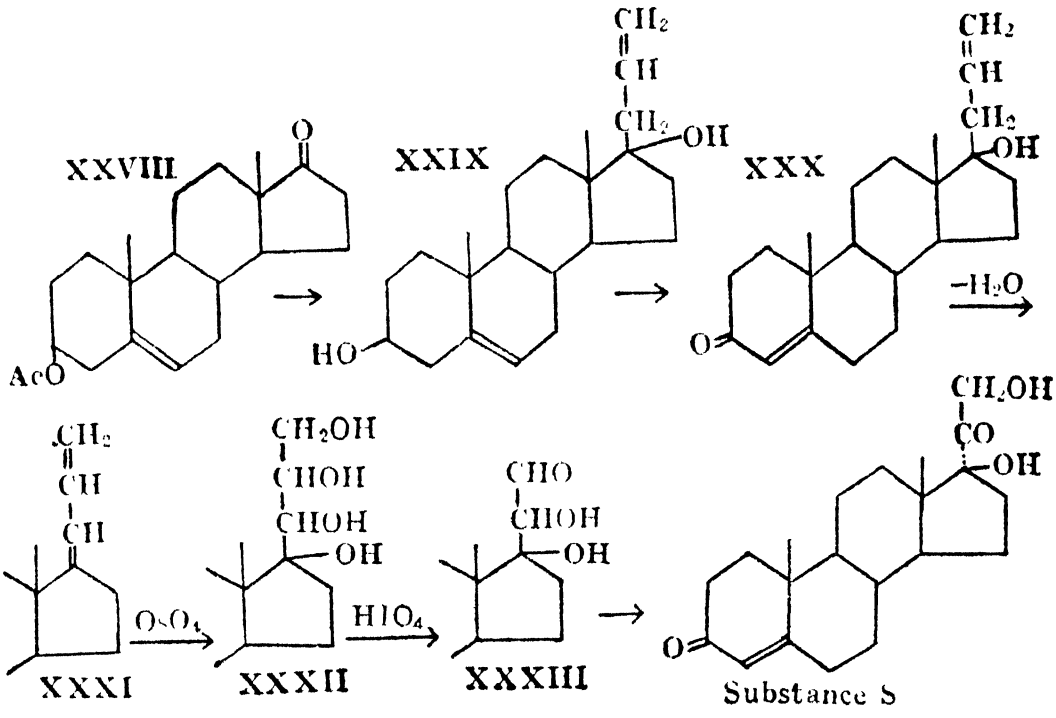
Among other methods of preparing deoxycorticosterone, the one starting from progesterone is simple though it gives poor yields. Erhardt et al. [11] found that progesterone could be oxidised directly with lead tetra-acetate to form deoxycorticosterone acetate.



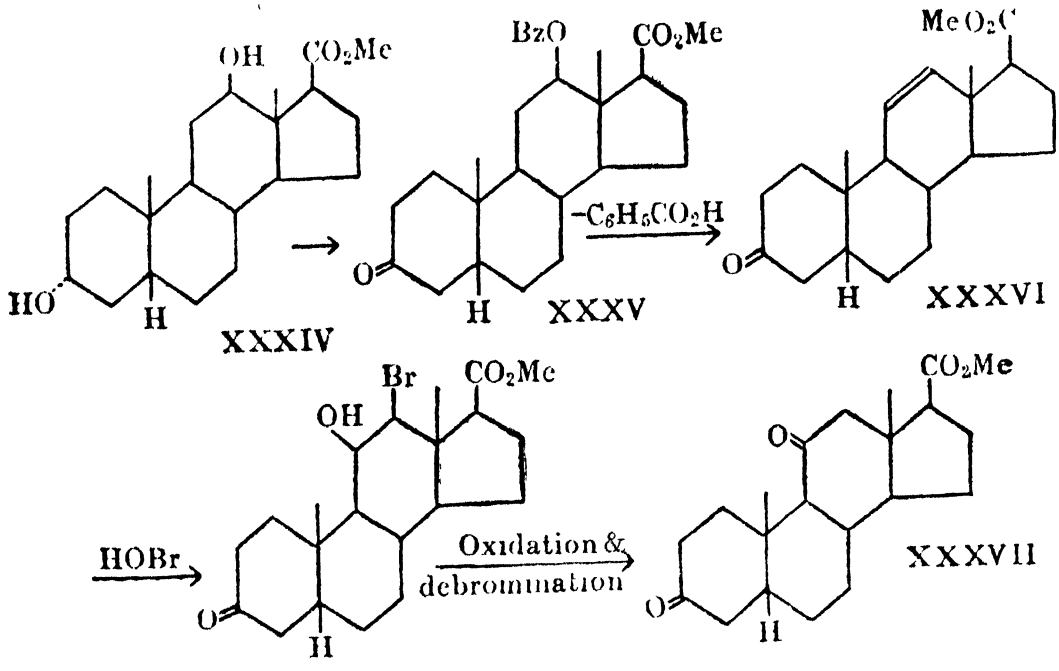
Progesterone — Deoxy-corticosterone acetate

Substance S. [12]:— Dehydroandrosterone acetate (XXVIII) was the starting point. By the action of allyl bromide and magnesium it was converted into 17-allyl-androstenediol (XXIX) which was oxidised with aluminium tert. butoxide and acetone to the corresponding $\alpha:\beta$-unsaturated ketone (XXX). Dehydration of this yielded the trienone (XXXI). It was first oxidised with osmium tetroxide to the tetrahydroxy compound (XXXII). Further controlled oxidation of the tetrol with periodic acid gave the hydroxy-aldehyde (XXXIII)

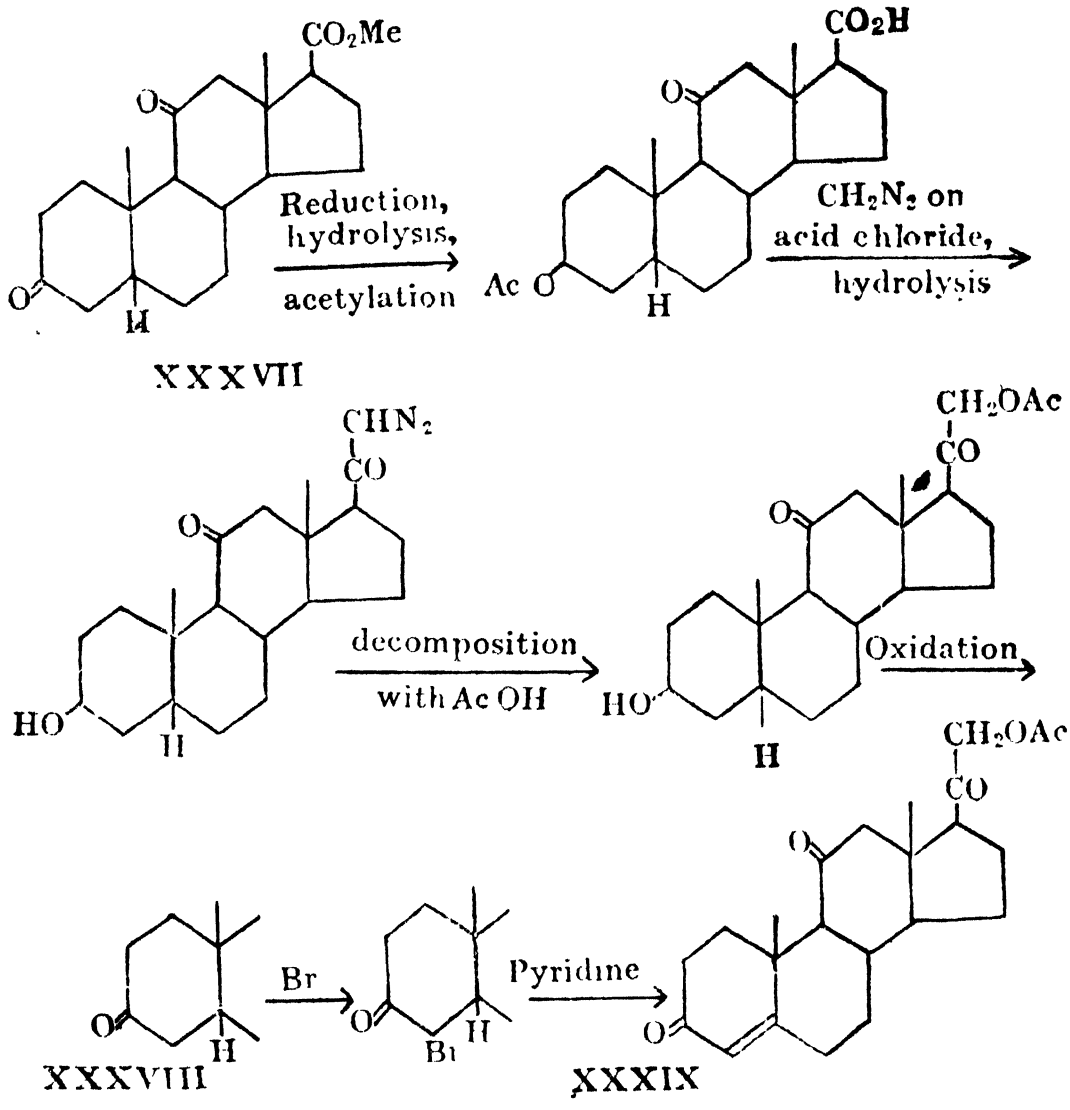
which underwent isomeric change to substance S when treated with pyridine.



Dehydrocorticosterone:—The recent synthesis of dehydro-corticosterone[13] (II) is important for several reasons. It provides unequivocal proof of the position of the oxygen atom in ring (C) of the oxygenated steroids I to IV). It further opens out possibilities of eventual synthesis of all of them and of their availability more readily for physiological examination. Hitherto they were obtained in poor yields with great difficulty by the extraction of the glands. The synthesis itself is the culmination of a large amount of persistent and difficult work. It is based on the utilisation of a 12-oxygenated bile acid or its degradation product and involves a large number of stages. The first stage is the conversion of methyl 3-α-12-β-dihydroxy-aetiocholanate (XXXIV) into methyl 3-keto-Δ^{11}-aetiocholenate (XXXVI) and involves partial oxidation and subsequent removal of water. The latter operation is effected indirectly by pyrolysis of the benzoate (XXXV). The second stage consists in the re-introduction of the oxygen atom, as a keto group in position 11 (XXXVII). This is effected by the addition of hypobromous acid, subsequent oxidation and debromination.



The third stage involves the building up of the hydroxy-ketone side chain. This is carried out in a way similar to that adopted in the case of deoxycorticosterone. The keto-group in the 3-position is catalytically reduced before this transformation and later re-established by oxidation (XXXVIII). As the last stage the Δ^4-double bond is introduced by bromination and treatment with pyridine. The product is dehydro-corticosterone acetate (XXXIX).



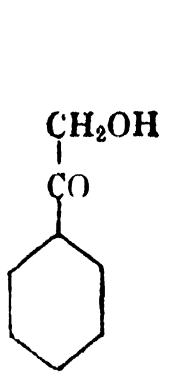
Dehydro-corticosterone acetate

Assay:—Cortex extracts and hormones can be assayed by determining the period of time they could keep test animals alive and well after removal of the suprarenals. Rats and mice have the advantage of cheapness, but they may develop accessory cortical tissue and survive indefinitely. Dogs have therefore been preferred. Drakes give the result in a shorter time since they normally survive less than ten hours after the removal of the adrenals.

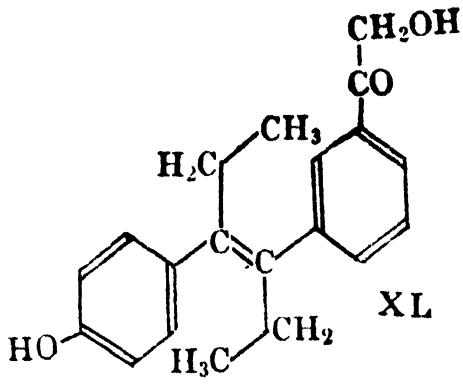
One rat unit is defined as the minimum daily dose sufficient to protect at least 80 per cent of the rats and produce an average growth of 20 grams for a 20 day period in 4 weeks old male rats of 50 to 60 grams weight. The dog unit is the minimum daily dose per kilogram

body weight, which will maintain the blood urea level and weight in normal condition when administered for a week. Other assay methods using sodium retention, carbohydrate metabolism and sensitivity to shock, cold, etc. have also been suggested. With cortex extracts, corticosterone and dehydro-corticosterone, administration by mouth or by injection has the same efficiency, whereas deoxycorticosterone is several times more efficient by injection than by oral supply. Differences therefore arise due to the method of administration and also due to the physiological criterion employed.

Constitution and physiological properties:—An examination of the constitution of the cortex hormones may lead to the conclusion that the minimum of structural characters required for physiological activity is embodied in the simplest of them, deoxycorticosterone. They are (1) the steroid skeleton, (2) the $\alpha:\beta$-unsaturated carbonyl group in ring A and (3) the hydroxy-ketone side chain. Regarding (2) it may be pointed out that a large number of steroid compounds that do not possess this grouping have no activity. Further the reduction of the Δ^4-double bond destroys activity in several cases. However there are some examples of synthetic active compounds in which this grouping is absent As regards (3) the hydroxyl group at C_{21} may appear to be essential for marked activity, since progesterone has only very feeble activity and 17-hydroxyprogesterone is inactive. There are exceptions even to this. It should be mentioned that even the steroid skeleton (1) does not seem to be absolutely essential for activity; non-steroid α-ketols[14] like benzoyl-carbinol and compound (XL) have been reported to exhibit cortin activity. Thus there does not seem to exist strict structural specificity in the cortex hormones. As already mentioned the oxygen atom at C_{11} is essential for activity in carbohydrate metabolism. An additional hydroxyl group at C_{17} enhances this property.



Benzoylcarbinol



XL

Very little is known regarding the mechanism of action of cortical hormones. There seems to be some evidence that they are involved in phosphorylation processes. Urinary steroids, particularly androgens, are considered to arise partly from cortex hormones by side chain oxidation and other modifications.

CHAPTER XXIV

HORMONES OF UNKNOWN CONSTITUTION

INSULIN

One of the most brilliant and useful discoveries in the field of hormones is that of insulin. The pancreas is the seat of two types of secretions: (1) the digestive juice (pancreatic juice) which is discharged into the duodenum and (2) an internal secretion which is confined to the so-called "Islets of Langerhans" and is directly poured into the blood stream. That the cause of diabetes mellitus is the improper functioning of the pancreas and that the internal secretion of this gland contains a hormone which can cure diabetes was definitely established by the work of Banting, Best, Collip and Macleod who isolated the hormone in 1921. In the beginning an extract of the pancreas was employed for the treatment of the disease and subsequently the hormone, insulin in a more or less pure condition came into use.

Isolation:—Insulin of commerce is made from the pancreas of the ox and the pig. The islet tissue of fish, particularly cod, is said to be a very good source. Since the hormone is of protein nature its purification is difficult and only under special conditions could it be crystallised. Fresh glands are directly employed; if they have to be stored, the action of proteolytic enzymes is inhibited by quick refrigeration and the hormone afterwards extracted by treatment with suitable solvents. Purification involves removal of fats and accompanying proteins. A number of methods have been described by different workers. The following is that of Scott and Parker [1].

The fresh glands are minced at a low temperature, soaked in acidified aqueous alcohol and allowed to stand overnight. The extract is separated by centrifugation, rendered alkaline with ammonia (p_H 8) and the heavy inactive precipitate filtered. The filtrate is then reacidified and the alcohol distilled off in vacuo. At this

stage most of the fat is thrown out and can be easily filtered off. The active protein material in the solution is liberated by salting out with 25% sodium chloride. The precipitate is subjected to several successive stages of purification: (1) dissolution in water and reprecipitation by the addition of sodium chloride to 15% concentration; (2) slow isoelectric precipitation at p_H 5 at a low temperature (2°.); (3) precipitation from solution in acidified water (p_H 2) by the addition of excess of alcohol and ether and (4) final purification by isoelectric precipitation at p_H 5. The product, when dissolved in dilute hydrochloric acid (p_H 2·8) and sterilized by means of bacterial filter (Seitz), can be used for therapeutic purposes. The yield is 2000 to 3000 international units per kilogram of pancreas which is roughly about 0·01%. On a manufacturing scale enamelled or earthenware apparatus has to be used and contact with metal avoided. As the result of great improvements in manufacture, insulin is now available for therapeutic purposes at a very moderate cost.

