CHEMISTRY and PHYSIOLOGY of the Vitamins

By

H. R. ROSENBERG, Sc.D.

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PREFACE TO THE REVISED REPRINT

The present volume is essentially a reprint of the "Chemistry and Physiology of the Vitamins" which was published in 1942. Although many new discoveries have been made since the manuscript for the first edition was written, practically none of these has been incorporated into the reprint. Only actual errors which have crept into the first edition and which have come to the attention of the author have been corrected. However, the "Recommended Daily Allowances" as published in January 1943 by the Food and Nutrition Board of the National Research Council have been added.

Wilmington, Del.
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H. R. ROSENBERG

PREFACE TO THE 1942 EDITION

This monograph, "Chemistry and Physiology of the Vitamins," has a long historical background. Exactly a decade ago it was the privilege of the author to be present at the meeting held by the Zürich section of the Swiss Chemical Society when Paul Karrer announced a few days prior to its publication the successful isolation of essentially pure vitamin A from fish liver oils and the establishment of the structure of this vitamin. It was then that the author felt the urge to compile the available data on the chemistry and physiology of all vitamins. The science of vitamins had by that time advanced to a state at which the existence of vitamins, brought into the limelight between 1906 and 1914, was no longer questioned. In 1926 B. C. P. Jansen and W. F. Donath had announced the isolation of pure, crystalline vitamin B_1 but the constitution of this vitamin remained unknown for many years to come. At the same time (1926), Pohl, Windaus, Hess, Rosenheim and Webster had recognized that the long known ergosterol could be converted into a vitamin D by activation with ultraviolet light; but the pure vitamin D was not obtained until 1931–1932, and the correct chemical constitution was not known. Thus, Karrer was the first to establish the true chemical constitution of a vitamin.

In the years following Karrer's announcement, the author, while working in the laboratories of L. Ruzicka and T. Reichstein, had the good fortune of being able to see and observe the progress made on the establishment of the structure and the synthesis of several vitamins and hormones. During that time Reichstein synthesized vitamin C, the first vitamin ever obtained by total synthesis. And, Karrer, in a laboratory only a few blocks removed, worked in a dramatic race against Kuhn in Germany on vitamin B_2, and was the first to announce the successful synthesis of this vitamin. From Ruzicka's laboratory the synthesis of the male sex hormones, androsterone and testosterone, by degradation of cholesterol was announced, and Reichstein started to investigate the hormones of the adrenal cortex.

The author was then tempted to plan the publication of a comprehensive volume on the chemistry and physiology of the vitamins and hormones. These two classes of compounds have so much in common that a review of one of them seemed to necessitate a review of the other. The extraordinary activity of research workers in all parts of the world, however, has resulted in the accumulation of such an enormous amount of scientific and practical material, that it became infeasible to combine a discussion of

both the vitamins and the hormones in one volume. Thus, this monograph is confined to a treatment of the vitamins exclusively.

Since the author left the hospitable Swiss Laboratories, he has been connected, at some time or other, with the development of many of the vitamins known today. Simultaneously, the need for an up-to-date presentation of the chemistry and physiology of the vitamins became more and more recognized, and this need is felt today by everyone who desires to inform himself or others on this subject. Short tabulations of the vitamins and scattered review articles do not answer the need. Books written for the general public are obviously of a different category than books of a purely technical character. Fortunately, there have been available in the English language a number of excellent books on the medical aspects of vitamin therapy, such as "The Vitamins," a symposium published under the auspices of the American Medical Association and "The Avitaminoses" by W. H. Eddy and G. Dalldorf. There is also a very satisfactory book on "The Biological Standardization of the Vitamins," written by K. H. Coward. In addition, there are monographs on special vitamins such as the book on "Vitamin B_1" by R. R. Williams and T. D. Spies; "Vitamin D" by C. I. Reed, H. C. Struck and I. E. Steck; "Vitamin K" by H. R. Butt and A. M. Snell and "Vitamin E," a symposium held under the auspices of The Food Group of the Society of Chemical Industry. The author, however, knows of no comprehensive treatment of the chemistry and physiology of all the vitamins.

The present monograph on the "Chemistry and Physiology of the Vitamins" begins with the presentation of a definition of the vitamins which distinguishes this group of compounds sharply from the hormones and from other essential and non-essential food constituents. A new classification for compounds which have the dual character of vitamins and structural building units or suppliers of energy is introduced. Each vitamin is then discussed separately, emphasis being laid upon the chemistry and the physiological action of these compounds. The chapters on each vitamin start with a review of the nomenclature and a tabulation of the historical development followed by a paragraph on the occurrence of the vitamin. The main discussions on the chemistry and physiology follow. Under the chemistry of each vitamin the procedures used for the isolation of the vitamin, the proof of the chemical constitution and the synthesis of the vitamin are reviewed separately. There are special paragraphs on industrial methods of preparing the vitamins and on their biogenesis. The specificity of the vitamin action is treated separately. The determination of the vitamins is subdivided into physical, chemical,

biochemical and biological methods, and is followed by a paragraph on the vitamin standards. The physiology of plants and microorganisms is separated from the animal physiology which is subdivided into the metabolism of the vitamin, the physiological action and the mechanism of the vitamin action. The relation of each vitamin to other vitamins, to hormones, and to inorganics is presented in special paragraphs. This is followed by a short review of the present-day knowledge of the pathological aspects, the hypovitaminoses, avitaminoses, hypervitaminoses and paravitaminoses, with a special section on clinical test methods. Finally the vitamin requirements are briefly stated. The book ends with a list and abstracts of vitamin patents which have issued in the United States of America, Great Britain, Germany and France, arranged in a manner similar to that of the main text.

The vitamins are presented according to the alphabetical order of nomenclature. This arrangement follows in general the incidental discovery of the vitamins. Eventually, a classification according to the function of the vitamins will probably prove to be more satisfactory, but cannot successfully be undertaken at this time due to the rather incomplete knowledge of the primary function of many members.

This volume has been prepared with the idea of covering all topics of vitamin research and especially the chemistry and physiology of the vitamins. In presenting this monograph to the public the hope is expressed that it may guide the student and the scholar through our present-day knowledge of the field and inspire further development. This is especially desirable and should be expedited as much as possible since the health and successful propagation of man are, to a considerable extent, dependent upon proper nutrition, and any advance in the knowledge of the vitamins can be utilized immediately for the benefit of mankind.

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H. R. ROSENBERG
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IN GENERAL

THE VITAMINS IN GENERAL

1. Definition of Vitamins

Vitamins are organic compounds which are required for the normal growth and maintenance of life of animals, including man, who, as a rule, are unable to synthesize these compounds by anabolic processes that are independent of environment other than air, and which compounds are effective in small amounts, do not furnish energy and are not utilized as building units for the structure of the organism, but are essential for the transformation of energy and for the regulation of the metabolism of structural units.

The science of nutrition classifies the alimentary constituents (according to their function) into two different groups: energy and building unit providing food, on the one hand, and protective food, on the other. The latter group comprises water, inorganic substances and certain organic compounds among which are the vitamins. The definition of vitamins, as given above, clearly differentiates this group of nutrients from all other food constituents. Vitamins are organic compounds, and water or inorganic substances cannot be classified as vitamins. Vitamins are required for the normal growth and maintenance of life of animals, including man. All known vitamins, with the probable exception of vitamin D, are synthesized by plants and, as far as is known, are used by them essentially for the same purposes as by man and animals. The latter, however, as a rule are unable to synthesize these compounds by anabolic processes which are independent of the environment with the exception of air. In other words, the inherently available mechanisms of organo-synthesis in animals and man are not provided with means to produce the vitamins. On the other hand, those compounds which are produced anabolically (or catabolically) and which otherwise conform to the definition for vitamins as given above are classified as hormones The apparent independence of cattle for certain members of the vitamin B complex and for vitamin K is due to bacterial synthesis of these vitamins in the rumen which is not an anabolic process. The production of vitamin D in the skin is not an anabolic process as defined since this process is not independent of the environ-

ment but requires energy from the outside. This energy consists of ultraviolet light and is normally supplied by sunlight.

Vitamins are compounds which are effective in small amounts and occur as traces in cells and body fluids. While the maximum concentration in tissues or fluids, with the exception of those which serve as storage places, has not been determined for all vitamins, it is estimated that the amount of any one vitamin is less than 5 γ per gram of dry weight.[1] Vitamins do not furnish energy and are thus distinguished from the energy-bearing food constituents. The amount of vitamins needed is too small to account for even a fraction of the total energy required. Actually, minute quantities of vitamins are burned in the organism, but the energy set free by this process is infinitesimal. The vitamins, furthermore, do not act as morphological or structural building units of the organism or its cells. The fact that excessive amounts of most vitamins are excreted unchanged indicates that they are not utilized as suppliers of energy or as structural building units. The vitamins are, however, essential for the transformation of energy and for the regulation of the metabolism of structural units, and are functional in systems which carry out these reactions. Such systems are quite complex and are, as far as is known, enzymatic in character. These enzyme systems consist of many different components, a few of which are vitamins.

Vitamins are required by animals, including man, as has been stated previously. As long as at least one animal species is known to be unable to synthesize a particular compound, that compound should be considered a vitamin provided it conforms with the definition for vitamins in other particulars. Actually it cannot be expected that all animals need exactly the same nutritional elements. In accordance with this thought it has, for example, been observed that the cockroach apparently does not need any vitamin A,[2] and it is conceivable that animals will be discovered which do not need any vitamin D. Ascorbic acid, on the other hand, while apparently needed by all animals, is synthesized by many of them, whereas other animals, such as the primates and the guinea pig, are dependent upon an outside source of this compound. Ascorbic acid is thus a vitamin according to the definition of this term.

The classification of the nutritional elements which exert vitamin A activity requires special consideration. Man and most animals obtain in their foods two different types of substances which bring about the same

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[1] D. E. Green, *Advances in Enzymology*, **1**, 177 (1941).

[2] R. E. Bowers and C. M. McCay, *Science*, **92**, 291 (1940).

physiological effect. Both groups belong to the same class of organic compounds, namely, the carotenoids. They differ from each other in that the one group contains 40 carbon atoms in the molecule, while the other group contains about 20 carbon atoms. The first group of substances is converted metabolically into the second group in the animal organism. The second group has acquired the term vitamin A while the first group has been designated as provitamin A. This terminology is not in strict agreement with the definition for vitamins, according to which both groups of compounds should be called vitamins. The term provitamin A has, however, been retained in this monograph in accordance with general usage in order to avoid further complication of the vitamin terminology, since different terms have to be applied to the two different types of compounds.

The definition of vitamins, as given above, has not been undisputed. The most severe criticism is born of the thought that the ingested essential nutrients exert no vitamin activity as such, but are active only after chemical transformation into other compounds. According to these views, the ingested compounds should be called provitamins unless it is established that they do not undergo transformation in the body. Nicotinamide and nicotinic acid according to this interpretation should be called provitamins, while nicotinamide-containing coenzymes, codehydrogenase I and II, should be called vitamins. Similarly vitamin B_1 (thiamin) and vitamin B_2 (riboflavin) should be called provitamins. This definition of the term vitamin, however, does not appear desirable. Compounds like nicotinamide and riboflavin are constituents of a number of different enzymes and a multitude of different vitamins would have to be postulated. As a result, it would be difficult in many cases to decide if the coenzyme or the entire enzyme system should be called a vitamin. The term "vitamin" would be dissociated from the science of nutrition which needs a term for these compounds. The term "vitamin" as defined on page 3 has served a useful purpose and it appears more logical to adopt a special term for enzymes which contain vitamins, such as, for example, the term "vitazyme," than to change radically the present-day vitamin definition which has been adopted generally.

There is another group of organic compounds which are protective foods. They, like the vitamins, are required for normal growth and maintenance of life of animals, including man, who, as a rule, are unable to synthesize these compounds by anabolic processes. They are also essential for the transformation of energy and for the regulation of the metabolism of structural units. These compounds differ from the vitamins in that they also

act as suppliers of energy or as structural building units. In view of the similarity of these compounds to the vitamins, but in recognition of the fact that they differ in one important functional aspect from them, it is suggested that this class of compounds be called "*vitagens.*" This term is broad and emphasizes that the compounds of this class are concerned with the production and maintenance of life. It is recommended that this terminology be adopted until the time when more precise information is available concerning the physiological action of these compounds and the vitamins.

The number of compounds which should be classified as vitagens is unknown. There is ample evidence that they include the essential fatty acids and the essential amino-acids. There may be some essential carbohydrates which conform to the vitagen concept. Choline and related compounds which supply the essential transferable methyl group are vitagens and it is suspected that some organic sulfur-containing compounds will be discovered which belong to this group. The actual vitagen nature of all these compounds, however, has not been demonstrated. The essential fatty acids are structural units in many phospholipids and their physiological action appears to be primarily a regulatory one. Some of the essential amino-acids have been shown to be structural building units for tissue and cellular and intracellular fluid constituents but are also constituents of enzyme systems involved in the metabolism of energy-bearing foods. For example, the essential amino-acids lysine, histidine, tryptophane, phenyl-alanine and arginine are constituents[3] of the apoenzyme of a riboflavin-enzyme system, and the possibility remains that some other essential amino-acid is present in this system. Choline and other compounds, which provide an essential transferable methyl group for the anabolism of the structure of the organism, also act as regulators, for example, in the distribution of fat.

A dietary deficiency of any one of the vitagens gives rise to specific clinical symptoms which are similar to those encountered in vitamin deficiencies. Obviously, they might be considerably complex since the vitagens have the double function of providing energy or building units on the one hand, and of being concerned with the transformation of energy or the regulation of the metabolism of structural units on the other hand. A deficiency of any one of the essential amino-acids which are constituents of a riboflavin-enzyme system, should eventually cause the occurrence of the same syndromes which are observed during times of riboflavin defi-

[3] R. Kuhn and P. Desnuelle, *Ber.*, 70, 1907 (1937).

ciency. Whether or not that is actually the case cannot be stated. It might be expected that during times of a vitagen deficiency the vitagen would be liberated in the organism from the structural materials and utilized functionally. This, however, does not seem to be the case. Thus a deficiency of choline in the diet becomes apparent from a disturbance of the distribution of fat in the liver in spite of the presence of very large amounts of choline in the phospholipids of the body.

2. History of the Discovery of the Vitamins[5]

Generally speaking, three distinctly different periods in the history of vitamins can be differentiated. First there was the period which is characterized by the recognition of the existence of nutrient materials different from those which are needed only for the maintenance of the energy and building unit supply. This period actually began many centuries ago but became a definite science at the turn of this century. The second period is that of the isolation of a great number of vitamins in pure form and the elucidation of their chemical structure which culminated in the synthesis of various vitamin compounds. This period started about in the middle of the 1920's when the first vitamins were obtained in crystalline form. The last period is characterized by the recognition that various compounds which were previously known to exert beneficial effects in the growth of lower organisms such as yeast and bacteria are also necessary for man and animals. Simultaneously an earnest effort is being made to understand the physiology and the mechanism of the vitamin action, which has resulted so far in the recognition of the part which various vitamins, especially those of the B-complex, play in a number of different enzyme systems.

Diseases of the human organism caused by a deficiency of vitamins are probably as old as the human race. Among the uncovered skeletons of prehistoric man are some which show definite signs of rickets (vitamin D deficiency) and of scurvy (vitamin C deficiency). The symptoms of these diseases and of beriberi (vitamin B_1 deficiency) and of night-blindness (vitamin A deficiency) were known to the physicians of ancient days, and were described in various manuscripts which were written in the first thousand years A.D. With the exception of a remedy for night-blindness no effective therapeutic methods were known at that time for curing or pre-

[4] F. X. Aylward, H. J. Channon and H Wilkinson, *Biochem. J.*, 29, 169 (1935).

[5] For more details of the historical development see: H. C. Sherman and S. L. Smith, *The Vitamins*, New York, 1931; Medical Research Council, *Vitamins: A Survey of Present Knowledge*, London, 1932; v. Brunn, *Münch. Med. Wochschr.*, 84, 223 (1937).

venting these diseases. The old Greek, Roman and Arab physicians recommended an internal and external therapy with livers of goats to overcome night-blindness. The cure of the blind Tobias by means of fish bile, as described in the Bible, points to an early knowledge of means for dealing with night-blindness. Effective therapeutic measures against other diseases which are now known to be caused by vitamin deficiencies were not discovered prior to modern times. In the middle of the 16th century oranges and lemon juice were recommended as a cure for scurvy, and in the 17th century oranges apparently were used by the Dutch navy to prevent the occurrence of scurvy. At the same time the curative effect of fresh vegetables was observed and horse-radish preserved in French brandy was used successfully to prevent scurvy during long voyages (Dietz, 1665). However, the beneficial effects of these materials were not generally recognized and in the following century the therapy of scurvy with fresh vegetables and with lemon juice was discovered anew and advocated (by the Austrian physician Kramer in 1720 and by the English physician Lind in 1757). During the middle ages, many different remedies were occasionally recommended for the treatment of rickets among which are some known today to be effective such as fresh air, sunshine, egg yolk and fried fish livers. In 1824 Schütte recommended the use of fish liver oils. No remedies were known for beriberi until 1882 when Takaki observed that the onset of this disease could be prevented in the Japanese Navy by dietary means.

The few isolated reports about the curative effect of nutritional materials upon special diseases remained as a whole unrecognized. Until about the end of the last century food was generally believed to consist solely of carbohydrates, fats, proteins, salts, and water. Previously only a few investigators expressed the view that diseases could possibly originate from faulty nutrition. In 1657 Hoefer and in 1754 von Bergen expressed the view that night-blindness is caused by malnutrition. In 1755 Roupp claimed that a deficiency of fresh vegetables in the nutrition caused scurvy and in 1785 Rosen von Rosenstein believed that rickets was caused by faulty nutrition. Prior to the existence of the vitamin concept the explanation was offered that if special foods really have a beneficial effect, which was doubted by the majority of the physicians, this effect is due solely to the ability of foods to overcome an unknown toxic material which was thought to cause the specific disease.

The impulse for the formation of the theory that certain foods contain special substances which are essential for life in addition to the carbohydrates, fats, proteins, salts and water came from experimental studies with

pound), vitamin E and vitamin K. The existence of other vitamins was postulated during this period but general knowledge did not proceed further than the recognition of the existence of further vitamins and the determination of more or less specific deficiency symptoms.

The third period in the history of vitamin research proceeds along somewhat different lines of thought. While in the second period the vitamin was isolated by tedious concentration procedures using the experimental animal as the tool for the determination of the progress made, a different course was followed in the next period. This originated as a development of the study of the nutritional elements necessary for microorganisms, especially yeast and certain bacteria. J. von Liebig already had observed in the last century that yeast cannot grow properly unless the culture medium contains, besides the known and accepted nutritional elements, some growth-stimulating material which was found to be present in meat extracts. This unknown substance was later called "bios" and proved to be a mixture of substances. The first compound isolated from bios concentrates was inositol (1928) which Woolley identified in 1940 as a vitamin. Elvehjem found that the bios factor, nicotinic acid, cured blacktongue, a deficiency disease of dogs. This was soon followed by the isolation of nicotinic acid from crude concentrates of the anti-blacktongue factor. Almost immediately the effectiveness of nicotinic acid in the cure of human pellagra was discovered. Several other vitamins were discovered in the same way. The yeast growth factor, pantothenic acid, proved to be the chicken antidermatitis vitamin; biotin was found to be identical with the postulated vitamin H and *p*-amino-benzoic acid, the growth factor for *Clostridium acetobutylicum*, was shown to exert vitamin activity for man and animals. This development is not necessarily terminated as yet, since further unknown growth factors for microorganisms apparently exist which may prove to be identical with vitamins for the animal organism.

As in the case of other natural sciences the first step in vitamin research is to recognize the various members. The next step is to study the physiological action, and the final step is to elucidate the mechanism of the action. It seems that the main task of the recognition of vitamins has been essentially accomplished. No doubt the list of vitamins is not complete as yet and considerably more work needs to be done before all the vitamins will be recognized. Nevertheless it appears that those vitamins which cause the most obvious deficiency diseases are known. While Lunin in 1881 and Hopkins in 1906 failed to keep animals alive on a diet composed of the then known nutritional elements, the situation is quite different today. It has been possible in a number of different laboratories to raise experimental

animals, rats for example, on a completely synthetic diet, which includes all the known vitamins, and to keep the animals alive over a period of several generations. Simultaneously essential work is in progress for clarifying the physiological behavior of the vitamins in the organism. Once the reactions which are carried out by the vitamins in the organism are known, attempts can be made to clarify the mechanism of this action. The gross physiological effect of each vitamin is evident from the symptoms caused by the deficiency of the vitamin. But these clinical symptoms may be of secondary nature and the elucidation of the primary reaction is quite difficult and requires new and special technics (see under Physiology of the vitamins, page 26). This work has shown that the vitamins are essential participants in the metabolism of energy-bearing food, of minerals and of water. The proof is in some cases a direct one, in other cases indirect and needs confirmation. The mechanism of the vitamin action has been traced definitely for three vitamins of the B-complex, namely for vitamins B_1, B_2 and nicotinic acid, which were found to take part in enzyme systems which are concerned with the processes of carboxylation and decarboxylation and, by transportation of hydrogen, with the oxidation-reduction mechanism.

3. Nomenclature

Hopkins[6] (1906-1912) called the nutritional elements which are required by the animal organism in addition to the carbohydrates, fats, proteins, salts and water, "accessory factors." In systematizing the knowledge of these accessory factors, Funk[7] in 1912 proposed the generic term "vitamine" because these compounds are essential to life (the Latin term "vita" meaning life) and because he believed the anti-beriberi compound to be an amine. Funk[8] differentiated an anti-beriberi vitamine, an anti-scurvy vitamine, an anti-rickets vitamine, etc., according to the diseases which occurred during the respective nutritional deficiencies. No statement could be made at that time as to whether these diseases are caused by a deficiency of just one compound or of a multitude of compounds. Osborne and Mendel,[9] and McCollum and Davis,[10] in 1915 distinguished two types of accessory factors by difference of their solubility and called them "Fat-Soluble A" and "Water-Soluble B." "Fat-soluble A" was shown to cure

[6] F. G. Hopkins, *Analyst*, **31**, 385 (1906); *J. Physiol.*, **49**, 425 (1912).
[7] C. Funk, *J. State Med.*, **20**, 341 (1912).
[8] C. Funk, *Die Vitamine*, Wiesbaden, 1914.
[9] T. B. Osborne and L. B. Mendel, *J. Biol. Chem.*, **20**, 379 (1915).
[10] E. V. McCollum and M. Davis, *Ibid.*, **23**, 181 (1915).

nutritional eye disease (the symptoms of which are xerophthalmia and keratomalacia) and to be necessary for growth of experimental animals. A deficiency of "water-soluble B" produced beriberi in pigeons. This terminology did not include a term for the anti-scurvy substance. Drummond[11] in 1920 proposed to combine the previously suggested terms and to drop the terminal "e" of vitamine to the generic term "vitamin" and called the fat-soluble growth and eye factor "vitamin A," the water-soluble anti-beriberi compound "vitamin B" and the anti-scurvy factor "vitamin C." This suggestion has generally been adopted by all workers in the field and the more recently discovered vitamins have been added to the list by using the letters of the alphabet consecutively. Thus, McCollum called the antirachitic compound "vitamin D." The anti-sterility compound was called "vitamin E," etc. The vitamins G, H and I were added later to the list of vitamins. The blood coagulation vitamin was called vitamin K because the term "Koagulation" is spelled with "K" in the German language. The postulated *l*actation vitamin is called "vitamin L" and the compound which prevents excessive *p*ermeability of cells is "vitamin P."

Considerable discussion arose from time to time as to whether or not the generic term "vitamin" should be maintained or changed to a term which would not indicate any relation to chemical or physiological properties of the compounds. The term "accessory factors" of foods was discarded because it was felt that this term is too modest since the compounds in question are essential food factors and not merely accessory food constituents. Other terms which have been suggested are "Advitant" and "Exogenous Hormones." Neither of these terms has, however, been generally accepted.

The further development of the vitamin terminology has unfortunately resulted in an illogical nomenclature which can only be understood from its historical development. What was originally called vitamin B proved to be a mixture of compounds which are referred to today as the "vitamin B complex" in accordance with the original nomenclature. This complex consists of an unknown number of different vitamins which have been designated arbitrarily as vitamin B_1, B_2, etc. The anti-beriberi vitamin which was first discovered is called vitamin B_1. So far eight different vitamins have been given vitamin B subnumbers. One of the difficulties which arose during the course of the vitamin research was that a deficient diet gave different symptoms in various animals although it was found later that some of these symptoms were caused by the absence of the same vitamin. Thus vitamin B_2 has also been called vitamin G. Originally vitamins B_2

[11] J. C. Drummond, *Biochem. J.*, **14**, 660 (1920).

and B_4 were believed to be necessary only for the rat while vitamins B_3 and B_5 were believed to produce growth only in the pigeon.

By the time that the vitamins were isolated as chemical compounds, they were given names which identified them according to the class of chemical compounds to which they belong. Thus, vitamin B_1 is called "thiamin," vitamin B_2 "riboflavin," vitamin B_6 "pyridoxin," etc. While the research on the isolation of the vitamins B_3, B_4 and B_5 was progressing only slowly, other vitamins were discovered through experimental work with different organisms. Thus, nicotinic acid, pantothenic acid and biotin which were known to be growth stimulants for yeast were also found to be vitamins for the animal organism. It has subsequently been discovered that pantothenic acid is probably identical with vitamin B_3 and nicotinic acid with vitamin B_5. Biotin, glycine and arginine, on the other hand, appear to be present in what was originally called vitamin B_4.

While the original vitamin B has been shown to consist of a number of chemically and physiologically different compounds which were differentiated by subnumbers, the fat-soluble vitamins A, D, E and K each have been found to occur naturally not as single compounds but as mixtures of compounds each of which exerts the same physiological action but differs from the others slightly in its chemical constitution. Thus, there exist two or three different vitamins A, at least six vitamins D, three vitamins E and two vitamins K. They are called vitamins A, D, E and K and are differentiated by subnumbers. Thus, for example, vitamin A_1 exerts essentially the same physiological effect as vitamin A_2. On the other hand, in the water-soluble class of vitamins each subnumber indicates a physiologically different vitamin with an entirely different mechanism of action as has been stated before.

The vitamins have been classified as "biocatalysts" together with the hormones and enzymes. Instead of the term "biocatalysts" the term "ergins" has been proposed by Ammon and Dirscherl.[12] On the other hand, Euler[13] advocated the term "ergons" for the vitamins, hormones and those coenzymes which contain as part of their structure vitamin or hormone molecules. Such coenzymes have also been called "vitazymes" and "hormozymes."

4. List of the Vitamins

Two classes of vitamins are differentiated today, namely, those which have been identified and those which have not been definitely identified.

[12] R. Ammon and W. Dirscherl, *Fermente Hormone Vitamine*, Leipzig, 1938.

[13] H. v. Euler, *Arkiv Kemi Mineral. Geol.*, **A11**, No. 12 (1934); **B11**, No. 45 (1934); **B12**, No. 11 (1935).

By identification is meant the recognition of a vitamin as a chemical compound the physical properties of which have been established.

THE IDENTIFIED VITAMINS

Vitamin	Name
The Group of Vitamins A	
Vitamin A	Axerophthol
Vitamin A_2	
Vitamin A_3 ?	
Vitamin B_1	Thiamin — Vitamin B Complex
Vitamin B_2	Riboflavin — Vitamin B Complex
Vitamin B_6	Pyridoxin — Vitamin B Complex
Nicotinic Acid (Vitamin B_5 ?)	Vitamin B Complex
Pantothenic Acid (Vitamin B_3 ?)	Vitamin B Complex
Inositol	Vitamin B Complex
p-Amino-Benzoic Acid	Vitamin B Complex
Vitamin C	Ascorbic acid
The Group of Vitamins D	
Vitamin D_2	Viosterol, calciferol
Vitamin D_3	
Vitamin D_4	
Vitamin D_5	
Vitamin D_6	
The Group of Vitamins E	Tocopherols
α-Tocopherol	
β-Tocopherol	
γ-Tocopherol	
Vitamin H	Biotin
The Group of Vitamins K	
Vitamin K_1	
Vitamin K_2	
Vitamin P	Citrin

THE NON-IDENTIFIED VITAMINS

Vitamin B_3 (Pantothenic Acid ?)
Vitamin B_4 (Biotin, Arginine, Glycine ?)
Vitamin B_5 (Nicotinic Acid ?)
Vitamin B_7—Vit min I
Vitamin B_8 (Adenylic Acid)
Vitamin B_9
Vitamin B_p (Anti-Perosis Vitamin)
Vitamin J
Group of Vitamins L (Lactation Vitamin)
Vitamin L_1
Vitamin L_2
Vitamin M
Factor T
Factor U
Folic Acid
Grass Juice Factor

A list of the vitagens is as follows:

THE VITAGENS

Essential fatty acids	Choline and related compounds and the essential transferable methyl group
Essential amino-acids	Essential organic sulfur-containing compounds
Essential carbohydrates	

5. Occurrence

The natural distribution of the vitamins is discussed in this monograph separately in the vitamin chapters under the heading of occurrence. Generally speaking, vitamins occur in plant materials and are found in the animal organism only as a result of food intake or of the anabolic activity of micro-organisms living in the intestinal tract. The vitamin content of various sources is of eminent importance for the proper selection of food for man and animals and is often the determining factor in the commercial preparation of vitamin concentrates. Nevertheless, quantitative data as to the vitamin content are given only occasionally in this monograph. For practical purposes it is not possible to assign definite data to the vitamin content of foods because the vitamin content is only to a limited extent a function of the vitamin concentration in the fresh vegetable, in the fresh fruit, etc. The amount of most vitamins contained in food after cooking or preservation procedures differs considerably from that of the untreated food. In most cases the vitamin content decreases during cooking, heating and washing procedures while, in some instances, the amount of available vitamins increases by liberation from protein material.

Among the many other factors which influence the vitamin content of vegetable foods are the species, the time of harvesting, and the soil. And the potency of animal foods depends somewhat upon the vitamin supply in the animal's diet.

Extensive studies have been made to learn the vitamin content of practically all foods and foodstuffs, treated and untreated. The available information has been collected in a number of monographs [14] which are recommended as an excellent guide for the approximate vitamin potency of foodstuffs.

[14] E. P. Daniel and H. E. Munsell, *United States Department of Agriculture, Misc. Publ.*, No. 275 (1937). L. E. Booher, E. R. Hortzler and E. M. Hewston, *United States Department of Agriculture, Circ.* No. 638 (1942). M. A. B. Fixsen and M. H. Roscoe, *Nutrition Abstracts and Reviews*, **9**, 795 (1940). A. L. Bacharach, *Ibid.*, **10**, 450 (1941). H. A. Waisman and C. A. Elvehjem, *The Vitamin Content of Meat*, Burgess Publ. Co., Minneapolis, 1941.

living organisms. The first investigator who conducted such experiments was probably J. von Liebig. He found that yeast does not ferment or grow properly unless some unknown material, present in extracts from animal materials, is added to the culture medium. Shortly afterwards von Bunge set up experiments with the object of proving or disproving the theory that carbohydrates, fats, proteins and salt and water are the only necessary food constituents. Forster had observed in 1873 that pigeons and dogs died within a short period of time when they were fed only carbohydrates, fats, proteins and water. Lunin in von Bunge's laboratory in 1881 experimented with mice and found that the addition of salts (obtained by ashing milk), which Forster had not offered to his animals, to the diet did not materially increase the survival time of the animals, while the addition of fresh milk to the diet kept the experimental animals alive. Lunin concluded that natural substances such as milk contain, besides known principles, small quantities of unknown substances essential to life. Thus the vitamin theory was essentially formulated. However, it took another generation to rediscover and prove this early concept.

It was generally assumed that the diseases which could be cured by dietary means were caused by some unknown germ, bacterium, toxin or the like. The clue that these diseases occur because of a deficiency of nutritional material came from studies with beriberi. Eijkman reported in 1897 that hens which had been fed on milled rice developed a disease which closely resembled human beriberi. Thus, for the first time, an experimental vitamin deficiency was produced in animals, although this was not recognized at that time. Eijkman found, furthermore, that the disease could be prevented or cured by feeding rice bran together with the milled rice, and that the substance responsible for the therapeutic effect could be extracted from the bran by water or alcohol. A few years later (1901), Grijns, a colleague of Eijkman, proclaimed that the anti-beriberi substance acts not because it counteracts an unknown producer of the disease, but because the diet lacks certain essential constituents.

In 1907 Holst and Frölich attempted to produce beriberi in guinea pigs experimentally just as Eijkman produced this disease in chickens. The guinea pig, however, did not develop beriberi, but another disease which was recognized as scurvy. The discovery of the experimental animal scurvy was soon used to study the distribution of the anti-scurvy compound in foodstuffs.

Two special pathological conditions, beriberi and scurvy, were thus traced to nutritional deficiencies. Hopkins (1906 and 1912) was the first to emphasize the physiological and universal importance of specific nutri-

tional constituents. He carried out experiments on rats by supplying them with a diet consisting of purified carbohydrates, fats, proteins, inorganic material and water. The animals did not flourish, but growth resulted when "astonishingly" small amounts of milk were added to the diet (similar to the earlier but less exact experiments of Lunin). In place of milk, the alcohol- or ether-soluble fraction of milk was found to bring about the same effect. In 1909 Stepp observed that similar growth-promoting substances are present in bread. Pekelharing had already emphasized in 1905 in a paper which was overlooked for many years that only very small amounts of these substances present in traces in milk and probably in all sorts of foodstuffs, both of vegetable and animal origin, are necessary to keep mice alive.

Funk in 1912 reviewed the then existing knowledge of the diseases caused by nutritional errors. They were beriberi, and scurvy and possibly also rickets, sprue and pellagra. Funk was apparently the first to recognize pellagra as a nutritional deficiency disease. The etiology of sprue is still unknown today. Funk attempted to isolate the compound which prevents beriberi and concluded that it is, chemically speaking, an amine. Funk did not succeed in isolating the pure substance but obtained nicotinic acid as a by-product which he showed to have some slight beneficial growth effect. (Today we recognize nicotinic acid as the pellagra preventive compound.) In systematizing the knowledge of the nutritional elements other than carbohydrates, fats, proteins, inorganics and water, Funk called this new class of compounds "vitamines," a term which was later changed to "vitamins" (see under Nomenclature of the vitamins, page 12). Thus the first period of the history of vitamins in which the existence of the vitamins was recognized and proved is concluded.

In the second period of vitamin research, various vitamins were isolated in the pure state, their chemical constitution established and their chemical synthesis achieved. This development was made possible by using experimental animals as tools in order to determine the presence and concentration of vitamins and for studying and differentiating the symptoms caused by special diets deficient in various constituents. The isolation, the determination of the chemical structure and the synthesis of the vitamins will be discussed later. At this point the historic development leads to a special history of each single vitamin. This will be presented in the discussion of each vitamin under "Chronology." In this period the following vitamins were elucidated: vitamin A (the compound which prevents night-blindness), vitamin B_1 (the anti-beriberi vitamin), vitamin B_2, vitamin B_6, vitamin C (the anti-scurvy substance), vitamin D (the anti-rickets com-

6. Isolation

The first step in the recognition of a new vitamin is to produce a nutritional deficiency in an experimental animal. The next step consists in the isolation of the compound which exerts vitamin activity. The procedure of isolating the unknown vitamin is very tedious and requires in many cases a million-fold concentration of the original material. In the beginning of the history of vitamin research no effective methods were available for the isolation of compounds which occur in such small amounts and in mixtures with such enormous quantities of other substances. New methods, therefore, had to be devised and many old methods which were effective enough for the isolation of other compounds had to be modified and refined. The most important new methods are the chromatographic adsorption technic and the high vacuum molecular distillation. Both of these methods have been developed to such an extent that they are now used for the isolation of many vitamins in the pure form and have found commercial applications.