Crystalline insulin:—Insulin is ordinarily amorphous. In view of its complex protein nature its crystallisation was considered to be an achievement. Crystalline insulin was first prepared by Abel in 1926 and subsequently by Harington and Scott in 1929. The method consisted in using a highly buffered medium (ammonium acetate buffer) and adjusting the p_H to about 6 (5·8 to 6·3). Abel further added large quantities of brucine and pyridine and Harington and Scott used saponin instead. It was considered that by these means the isoelectric point could be slowly and correctly reached and supersaturated solutions obtained. The conditions were thus favourable for the separation of insulin in a crystalline form.

Scott[3] later examined this subject more thoroughly. He found that the crystals contained zinc whose origin was traced to the pancreas; bovine pancreas contains about 20 mg. of zinc per kilogram of fresh tissue. The presence of this metal appeared to be essential for crystallisation, because insulin, specially purified by electrodialysis and precipitation from acid solution by means

of alcohol and ether and containing very little mineral matter, could not be crystallised. Further, the addition of a small amount of zinc chloride to the medium again rendered crystallisation possible. According to him the simplest and most rapid method is to start with a phosphate buffer having p_H 7·5 and to bring the p_H to 2·3 by the addition of N hydrochloric acid. The insulin solution is added to this along with a small volume of dilute zinc chloride solution and some acetone. The p_H is then adjusted to 6·2 by adding N ammonia. The clear solution deposits crystals slowly when stirred and allowed to stand in a refrigerator for two days and they are separated by means of a centrifuge.

Crystalline insulin is generally rhombohedral; but its habit varies considerably. It is considered to be dimorphic. By analysing a number of carefully recrystallised samples Scott showed that the percentage of zinc is constant, being 0·52 and hence considered that it is held in chemical combination. Since, however, the crystals do not differ in several of their properties from an ash-free amorphous sample of insulin, zinc may not be involved in salt formation, but be present in a co-ordinate complex. They are therefore appropriately called zinc-insulin crystals. Later work indicates that the percentage of zinc is variable, depending upon the p_H of crystallisation, and the preparation of crystals containing much smaller amounts of zinc has been reported[3].

Properties and constitution[4]:—When crystaline insulin is heated it turns brown at 216° and melts rather sharp at 233° with decomposition. It is optically active and leavorotatory, the specific rotation varying with the p_H of the solution. It exhibits a characteristic absorption band in the ultraviolet between 250 and 290 mμ. and this is ascribed to the presence of cystine and tyrosine units in the molecule. The isoelectric point is at p_H 5·3; but the optimum p_H for crystallisation is 5·8 to 6·3 probably due to the product being a zinc complex.

Insulin is soluble in acids and alkalies and is sparingly soluble in most other solvents. It is unstable to alkali but relatively more stable to acids. It gives the

precipitation and colour reactions of a typical protein The elementary composition is that of an average simple protein, nitrogen being 16 per cent. It is exceptional in having a comparatively high sulphur content (3·2%) there is no phosphorus. Special reactions indicate the presence of arginine, tyrosine and cystine and the absence of tryptophane, sulph-hydryl and carbohydrate units. By the action of reducing agents - SH groups seem to be produced. Besides its sensitiveness to proteolytic enzymes it is also easily decomposed by oxidising as well as by reducing enzymes.

As in the case of other proteins there is great difficulty in obtaining information about the chemical composition and molecular weight of insulin. Many of the data are only approximate. The chemical composition seems to be best represented by the formula $C_{45}H_{75}O_{17}N_{11}S$ (Abel); the empirical formula weight should therefore be 1073. The molecular weight is found to be 20,000 by the chemical method. As determined by the ultra-centrifuge method at p_H 7·0 it is 35,000 and by the X-ray method it is 37,000. Viscosity measurements give values ranging between 40,000 and 50,000. Analytical study has not so far disclosed any special constituent chemically different from ordinary amino acids. The component amino-acids are cystine, 12%; leucine, 30%; glutamic acid, 21% (30%)), tyrosine, 12%; histidine, 4% (8%); arginine, 3%; lysine, 2%; proline, 10% and phenyl-alanine, 1%. Serine and threonine have also been found to be present. On the basis of the molecular weight of 35,700 the number of peptide bonds per molecule is estimated to be 292 ± 10.

As a protein insulin is remarkable for the very large number of ionisable groups it contains. It has been estimated that the acid-binding groups (basic groups) are 43 ± 2 and the base-binding groups (acid groups) $60-70$. Of the latter, carboxyls number 30-35, the rest being phenolic. The percentage of tyrosine corresponds to 24 tyrosine groups per molecule. Iodination of insulin is easy; the amount of iodine taken up corresponds to the formation of 3 : 5-di-iodotyrosine units and indicates that the tyrosine phenolic groups are free. No other change

in the molecule seems to take place. The iodinated insulin retains only about 5 to 10% of the physiological activity of insulin and partial removal of iodine restores the original potency proportionately. The phenolic groups seem to be therefore essential for activity[5]. Similar reduction in activity is produced by acetylation involving hydroxyl and amino groups and esterification wherein carboxyl groups are involved.

It was originally considered possible that the characteristic physiological property of insulin may be due to a specific component of the protein, just as in the case of thyroglobulin whose activity is due to the presence of thyroxine. However all attempts to separate an active substance of low molecular weight from insulin have been unsuccessful. Even the mildest degradation of the molecule by proteolytic enzymes or by other methods has invariably resulted in complete loss of activity. An alternative possibility is that the physiological activity is a property characteristic of the molecule as a whole. The isolation in recent years of various enzymes, hormones and bacterial toxins and the recognition of many of these as characteristic proteins strongly suggest that their specific qualities originate in the manner in which the component amino-acids are linked rather than in the presence of specific constituents. However, experiments on the physiological inactivation of insulin indicate that glutamine and cystine units are most closely involved in the change. Attempts have been made to synthesise some relatively simple compound possessing these active groups and having hypoglycæmic power similar to that of insulin ; but they have not so far been fruitful[6].

Physiological role :—Insulin helps to control the metabolism carbohydrates, but the precise mechanism is still undetermined. There is strong evidence that it is concerned with the conversion of glucose into glycogen in the liver and in the muscle ; its action is thus antagonistic to that of adrenaline which favours the change from glycogen to glucose (glycogenolysis). It also accelerates the oxidation of glucose in the tissues. When insulin is injected into a normal animal the blood sugar

rapidly falls leading to the condition known as hypoglycæmia and the animal exhibits convulsions. Conversely, if the pancreas is removed, the resulting acute insulin deficiency leads to greatly increased blood sugar levels (hyperglycæmia) and to the appearance of sugar in the urine (glycosuria) and the animal dies. Diabetes mellitus in human beings is the chronic condition which corresponds to this acute insulin deficiency in the case of the experimental animal. In a diabetic the total daily excretion of sugar in the urine is sometimes 15 to 30 grams and blood sugar ranges between 0·28 and 0·33% (normal value, 0·08%); the glycogen content of liver is poor and acetone bodies appear in blood and urine. Besides maintaining blood sugar level insulin prevents the formation of ketonic substances also and hence their presence in blood and urine is an indication of insulin deficiency.

Uses :—The chief use of insulin is in the treatment of diabetes mellitus. As the hormone is destroyed by proteolytic enzymes it cannot be given orally and should be administered by injection. The administration of insulin in adequate doses keeps the blood sugar within normal limits and thereby prevents glycosuria. It is usually supplied as 'insulin in solution' which is a solution of the dry powder of insulin in acidified water (p_H 3—4) containing an antiseptic to prevent the growth of bacteria. It should be stored at a temperature below 20° and is stable for a considerable number of months. The hormone is also available in tablet form which consists of sterile tablets of dry insulin mixed with a neutral substance such as lactose. Whatever may be the form, the material is packed in sterile containers and labelled with the number of units contained therein.

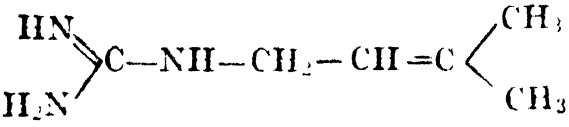
Assay :—The assay of insulin is biological. It is made by comparing the dose of the test sample necessary to produce hypoglycæmia (low level of blood sugar) in rabbits or convulsions in mice, with the dose of a standard preparation which will produce the same effects. The international standard consists of a crystalline zinc-insulin preparation to which has been assigned a potency of 22 international units per milligram. One internation-

al unit of insulin is defined as the amount capable of lowering the blood sugar (0·133%) of a normal rabbit of about 2 kilogram weight and starved for 24 hours, to the convulsive level (0·045%) within the course of 5 hours. Since this unit is too high for the treatment of human diabetes the clinical unit adopted is one third of it.

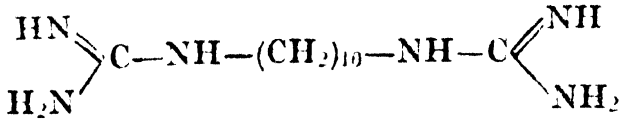
Insulin Products and Insulin Substitutes:—Marked advance in the treatment of diabetes has been made by the discovery of insulin derivatives which, after injection into the body, exert their activity for a much longer period than does ordinary insulin. Compounds or mixtures of insulin with saponin, bile acid, blood serum and proteins have had some success. 'Protamine insulinate' is a compound of insulin hydrochloride with the protamine obtained from the sperm of the rainbow trout (salmiridin). Since this preparation is more slowly absorbed than insulin hydrochloride, its effect on blood sugar is more even and more prolonged. Addition of a small amount of zinc chloride to insulin before adding the protamine, further markedly prolongs the hypoglycæmic action of the preparation and this product known as 'protamine-zinc-insulin'[7] has been extensively used for the treatment of diabetes. 'Zinc-histone-insulin' crystals have also been prepared and patented.

In contrast to insulin a number of substances have been found to have hypoglycæmic action when administered orally. The liquid extract of Galega officinalis and the alkaloid galegin obtained from this plant have both been employed in this manner. The constitution of galegin is represented by formula (I). The synthetics, 'synthalin' and 'neo-synthalin' (synthalin B), were discovered as the result of an investigation which started with para-thyroid tetany, a disease having no connection with diabetes. It is therefore interesting to note that they have constitutions similar to galegin. Synthalin is decamethylene-diguanidine dihydrochloride (II) while neo-synthalin is the corresponding dodecamethylene compound (III). Both have been recommended for mild cases of diabetes or for use in conjunction with insulin. Neo-synthalin, though milder in action than synthalin, is better tolerated. A

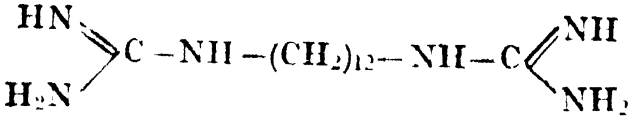
preparation containing decamethylene diguanidine bitartrate, pancreas ferment (lipase) and sodium phosphate is known as 'anticoman'. Glucosone (IV) is another synthetic compound that has been found effective in reducing hyperglycæmia and glycosuria when given orally. It should, however, be mentioned that the synthetic substitutes for insulin are frequently toxic; the mechanism of their action is also different and none of them has yet been found to be entirely satisfactory.



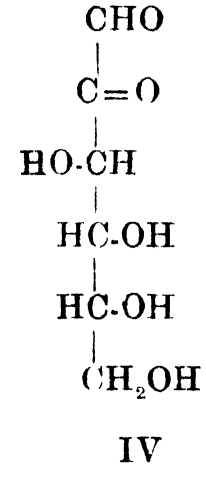
I Galegin



II Synthalin



III Neo-synthalin



IV

OTHER HORMONES.

Besides insulin there are a number of other hormones about which we have only meagre information. The hormone of the parathyroids (small organs attached to the thyroid and usually numbering four) frequently referred to by a trade name 'parathormone', is also a protein. It is decomposed by trypsin and hence has no effect when given orally. Its main effect is on the metabolism of calcium and inorganic phosphate and in normal persons the gland performs the important function of keeping the blood calcium level constant. The removal of parathyroid glands in animals brings about a sharp fall in the blood calcium concentration and this leads to a condition known as tetany which is characterised by muscular spasms. Injection of the hormone

restores the blood calcium level and abolishes the tetany. Large doses raise the blood calcium with delayed rise in the inorganic phosphate and the excretion of calcium is encouraged resulting in its loss from the bones.

The thymus gland seems to be concerned in some way with the general development of the animal and the injection of extracts of the thymus causes precocious development. It is interesting that the effects of a series of injections are cumulative and can even be carried from generation to generation.

A large number of hormones are associated with the digestive system. Secretin is the substance liberated from the mucous membrane of the upper part of the small intestine. It is carried by the blood stream to the pancreas where it causes the secretion of the pancreatic juice. Secretin has been crystallised and shown to be a poly-peptide containing sulphur and having a molecular weight of about 5000. The secretion of the gastric juice is stimulated by gastrin which resembles histamine. The gall bladder is subject to stimulation by cholecystokinin and the activity of intestinal villi is stimulated by villikinin. The pituitary hormones which also come under this category have already been mentioned.

CHAPTER XXV

THE PLANT HORMONES

AUXINS

The auxins form a further addition to the group of highly reactive organic compounds and they are associated with plants. Though the existence of a growth hormone in plants was known from about the year 1910, success in extracting it from the coleoptile tip was first achieved in 1928 by F.W. Went[1] who also evolved a method for its assay. He used agar blocks for the extraction; the activated blocks could be used again for promoting growth. Two well known characteristics of plant stems, (1) geotropism or capacity to grow up against gravity and (2) phototropism (heliotropism) or capacity to bend towards sunlight are due to the presence of this hormone and the way it is transported. It has been shown that it is produced in the growing tips and that it moves downwards encouraging the lower cells to elongate. The bending of stems is due to localised elongation of cells on one side of the stem. The method of estimating hormonal preparations involves the use of oat seedlings and the estimation is made in terms of oat units. The quantity necessary for causing a bending of 10° in a seedling represents one oat unit.

Though in plants there is no blood stream efficiently conveying hormones from the source to the different parts of the system, auxin is definitely transported from the coleoptile tip to places where elongation has to take place. Like animal hormones it is quite active in very small doses; the minimum dose for auxin is 0·0001γ per gram (plant), the minimum dose for thyroxine being 0·01γ per gram (man). But unlike animal hormones it does not seem to have strict specificity of function. Besides cell elongation it also promotes root growth, inhibits lateral bud formation and accelerates cambial activity (cell division).

Occurrence:—In addition to the real hormone called auxin-*a* produced by the apical portion of growing stems, two other substances having hormonal properties occur in nature. The second is called auxin-*b* and is chemically very similar to auxin-*a*. The third substance is more widely distributed and is somewhat different chemically; it is called heteroauxin. The first is the most potent physiologically, and though the others are somewhat less powerful they have the same order of potency. In addition to growing stems a large number of plant materials contain one or more of the three entities. Grains such as maize, peas, beans and lentils, vegetables such as asparagus, fruits such as tomatoes, oranges and lemons, malt, cultures of bacteria and yeast could be specially mentioned. Many animal organs yield fractions having hormonal activity. The discovery by Kögl and Haagen Smit (1931) that urine is a good source for the plant hormone marked again a great advance in its chemical study. As compared with oat seedlings which were originally employed, urine contains twenty-five times the amount of the hormone and is more easily extracted. This large quantity of auxin in the urine is derived partly from the food and partly from the action of bacteria in the intestine; the urinary system therefore plays an important part in bringing about this concentration.

Auxin-*a*: Extraction[2]: —The extraction of the hormone and its purification involve several complicated processes. They make use of the acid nature of the compound. its solubility in ether and insolubility in petroleum ether and its capacity to form a sparingly soluble lead salt and an easily soluble calcium salt. It can be distilled in high vacuum. Though only small quantities of the substance could be obtained, still an astonishingly thorough chemical examination was made by Kogl and his coworkers during the years 1933-35. This is a tribute to the efficiency of microchemical methods. The stages in the extraction of auxin-*a* are given below.

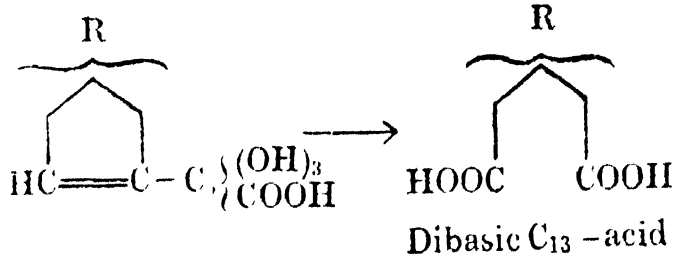
Urine concentrate was extracted with ether and the ether solution extracted with aqueous sodium bicarbonate. The acid fraction thus obtained was treated with

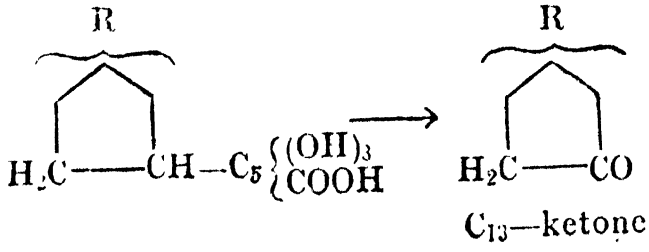
petroleum ether and ligroin which removed certain impurities. The residue was then purified by partition between benzene and alcohol, fractionated by lead salt and calcium salt formation and finally heated with 1·5% hydrogen chloride in methanol. A neutral substance was thus obtained. It was originally considered to be the ester, but was later found to be a lactone. When distilled in vacuo the main fraction passed over between 125° and 135°. Final recrystallisation from aqueous acetone yielded both the acid melting at 196° and the lactone melting at 173°. Both had the same potency, 50 million oat units per gram. The extent of concentration amounted to 20 to 50 thousand times and the yield of the pure material was 3 to 6%.

Properties and constitution[3]:—Auxin-*a* is a complex mono-carboxylic acid melting at 196° and having the molecular formula $C_{18}H_{32}O_5$. It is soluble in alcohol and ether and insoluble in light petroleum. When heated it readily forms a lactone whose properties agree with those of a δ-lactone. It undergoes reduction forming a dihydro derivative which also is capable of forming its lactone. Amongst other derivatives may be mentioned the methyl ester and the dinitrobenzoyl derivative in which three dinitrobenzoate groups are present. From the above properties it is clear that the compound has one carboxyl group, one double bond and three hydroxyl groups. It can be concluded that none of these hydroxyls exists in the γ-position, since otherwise, a stable γ-lactone would be readily formed. From the molecular formula and the fact that there is only one double bond it could also be guessed that the molecule contains one carbocyclic ring.

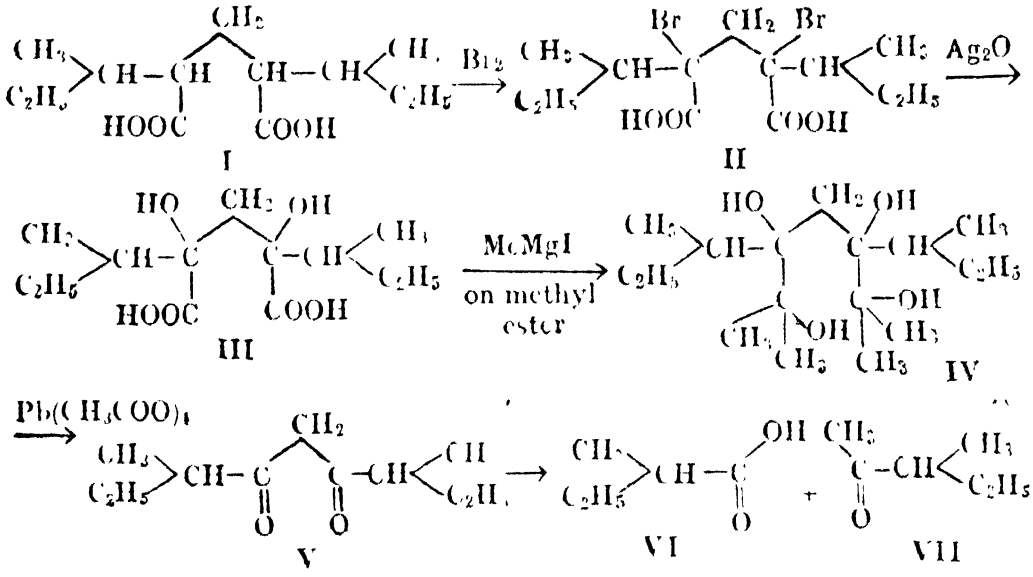
Important information about the structure of the auxin molecule was obtained by oxidation with potassium permanganate. The resulting product was a dibasic acid of the formula $C_{13}H_{24}O_4$. It had no hydroxyl groups or carbonyl groups and was a saturated acid. It readily formed an anhydride but its salts did not give rise to a cyclic ketone on distillation. It thus resembled glutaric acid. Dihydro-auxin, on the other hand, gave rise to a ketone on oxidation and it had the formula

$C_{13}H_{24}O$ The above observations find simple explanation if it is assumed that the ring consists of five carbon atoms and the double bond is present in it and that there is a side chain of five carbon atoms carrying in it the carboxyl group and all the hydroxyl groups. During the oxidation of auxin-*a* the original carboxyl and hydroxyl groups are lost along with the side chain and the ring opens at the double bond. In the case of the dihydro compound the ring is intact and at the point of linking of the side chain a ketone group is formed. The reactions can be represented briefly as below :

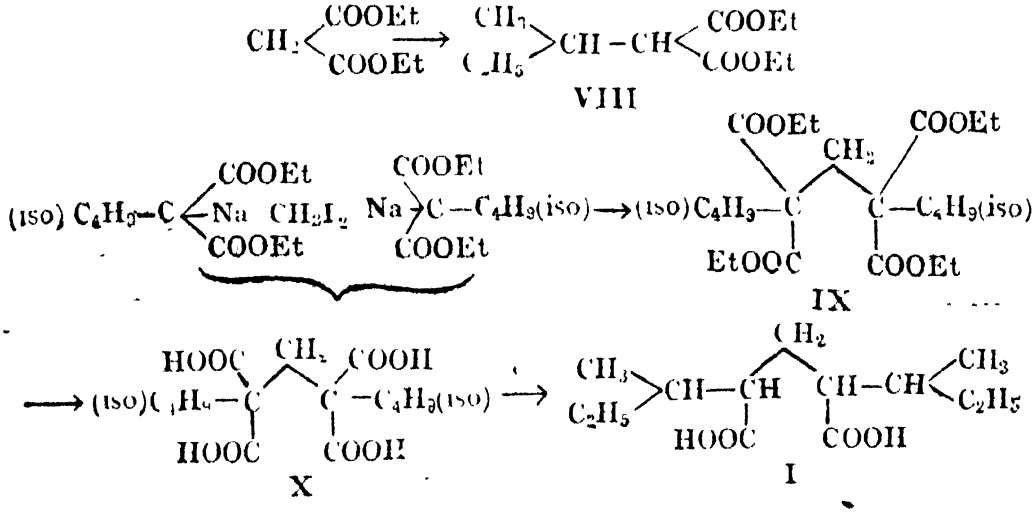




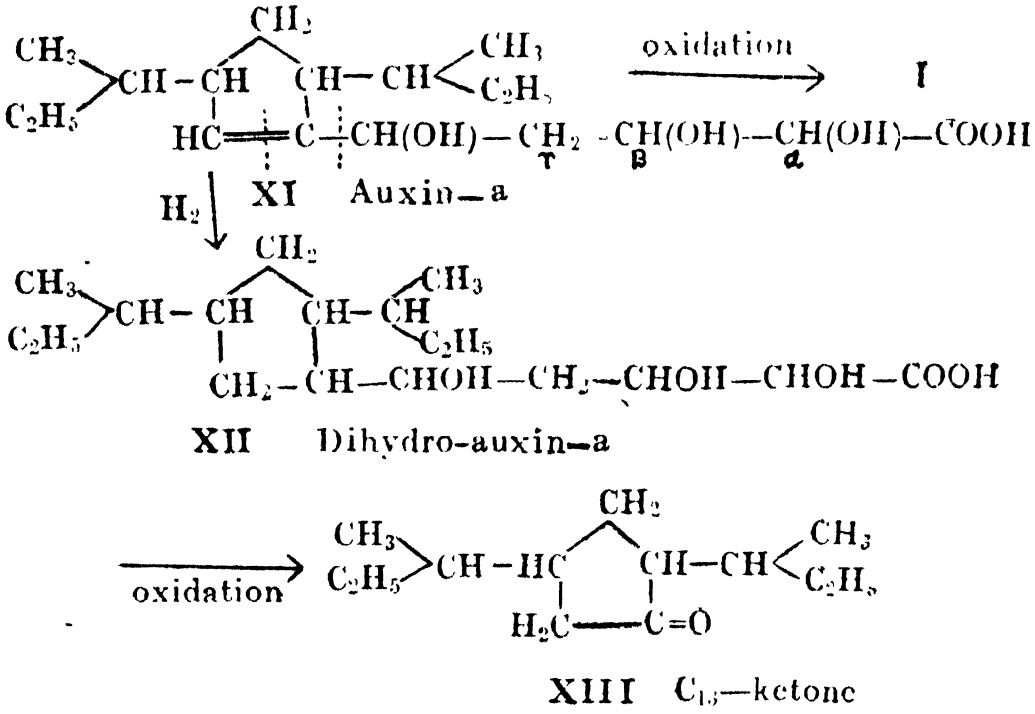
Amongst the large number of possibilities for the constitution of the C_{13} acid the correct one (I) was determined in the following manner. Bromination introduced two bromine atoms on the *a*-carbon atoms (II) and these were subsequently exchanged for hydroxyl groups by means of silver oxide (III). The methyl ester of the resulting acid was treated with methyl magnesium iodide. Oxidation of the product (IV) with lead tetra-acetate yielded a diketone (V) which on fission gave a molecule each of *a*-methyl-butyric acid (VI) and methyl secondary butyl ketone (VII). From these reactions the constitution of the C_{13} acid was concluded to be *a* : *a'*-diisobutyl-glutaric acid (I). The series of reactions may be represented as below :



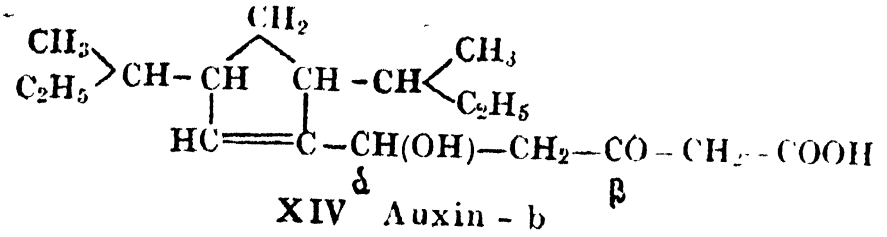
The constitution of the acid was finally established by synthesis. The starting point was diethyl malonate which was converted into the mono-iso-butyl derivative (VIII). Two molecules of the sodium compound of this were condensed with methylene iodide. The product (IX) was then hydrolysed (X) and decarboxylated, whereupon the required acid (I) was produced. It was identical with the oxidation product of auxin-*a*.



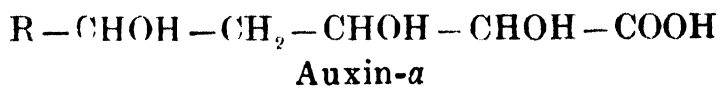
Once the constitution of the C_{13} acid was settled, that of auxin followed as given in (XI). The main point to be remembered in connection with the nature of the side chain is the absence of a hydroxyl group on the γ-carbon atom, since no γ-lactone is formed. The various transformations of the auxin are represented below:



Auxin-*b* [1]:—Auxin-*b* accompanies auxin-*a* in maize-germ oil and malt and was discovered in 1934. It melts at 183° and has the formula $C_{18}H_{30}O_4$ and thus appears to be a dehydration product of auxin-*a*. It is also a monobasic acid but contains only one hydroxyl group forming a monodinitrobenzoyl derivative and has one ketonic group giving rise to a semicarbazone. The reactions of the compound indicate the probable existence of the hydroxyl in the δ-position and the carbonyl in the β-position, since it loses carbon dioxide easily on heating and becomes inactive on keeping. On hydrogenation it also forms a dihydro compound and the oxidation of auxin-*b* and its reduction product gives rise to the same C_{13} acid (I) and ketone (XIII) respectively as were obtained from auxin-*a* and its dihydro derivative. Thus the two auxins are closely related and the relationship is similar to that existing between oestriol and oestrone (XV). The conversion of one into the other, however, has not been achieved in the laboratory. Probably it is taking place in living organisms. Embodying all these observations the constitution of auxin-*b* is represented as in formula (XIV).

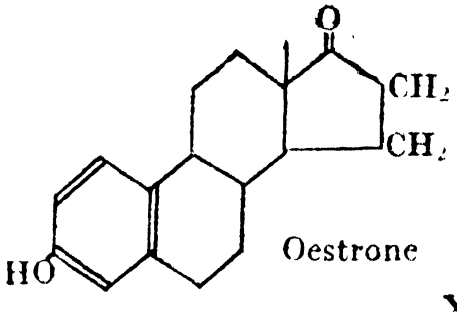


XIV Auxin - b



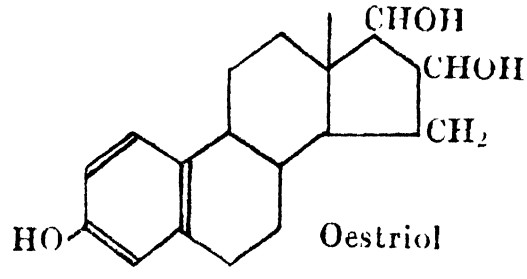
Auxin-*a*

(R = cyclopentene ring of auxins)



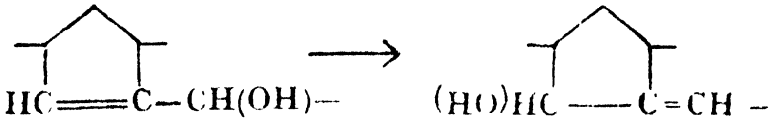
Oestrone

XV



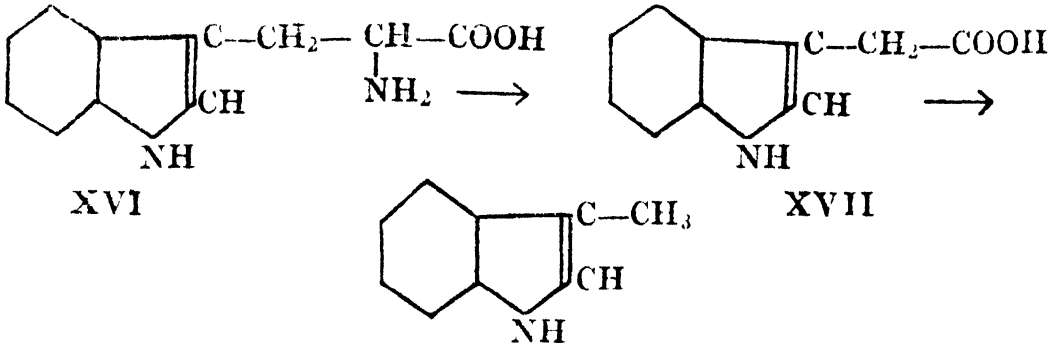
Oestriol

Auxins-*a* and -*b* undergo change on long standing into inactive isomers, called pseudo-auxins. This takes place even in the solid state, in vacuo and even in the absence of light. The change involves the migration of the δ-hydroxyl group and the double bond as shown below. This inactivation is probably used by the plant when there is no more need for the hormone.