Under the heading "Isolation" the methods used for the isolation of each vitamin are discussed separately in the respective chapters.

7. Chemical Constitution

Once a vitamin has been isolated in the pure or essentially pure state, efforts are made to elucidate the chemical constitution. The methods for the analysis and determination of the structure are principally the same as those which have been used for the determination of the structure of many organic compounds of non-vitamin character. They consist essentially in the degradation of the unknown compound to smaller molecules until derivatives of known structure are obtained. The structure of the original vitamin molecule is then reconstructed by skillful organic chemical reasoning.

Since each of the vitamins has a structure which is entirely different from that of any other vitamin, it is necessary to present the work carried out in determining the chemical constitution of the vitamins separately for each vitamin. Therefore the results of the experimental work which led to the postulation of the structure of the compound are reported under the individual vitamins.

8. Synthesis

The normal consequence of a postulation of the constitution of a vitamin is a challenge to synthesize the vitamin. Actually, all vitamins which

have been isolated have been synthesized with the exception of biotin (for which no structural formula has been suggested as yet due to the fact that sufficient amounts of the pure vitamin have not been available for chemical studies). A total synthesis is regarded as the final proof for the formula which has been postulated on the basis of degradation reactions. The methods used for the synthesis of the various vitamins are presented in special chapters. No total synthesis of any one of the vitamins D has been accomplished as yet, but some of the provitamins D have been obtained synthetically by hemi-synthesis from various sterols.

9. Industrial Methods of Preparation

An important feature in the development of vitamin research has been the availability of the vitamins to the clinician and practitioner for studies on experimental animals, for use in human therapy and finally for incorporation into foodstuffs for man and animals. The first industrial methods which were used in the production of vitamin preparations for the public were extractions from natural sources. Originally crude concentrates were offered to the trade, but recently the methods of isolation have been refined to such a degree that it is possible to offer commercially practically all the vitamins in essentially pure state. Besides the isolation of the natural compound, synthetic methods of preparing the vitamins have been studied and developed to such an extent that it is now more economical to produce most vitamins on a commercial scale by synthesis than by extraction from natural sources.

The methods used in industry for the manufacture of vitamin preparations are known in general but the details are usually kept secret. Under the heading of industrial methods of preparing vitamins the procedures used commercially are briefly reviewed for each vitamin. The result of industrial research and development work is reflected in patents which have been taken out to protect the manufacturers and the public, both of whom are interested in obtaining the best possible products and preventing unskilled and dishonest manufacturers from exploiting the market.[15] A list of the issued patents from the main industrialized countries, United States of America, Great Britain, Germany and France, will be found at the end of this monograph arranged according to subject. In isolated cases, patents issued in Switzerland, Holland, Belgium, Canada, Japan and Russia have also been incorporated.

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[15] A. G. Connolly, *Science*, **86**, 383 (1937).

10. Biogenesis

The methods used in the plant organism for synthesizing vitamins, which are complex compounds, are very interesting from a physiological standpoint. While up to the present time these methods are not known for all the vitamins and only a few theoretical approaches have been made, it is worth noting that the building principles for the synthesis of vitamins are the same as those used by the plant organism for the synthesis of many structural units. Thus, we find there is a vitamin represented among practically all types of compounds synthesized in plants. Further research will elucidate the reaction mechanism used by the plant organism for the synthesis of the structural building units and hence for vitamins in particular. Ultimately, the physiologist wishes to determine why vitamins are synthesized, why they are produced only in such minute quantities and how the mechanism for synthesizing these particular compounds differs from that for other, chemically very closely related substances which have no vitamin action.

11. Specificity

One of the most fascinating studies in biochemical research is the determination of the specificity of the vitamins. Generally speaking, two types of specificity must be differentiated, namely, compound specificity and species specificity. By compound specificity is meant the qualitative and quantitative differences in physiological behavior of various compounds on the same animal organism. Species specificity is defined as the difference in physiological response of one or more compounds on different species of animals. The various forms of vitamin D may serve as an illustration for both the compound and species specificity. Vitamin D_2 (activated ergosterol) and vitamin D_3 (activated 7-dehydro-cholesterol) have qualitatively and quantitatively the same antirachitic activity when tested on rats. Vitamin D_4 (activated 22-dihydro-ergosterol) has qualitatively the same antirachitic efficacy but quantitatively the effect is only half as great as that of the vitamins D_2 and D_3 on the molecular weight basis. This effect demonstrates compound specificity. When the same three vitamins D are tested on chicks under standardized conditions on the basis of Rat Units and compared with cod liver oil, it is observed that vitamin D_3 is as active as the vitamin D in cod liver oil and this activity is arbitrarily designated as 100% activity. On the other hand, when vitamin D_2 is tested under the same conditions an activity of only a few per

cent is obtained. Vitamin D_4 exerts an activity of about 20% under the same conditions. This effect demonstrates species specificity.

One of the objects of studying the specificity of vitamins is to determine whether or not the vitamin activity is due to the structure of the entire molecule or to a special group. This type of research has proved to be of extreme importance in the study of many pharmaceutical compounds. A well-known example in the field of chemotherapeutical compounds is the research which followed the discovery that "prontosil" counteracts streptococci.[16] Investigations revealed that the bactericidal action is due solely to the sulfanilamide portion of the molecule.[17] Research of this type in the vitamin field brought to light that the activity exerted by choline is due to the available methyl groups of the compound and resulted in the recognition of the available methyl group as a vitamin (or vitagen) factor. Specificity studies on the group of vitamins K showed that while the naturally occurring vitamins K_1 and K_2 are 2-methyl-1,4-naphthoquinone derivatives with long side chains, the vitamin activity is due only to the 2-methyl-1,4-naphthoquinone portion of the molecule.

Specificity studies in other fields have revealed that it is possible to alter the properties of the active compound so as to make them more suitable for therapeutic use. One of the best known examples in the hormone field is the use of methyl-testosterone[18] which does not occur naturally but which is effective when given by mouth[19] whereas the naturally occurring testosterone is active only by injection. Alterations of chemical or physical properties without alteration of physiological action in the vitamin field are of importance, for example, in order to make fat-soluble vitamins water-soluble or water-soluble vitamins fat-soluble. The most important practical problem of this type concerns vitamin K. K-avitaminosis occurs in patients with obstructive jaundice due to the fact that the bile does not reach the intestinal tract and thus prevents the vitamin from being absorbed. On the other hand, water-solubilized forms of vitamin K do not require the presence of bile for absorption from the intestinal tract and are thus active as such when taken orally.

From the vast amount of work which has been done to elucidate the compound specificity of the vitamins, it can be concluded that the physiological action of vitamins is very specifically a function of the entire molecule. In the class of water-soluble vitamins it is observed that lower or higher

[16] G. Domagk, *Deut. Med. Wochnschr*, **61**, 250 (1935).
[17] J. Tréfouel, J. Tréfouel, F. Nitti and D. Bovet, *Compt rend. soc. biol.*, **120**, 756 (1935)
[18] L. Ruzicka, M. W. Goldberg and H. R. Rosenberg, *Helv. Chim. Acta*, **18**, 1487 (1935)
[19] K. Miescher and L. Tschopp, *Schweiz. Med. Wochschr*, **68**, 1258 (1938); L. G. Foss, *Brit. Med. J.*, **1939**, July 1, No. 4095, 11.

homologs have either much lower activity than the vitamin proper or have no vitamin activity whatsoever. This is not the case in the class of fat-soluble vitamins. Actually, they occur naturally as a mixture of various homologs. Geometrical isomers of the vitamins are usually considerably less active than the vitamin itself. Thus, activated epi-7-dehydro-cholesterol is only about one-tenth as active as activated 7-dehydro-cholesterol. Another example of the high specificity of the vitamins is the specific effect of *d*-ribose in the vitamin B_2 molecule. This sugar compound cannot be replaced by any other sugar without essential loss of activity.

Under the heading "Specificity" the results obtained from the experimental studies and the conclusions reached will be presented separately for each vitamin.

12. Determination

One of the most fruitful developments in the biological field has been the study of methods for measuring the potency of vitamin preparations. There are principally a number of different procedures for the determination of vitamins. These comprise physical, chemical, biochemical and biological methods. Generally speaking, any method has to be investigated thoroughly in order to determine the specificity afforded by the test, the types of compounds which inhibit an evaluation of the procedure and finally the sensitivity of the procedure.

Among the physical methods for the determination of vitamins, spectroscopic studies are outstanding. These include determinations of the absorption spectrum in the ultraviolet or visible light region. The fluorescence spectrum is used successfully in specific cases. These tests are based on the presence of a specific chemical group in the vitamin molecule. Therefore, this procedure cannot be specific for any vitamin since the same physical phenomenon is given by any other chemical compound with the same characteristic group. Nevertheless, the physical methods are quite accurate when appropriately used and are, in general, more rapid than other methods.

The basis for all determinations of absorption spectra is Beer's law:

$$I = I_0 \cdot 10^{-\epsilon \cdot c \cdot d}$$

In this formula I_0 stands for the intensity of the incident beam, I for the intensity of the transmitted beam, c for the concentration (expressed in mols per liter), d for the depth of the solution (in cm.), and ϵ for the extinction coefficient. There is, however, no uniformity in the use of the units.

As long as a compound has not been isolated in the pure form, merely transmissions of solutions of known concentration are plotted and expressed as $\log I_0/I = \epsilon \cdot c \cdot d$. The adoption of a solution of 1 cm. depth standardized to contain 1% of the substance and expressed as *extinction* $E^{1\%}_{1cm.} = \log I_0/I$ is a frequently used refinement in the study of absorption characteristics. The *absorption constant* K has also frequently been used and is defined in the formula:

$$I = I_0 \cdot e^{-K \cdot c \cdot d}$$

(e is the basis of the natural logarithm). When the molecular weight of a compound is known, the extinction of the absorption per mol is measured and expressed according to Beer's law either as

$$\epsilon = \frac{1}{c \cdot d} \cdot \log \frac{I_0}{I}$$

or

$$\kappa = \frac{2.3}{c \cdot d} \cdot \log \frac{I_0}{I} \left[\frac{10^3 \cdot \text{cm.}^2}{\text{g.} \cdot \text{mol.}}\right]$$

The chemical methods for the determination of vitamins are based upon certain chemical reactions which the vitamin molecule undergoes with specific reagents. Usually those types of reactions are used for the determination of vitamins which develop a color which can be measured quantitatively. Such methods have been worked out for practically every vitamin and can be carried out relatively quickly. However, they have the disadvantage that the reactions are not specific for the vitamin since the same or similar color reactions are also given by a series of chemically similar and different compounds.

Biochemical methods for the determination of vitamins are defined as procedures in which the vitamin is determined by the part it takes in certain biochemical reactions. An illustration is the action of vitamin B_1 in a specific enzyme system which decarboxylates pyruvic acid. These procedures are very specific for the particular vitamin which is determined but require special skill in handling enzyme systems and are usually quite tedious. They are used in special cases with considerable advantage over any of the other procedures.

All physical, chemical and to a certain extent also the biochemical methods for the determination of vitamins are valid only if their results can be related to actual biological values. Thus, the biological methods, which

have generally been used as the primary tool in the discovery of the vitamins, must still be resorted to today as the final criteria for qualitative and quantitative vitamin assays, and for standardization and determination of the accuracy of other methods. Biological methods are very time-consuming and costly. The primary requirement is to feed a group of comparable animals, which are used for the determination of vitamins, a special diet which is deficient only in the vitamin to be tested and is otherwise well balanced in regard to the other vitamins and the energy- and building unit-bearing and protective food constituents. Multiple deficiencies, that is, the concurrence of avitaminoses caused by lack of more than one vitamin should generally be avoided in biological vitamin assays. The actual determination can be carried out either on a curative or on a prophylactic basis. The curative methods are usually preferred since it is possible to use for the actual test only those animals which have been inflicted successfully with the deficiency disease. It is often necessary to carry out simultaneously with the vitamin determination check experiments with animals which have not obtained the vitamin supplement. The highest possible degree of accuracy is achieved by comparing in parallel experiments the activity of the unknown material with the activity of a standard preparation. The results of biological assays are evaluated statistically. Mathematicians and biologists have given much thought as to the method of carrying out this statistical evaluation most efficiently in order to achieve a high degree of accuracy with a minimum of experimental animals.

A special type of biological method for the determination of vitamins is the so-called microbiological method in which microorganisms are used as test objects. Thus, practically all members of the vitamin B complex can be determined by the growth effect which they exert on microorganisms, such as yeast and different strains of bacteria. The amounts of vitamin required for such microbiological assays are considerably smaller than are needed in experiments with higher animals. The microbiological tests are relatively inexpensive and can be carried out in a relatively short period of time. Especially sensitive are such microbiological methods which are based not on the growth of the organism but on the action of the organism in special culture media. Thus, those bacteria are especially useful which produce acids, for example, lactic acid. The actual determination of the vitamin effect involves in such cases a titration of the amount of acid formed. This type of biological vitamin assay has proved to be of high accuracy in the determination of pantothenic acid, nicotinic acid and biotin.

The special methods which have been used and recommended for the determination of each vitamin are presented and discussed separately under each vitamin.

13. Standards

Standards of vitamins are necessary for the determination of these protective food constituents and for proper dosage. Vitamins originally were defined in terms of biological units. However, as soon as a vitamin is obtained in crystallized form and is easily accessible in that form, the standard is expressed on the weight basis of the crystallized material.

The term "biological unit" is a generic term for specific animal units such as Rat Unit, Chick Unit, Mouse Unit, etc. The biological units of non-identified vitamins are of relatively uncertain definition. They represent the reciprocal of a dose which had a certain effect on a certain animal at a certain time. Thus, a biological unit refers to the action of an animal while a standard based on the weight unit refers to the amount of the vitamin.

During the last two decades national and international standards have been set up for many vitamins, especially for the non-identified vitamins. By the time a vitamin is available as a stable crystallized compound, a new standard is set up which defines the unit in terms of actual weight of the vitamin. Usually that amount of crystallized vitamin is defined as one unit which corresponds on a comparative basis to the effect of the original biological unit.

The standards as adopted or as recommended for adoption are presented for each vitamin separately in the corresponding chapters.

PHYSIOLOGY

The physiology of vitamins comprises a study of the vitamin action in plants and animals. Since by definition specific organic compounds which are indispensable to the animal organism are called vitamins, and since the definition contains no statement concerning plants, it is obvious that the physiological action of the vitamin compounds in the animal organism is considered to be of primary importance. Nevertheless, the physiological action of these compounds in plants, where most vitamins are synthesized, must be incorporated in a complete study of the vitamins. Animal physiology is subdivided with respect to the action of the vitamins in the organism into (*a*) the metabolism of the vitamins, (*b*) the physiological action of the vitamins, (*c*) the mechanism of the vitamin action and (*d*) the relation of the vitamins to each other, to hormones and minerals.

The sections on the physiology of the vitamins are presented in a somewhat different manner from that of the sections on the chemistry of the vitamins due to the different character of the subject. The knowledge of both the chemistry and the physiology is based on experimental work. There is no, or practically no, misunderstanding possible in the conclusions drawn from intelligently planned chemical experiments. This is due to the fact that chemical experiments deal generally with systems, the constituents and the reactions of which are known and contain as the only unknown, the vitamin. The situation is entirely different in experiments designed to elucidate the physiology of the vitamins or the mechanism of the vitamin action. Here the vitamin acts in a very complicated and largely unknown system, namely, the living organism. As a result all experimental data obtained from studies on the living organism must be qualified as to the exact conditions used in the experiments. The multiplicity of possible interpretations of these results then causes difficulties. The same physiological experiment carried out under only slightly different conditions may give entirely different results. The interpretations of these results are then bound to differ markedly. By the time the knowledge of the chemistry and the physiology of the vitamins and the living organism expands it is evident that conclusions drawn from earlier experiments must often be modified due to the fact that all statements regarding physiological reactions are based on certain assumptions and hypotheses regarding the equivalency of principal physiological reactions under various reaction conditions.

The result of all these factors is that a tremendous amount of work is necessary to establish the true physiological action of the vitamins. In a few cases this has already been acomplished. In most cases a final understanding has not been reached. A review of these latter cases is extremely difficult since from the mass of data and interpretations available only a limited amount can be presented in this monograph. The discussion of the experimental data is usually followed by interpretations which are mostly subjective in nature. Efforts have been made to present the material in the form of a well-rounded picture, but it appears possible that just this attempt has led to the presentation of conclusions which ultimately may prove to be erroneous. A few examples of somewhat questionable views may be cited. The existence of a combined form of ascorbic acid, "ascorbigen," is accepted as proved in at least a few instances although many references could be cited to disprove the theory of the existence of ascorbigen. The view is taken that vitamin D_2 is less efficacious for human beings than is vitamin D_3, although statements disproving this thesis can be found

in the literature. In most cases in which a distinct difference of opinion exists between various groups of workers, both sides are cited.

14. Physiology of Plants and Microorganisms

Most vitamins are synthesized in plants. There is apparently a special mechanism for the synthesis of each of the vitamin compounds. Very little is known about the physiological behavior of these compounds in plants, but it has been shown in a few isolated cases that the vitamins are essential growth-promoting substances for the plant organism. It is assumed that the vitamins act in the plant organism principally in the same manner in which they act in the animal organism.

The information available concerning the physiological action of the vitamins in microorganisms is very meager. The most interesting discovery in this field is the observation that while some microorganisms are able to provide their own needs like the plants, there are others which rely upon an outside supply of the vitamins. Curiously enough, there are also quite a number of microorganisms which do not have the power of synthesizing the vitamins completely, but which are able to synthesize part of the vitamin molecule and are able to build up the entire molecule when the missing component is supplied.

15. Animal Physiology

(*a*) *Metabolism of the Vitamins*

Vitamins are by definition compounds which cannot be synthesized in the organism of man or animals. With one single exception (vitamin D) the vitamins are always supplied to the human and animal organism by oral administration as normal food constituents. In the properly functioning organism they are readily absorbed in the intestinal tract. The water-soluble vitamins are absorbed as such at least when they are supplied in the free form. In combined form they have to be hydrolyzed to the free vitamin before absorption can take place. The fat-soluble vitamins, on the other hand, require the presence of bile for proper absorption. Following the absorption the vitamins are carried through the organism by means of the blood stream and are carried to the tissues and organs which need them.

The animal organism does not have special storage organs for the deposition of vitamins. A certain level of vitamins is usually maintained in tissues and body fluids and some special organs contain increased amounts. The reason for the presence of these increased amounts is not obvious,

but is believed to be a necessity for the proper functioning of that particular organ. This conclusion has been reached in recognition of the fact that during times of low vitamin intake these organs are not depleted of their vitamin content, although the actual amount is usually somewhat reduced. Simultaneously, symptoms of vitamin deficiencies occur long before the amount present in these special organs is exhausted. Thus, the amount of vitamins present in such special organs cannot be considered as a storage of the vitamin for distribution over the entire organism.

The physiological reason for maintaining a certain "normal" vitamin concentration in the organism is apparently a certain safety factor for times of low vitamin intake. To a limited extent the body appears to contain a self-regulatory mechanism for the utilization of the vitamins. This is apparent from the fact that, while the healthy organism on a normal diet excretes certain amounts of the vitamins, they are not excreted, or are excreted only to a limited extent at times of low vitamin intake even when abundant quantities are present in the organism.

Administration of excessive amounts of the vitamins usually causes their excretion within a relatively short time. The water-soluble vitamins are excreted mainly through the urine. The fat-soluble vitamins are excreted through the feces. A certain amount is destroyed in the organism. It appears that once a vitamin molecule is destroyed it is completely burned since it has not been possible so far to find any degradation products in the excretions.

In accordance with the life-maintaining function of the vitamins it is obvious that these protective compounds are secreted in the nutritional materials necessary for proper growth and development of the offspring. Thus, small but definite amounts of all vitamins are found in milk and eggs.

(*b*) *Physiological Action of the Vitamins*

The principal physiological action of vitamins has become a part of the vitamin definition. They "do not furnish energy and are not utilized as building units for the structure of the organism, but are essential for the transformation of energy and for the regulation of the metabolism of structural units."

Vitamins are necessary for the animal organism as a whole, not for the individual cells, although the reactions of the vitamins are carried out predominantly in cells. Cells deprived of vitamins can survive and multiply while the entire organism cannot.[20]

[20] S. B. Wolbach, *Science*, **86**, 569 (1937).

The primary physiological action of most vitamins is scarcely known. The observations made in experimental studies are in many cases secondary symptoms or secondary reactions, and it is very difficult to decide in many cases whether the observed actions of the vitamins are of primary or of secondary nature. Whatever they may be, it seems that, at least on the basis of the present-day information, a certain difference exists between the physiological action of the water-soluble and fat-soluble vitamins. The fat-soluble vitamins and also water-soluble vitamin C apparently maintain the "regulation of the metabolism of structural units." From the experimental data available it may be concluded that vitamin A is concerned with the building of the cell nucleus and vitamin E with the maturation and differentiation of cells. Vitamin C enables the cells to produce supporting tissues and to maintain intercellular substances. Vitamin D plays an essential role in the process of bone calcification, which process is a deposition of calcium phosphate in bone matrix. Vitamin K maintains the level of certain structural (or at least potentially structural) building units of blood which initiate the process of blood coagulation under proper conditions. In contradistinction from the action of these vitamins, the water-soluble vitamins of the "B complex" are concerned mainly "with the mechanism of the transformation of energy." Thus, the vitamins B_1, B_2 and nicotinic acid have been shown to take part in certain reactions which regulate the carbohydrate metabolism. Vitamin B_6 is apparently involved in the amino-acid metabolism.

The observations and conclusions reached from experimental data concerning the physiological action of the vitamins in the animal organism are presented separately for each vitamin in the corresponding chapters.

(*c*) *Mechanism of the Vitamin Action*

Ultimately, the mechanism of the action of each vitamin will be understood. So far the reaction mechanism of only a few vitamins is known. The primary requisite for studies of the mechanism of the vitamin action is a proper understanding of the vitamin action as such. As has been pointed out in the previous paragraph, the physiological action of the vitamins is, as a whole, not properly understood. As a result the mechanism is unknown. In the few cases in which the primary physiological action of a vitamin has been elucidated, efforts have been made to determine the mechanism of the vitamin action. Thus, in the cases of the vitamins B_1, B_2 and nicotinic acid of the vitamin B complex the relation of the vitamin to the carbohydrate metabolism has been the subject of many experimen-

tal studies and theoretical considerations. These have culminated in the recognition of the fact that these three vitamins are part of enzyme systems which can be essentially separated from the animal organism and can thus be subjected to experimental studies under defined conditions in contradistinction to the reactions in the organism where the conditions are not defined, at least not from an experimental point of view. As a result of this the mechanism of the action of these three vitamins is at least partly understood.

The mechanism of the action of the other vitamins is not known but various theories have been presented which attempt to explain the experimentally observed phenomena.

(*d*) *Relation of the Vitamins to Each Other, to Hormones and Minerals*

An integral part of the physiological action of the vitamins is their influence on the secretion of hormones and on the metabolism of inorganic compounds. However, no physiological relation exists between any one vitamin and another.

Shortly after the recognition of the existence of vitamins, a theory of synergism and antagonism of the vitamins was postulated. According to this theory the elimination of one vitamin from the food causes a change of the action of the remaining vitamins. Certain experimental facts have been cited to prove this conception but a logical consideration of the vitamin concept disproves the existence of this theory. If it were true that the elimination of one vitamin changes the action of the remaining vitamins, it would be impossible to observe specific vitamin deficiencies. It must be remembered that the vitamin intake of the average human being changes considerably from day to day. It is quite certain that the amount of vitamins administered every day changes markedly. Thus, on one day a considerable amount of one vitamin may be consumed while the diet of the next day may not contain any of this vitamin. If the theory of synergism and antagonism of vitamins were correct, each vitamin would thus exert a different type of reaction practically every day. The knowledge of the vitamin action and of the mechanism of the vitamin activity of certain members of the vitamin B complex makes it appear improbable if not impossible that one vitamin could replace another in these reactions.

Nevertheless, the theory of antagonism and synergism of vitamins has been generally accepted although the experiments on which such a theory is based are not conclusive and have not been carried out under convincingly well-chosen conditions. Recent carefully controlled investiga-

tions[21] have indicated that the vitamins A, B_1, B complex and D do not act according to the postulated theory, at least as long as administered in amounts which correspond to the optimal requirement of these vitamins. It has thus been shown experimentally that a definite relation of one vitamin to another does not exist.

It has been found that certain vitamins, especially vitamin C, but also vitamin A and vitamin B, are able to exert in addition to their vitamin action also other therapeutic effects. Thus, vitamin C has been found to counteract intoxications caused by many organic and inorganic compounds, toxins, etc. This action is not specific for vitamin C but is a function of a particular group in the molecule. Other compounds with similar chemical groups are able to exert the same or similar effects. This detoxifying action of vitamin C has also been observed in certain experimental animals when excess toxic doses of fish liver oils have been administered. The beneficial effect of vitamin C has in this case been interpreted as indicating an antagonism of vitamin C to the vitamins present in fish liver oils. Actually, however, vitamin C acts as a detoxifying agent for the harmful components of the liver oil regardless of whether or not they are vitamins.

Although the author of this monograph is of the definite opinion that the theory of synergism and antagonism of vitamins is incorrect and that such actions do not exist, theories concerning these phenomena and experimental data obtained in studies of these effects are presented in the corresponding section of each vitamin. This presentation may assist in the ultimate recognition of the non-existence of functional relations of one vitamin to another.

In contradistinction to the relations of vitamins to each other there is the possibility that vitamins may influence the secretion or action of hormones. Since the vitamins maintain animal life, they may as part of their action influence secretions within the organism. Thus, a relation of vitamins to hormones is not illogical although not necessary *a priori*. Thus, it can be generally observed that in animals which have been deprived of vitamins for a long period of time, not only the entire organism suffers but that specifically the glands lose vitality and as a result of this the effective secretion is reduced. This has been especially obvious, for example, in the case of the germinal glands. In addition to this general effect of vitamins on the secretion of hormones, there has also been postulated a theory of synergism and antagonism of vitamins to hormones. In a few isolated cases indications for such relations have been observed. Thus,

[21] P. E. Simola, *Ber. ges. Physiol. expil. Pharmakol.*, **83**, 312 (1936). A. Scheunert, *Naturwissenschaften*, **28**, 297 (1940).

there is what has been interpreted as a synergism between vitamin C and the hormones of the adrenal medulla and cortex. An antagonism between vitamin C and the thyroid hormone has also been postulated. It is extremely difficult to prove or disprove experimentally the existence of relations of this type between vitamins and hormones. For a long time such a relation had been assumed to exist between the hormone of the thyroid gland and vitamin D, but more thorough studies have indicated that such a relation actually does not exist.

It has already been stated under "Physiological Action of the Vitamins" (page 27) that they are concerned with "the regulation of the metabolism of the structural units." Thus, a relation of the vitamins to those inorganics which act as structural building units is obvious. In addition a relation of the vitamins to the functional inorganic building units of the organism exists. Such a relation is, however, indirect in the sense that certain inorganics act "vitamin-like" in addition to the vitamins and in cooperation with them. An antagonism or synergism is not known. As an example of such a cooperative action may be cited the essential action of manganese in enzyme systems which contain vitamin B_1 and the action of copper in ascorbic oxidase.

PATHOLOGY

The study of the pathological effects caused by vitamins comprises a study of the effects caused by an administration of insufficient amounts of the vitamins and of excess doses. The symptoms caused by the former conditions are called avitaminoses and hypovitaminoses, whereas the symptoms caused by the latter conditions are called hypervitaminoses.

16. Avitaminosis and Hypovitaminosis

During times of vitamin deficiencies, two types of clinical symptoms can be observed, namely, specific deficiency syndromes and general non-specific effects. The latter comprise what is generally known as "ill health" and constitute minor aches and a feeling of discomfort. In experimental animals a premature aging also is observed. Such conditions are usually not specific for a particular vitamin but may be caused by a deficiency of practically any vitamin. The specific deficiency syndromes are classified according to the severity of the cases into hypovitaminosis and avitaminosis. The state of hypovitaminosis is characterized by a partial vitamin deficiency, that is, a state at which a vitamin is administered in suboptimal amounts. Under these circumstances the general health of the organism

decreases and specific deficiency syndromes set in. These are usually not very severe and can be cured easily by the administration of the proper vitamin. A total deficiency of a vitamin causes the appearance of specific clinical symptoms in a much more severe state and is called avitaminosis. These diseases can usually be cured at an early stage. Organisms which have suffered from avitaminoses for a prolonged period of time cannot always be repaired completely. The clinical symptom which remains after treatment with vitamins is called paravitaminosis.[22]

The special symptoms of hypovitaminosis and avitaminosis in human beings and in experimental animals are described separately for each vitamin in the corresponding chapters.

(*a*) *Clinical Test Methods*

While it is usually easy to diagnose an avitaminosis, the recognition of a hypovitaminosis is often difficult due to the fact that the symptoms are not very specific. Methods of determining the state of hypovitaminosis are, therefore, of considerable importance. Hand in hand with this problem goes the problem of determining the average or individual requirement for a specific vitamin in order to insure the success of a prescribed vitamin therapy. Tests have therefore been developed and are being perfected in which the concentrations of vitamins in the organism can be determined quantitatively. In general, such tests deal with the determination of the vitamin in blood where a certain level usually is maintained and in the urine or feces provided the vitamin is regularly excreted. Special saturation tests are being developed for many vitamins in which either the amount of vitamin necessary or the time consumed is measured until a certain minimum concentration of the vitamin occurs in blood or in the excretions. In addition to these general methods a number of specific tests have been developed for special vitamins which are based on particular functions of such vitamins, such as, for example, the dark adaptation test for the determination of the presence of vitamin A and the capillary resistance test for the determination of the presence of vitamin C (or vitamin P).

17. Hypervitaminosis

Hypervitaminosis is according to the definition of the term the clinical syndrome which occurs when toxic amounts of a vitamin are administered.

[22] G. Mouriquand, *Bull acad. méd.*, 119 (3), 102, 678 (1938).

A study of the hypervitaminoses constitutes, therefore, a study of the pharmacological effects of the vitamins. All vitamins in the pure state are essentially non-toxic even at doses which are many times as great as those of the daily food intake. The vitamins also exert no, or practically no, specific pharmacological effects other than the specific vitamin action.

In experimental studies designed to determine the toxic level of vitamins, it has been found that amounts which are as high as a thousand or million times that of the daily requirement may cause the occurrence of specific syndromes of hypervitaminosis. Specificity studies of the vitamins are especially interesting from the toxicological standpoint since it has been observed that compounds which differ chemically from the vitamins only slightly may be considerably more toxic than the vitamin itself. The pharmacological study of chemical compounds usually comprises also a determination of the lethal dose. The lethal dose of most vitamins is unknown due to the fact that the vitamins are essentially non-toxic.

18. Requirements

The requirements of vitamins by man and various species of animals are most important from a practical point of view. The determination of the actual amount needed proved to be a very difficult task due to the fact that the vitamin requirements vary with environment and other factors. Efforts are being made to correlate the necessary amount to either body weight or food intake according to the function of the vitamin.

Research is being directed to and national and international organizations are concerned with the problem of setting up standards for the requirements of the vitamins. The latest and most outstanding efforts of this type have precipitated the "Recommended Dietary Allowances" as formulated by the Food and Nutrition Board of the National Research Council. The information published by this organization is reprinted on page 613 of this monograph. These allowances fix desirable levels and are considered to be relatively liberal. They constitute norms fixed by a body of distinguished American students of nutrition available to the rest of the world for discussion and study. They furnish a guide for consideration and help to focus the aims of further experiment in the light of which further revision of the standards is to be anticipated.[22a]

The amount of vitamins which is recommended is expressed as the optimum quantity under average conditions. There was a tendency at the beginning of vitamin research to recommend as a daily dose the mini-

[22a] R. A. Williams, *J. Franklin Inst.*, 237, 21 (1944).

mum amount of vitamin which is necessary to prevent the occurrence of clinical symptoms of a deficiency disease. It was observed in the following years that the minimum amount as defined previously was not always the optimum amount which should not only prevent the occurrence of specific clinical symptoms of an avitaminosis but should also provide for certain safety factors. Generally speaking, the vitamin requirements vary with age, environment and individual utilization of nutrients. Babies, generally, need increased amounts, that is, the vitamin requirements of babies cannot be correlated to body weight or food intake on the same basis as expressed for adults. When children grow older they still need increased amounts of vitamins but somewhat less than adults. There are some indications that aged people need increased amounts of vitamins, which seems to be due to the fact that the organism of older people utilizes food less well than adults. The influence of environment upon the vitamin requirements is especially evident for those vitamins, the average requirement for which is correlated to food intake. Thus, it has been found that approximately twice as much of vitamins B_1, B_6 and pantothenic acid are required by rats at 91° F. than at 65° F.[23]

The health and general well-being of a nation is dependent to a considerable extent upon the proper vitamin consumption of its people. Extensive investigations have shown that the normal food of the average man in most countries is deficient in many vitamins. It has, therefore, been necessary to take definite steps for providing an adequate vitamin supply to everyone. This can be achieved by supplying with the necessary vitamins such foods as are generally used. Thus the Food and Nutrition Board of the National Research Council considers it good practice to fortify margarine with vitamin A and milk with vitamin D. It is also recommended and in some countries ordered to reconstitute white flour to the original content of the vitamin B complex as found, for example, in whole wheat grain. In the United States of America "enriched" flour must contain per pound 2.0–2.5 mg. of vitamin B_1, 1.2–1.5 mg. of vitamin B_2 and 16–20 mg. of nicotinic acid, and may contain 250–1000 U.S. Pharmacopoeia Units of vitamin D.[24]

[23] C. A. Mills, *Proc. Am. Physiol. Soc.*, **1941**, *P* 202.

[24] *Federal Register, The National Archives of the United States*, July 3, 1943.

THE GROUP OF VITAMINS A

THE GROUP OF VITAMINS A

1. Chronology

1831 WACKENRODER isolated carotene from carrots.

1904 Xerophthalmia was observed as an epidemic disease in Japan.

1906 The empirical formula of carotene was established by WILLSTÄTTER. HOPKINS and STEPP (1909) discovered the indispensability of certain fat-soluble substances for the growth of mice and rats.

1913–1915 McCOLLUM and DAVIS, and OSBORNE and MENDEL ascertained in differentiation from other compounds, the presence of a growth factor, "fat-soluble A," in cod liver oil and in butter.

1919 STEENBOCK discovered the vitamin A activity of carotenoids. (His results could not be verified until 1929 when H. v. EULER showed that vitamin D must be added to the diet of animals.)

1920 STEENBOCK recognized that vitamin A is found in the unsaponifiable parts of fish oils.

1928–1930 ZECHMEISTER, KARRER and KUHN established the constitution of carotene.

1930 MOORE disclosed the conversion of provitamins A to vitamin A in the animal organism and storage of vitamin A in the liver after large doses of carotene.

1931 KARRER obtained a very highly concentrated vitamin A preparation and determined the structure of the vitamin.

1933 KARRER synthesized perhydro-vitamin A.

1937 KUHN and MORRIS announced a synthesis of vitamin A. HOLMES and CORBET obtained vitamin A for the first time in crystalline form. In this year, the existence of a second form of this vitamin, called vitamin A_2, was recognized by LEDERER and EDISBURY and their respective co-workers.

2. The Group of Vitamins A

The physiological effect of vitamin A is brought about in man and in animals by a number of different, naturally occurring and synthetic compounds. Whereas all these substances appear to react physiologically alike, they differ from each other chemically. The compounds found in plants, with vitamin A activity for animals, belong to the class of carotenoids with 40 carbon atoms. They are apparently not used as such by the organism (at least not as vitamin A), but are converted into other substances to produce vitamin A activity. These new compounds are stored to a certain extent in the animal organism in special storage organs but

have never been found in plants. Chemically, they are degradation products of the carotenoids. The most familiar of these is the one known as "vitamin A" which possesses the empirical formula $C_{20}H_{30}O$. All degradation products of carotenoids which occur in the animal body and react physiologically alike are called "vitamins A." The carotenes, the precursors of the vitamin A, are called "provitamins A." It should be pointed out that the term provitamin is applied properly only to the specific organism for which the physiological activity has been ascertained. Rats are usually used for provitamin A studies and it is assumed, but not proved, that compounds of provitamin activity for rats also exhibit provitamin A activity for man. Nine different naturally occurring provitamins A and two vitamins A are known. However, there are many reasons for believing that more provitamins and, especially, more vitamins A occur in nature.

The true physiological action and the mechanism of the vitamin A activity are essentially unknown. A few approaches have been made, however, toward solving this problem. Furthermore, a number of different diseases of man and animals are known, which are caused by a vitamin A deficiency and which can be cured by the administration of the vitamin.

PROVITAMINS A

3. Occurrence

The provitamins A occur in plants together with chlorophyll. They are generally absent from the animal organism, since they are not stored as such but are converted into vitamins A. A few exceptions to this general rule must, however, be noted. Traces of provitamins A have been found in fat deposits of animals. A special type of this class of compounds plays an important role in the visual purple of the eye and will be discussed later (see page 89). Milk and butter contain small amounts of carotenes. Egg yolk, however, contains only traces. Almost pure β-carotene (and some α-carotene) has been found also in the *corpus luteum*[1] and the *corpus rubrum* of cows,[2] in the human placenta,[2] the testes of bulls[3] and the adrenal gland of practically all mammals.[4] Carotenoids, the provitamin

[1] R. Kuhn and E. Lederer, *Z. physiol. Chem.*, **200**, 246 (1931). P. Karrer and W. Schlientz, *Helv. Chim. Acta*, **17**, 8 (1934).

[2] R. Kuhn and H. Brockmann, *Z. physiol. Chem.*, **206**, 64 (1932).

[3] R. Netter, *Bull. soc. chim. biol.*, **14**, 1555 (1932).

[4] A. A. H. van den Bergh, P. Muller and J. Broekmeyer, *Biochem. Z.*, **108**, 279 (1920). C. L. Connor, *J. Biol. Chem.*, **77**, 619 (1928); *Am. J. Path.*, **4**, 293 (1928). H. v. Euler and E. Virgin, *Biochem. Z.*, **245**, 252 (1932). H. v. Euler, U. Gard and H. Hellström, *Svensk Kem. Tid.*, **44**, 191 (1932). L. Zechmeister and P. Tuzson, *Z. physiol. Chem.*, **231**, 259 (1935). O. Bailly and R. Netter, *Compt. rend.*, **193**, 961 (1931).

A character of which has not definitely been established, occur also in the yellow bone marrow. The sex glands of sea urchins contain a provitamin A, echinenone, which has so far not been found in plant material

The most important sources of vitamin A for man and animals are the provitamins present in all green or yellow parts of vegetables Carrots, apricots,[5] lettuce, cabbage and spinach are especially rich in carotenes. Tomatoes and bananas contain a lesser amount. Yellow corn contains a considerable quantity of a special provitamin A, cryptoxanthene,[6] predominantly in the form of its ester. The presence of carotenes in certain vegetable oils, especially red palm oil,[7] is technically important. The provitamins myxoxanthin and aphanin occur in algae, especially blue-green algae.

The total amount of carotenes in vegetables is rather small Fresh carrots contain approximately 0.01% carotenes and the best source of palm oil 0.15–0.20%.[8]

The provitamins A occur in nature only to a limited extent in the free form. They are mainly bound in symplex form to proteins as has been demonstrated for carotene both in plant (carrots[9]) and in animal (serum[10]) materials.

4. Properties

There are nine different naturally occurring compounds known as provitamins A, namely α-, β- and γ-carotene, cryptoxanthene, echinenone, myxoxanthin, leprotene, aphanin and aphanicin. It is quite possible that other provitamins A occur in nature which have not been discovered as yet. All these substances crystallize in deep red prisms of high melting points (above 160°). They exhibit typical absorption spectra (listed below), the positions of the maxima of which differ with the solvent used. The theory has been advanced that this shift is due to a change of the equilibrium of *cis-trans*-isomers.[11]

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[5] H Brockmann, *Z physiol Chem* **216**, 45 (1933)

[6] R Kuhn and C Grundmann, *Ber* , **67**, 593 (1934)

[7] R Kuhn and H Brockmann, *Z physiol Chem.*, **200**, 255 (1931)

[8] O Ungnade, *Chem Ztg* , **63**, 9 (1939)

[9] R Willstätter and H H Escher, *Z physiol Chem* , **64**, 47 (1910). R Kuhn and H J Bielig, *Ber.*, **73**, 1080 (1940)

[10] L S Palmer, *J. Biol Chem.*, **23**, 261 (1915) *Carotinoids and Related Pigments*, New York, 1922 p 208

[11] E L Smith, B E Stern and F E Young, *Nature*, **141**, 551 (1938).

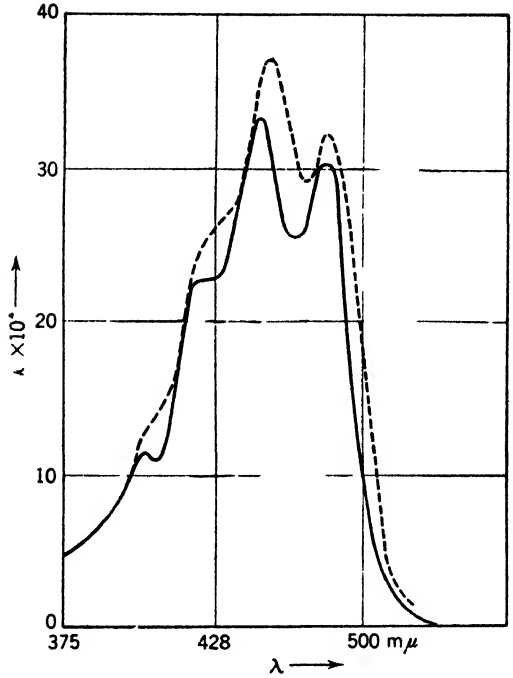


Fig 1 — Absorption spectra of α-carotene (——) and of β carotene (– – – –) in hexane solutions (H. Rudy, according to Kuhn and Brockmann)

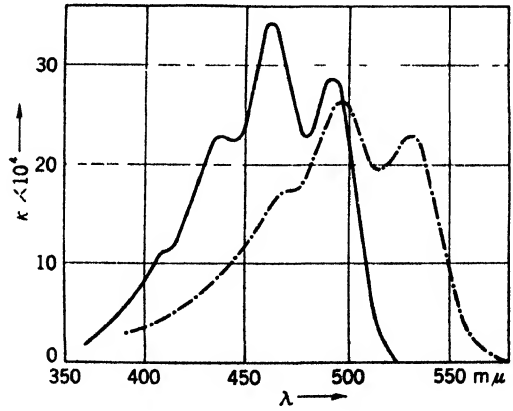
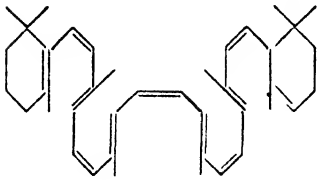


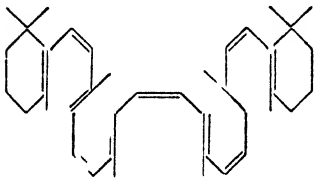
Fig 2 —Absorption spectrum of γ-carotene in hexane (——) and in CS_2 (–·–·–) solution. (R. Kuhn and H. Brockmann)

α-Carotene (α,β-Carotene):



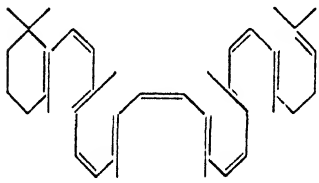
M. p. 187°. $[\alpha]_{844} = +323°$. 511, 478, 452 mμ (CS_2). 485, 447.5 mμ (Benzene). R. Kuhn and co-workers, *Ber.*, **64,** 1349 (1931); *Z. physiol. Chem.*, **200,** 255 (1931). P. Karrer and co-workers, *Helv. Chim. Acta*, **15,** 1158 (1932); **16,** 641 (1933); *Nature*, **132,** 171 (1933).

β-Carotene (β,β-Carotene).



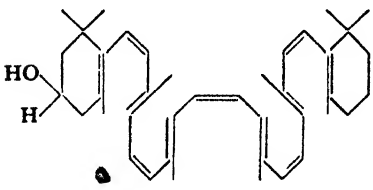
M. p. 184°. 520, 484, 452 mμ (CS_2). 485, 452, 424 mμ (Benzene). P. Karrer and co-workers, *Helv. Chim. Acta*, **12,** 1142 (1929); **13,** 1084 (1930); **14,** 1083 (1931).

γ-Carotene (β-Lyco-β-carotene).



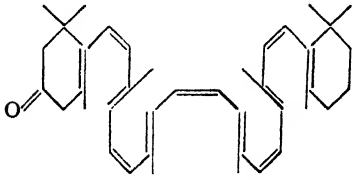
M. p. 178°. 533, 496, 463 mμ (CS_2). 495, 462, 431 mμ (Benzene). R. Kuhn and H. Brockmann, *Ber.*, **66,** 407 (1933); A. Winterstein, *Z physiol. Chem.*, **219,** 249 (1933).

Cryptoxanthene (3-Hydroxy-β,β-carotene):

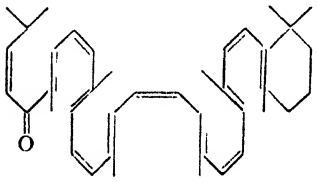


M. p. 169°. 520, 484, 452 mμ (CS_2). 485, 452, 424 mμ (Benzene). R. Kuhn and C. Grundmann, *Ber.*, **66,** 1746 (1933); **67,** 593 (1934); P. Karrer and W. Schlientz, *Helv. Chim. Acta*, **17,** 55 (1934); R. Yamamoto and S. Tin, *Sci. Papers Inst. Phys. Chem. Research Tokyo*, **20,** 411 (1933); L. Zechmeister and L. Cholnoky, *Ann.*, **509,** 269 (1934).

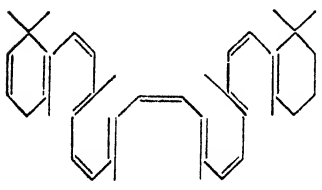
Echinenone: Structure questionable! M. p. 192–193°. 520, 488, 450 mμ (CS_2). E. Lederer, *Compt. rend.*, **201,** 300 (1935); E. Lederer and T. Moore, *Nature*, **137,** 996 (1936).



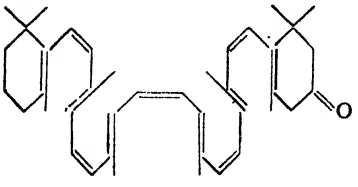
Myxoxanthin: M. p. 168–169°. 488 mμ (CS_2). 473 mμ ($CHCl_3$). 470 mμ (C_2H_5OH). 465 mμ (Light petroleum). I. M. Heilbron and B. Lythgoe, *J. Chem. Soc.*, **1936,** 1376.



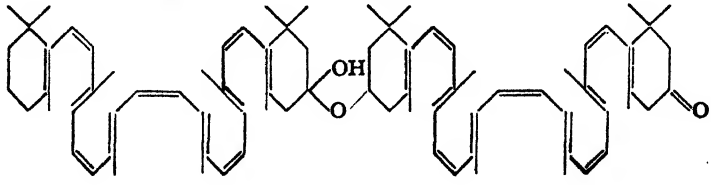
Leprotene: Structure and provitamin A action questionable! M. p. 189–200°. 517, 479, 447 mμ (CS_2). **484,** 452, 425 mμ (Benzene). C. Grundman and Y. Takeda, *Naturwissenschaften*, **25,** 27 (1937); Y. Takeda and T. Ohta, *Z. physiol. Chem.*, **258,** 6 (1939); **262,** 168 (1939); **265,** 233 (1940); **267,** 171 (1941).



Aphanin. Structure questionable! M. p. 180°. 533.5, 494 mμ (CS_2). 504, 474 mμ ($CHCl_3$). 505, 472 mμ (Benzene). 494, 460 mμ (Petroleum). 507.5, 477 mμ (Pyridine). 491.5, 457 mμ (Methanol). J. Tisher, *Z. physiol. Chem.*, **251,** 109 (1938); A. Scheunert and K. H. Wagner, *Ibid.*, **260,** 272 (1939).

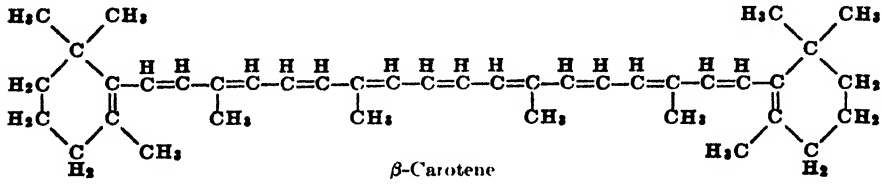


Aphanicin: Structure questionable! M. p. 195°. 533, 494 mμ (CS_2). 504, 474 mμ ($CHCl_3$). 505, 474 mμ (Benzene). 494, 462 mμ (Petro-



leum). 507.5, 478 mμ (Pyridine). 491.5, 457 mμ (Methanol). A. Scheunert and K. H. Wagner, *Z. physiol. Chem.*, **260**, 272 (1939).

The aliphatic part of the molecule is written in the manner of Ruzicka in the form of "open rings." This formula is not intended to indicate the true spatial arrangement of the aliphatic chain, but to stress the relationship to similar compounds and to impress the memory Textbooks commonly use the "long chain" formula:



β-Carotene

Provitamins A are extremely sensitive to oxidation, autoxidation and light. They are quite stable to heat in inert atmosphere.

α-Carotene is the only provitamin A with an asymmetric carbon atom and is, therefore, optically active.

The solubilities of the provitamins A are very similar. They dissolve readily in chloroform, carbon disulfide and benzene, but more difficultly in petroleum ether. They are practically insoluble in alcohol. Cryptoxanthene, due to the presence of a hydroxyl group in the molecule, is somewhat soluble in alcohols. All provitamins A are soluble in fats.

5. Isolation

Crude preparations of mixtures of provitamins A are obtained by first separating the provitamins from the protein material to which they are usually bound in symplex form. This is achieved by rapid heating,[12] for example, to 40–60° C. or by reaction with "invert-soaps" such as lauryl-dimethyl-benzyl-ammonium-bromide.[13] In the case of isolating provitamins A from vegetable oils an initial saponification with alcoholic potassium hydroxide is carried out. In either case an extraction with organic solvents such as petroleum ether follows. From the petroleum ether residue the crude mixture of carotenoids is either crystallized as such or a saponification may now be carried out in case this procedure was not employed in the beginning.

[12] R. Willstätter and H. H. Escher, *Z. physiol. Chem.*, **64**, 47 (1910).
[13] R. Kuhn and H. J. Brelig, *Ber.*, **73**, 1080 (1940).

As stated, mixtures of carotenoids are obtained according to these procedures. The single compounds are obtained by methods which will be described separately for each provitamin.

α-Carotene seldom occurs as the only carotenoid in crude provitamin A preparations. It is usually accompanied by its isomer, β-carotene, and sometimes by γ-carotene. Other carotenoids may be present. The total amount of α-carotene in crude carotene preparations varies between 0% and 40%. The highest concentration of α-carotene is found in red palm oil. Almost pure α-carotene has been found in leaves of tea from Formosa.[14] The progress in separation of the α-isomer from the other carotenoids can be followed by measuring the optical activity of the preparations. α-Carotene can be isolated, for example, by fractional precipitation with iodine in the form of a di-iodo-α-carotene[15] followed by regeneration of α-carotene by means of sodium thiosulfate.[16] Fractional adsorption o aluminum oxide[17] or on fuller's earth[18] is a much better method. α-C tene can be isolated quantitatively by chromatographic adsorption or cium hydroxide or on magnesium oxide[19] from a solution of a crude prep tion in petroleum ether. From a mixture of the α- and β-isomers an alm quantitative separation can be achieved by a single adsorption. A layer of β-carotene and a lower, more yellow layer of α-carotene,[20] are tained.

β-Carotene is the easiest of all provitamins A to obtain in pure It is the only provitamin A in many plants. Its purity can easily b checked, since β-carotene is optically inactive, whereas the α-isomer ha strong, positive optical rotation. Small amounts of the γ-isomer can b separated by adsorption on calcium carbonate or calcium hydroxide. The separation of all provitamins A, and especially of β-carotene, from xantho phyll, a yellow chloroplast pigment of green leaves, can easily and quantitatively be achieved by distribution between petroleum ether and 90% methanol.[21] Carotene remains almost entirely in the petroleum ether phase, while the xanthophyll passes into the methanol layer. Xanthophyll is 3,3′-dihydroxy-α-carotene.

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[14] R. Yamamoto and T. Muraoka, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **19**, 127 (1932).
[15] R. Kuhn and E. Lederer, *Ber.*, **64**, 1349 (1931).
[16] R Kuhn and E. Lederer, *Ibid.*, **65**, 637 (1932).
[17] R. Kuhn and E. Lederer, *Z. physiol. Chem.*, **200**, 246 (1931).
[18] R. Kuhn and H. Brockmann, *Ibid.*, **200**, 255 (1931).
[19] H. H. Strain, *J. Biol. Chem.*, **105**, 525 (1934); **111**, 85 (1935).
[20] P. Karrer and O. Walker, *Helv. Chim. Acta*, **16**, 641 (1933).
[21] R. Willstätter and A Stoll, *Untersuchungen über Chlorophyll. Methoden und Ergebnisse*. Berlin 1913, pp. 133, 237.

γ-Carotene occurs only in very small quantities and is found together with β-carotene. Most technical carotene preparations contain about 0.001% of the γ-isomer. Occasionally, however, γ-carotene occurs in larger quantities. The carotenoids of *Gonocaryum pyriforme* fruit peels consist of 50 60% γ-carotene.[22] It can be separated from other carotenoids by chromatographic adsorption on aluminum oxide.

Cryptoxanthene seems to occur frequently together with β-carotene and other carotenoids, especially in red blossoms and fruits. Especially high in cryptoxanthene are the calyx of *Physalis alkekengi* and *Physalis franchetti* (about $^1/_3$ of the total pigment) and the pigments of yellow corn and of paprika. In all these cases, cryptoxanthene does not occur in the free form and must be saponified before isolation. Since cryptoxanthene has almost the same properties as β-carotene, fractionation does not result in a separation. A number of different methods are available for separating β-carotene from cryptoxanthene. Distribution between petroleum ether and 90% methanol does not bring about any separation; distribution between petroleum ether and 95% methanol, however, causes the cryptoxanthene to pass slowly into the alcohol layer. From a benzene solution of β-carotene and cryptoxanthene only the latter is adsorbed on calcium carbonate. The best method for the preparation of pure cryptoxanthene is the chromatographic adsorption on aluminum oxide.

Leprotene has been isolated from bacilli, namely, from *Mycobacterium lepi* and from another bacillus of unknown species found in lepra diseases. It is strongly adsorbed on aluminum oxide and can therefore be separated from the less strongly adsorbed β-carotene.

Echinenone, found in the sex glands of sea urchins, occurs together with β-carotene, from which it can be separated by the chromatogram method.

Myxoxanthin has been found in algae of the class *Myxophyceae.* A convenient source is the fresh water species, *Oscillatoria rubrescens.* It has been separated from accompanying carotenoids, especially from β-carotene, by chromatographic adsorption on aluminum oxide, on which it is more strongly adsorbed than β-carotene.

Aphanin has been isolated from blue-green algae of the Aphanizomenon family by chromatographic adsorption on aluminum oxide whereby it can be separated from other carotenoids, for example, β-carotene, which is less strongly adsorbed than aphanin.

Aphanicin occurs together with aphanin and can be separated from

[22] A. Winterstein, *Z. physiol. Chem.*, 219, 249 (1933).

this and from β-carotene since it is more strongly adsorbed on aluminum oxide than either of the other accompanying carotenoids.

6. Chemical Constitution

The provitamins A belong chemically to a special class of polyenes, the carotenoids. They are characterized by a long aliphatic chain containing a continuous system of conjugated double bonds[23] which is responsible for their deep red color. Many naturally occurring polyenes are known but only a few of them are provitamins.

The chemical structure of all provitamins A is identical in their middle part, the symmetrical aliphatic chain of 18 carbon atoms with the continuous system of conjugated double bonds and four methyl groups constituting side chains. They differ from each other by the structure of the groups on both ends of the aliphatic chain. β-Carotene has on both ends rings of the same structure as the ring of β-ionone (Δ^5-1,1,5-trimethyl-cyclohexene). β-Carotene, therefore, is called β,β-carotene. α-Carotene (α,β-carotene) has on one side a ring with the β-ionone structure and on the other a ring with the α-ionone structure (double bond in 4,5-position). γ-Carotene has on one side a ring of the β-ionone structure and on the other end no ring, but the same number of carbon atoms as all other provitamins, only in the form of an aliphatic chain (pseudo-ionone structure), as in lycopene, the polyene from tomatoes. In analogy with the other carotenes, γ-carotene may be called β-lyco-β-carotene. Cryptoxanthene is 3-hydroxy-β,β-carotene. The methyl groups, which form side chains on the molecule, are in 1,5-position to each other, corresponding to the natural principle of the structure of most terpenes, which seem to be built up from isoprene residues by dehydrogenation.[24] Only in the middle of the chains, two methyl groups are in 1,6-position, thus dividing the molecule into two symmetrical halves.[25]

Through catalytic reduction, β-carotene takes up 11 mols of hydrogen, indicating the presence of 11 double linkages.[26] Ozonization of β-carotene gives geronic acid in the same yield as obtained by ozonization of two molecules of β-ionone. From α-carotene an equal amount of geronic and isogeronic acid was obtained. γ-Carotene yielded only geronic acid, in an

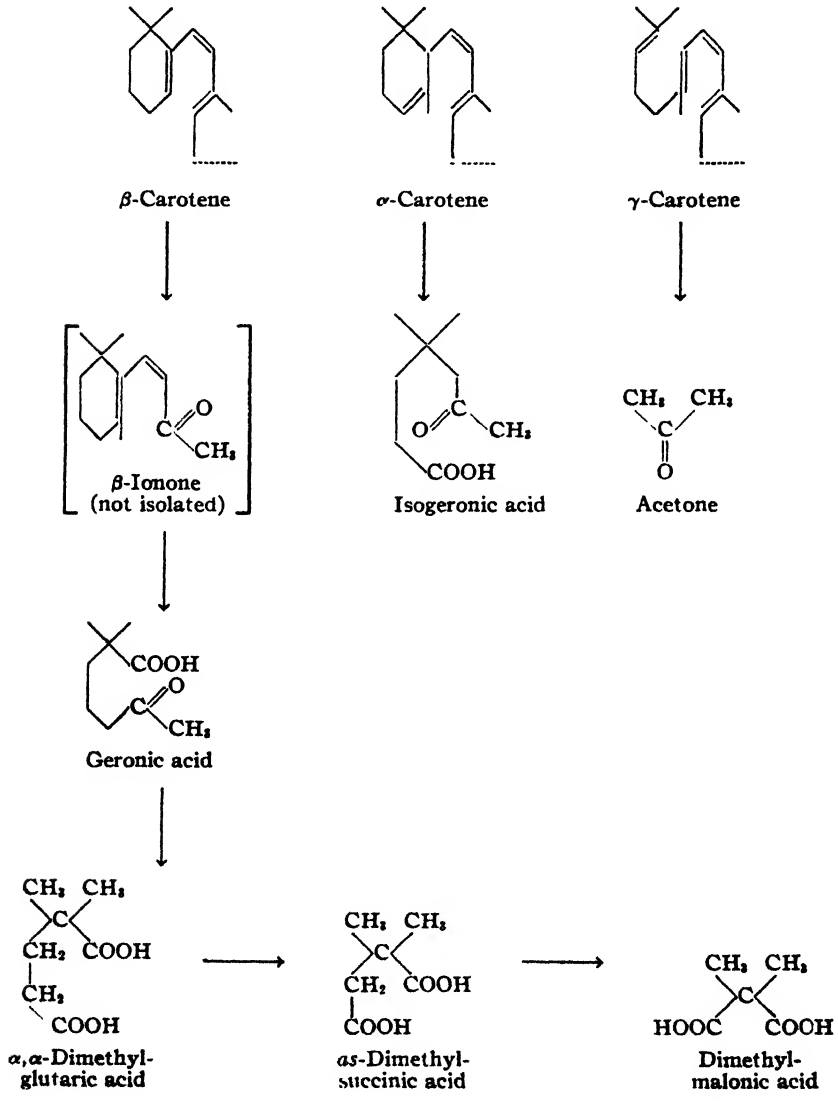
[23] R. Kuhn and A. Winterstein, *Helv. Chim. Acta*, 11, 428, 718 (1928). P. Karrer and H. Salomon, *Ibid.*, 11, 515 (1928).

[24] R. Willstätter and W. Mieg, *Ann.*, 355, 1 (1907). R. Kuhn and A. Winterstein, *Helv. Chim. Acta*, 11, 430 (1928).

[25] P. Karrer and co-workers, *Helv. Chim. Acta*, 13, 1087 (1930); 15, 1405 (1932).

[26] L. Zechmeister, L. Cholnoky and V. Vrabely, *Ber.*, 61, 566, 1534 (1928).

amount corresponding to the presence of one ring of the β-structure. Besides the geronic acid, ozonization of γ-carotene yields one molecule of acetone, split off from the aliphatic end of the molecule. γ-Carotene has 12 double bonds, one more than α- or β-carotene. Degradation of β-carotene with potassium permanganate gives four mols of acetic acid,

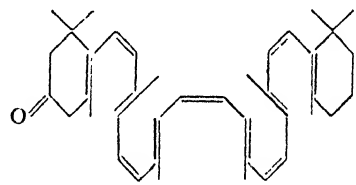


with chromic acid six mols. This proves the presence of six methyl groups in the molecule. Four of these, the ones giving acetic acid with permanganate, belong to the aliphatic chain, the other two came from methyl groups in the two rings at the ends.

Permanganate oxidation of β-carotene yields α,α-dimethyl-glutaric acid, *as*-dimethyl-succinic acid and dimethyl-malonic acid.

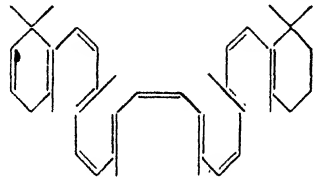
The presence of a secondary hydroxyl group in **cryptoxanthene** has been established by the presence of one active hydrogen atom and the formation of crystallized esters, for example, the acetate, which shows the same absorption spectrum as cryptoxanthene itself. The esters cannot be adsorbed on calcium carbonate nor can they be extracted from a petroleum ether solution by means of 95% methanol. The hydroxyl group, the exact position of which has not been determined, is assumed to occupy the carbon atom three in analogy to a number of other naturally occurring carotenoids, especially lycopene.

Echinenone is a monoketone of the probable formula $C_{40}H_{56}O(\pm H_2)$. Since it shows provitamin A activity, one part of the molecule is assumed to contain the β-ionone structure. It is perhaps a dehydro-cryptoxanthene of the following structure:



Myxoxanthin is a monoketone of the formula $C_{40}H_{54}O$ and forms an oxime. By hydrogenation 12 mols of hydrogen are taken up and 13 mols are absorbed by hydrogenation of the oxime, indicating the presence of 12 ethenoid linkages. Since myxoxanthin possesses growth-promoting properties, one part of the molecule probably has the β-ionone configuration. Myxoxanthin is, therefore, monocyclic and must be classified as a derivative of γ-carotene. By reduction of the keto group to the alcohol group with aluminum isopropoxide, myxoxanthol is obtained which is spectroscopically identical with γ-carotene.

Leprotene probably has the constitution of a dehydro-β-carotene. Since it contains two hydrogen atoms less than β-carotene, but exhibits the same absorption spectrum characteristics, the following formula is most probable:



Aphanin is a monoketone of the formula $C_{40}H_{54}O$. The presence of the keto group has been established by the formation of a crystallized oxime. From the absorption spectrum of this oxime, which is the same as that of aphanin, it is concluded that the keto group is not in conjugation with the system of double bonds. The negative result of an isopropylidene determination excludes the possibility of an open chain structure at one end of the molecule. Catalytic hydrogenation indicates the presence of 12 double bonds, one of which belongs to the keto group.

Aphanicin is a dicarotenoid consisting of two molecules of aphanin linked together by an oxygen bridge. The analysis conforms with the empirical formula $C_{80}H_{106}O_3$. The presence of a keto group was ascertained by the formation of an oxime, and the molecular weight is indicated by the physiological effect of aphanicin as provitamin A.

7. Synthesis

None of the provitamins A has been synthesized as yet.

8. Industrial Methods of Preparation

The technical isolation of provitamins A from plant material is simple compared with the difficulties of manufacturing some of the other vitamins. Two different starting materials are chiefly used in industry: fresh plant material, such as carrots, nettle, lucerne, grass, etc., or vegetable oils, among which red palm oil is one of the richest sources of provitamins A.

The main difficulty in extracting carotene from fresh plant material is the presence of large amounts of water, which hinders the extraction with organic solvents. Fresh plant material must therefore be dried, which process is always attended by some loss of provitamins. The best results are obtained by a rapid, short drying process, preferably at low temperature and under vacuum. Another method is the addition of water-binding agents to the freshly ground plant material. As water-binding agents, sodium sulfate, sodium carbonate, anhydrous calcium sulfate, etc., are recommended. Another method, which, however, proved to be too ex-

pensive, is the extraction of fresh plant material with alcohol, which takes up all the water, but leaves the carotenes undissolved.

The extraction of carotene from the dried material is brought about by organic solvents, such as benzene, petroleum ether, acetone, etc. These solvents dissolve, besides the carotene, all fats present. In order to obtain carotene free from other materials, and especially free from fat, a saponification of the extract must be carried out. An alternative method is the saponification of the total plant material before extraction. In most cases this latter procedure is too expensive. Since carotenes are somewhat sensitive to alkali, especially at higher temperatures (rearrangement of double bonds), the use of large excesses of alkali is carefully avoided.

The juice of plant material, for example, of carrots, is also used as starting material for carotene manufacture. The juice contains large amounts of protein materials, which adsorb the carotene when coagulated. The coagulation is carried out by the different known procedures, for example, by heating, by acids, by precipitation with lead acetate, magnesium chloride, etc. The precipitates are in turn extracted with organic solvents to isolate the carotenes.

It is not always of technical importance to prepare crystalline carotenes. Fat concentrates or dried grass and similar products are often used for food supplements.

The preparation of pure, single provitamins A is seldom carried out technically, although two different methods can be applied, namely, the precipitation of β-carotene with iodine and the separation by chromatographic adsorption.

9. Biogenesis

Little actual knowledge exists about the biogenesis of provitamins A although this problem has attracted widespread attention. As far as is known, only plants and certain microorganisms have the power of synthesizing provitamins A. Their constitution leads to the supposition that the building units of the provitamins are the same as those of most terpenes and polyterpenes. All these compounds appear to be built up from a simple, five carbon atom containing substance of the general formula of isoprene. Thus, all provitamins can be regarded as resulting from a condensation[27] together with a simultaneous dehydrogenation[28] of isoprene molecules. Actually, however, neither isoprene nor any other similar

[27] R. Willstätter and W. Mieg, *Ann.*, 355, 1 (1907).
[28] R. Kuhn and A. Winterstein, *Helv. Chim. Acta*, 11, 427 (1928).

compound has ever been found in plants. This can be explained, of course, on the basis that these compounds are anabolic intermediates which are not stored as such but are used immediately for the synthesis of other compounds. Besides isoprene, other compounds have been suggested as potential intermediates. Among these, β-methyl-crotonaldehyde,[29] levulinic acid[30] and methyl-vinyl-ketone are outstanding because of their obvious ability to condense with other molecules.

Without any doubt carotenoids are built up by total synthesis in plant tissues. It is questionable, however, whether or not the provitamins A are synthesized according to the above outlined scheme. The possibility that they might be formed by dehydrogenation of compounds of similar structure has also been discussed. Thus, for example, phytol[31] or a related compound[32] may serve as a starting material.

The synthesis of provitamins A appears to be catalyzed by light. Generally speaking, the carotene content of plants grown in light is several times higher than the content of plants raised in the dark. Tissues of plants grown in the absence of light contain compounds which are believed to be precursors of the provitamins A. The former, however, are essentially colorless (perhaps saturated compounds) and differ from the provitamins in many other respects such as water solubility.[33] On the other hand, it appears remarkable that carrot roots, for example, contain a more abundant amount of provitamins A than the leaves. It might be assumed, of course, that these provitamins were synthesized in the leaves and transported into the roots. This hypothesis is, however, in contradiction to the general belief that plants do not have a mechanism for the transportation of fat-soluble, that is, non-water-soluble materials. The additional hypothesis might then be considered that a water-soluble precursor is synthesized in the leaves, transported to the roots and there transformed (possibly by dehydration or dehydrogenation) into provitamins. On the other hand, the possibility cannot be excluded that these provitamins A are totally synthesized in the roots even in the absence of light.

10. Determination

The problem of determining quantitatively the amount of provitamins A in natural materials is quite difficult. In the analysis of plant material the

[29] H. v. Euler and E. Klussmann, *Svensk Kem. Tid.*, **44**, 198 (1932).
[30] H. Emde, *Helv. Chim. Acta*, **14**, 881 (1931).
[31] L. Zechmeister and L. Cholnoky, *Ann.*, **465**, 288 (1928).
[32] P. Karrer, H. Helfenstein, H. Wehrli and A. Wettstein, *Helv. Chim. Acta*, **13**, 1084 (1930).
[33] R. Willstätter and A. Stoll, *Untersuchungen über die Assimilation der Kohlensäure*, Berlin, 1918, p. 134.

problem is somewhat simplified by the absence of vitamin A, which prevails in animal tissues. The methods used for the analysis of provitamins A in the presence of vitamin A are described on page 80.

The only exact method for the provitamin A assay is the biological method using pure β-carotene as a standard reference. This method gives average values of the total carotenoid mixture expressed as β-carotene equivalent (see page 82).

Approximate provitamin A values can be obtained by either physical or chemical methods. They involve a separation of the provitamins A from other carotenoids present, especially xanthophyll (lutein) and other phytoxanthins. Such a separation is based on the fact that α-, β- and γ-carotenes are hydrocarbons and the phytoxanthins are oxygenated carotene derivatives, that is, alcohols and ketones. They show different solubility characteristics and can therefore be separated to a certain extent. These separations do not bring about a separation of provitamins A which contain hydroxyl or keto groups from non-provitamin A carotenoids with such functional groups. Fortunately, the average provitamin A analysis in foodstuffs can disregard all provitamins A other than the carotenes proper, since they are found only infrequently.

The actual assay procedure involves, first, the separation of the provitamin A from the protein to which it is bound in animal and plant materials. This is accomplished either by heating to 40–60° C. or by the addition of "invert-soaps" such as lauryl-dimethyl-benzyl-ammonium-bromide.[34] This is followed by an extraction from the natural source, either before or after a saponification. The alcohols, ethers, petroleum ether, hexane, acetone, pyridine and mixtures, such as petroleum ether and methanol or petroleum ether and acetone, have been recommended as solvents. The saponification is carried out with potassium hydroxide in alcohol, preferably at room temperature although the application of heat is recommended when entire tissues are to be saponified. Saponification with aqueous potash has also been advocated. The main object is to bring the pigments in the saponified form into a solution of petroleum ether. This solution is then washed with dilute alkali and water to effect a separation from the chlorophyll. The actual separation of the carotenoids is then accomplished by shaking the petroleum ether solution with 92% methanol (by volume). The hydrocarbons remain in the petroleum ether layer while the oxygenated xanthophylls go into the methanol phase.[35] After repeated extractions, the pe-

[34] R. Kuhn and H. J. Bielig, *Ber.*, 73, 1080 (1940).