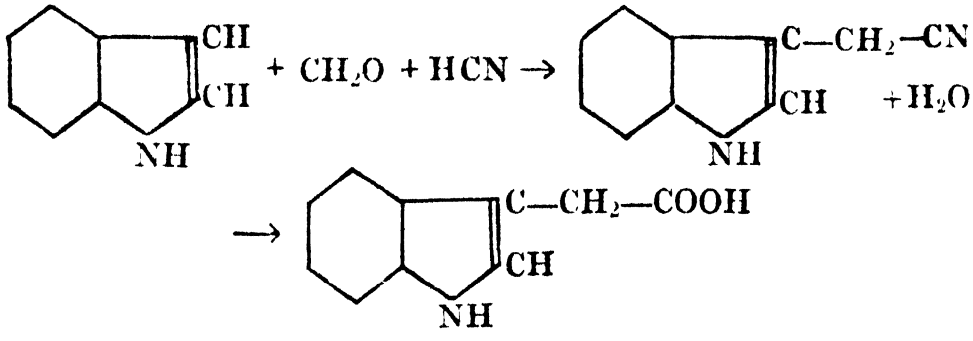


Hetero-auxin[5] :—The third member of the auxin group was obtained later from urine. When Kögl and his co-workers developed in 1934 a charcoal adsorption method for removing the active substances from urine, they met with a substance which underwent destruction in the course of the treatment for lactonisation, (i.e.) heating with methanolic hydrogen chloride. Its nature was considerably simpler and it was quickly identified as indolyl-3-acetic acid (XVII). It was already known to occur in nature particularly in fermentation products and it yields skatole on decarboxylation. It is slightly less potent than auxins-*a* and -*b*, but its growth-promoting activity is quite considerable and is of the same

order. It is considered to be produced by the degradation of the important amino-acid, tryptophane (XVI).

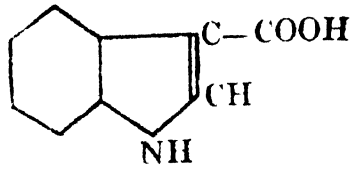


Hetero-auxin crystallises from chloroform in the shape of colourless plates and melts at 164°. It is laevorotatory, the specific rotation being −3·8°. An easy method of synthesis[6] has recently been patented. It consists in treating indole with formaldehyde and a cyanide in alcoholic solution. The indolyl-3-acetonitrile thus formed is then hydrolysed to the required indolyl-3-acetic acid.



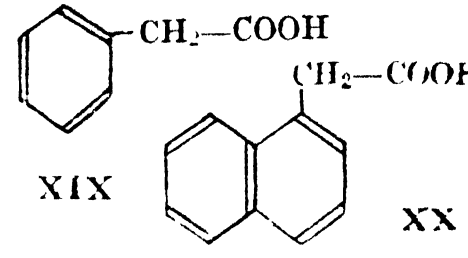
Structure and Hormone Properties[7]:—A large number of compounds which are derivatives, isomers or analogues of auxins have been prepared and examined. The results provide information regarding the structural features essential for phytohormone properties. They are (1) unsaturation due to a double bond or aromatic ring, (2) a free carboxyl group, if esterified readily hydrolysable, (3) a ring system, either a simple 5-membered one as in auxins-*a* and -*b*, or aromatic and condensed as in hetero-auxin and related compounds and (4) a minimum distance of at least one carbon atom between the carboxyl and the ring. Regarding (2) it may be stated that esters of auxin-*a* are inactive and this inactive ester-form

probably occurs in a number of natural products, particularly oils. As an example for (4), indole-3-carboxylic acid (XVIII) has been shown to be inactive whereas indole-3-propionic and -butyric acids are active, though comparatively much less than hetero-auxin. The substituted acetic acids are the most promising. Phenylacetic acid (XIX) is the simplest of these compounds and since it can be easily prepared it is now used very commonly in horticultural practice for stimulating the growth of seedlings. Though α-naphthylacetic acid (XX) is more difficult to prepare, it is more powerful and hence it also finds use.



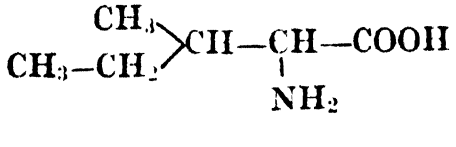
XVIII

(Inactive)

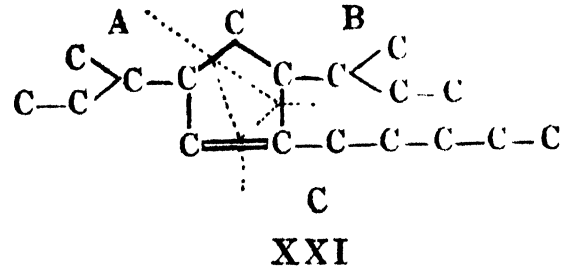


XIX XX

Biogenesis:—The biochemical origin of auxins-*a* and -*b* does not seem to have received much attention. Though they are carboxylic acids with 18 carbon atoms they are definitely different from ordinary fatty acids and are not related to them. It is possible to divide the auxin molecule into 3 units of 6 carbon atoms each as shown in formula (XXI). Two of these units, (A) and (B) may be considered to arise from isoleucine and the third oxygen-rich unit (C) from a molecule of hexose, say glucose. An early stage may be the deamination of isoleucine and reduction, providing the required branched chain 6-C unit. But there seem to be no chemical analogies to suggest possible details of the processes involved.



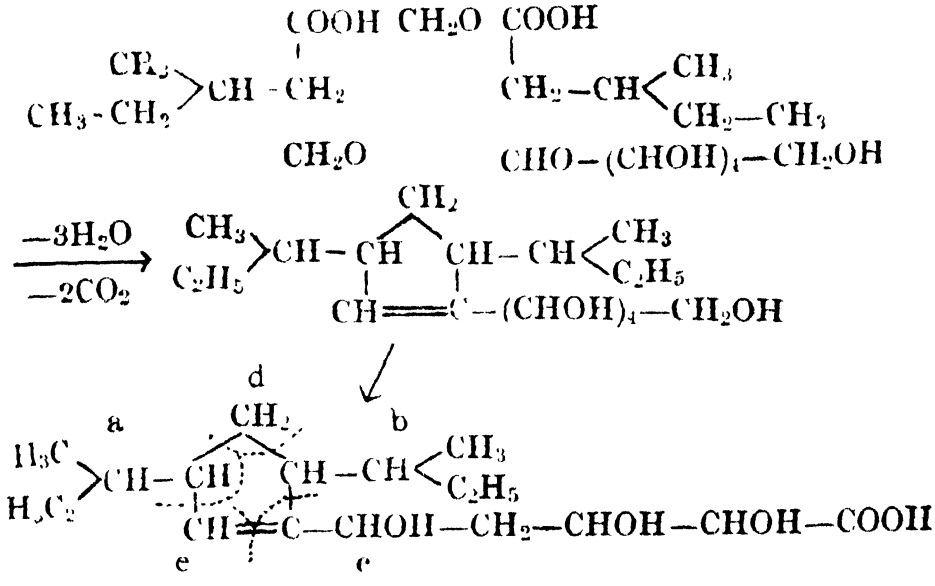
Isoleucine



XXI

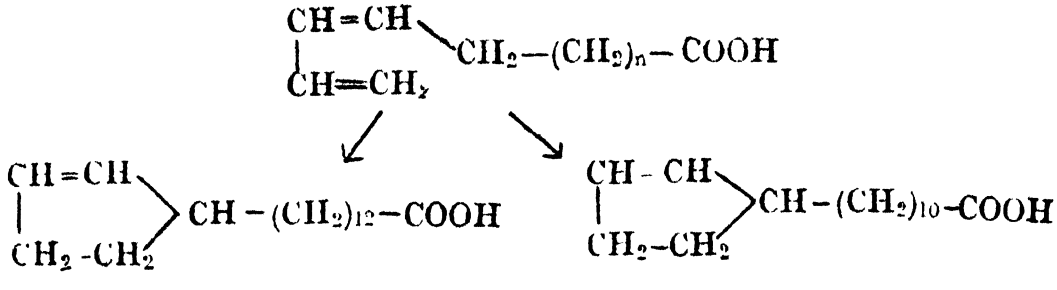
A more easily understood picture can be obtained if the auxin molecule is split up as shown in (XXII). In

this case the C_5 units (a and b) can be easily derived from isoleucine by deamination and decarboxylation, processes which are known to take place with readiness in the living system. The odd carbon atoms (d) and (e) should then arise from formaldehyde units, the oxygen-rich 6-C unit (c) being derived from a hexose.



XXII Auxin-a

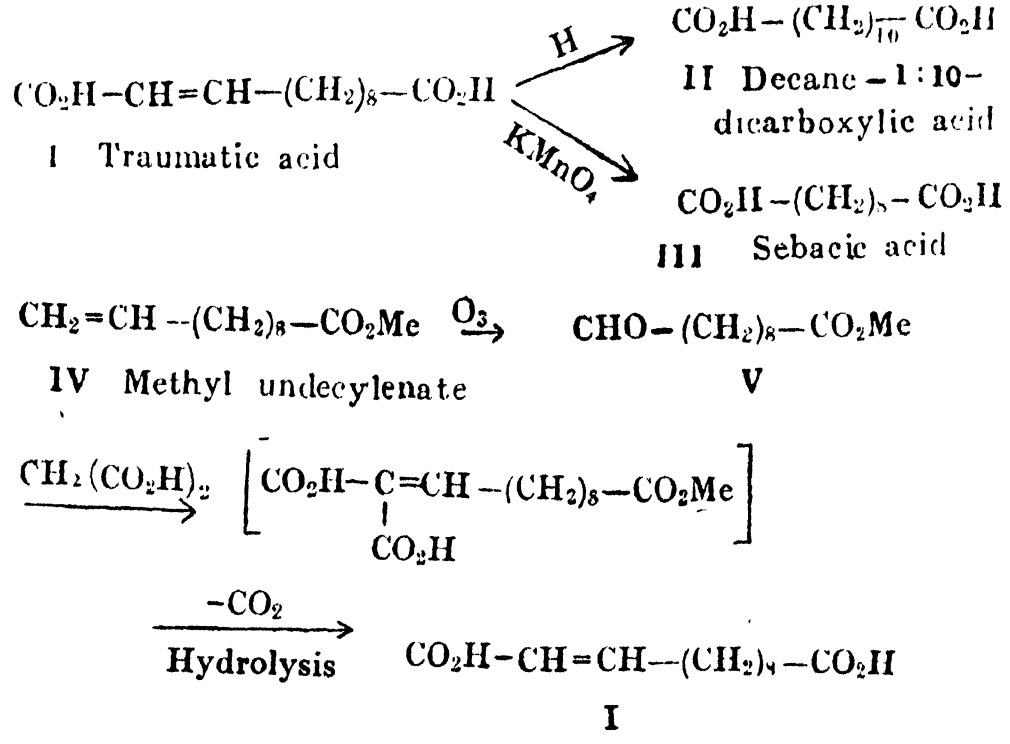
As examples of fatty acids with rings and having physiological properties may be mentioned chaulmoogric and hydnocarpic acids and their homologues which occur in chaulmoogra and hydnocarpus oils. They have been used in the treatment of leprosy. They are considered to be related to ordinary fatty acids with 18 and 16 carbon atoms and to be evolved in a simple way[8] from unsaturated straight chain fatty acids by ring closure at one end as shown below :



Chaulmoogric acid Hydnocarpic acid

WOUND HORMONES—TRAUMATIC ACID.[2]

When plant cells suffer injury the adjacent uninjured cells divide in order to repair the damage. The abnormal activity of the cells is due to substances which are formed or liberated at the injured surfaces; these substances are called wound hormones. From an aqueous extract of ground bean-pods English and coworkers isolated in 1939 a compound now called traumatic acid. Analysis and molecular weight determinations gave the formula $C_{12}H_{22}O_4$ while a determination of the equivalent weight by direct titration showed that it was a dibasic acid. The molecule contained one double bond. Catalytic hydrogenation produced decane-1:10-dicarboxylic acid (II), while oxidation by permanganate in acetone yielded sebacic acid (III). These reactions indicated the structure, Δ^1-decene-1 : 10-dicarboxylic acid (I) for traumatic acid. This was confirmed by synthesis starting from methyl undecylenate (IV). Ozonisation of (IV) yielded the half aldehyde-ester of sebacic acid (V) which condensed readily with malonic acid in pyridine with evolution of carbon dioxide. Hydrolysis of the product yielded traumatic acid (I) identical with the natural product.



A large number of organic compounds were tested for similar activity by English and coworkers (1939), but among them the only active substances were certain homologues and analogues of traumatic acid. Compounds (II) and (III) had about half the activity of traumatic acid. The saturated dibasic acids containing 7 or fewer carbon atoms were without activity while suberic (8 C atoms) and azelaic (9 C atoms) acids possessed feeble activity.

NOTES

Chromatographic analysis

This method is one of the important recent additions to the resources of the chemist and is very useful for the separation of complex organic mixtures. It has been of great help in the investigation of natural products when the required compounds, contaminated with large quantities of unwanted substances, have to be concentrated or when mixtures of compounds of similar chemical nature have to be analysed into their components. The essential principle of chromatographic analysis is that the affinity between a substance and an adsorbent depends on the nature of both, and so also the affinity of a solvent for a solute is a specific property depending on both of them. When a mixture of substances is dissolved in a solvent and treated with an adsorbent, the relative tenacity with which each of them will be held either by the solvent or by the adsorbent will show a progressive variation.

In practice a solution of the mixture to be separated is allowed to flow down a column packed with the adsorbent. The solute mixture gets concentrated in a zone at the top of the column. The adsorption picture is then developed by allowing more of the pure solvent to pass through the same column ; the constituents travel downwards but at different rates and eventually become localised in distinct zones. The banded tower of adsorbent is then termed a chromatogram. For getting out the different components, one of two procedures may be followed. The column may be washed with more solvent and each component collected separately as it reaches the lower end of the column and is released. Or each of the adsorption zones may be mechanically removed and the adsorbate eluted with a suitable solvent.

The above represents an ideal case ; in actual practice, however, several difficulties are met with. Nevertheless the method of chromatographic analysis has yielded some of the most fruitful results in the field of biological chemistry. Choice of adsorbent and solvent may have

to be made by trial. For the majority of cases Brockmann's activated alumina has proved to be a successful adsorbent. Calcium hydroxide, calcium carbonate, magnesium oxide and cane sugar have also been found useful.

Molecular Distillation

The idea of distilling substances under very high vacua was first employed by Bronsted and Hevesy in order to separate the isotopes of mercury and has since been developed into the art of molecular distillation. With the help of this technique materials which were originally considered non-volatile and non-distillable have been brought into the realm of volatile liquids.

When a gas is held at atmospheric pressure, there exist some 10^{19} molecules in each cc. of the gas, and no molecule can move in a straight line for more than 10^{-4} mm. without meeting another molecule. This is called the mean molecular free path. When the pressure of the gas is reduced to 10^{-7} mm. of mercury, each molecule is able to move about in this relative vacancy over much longer distances before colliding with another molecule. If therefore molecules are given off from a warm surface into a vacuum of the order mentioned above, and if a relatively cold surface is placed at a distance from the warm surface which is less than the mean free path of the molecules, then every molecule that darts in the direction of the cool surface strikes that surface and gets condensed. This is the principle of molecular distillation. In a molecular still the material to be distilled is caused to flow evenly over a heated surface. Directly opposite to it is a relatively cooler surface, annular in shape, and between the two there is very high vacuum. The distance between the heated liquid film and the condensing surface is adjusted to be less than the mean free path of the molecules to be distilled. The molecules jump across the gap from the warmer to the cooler surface and the distillate is collected.

The special merit of molecular distillation is that by means of it, it is possible to distil several materials like glycerides, resins, petroleum residues and the un-

saponifiable fraction of fats without appreciable decomposition, and distillation can take place at comparatively low temperatures. It has been of great utility in the preliminary concentration of heat-labile substances which cannot be processed by conventional methods. The most useful results have been obtained in the case of fats and oils which can conveniently be divided into three fractions, the boiling point progressively rising with increasing molecular weight of the distilling component. The components responsible for odour and taste generally distil first; sterols, vitamins, hydrocarbons etc. which form the unsaponifiable matter distil next (from about 100° to 180°) and the triglycerides distil last (between 220° and 260°). The methods previously employed in the treatment of oils left the refined glycerides as a residue whereas in molecular distillation we get them as a distillate. Again, in the concentration of the vitamins present in animal and vegetable oils the earlier practice was to saponify the oils and then to extract the unsaponifiable matter which contained the vitamins. This procedure gave the vitamins in the form of the free alcohols and there was always a certain amount of inevitable loss. By adopting molecular distillation for the concentration there is little loss and the vitamins are isolated in the form in which they occur in the source In fact through the adoption of this technique it was possible to prove that vitamins A and D exist largely as esters in the original oils.

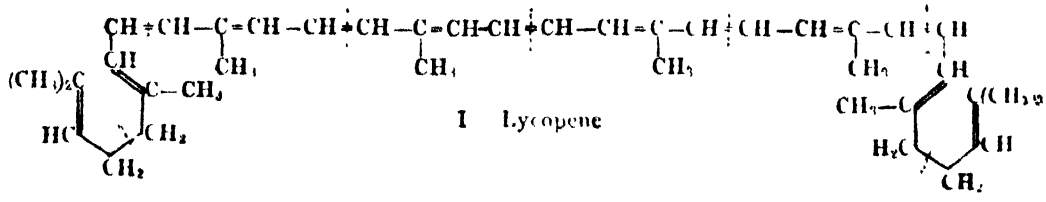
Carotenes

Carotenes form an important group of orange red hydrocarbons widely occurring in plants and having the formula $C_{40}H_{56}$. They and their oxy-compounds (alcohols, acids, etc) classed under the general name carotenoids exhibit characteristic colour reactions. With concentrated sulphuric acid they give a blue, bluish violet or greenish blue colour. The colour is better observed using a chloroform solution and shaking with sulphuric acid. The blue colour with antimony trichloride in chloroform solution is also another characteristic of carotenoids.

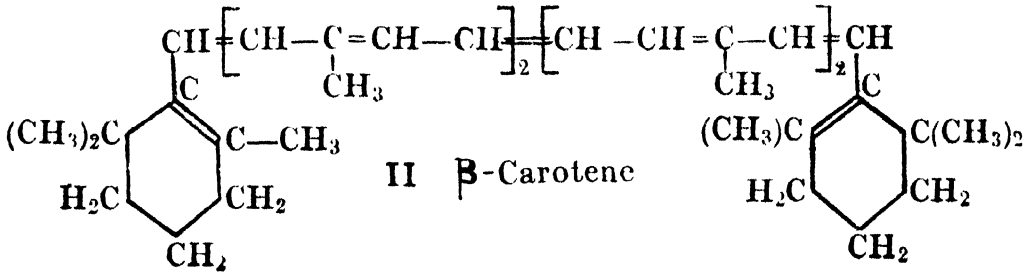
Of the carotenes, β-carotene (II) is the most important and most widely occurring and it possesses the highest vitamin A value. When pure it is obtained in the form of dark red plates melting at 184°. It is easily soluble in carbon disulphide and benzene, sparingly soluble in alcohol, ether and chloroform and insoluble in water. In carbon disulphide it has absorption bands at 521, 485 and 450 mμ. It is optically inactive and in this respect differs from the α-variety. With antimony trichloride it gives a greenish blue colour and the solution shows maximum absorption at 520 mμ. α-Carotene (III) melts at 187°, exhibits optical activity, $[\alpha]_D$, + 364° in benzene solution and it has absorption bands in carbon disulphide at 511 and 476 mμ. The following represents the carotene composition of some of the vegetable sources:—palm oil, 30-40% α and the rest β; carrot, 10-20% α and the rest β; stinging nettle, spinach, grass and chillies contain only β- and no α-carotene.

γ-Carotene (IV) forms, in general, only a very small proportion of the total carotene of plants (about 0·1%). Larger quantities occur in the leaves of the lily of the valley and in the fruits of certain Dutch East Indian plants while the marsh dodder (Cuscata Salina) is a rich source of the hydrocarbon. It melts at 174° and is optically inactive.

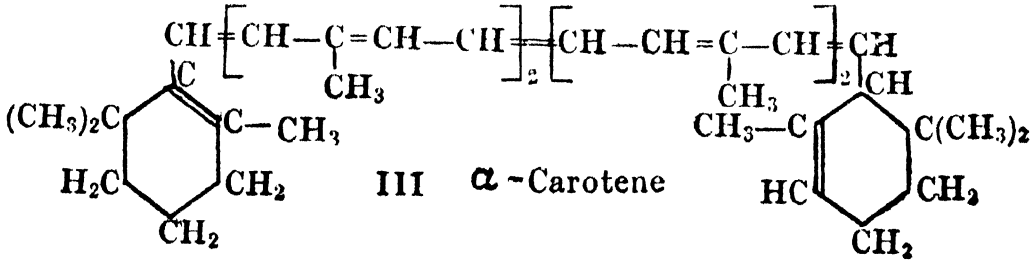
The above three carotenes exhibit provitamin activity. A ζ-carotene has been described by Winterstein. Nothing definite is known about its vitamin A activity. There is a fifth isomer called isocarotene which is obtained from β-carotene by treating it with antimony trichloride and chloroform and pouring the resulting solution into water. When crystallised, it comes out as needles or leaflets with a steel blue reflex, melting at 192°. It is optically inactive and has no provitamin A activity. Another compound having the same molecular formula ($C_{40}H_{56}$) as the carotenes is lycopene (I) which occurs in the red tomato. It melts at 175°. It has no ring in its molecule and has no provitamin A activity. It may be considered to be the parent hydrocarbon from which the carotenes are derived. The structural relationship between these compounds is represented below. They are all built up of units of isoprene (C_5H_8).



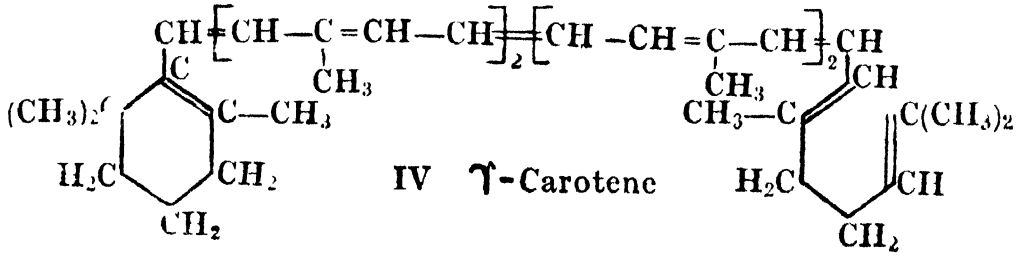
I Lycopene



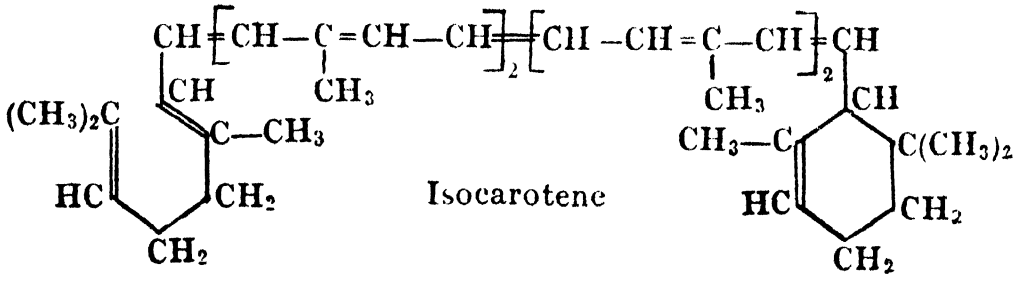
II β-Carotene



III α-Carotene



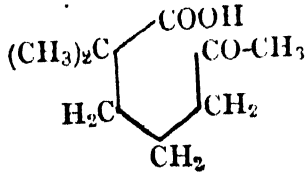
IV γ-Carotene



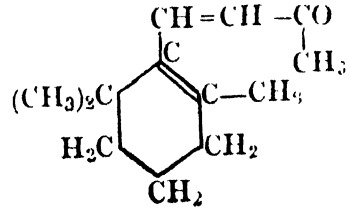
Isocarotene

The existence of a large number of conjugated double bonds is responsible for the marked colour of these compounds. The number of double bonds is found by catalytic hydrogenation. Important information about their structures has been obtained from oxidation experiments. β-Carotene yields geronic acid (V) on oxidation with permanganate. This reaction gave the first indication that the molecule contains β-ionone units (VI). In Karrer and Helfenstein's experiment there was a strong odour

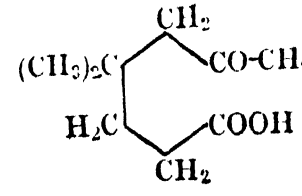
of ionone which filled the whole room when the products of permanganate oxidation of this carotene were being concentrated and thus there was no doubt regarding the above conclusion. Ozonolysis yielded more detailed knowledge. β-Carotene forms two molecular proportions of geronic acid showing that it contains two β-ionone units and is therefore symmetrically built. α-Carotene yields both geronic and isogeronic (VII) acids and γ-carotene geronic acid and acetone, thus showing that these two have only one β-ionone ring system and the other part contains an isomeric ring in α-carotene and an aliphatic portion in γ-carotene.



V

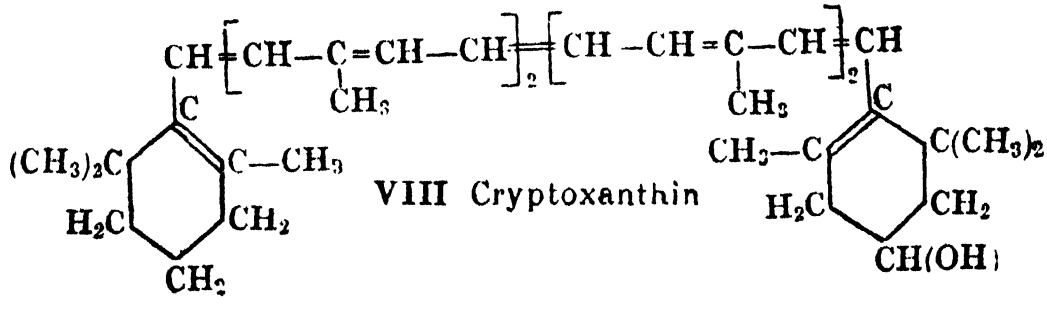


VI

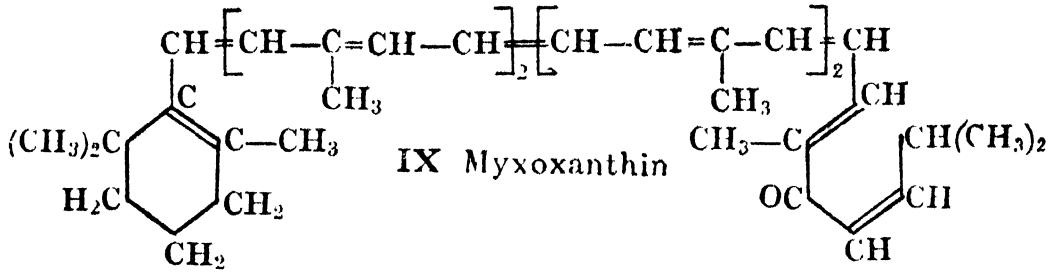


VII

Several other carotenoid pigments have also been found to be capable of acting as provitamin A. The most important of these is cryptoxanthin occurring in yellow maize, paprika and certain other yellow vegetables. It is best obtained from ground cherry; it crystallises as reddish violet prisms and melts at 169°. It has one hydroxyl group, eleven double bonds and two rings and has been shown to be 3-hydroxy-β-carotene (VIII). Myxoxanthin which is obtained from algae and which also exhibits provitamin A activity is a monoketone having the structure represented in (IX).

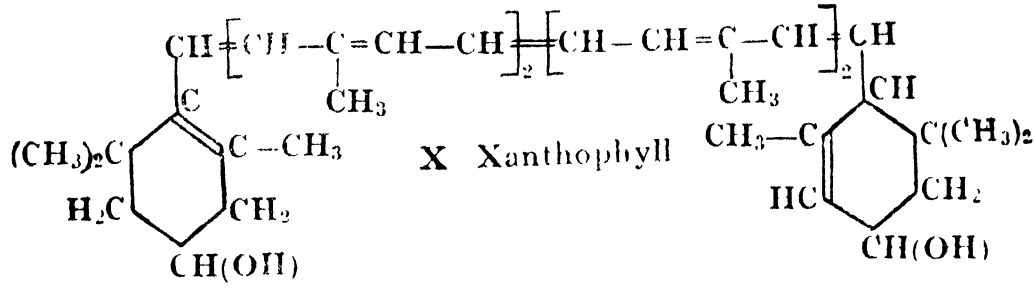


VIII Cryptoxanthin

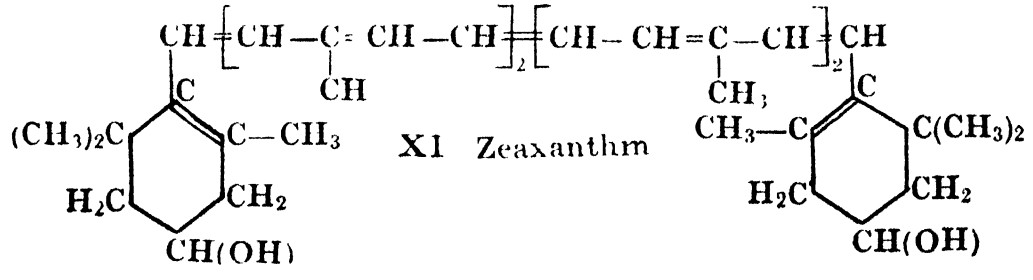


IX Myxoxanthin

In all the foregoing compounds with provitamin A activity the existence of at least one unmodified β-ionone ring is apparent. Indeed this is now definitely considered to be a prerequisite, since xanthophyll or lutein (3 : 3'-dihydroxy- -carotene X) and zeaxanthin (3 : 3'-dihdroxy-β-carotene, XI) which are lacking in it, are both devoid of provitamin A activity. Several degradation products of β-carotene are active due to their capacity to form vitamin A. They also have the β-ionone unit in their molecules.



X Xanthophyll



XI Zeaxanthin

In view of the great value of carotenes as provitamins their preparation from cheap and easily available sources is of considerable importance. They have been obtained in substantial quantities from a variety of sources like carrot, stinging nettle, capsicum and palm oil. As has been mentioned earlier, they occur almost invariably as mixtures of the α-, β- and γ- forms and are often associated with chlorophyll, xanthophyll or

other carotenoid pigments. Carrot is by far the most convenient source for isolation since it contains the carotenes as practically the sole colouring matter and in the highest percentage and the isolation of the pigments is therefore simple and cheap.

Dried carrot powder is extracted with petroleum ether and the concentrated extract treated with carbon disulphide. By the regulated addition of alcohol to the resulting solution, colourless impurities are first removed and subsequently the carotene portion is obtained as a precipitate. The crude material is further purified by solution in carbon disulphide, precipitation with alcohol and final crystallisation from petroleum ether.

The preparation of carotenes from stinging nettle is a little more difficult as it involves the elimination of chlorophyll and xanthophyll. The material is exhausted with 80% acetone and the extract poured into petroleum ether. When water is subsequently added to the mixture, separation into two layers takes place and the pigments are now found in the petroleum ether layer. The aqueous acetone layer is separated and rejected, while the petroleum ether layer is agitated with 80% methanol which removes xanthophyll and part of chlorophyll. When the petroleum ether layer is subsequently washed with water, the remaining chlorophyll is also precipitated in a finely divided state. The turbid petroleum ether layer is shaken with anhydrous sodium sulphate and talc and then filtered through a layer of talc. The filtrate which contains all the carotenes, is concentrated and the residue crystallised from 95% alcohol.

Separation of mixtures into α-, β- and γ-carotenes has been effected mainly by chromatographic adsorption from bezene or petroleum ether solutions on a column of calcium hydroxide or carbonate or alumina. Sometimes simple adsorption on Fuller's earth or 'Faserto-nerde' and elution has been found to be quite adequate. It may however be mentioned that separation is seldom necessary for nutritional purposes.

Substances related to vitamin A and present in liver oils

Certain substances related to vitamin A occur in

small quantities in fish liver oils. Though they have very little or no vitamin activity they closely resemble the vitamin in optical properties. If therefore they should be present in appreciable amounts, the ultraviolet and antimony trichloride methods of estimation become unreliable.

The term 'cyclised' vitamin A has been used for a substance which occurs in traces in all vitamin A-bearing oils. It is also formed when the vitamin is subjected to chemical treatments. The substance is generally prepared by treating an alcoholic solution of the vitamin with anhydrous hydrogen chloride. It has been obtained in a crystalline form (yellow rhombs) melting at 77°. Its composition agrees with the formula $C_{20}H_{28}$ and it is found to be devoid of a hydroxyl group; hence it is more appropriately called 'anhydro-vitamin A'. It is rather unstable and is weakly adsorbed on most chromatographic adsorption agents. The absorption maximum of the antimony trichloride reaction is nearly the same as for vitamin A (620 mμ.) being displaced only by 2-3 mμ. towards the red. The value of $E^{1\%}_{1\,cm.}$ is 5,500 whereas it is 4,800 for vitamin A. Its ultraviolet absorption bands are however at 351, 371 and 392 mμ. and not at 328 mμ. as given by vitamin A.