[35] R. Willstätter and A. Stoll, *Untersuchungen über Chlorophyll*, Berlin, 1913.

troleum ether solution is used for direct determinations.[36] Thus, the total amount of carotenes can be determined spectrophotometrically and is usually expressed in terms of β-carotene. Determinations are usually made of only one wave length using the 450 mμ band of β-carotene, for which $E^{1\%}_{1\,cm.} = 2500$ (in petroleum ether). It has also been suggested that the provitamins A be determined colorimetrically by comparison with the color of standard dye solutions, for example, of azobenzene of known concentrations[37] or of a 0.1% solution of $K_2Cr_2O_7$.[38, 39]

Another method is to develop a blue color according to the method of Carr and Price (see page 78) and to determine the color by spectrophotometric methods.

Qualitative estimations for the presence of individual provitamins A can be made by using the chromatographic adsorption technique for the separation from other carotenoids. The methods used are the same as described for the preparation of the individual provitamins A. The principle of preferential adsorption has also been developed for the quantitative estimation of carotenes. Thus, a specially prepared magnesium carbonate has been developed to absorb xanthophyll but not carotene,[40, 41] and to absorb lycopene but not carotene.[41, 42]

CONVERSION OF PROVITAMINS A INTO VITAMINS A

Most substances ingested by human beings and by animals, which bring about vitamin A action, are not identical with the vitamins A found in animal organisms. They are provitamins A. In 1919 Steenbock[43] found that A-avitaminosis in rats can be cured with fresh, green plant materials and that the healing action corresponded to the amount of carotene present. This result could not be repeated until it was demonstrated ten years later[44] that the avitaminotic animals needed also vitamin D to regain health. It was furthermore discovered that the vitamin A content of the liver of

[36] "Tentative method of the Association of Official Agricultural Chemists for the determination of carotene," *J. Assoc. Official Agr. Chem.*, **22**, 79 (1939).

[37] P. Karrer and K. Schöpp, *Helv. Chim. Acta*, **15**, 745 (1932). R. Kuhn and H. Brockmann, *Z. physiol. Chem.*, **206**, 41 (1932).

[38] H. R. Guilbert, *Ind. Eng. Chem., Anal. Ed.*, **6**, 452 (1934).

[39] V. E. Munsey, *J. Assoc. Official Agr. Chem.*, **22**, 664 (1939).

[40] G. S. Fraps and A. R. Kemmerer, *Ibid.*, **22**, 190 (1939).

[41] G. S. Fraps, A. R. Kemmerer and S. M. Greenberg, *Ibid.*, **23**, 422 (1940)

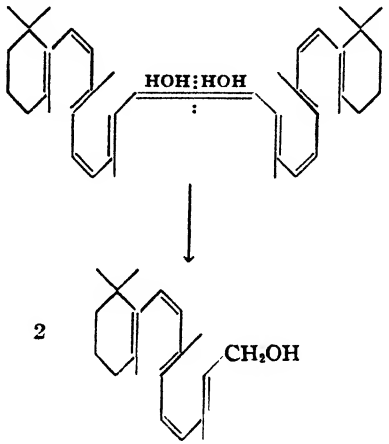
[42] G. S. Fraps, A. R. Kemmerer and S. M. Greenberg, *Ibid.*, **23**, 659 (1940)

[43] H. Steenbock, *Science*, **50**, 352 (1919); H. Steenbock and P. W. Boutwell, *J. Biol. Chem.*, **41**, 81 (1920). H. Steenbock, M. T. Sell, E. M. Nelson and M. V. Buell, *Ibid.*, **46**, 32 (1921). H. Steenbock and E. G. Gross, *Ibid.*, **51**, 63 (1922).

[44] B. v. Euler, H. v. Euler and P. Karrer, *Helv. Chim. Acta*, **12**, 278 (1929). H. v. Euler, V. Demole, P. Karrer and O. Walker, *Ibid.*, **13**, 1078 (1930).

rats decreased[45] rapidly and finally disappeared completely when the animals were kept on a vitamin A-free diet. After the addition of carotene to the diet, the vitamin A content of the liver increased again rapidly.

At the same time, vitamin A was isolated from liver oils and its formula was established.[46] Vitamin A contains just half as many carbon atoms as carotene. Theoretically, two mols of vitamin A must split out two mols of water to become β-carotene.



The hypothesis was then advanced that β-carotene is split in the organism with the addition of two molecules of water to yield two molecules of vitamin A. This theory was strongly supported by the difference in the quantitative physiological efficacy of α- and β-carotene. There exist, however, trustworthy observations[47] which indicate that even under optimum conditions an unsymmetrical fission of provitamin A occurs, which in the case of β-carotene yields a maximum of only one mol of vitamin A plus other decomposition products. This is in agreement with the observed potency of provitamin A in many cases (see page 75).

The mechanism of the conversion of provitamins A to vitamins A is not known. It is assumed that the conversion is effected by an enzyme, called carotenase. It is not known with certainty in which organ the conversion takes place, but much evidence exists that it is in the liver.[48] Furthermore, the pancreas appears to be involved since in human beings with

[45] T. Moore, *Biochem. J.*, **24**, 692 (1930).

[46] P. Karrer, R. Morf and K. Schöpp, *Helv. Chim. Acta*, **14**, 1036, 1431 (1931).

[47] S. W. F. Underhill and K. H. Coward, *Biochem. J.*, **33**, 589 (1939). H. v. Euler, P. Karrer and A. Zubrys, *Helv. Chim. Acta*, **17**, 24 (1934).

[48] H. S. Olcott and D. C. McCamm, *J. Biol. Chem.*, **94**, 185 (1931). B. Ahmad, *Biochem. J.*, **25**, 1195 (1931). J. L. Rea and J. C. Drummond, *Z. Vitaminforsch.*, **1**, 177 (1932).

juvenile diabetes mellitus an inability to convert carotene into vitamin A was demonstrated.[49]

Not all animals are able to convert provitamins A into vitamin A with the same efficiency. Rats are the most efficient;[50] chickens,[51] guinea pigs,[52] rabbits,[52] pigs[53] and cattle[54] are less efficient, and cats are apparently incapable of this conversion. Foxes can utilize provitamins A only to a very limited extent.[55] It seems that all carnivorous animals find enough vitamin A in their food and, therefore, do not need the ability to convert the provitamins A. The ability of man to utilize provitamins A efficiently has not been proved beyond any doubt. While there are good indications that man converts carotenes into vitamin A, there are also good indications that human beings may have difficulty in utilizing cryptoxanthene as a source of vitamin A.[56]

According to this species difference only those carotenoids are provitamins A which can be converted into vitamin A in a non-carnivorous organism, for example in the rat. There are indications that fish are able to produce vitamin A from carotenoids other than from the provitamins A as just defined. For a discussion of the synthesis of vitamin A in fish see page 73. Furthermore, it has been shown that in fresh water fish the feeding of provitamins A gives rise to a considerable increase not only of vitamin A but also of vitamin A_2.[57] Thus, at least α- and β-carotene are also provitamins A_2.

The provitamins A are not converted quantitatively into vitamin A under normal conditions. The organism usually obtains much more provitamins A than is necessary. The excess amounts are metabolized.

It must be assumed[58] that the conversion of provitamins A to vitamin A is regulated by a special mechanism which takes care of a rapid and quantitative transformation when needed and which, on the other hand, permits only a certain amount of provitamin to be converted if excessive amounts of provitamin A are taken in by the organism. It is estimated that small amounts of provitamins are converted to about 70–80% into vitamin A. Large quantities are less well utilized.[59]

[49] J. G. Brazer and A. C. Curtis, *Arch. Internal Med.*, **65**, 90 (1940).
[50] B. Ahmad and K. Malik, *Indian J. Med. Research*, **20**, 1033 (1933).
[51] N. S. Capper, I. M. W. McKibbin and J. H. Prentice, *Biochem. J.*, **25**, 265 (1931).
[52] H. Brockmann and M. L. Tecklenburg, *Z. physiol. Chem.*, **221**, 117 (1933).
[53] T. Moore, *Biochem. J.*, **25**, 2131 (1931).
[54] T. Moore, *Ibid.*, **26**, 1 (1932).
[55] A. I. Coombes, G. L. Ott and W. Wisnicky, *North Am. Vet.*, **21**, 601 (1940).
[56] A. G. van Veen and J. C. Lanzing, *Proc. Acad. Sci. Amsterdam*, **40**, 779 (1938).
[57] R. A. Morton and R. H. Creed, *Biochem. J.*, **33**, 318 (1939).
[58] See L. Zechmeister, *Ergeb. Physiol. biol. Chem. exptl. Pharmakol.*, **39**, 148 (1937).
[59] C. A. Baumann, B. M. Riising and H. Steenbock, *J. Biol. Chem.*, **107**, 705 (1934).

The question of whether or not there is only one mechanism which controls the conversion or if there is a series of mechanisms which control the chemical conversion and the conversion rate, etc., cannot be answered. The latter seems more likely from a physiological point of view. Only a few isolated facts are known which, however, do not permit any definite conclusion. The rate of transformation of carotene is diminished during diabetes,[60] during liver poisoning, brought about by phosphorus,[61] and during certain diseases of the liver. In such cases provitamin A accumulates in the system, especially in the Kupffer cells in the liver.

The conversion of provitamins A into vitamin A can be demonstrated experimentally by following the levels of carotenes and vitamin A in blood. Each person, for example, usually maintains a definite level of vitamin A in the blood, whereas the carotene level depends somewhat upon the intake of carotenes. Thus, during times of complete depletion of any form of vitamin A the vitamin A level in blood does not fall appreciably until the vitamin A stored in the organism is reasonably depleted and the available carotenes have been converted into vitamin A. The carotene level in the blood has been found to fall constantly until practically no more provitamins A are present. Then the vitamin A content of the blood begins to decrease.[62]

Ever since it became evident that provitamins A are converted in the liver into vitamin A, experiments have been made to bring about this reaction *in vitro*.[63] Recently such a conversion was apparently carried out successfully[64] by the use of liver tissue. This conversion was indicated by a faster rate of growth of cultures of fibroblasts than is obtained in the absence of liver tissue.

Some thought has also been given to the possibility of a conversion, in the organism, of vitamin A to carotenoids of double the amount of carbon atoms—in other words, an inverted process to the formation of vitamin A from provitamins A. Whereas there is at present no definite proof for such a reaction mechanism there are a few observations which may indicate that under special conditions such condensations occur. The visual purple

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[60] E. P. Ralli, A. C. Pariente, H. Brandaleono and S. Davidson, *J. Am. Med. Assoc.*, **106**, 1978 (1936).

[61] J. D. Greaves and C. L. A. Schmidt, *Am. J. Physiol.*, **111**, 502 (1935).

[62] C. D. May, K. D. Blackfan, J. F. McCreary and F. H. Allen, *Am. J. Diseases Children*, **59**, 1167 (1940).

[63] H. S. Olcott and D. C. McCann, *J. Biol. Chem.*, **94**, 185 (1931). J. L. Rea and J. C. Drummond, *Z. Vitaminforsch.*, **1**, 177 (1932). H. v. Euler and E. Klussmann, *Arkiv. Kemi, Mineral. Geol.*, **B11**, 6 (1932). B. Woolf and T. Moore, *Lancet*, **1932, II**, 13.

[64] K. Willstaedt, *Enzymologia*, **3**, 228 (1937). L. E. Baker, *Proc. Soc. Exptl. Biol. Med.*, **33**, 124 (1935).

(see page 89) appears to contain a carotenoid of more than 20 carbon atoms which is built up in the organism from vitamin A. There is also the remarkable fact that in rats the incisor teeth are white during vitamin A deficiency but contain a deep orange pigment in the enamel following the administration of vitamin A and during times of normal nutrition.[65] Neither of these colored compounds has as yet been sufficiently analyzed to state their constitution.

VITAMIN A

11. Nomenclature and Survey

Names:

Axerophthol.[66]
Vitamin A_1.
The epithelium-protecting and antixerophthalmic vitamin.

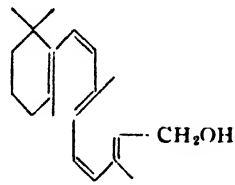
Historical names:

Fat-soluble A (McCollum and Davis[67]).
Biosterol (Takahashi, 1922).
Vitamin A_2 (v. Euler, 1924).
Ophthalamin (Jones, 1928).
Anti-infective vitamin (1929).

Chemical name:

θ-(2,2,6-Trimethyl-Δ^6-cyclohexenyl)-β,ζ-dimethyl-$\Delta^{\alpha,\gamma,\epsilon,\eta}$-octatetraene-$\alpha$-carbinol.

Structure:



Empirical formula:

$C_{20}H_{30}O$.

Efficacy:

4,500,000 International Units per gram.

[65] J. T. Irving and M. B. Richard, *Nature*, **144**, 908 (1939).
[66] Name proposed by P. Karrer, *Rapports et discussion sur les vitamins et les hormones. Institut international de chimie Solvay, sixieme-conseil de chimie*, Paris, 1938.
[67] E. V. McCollum and M. Davis, *J. Biol. Chem.*, **19**, 245 (1914).

12. Occurrence

Vitamin A occurs only in the animal organism; it has not been found in plants.

The most important source of vitamin A is liver oils, because the organism stores most of the excess vitamin A in the liver. The amount of vitamin A in the liver varies greatly, according to the type of food consumed, general living conditions, season, etc. Fish liver oils from cod, halibut, tuna, etc., contain vitamin A in fair amounts. Especially rich in vitamin A are the liver oils from *Hippoglossus hippoglossus*, *Scombresox saurus*, *Rhombus maximus* and from the Japanese fish, *Stereolepis ishnagi*, which contain from 200 to 2000 times as much vitamin A as the common cod liver oil.

TABLE I

DISTRIBUTION OF VITAMIN A IN VARIOUS FISH LIVER OILS

Source of oil	Zoological name	Potency I. U. per gram
Haddock	*Gadus aglefinus*	65
Cod	*Gadus morrhua*	600
Striped bass	*Roccus lineatus*	4,500
Jack smelt	*Atherinopsis californiensis*	10,000
Albacore	*Germo alalunga*	18,000
Grouper	*Epinephelus morio*	25,000
Boston mackerel	*Scomber scombrus*	30,000
Barracuda	*Sphyraena argentea*	40,000
Skipjack tuna	*Katsuwonus pelamis*	40,000
Yellowtail	*Seriola dorsalis*	50,000
White sea-bass	*Cynoscion nobilis*	50,000
Red snapper	*Lutianus campechanus*	60,000
Totuava	*Eriscion macdonaldi*	60,000
Bluefin tuna	*Thunnus thynnus*	60,000
Yellowfin tuna	*Neothunnus macropterus*	70,000
Pacific mackerel	*Pneumatophorus diego*	80,000
Bonito	*Sarda chiliensis*	120,000
Cabrilla pinta	*Epinephelus analogus*	170,000
Swordfish	*Xiphias gladius*	250,000
Ishnagi	*Stereolepis ishnagi*	300,000
Black sea-bass	*Stereolepis gigas*	600,000

Besides the liver, other organs contain some vitamin A, the most important of which are the pyloric ceca of a number of fish species. These contain an amount equal to that found in the liver.[68] The rest of the intes-

[68] J. R. Edisbury, R. A. Morton, G. W. Simpkins and J. A. Lovern, *J. Biol. Chem.*, 32, 118 (1938).

tinal tract contains smaller amounts. Considerable quantities are present in the retina and the corpora lutea, small amounts in lungs and kidney.[69] Vitamin A is present also in egg yolks (one hen egg contains about 20 U. S. Pharmacopoeia Units),[70] milk (3 U. S. Pharmacopoeia Units per gram)[71] and milk products (butter, 50 U. S. Pharmacopoeia Units per gram). Colostrum of man and of cows is from two to ten times richer in vitamin A than the milk. Stored fats contain small amounts of vitamin A, especially during pregnancy.

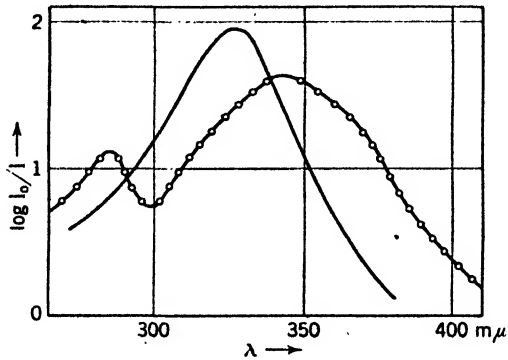


Fig. 3.- Absorption spectra of vitamin A (——) and of vitamin A_2 (o–o–o). (A. E. Gillam, I. M. Heilbron, W. E. Jones and E. Lederer.)

Vitamin A occurs both as free alcohol and predominantly in esterified form in the animal organism.[72] Vitamin A palmitate has been isolated from the liver oil of *Stereolepis ishnagi*[73] and from cod liver oil[74] in the form of an addition product with maleic anhydride. The free vitamin A has been separated[75] from its esters by molecular distillation. By the use of the same method it has also been shown that different esters are present in halibut liver oil and cod liver oil.

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[69] T. Moore, *Biochem. J.*, **25**, 275 (1931).

[70] H. v. Euler and E. Klussmann, *Biochem. Z.*, **219**, 215 (1933).

[71] R. A. Morton and I. M. Heilbron, *Biochem. J.*, **24**, 860 (1930). T. Moore, *Biochem. J.*, **26**, 1 (1932).

[72] A. L. Bacharach and E. L. Smith, *Quart. J. Pharm.*, **1**, 539 (1928). L. Reti, *Compt. rend. soc. biol.*, **120**, 577 (1935).

[73] S. Hamano, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **26**, 87 (1935). K. Kawakami, *Ibid* **26**, 77 (1935).

[74] K. C. D. Hickman, *Ind. Eng. Chem.*, **29**, 968, 1107 (1937).

[75] A. O. Tischer, *J. Biol. Chem.*, **125**, 475 (1938).

13. Properties

Vitamin A crystallizes from methanol in pale yellow plates, containing solvent of crystallization and melting at 8° C.[77] A purer form of vitamin A crystals, melting at 63–64° C., is obtained from ethyl formate or from propylene glycol.[76] Vitamin A was found to distill at 137–138° C. at 10^{-5} mm.,[78] but later workers found the material to evaporate at 120–125° C. at 5×10^{-3} mm.[79] The natural vitamin A esters distill from 200–240° C. at 10^{-3} mm. The absorption spectrum of vitamin A in the ultraviolet shows a sharp band at the wave length 328 mμ (Fig. 3), with an extinction coefficient $E^{1\%}_{1\,cm} = 1725$.[77] The biological activity is 4,500,000 International Units per gram.[80]

Vitamin A is sensitive to oxidation and autoxidizes readily. It is quite heat-stable in inert atmosphere and is readily stabilized in solution in oil. The esters of vitamin A are more stable than the free substance. Vitamin A is destroyed by ultraviolet light and is optically inactive. Vitamin A crystals appear to be isotropic. Vitamin A is soluble in most organic solvents, but is insoluble in water. It has, therefore, been classified as a member of the fat-soluble vitamins.

14. Isolation

Vitamin A can be isolated from the unsaponifiable parts of animal fats, especially liver oils. Three different methods have been used for the isolation, namely, chromatographic adsorption, vacuum distillation and fractional crystallization at low temperatures.

The use of the chromatographic adsorption method[81] brought about the first preparation of almost pure material. The liver oils of *Hippoglossus* and of the mackerel *Scombresox saurus* were saponified and the unsaponifiable part freed from sterols by cooling to −70° C. The non-crystallized material possesses the vitamin A activity. By adsorption on aluminum oxide followed by differential adsorption on calcium hydroxide a concentrate was obtained, which by repeated adsorption could not be further

[76] J. G. Baxter and C. D. Robeson, *Science*, **92**, 202 (1940)
[77] H. N. Holmes and R. E. Corbet, *J. Am. Chem. Soc.*, **59**, 2042 (1937).
[78] I. M. Heilbron, R. N. Heslop, R. A. Morton, E. T. Webster, J. L. Rea and J. C. Drummond, *Biochem. J.*, **26**, 1178 (1932). F. H. Carr and W. J. Jewell, *Nature*, **131**, 92 (1933)
[79] K. C. D. Hickman, *Ind. Eng. Chem.*, **29**, 968, 1107 (1937).
[80] J. G. Baxter, P. L. Harris, K. C. D. Hickman and C. D. Robeson, *J. Biol. Chem.*, **141**, 991 (1941).
[81] P. Karrer, R. Morf and K. Schöpp, *Helv. Chim. Acta*, **14**, 1036, 1431 (1931).

purified.[82] It consisted of a viscous yellow oil, which was used for the successful determination of the chemical structure.

The disadvantage of this method lies in the fact that due to the sensitivity of the vitamin A molecule partial destruction occurs during the adsorption procedure. Rearrangements of the double bonds and slight oxidation might be the chief reasons for the change of the compound.

By the use of the vacuum distillation process vitamin A concentrates have been obtained[83, 84, 85, 86] of the same properties as obtained by the use of the chromatographic adsorption method. A decided improvement is the use of the short-path high-vacuum distillation which has resulted in the isolation of vitamin A and its esters in relatively pure form and has enabled the determination of exact boiling points and distillation characteristics. This has been accomplished by the use of the cyclic molecular still and certain adjuncts such as "constant yield" oils and "pilot" dyes. The saponified vitamin concentrate is dissolved in a mixture of neutral residue oil and constant yield oil and is distilled in stepwise fashion over long ranges of temperatures. The concentration of vitamin A in the distillates is plotted against temperature and results in an "elimination curve" with a distillation maximum on the temperature axis. Using this method, vitamin A is shown to distill at 123° as compared with celanthrene red distilling at 125° C.[87]

Vitamin A was finally crystallized[88] by the use of fractional freezing and cold filtration after the isolation of the unsaponifiable material. Vitamin A crystals are best obtained by crystallization from ethyl-formate or from propylene-oxide.[89] From methanol, vitamin A crystallizes with solvent of crystallization. Pure vitamin A melts at 63 64° C.[89]

15. Chemical Constitution

The constitution of vitamin A has been determined mainly by Karrer, who suggested the following structure:

[82] P. Karrer and R. Morf, *Helv. Chim. Acta*, 16, 625 (1933).

[83] I. M. Heilbron, R. N. Heslop, R. A. Morton, E. T. Webster, J. L. Rea and J. C. Drummond, *Biochem. J.*, 26, 1178 (1932).

[84] F. H. Carr and W. J. Jewell, *Nature*, 131, 92 (1933).

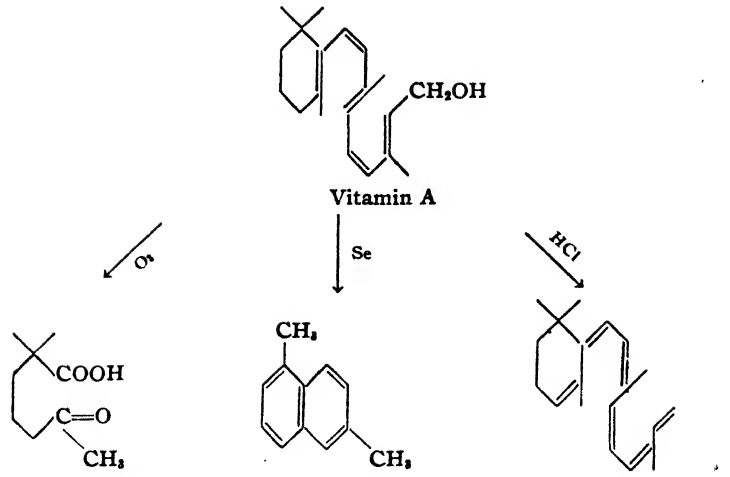
[85] J. C. Drummond, H. J. Channon and K. H. Coward, *Biochem. J.*, 19, 1047 (1925).

[86] Vitamin A prepared according to this method was used in 1935, to set up a provisional standard. E. M. Hume and H. Chick, *Med. Research Council Rept.*, IV, *The Standardization and Estimation of Vitamin A*, 1935.

[87] K. C. D. Hickman, *Ind. Eng. Chem.*, 29, 968, 1107 (1937). D. H. Killeffer, *Ibid.*, 29, 966 (1937). N. D. Embree, *Ibid.*, 29, 915 (1937). J. G. Baxter, E. L. Gray and O. A. Tinker, *Ibid.*, 29, 1112 (1937).

[88] H. N. Holmes and R. E. Corbet, *J. Am. Chem. Soc.*, 59, 2042 (1937).

[89] J. G. Baxter and C. D. Robeson, *Science*, 92, 202 (1940).



Geronic acid | 1,6-Dimethyl-naphthalene | Dehydrated vitamin A (Spurious vitamin A?)

The empirical formula is $C_{20}H_{30}O$. Various crystallized esters, for example, the beta-naphthoate, m. p. 76° C., the anthraquinone-β-carboxylic acid ester, m. p. 126° C.,[90] the acetate, m. p. 56–58° C., the palmitate, m. p. 26–28° C. and the disuccinate, m. p. 73–75° C., have been prepared and indicate the alcoholic function of the oxygen. Molecular weight determinations proved to be extremely difficult to carry out due to rapid changes of the vitamin in solution. The best experimental values obtained show an average molecular weight of 294, but it seems probable that the correct weight must be a little less.[91] (Karrer's formula calls for 286.)

Oxidation of vitamin A with ozone yields geronic acid which proves the presence of the β-ionone ring. The methyl-groups in the side chain are converted into acetic acid by oxidation with permanganate, in analogy to the results obtained by the oxidation of the provitamins A. Dehydrogenation of vitamin A with selenium forms 1,6-dimethyl-naphthalene by closing a second ring and splitting off two methyl groups and part of the side chain.[92] By the action of mineral acids on vitamin A a transformation occurs, which is indicated by a change of the absorption spectrum. It has been suggested[93] that under the influence of acids a second ring is formed.

[90] S. Hamano, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **28**, 69 (1935); **32**, 44 (1937). T. H. Mead, *Biochem. J.*, **33**, 589 (1939).

[91] H. R. Bruins, J. Ovahoff and L. K. Wolff, *Biochem. J.*, **25**, 430 (1931). H. N. Holmes and R. E. Corbet, *J. Am. Chem. Soc.*, **59**, 2042 (1937).

[92] I. M. Heilbron, R. A. Morton and E. T. Webster, *Biochem. J.*, **26**, 1194 (1932).

[93] J. R. Edisbury, A. E. Gillam, I. M. Heilbron and R. A. Morton, *Ibid.*, **26**, 1164 (1932).

It is more plausible that a dehydration may take place. A compound apparently identical with dehydrated vitamin A and often referred to as "spurious vitamin A" has also been found to be present as a normal constituent of fish liver oils. (The absorption maxima occur at 350, 368 and 379 mμ.[94])

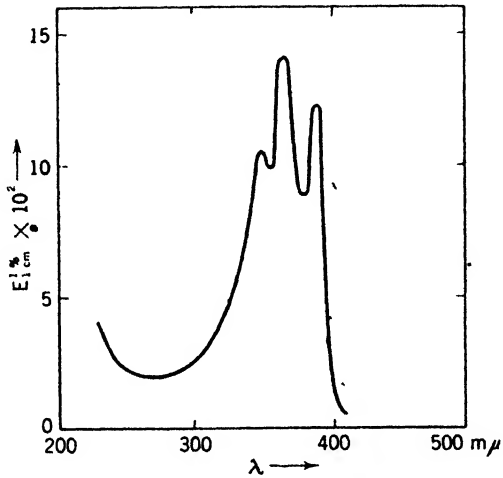


Fig. 4.—Absorption spectrum of anhydro-vitamin A. (N. D. Embree.)

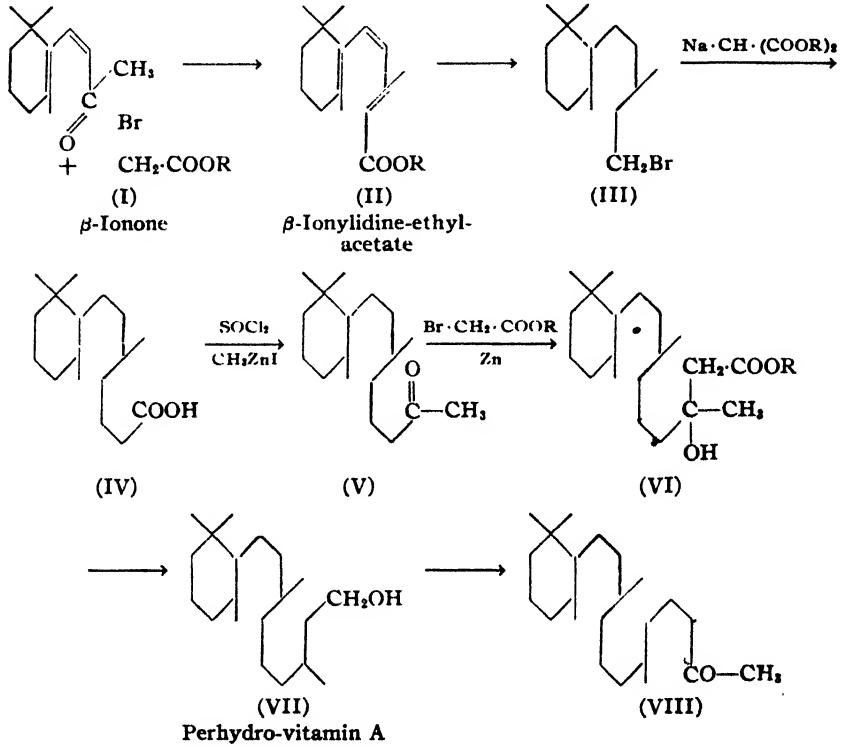
Vitamin A contains a continuous series of five conjugated double bonds. Maleic and citraconic anhydrides form addition products of unknown constitution.[95] By catalytic hydrogenation of vitamin A five mols of hydrogen are absorbed. The reaction product, perhydro-vitamin A, has been prepared synthetically according to the following scheme[96]: β-Ionone and bromo-ethyl-acetate were condensed with zinc according to Reformatsky forming β-ionylidene-ethyl-acetate (II). By catalytic hydrogenation the double bonds were saturated and by reduction with sodium and alcohol according to Bouvault-Blanc the ester group was converted into an alcohol.

[94] D. C. Castle, A. E. Gillam, I. M. Heilbron and H. W. Thompson, *Biochem. J.*, **28**, 1702 (1934). I. M. Heilbron, R. N. Heslop, R. A. Morton, J. L. Rea and J. C. Drummond, *Ibid.*, **26**, 1178 (1932). H. Pritchard, H. Wilkinson, J. R. Edisbury and R. A. Morton, *Ibid.*, **31**, 258 (1937). N. D. Embree, *J. Biol. Chem.*, **128**, 187 (1939).

[95] S. Hamano, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **25**, 536, 538 (1934); **26**, 87 (1935). K. Kawakami, *Ibid.*, **26**, 77 (1935). Z. Nakamiya, *Ibid.*, **27**, 42 (1935).

[96] P. Karrer and R. Morf, *Helv. Chim. Acta*, **16**, 557, 625 (1933).

The corresponding bromide (III) was obtained by the action of hydrobromic acid. Condensation of the bromide (III) with sodium-malonic-acid-ester followed by decarboxylation yielded the acid (IV).



Through the acid chloride the higher ketone (V) was obtained by the reaction with methyl-zinc-iodide. The Reformatsky reaction of the ketone (V) with bromo-ethyl-acetate gave the tertiary alcohol (VI). The hydroxyl group was converted into the bromide and by reduction with zinc and acetic acid replaced by hydrogen. Reduction with sodium and alcohol finally yielded the perhydro-vitamin A (VII). This, however, like the perhydro-vitamin A from the natural vitamin A, proved to be an oil. To obtain a crystallized derivative, the higher ketone (VIII) was prepared. The alcohol, perhydro-vitamin A, was converted into the bromide, the bromide condensed with sodium-malonic-acid-ester and the reaction product decomposed by saponification. The obtained acid was converted into the acid chloride and condensed with methyl-zinc-iodide, yielding finally the ketone (VIII). All these steps represent the same reactions as

described for the synthesis of the ketone (V) from the bromide (III). The ketone (VIII) gave a semicarbazone identical with the semicarbazone from the ketone (VIII) prepared from the perhydro-vitamin A of natural origin

The four conjugated double bonds in the side chain of vitamin A may theoretically bring about 16 *cis-trans*-isomers. The configuration of the crystallized vitamin A is unknown. The natural vitamin A might easily be a mixture of different isomers, although nothing is known about the biological activity of isomeric compounds of this type, nor under what conditions rearrangements of the *cis-trans*-isomers in a compound of the structure of vitamin A occur. The fact that the absorption bands of vitamin A vary somewhat in different solvents both in position and magnitude suggested to some workers[97] that different equilibria of *cis-trans*-isomers are formed.[98] This theory has not yet been proved or abandoned.

Vitamin A (and also carotene, although somewhat more slowly) is easily destroyed by ultraviolet light. The typical absorption band at 325–328 mμ disappears. From the reaction product three compounds have been isolated. One, substance A′, has an absorption maximum at 312 mμ. Substance A″ is converted by further irradiation into another substance with the absorption maximum at 290 mμ (like β-ionone). This new substance is probably a ketone. It forms a semicarbazone (absorption spectrum identical with that of β-ionone-semicarbazone). None of those substances has been isolated in pure form. They all are biologically inactive.[99]

16. Synthesis

Many attempts have been made to synthesize vitamin A.[100] Kuhn and Morris [101] succeeded in the following way: β-Ionylidene-ethyl-acetate[102] was reacted with *o*-toluidine-magnesium-iodide, yielding the *o*-toluidide (II). By conversion through the imide-chloride (III) and the Schiff's base (IV), β-ionylidene-acetaldehyde (V) was finally obtained (chromous-chloride method of v. Braun[103]). This aldehyde (V) underwent polyene

[97] A. E. Gillam and M. S. El Ridi, *Biochem. J.*, **32**, 820 (1938).

[98] E. L. Smith, B. E. Stern and F. E. Young, *Nature*, **141**, 551 (1938). D. C. Garratt, *Ibid.*, **142**, 76 (1938).

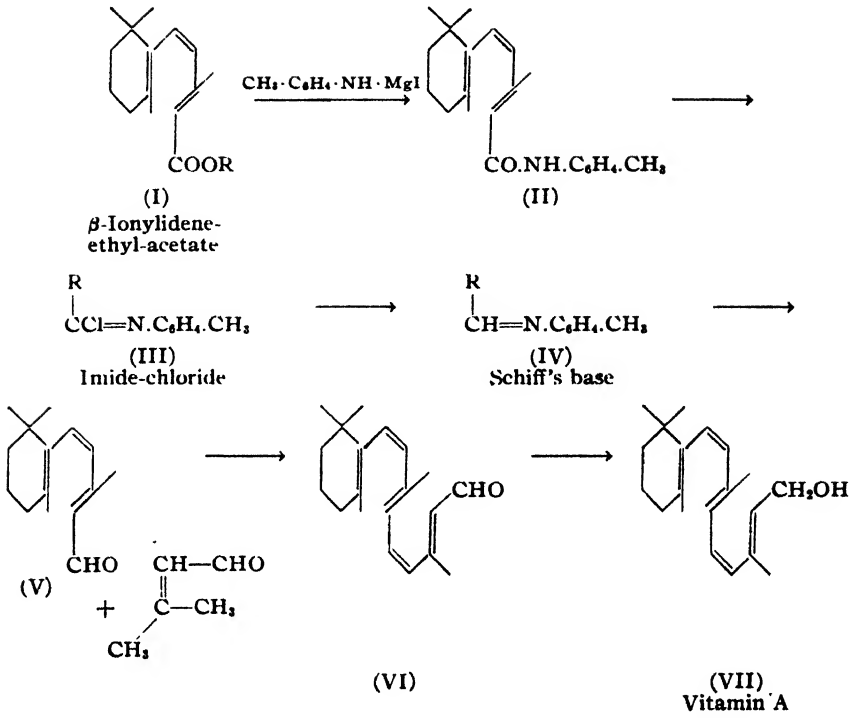
[99] W. J. Dann, *Biochem. J.*, **27**, 274 (1933). A. Chevallier and co-workers, *Compt. rend. soc. biol.*, **117**, 10 (1934); *Compt. rend.*, **198**, 2207 (1934); *Bull. soc. chim. biol.*, **18**, 703, 1115 (1936); *Compt. rend. soc. biol.*, **121**, 1495 (1936); *Bull. soc. chim. biol.*, **17**, 530 (1935).