Another substance called 'subvitamin A' occurs to a small extent in most liver oils. It is more strongly adsorbed by alumina than vitamin A and is probably an oxygenated derivative of the vitamin. Besides affecting the antimony trichloride colour it is found to be responsible for the distortion of the ultraviolet absorption maximum of vitamin A at 328 mμ. on the side of the short wave lengths.

A more remarkable compound occurring as fatty acid esters is 'kitol'. It is most plentiful in whale liver oil (kitos = whale) which is considered to be a poor source of vitamin A. It has been obtained as a viscous pale yellow oil or glassy solid having the probable formula $C_{40}H_{58}(OH)_2$. It has an absorption band at 290 mμ. and no vitamin potency. However, on distillation it decomposes to yield vitamin A. From its composition it appears

to be divitamin A, but it yields only one molecule of the vitamin by pyrolysis. It may be considered to be another provitamin A, but unlike the carotenoids which undergo conversion only in the animal organism, kitol can be converted into the vitamin by a simple laboratory process. It has been suggested that kitol is a form in which superfluous vitamin A is disposed of by the whale, and even otherwise it should be considered to be a post-vitamin A.

Sterols

The vitamins of the D group and a number of hormones are closely related to the sterols. These are monohydric alcohols which are widely distributed both in plant and animal tissues, either free or as esters with higher fatty acids. All fats and waxes contain them in more or less quantities. When these materials are saponified with alkali, the sterols are found in the unsaponifiable portion and can be extracted with ether or similar solvent. Usually they occur as mixtures of related compounds and have to be purified by the method originally introduced by Windaus and Hauth in 1906. The mixture is acetylated and the acetates treated with excess of bromine. The bromoacetates are subjected to fractional crystallisation and thus the separation of the mixture is effected. The pure entities are then debrominated using zinc and acetic acid, deacetylated with alkali and crystallised from suitable solvents.

Sterol mixtures from plants are considered to be more efficiently separated by the crystallisation of their benzoates and nitrobenzoates. More recent work utilises chromatographic adsorption on alumina. It has been found advantageous to oxidise the sterols to the corresponding $\alpha : \beta$-unsaturated ketones and then effect the separation using adsorption.

There are a few colour reactions which have been found very useful for the preliminary identification and quantitative estimation of sterols, though they are not specific. In the Salkowski reaction a chloroform solution of a sterol is shaken with an equal volume of concentrated sulphuric acid. After separation of the two

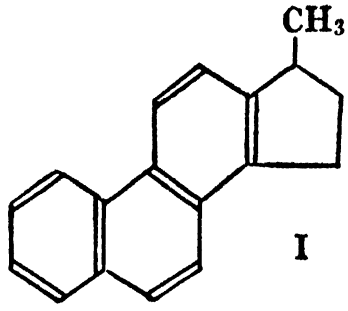
layers, a red colour is generally present in the chloroform layer and a green fluorescence in the sulphuric acid layer. Some of the sterols give a red colour in the acid layer and no colour in the chloroform layer; this is known as the reverse Salkowski reaction. In the Liebermann-Burchard reaction a chloroform solution of a sterol is treated with a few drops of acetic anhydride and concentrated sulphuric acid is slowly added with cooling. A colour soon develops in the solution passing rapidly from rose-red through blue to green.

Sterols are broadly classified as (1) zoosterols from animals *e.g.*, cholesterol, (2) phytosterols from plants *e.g.* stigmasterol and (3) mycosterols from yeasts and fungi *e.g.*, ergosterol. A brief account of the more important of the sterols is given below.

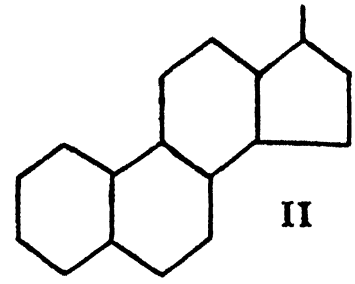
Cholesterol is the most important and the most fully investigated of the sterols. It occurs in all nerve tissue either free or in the form of esters. It is also present in lanolin or wool fat, egg-yolk and bile. Gall stones may sometimes consist of almost pure cholesterol. It melts at 148° and is optically active, $[\alpha]_D$ in chloroform being $-29{\cdot}5°$. It is a secondary alcohol having the molecular formula $C_{27}H_{46}O$ and contains one double bond in the molecule. The elucidation of its structure, which incidentally helped to solve the problem of the constitution of other sterols also, was a remarkable feat which was accomplished only in 1932 by the united effort of a number of eminent chemists of different nationalities. In what follows, only the most important aspects of the chemistry of cholesterol, those relating to the carbon skeleton, are mentioned. Like the carotenoids the sterols also are considered to be made up of isoprene units.

The most valuable information regarding the ring system of cholesterol was obtained from dehydrogenation experiments. Using selenium at 360° Diels obtained a hydrocarbon, (known as Diels' hydrocarbon) having the formula $C_{18}H_{16}$ which has been proved to be 3'-methyl - 1 : 2 - cyclopentenophenanthrene (I). Hence cholesterol should contain a reduced phenanthrene ring system to which a cyclopentane ring has been fused. Further, it should carry a side chain in the position

corresponding to that of the methyl group in Diels' hydrocarbon as shown in (II).

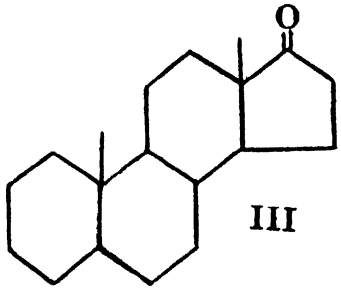


I



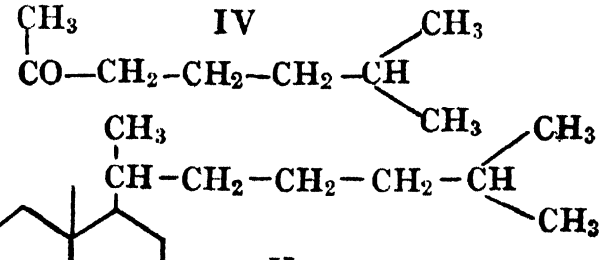
II

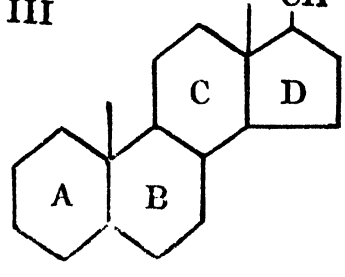
Evidence as to the exact nature of the side chain and the relationship between cholesterol and the well known group of compounds known as bile acids was obtained as follows. On hydrogenation cholesterol was first converted into dihydro-cholesterol as one of the products and subsequently to a hydrocarbon known as cholestane. Vigorous oxidation of this hydrocarbon with chromic acid gave rise to two ketones, one having the formula $C_{19}H_{30}O$ and the structure (III) and the other being identical with methyl iso-hexyl ketone (IV); cholestane was therefore represented as shown in (V).



III

IV

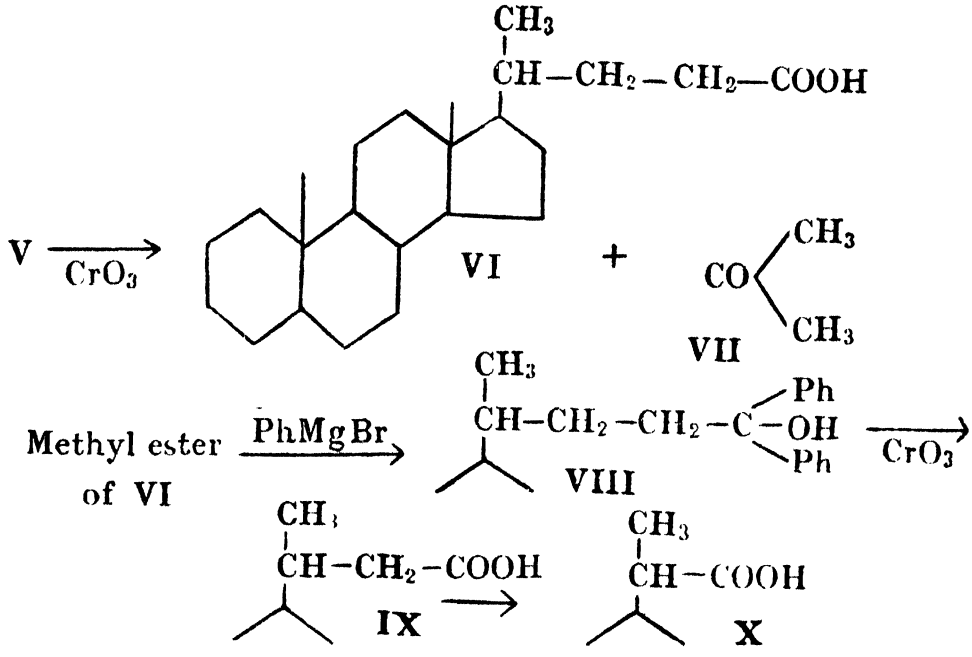




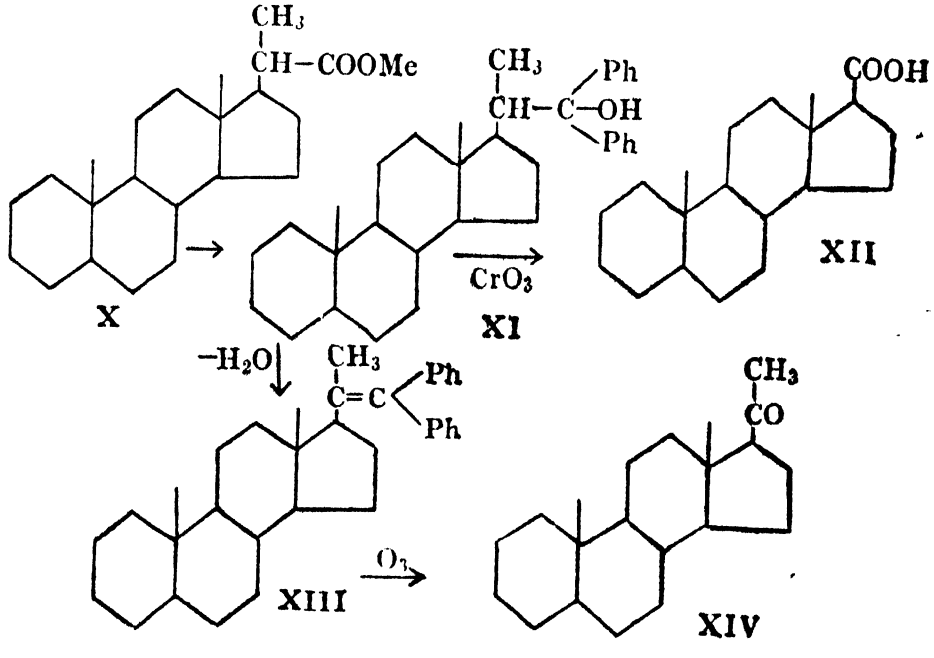
V

An alternative method of study which was of particular importance in connection with the establishment of the relationship between cholesterol, stigmasterol and ergosterol involved stepwise degradation of cholestane under milder conditions. By mild chromic acid oxidation cholestane gave acetone (VII) and allocholanic acid (VI)

which by a process known as Wieland's degradation could be converted successively into nor-allocholanic acid (IX) and bis-nor-allocholanic acid (X).



The methyl ester of the last was converted into a diphenyl carbinol (XI) which gave either œtioallocholanic acid (XII) by direct oxidation or a ketone ($C_{21}H_{34}O$) (XIV) by dehydration followed by ozonolysis.

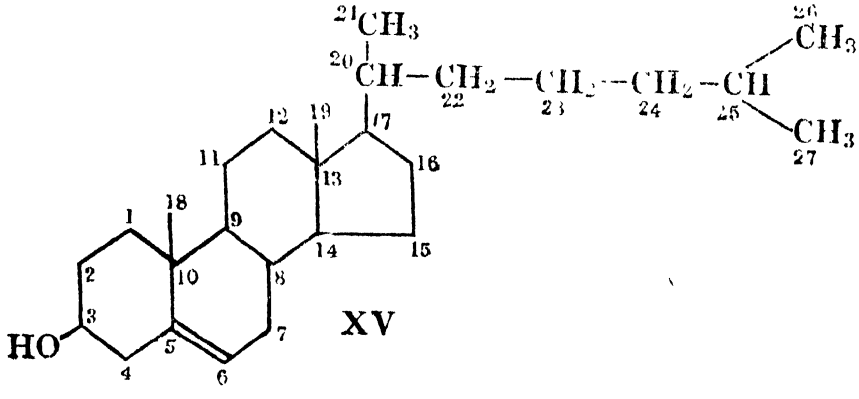


The location of the hydroxyl (3 position) and of the double bond (5 : 6- position) in cholesterol was far more

difficult. This was arrived at from a number of degradation experiments involving mainly oxidation.

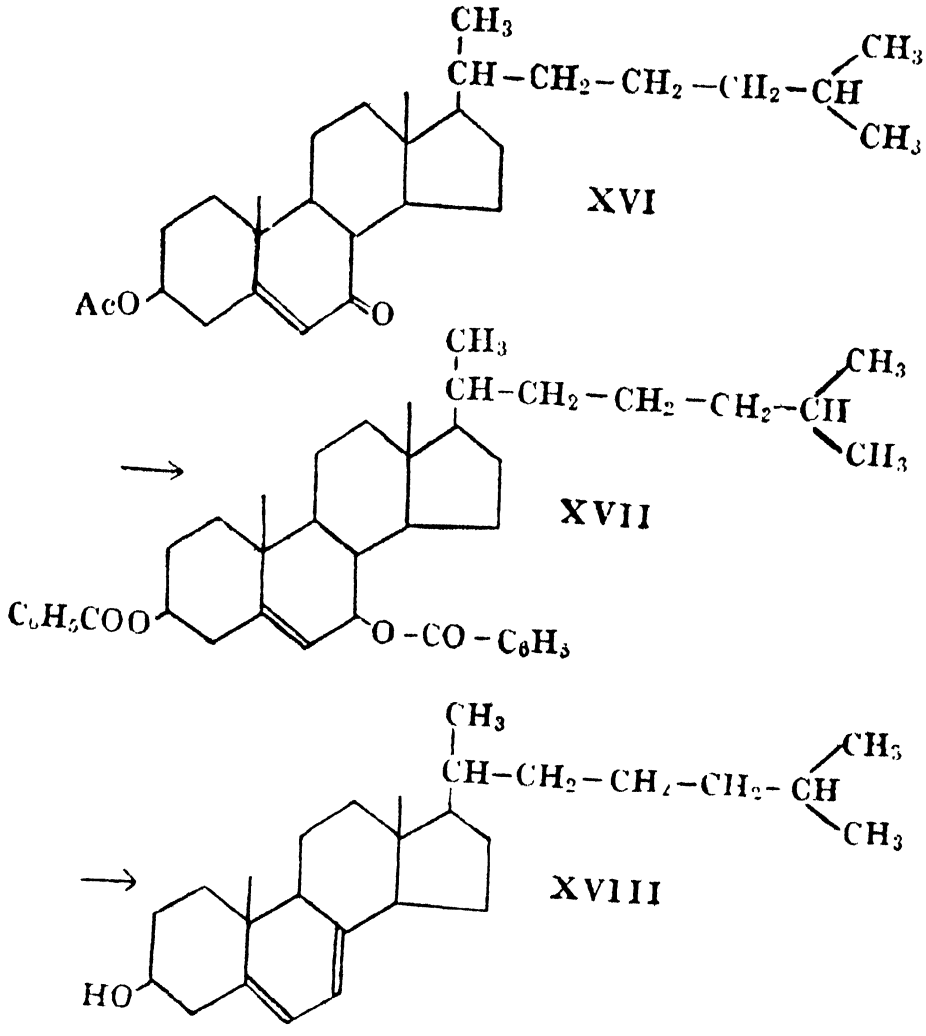
The stereochemistry of the sterols is rather complex. The main factors involved are (1) the manner of fusion of the four rings to one another and (2) the spatial arrangement of the 3-hydroxyl group with reference to the angular methyl group at C_{10}.

In cholestane (V) the relationship between the different rings is as follows: A/B trans; B/C trans; C/D trans. Cholesterol may be considered to be derived from the parent hydrocarbon cholestane by the introduction of a double bond in the 5-6 position and a hydroxyl in the 3-position. The hydroxyl is cis with reference to the angular methyl group in position 10. It can therefore be designated as 3-cis-hydroxy-Δ^5-cholestene (XV).



XV

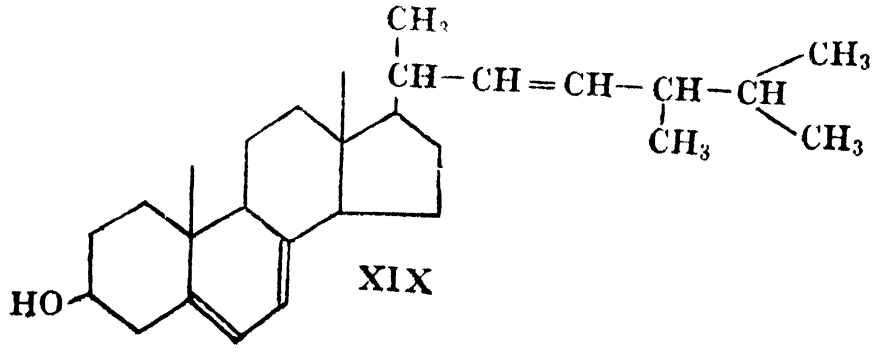
7-Dehydrocholesterol (3-Cis-hydroxy-$\Delta^{5,7}$-chloestadiene). It has the formula $C_{27}H_{44}O$ and contains two double bonds. It was first prepared synthetically by Windaus, Lettree and Schenck in 1935 starting from cholesterol. The method consisted in oxidising cholesteryl acetate with chromic acid when 7-ketocholesteryl acetate (XVI) was obtained. It was reduced with aluminium isopropoxide and the diol, 7-hydroxy-cholesterol was isolated as the dibenzoate (XVII). When heated to 200°, the dibenzoate lost one molecule of benzoic acid and gave the benzoate of 7-dehydrocholesterol. This on saponification yielded the free sterol (XVIII). A recent improvement in the process consists in treating the dibenzoate with boiling dimethylaniline instead of pyrolysis.



7-Dehydrocholesterol melts at 150° and is optically active, $[\alpha]_D$ in chloroform being − 113 6°. It has since been found to be widely distributed in nature, and it occurs particularly in skin fats. It is best obtained from the skin fat of pigs. It is believed that it is produced in animals from cholesterol and it is therefore a constant impurity in cholesterol. More recently it has been obtained from certain invertebrate organisms like mussels and earthworms also. The mixed sterols got from them yield on fractionation 7-dehydrocholesterol.

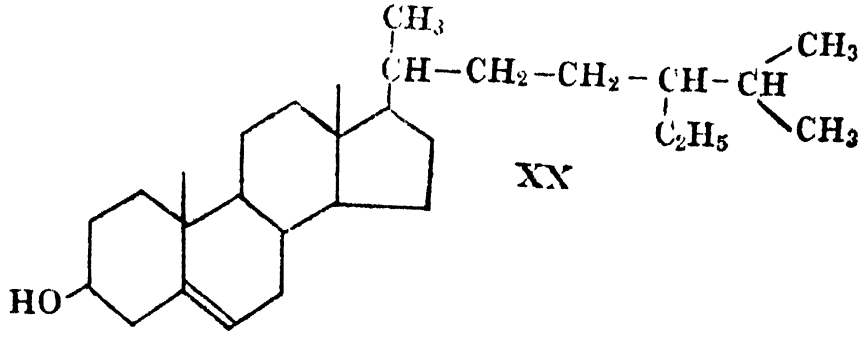
Ergosterol was originally obtained from the fat of ergot which is a fungus growing on rye. It is now produced on a large scale from yeast in which it occurs up to 2% of the dry weight. It melts at 168°, exhibits laevo rotation, $[\alpha]_D^{20°}$, − 133° in chloroform solution, and

is sensitive to light. Its absorption spectrum contain four bands with maxima at 260, 270, 282 and 293 mμ. It is also a secondary alcohol having the formul $C_{28}H_{44}O$ and contains three double bonds. It is 3-cis hydroxy-24-methyl-$\triangle^{5,7;22}$-cholestatriene and its cons titution is represented as below (XIX).



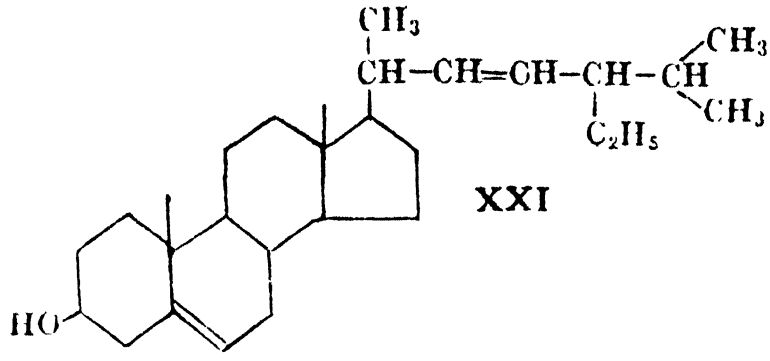
XIX

Sitosterol:—This is the most widely distributed stero of higher plants. It is the main sterol of wheat oil Depending upon the source it has a varying melting point (132° to 142°) and rotation (− 26° to − 38°) and i obviously heterogeneous Recent work has establishe the existence of at least three forms (α-, β- and γ-); a mixture of these is difficult to separate. Sitosterol ha the formula $C_{29}H_{50}O$. The main structure and the posi tion of the double linking are those of cholesterol; it side chain differs in containing two more carbon atom as shown in (XX) below:



XX

Stigmasterol $C_{29}H_{48}O$:—This sterol occurs in calaba and soya beans and in many other legumes; in genera it accompanies sitosterol in varying proportions. It i very similar to sitosterol and differs from it in having a second double bond located in the side chain (formul XXI).



XXI

Allophanates

When compounds containing hydroxyl groups are treated with cyanic acid, esters known as allophanates are produced in the following manner.

$$2\ HNCO + ROH \rightarrow H_2N\text{-}CO\text{-}NH\text{-}COOR$$

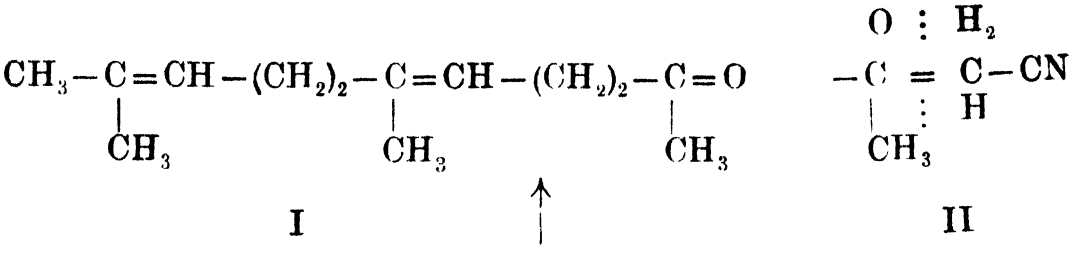
They are really esters of urea carobxylic acid. On account of the existence of the carbamide grouping they have higher melting points than the more common derivatives and are also less soluble. Consequently they are very useful for the purification and characterisation of hydroxy compounds.

For preparing the allophanate, the hydroxylic body is dissolved in an anhydrous solvent like benzene and the solution saturated with cyanic acid gas generated by heating cyanuric acid. It is convenient to carry it over to the solution in a slow stream of carbon dioxide. Some amount of cyamelide is also formed along with the allophanate, but it is readily separated.

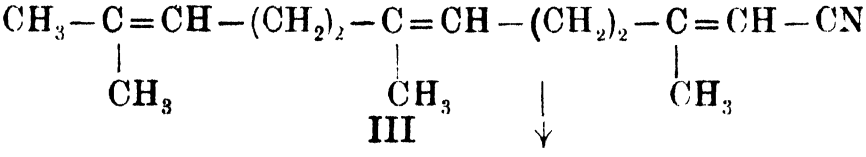
Farnesol

Farnesol is a sesquiterpene alcohol which occurs in the flowers of certain acacias (*e.g.* Acacia farnesiana) and some other plants and in musk kernels. It is of great importance in perfumery on account of its lasting and pleasant smell. It has the composition $C_{15}H_{26}O$ and has three double bonds in the molecule. On oxidation it yields an aldehyde, farnesal having the same number of carbon atoms ($C_{15}H_{24}O$). Hence farnesol should be a primary alcohol. The oxime of farnesal, when dehydrated, yields a nitrile which on hydrolysis gives rise to an acid, farnesenic acid, and also a ketone containing

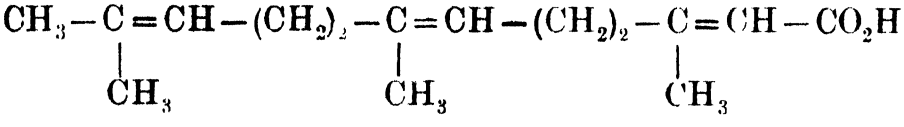
two carbon atoms less ($C_{13}H_{22}O$). This ketone is identical with dihydro-ψ-ionone (I). Its formation is evidently due to the addition of the elements of water to a double bond as shown in (II). Hence the nitrile can be represented by formula (III) and farnesenic acid and farnesol by (IV) and (V) respectively.



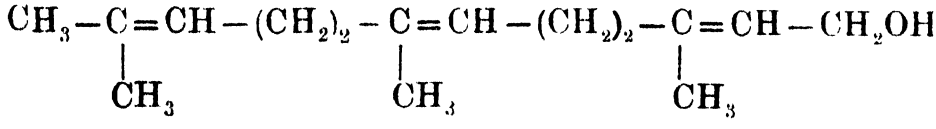
I II



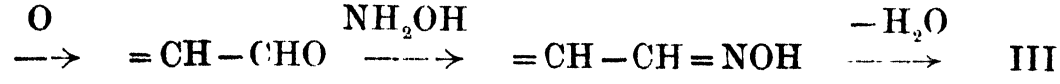
III



IV

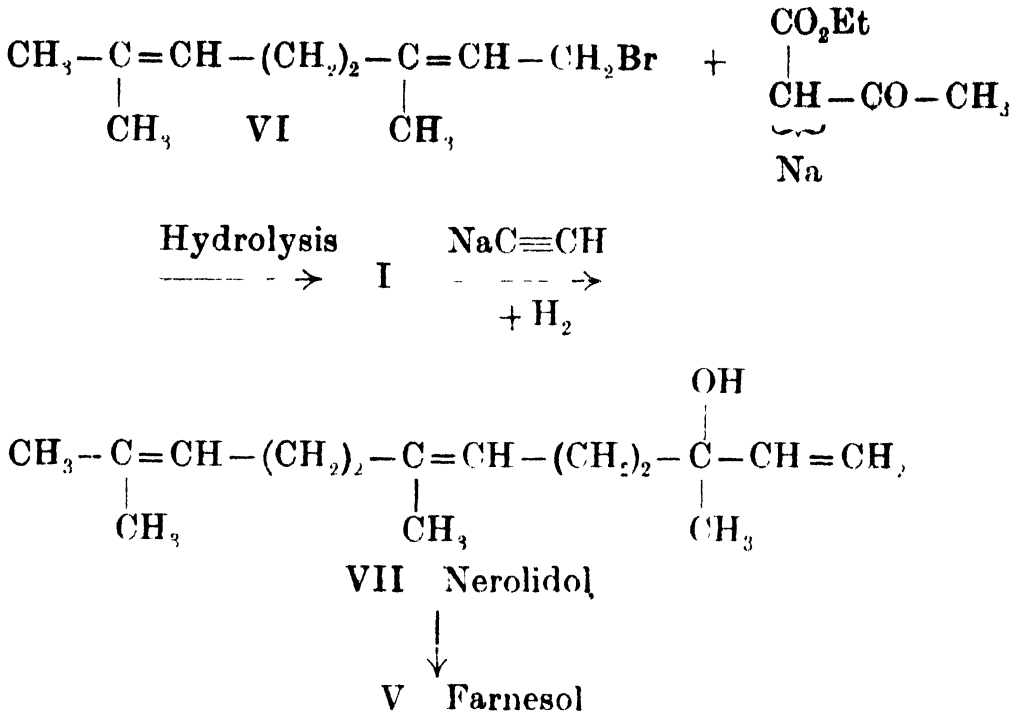


V Farnesol



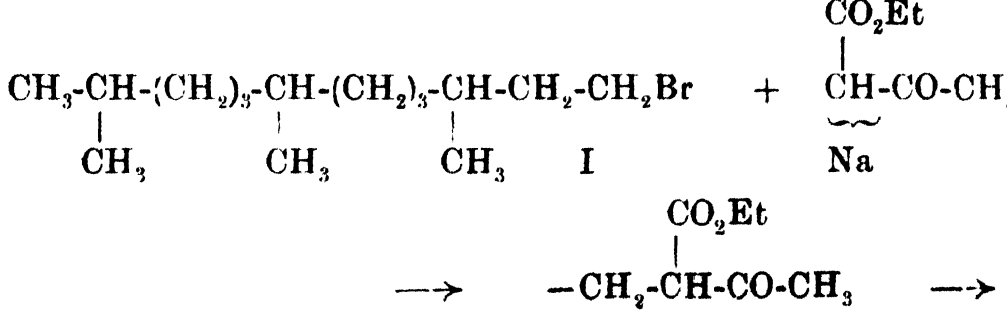
III

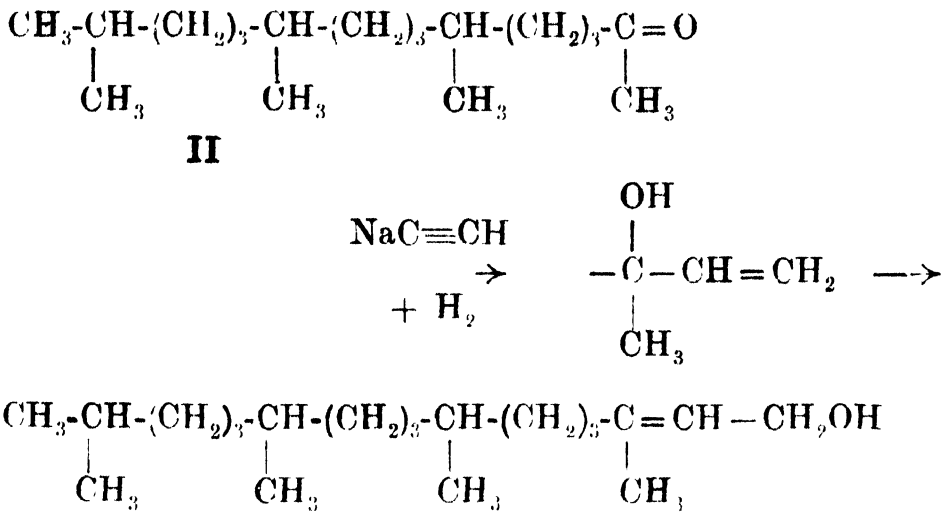
Farnesol has been prepared synthetically starting from geraniol. Geranyl bromide (VI) is condensed with ethyl acetoacetate leading to the production of dihydro-ψ-ionone. By the condensation of this ketone with sodium acetylide and partial reduction of the product dl-nerolidol (VII) is formed. The optically active form of this alcohol occurs in Peru balsam. Boiling with acetic anhydride and sodium acetate yields farnesyl acetate (isomeric change) which is hydrolysed to farnesol.