[100] See, for example, the series of papers by I. M. Heilbron and co-workers, *J. Chem. Soc.*, **1935** and the following years. R. C. Fuson and R. E. Christ, *Science*, **84**, 294 (1936). I. M. Heilbron and W. E. Jones, *Chemistry & Industry*, **55**, 813 (1936). F. B. Kipping and F. Wild, *Ibid.*, **58**, 802 (1939).

[101] R. Kuhn and C. J. O. R. Morris, *Ber.*, **70**, 853 (1937).

[102] P. Karrer, H. Salomon, R. Morf and O. Walker, *Helv. Chim. Acta*, **15**, 878 (1932).

[103] J. v. Braun and H. Rudolph, *Ber.*, **67**, 269, 1755 (1934).



synthesis with β-methyl-crotonaldehyde, to give the five-times unsaturated aldehyde (VI), which was reduced to the corresponding alcohol, that is, vitamin A. The pure substance was, however, not isolated. After purification by chromatographic adsorption a preparation was obtained which, by physical and biological tests, showed a vitamin A activity corresponding to a vitamin A content of 7.5%.[104]

17. Industrial Methods of Preparation

Vitamin A is commercially of considerable importance as a food supplement for man and animals, and as a therapeutic in the hands of physicians. A great number of technical methods for the manufacture of vitamin A preparations have therefore been developed. Pure crystallized vitamin A is marketed, but is not of great commercial significance, since vitamin A concentrates are more easily available and are satisfactory for clinical use.

[104] See also the critical discussion by P. Karrer and A. Rüegger, *Helv. Chim. Acta*, **23**, 284 (1940).

The starting materials for most technical vitamin A preparations are fish livers and other viscera, which contain, besides vitamin A, considerable, but varying amounts of vitamin D. A great industry has been developed for manufacturing concentrates which contain either vitamin A alone or in mixture with vitamin D.

There are three commercial methods for preparing vitamin A concentrate from a fish oil. The first and oldest employs saponification of the oil by alkali. The second method starts with hydrolysis but follows with the molecular distillation of the non-saponifiable matter from which the sterols have previously been removed by freezing. There results a distillate of vitamin A-alcohol containing 1,000,000–2,000,000 units per gram. The third process which is rapidly becoming the most important technical method for extracting vitamin A subjects the crude fish oil itself to molecular distillation and volatilizes, besides the free vitamin A-alcohol, the natural vitamin A-esters leaving the main body of the oil undistilled. The residue is a bland oil which is used in the leather industry or is converted into turkey-red oil. The molecular distillates contain the natural esters of vitamin A, chiefly palmitic, accompanied by esters of C_{14} and C_{20} acids. The non-saponifiable matter ranges from 10% to 30% and the potency of the vitamin A distillate from one hundred to half a million units per gram from a single step of distillation. This method developed by Hickman and co-workers has made available for food fortification and drug use a large range of low potency oils which hitherto were useless for this purpose.

Much effort has been spent on the development of methods for the preparation of concentrates of vitamin A and vitamin D from fish liver oils. Although the liver oils of many species contain considerable amounts of both vitamins, halibut liver oil is the preferred commercial source of highly potent vitamin A preparations. Special care must be taken for the preservation of these vitamins in the fresh livers. Vitamin A is more sensitive to oxidation and heat than is vitamin D. Fish livers are generally stored in the cold and protected by sterilization against destruction. The oil is obtained by application of pressure, by treatment with steam whereby the oil collects on the surface, or by extraction of the livers with organic solvents.

In order to concentrate the vitamins, the oil is saponified. The vitamins are then in the unsaponifiable portion. It is essential not to apply too much heat or too much alkali. Therefore, some methods use only partial saponification which, after separation of the free fatty acids, brings about a considerable concentration. The saponification is usually carried out in alcohol, ethylene glycol or acetone at room temperature or slightly elevated

temperatures in inert atmosphere. The next step is the extraction of the unsaponifiable part. Good solvents are ether, petroleum ether, ethylene chloride, benzene, etc. The solvents are distilled off and the residue used as such.

As alternative steps in the above procedure for concentrating the vitamins A and D the entire liver can be saponified instead of saponifying the liver oils, or in place of saponification the liver oils can be extracted with a non-miscible solvent, such as alcohol.

Methods for overcoming the fishy odor and taste of the oils are technically important. Removal of the odorous compounds by distillation has already been mentioned. A partial hydrogenation of the oils has been recommended repeatedly.

The use of proper oils for blending to the desired vitamin potency is quite important. Oils containing high amounts of unsaturated fatty acids are undesirable, since they tend to form peroxides which in turn oxidize the vitamins. Oils of satisfying properties are olive oil, coconut oil (recommended for vitamin A standards), linseed oil and, to a certain extent, corn oil. All these oils help to overcome the fishy odor and taste and contain natural antioxidants. For some purposes special antioxidants, for example, hydroquinone or vitamin E, are added.[105]

VITAMIN A_2

Whereas only vitamin A seems to occur in salt water fish and in mammals, fresh water fish contain besides vitamin A a slightly different substance, provisionally called vitamin A_2[106] (maximum ratio vitamin A_2:vitamin A = 2.67:1). It has been found in the eyes, livers, viscera and intestines.

Vitamin A_2 has not been isolated in pure crystalline form. Its separation from vitamin A cannot be achieved by either chromatographic adsorption or vacuum distillation. Therefore it has not been possible to determine with certainty the constitution of vitamin A_2. Vitamin A_2 can be differentiated from vitamin A by:

1. The ultraviolet absorption spectrum in alcohol. Vitamin A exhibits a maximum between 325 and 328 mμ compared with 345–350 mμ (subsidiary maximum at 285 mμ) for vitamin A_2 (see Fig. 3 on page 59).

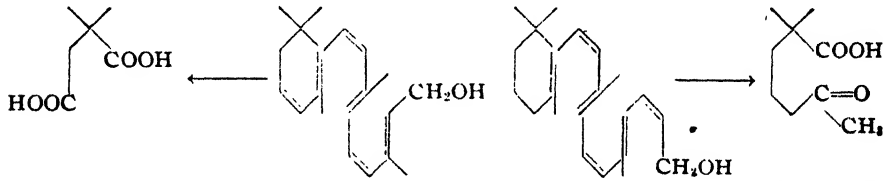
[105] The Council of Pharmacy of the Am. Med Assoc. does not accept the use of hydroquinone. *J. Am. Med Assoc.*, **109**, 1454 (1937)

[106] E. Lederer and V. Rosanova, *Biokhimiya*, **2**, 293 (1937). J. R. Edisbury, R. A. Morton and G. W. Simpkins, *Nature*, **140**, 234 (1937). A. E. Gillam, I. M. Heilbron, E. Lederer and V. Rosanova, *Ibid.*, **140**, 233 (1937).

2. The spectral absorption of the blue solutions brought about by antimony trichloride dissolved in alcohol. Vitamin A gives bands mainly at 610–620 mμ, whereas vitamin A_2 shows maximum absorption at 693 –697 mμ. (For details of the determination methods see page 79.)

Vitamin A_2 contains six conjugated ethylene linkages, whereas vitamin A contains only five. By hydrochloric acid treatment, vitamin A_2 appears to "cyclize" in the same manner as does vitamin A, suggesting a close chemical relationship of both compounds. The reaction products of both vitamins show the same absorption bands in the ultraviolet.

Formulas (I) and (II) have been suggested as possible constitutions of vitamin A_2. Formula (I) represents the formula of vitamin A plus an additional double bond in the ring. Formula (II) is the C_{22}-homolog of vitamin A and is called β-apo-5-carotinol according to Karrer's nomenclature.



α, α-Dimethyl-succinic acid (I) (II) Geronic acid

On the basis of the available experimental evidence it cannot be decided which of the formulas is the correct one. By oxidation with ozone, geronic

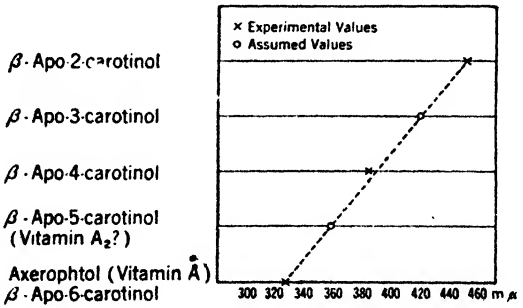


Fig. 5.—Position of the absorption maxima of the homologs of vitamin A. (P. Karrer, A. Rüegger and A. Geiger.)

acid is obtained in an amount corresponding to the C_{22}-formulation. No α,α-dimethyl-succinic acid, a degradation product of a compound of formula

(I), could be detected in the ozonolysis product. On the other hand, the elimination curves of vitamin A and vitamin A_2 determined by stepwise short-path high-vacuum distillation resemble each other very closely, vitamin A_2 distilling less than 3° C. higher than vitamin A,[107] as would be expected in case vitamin A_2 has the formula (I),[108] whereas a compound of the formula (II) would be expected to distill at an appreciably higher temperature.

By mild oxidation of β-carotene, a series of aldehydes has been obtained, which after reduction yields a series of "homologs" of vitamin A.[109] From the data of the absorption maxima of these compounds and of the maxima produced by $SbCl_3$ it is possible to predict the absorption characteristics of β-apo-5-carotinol of formula (II) (see Fig. 5). Since these data do not correspond with the data of vitamin A_2, it appears questionable that vitamin A_2 has the constitution of (II).[110]

On oxidation with aluminum-*tert*-butoxide in the presence of acetone, both vitamins yield ketones, which contain three carbons more than the starting material. These are formed by condensation of the originally formed vitamin aldehyde with acetone.[111] The ketones differ from each other by their absorption maxima in the ultraviolet: the ketone from vitamin A shows a maximum at 401 mμ, the ketone from vitamin A_2 at 413 mμ (in alcohol). These ketones are oils; however, the *p*-chloro-benzoyl-hydrazones are crystalline substances. Analysis of the compound obtained from vitamin A_2 supports the view that vitamin A_2 is the C_{22}-homolog of vitamin A.[112]

A further proof for the close chemical relationship of vitamin A_2 and vitamin A is seen in the fact that both vitamins appear in the organism of fish (perch and dace) when carotene is fed. Carotene, therefore, is also a provitamin A_2.[113]

The physiological significance of the presence of two chemically different vitamins A is not known at present. Comparative studies of various organs in different fish lead to the conclusion that vitamins A and A_2 probably do not replace each other with equal readiness in all functions.[114]

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[107] E. L. Gray and J. D. Cawley, *J. Biol. Chem.*, **134**, 397 (1940). E. L. Gray, *Ibid.*, **131**, 317 (1939). J. A. Lovern, T. H. Mead and R. A. Morton, *Biochem. J.*, **33**, 338 (1939).

[108] E. L. Gray and J. D. Cawley, *J. Biol. Chem.*, **134**, 397 (1940).

[109] For a more detailed description of this reaction and its reaction products see page 76.

[110] H. v. Euler, P. Karrer and U. Solmssen, *Helv. Chim. Acta*, **21**, 211 (1938). P. Karrer, A. Rüegger and A. Geiger, *Ibid.*, **21**, 1171 (1938).

[111] J. W. Batty, A. Burawoy, S. H. Harper, I. M. Heilbron and W. E. Jones, *J. Chem. Soc.*, **1938**, 175.

[112] A. E. Gillam, I. M. Heilbron, W. E. Jones and E. Lederer, *Biochem. J.*, **32**, 405 (1938).

[113] R. A. Morton and R. H. Creed, *Ibid.*, **33**, 318 (1939).

[114] J. A. Lovern, R. A. Morton and J. Ireland, *Ibid.*, **33**, 325 (1939).

Attempts have been made to correlate the different vitamin A and A_2 concentrations of various tissues with possible functions of these vitamins. As yet, no definite conclusions as to the difference in the participation of these vitamins in fat exchange or assimilation can be reached.[115] Rats, frogs and birds can store vitamin A_2 in the liver when it is ingested in the food.[116]

Up to the present, there is no indication that vitamin A_2, although it might have a biological value equal to vitamin A, plays any significant role in mammalian nutrition.[117]

OTHER VITAMIN A FACTORS—VITAMIN A_3

It is important to know how many different vitamins A occur in nature. Only two forms are definitely known—the previously described vitamins A and A_2. There are many reasons, however, for assuming that some other vitamins A exist. The data of most fish liver oils differ from each other in their relation of biological activity, absorption spectrum and absorption of the color produced by antimony trichloride. These differences might possibly be explained by errors of determination, foreign substances influencing the absorption and perhaps some synergistic factors of unknown constitution. On the other hand, critical evaluation of these factors and various experimental evidences lead to the assumption that other vitamins A exist. Isomers and "homologs" of vitamin A might possibly be active ingredients of fish liver oils. All these questions are more or less speculative at present, but require serious consideration.

The possibility of an occurrence of a *cis-trans*-isomeric factor of vitamin A in concentrates was suggested when it was established that the vitamin A absorption bands of fish liver oils and concentrates vary somewhat with change of solvent, both in position and in magnitude.

It has also been observed that short irradiation of solutions of vitamin A sometimes increased, sometimes decreased the absorption maximum at 328 mμ, but that the absorption returned almost to the original value after the irradiated solution had stood in the dark. Variations in the proportions of *cis-trans*-isomers might explain these results[118] and also some of the spontaneous increases as well as decreases in absorption of liver oils and con-

[115] J. R. Edisbury, R. A. Morton, G. W. Simpkins and J. A. Lovern, *Biochem. J.*, **32**, 118 (1938). J. A. Lovern and R. A Morton, *Ibid.*, **33**, 330 (1939).

[116] A. E. Gillam, *Ibid.*, **32**, 1496 (1938). E. Lederer and F. H. Rathman, *Compt. rend.*, **206**, 781 (1938); *Biochem. J.*, **32**, 1252 (1938).

[117] A. Wormall, *Annual Reports on the Progress of Chemistry for 1938*, **35**, 332 (1939).

[118] E. L. Smith, B. E. Stern and F. E. Young, *Nature*, **141**, 551 (1938). A. E. Gillam and M. S. El Ridi, *Biochem. J.*, **32**, 820 (1938).

centrates during the period of storage.[119] Indications along the same lines come from comparative studies of the ratio of the adsorption at 328 mμ and the $SbCl_3$ color test maximum at 620 mμ of different fractions of distilled halibut intestinal oil which varies between 0.21 and 0.38.[120]

Different values for the absorption maxima are obtained when liver oils are saponified in the cold than when heat is applied. The absorption is appreciably higher after cold saponification.[121] A biological comparison of these samples has not been reported. The existence of a heat labile isomer of vitamin A, however, has been postulated.

The existence of other forms of vitamins A is also shown by the following experimental evidence. By adsorption of a vitamin A concentrate on calcium hydroxide, a small amount of a foreign substance has been obtained with an absorption maximum at about 270 mμ.[122] This substance, called "hepaxanthene," has not been obtained in pure form and its biological activity is uncertain. The new compound is regarded by some workers as an isomeric vitamin A, while others discuss the possibility of an artifact arising from decomposition of vitamin A. A non-quantitative separation of vitamin A from other active substances has recently been achieved by an extraction with 83% alcohol.[123] The soluble material resembles the substance known as vitamin A in biological and physical properties, whereas the insoluble material appears to be distinctly different. It shows a considerably greater biological value than would be anticipated from the reaction with antimony trichloride or from the spectroscopic examination. The material exhibits a maximum at 285–290 mμ often without even an inflection at 328 mμ. Material of the same characteristics has been obtained from mammalian liver oil. ($E^{1\%}_{1\,cm.}$ 290 mμ = 240. $SbCl_3$ color test $E^{1\%}_{1\,cm.}$ 594 mμ = 180, 496 mμ = 172.) If this material proves to be a real vitamin A, it must be chemically different from the vitamins A and A_2. Besides the already mentioned different solubility, it is apparently not affected by hydrogen chloride, as indicated by the unchanged absorption spectrum and biological activity (18,000 International Units per gram). Material of the same characteristics has also been obtained from whale liver oil by chromatographic adsorption on calcium hydroxide.[124]

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[119] H. N. Griffiths, T. P. Hilditch and J. Rae, *Analyst*, **56**, 65 (1933). I. M. Heilbron, A. E. Gillam and R. A. Morton, *Biochem. J.*, **25**, 1352 (1931). H. N. Holmes and R. E. Corbet, *J. Am. Chem. Soc.*, **59**, 2042 (1937).

[120] R. A. Morton, *Nature*, **141**, 552 (1938).

[121] D. C. Garratt, *Ibid.*, **142**, 75 (1938).

[122] P. Karrer, *Ibid.*, **132**, 26 (1933). P. Karrer and K. Schöpp, *Helv. Chim. Acta*, **16**, 625 (1933). H. v. Euler, P. Karrer and A. Zubrys, *Ibid.*, **17**, 24 (1934).

[123] H. Pritchard, H. Wilkinson, J. R. Edisbury and R. A. Morton, *Biochem. J.*, **31**, 258 (1937).

[124] H. Willstaedt and H. B. Jensen, *Nature*, **143**, 474 (1939).

Another indication for the possible existence of additional vitamin A factors is the discovery that the visual purple in some marine fish has different absorption characteristics than either rhodopsin or porphyropsin (see page 89). Furthermore, after the administration of α-carotene to rats, a compound of unknown biological activity was observed in the liver oils which exhibited upon reaction with antimony trichloride an absorption maximum at about 540 mμ.[125] By comparison of the different characteristics of this material with those of the vitamins A and A_2 (see Table II) it seems possible that the new substance, which probably will be designated as vitamin A_3, contains one double bond less than vitamin A. If that is the case, it seems possible that the ethenoid linkage in the ring of vitamin A is replaced by a single bond. It will be recalled that vitamin A_2 which has one double bond more than vitamin A might have the additional double bond in the ring. On the other hand, if it can be substantiated that vitamin A_3 is not dehydrated by the action of mineral acids, it might be concluded that this compound has not an allyl-alcohol-constitution.

TABLE II

	Vitamin A_3 (?)	Vitamin A	Vitamin A_2
	4 double bonds	5 double bonds	6 double bonds
Absorption maximum:	285–290 mμ	325–328 mμ	345-350 mμ
$SbCl_3$ color reaction:	496 and 594 mμ	580 and 620 mμ	645 and 693 mμ

VITAMINS A

18. Biogenesis

The formation of vitamin A in the organism of mammals and birds has previously been discussed (see page 53). The ingested provitamins are converted into vitamin A, probably in the liver. The origin of the tremendous quantities of vitamins A in fish livers and viscera is a much discussed question. Principally there are only two possible explanations—either the fish eat the vitamin A or they make it themselves. The former theory appears somewhat remote. Fish, at least many species, live from plankton which is devoid of vitamin A, but may contain vitamin A precursors. Other species ingest vitamin A in that they live from fish, but no fundamental difference has been observed in the vitamin A content of the

[125] R. Kuhn and M. L. Tecklenburg, *Z. physiol. Chem.*, **221**, 117 (1933).

livers of these two classes of fish. The theory, then, that fish produce the vitamin which they store in their body appears to be much more plausible. The vitamin may, of course, be produced by the same or similar methods which the plant uses in building up provitamins A (see page 50). Thus it is possible that vitamin A is produced by total synthesis or by transformation of some ingested vitamin A precursor. The latter theory has been favored in recent years by many investigators. It may well be that fish are able to convert, besides provitamins A, also other carotenoids into vitamin A. Since fish consume considerable amounts of *Crustacea* and since there is evidence[126] that fish are able to absorb the principal carotenoid of *Crustacea*, astaxanthin, it has been thought possible that fish possess a peculiar power of transforming these carotenoids into vitamin A.[127]

Whatever the actual mechanism may be, there is ample evidence that fish have a somewhat different way of producing vitamin A than mammals. This is obvious, for example, from the occurrence of vitamin A_2 in fish. This vitamin has, at least so far, not been observed in the tissues of mammals or of birds.

19. Specificity of the Vitamin A Action

The vitamin A action is, as demonstrated in experiments with rats, the result of the structure of the entire molecule. In the provitamins A, the presence of at least one ring of the β-ionone structure in the molecule is essential. Carotenoids with a ring structure corresponding to that of α-ionone are inactive. Thus, for example, β-carotene, which contains two rings of the β-configuration, is under optimum conditions twice as active as α-carotene, which contains only one ring of the β-configuration, the second ring being of the α-configuration.[128] The same is true for all the other provitamins A. They all have only one ring of the β-structure. In agreement with this aphanicin which is only about one-fourth as active as β-carotene has been assigned a constitution of double the molecular weight of the other carotenes and containing only one ring of the β-ionone configuration. The minimum dose necessary to induce growth in rats under optimum conditions is 2.5 γ for β-carotene while the minimum amount of aphanicin is 10 γ and that of the other provitamins A 5 γ. While not all workers in the field agree as to the minimum dose of the provitamins A

[126] G. Wald and H. Zussman, *Nature*, **140**, 197 (1937).
[127] R. A. Morton, *Chemistry & Industry*, **59**, 301 (1940).
[128] R. Kuhn and H. Brockmann with A. Scheunert and M. Schieblich, *Z. physiol. Chem.*, **221**, 129 (1939)

there is general agreement that somewhat smaller doses of β-carotene are necessary than of all the other provitamins.

On the other hand, all provitamins are less active than vitamin A. In a series of carefully conducted experiments on rats, the activity of vitamin A was found to be twice as high as the activity of β-carotene. According to these findings, vitamin A exhibits an activity of 3.32×10^6 International Units per gram while β-carotene contains 1.667×10^6 International Units per gram.[129] This indicates that one mol of β-carotene yields one mol of vitamin A, probably due to an asymmetrical fission of the β-carotene molecule. Other investigators have reported similar findings.[130, 131, 132]

Esters of vitamin A, for example, the crystalline anthraquinone-2-carboxylate and the 2-naphthoate, show the full biological activity of the free alcohol on the molecular weight basis.[129]

The complete series of conjugated double bonds is also necessary. The biological activity of the α- and β-dihydro-carotene (minimum daily dose 100γ) is not certain and is probably due to the presence of unreduced carotenes or to a biological dehydrogenation yielding carotene.[133] Tetrahydro-vitamin A, 1-(β-cyclo-geranyl)-geraniol,[134] and perhydro-vitamin A[135] show no activity. Some degradation products of β-carotene show vitamin A activity in a daily dose of 5 γ (like α-carotene) as long as one part of the molecule still contains the β-ionone structure. Thus by mild oxidation of β-carotene with chromic acid a biologically active oxy-β-carotene of unknown constitution is obtained.[136] Naturally occurring carotenoids with hydroxyl groups in both ring systems are inactive. Another oxidation product, β-semi-carotenone (I), a diketone,[137] and its mono-oxime (II) are active. By ring closure of the diketone with alkali the β-dehydro-semi-carotenone (III) is obtained, which is still active.[138] When, by further oxidation, the second ring with β-ionone structure is also opened to form a tetraketone[139] the activity disappears. By oxidation of β-carotene with perbenzoic acid β-carotene-oxide is obtained[140] which is biologically active

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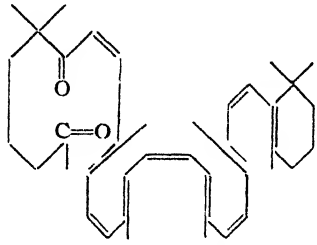
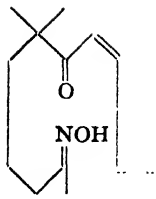
[129] S. W. F. Underhill and K. H. Coward, *Biochem. J.*, **33**, 589 (1939).
[130] H. v. Euler, P. Karrer and A. Zubrys, *Helv. Chim. Acta*, **17**, 24 (1934).
[131] R. N. Holmes and R. E. Corbet, *J. Am. Chem. Soc.*, **59**, 2042 (1937).
[132] J. G. Baxter and C. D. Robeson, *Science*, **92**, 202 (1940).
[133] J. H. C. Smith, *J. Biol. Chem.*, **90**, 597 (1931). H. v. Euler, P. Karrer, H. Hellström and M. Rydbom, *Helv. Chim. Acta*, **14**, 839 (1931). R. Kuhn and H. Brockmann, *Z. physiol. Chem.*, **213**, 1 (1932).
[134] L. Ruzicka and W. Fischer, *Helv. Chim. Acta*, **17**, 633 (1934).
[135] H. v. Euler, V. Demole, P. Karrer and O. Walker, *Ibid.*, **13**, 1082 (1930).
[136] R. Kuhn and H. Brockmann, *Z. physiol. Chem.*, **213**, 1 (1932). *Ber.*, **65**, 894 (1932).
[137] R. Kuhn and H. Brockmann, *Ber.*, **66**, 1319 (1933).
[138] R. Kuhn and H. Brockmann, *Ann.*, **516**, 95 (1935).
[139] R. Kuhn and H. Brockmann, *Ber.*, **65**, 894 (1932).
[140] H. v. Euler, P. Karrer and O. Walker, *Helv. Chim. Acta*, **15**, 1507 (1932).

in a daily dose of 5 γ. Carotene iodide is also active in a daily dose of 40 γ, probably due to a regeneration of carotene.[141]



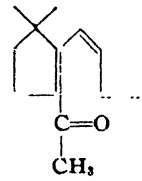
M. p. 119° C.
501, 470 mμ (Benzene)

(I)
β-Semi-carotenone



M. p. 135° C.
501, 470 mμ (Benzene)

(II)
β-Semi-carotenone-monoxime

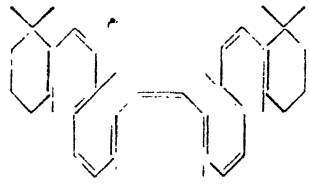


M. p. 176° C.
512, 480 mμ (Benzene)

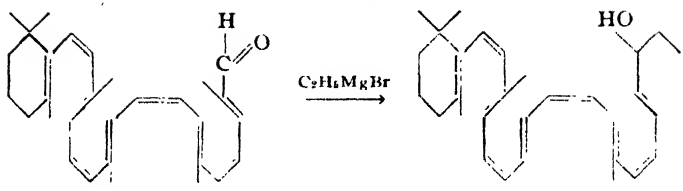
(III)
β-Dehydro-semi-carotenone

Some of the degradation products of β-carotene are biologically active, whereas the corresponding derivatives of α-carotene appear to be inactive. By mild oxidation of β-carotene with permanganate, a series of aldehydes was obtained, by fission of various double bonds. Thus, the main reaction product, β-apo-2-carotinal[142] (formula (II) on page 77) is obtained by an oxidation of the double bond in the aliphatic chain of the molecule adjacent to one ring. This compound as well as its oxime proved to be active in a daily dosage of 5 γ.[143] The vitamin A produced from β-apo-2-carotinal in rats resembles in all characteristics the vitamin A_1 or axerophthol.[144] In other words, this degradation product of β-carotene is apparently not a vitamin A, but a provitamin A. By reduction with aluminum isopropylate, β-apo-2-carotinal can be converted into the corresponding alcohol, β-apo-2-carotinol, which compound probably also is a provitamin A. By condensation of β-apo-2-carotinal with ethyl-magnesium-bromide, a tertiary alcohol of the formula (III) is produced, which is also active.[145]

[141] P. Karrer and M. Rydbom, *Ber.*, **62**, 2445 (1929). T. Moore, *Lancet*, **1929, II**, 219.
[142] P. Karrer and U. Solmssen, *Helv. Chim. Acta*. **20**, 682 (1937).
[143] H. v. Euler, P. Karrer and U. Solmssen, *Ibid.*, **21**, 211 (1938).
[144] H. v. Euler, G. Günther, M. Malmberg and P. Karrer, *Ibid.*, **21**, 1619 (1938).
[145] P. Karrer, A. Rüegger and A. Geiger, *Ibid.*, **21**, 1171 (1938). H. v. Euler, G. Günther, M. Malmberg and P. Karrer, *Ibid.*, **21**, 1619 (1938)

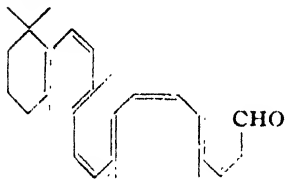


(I)
β-Carotene

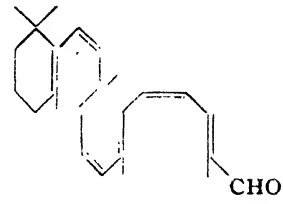


(II)
β-Apo-2-carotinal

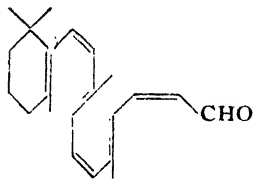
(III)



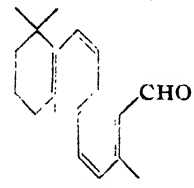
(IV)
β-Apo-3-carotinal



(V)
β-Apo-4-carotinal



(VI)
β-Apo-5-carotinal



(VII)
β-Apo-6-carotinal
Vitamin A-aldehyde

The next lower "homolog" of β-apo-2-carotinal is β-apo-3-carotinal (formula (IV)), which is present in the oxidation products in small quantities, but which has not been isolated. β-Apo-4-carotinal has been obtained, however, in the form of its crystallized oxime, which is biologically active. The corresponding alcohol has also been prepared. The next lower "homolog" would be β-apo-5-carotinal, the alcohol of which has the formula which has tentatively been assigned to vitamin A_2. This compound has not yet

been isolated from the oxidation products of carotene, although lower homologs are present. The absorption spectra of the series of "homolog" alcohols of axerophthol indicate a constant shift of the maxima toward higher wave lengths with an increasing number of double bonds present (see Fig. 5). From these data it appears questionable that vitamin A_2 has the formula of a β-apo-5-carotinol.

20. Determination

(a) *Chemical Methods*

A great number of different color reactions have been proposed for the determination of vitamin A. These color tests are based on three different types of reactions: reaction with phenols, with acids, and with inorganic chlorides, which in aqueous solution show an acidic reaction.

The method of Carr and Price[148] is the only one in general use: A 20–25% solution of antimony trichloride in chloroform is added to a solution of vitamin A in chloroform. To insure greatest possible accuracy in the determination the antimony-trichloride reagent should be free of any traces of ferric chloride.[146] A blue color develops which shows its maximum after 10 seconds and then immediately begins to fade. The color is measured in a colorimeter, for example, in the Lovibond tintometer (visual or photoelectric[147] observation), or by spectrophotometric analysis. The values obtained may be checked against standardized solutions of copper sulfate[149] and cobalt nitrate or against certain color filters. The value of a 20% solution of the vitamin-containing material in chloroform is called a "Lovibond Unit." For international use a 2% solution is recommended. The values obtained by this latter method are called "C. L. O." (cod liver oil) Units. Various other manners of expressing the color value have been recommended, for example, the Carr-Price value,[148] the Moore[150] blue units and the Dann-Evelyn[151] L-620 mμ value. A noteworthy variation is the Anderson-Nightingale[152] dilution test which consists in diluting the material to be analyzed for its vitamin A content with chloroform until the blue color is just visible. (For comparison of different units see also page 83.)

[146] J. R. Edisbury, *Analyst*, **65**, 484 (1940).
[147] R. L. McFarlan, J. W. Reddie and E. C. Merrill, *Ind. Eng. Chem., Anal. Ed.*, **9**, 324 (1937).
[148] F. H. Carr and E. A. Price, *Biochem. J.*, **20**, 498 (1926)
[149] H. Brockmann and M. L. Tecklenburg, *Z. physiol. Chem.*, **221**, 117 (1932).
[150] T. Moore, *Biochem. J.*, **23**, 1267 (1929); **31**, 155 (1937).
[151] W. J. Dann and K. A. Evelyn, *Ibid.*, **32**, 1008 (1938).
[152] A. Andersen and E. Nightingale, *J. Soc. Chem. Ind.*, **48**, 139T (1929).

The values obtained with the Carr-Price method for vitamin A from liver oils are in fairly good agreement with the results of biological tests. Crude liver oils contain substances which interfere with the color reaction,[154] and should therefore be saponified under anaerobic conditions and in the absence of sunlight before testing. Some naturally occurring acids decrease the value.[155] Some carotenoids, for example, cyclized vitamin A, increase the value. It is possible, however, to differentiate by the use of selective filters between the colors produced by vitamin A and by carotenes by measuring the absorption, the maximum being at 620 mμ for vitamin A and at 590 mμ for carotene. The maximum for vitamin A_2 lies at 693 mμ. Impurities and mixtures of vitamins A and carotenes may change the wave length of the maximum of absorption.[156] Visually, the colors produced by carotene and vitamin A can be differentiated since the blue obtained from carotene persists without fading, while the blue from vitamin A fades in two to five minutes. A mixture is indicated when the color fades rapidly at first but remains constant later on. A red color is caused by the presence of sterols.[153] The Carr-Price color reaction cannot be used for the determination of biologically active material in synthetic vitamin A preparations.

Antimony trichloride causes a change in the chemical structure of the carotenoids, the nature of which is unknown. The reaction product is biologically inactive.[157]

A number of modifications of the Carr-Price test have been proposed, for example, the addition of a 0.5% pyrocatechol,[158] the addition of arsenic trichloride and hydroquinone,[159] determination at low temperature,[160] determination of the blue value minus the yellow value,[161] etc.[162, 163]

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[153] Y. Raoul and P. Meunier, *J. pharm. chim.*, **29**, 112 (1939).

[154] E. R. Norris and A. E. Church, *J. Biol Chem.*, **85**, 477 (1929), **87**, 139 (1930), **89**, 421 (1930). E. L. Smith and V. Hazley, *Biochem J.*, **24**, 1942 (1930) K. H. Coward, F. J Dyer, R. A. Morton and J. H. Gaddum, *Ibid.*, **25**, 1102 (1931). R. S. Morgan, *Ibid.*, **26**, 1144 (1932).

[155] A. Emmerie, *Nature*, **131**, 364 (1933); *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **2**, 156 (1933); *Proc. Roy. Soc. Amsterdam*, **35**, 1347 (1932).

[156] H. v. Euler, P. Karrer, E. Klussmann and R. Morf, *Helv. Chim. Acta*, **15**, 502 (1932). H. Brockmann and M. L. Tecklenburg, *Z. physiol. Chem.*, **221**, 117 (1933). I. M. Heilbron, A. E. Gillam and R. A. Morton, *Biochem. J.*, **25**, 1352 (1931). M. van Eekelen, A. Emmerie, H. W. Julius and K. L. Wolff, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **1**, 8 (1931).

[157] A. E. Gillam, I. M. Heilbron, R. A. Morton and J. C. Drummond, *Biochem. J.*, **26**, 1174 (1932).

[158] J. Rosenthal and J. Erdélyi, *Ibid.*, **28**, 41 (1934).

[159] G. Gutzeit, *Arch. sci. phys. nat.*, **9**, 155 (1927).

[160] E. R. Norris and co-workers, *J. Biol. Chem.*, **85**, 477 (1929); **89**, 421 (1930); *J. Nutrition*, **5**, 495 (1932).

[161] R. S. Morgan, *Biochem. J.*, **26**, 377 (1932).

[162] E. L. Smith and V. Hazley, *Ibid.*, **24**, 1942 (1930); **27**, 17 (1933)

[163] T. Moore, *Ibid.*, **24**, 692 (1930).

The Carr-Price method uses the principle of developing a color of vitamin A with an inorganic chloride in anhydrous solvents. The same principle is used in tests with aluminum chloride, arsenic chloride,[164] ferric chloride or stannic chloride.[165] The principle of using acids to produce a measurable color with vitamin A has been recommended. Sulfuric acid, phosphoric acid,[166] chloric acid,[167] molybdenum phosphotungstic acid[168] and trichloroacetic acid[169] are used either alone or in combination with the phenol principle, to produce colors.[167, 169] The latter principle is also used alone, for example, by means of pyrogallol.[169, 170]

The phenol principle has also been applied in conjunction with inorganic chlorides.[171]

All these methods are equally as accurate or less so than the Carr-Price reaction. None of these has been adopted for the determination of vitamin A by any official organization.

Carotene in the presence of vitamin A can be determined by either chromatographic adsorption[172] or by colorimetric measurements[173] of the color developed by the Carr-Price reaction (see above) or by direct spectroscopical examination.

The amount of free vitamin A in mixture with vitamin A esters can be estimated by the behavior of the free vitamin toward methanol: From an oily solution, containing both the free and the esterified vitamin A, about 40–60% of the free vitamin, but no esters, is extracted with methanol.[174] Molecular distillation is another method for the separation of the two forms.[175]

(*b*) *Physical Methods*

The extinction coefficient of the characteristic absorption band at 325–328 mμ can be used for the quantitative determination of vitamin A.[176]

[164] O. Rosenheim and J. C. Drummond, *Biochem. J.*, **19**, 753 (1925).

[165] F. H. Carr and E. A. Price, *Ibid.*, **20**, 497 (1926).

[166] E. Kobayashi and K. Yamamoto, *J. Soc. Chem. Ind., Japan*, **27**, 1060 (1924).

[167] A. E. Pacini and M. H. Taras, *J. Am. Pharm. Assoc.*, **26**, 721 (1937).

[168] N. Bezssonoff, *Bull. soc. chim. biol.*, **11**, 294 (1929).

[169] W. R. Fearon, *Biochem. J.*, **19**, 888 (1925).

[170] T. Moore, *Lancet*, II, 219 (1929). S. G Willimott and F. Wokes, *Ibid.*, II, 8 (1927). O. Rosenheim and T. A. Webster, *Ibid.*, II, 806 (1926); *Biochem. J.*, **20**, 1342 (1926).

[171] J. Rosenthal and J. Erdélyi, *Biochem. Z.*, **271**, 414 (1934).

[172] P. Karrer and K. Schöpp, *Helv. Chim. Acta*, **15**, 745 (1932).

[173] R. Kuhn and H. Brockmann, *Z. physiol. Chem.*, **206**, 41 (1931).

[174] V. Ritsert, G. P., 636,227.

[175] K. C D. Hickman, *Ind. Eng. Chem.*, **29**, 968, 1107 (1937).

[176] R. A. Morton, *Practical Aspects of Absorption Spectrophotometry*, Institute of Chemistry, 1938. I. M. Heilbron and co-workers, *Biochem. J.*, **22**, 987 (1928), **24**, 870 (1930). A. Chevallier and P. Chabre, *Ibid.*, **27**, 298 (1933). K. H. Coward, F. J. Dyer, R. A. Morton and J. H. Addum. *Ibid.*, **25**, 1102 (1931). A. Chevallier and P. Doubouloz, *Bull. soc. chim. biol.* **18**, 190 (1936).

Care must be taken in choosing the solvent; that is, either cyclohexane, ethyl or isopropyl alcohol has been recommended. The absorption bands vary somewhat both in position and in magnitude with change of solvents. As much as 10% increase (in ether) or 10% decrease (in chloroform) has been observed in comparison with the extinction coefficient of the same sample of vitamin A in alcohol.[177] It has been recommended[180] to check the instruments used against a suitable potassium chromate solution as a standard before and after each determination in order to increase the accuracy of the determinations. The factor for converting $E^{1\%}_{1\,cm.}$, 328 mμ, into International Units of vitamin A per gram is 1600, as accepted by the International Vitamin Conference 1934 ("conversion factor" = I. U./ $E^{1\%}_{1\,cm.}$).[178] The value for the "conversion factor" for pure vitamin A is believed to be about 2000–2100,[179] or even 2375, but not enough experimental data are available to decide which value is correct.[182, 183] For vitamin A preparations from whale livers a conversion factor $E^{1\%}_{1\,cm.}$, 325 mμ, of 1200 has been recommended[184] due to the presence of considerable amounts of materials such as vitamin A_3.

A certain amount of the observed total absorption at 325–328 mμ may be due to carotenoids other than vitamin A, and especially to glycerides. This is of importance when determinations of low potency materials are made, whereas the error becomes negligible with preparations of high vitamin A potency.

As stated before, vitamin A and the provitamins are destroyed by ultraviolet light. Therefore, the determination of the absorption band is somewhat inexact. At very low temperatures a differentiation between the band of vitamin A and the irradiation product of carotene is possible.[181] The selective decrease of absorption at 325 mμ produced by irradiating an alcoholic solution of a marine fish liver oil with mercury light of 365 mμ, which destroys vitamin A, is said to be an accurate measure of the vita-

[177] E. L. Smith, B. E. Stern and F. E. Young, *Nature*, **141**, 552 (1938). E. M. Hume and H. Chicks, *Med. Research Council Brit. Special Rept. Series*, **1935**, No. 202.

[178] See also E. M. Hume, *Nature*, **139**, 467 (1937).

[179] H. N. Holmes and R. E. Corbet, *J. Am. Chem. Soc.*, **59**, 2043 (1937). T. H. Mead, S. W. F Underhill and K. H. Coward, *Biochem. J.*, **33**, 589 (1939).

[180] J. B. Wilkie, *J. Assoc. Official Agr. Chem.*, **22**, 465 (1939).

[181] F. P. Bowden, S. D. D. Morris and C. P. Snow, *Nature*, **131**, 582 (1933).

[182] This high factor could, for example, be caused by a hyper-activity of the U. S. Pharmacopoeia Reference Oil or by an inadequate utilization of carotene (less than 50%) when the U. S. Pharmacopoeia Reference Oil was initially standardized. It has also been debated if the values are not exaggerated due to deterioration of the Standard Reference Oil. J. R. Edisbury, *Analyst*, **65**, 484 (1940).

[183] J. G. Baxter and C. D. Robeson, *Science*, **92**, 202 (1940).

[184] J. R. Edisbury, *Analyst*, **65**, 484 (1940).

min.[185] The effect on other constituents, particularly carotenoids, is, however, unpredictable.

Fluorescence offers a possible method of differentiating between vitamins A_1 and A_2. Vitamin A_1 exhibits a characteristic green fluorescence, vitamin A_2 a reddish fluorescence.[186] Upon irradiation both fluorescences fade.

(c) *Biological Methods*

The efficacy of vitamin A preparations is tested on young rats. In the prophylactic method the substance with the unknown vitamin A content is added to a vitamin A-free diet, and the growth of rats is compared with the growth rate of normally growing animals. Another method uses young rats which have ceased growing due to a vitamin A deficiency, but gain weight after the addition of vitamin A. The results are compared with an International Standard preparation of β-carotene which, as has been pointed out before, is converted under suitable conditions in the rat into an equimolecular quantity of vitamin A. The latter method is recommended by the U. S. Pharmacopoeia.

In addition, the appearance of a cornified epithelium in the vaginas of spayed rats (colpokeratosis) on a vitamin-free diet and its rapid disappearance following vitamin A feeding have been used for the determination of vitamin A.

Another biological test which has been used for determining the "physiological minimum" requirement is the evaluation of the dark adaptation of the eye after exposure to bright light. Individuals who have a partial or complete deficiency of this vitamin show some degree of "night blindness."[187]

21. Standards

The International Standard for vitamin A was adopted by the International Vitamin Conference 1934 and subsequently adopted for the United States Pharmacopoeia.

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[185] A. Chevallier, *Z. Vitaminforsch.*, **7**, 10 (1938). N. K. De, *Indian J. Med. Research*, **24**, 3 (1937). O. Notewark, *Biochem. J.*, **29**, 1227 (1935).

[186] R. Greenberg and H. Popper, *Proc. Am. Physiol. Soc.*, **1940**, 71.

[187] See chapter on the visual purple. This method was originally described by P. C. Jeans and J. Zentmire, *J. Am. Med. Assoc.*, **102**, 892 (1934) and has been modified by several workers.

1 I. U. (International Unit) = I U. S. P. Unit
= 0.6 γ[188] of pure β-carotene, m. p. 184°, optically inactive, dissolved in coconut oil[189] with addition of hydroquinone
= 1.5–2 Sherman Units.

1 g. of U. S. P. cod liver oil must contain at least 850 U. S. P. Units of vitamin A.

1 g. pure carotene contains 1,670,000 I. U.

1 g. pure vitamin A contains 4,500,000 I. U.

1 C. L. O. Unit (Cod Liver Oil Unit) = 125 γ β-carotene
= 208 U. S. P. Units
= 10 "Lovibond Units"
= 50 Lovibond Units (Wolff)
= 550 blue units (Moore).

1 blue unit (Moore) = Carr-Price value 0.0182
= $E^{1\%}_{1\,cm.}$, 328 mμ 0.000373
= 60 I. U.

The ratio of $E^{1\%}_{1\,cm.}$, 620 mμ to $L^{1\%}_{1\,cm.}$, 620 mμ (L—620 mμ value) is 1.30 ± 0.03.

The ratio of the activity of β-carotene and of vitamin A as reported above has been determined under the specified conditions of the biological test. An equality of an International Unit of vitamin A and of carotene can be claimed only under those conditions and is actually quite different for the metabolism of a normal growing organism. To express the requirement of an organism, double standards must be recognized, one for carotene and another one for vitamin A (see also page 95). (For a discussion of the various influences on vitamin A and on carotene utilization by the organism, see page 84.)

At the level of a physiological optimum the ratio of efficiency of vitamin A to carotene by weight is about 6:1 and at a level that assures significant storage and successful reproduction about 10:1.

It would be well to express the values of vitamin A potency not only in units but it should be required to indicate the method which was used for the determination, for example, U. S. Pharmacopoeia method, spectroscopic method, etc.

22. Physiology of Plants and Microorganisms[190]

Plants and also microorganisms contain only provitamins A. The presence of vitamin A has never been demonstrated and there is ample

[188] $1\gamma = 0.001$ mg. $= 10^{-6}$ g.

[189] The standard of 0.6 γ β-carotene as one International Unit replaced the earlier standard, set up in 1931. One Standard Unit was then defined by agreement to represent 1 mg. of carotene, m. p. 179°, prepared according to the method of Willstätter. When it became evident that carotene is a mixture of varying amounts of isomeric compounds, the biological equivalent of the earlier standard, expressed in weight of the pure β-carotene was adopted as International Standard.

[190] See also O. A. Bessey and S. B. Wolbach, *J. Am. Med. Assoc.*, **110**, 2072 (1937).

evidence that plants and many microorganisms do not need this vitamin. Whatever the function of this vitamin may be in the animal organism, either plants do not need this function or they take care of it by some other means. On the other hand, the relatively high concentrations of carotenoids and especially of provitamins in plants are striking. These compounds generally appear in all growing parts of the plant and especially in the shoots. It thus appears that provitamins A have a very definite purpose in the plant organism. What this is, has, however, not been elucidated. It is suspected that plants need provitamins A for proper growth. It has also been assumed that at least part of the action of these compounds is related to the efficient utilization of light by the plant organism. Plants are definitely photosensitive and phototropic responses have been shown to be associated with the presence of carotenoids.[191]

23. Animal Physiology

(*a*) *Metabolism of Provitamins A and of Vitamins A*

Both provitamins A and vitamins A are preferably administered orally, although they can be applied parenterally, for example, by subcutaneous or intramuscular injection. They are also efficiently absorbed through the skin.[192]

Provitamins A and vitamins A are absorbed from the intestinal tract. Carotenes in colloidal water solutions are efficiently utilized, for example, by the rat,[193] to prevent vitamin A-deficiency symptoms. Proper absorption of vitamin A is, on the other hand, related to the presence of fat. The nature of the fat or oil, which is used, for example, for dissolving the vitamin, determines to a certain extent the degree of utilization of the vitamin. The more unsaturated the oil is, the better the absorption. From mineral oils, vitamin A is absorbed only sparingly. The absorption of provitamins A and of vitamins A is furthermore linked to the presence of bile acids and apparently also of pancreatic lipase. In jaundice and in choledochocolonostomized dogs carotene is not absorbed.[194] There is evidence that the absorption mechanism of birds is somewhat different in that these animals need some external supply of fat for proper utilization of

[191] E. S. Johnston, *Smithsonian Inst. Pub.*, **92**, 11 (1934). G. Wald and H. G. du Buy, *Science*, **84**, 247 (1936).

[192] W. v. Drigalski, *Z. Vitaminforsch.*, **3**, 200 (1934). H. J. Lauber and H. Rocholl, *Klin. Wochschr.*, **II**, 1143, 1702 (1935).

[193] B. N. Majumdar, *Ind. J. Med. Research*, **27**, 413 (1939).

[194] J. D. Greaves and C. L. A. Schmidt, *Am. J. Physiol.*, **111**, 492, 502 (1935)

carotenes while vitamin A is apparently absorbed efficiently even when practically no fat is given in the diet.[195]

The absorption of provitamins and of vitamins A from the intestinal tract is a rapid process. The absorption of vitamin A in rats and in man reaches a maximum in three to five hours after administration, as shown by the disappearance from the intestinal tract and the rise of the vitamin A concentration in the blood.[196] The rate of absorption of the provitamins A is somewhat slower and reaches its maximum at seven to eight hours after administration. Vitamin A esters are hydrolyzed in the gut prior to absorption,[197] but it seems that the vitamin is re-esterified during or soon after the absorption.[198]

Both provitamins A and vitamins A are transported through the organism by the blood. The carotenoids are present in the blood serum while the erythrocytes are devoid of these compounds. They appear to be solubilized by the formation of loose protein addition compounds. There seems to exist a minimum normal blood level of vitamin A and perhaps also of provitamin A. This level is independent of the amount of stored vitamin or provitamin and is only temporarily increased during times of absorption from the intestines. In vitamin A-depleted rats, for example, the vitamin A content of the blood plasma is zero. During times of low vitamin A intake, the level in the blood is directly related to the amount fed. After the concentration in the serum has reached what appears to be an optimal level, that is, about 100 International Units per 100 cc. of plasma, the concentration does not increase in the blood even when excessive amounts are given. On the other hand, no vitamin A is stored during low vitamin A intake while this vitamin is rapidly deposited in the liver[199] when optimal doses are administered.

Provitamins A and vitamins A are removed from the blood by the reticulo-endothelial system.[200] Large amounts of vitamin A are stored in the liver. There are no quantitative data available which would indicate what the minimum amount stored in the livers of any species or of man should be. The vitamin is present in the liver mainly in the Kupffer and epithelial cells, in the latter in lipoid droplets and diffusely in the cytoplasm. Vitamin A is also stored to a certain extent in the adrenal cortex

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[195] W. C. Russell, M. W. Taylor, H. A. Walker and L. J. Polskin, *Proc. Am. Soc. Biol. Chem.*, 1941 CIX.

[196] S. W. Clausen, *J. Am. Med. Assoc.*, 101, 1384 (1933).

[197] E. L. Gray, K. Morgareidge and J. D. Cawley, *Proc. Am. Soc. Biol. Chem.*, 1940, XXXVII.

[198] J. C. Drummond. M. E. Bell and E. T. Palmer, *Brit. Med. J.*, I, 1208 (1935).

[199] J. M. Lewis, O. Bodansky, K. F. Falk and G. McGuire, *Proc. Soc. Exptl. Biol. Med.*, 46, 248 (1941).

[200] B. Ahmad, *Current Sci.*, 2, 477 (1934). F. Lasch and D. Roller, *Klin. Wochschr.*, 15, 1636 (1936).

in the form of small globules, in the *corpus luteum* and in the lutein cells of the ovary. Generally, fat cells contain small amounts. Traces may be found in the interstitium of the renal cortex and papilla, in the alveolar septum of the lungs and the intermediary part of the pituitary.[201]

Vitamin A is stored in the body mainly in esterified form, although small but definite amounts of the free vitamin are always present. The stored vitamin A esters are derivatives of a series of saturated and unsaturated fatty acids. Whether or not certain specific acids are preferentially selected by the organism for esterification purposes is not known.[202]

The ability of the animal organism to store large amounts of vitamin A has been discussed. The rat, for example, may store in a few days enough vitamin A to supply its nutritional needs for several months.[203] In the mammal this storage is very efficient after times of vitamin A depletion or of low vitamin A intake. The efficiency of this storage decreases, however, with an excess intake over the minimum physiological requirement.[204] On the other hand, the stored vitamin A is used rapidly when the food intake is lacking in this vitamin until a certain apparently critical vitamin A level in the liver is reached. Thereafter, the rate of depletion is much slower.[205] These observations suggest that a special vitamin A utilization mechanism exists in the organism. Provitamins A are stored in small but definite amounts. Examples have been given in the section on the occurrence of provitamins A (see page 38).

Vitamin A, and to a certain but considerably smaller extent also provitamins A, are secreted into milk. In cow's milk, the ratio of vitamin A to provitamin A varies considerably with the species, breed, nutrition, etc., but the total is of the same order of magnitude regardless of the breed.[206] The vitamin A activity of the milk is fairly constant regardless of the time of the day when the milk is secreted, but is to a certain extent influenced by the vitamin A potency of the food. Human milk is about 5 to 10 times as rich in vitamin A potency as cow's milk. The colostrum of all species investigated contains a much higher vitamin A potency than the milk. In the case of humans the colostrum contains 2 to 3 times, and in the case of cows 10 to 100 times the amount present in milk.[207] Both provitamins and vitamins A are also secreted in eggs. A special mechanism apparently

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[201] H. Popper and R. Greenberg, *Proc. Am. Physiol. Soc.*, **1940**, 146.
[202] E. L. Gray, K. C. D. Hickman and E. F. Brown, *J. Nutrition*, **19**, 39 (1940).
[203] A. W. Davies and T. Moore, *Biochem. J.*, **31**, 172 (1937).
[204] L. E. Booher and M. B. Porter, *Proc. Am. Soc. Biol. Chem.*, **1941**, XCI.
[205] A. W. Davies and T. Moore, *Biochem. J.*, **29**, 147 (1935).
[206] C. A. Baumann, H. Steenbock, W. M. Deeson and I. W. Rupel, *J. Biol. Chem.*, **105**, 1167 (1934).
[207] W. J. Dann, *Biochem. J.*, **30**, 1644 (1936).

exists for the passage of vitamin A through the placental wall. The vitamin A content of all newborn animals is low, but a vitamin A deficiency has never been found when the mothers have had an ample supply of this vitamin. After birth, the vitamin A content rises rapidly and reaches the normal level of the adult within a few days or weeks.

Both provitamins and vitamins A are in general readily metabolized. No excretion takes place through the kidneys. In the feces a certain proportion of the ingested provitamins may be found, especially when excessive amounts are fed or when the absorption mechanism is hampered.

(*b*) *Physiological Action of Provitamins A and of Vitamins A*

The physiology of man and of animals as influenced by vitamin A must ultimately be traced to the mechanism of the vitamin A action. This mechanism is for the most part unknown.

It has already been discussed that provitamins A are absorbed in the intestinal tract and to a certain extent stored in the animal organism. It has also been shown that provitamins A are converted in the organism into vitamin A. Except as precursor of vitamin A, carotenes as such are not known to be active in the animal economy. The possibility should, however, not be overlooked that provitamins may act in a specific way of their own. Thus, it may be significant that provitamins A are stored in man and in animals in a number of special organs, primarily in the glands which are concerned with the functions of reproduction. In invertebrata, which are able to synthesize their own specific carotenoids,[208, 209] these carotenoids apparently play a definite and important role in metabolic processes. It has also been suggested that, for example, in mussels, carotenoids may play some role in gametogenesis.[209] In this connection it is well to recall the action of various carotenoids of algae for which these compounds are secretion products concerned with the development of motility and with conjugation of the gametes.[210] While these carotenoids in lower animals are not necessarily provitamins A, they are closely related to them. Thus, it seems possible that the provitamins A have an influence upon the maintenance of a normal mechanism of the sex apparatus. It has, furthermore, been shown repeatedly that the sex organs of rats kept on a vitamin A-depleted diet degenerate. This may of course be explained as meaning that either vitamin A or provitamins A are necessary. It is, however, also possible that the sex glands do not utilize the provitamins A proper but

[208] E. Lederer, *Bull. soc. chim. biol.*, **20**, 554, 567, 611 (1938).
[209] B. T. Scheer, *J. Biol. Chem.*, **136**, 275 (1940)
[210] F. Moewus, *Naturwissenschaften* **27**, 97 (1939).

have a mechanism of their own for converting the provitamins into active compounds such as, for example, vitamin A.

Another unsettled question of physiological importance is the problem of whether or not the vitamin A itself acts in the organism. It has previously been stated that vitamin A is stored in the liver mainly in the esterified form, but that some free vitamin A can always be found. Thus it is possible that the vitamin is esterified only for the purpose of storage and it is probable that the vitamin acts in the free form. This question cannot be decided definitely until the mechanism of the vitamin A action has been elucidated.

A possible clue to this mechanism may be seen in the increase of purines in the growing vitamin A-depleted tissue after this vitamin has been administered.[211] Purines are necessary building units of cell nuclei. Actually all primary and secondary symptoms of a vitamin A deficiency can be explained on this basis. They all constitute cellular changes in the most sensitive parts of the body, such as in the respiratory mucosa, the salivary glands, the intestinal tract and finally in the skin in general. Thus, the principal role of vitamin A is to stimulate the building of cells.

Beyond this fundamental and specific action of vitamin A a general influence on the basic metabolism has also been considered. A possible connection with the oxidation mechanism has been suggested and was apparently supported by experiments which showed that the oxygen consumption of livers in the presence of iron-containing porphyrines increases with the amount of vitamin A present.[212] This theory has not, however, been further advanced and appears to be somewhat doubtful and unspecific in view of the fact that it has been possible to connect some of the other vitamins with definite stages of the oxidation-reduction mechanism of the living tissue. This has, however, not been possible in the case of vitamin A.

Vitamin A seems to exert, however, some non-specific functions upon the fat and carbohydrate metabolism. The necessity of the presence of fat has been discussed in connection with the absorption and storage of this vitamin. Furthermore, during avitaminosis the amount of fat deposited in the organism decreases slowly, but the fat deposits are restored upon administration of vitamin A. Similarly the cholesterol content in the organism decreases during times of low vitamin A intake. On the other hand, excess doses of vitamin A cause an appreciable increase of the cholesterol content in blood and in the brain. Similar relations have been

[211] H. v. Euler and G. Schmidt, *Z. physiol. Chem.*, 223, 215 (1934).
[212] H. v. Euler and L. Ahlström, *Ibid.*, 204, 168 (1932).

found for the carbohydrate metabolism, for example, for the glycogen content of the liver.

Vitamin A acts, however, in the body also in some other specific way. In conjunction with a specific protein, vitamin A plays an essential role in the visual purple. This will be discussed in a special section (see below). Whether or not vitamin A acts in conjunction with other proteins in some other specific functions is not known. It has been found experimentally that in vitamin A deficiency a marked decrease in the concentration of blood serum esterase, an appreciable decrease in hepatic esterase and a marked increase in hepatic lipase occur.[213] These findings need confirmation and should not necessarily be interpreted as indicating a relationship of vitamin A to specific enzyme systems.

Vitamin A is mobilized in the organism in a very special way whenever a state of disease occurs. Thus the intake of ethyl alcohol by dogs[214] or by human beings causes a specific mobilization of the vitamin A stored in tissues since both the blood level[215] and the dark adaptation test[216] indicate higher vitamin A concentrations than normal. In many diseases such as fever, etc., an increased demand for vitamin A has been demonstrated.[217] The turnover of vitamin A in the liver can also be effected by injection of carcinogenic compounds, such as dibenzanthrene, benzopyrene and methylcholanthrene.[218] This effect is not necessarily specific for vitamin A, although it appears to be.

(c) *The Visual Purple*

The energy of dim light, which strikes the eye, is transformed into nerve impulses by the visual purple on the outside end of the retina. Color and light of high intensity are perceived by the cones of the retina.

Rhodopsin, the visual purple, bleaches out under the influence of light and regenerates in the absence of light. Rhodopsin is a carotenoid-albumin, with an absorption maximum at 500 mμ. The prosthetic group, retinene, is a carotenoid of unknown composition,[219] but related to a form

[213] B. Sure, M. C. Kik and K. S Buchanan, *Proc. Soc. Exptl. Biol. Med.*, **35**, 209 (1936).

[214] S. W. Clausen, W. S. Baum, A. B. McCoord, J. O. Ryden and B. B. Breese, *Science*, **91**, 318 (1940).

[215] S. W. Clausen, B. B. Breese, W. S. Baum, A. B. McCoord and J. O. Ryden, *Ibid.*, **93**, 21 (1941).

[216] L. B. Pett, *Ibid.*, **92**, 63 (1940).

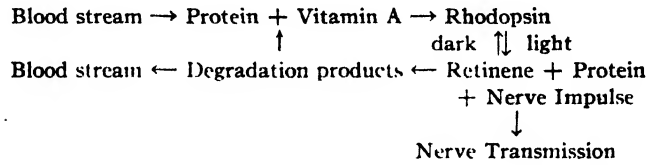
[217] S. W. Clausen, *J Am. Med. Assoc.*, **111**, 144 (1938).

[218] C. A. Baumann and E. G. Foster, *Proc. Am. Soc. Biol. Chem.*, 1941, XII.

[219] L. S. Fridericia and E. Holm, *Am. J. Physiol.*, **73**, 63 (1925). E. Holm, *Ibid.*, **73**, 79 (1925). S. Hecht, A. M. Chase, S. Shlaer and C. Haig, *Science*, **84**, 331 (1936). S. Hecht, *Physiol. Rev.*, **17**, 239 (1937). S. Hecht, A. M. Chase and S. Shlaer, *Science*, **85**, 567 (1937). C. Haig, S. Hecht and A. J. Patek, *Ibid.*, **86**, 534 (1937). S. Hecht and J. Mandelbaum, *Ibid.*, **88**, 219 (1938). S. Hecht and J.

(*Footnote continued on page 90.*)

of carotene. Under the influence of light the red color disappears and an unstable orange compound, "transient orange," is formed. This changes, probably in a dark process, to "indicator yellow," which appears to be a loose protein compound of retinene. Retinene can be extracted from the visual yellow with chloroform, is ligroin-soluble and has an absorption maximum at 385 mμ in chloroform ($SbCl_3$ color reaction yields a maximum at 664 mμ). Retinene can also be obtained from rhodopsin through acid or alkali hydrolysis. The visual yellow breaks down further to vitamin A and a specific protein. In the dark, a partial resynthesis of rhodopsin occurs. The pigmented layers of the eye contain large amounts of vitamin A, which is used for the synthesis of rhodopsin. Vitamin A deficiency therefore causes, as one of its first symptoms, a decreased ability for dark adaptation, since not enough vitamin A is available for the synthesis of rhodopsin.



Vitamin A is replaced in fresh water fish by vitamin A_2, which replaces also the vitamin A in the visual purple, and is called porphyropsin (absorption maximum at 525 mμ).[220] In some marine fish, compounds of different absorption characteristics are found, which indicate the presence of additional factors A (or mixtures of rhodopsin and porphyropsin).[221]

(*d*) *Relation of Vitamins A to Other Vitamins, Hormones, etc.*

In the general chapter on the interrelationship between the vitamins and the hormones, it has been pointed out that the physiological equilibrium of any organism is disturbed if one of the vitamins is not supplied regularly and adequately.

Of special interest in the case of vitamin A are the claims concerning an antagonism with the other fat-soluble vitamins, especially vitamin D, and the antagonism with some of the water-soluble vitamins, especially vitamin C. It has been stated that a vitamin A deficiency causes similar

[219] (*Continued*).
Mandelbaum, *Am. J. Physiol.*, **130**, 651 (1940). G. Wald, *J. Gen. Physiol.*, **18**, 905 (1935). G. Wald and A. B. Clark, *Am. J. Physiol.*, **116**, 157 (1936). G. Wald, *J. Gen. Physiol.*, **19**, 351, 781 (1935); **20**, 45 (1936). *Nature*, **139**, 587 (1937). R. J. Lythgoe, *J. Physiol.*, **89**, 331 (1937).

[220] G. Wald, *Nature*, **139**, 1017 (1937)

[221] G. Wald, *Ibid.*, **139**, 1017 (1937). R. J. Lythgoe and K. Tunsley, *Proc. Roy. Soc.* (*London*), **B120**, 95 (1936).

disturbances as an excess supply of vitamin D. The vitamins A and D, however, do not exert any synergistic or antagonistic action at the normal optimal concentrations.[222] The natural combination of vitamins A and D in liver oils prevents any signs of hypervitaminosis even if given in large amounts due to the alleged antagonism of these vitamins, which in this case even represents a synergism. Antagonism of vitamins A and C is seen when both vitamins are given in excess at the same time since no vitamin A hypervitaminosis develops.[223]

There is apparently a relationship between the vitamin A metabolism and vitamin E. During vitamin E deficiency in the rat, no vitamin A is stored in the liver and the amount of vitamin reserves in the liver is markedly reduced, even when ample quantities of vitamin A are administered.[223a] Thus an acute secondary deficiency of vitamin A develops. Whether this is due only to the non-specific antioxidant power of vitamin E compounds or to a specific physiological effect is not known.

Among the relations of vitamin A to hormones the alleged antagonism to the hormone of the thyroid gland, thyroxine, has been extensively studied.[224] Toxic effects of excesses of this hormone are said to be overcome by the administration of large amounts of vitamin A. Rats given thyroxine were depleted of vitamin A much more rapidly than normal rats.[225] No evidence for a specific antagonism could be obtained, however,[226] but a temporary disturbance in the metabolic rate has been found upon administration of large doses of vitamin A to normal and thyroidectomized rats.[227]

The secretion of the hormones of the anterior lobe of the pituitary gland seems to be related to the vitamin A metabolism since in frogs the glycogenotropic hormone could be detected in the liver only after intake of vitamin A[228] together with vitamin E.

24. Avitaminosis and Hypovitaminosis

The action of vitamin A can be regarded essentially as a stimulus to the building of new cells. Thus vitamin A deficiency causes retarded growth. This symptom is, however, not specific for a vitamin A deficiency.

Specifically, lack of vitamin A causes atrophy of the epithelium with substitution of a stratified keratinized epithelium for the normal epithelial

[222] A. Scheunert, *Naturwissenschaften* **28**, 297 (1940).
[223] H. Wendt and H. Schroeder, *Z. Vitaminforsch.*, **4**, 206 (1935).
[223a] T. Moore, *Biochem. J.*, **34**, 1321 (1940). A. W. Davies and T. Moore, *Nature*, **147**, 794 (1941).
[224] H. v. Euler and E. Klussmann, *Z. physiol. Chem.*, **213**, 21 (1932).
[225] J. D. Greaves and C. L. A. Schmidt, *Am. J. Physiol.*, **116**, 456 (1936).
[226] C. H. Baumann and T. Moore, *Biochem. J.*, **33**, 1639 (1939).
[227] R. F. Sheets and H. C. Struck, *Proc. Am. Physiol. Soc.*, **1941**, 256.
[228] L. Képinov, *Compt. rend.*, **209**, 358 (1939); **210**, 188 (1940).

structure.[229] This is first observed in the respiratory mucosa. The mucosa in the mouth and the salivary glands is next affected, causing in turn a greater susceptibility to infections. Finally the epithelial mucosa of the eyes, the intestinal tract, the urethra, the kidney, etc., is degenerated. The epithelium of the vagina is especially sensitive to vitamin A deficiency causing the appearance of colpokeratosis, and of the so-called senile vaginitis.[230] In man a general dryness of the skin and a hyperkeratosis of the hair follicles are observed. The hair becomes dry and lusterless. Dermatosis, a form of keratosis or cutaneous eruption, occurs on the forearms and thighs and later practically all over the body.[231] Gastrointestinal disorders may occur[232] and stones may be formed in the bladder and in the kidney (urinary calculi).[233] In some experimental animals, for example, in pigs and in rats, certain neurological lesions also were observed.[234]

The various eye lesions caused by vitamin A deficiency are especially significant. An early symptom of a hypovitaminosis is often the so-called night blindness (nyctalopia or functional hemeralopia).[235] At later stages of the deficiency a softening of the cornea, followed by perforation (keratomalacia) and a dry, lusterless condition with white deposits on the scleral conjunctiva (nutritional xerophthalmia) occur. Vitamin A deficiency may also contribute to the development of myopia.[236] In pigs from sows kept on a vitamin A-depleted diet during pregnancy total blindness was observed and some animals had no eyes at all.[237] Lack of vitamin A may cause blindness by a constriction of the optic nerve associated with a stenosis of the optic canal.[238]

During times of vitamin A deficiency, the normal functioning of the reproductive system is hampered. In the female rat, the vaginal mucous membrane becomes cornified, as previously stated. There is also some adverse influence on the ovary, and normal reproduction does not occur.

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[229] S. B. Wolbach and P. R. Howe, *J. Exptl. Med.*, **42**, 753 (1925).

[230] J. W. Simpson and K. E. Mason, *Am. J. Obstet. Gynecol.*, **32**, 125 (1936).

[231] L. J. A. Löwenthal, *Arch. Dermat. Syphilol.*, **28**, 700 (1933). C. N. Frazier and C. K. Hu, *Ibid.*, **33**, 825 (1936). J. B. Youmans and N. B. Corlette, *Am. J. Med. Sci.*, **195**, 644 (1938).

[232] R. Roller, *Z. Klin. Med.*, **130**, 163 (1936). G. Will, *Klin. Wochschr.*, **15**, 1281 (1936).

[233] W. M. Kerns, *Wisconsin Med. J.*, **36**, 170 (1937). C. C. Higgins, *J. Urol.*, **36**, 168 (1936); *Surg. Gynecol. Obstet.*, **63**, 23 (1936). M. Meltzer, *N. Y. State J. Med.*, **37**, 865 (1937).

[234] S. B. Wolbach and O. A. Bessey, *Science*, **91**, 599 (1940).

[235] I. O. Park, *J. Oklahoma Med. Assoc.*, **28**, 357 (1935); **29**, 129 (1936). P. C. Jeans and Z. Zentmire, *J. Am. Med. Assoc.*, **106**, 996 (1936). P. C. Jeans, E. Blanchard and Z. Zentmire, *Ibid.*, **108**, 451 (1937). H. Frandsen, *Acta Ophthalmol.* (Supplements), 4 (1935). H. Jeghers, *New Engl. J. Med.*, **216**, 51 (1937); *Ann. Internal Med.*, **10**, 1304 (1937).

[236] H. Miller, *Am. J. Opthalmol.*, **23**, 296 (1940).

[237] J. L. Novaes, *Hora Medica*, **11**, 84 (1939).

[238] L. A. Moore, *J. Nutrition*, **17**, 443 (1939).

In the male rat, the testes degenerate in severe cases of A-avitaminosis.