Phytol

Phytol is a long chain aliphatic alcohol obtained by the saponification of chlorophyll. It is a thick oil which boils at 204° under 10·0 mm. pressure. It has the molecular formula $C_{20}H_{40}O$ and has one double bond. Its constitution was established from the following degradations. Oxidation with chromic acid yielded a saturated ketone (now called 'phytol ketone') having two carbon atoms less, $C_{18}H_{36}O$. By the action of ozone, however, the above ketone was obtained along with glycollic aldehyde. The existence of an $\alpha:\beta$-unsaturated alcoholic grouping as in allyl alcohol was thus established. The nature of phytol ketone became clear when it was found to be identical with the ketone (II) synthesised from hexahydro-farnesol. The bromide of the alcohol (I) was condensed with the sodium derivative of ethyl acetoacetate and the product hydrolysed.





II

III Phytol

The constitution of phytol should therefore be 3 : 7 : 11 : 15 - tetramethyl-Δ^2-hexadecenol (III). It is a diterpene alcohol built up of isoprene units, but compared with other terpenes it is considerably hydrogenated. It can be synthetically prepared from the above ketone (II) by condensation with sodium acetylide, the procedure being just the same as for the synthesis of farnesol from dihydro-ψ-ionone (see page 300).

A new synthesis of phytol ketone has recently been effected by Smith and Sprung starting from citral which is more easily available as a convenient raw material (see page 51). This makes synthetic phytol considerably cheaper than before.

Clotting of blood

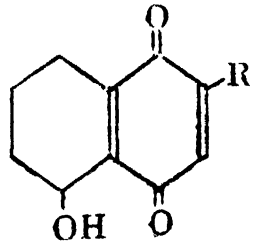
When blood is freshly drawn from an artery or vein it forms a jelly-like mass within a few minutes. This is known as clotting. This property which is inherent in blood is quite essential for preventing undue loss of this precious fluid when blood vessels are injured by accident. The blood clot is made up of a close mesh of fibrin, a protein, and encloses within it the blood corpuscles and the blood serum. The phenomenon of clotting may be described as taking place somewhat in the following manner. Blood contains fibrinogen and prothrombin. In the presence of calcium ions and under the influence of thrombokinase, prothrombin is converted into thrombin,

an enzyme, which then acts on fibrinogen to produce the clot of fibrin. Thus for the satisfactory clotting of blood, both calcium ions and prothrombin are necessary. Removal of calcium ions normally present in the blood, by means of oxalate or fluoride or citrate, prevents clotting, while an inadequate amount of prothrombin in the blood delays clotting.

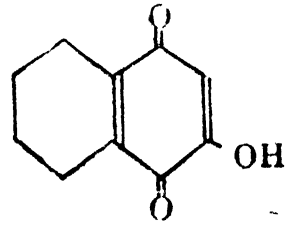
Prothrombin behaves like a globulin. It is formed in the liver and for its formation vitamin K is essential. Consequently an adequate supply of this vitamin is necessary for satisfactory clotting.

Naphthoquinones

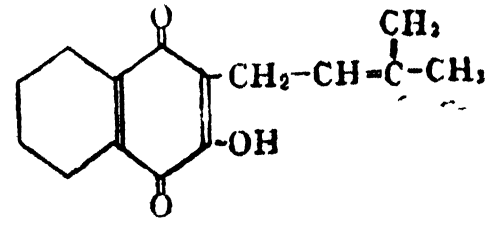
The naphthoquinone nucleus has been found to occur in several dye-yielding plants. Juglone which is present as a leuco-compound in unripe walnut shells is 5-hydroxy-1 : 4-naphthoquinone, and plumbagin, the yellow pigment from the roots of plumbago species, is 2-methyl-juglone. Lawsone isolated from Lawsonia alba is an isomer of juglone, being 2-hydroxy-1 : 4-naphthoquinone. Lapachol which is obtained from the heart-wood of various plants belonging to the family Bignoniaceæ and lomatiol, the pigment surrounding the seeds of Australian Lomatia are two closely related compounds, the latter being hydroxy-lapachol.



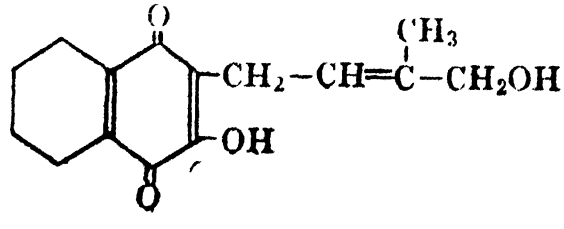
Juglone R = H
Plumbagin R = CH_3



Lawsone

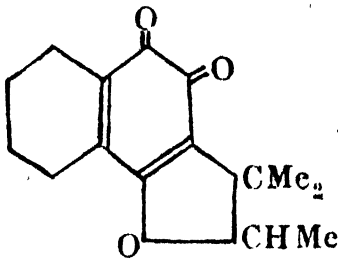


Lapachol

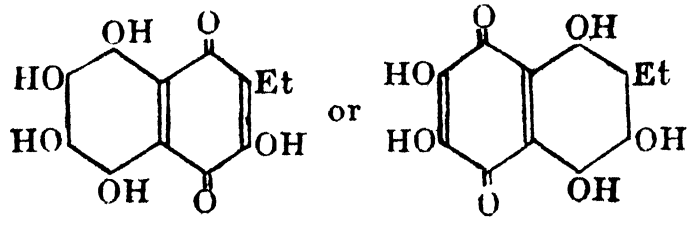


Lomatiol

More recent additions to the list of naphthoquinone pigments are dunninone and echinochrome A which were isolated from Streptococcus Dunnii and the eggs of Arbacia pustulosa respectively, and whose constitutions have been represented as given below:



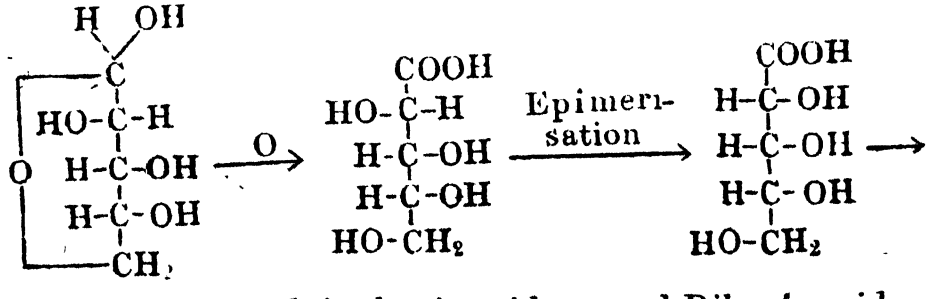
Dunninone



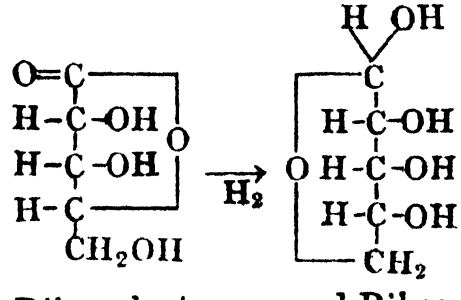
Echinochrome A

d-Ribose

d-Ribose is an important component of nucleic acids and coenzymes; from the first source it can be prepared by hydrolysis with mineral acids. It crystallises in colourless plates melting at 87° and has a specific rotation of −23·7° after mutarotation. Its constitution was established by its synthesis from d-arabinose which was converted into d-arabonic acid and subjected to epimerisation whereby d-ribonic acid was obtained. The lactone of the acid was subsequently reduced with sodium amalgam whereupon d-ribose was produced. This method has recently been improved by Steiger (1936) to give better yields.

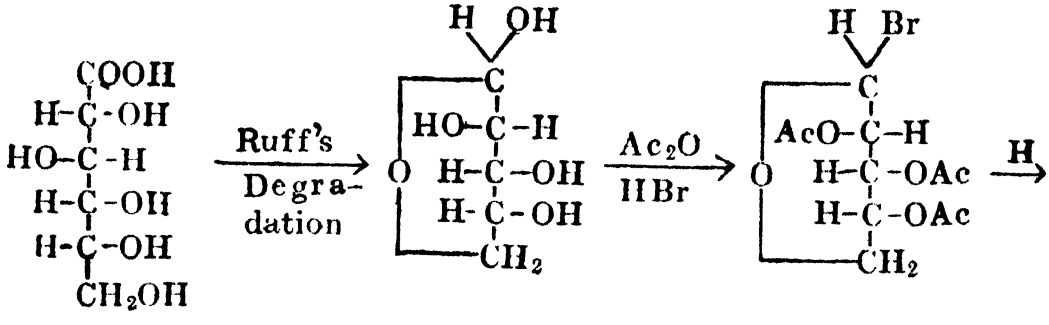


d-Arabinose — d-Arabonic acid — d-Ribonic acid

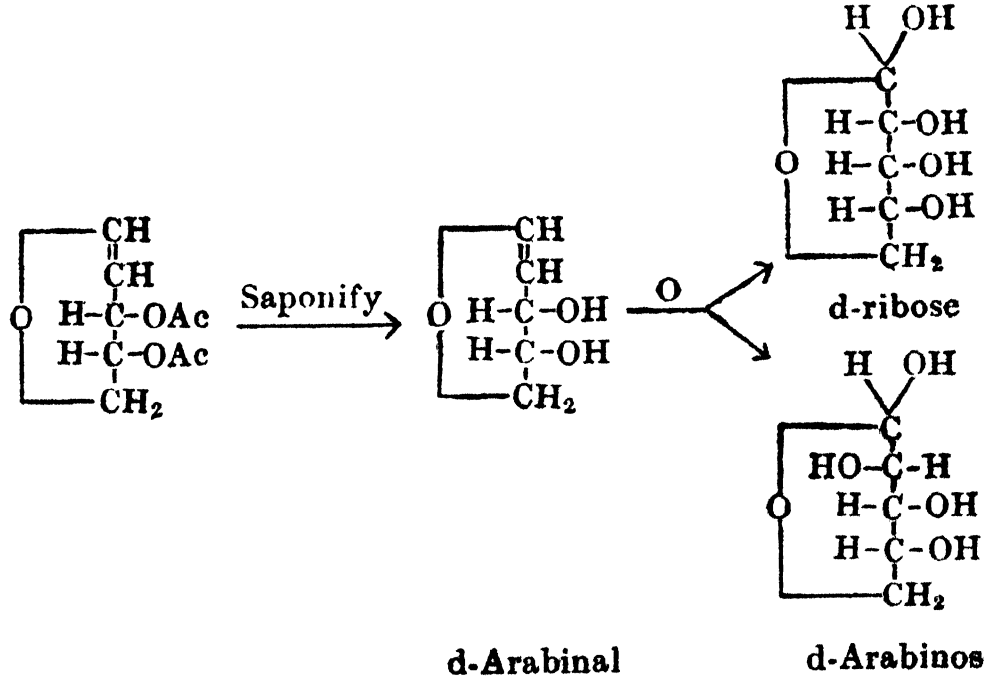


d-Ribonolactone — d-Ribose

A more efficient method of preparing d-ribose was described by Kuhn and by Karrer in connection with their synthesis of riboflavin. The starting point was d-glucose which was converted into gluconic acid by oxidation either by means of micro-organisms or by electrolytic methods. The calcium salt was subjected to Ruff's degradation. This involved the use of hydrogen peroxide for the oxidation in the presence of ferric acetate formed by the double decomposition of ferric sulphate and barium acetate in aqueous solution. The product was d-arabinose. By the action of acetic anhydride and hydrogen bromide the triacetyl bromide of d-arabinose was produced. Reduction with zinc and acetic acid along with a little zinc-copper couple yielded diacetyl-d-arabinal, which on saponification gave d-arabinal. Oxidation of this product with perbenzoic acid in ethyl acetate re-



d-Gluconic acid d-Arabinose



d-Arabinal d-Arabinose

sulted in a mixture of d-ribose (major product) and d-arabinose (minor product) which were separated. The yield of d-ribose by this method is claimed to be 53 grams from 2 kilograms of calcium gluconate which is commercially available.

Sorbitol and Sorbose

d-Sorbitol (ordinary sorbitol) is obtained by the reduction of d-glucose either by the action of sodium amalgam in faintly alkaline medium or by catalytic reduction. It is sold in the market as a sweet substitute for sugar for diabetics. It occurs in various fruits and in considerable quantities in the berries of the mountain-ash. Improved methods of reduction of d-glucose make use of a special catalyst (nickel-cobalt-chromium).

l-Sorbose is present as an ester in the juice of the mountain-ash berries in which it occurs along with d-sorbitol; this association is common in the Rosaceæ. The oxidation of d-sorbitol to l-sorbose was originally effected with Bacterium xylinum but more recently Acetobacter suboxydans has been found to be capable of bringing about the same reaction in a much shorter period, 3-5 days as against 6 weeks required by the older method.

Basal Metabolism

Metabolism is the term employed for denoting all the several chemical changes that take place in the nature of any food material taken by an animal from the time of its absorption into the general circulation to the time of its complete utilisation or disposal otherwise. All nutrient materials, proteins, fats and carbohydrates, are ultimately oxidised in the tissues with the liberation of energy which is utilised by the organism for the maintenance of body temperature, for the functional activity of the vital organs, for the performance of mechanical work and in other less important ways.

When the body is completely at rest without food the production of energy is reduced to a low level and the organism is then said to be having its basal metabolism. Basal metabolism is conventionally defined as the heat production of the resting organism which so balances

the heat lost by radiation and by other means, that a constant temperature results in the organism. This heat production has been found to be directly proportional to the body surface which in turn depends on both the height and weight. Basal metabolic rate has a remarkably constant value for the same species under identical conditions. Thus for young adult males (man) it is 39·5 calories per hour per square metre of body surface and for women 37·0 calories. The younger growing individual produces relatively more heat and with increasing age there is a slow fall in heat production. Mental effort, physical exercise, digestion and absorption of food all raise the total heat production of the body above the basal metabolic rate. Sleep, and more markedly prolonged under-nutrition lower the total metabolism below the basal figure.

Metabolism can be measured either by a direct determination of the heat produced in a given time or indirectly from the amount of oxygen consumed or the carbon dioxide given out in a given time. The measurements are usually carried out in calorimeters specially designed for the purpose, such as the bed calorimeter, the respiration calorimeter and the metabolism chamber.

Determinations of basal metabolism are of considerable clinical value, particularly in diseases of the thyroid gland. Over-activity of the gland results in increased tissue oxidation all over the body and hence increased heat production, while diminished activity has the opposite effect, there being, in the total absence of thyroid activity, only about 60% of the normal heat production.

Carbohydrate metabolism

The carbohydrate content of an average meal generally exceeds the equivalent of 100 grams of glucose. If all this amount were suddenly thrown into the circulation of an adult, there would result an increase of about 1·6% in the concentration of the blood sugar. Actually however it seldom rises considerably above the fasting figure, viz., 0·08%. It usually rises to 0·12–0·14% after half to one hour following the meal, then falls steadily and about two hours later it is back again at the fasting

level. This shows that glucose is rapidly withdrawn from the blood. The most important organs that are involved in this process are the liver and muscle, which store it up in the form of glycogen. The liver of a well-nourished man usually contains about 100 grams of glycogen and a still greater amount is present in the muscles. It is this reserve store of glycogen that is responsible for the maintenance of blood sugar at the normal level even during fasting, in spite of the fact that blood is continually depleted of its sugar for supplying energy.

The mechanism by which the body makes use of its store of glycogen is as follows. Each of the two tissues, liver and muscle, has its own phosphorylase which converts glycogen into glucose-1-phosphate, the reaction being reversible.

$$\text{Glycogen} + \text{Inorganic phosphate} \rightleftharpoons \text{Glucose-1-phosphate}$$

The subsequent changes differ in the liver and muscle. In the former a phosphatase splits glucose-1-phosphate into phosphate and free glucose, the latter being immediately carried by the circulation to the tissues where it is needed. In the case of muscle, however, there is no phosphatase and hence no free glucose can be formed. On the other hand the glucose-phosphate is degraded by a series of complex reactions to lactic acid. This change from muscle glycogen to lactic acid is associated with muscle contraction. During the subsequent period of relaxation about one-fourth to one-fifth of the lactic acid is oxidised to carbon dioxide and water. Part of the energy derived from this oxidation is utilised for the performance of work, the rest being used for the re-synthesis of muscle glycogen from the remaining lactic acid.

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Special Abbreviations used

J.B.C.	Journal of Biological Chemistry
Z. Physiol. C.	Zeitschrift for Physiologische Chemie
Biochem. *Z.*	Biochemische Zeitschrift
Helv.	Helvetica Chimica Acta

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