Vitamin A deficiency during the period of tooth development can impair tooth structure by causing an atrophy and metaplasia of the enamel organ.[239] Thus a hypoplastic tooth with thin, defective enamel is formed. The rate of apposition of dentine is altered in vitamin A deficiency while the life span of the formative cells is not affected.[240] In rats the incision teeth lose the deep orange pigment in the enamel, which is restored upon administration of vitamin A.[241]

An early symptom of vitamin A deficiency is the decrease of the normal vitamin A and carotenoid level in blood. The normal level for children, when determined under specified conditions, is from 5.5 to 27.3 units of vitamin A per cc. of blood and the carotene level is from 3.1 to 75 units per cc. A vitamin A level of less than 3 units of vitamin A indicates avitaminosis and a value of less than 3 units of carotenes points to a state of hypovitaminosis.[242] During avitaminosis vascular lesions in practically the entire arterial system have also been observed.[243]

The state of hypovitaminosis or avitaminosis may be caused, besides, by an insufficient intake of vitamin A, also by an impaired intestinal absorption as has been found in cases of congenital obliteration of the bile ducts, fibrosis of the pancreas, celiac disease and others.[242]

(*a*) *Clinical Test Methods*

1. The Dark Adaptation Test. Night blindness, in the absence of eye disease and of a hereditary tendency in that direction, may often be a manifestation of vitamin A deficiency.[244] For actual diagnosis a number of different adaptometers,[245] which are modified photometers, are available on the market. The test is carried out by one of two methods. The patient, after a preliminary period in the dark, is exposed to subdued light and the minimum amount of light that is visible is measured. This method has been found especially useful for the determination of a vitamin A deficiency in infants.[246] In the other method the visual purple in the

[239] S. B. Wolbach and P. R. Howe, *J. Exptl. Med.*, **42**, 753 (1925).

[240] L. Schour, M. C. Smith and M. M. Hoffman, *Proc. Soc. Exptl. Biol. Med.*, **39**, 447 (1938).

[241] J. T. Irving and M. B. Richards, *Nature*, **144**, 908 (1939).

[242] C. D. May, K. D. Blackfan, J. F. McCreary and F. H. Allen, *Am. J. Diseases Children*. **59**, 1167 (1940).

[243] L. Opper, *Proc. Soc. Exptl. Biol. Med.*, **40**, 449 (1939).

[244] J. B. Feldman, *Arch. Ophthalmol.*, **12**, 81 (1934); **15**, 1004 (1936); **17**, 648 (1937); **18**, 821 (1937).

[245] G. S. Derby, P. A. Chandler and L. L. Sloan, *Arch. Ophthalmol.*, **3**, 31 (1930). S. Hecht and J. Mandelbaum, *J. Am. Med. Assoc.*, **112**, 1910 (1939). S. Hecht and S. Shlaer, *J. Optical Soc. Am.*, **28**, 269 (1938).

[246] C. Friderichsen and C. Edmund, *Am. J. Diseases Children*, **53**, 89, 1179 (1937).

retina is bleached by means of bright light, and the minimum time necessary to recover clear vision is then determined.[247]

2. Determination of the Vitamin A Content of Blood. A number of different methods have been developed for the determination of the vitamin A content of blood. Assays can be made with the serum alone since the blood corpuscles do not contain significant amounts of this vitamin. The actual determination can be carried out spectroscopically in alcohol solution after the blood has been treated with sodium sulfate.[248] It has also been recommended to test for vitamin A in blood by determining the amount of material destroyed by ultraviolet light.[249] For this purpose the blood is hydrolyzed and the non-saponifiable fraction is determined spectroscopically for its apparent vitamin A content. Irradiation with monochromatic light of 365 mμ causes specific changes of the absorption maximum of vitamin A, from which the actual amount of vitamin A present in the solution can be calculated. This method cannot be used, however, if appreciable amounts of carotene are present. The best method for determining both vitamin A and provitamins A in blood consists in a combination of a spectroscopical determination with the Carr-Price color reaction.[250] For this purpose, blood serum is extracted with a mixture of ethanol and petroleum ether.[251] The petroleum ether extract is used for a spectroscopical determination of the carotenoids[252] since bile pigments are not extracted under the specified conditions. For practical purposes the total carotenoid value is considered to consist of a 50–50 mixture of β-carotene with the inactive xanthophyll. The vitamin A content of the extract is then determined according to the Carr-Price technic (see page 78). The vitamin A content of blood can, of course, also be determined by the biological methods described for the determination of vitamin A.

3. Demonstration of Keratinized Epithelial Cells. In this test, the presence of cornified cells in scrapings from the cornea, nose and mouth and in secretions from the trachea, bronchi, kidneys and vagina is investigated by special staining methods which give the keratinized cells definite colors while leaving normal cells colorless. Several reagents have been suggested, among which is 1% methylene blue in a 3% acetic

[247] L. B. Pett, *J. Lab. Clin. Med.*, **25**, 149 (1939).

[248] A. Chevallier and Y. Choron, *Compt. rend. soc. biol.*, **118**, 889 (1935).

[249] A. Chevallier, Y. Choron and R. Matheron, *Ibid.*, **127**, 541 (1938).

[250] C. D. May, K. D. Blackfan, J. F. McCreary and F. H. Allen, *Am. J. Diseases Children*, **59**, 1167 (1940).

[251] S. W. Clausen and A. B. McCoord, *J. Pediatrics*, **13**, 635 (1938).

[252] W. S. Ferguson, *Analyst*, **60**, 680 (1935).

acid-water solution (Mallory's stain for diphtheria bacilli) which dyes the pathological cells deep red.[253]

4. Vitamin A Absorption Test. The efficiency of the intestinal absorption of vitamin A is studied in this test.[254] A standardized test dose of vitamin A is given by mouth and the vitamin level of the patient's blood is assayed before and at definite intervals after the vitamin intake. An average rise of at least 50 to 130 units of vitamin A per cc. should be observed in from three to five hours, otherwise a subnormal efficiency of the intestinal absorption is indicated.

25. Hypervitaminosis

No indications are known for any toxic effects of excessive doses of provitamins A. It can be assumed that a regulatory mechanism in the body takes care of handling amounts of provitamin A which are in excess of the animal's actual needs.

The toxicity of pure crystalline vitamin A has not been investigated so far. Using vitamin A concentrates, doses in excess of 100,000 units per day are harmful for rats.[255, 256] The pathological changes observed include retarded growth, hemorrhages, especially in the mucous membranes, and abnormal rarefaction and fragility of the bones.

26. Vitamin A Requirements

All mammals, birds and fish which have been investigated, utilize vitamin A. This does not mean, however, that all animals need this factor. Thus, it has been demonstrated[257] that the cockroach (*Blatella germanica* L.) needs no provitamin or vitamin A in the diet and is able to function normally throughout life without this vitamin. It is, therefore, suspected that other animals, such as certain insects, do not require vitamin A.

Double requirement standards must be recognized, one for vitamin A and one for carotene (provitamin A) since the International Units of carotene and of vitamin A are equal only under the specified conditions of the biological test, but not in the metabolism of a normal growing organism.[258]

The vitamin A requirements of adult mammals can be correlated to the body weight. Fairly uniform requirements have been established for

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[253] K. D. Blackfan and S. B. Wolbach, *J. Pediatrics*, **3**, 676 (1933).
[254] J. Chesney and A. B. McCoord, *Proc. Soc. Exptl. Biol. Med.*, **31**, 887 (1934).
[255] E. B. Vedder and C. Rosenberg, *J. Nutrition*, **16**, 57 (1938).
[256] I. Ikegaki, *Z. Vitaminforsch.*, **7**, 113 (1938).
[257] R. E. Bowers and C. M. McCay, *Science*, **92**, 291 (1940).
[258] H. R. Guilbert, C. E. Howell and G. H. Hart, *J. Nutrition*, **19**, 91 (1940).

man,[259] horse,[258] dog,[260] cattle,[258, 261] sheep,[258, 261] swine,[258, 261, 262] rabbit,[263] rat[264] and hedgehog.[265] All show approximately the same requirements, namely, 25 γ of β-carotene (corresponding to 40 International Units) or 4 γ (20 International Units) of vitamin A per kilogram of body weight.[258, 261, 266] These values represent the minimum for normal growth without showing any clinical symptoms of vitamin A deficiency, but little or no storage of the vitamin occurs at these levels. About three times the minimum amount of vitamin A (12 γ or 60 International Units) and five times the minimum amount of β-carotene (125 γ or 200 International Units) is considered minimum for significant storage and reproduction. The optimum vitamin A requirement for an average adult is then about 5000 International Units of vitamin A or 15,000 International Units of carotene. Increased amounts are recommended for pregnant and nursing women and during adolescence.[267] The allowances as recommended by the Food and Nutrition Board of the National Research Council are reprinted on page 613.

The vitamin A requirements of animals other than mammals are less well known. Birds apparently need vitamin A in an amount of the same order of magnitude as mammals. Growing chicks need about 95 to 125 γ of carotene per day[268] (or about 1800 International Units of vitamin A per pound of feed) but for laying stock 200–500 γ of carotene have been recommended.[269] (This amount corresponds to a diet of yellow corn or to an addition of 2.5–5% of a good grade alfalfa meal.)

[259] See the discussion by L. E. Booher, *J. Am. Med. Assoc.*, **110**, 1920 (1938).

[260] P. D. Crimm and D. M. Short, *Am. J. Physiol.*, **118**, 477 (1937).

[261] H. R. Guilbert and G. E. Hart, *J. Nutrition*, **10**, 409 (1935) H. R. Guilbert, R. F. Miller and E. H. Hughes, *Ibid.*, **13**, 543 (1937).

[262] H. Mollgaard, *Biedermanns Zentr.* (B. Tierernähr.), **10**, 214 (1938).

[263] P. H. Phillips and G. Bohstedt, *J. Nutrition*, **15**, 309 (1938).

[264] R. Kuhn and H. Brockmann, *Klin. Wochschr.*, **12**, 972 (1933).

[265] P. Swomalainen, *Skand. Arch. Physiol.*, **83**, 104 (1939).

[266] J. T. Irving and M. B. Richards, *Nature*, **144**, 908 (1939).

[267] Technical Commission for the Study of Nutrition, Health Organization of the League of Nations, *Geneva Bulletin*, **7**, April, 1939.

[268] R. M. Sherwood and G. S. Fraps, *Texas Agr. Exptl. Station Bull.*, **M528**, Sept., 1936

[269] J. K. Williams, C. E. Lampman and D. W. Bolin, *Poultry Sci.*, **18**, 268 (1939).

VITAMIN B_1—
THIAMIN

VITAMIN B_1—THIAMIN[1]

1. Nomenclature and Survey

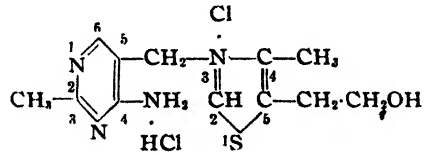
Names:

Thiamin: American nomenclature.[2]
Aneurin: European nomenclature.[3]
Oryzanin: Japanese nomenclature.[4]
Torulin: Historical name.[5]
Polyneuramin: Historical name.[6]
Vitamin F: Abandoned term.[7]
The antineuritic, anti-beriberi vitamin.

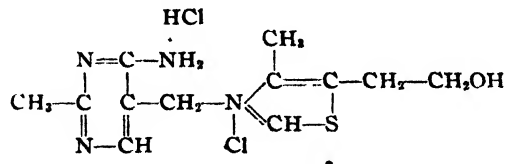
Chemical name:

4-Methyl-5-β-hydroxy-ethyl-*N*-{[2-methyl-4-amino-pyrimidyl-(5)]-methyl}-thiazolium-chloride-hydrochloride.

Structure:*



* Some authors prefer to write the formula of vitamin B_1 as follows:



No agreement exists as to the manner of writing the pyrimidine nucleus, except in purine derivatives. The preferred form is the hexagonal, to which the eye is accustomed and which best illustrates the conversion of vitamin B_1 to thiochrome.

[1] R. R. Williams and T. D. Spies, *Vitamin B_1*, New York, 1938.
[2] "Council on Pharmacy and Chemistry," *J. Am. Med. Assoc.*, **109**, 952 (1937).
[3] B. C. P. Jansen, *Nature*, **135**, 267 (1935).
[4] U. Suzuki, T. Shimamuri and S. Odake, *Biochem. Z.*, **43**, 89 (1912).
[5] C. S. Edie, W. H. Evans, B. Moore, G. C. E. Simpson and A. Webster, *Biochem. J.*, **6**, 234 (1912).
[6] R. L. Jones, *Science*, **68**, 480 (1928).
[7] H. C. Sherman and J. H. Axtmayer, *J. Biol. Chem.*, **75**, 207 (1927).

Empirical Formula:

$C_{12}H_{17}ON_4SCl \cdot HCl$.

Efficacy:

1 g. = 333,000 International Units.

2. Chronology

1885 TAKAKI[8] prevented the occurrence of beriberi in the Japanese navy by changing the dietary ration.

1893-1897 EIJKMAN[9] produced experimental polyneuritis (beriberi) in fowls by a diet consisting exclusively of polished rice and prevented the disease by dietary means.

1912 FUNK[10] cured rats with dietary polyneuritis by the administration of water extracts of rice bran.

1926 JANSEN and DONATH[11] isolated the vitamin in crystalline form from rice bran.

1931 WINDAUS, TSCHESCHE, RUHKOPF, LAQUER and SCHULTZ[12] isolated the *pure* vitamin B_1 from yeast and established its empirical formula.

1936 WILLIAMS and independently GREWE elucidated the chemical structure of vitamin B_1. In the same year, the synthesis of this vitamin was accomplished by WILLIAMS and CLINE, and by ANDERSAG and WESTPHAL.

1937 LOHMANN and SCHUSTER isolated crystalline cocarboxylase from yeast and proved its constitution as the pyrophosphoric ester of vitamin B_1.

3. Occurrence

Vitamin B_1 is present in many plants. Vegetables, fruits and nuts contain small amounts; ripe peas and beans are rich sources; but vitamin B_1 is found most prevalently in outside bran coats of grains (rice) and in yeast (baker's yeast and brewer's yeast). Generally speaking, vitamin B_1 is present in high concentration in seeds, that is, in the nutritive material for the plant germ (see page 133). In most leaves the concentration of vitamin B_1 amounts to a constant value of about 25 International Units per 100 g., regardless of the botanical family.[13]

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[8] K. Takaki, *Sei-i-Kai Med. J.*, August, 1885; April, 1886; 6, 73 (1887); *Lancet*, 1906, I, 1369, 1451, 1520.

[9] C. Eijkman, *Arch. path. Anat. (Virchow's)*, 148, 523 (1897).

[10] C. Funk, *J. Physiol.*, 45, 75 (1912).

[11] B. C. P. Jansen and W. F. Donath, *Mededeel. Dienst Volksgesondheid Nederland.-Indië*, 1926 (Pt. I), 186; *Chem. Weekblad*, 23, 201 (1926); *Koninkl. Akad. Wetenschappen Amsterdam, Wisk. Naturk. Afd.*, 35, 923 (1926).

[12] A. Windaus, R. Tschesche, H. Ruhkopf, F. Laquer and F. Schultz, *J. physiol. Chem.*, 204, 123 (1932); *Nachr. Ges. Wiss. Göttingen., Math.-physik. Klasse*, 1932, 207, 342.

[13] M. Pyke, *Biochem. J.*, 34, 330 (1940).

Some microorganisms are able to synthesize vitamin B_1. For example, certain bacteria exist in the intestinal tract of rats[14] (especially in the colon) and of nursing children (especially in the great gut) that synthesize vitamin B_1, which is then found in the feces. Bacteria which produce vitamin B_1 also live in the rumen of cattle, sheep, etc.[15]

Vitamin B_1 is widely distributed in the animal organism in different organs (liver, kidney) and in muscles (especially in the heart).[16] The actual amount is very small and varies greatly in different species. Pork muscles, for example, contain about eight times as much vitamin B_1 as beef muscles.[17] Storage of large amounts does not occur in the animal organism.

Vitamin B_1 occurs in nature as the free compound or in the form of its salts, as vitamin B_1-protein complex,[18] as vitamin B_1-pyrophosphoric acid ester (cocarboxylase) and as vitamin B_1-phosphorus-protein complex It is suspected that other vitamin B_1-containing compounds, for example, the monophosphate and other esters exist in tissues. The relative amounts of these forms vary considerably in different sources. Milk contains predominantly the free vitamin and the vitamin-protein complex.[19] The first colostrum contains practically no protein complexes. In skeletal and in heart muscle the amount of the free vitamin is greater than the phosphorylated compound, whereas in brain and liver the cocarboxylase (and its protein complex) occurs predominantly.[20]

4. Isolation

Since vitamin B_1 is water-soluble it is extracted, for example, from rice polish or yeast with cold water, which is brought to pH 4.5 by the addition of mineral acid. From the aqueous solution, the vitamin is adsorbed on activated fuller's earth or charcoal at pH 6.5.[21] Riboflavin, which is also present in the water extract, is not adsorbed under these conditions.[22] The vitamin is then extracted from the charcoal with dilute acids or from the fuller's earth with dilute alkali. By these methods, however, a con-

[14] *J. Hyg.*, **27**, 70 (1927).

[15] S. I. Bechdel, H. E. Honeywell, R. A. Dutcher and M. H. Knutsen, *J. Biol. Chem.*, **30**, 231 (1928).

[16] H. G. K. Westenbrink, *Arch. néerland. physiol.*, **17**, 560 (1932); **19**, 116 (1932).

[17] C. A. Elvehjem, W. C. Sherman and A. Arnold, *J. Biol. Chem.*, **109**, XXIX (1935). R. Hoagland, *J. Agr. Research*, **83**, 431 (1929).

[18] J. Houston and S. K. Kon, *Nature*, **143**, 558 (1939).

[19] J. Houston, S. K. Kon and S. Y. Thompson, *J. Soc. Chem. Ind.*, **58**, 651 (1939). J. Houston and S. K. Kon, *Nature*, **143**, 558 (1939).

[20] S. Ochoa and R. A. Peters, *Biochem. J.*, **32**, 1501 (1938).

[21] H. W. Kinnersley, J. R. O'Brien and R. A. Peters, *Ibid.*, **27**, 232 (1933).

[22] S. Ohdake, *J. Agr. Chem. Soc. Japan*, **10**, 409 (1934). R. R. Williams, R. E. Waterman and J. C. Keresztesy, *J. Am. Chem. Soc.*, **56**, 1187 (1934).

siderable amount of vitamin is lost. If, however, quinine salt solutions or salts of other organic bases are used, about five times as much vitamin is obtained[22] (approximately 90% of the vitamin B_1 originally present).

Impurities can be precipitated by barium hydroxide,[22] benzoyl chloride in the presence of an excess of sodium bicarbonate,[23] silver nitrate in acid solution[24] and by many other reagents. The vitamin itself is precipitated by silver nitrate at pH 7.5,[22, 24] phosphotungstic acid at pH 4.5–5.5,[24, 25, 26] silicotungstic acid[27, 28] benzene-sulfo-chloride,[29] picrolonic acid,[30, 31, 32] Rufian acid,[30] Reinecke acid,[30] gold chloride in aqueous solution,[24, 30, 33, 34] platinum chloride in alcoholic solution,[24, 30, 35] and mercuric chloride in the presence of sodium acetate or carbonate,[30] (but not by mercuric sulfate in acid solution,[36]) etc.

After the elution of the vitamin from the adsorbent, a combination of different precipitations is carried out. The vitamin B_1 is thus obtained as the hydrochloride. Four hundred and fifty pounds of rice polish or 2000 lbs. of yeast yield about 1 g. of the vitamin. For practical isolation procedures see page 118.

Vitamin B_1 and riboflavin, which usually occur together, are separated by precipitation of the riboflavin from an aqueous neutral or slightly alkaline solution with lead acetate, whereby vitamin B_1 remains in solution.[35, 37, 38] Another method is a fractional adsorption, first on charcoal at pH 4–5, which adsorbs all the riboflavin, and then on fuller's earth, which adsorbs all the vitamin B_1.[39] On the other hand, vitamin B_1 may first be adsorbed from solutions of pH 3 on silica gel[40] or fuller's earth.[41] The riboflavin remains in solution and can be recovered from the filtrate.

[22] A. Seidell, *J. Biol. Chem.*, **82**, 633 (1929).
[23] B. C. P. Jansen and W. F. Donath, *Mededeel. Dienst Volksgezondheid Nederland.-Indië*, **1926** (Pt. I) 186; *Chem. Weekblad*, **23**, 201 (1926); *Koninkl. Akad. Wetenschappen Amsterdam, Wisk Naturk. Afd.*, **35**, 923 (1926).
[24] H. W. Kinnersley, J. R. O'Brien and R. A. Peters, *Biochem. J.*, **27**, 232 (1933).
[25] H. W. Kinnersley and R. A. Peters, *Ibid.*, **24**, 1856 (1930).
[26] B. C. P. Jansen, J. P. Wibaut, P. J. Hubers and P. W. Wiardi, *Rec. trav. chim.*, **52**, 366 (1933).
[27] B. C. P. Jansen, *Ibid.*, **48**, 984 (1929).
[28] A. G. van Veen, *Z. physiol. Chem.*, **208**, 125 (1932).
[29] A. Windaus, R. Tschesche, H. Ruhkopf, F. Laquer and F. Schultz, *J. physiol. Chem.*, **204**, 123 (1932); *Nachr. Ges. Wiss. Göttingen, Math.-physik. Klasse*, **1932**, 342.
[30] S. Ohdake, *Proc. Imp. Acad. (Tokyo)*, **7**, 102 (1931); **8**, 179 (1932); **10**, 95 (1934).
[31] A. Seidell and M. I. Smith, *J. Am. Chem. Soc.*, **55**, 3350 (1933).
[32] B. C. Guha, *Biochem. J.*, **25**, 931 (1931).
[33] B. C. Guha and J. C. Drummond, *Ibid.*, **23**, 880 (1929).
[34] B. C. P. Jansen, J. P. Wibaut, P. J. Hubers and P. W. Wiardi, *Rec. trav. chim.*, **52**, 366 (1933).
[35] B. C. P. Jansen, H. W. Kinnersley, R. A. Peters and V. Reader, *Biochem. J.*, **24**, 1824 (1930).
[36] H. Chick and M. H. Roscoe, *Ibid.*, **23**, 504 (1929).
[37] J. L. Rosedale, *Ibid.*, **21**, 1266 (1927).
[38] R. D. Greene and A. Black, *J. Am. Chem. Soc.*, **59**, 1395 (1935).
[39] P. A. Levene, *J. Biol. Chem.*, **79**, 465 (1928); *Science*, **71**, 668 (1930).
[40] W. D. Salmon, N. B. Guerrant and I. M. Hays, *J. Biol. Chem.*, **80**, 91 (1928).

5. Properties

Vitamin B_1-hydrochloride is soluble in water (1 g. in 1 cc.) and alcohols (1 g. in 100 cc. of 95% alcohol or in 315 cc. absolute alcohol, or in 18 cc. of glycerin), but insoluble in ether, chloroform, benzene and acetone.[24] It is optically inactive.[30] Vitamin B_1-hydrochloride crystallizes from alcoholic aqueous solutions as the hemihydrate (colorless monoclinic needles), melting at 248–250°.[42] The bromide-hydrobromide hemihydrate occurs as rosettes of needles that melt at 229–231°.

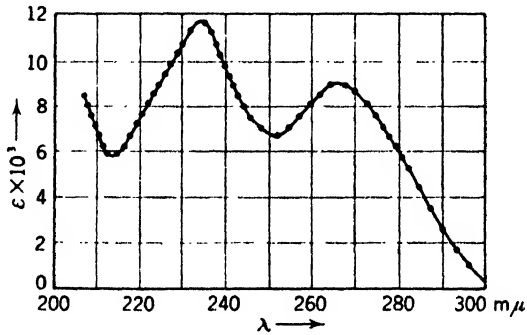

Fig. 6.—Absorption spectrum of vitamin B_1. (O. Wintersteiner, R. R. Williams and A. E. Ruehle.)

The melting points of various other salts are as follows: sulfate, m. p. 203° and 276–278°;[43] nitrate, m. p. 164–165°;[43] picrolonate (dimorphous), m. p. 165° or 229°; picrate, m. p. 208°; gold salt, m. p. 189°; rufinate, m. p. 291°.[44] All melting points are somewhat uncertain because of attendant decomposition. The free vitamin base may be obtained from the chloride in amorphous form by treatment with silver oxide. On standing in air of average humidity vitamin B_1 crystals absorb humidity in an amount of one mol. Vitamin B_1 crystals and solutions have a slight yeast-like or nutty odor.

The ultraviolet absorption spectrum of the vitamin hydrochloride shows two bands at a pH of 7 (or greater) at 235 mμ and 267 mμ, respectively,[45]

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[42] J. K. Cline, R. R. Williams and J. Finkelstein, *J. Am. Chem. Soc.*, **59**, 1052 (1937). R. R. Williams and J. K. Cline, *Ibid.*, **59**, 216 (1937).

[43] H. W. Kinnersley, J. R. O'Brien and R. A. Peters, *Biochem. J.*, **29**, 701 (1935).

[44] R. R. Williams, *Ergeb. Vitamin Hormonforsch.*, **1**, 217 (1938).

[45] O. Wintersteiner, R. R. Williams, and A. E. Ruehle, *J. Am. Chem. Soc.*, **57**, 517 (1935).

and only one band at pH 5.5 (or less) at 245 mμ to 247 mμ.[46, 47] Thus, the absorption spectrum of vitamin B_1 is a function of the hydrogen-ion concentration.[48] This behavior must be attributed to the pyrimidine component of the vitamin molecule.[49, 50]

Vitamin B_1 is quite stable in strongly acid solution. The pure vitamin B_1-hydrochloride in aqueous solution of pH 3.5 can be heated to 120° C. without any decomposition.[51] In weak acid solutions, however, the molecule decomposes. At pH 5–6, for example in a sulfite solution, vitamin B_1 undergoes a cleavage, which will be described later, and loses its activity. The same is true for sodium acetate or barium nitrite solutions.[52] In neutral and alkaline solution vitamin B_1 is extremely sensitive to heat. The amount of antineuritic activity in foods, however, is destroyed only to a small degree by cooking. This may be due to the fact that the vitamin occurs in combined form to a greater extent than in the free form.

Vitamin B_1 is very sensitive to both oxidation and reduction. By mild oxidation, even by allowing an alcoholic solution to stand for several months, thiochrome is formed (see page 120).[53] Under physiological conditions a disulfide is obtained (see page 143). By mild reduction a dihydro-compound is formed, which will be discussed later (see page 144).

6. Chemical Constitution and Synthesis

The empirical formula of vitamin B_1-hydrochloride is $C_{12}H_{18}ON_4SCl_2$. During the attempts to isolate the vitamin, it was observed that in the presence of sodium sulfite the vitamin activity disappears.[54, 55] When the vitamin B_1-hydrochloride is kept at room temperature in contact with a solution of sodium sulfite containing sufficient excess of sulfurous acid to bring the solution to pH 4.8–5, the vitamin molecule undergoes fission, giving quantitatively a sparingly soluble acidic product, $C_6H_9O_3N_3S$ (II), and a chloroform-soluble basic product, C_6H_9ONS (III).

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[46] A. Smakula, *Z. physiol. Chem.*, **230**, 231 (1934).

[47] R. A. Peters and I. St. Philpot, *Proc. Roy. Soc. (London)*, **B113**, 48 (1933).

[48] E. R. Holiday, *Biochem. J.*, **29**, 718 (1935).

[49] R. R. Williams, A. E. Ruehle and J. Finkelstein, *J. Am. Chem. Soc.*, **59**, 526 (1937).

[50] F. M. Uber and F. Verbrugge, *J. Biol. Chem.*, **134**, 273 (1940).

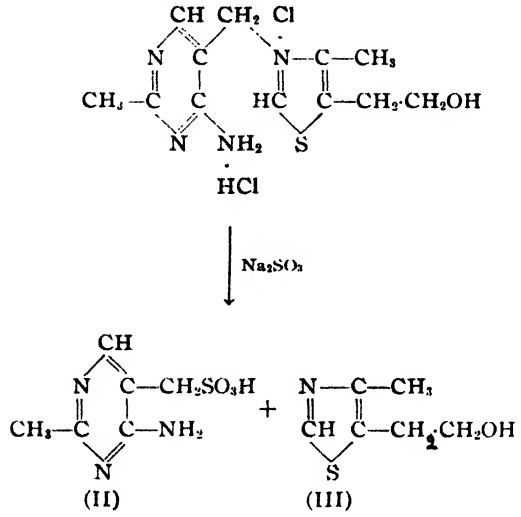
[51] H. Molitor and W. L. Sampson, *Merck Jahresberichte*, **1936**.

[52] R. R. Williams, "Chemistry of thiamin," *J. Am. Med. Assoc.*, **110**, 730 (1938).

[53] H. W. Kinnersley, J. R. O'Brien and R. A. Peters, *Biochem. J.*, **29**, 701 (1935).

[54] R. R. Williams, *J. Am. Chem. Soc.*, **57**, 229 (1935).

[55] R. R. Williams, R. E. Waterman, J. C. Keresztesy and E. R. Buchman, *Ibid.*, **57**, 536 (1935).



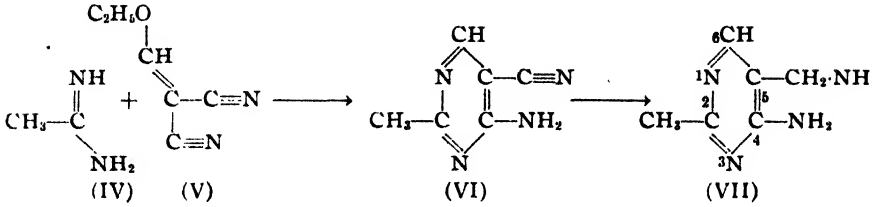
(a) *The Pyrimidine Part*

The amino-sulfonic acid II has the empirical formula $C_6H_9O_3N_3S$ and is insoluble in organic solvents, sparingly soluble in water and infusible up to 440° C. On reaction with hydrochloric acid, ammonia is split out with the formation of an oxy-sulfonic acid, thus indicating the presence of a primary amino-group in the amino-sulfonic acid. On heating with water at 200°, sulfuric acid is obtained and on fusing with alkali, sulfurous acid is obtained, both reactions indicating the presence of a sulfonic acid group. This group is not present in the vitamin, but is introduced during the sulfite cleavage.

The presence of the pyrimidine ring was suspected by comparison with synthetic imidazole[56] and pyrimidine[57] compounds. The ultraviolet absorption spectrum of the cleavage product showed the characteristics of a 4-amino-pyrimidine.[58, 59, 60] By oxidation of vitamin B_1-sulfate with barium permanganate or by the action of liquid ammonia[61] a diacidic base, $C_6H_{10}N_4$ (VII), was obtained,[62] which by synthesis[63] was identified as 2-

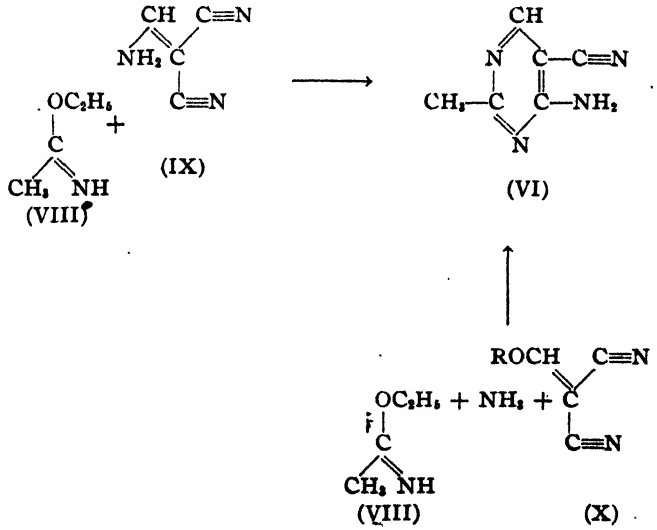
[56] A. Windaus, R. Tschesche and R. Grewe, *Z. physiol. Chem.*, **228**, 27 (1928).
[57] R. R. Williams, E. R. Buchman and A. E. Ruehle, *J. Am. Chem. Soc.*, **57**, 1093 (1935).
[58] R. R. Williams, *Ibid.*, **57**, 229 (1935).
[59] A. Smakula, *Z. physiol. Chem.*, **230**, 231 (1934).
[60] F. F. Heyroth and J. R. Loofbourow, *Bull. Basic Sci. Research*, **4**, 35 (1932).
[61] R. R. Williams, *J. Am. Chem. Soc.*, **58**, 1063 (1936). J. K. Cline, R. R. Williams, A. E. Ruehle and R. E. Waterman, *Ibid.*, **59**, 530 (1937).
[62] A. Windaus, R. Tschesche and R. Grewe, *Z. physiol. Chem.*, **237**, 98 (1935).
[63] R. Grewe, *Ibid.*, **242** 89 (1936)

methyl-4-amino-5-amino-methyl-pyrimidine. The synthesis was carried out as follows:



Acetamidine (IV) was reacted with ethoxy-methylene-malonic-nitrile (V), yielding 2-methyl-4-amino-5-cyano-pyrimidine (VI), which compound was converted into 2-methyl-4-amino-5-amino-methyl-pyrimidine (VII) by catalytic hydrogenation.

Variations of this synthesis consist in condensing acetimido-ethyl-ether-hydrochloride (VIII) with amino-methylene-malonic-nitrile (IX) to give 2-methyl-4-amino-5-cyano-pyrimidine (VI).[64] Or acetimido-ethyl-ether-hydrochloride is condensed with ammonia and ethoxy-methylene-malonic-nitrile (X).[65]

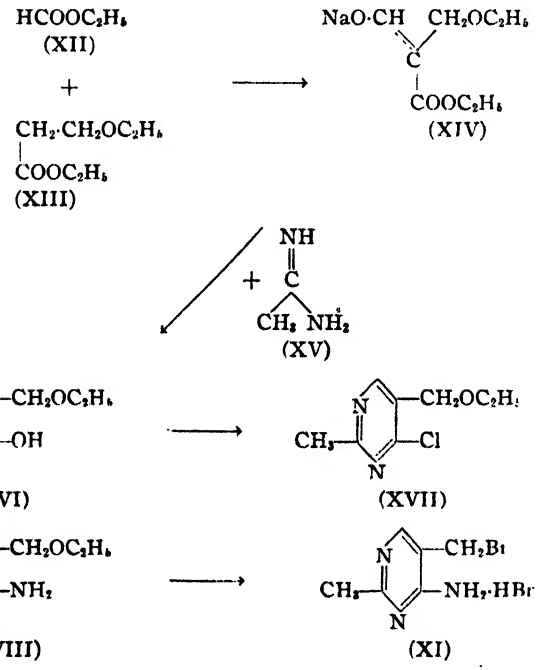


A number of different methods have been worked out for synthesizing 2-methyl-4-amino-5-bromo-methyl-pyrimidine (XI), which is used in the

[64] O. Hromatka, G. P. 667,990.
[65] O. Hromatka, G. P. 670.635.

synthesis of. vitamin B_1 (see page 116). Grewe converted the 5-amino-methyl-compound (VII) (see above) into the corresponding 5-bromo-methyl-compound (XI).[66].

Cline, Williams and Finkelstein obtained the compound (XI) in the following way:[67] By condensing ethyl-formate (XII) and β-ethoxy-ethyl-

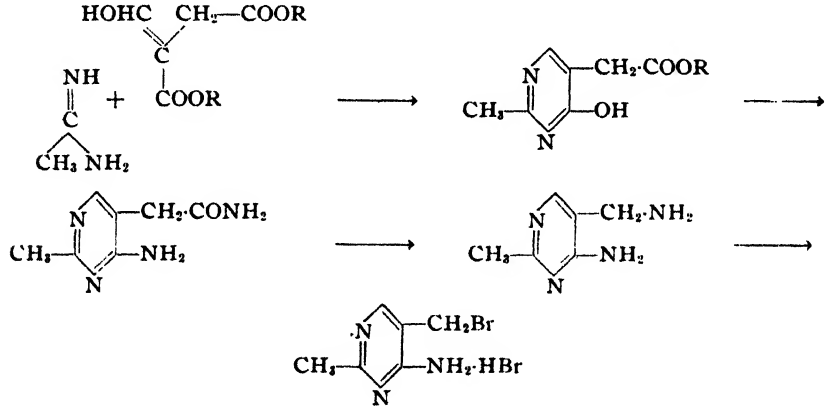


propionate (XIII) with sodium, sodium-formyl-β-ethoxy-ethyl-propionate was obtained (XIV), which by condensation with acetamidine-hydrochloride (XV) yielded 2-methyl-4-hydroxy-5-ethoxy-methyl-pyrimidine (XVI). The hydroxyl-group in 4-position was converted into the chloride (XVII) by phosphorus-oxychloride, and finally into an amino-group (XVIII) by ammonia in alcohol. By the action of hydrobromic acid, the

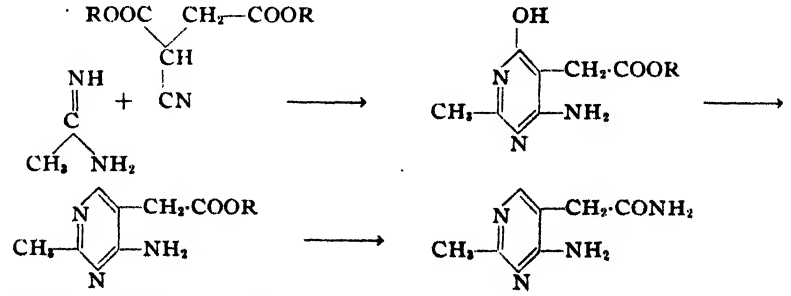
[66] See also T. Imai and K. Makino, *Z. physiol. Chem.*, 252, 76 (1938).
[67] J. K. Cline, R. R. Williams and J. Finkelstein, *J. Am. Chem. Soc.*, 59, 1052 (1937).

ethoxy-group in the methyl in 5-position was replaced by bromine, yielding the 2-methyl-4-amino-5-bromo-methyl-pyrimidine-hydrobromide (XI).

Andersag and Westphal have chosen another route:[68] By condensation of acetamidine with formyl-ethyl-succinate, 2-methyl-4-oxypyrimidine-5-ethyl-acetate is formed. The hydroxyl-group is replaced by chlorine and the chloro-compound on reaction with liquid ammonia yields 2-methyl-4-amino-pyrimidine-5-acetamide. By a Hoffmann degradation 2-methyl-4-amino-5-methyl-amino-pyrimidine is obtained nearly quantitatively. By the action of nitrous acid on the diamine, only the aliphatic amino-group is converted into the corresponding alcohol. Hydrobromic acid treatment yields finally the 2-methyl-4-amino-5-bromo-methyl-pyrimidine-hydrobromide.



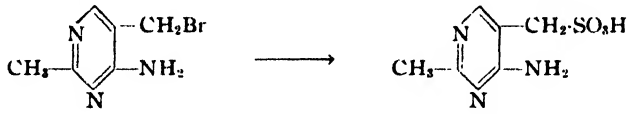
A modification of this method consists in the condensation[69] of acetamidine with ethyl-cyano-succinate, yielding 2-methyl-4-amino-6-oxypyrimidine-5-ethyl-acetate. The hydroxyl-group in 6-position is converted into a



[68] H. Andersag and K. Westphal, *Ber.*, 70, 2035 (1937).
[69] H. Andersag and K. Westphal, G. P. 671,787.

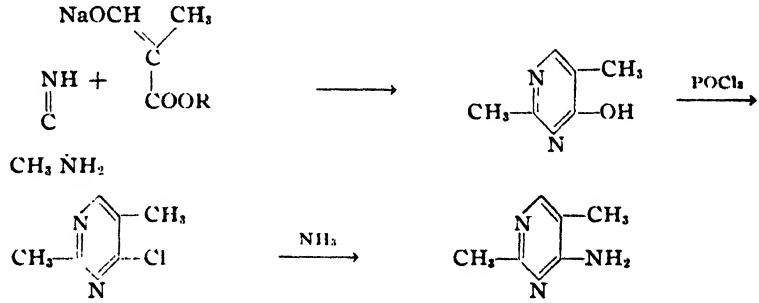
chlorine group and the chlorine is eliminated by zinc dust yielding the 2-methyl-4-amino-pyrimidine-5-ethyl-acetate. Ammonia treatment yields then the 5-acetamide-compound of the previously mentioned synthesis.

As has been mentioned before, the sulfite cleavage product of vitamin B_1 that contains the pyrimidine ring has the formula (II). This acid was synthesized by Grewe[70] from the previously described 2-methyl-4-amino-5-bromo-methyl-pyrimidine by heating with an acid bisulfite solution:



(II)

By reduction with sodium in liquid ammonia, the amino-sulfonic acid (II) gives a base,[71] $C_6H_9N_3$, the picrate of which proved to be identical with that of 2,5-dimethyl-4-amino-pyrimidine. The latter compound was synthesized[72] by condensing acetamidine and sodium-formyl-ethyl-propionate, transforming the obtained 2,5-dimethyl-4-hydroxy-pyrimidine into the corresponding 4-chloro-compound and exchanging the chlorine with ammonia:



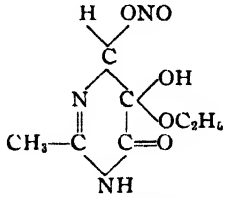
The oxy-sulfonic acid, 2-methyl-4-hydroxypyrimidine-5-methyl-sulfonic acid, mentioned before as the reaction product of hydrochloric acid with the amino-sulfonic acid (II), has been obtained by reacting the synthetically obtained 2-methyl-4-hydroxy-5-ethoxy-methyl-pyrimidine with sodium sulfite.[71]

[70] R. Grewe, *Z. physiol. Chem.*, 242, 89 (1936).

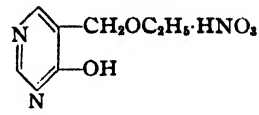
[71] J. K. Cline, R. R. Williams, A. E. Ruehle and R. E. Waterman, *J. Am. Chem. Soc.*, 59, 530 (1937).

[72] R. R. Williams, A. E. Ruehle and J. Finkelstein, *Ibid.*, 59, 526 (1937).

By mild oxidation of vitamin B_1 with nitric acid another pyrimidine derivative is obtained. It is an ethyl ester of the formula $C_7H_{11}N_3O_5$, and may have the constitution of (XIX) according to Grewe[73, 74] or of (XX) according to Williams.[75]



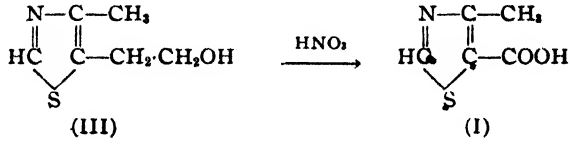
(XIX)



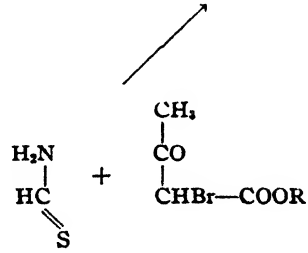
(XX)

(*b*) *The Thiazole Part*

The other reaction product of the sulfite cleavage of vitamin B_1 is the base C_6H_9ONS (III). The sulfur in this compound is, in differentiation to the sulfur in the pyrimidine part of the cleavage product, in the same form as in the vitamin. By oxidation of the base with nitric acid, the side chain is degradated, yielding the acid (I), which is also obtained by direct



(III) (I)



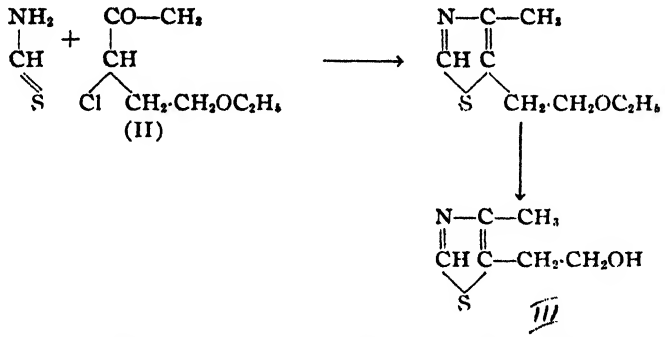
nitric acid oxidation of the vitamin,[76] and is identical with the synthetically [77, 78] prepared 4-methyl-thiazole-5-carboxylic acid.[79, 80] This syn-

[73] A. Windaus, R. Tschesche and R. Grewe, *Z. physiol. Chem.*, **237**, 98 (1935).
[74] R. Grewe, *Ibid.*, **242**, 89 (1936).
[75] R. R. Williams, *J. Am. Chem. Soc.*, **58**, 1063 (1936).
[76] A. Windaus, R. Tschesche and R. Grewe, *Z. physiol. Chem.*, **228**, 27 (1928).
[77] M. Wohmann, *Ann.*, **259**, 299 (1890).
[78] M. L. Tomlinson, *J. Chem. Soc.*, 1935, 1030.
[79] H. T. Clarke and S. Gurin, *J. Am. Chem. Soc.*, **57**, 1876 (1935).
[80] R. R. Buchman, *Ibid.*, **58**, 1803 (1936).

thesis can be carried out by condensing thioformamide with α-bromo-acetoacetic-ester.

The presence of a thiazole ring in vitamin B_1 was indicated by ultraviolet absorption studies, by the stability against nitric acid and the loss of sulfur by the action of alkaline plumbite.

The thiazole part of the sulfite cleavage of vitamin B_1 is an alcohol and has one carbon atom more than the acid obtained by its nitric acid oxidation. The alcohol is 4-methyl-5-β-hydroxy-ethyl-thiazole (III). The presence of the hydroxyl-group was proved[81] by the formation of a chloride and a *p*-nitrobenzoate. Since this benzoate was found to be basic, the presence of a tertiary nitrogen was suspected. This was further proved by the formation of a quaternary salt with methyl-iodide. The primary nature of the alcoholic group was indicated by the result of its nitric acid oxidation, by the optical inactivity of the alcohol and the failure of the iodoform test. These indications were substantiated by synthesis. The alcohol has been synthesized[82] by condensation of thioformamide with the chloro-ketone (II) (methyl-α-chloro-γ-ethoxy-propyl-ketone) and hydrolysis to the corresponding alcohol.



A variety of modifications of this method are also possible. Todd and co-workers[83, 84, 85] used, instead of the chloro-ethyl-ether (II), the corresponding chloro-ethyl-acetate, the ester group of which can be saponified with more facility than the ether group. Buchman[86] simplified the preparation by condensing acetoacetic acid ester with ethylene oxide to α-acetyl-butyro-lactone, chlorinating the lactone in α-position with sulfuryl

[81] E. R. Buchman, R. R. Williams and J. C. Keresztesy, *J. Am. Chem. Soc.*, **57**, 1849 (1935).

[82] H. T. Clarke and S. Gurin, *Ibid.*, **57**, 1876 (1935).

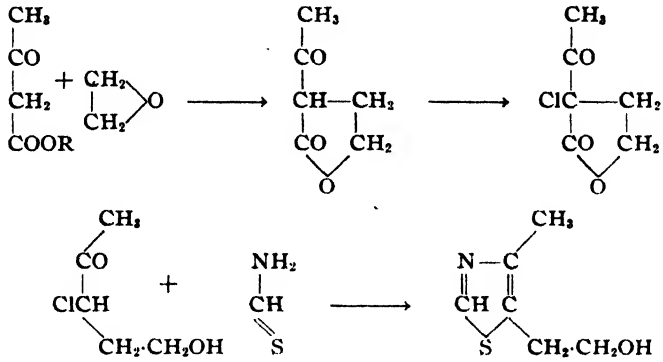
[83] G. Barger, F. Bergel and A. R. Todd, *Nature*, **136**, 259 (1935); *Ber.*, **68**, 2257 (1935).

[84] A. R. Todd, F. Bergel and Karimullah, *Ber.*, **69**, 217 (1936).

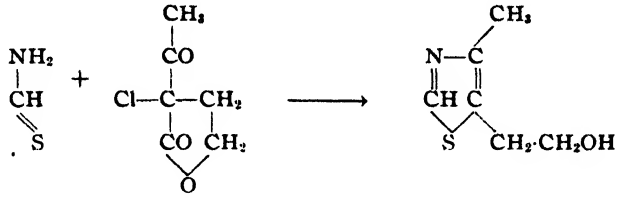
[85] A. R. Todd, F. Bergel, Karimullah and R. Keller, *J. Chem. Soc.*, **1937**, 361. A. R. Todd, F. Bergel and A. Jacob, *Ibid.*, **1936**, 1555.

[86] E. R. Buchman, *J. Am. Chem. Soc.* **58**, 1803 (1936).

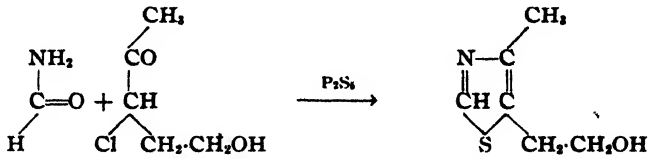
chloride and obtaining by hydrolysis γ-chloro-γ-acetyl-propyl-alcohol, which by condensing with thioformamide yielded the 4-methyl-5-β-hydroxy-ethyl-thiazole.



A further simplification was made by Wenz,[87] who found that the α-chloro-α-acetyl-butyro-lactone can be condensed with thioformamide directly to the thiazole compound:



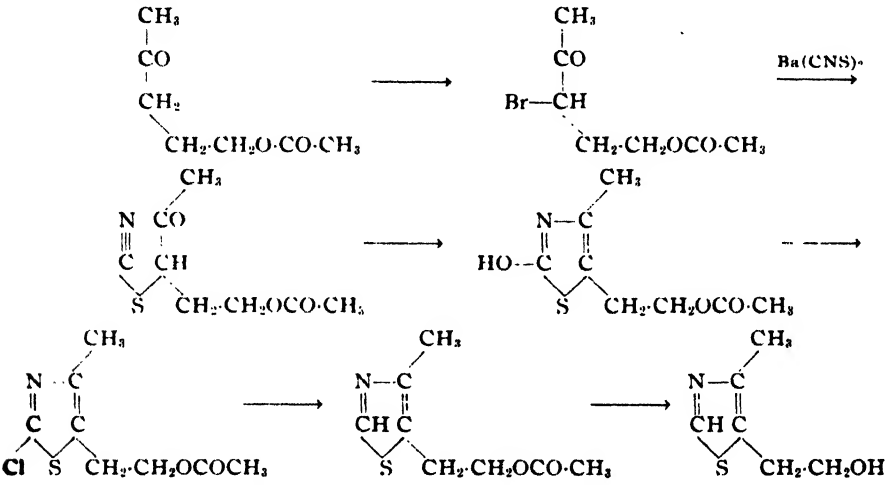
Another modification, by Hromatka,[88] was the condensation of formamide with γ-chloro-γ-acetyl-propyl-alcohol in the presence of phosphorus-pentasulfide:



A different synthesis was announced by Andersag and Westphal:[89] γ-aceto-propyl-acetate is brominated and the bromo-compound condensed with barium-thiocyanate, forming γ-thiocyano-γ-aceto-propyl-acetate. This rearranges in acid solution to the isomeric 2-hydroxy-4-methyl-5-

[87] A. Wenz, G. P. 664,789.
[88] O. Hromatka, U. S. P. 2,160,867.
[89] H. Andersag and K. Westphal, *Ber.*, 70, 2035 (1937).

acetoxy-ethyl-thiazole. The hydroxyl-group in 2-position is converted into chlorine by phosphorus-oxychloride and the chloride by means of zinc and acetic acid is transformed into 4-methyl-5-acetoxy-ethyl-thiazole, which by saponification yields 4-methyl-5-β-hydroxy-ethyl-thiazole.



(c) *The Connection of the Pyrimidine and the Thiazole Part*

The pyrimidine compound and the thiazole compound are connected in the vitamin B_1 molecule by a methylene bridge. The presence of the methylene group was suspected by the ultraviolet absorption of the vitamin,[90] and was proved by synthesis. The pyrimidine portion is connected with the carbon atom to which the sulfonic acid group is added by the sulfite cleavage process. The thiazole portion is connected with the tertiary nitrogen atom, since in the vitamin itself the nitrogen is quaternary. This has been shown by the already-mentioned addition of methyl-iodide to the thiazole compound and by potentiometric titrations of the quaternary methyl-iodide compound and of the vitamin-hydrochloride[91–94] (see Fig. 7).

Williams states:[95] "On titrating thiamin hydrochloride with alkali, we find a sharp rise when 1 mol is reached corresponding to the formation of the neutral or mono-acid salt, but there is no further break until a total of

[90] K. Makino and T. Imai, *Z. physiol. Chem.*, **239**, 1 (1936).
[91] H. T. Clarke and S. Gurin, *J. Am. Chem. Soc.*, **57**, 1876 (1935).
[92] T. W. Birch and L. J. Harris, *Nature*, **135**, 654 (1935).
[93] R. C. G. Moggridge and A. G. Ogsten, *Biochem. J.*, **29**, 866 (1935). A. G. Ogsten and R. A. Peters, *Ibid.*, **30**, 736 (1936).
[94] R. R. Williams and A. E. Ruehle, *J. Am. Chem. Soc.*, **57**, 1856 (1935).
[95] R. R. Williams and T. D. Spies, *Vitamin B_1*, New York, 1938, p. 163.

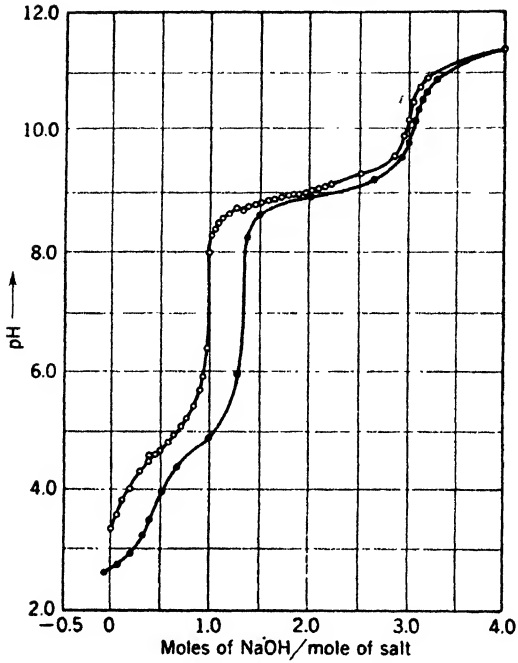
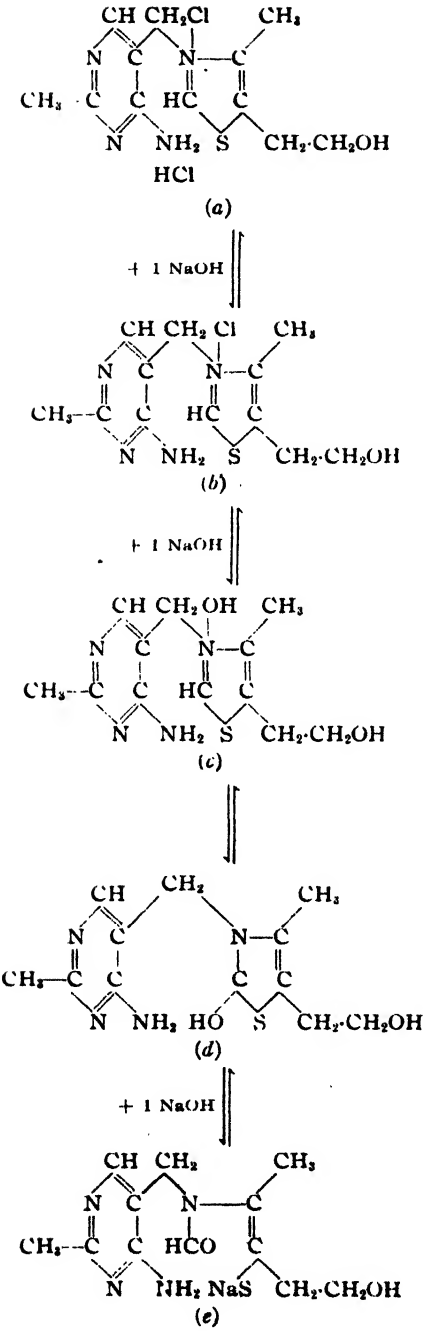


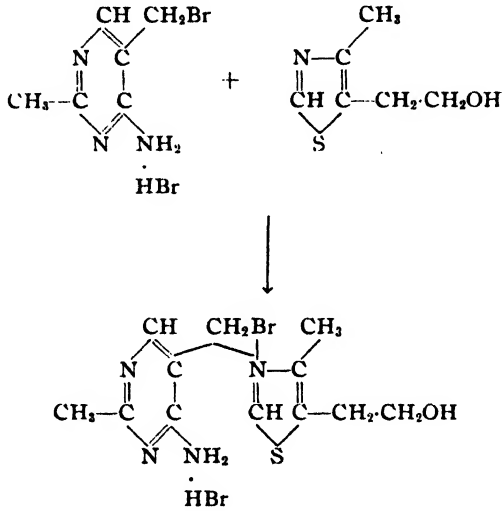
Fig. 7. - Titration of vitamin B_1 chloride with NaOH (o—o) and back titration with HCl (●—●). (R. R. Williams and A. E. Ruehle.)

3 mols has been added. Apparently the addition of 1 mol of alkali liberates the monochloride (*b*). Further addition of alkali begins to liberate the strong quaternary base (*c*) which goes over rapidly into the neutral pseudo base or carbinol (*d*). This does not occur instantaneously, as one can see during titration by the momentary rise and subsequent slow fall of pH after each addition of alkali. Such process of the migration of hydroxyl is well known in cyclic quaternary bases and in effect uses up hydroxyl ions. Nor does the pH rise even when 2 mols have been added, because quaternary thiazoles such as this undergo ring-opening in alkali solution, forming an acidic sulfhydryl group.[96] Only after this is neutralized with a third mol of alkali to form (*e*) does the alkalinity rise sharply, indicating free NaOH. The reverse arrows indicate reversal of the sequence and the regeneration of the vitamin upon back titration. If the solution stands in alkaline condition for some time, there is some permanent destruction of the vitamin."

[96] W. H. Mills, L. M. Clark and J. A. Aeschlimann, *J. Am. Chem. Soc.*, 123, 2353 (1923).


CH CH2Cl CH3
N C N C
CH3 C C HC C
N NH2 S CH2·CH2OH
HCl
(a)
+ 1 NaOH
CH CH2Cl CH3
CH3 C C HC C
N NH2 S CH2·CH2OH
(b)
+ 1 NaOH
CH CH2OH CH3
CH3 C C HC C
N NH2 S CH2·CH2OH
(c)
CH CH2 CH3
CH3 C C C C
N NH2 HO S CH2·CH2OH
(d)
+ 1 NaOH
CH CH2 CH3
CH3 C C HCO C
N NH2 NaS CH2·CH2OH
(e)

The final proof for the connection of both fragments is given by the total synthesis of vitamin B_1. 2-Methyl-4-amino-5-bromo-methyl-pyrimidine-hydrobromide upon condensation with 4-methyl-5-β-oxy-ethyl-thiazole yields the vitamin B_1 bromide-hydrobromide.[97, 98, 99]

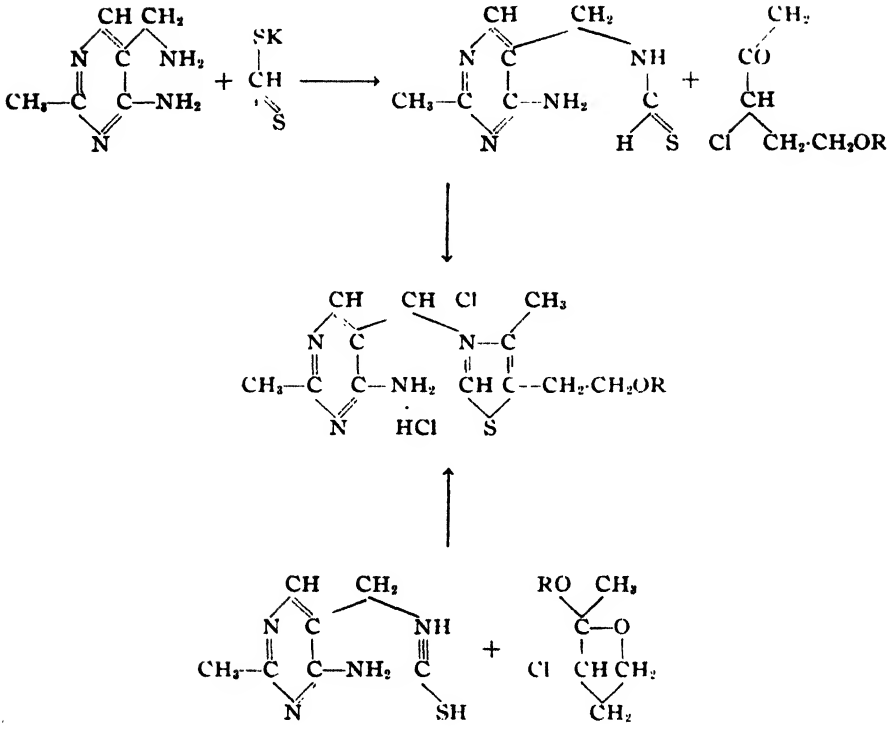


The bromide-hydrobromide can be converted into the chloride-hydrochloride either by shaking with silver chloride[98] or by preparing the sparingly soluble picrate followed by digestion with 10% hydrochloric acid.[99]

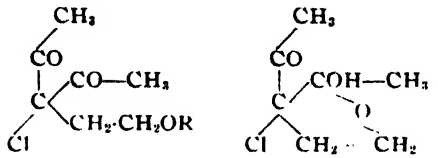
An improvement[100] in this method consists of condensing the hydrochloride of the 5-hydroxymethyl-pyrimidine compound with the hydrochloride of the thiazole compound, giving the vitamin chloride-hydrochloride directly.

An entirely different method of synthesizing vitamin B_1 has been effected[101, 102] by condensing 2-methyl-4-amino-5-thioform-amido-methyl-pyrimidine, which can be obtained from the 5-amino-methyl-compound by condensation with potassium dithio-formate or with ethyl-formate in the presence of phosphorus-pentasulfide, with γ-chloro(or bromo)-γ-aceto-propyl-alcohol (or acetate or benzoate):

[97] R. R. Williams and J. K. Cline, *J. Am. Chem. Soc.*, **58**, 1504 (1936).
[98] J. K. Cline, R. R. Williams and J. Finkelstein, *Ibid.*, **59**, 1052 (1937).
[99] H. Andersag and K. Westphal, *Ber.*, **70**, 2035 (1937).
[100] O. Zima, G. P. 669,187.
[101] A. R. Todd and F. Bergel, *J. Chem. Soc.*, 1937, 364.
[102] H. Andersag and K. Westphal, *Ber.*, **70**, 2035 (1937).



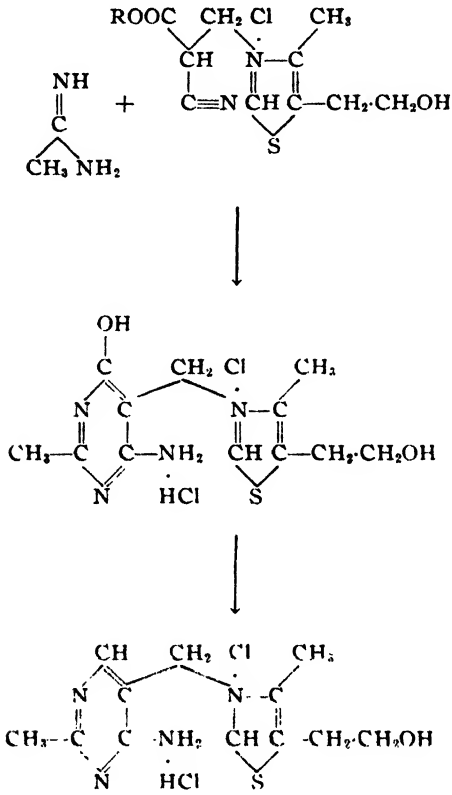
Instead of the γ-halogen-γ-aceto-propyl-alcohol, also γ,γ-diaceto-γ-halogeno-(or mercapto)-propyl-alcohol or its inner hemiacetale might be used.[103]



A modification of this method, which is said to give much better yields[104] consists in the condensation of 2-methyl-4-amino-5-thioform-amido-methyl-pyrimidine with 2-methyl-2-alkoxy-3-chloro-tetrahydrofurane, which can be obtained from the previously described α-chloro-α-aceto-butyro-lactone and a primary alcohol in the presence of sulfuric acid.

[103] T. Matukawa and M. Ohta, U. S. 2,184,720; Indian 25,808.
[104] M. Klingenfuss, U. S. P. 2,127,446; G. P. 676,980; F. P. 831.110.

A different method of synthesizing vitamin B_1 should finally be mentioned.[105] It consists in building the pyrimidine nucleus on the thiazole nucleus and is best described by the following series of formulas:



7. Industrial Methods of Preparation

Vitamin B_1 is manufactured today by total synthesis according to the methods described before. The isolation from natural sources, which is more expensive, is, however, still carried out technically. The latter method has been worked out systematically.[106] The vitamin is adsorbed on fuller's earth or synthetic zeolites,[107, 108, 109] extracted with acid salts

[105] H. Andersag and K. Westphal. F. P. 816,432.
[106] R. D. Greene and A. Black, *J. Am. Chem. Soc.*, **59**, 1395 (1935).
[107] L. R. Cerecedo and D. J. Hennessy, *Ibid.*, **59**, 1617 (1937).
[108] L. R. Cerecedo and F. J. Kaszuba *Ibid.*, **59**, 1619 (1937).
[109] L. R. Cerecedo and J. J. Thornton, *Ibid.*, **59**, 1621 (1937).

of organic nitrogen-containing bases, such as pyridine or quinine, the adventitious matter removed by benzoylation in soda-alkaline solution and chloroform extraction, followed by precipitation of the vitamin with silver nitrate, barium hydroxide and phosphotungstic acid. Vitamin B_1 is finally recrystallized from acidified organic solvents, such as combinations of phenol and butyl alcohol or HCl-alcohol. From one ton of rice polishings 5–10 g. of vitamin B_1[110] can be obtained by this method.

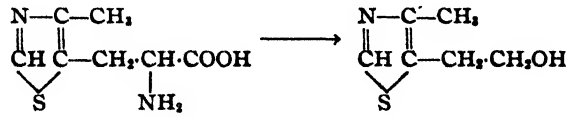
Commercial vitamin B_1 contains about 4% water which can be removed by drying at 100° C. or by standing in a vacuum over sulfuric acid. Elixirs of thiamin chloride are marketed which contain a wine base. Since tannic acid precipitates vitamin B_1, the wine is completely freed from tannic acid by the use of freshly prepared casein or completely defatted and dealbuminized milk.[111]

The preparation of the vitamin B_1-pyrophosphate, which will be described later, is also commercially important.

8. Biogenesis

The natural precursors of vitamin B_1 and the reactions leading to the synthesis of vitamin B_1 in the plant organism are unknown. The hypothesis has been advanced[112, 113] that the particular thiazole part of the vitamin B_1 molecule might arise during yeast fermentation from α-amino-β-(4-methyl-thiazole-5)-propionic acid (I), which could have been built up from methionine, acetaldehyde and ammonia, by a reaction analogous to the formation of fusel oil in alcoholic fermentation. Yeast has been found to be able to convert this amino-propionic acid derivative of thiazole (I) into the 4-methyl-5-(β-hydroxy-ethyl)-thiazole (II) of vitamin B_1.

(For the influence of light and chlorophyll upon the vitamin B_1 synthesis and for the site of the synthesis in higher plants see page 133).



(I) (II)

[110] R. R. Williams, R. E. Waterman and J. C. Keresztesy, *J. Am. Chem. Soc.*, **56**, 1187 (1934).

[111] L. Greengard, *J. Am. Pharm. Assoc.*, 1, 230 (1940).

[112] C. R. Harington and R. C. G. Moggridge, *Biochem. J.*, **34**, 685 (1940); *J. Chem. Soc.*, **1939**, 443.

[113] J. Bonner and E. R. Buchman, *Proc. Natl. Acad. Sci. U. S.*, **24**, 431 (1939).

9. Thiochrome

By oxidation, vitamin B_1 is converted into thiochrome, a yellow substance of intense blue fluorescence.[114] This conversion occurs also when alcoholic solutions of the vitamin stand at room temperature for several months.[115] Very little thiochrome is formed at pH 2, but it is produced more rapidly as the pH approaches 7. Thiochrome can be obtained from vitamin B_1 by oxidation with permanganate or manganese oxides at pH 7,[116] by oxidation of alkaline solutions of vitamin B_1 with potassium ferricyanide,[117] by hydrogen peroxide, selenium dioxide, etc.[118] The same substance has been isolated from yeast by Kuhn and co-workers[119] who proposed the name thiochrome. The compound melts at 227–228° and exhibits absorption maxima at 358 and 375 mμ (see Fig. 8). The isolation of thio-

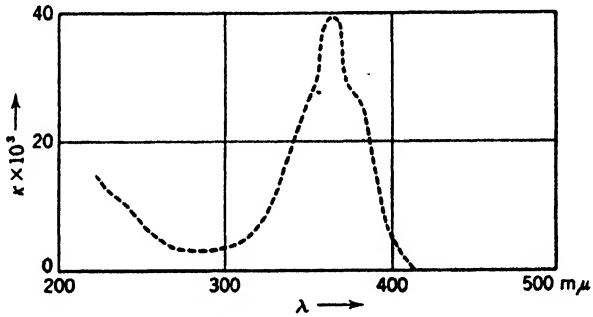
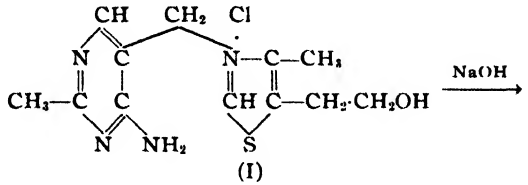


Fig. 8.—Absorption spectrum of thiochrome. (R. Kuhn and H. Vetter.)

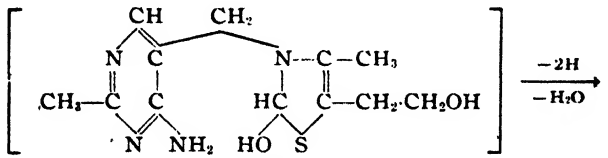
chrome has been carried out by adsorption on acid silicates, followed by elution and precipitation with silver nitrate. From 8100 lbs. yeast, about 250 mg. thiochrome are obtained. Thiochrome probably does not occur as such in yeast, but is formed when the yeast is worked up. Sodium hydrosulfite reduces thiochrome in neutral or alkaline solutions to a leuco-compound, which does not fluoresce. By air oxidation, thiochrome is regenerated. In alkaline solutions, thiochrome is sensitive to light and the

[114] R. A. Peters, *Nature*, **135**, 107 (1935).
[115] H. W. Kinnersley, J. R. O'Brien and R. A. Peters, *Biochem. J.*, **29**, 701 (1935).
[116] H. W. Kinnersley, J. R. O'Brien and R. A. Peters, *Ibid.*, **29**, 2369 (1935).
[117] G. Barger, F. Bergel and A. R. Todd, *Nature*, **136**, 259 (1935); *Ber.*, **68**, 2257 (1935).
[118] R. Kuhn and H. Vetter, *Ber.*, **68**, 2375 (1935).
[119] R. Kuhn, T. Wagner-Jauregg, F. W. von Klaveren and H. Vetter, *Z. physiol. Chem.*, **234**, 196 (1935).

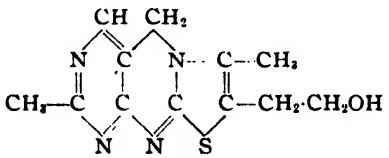
fluorescence disappears irreversibly. The mechanism of the dehydrogenation of vitamin B_1 to thiochrome is not known with certainty. It is, however, very probable that at first an intermediate is formed of the following formula (II):



(I)



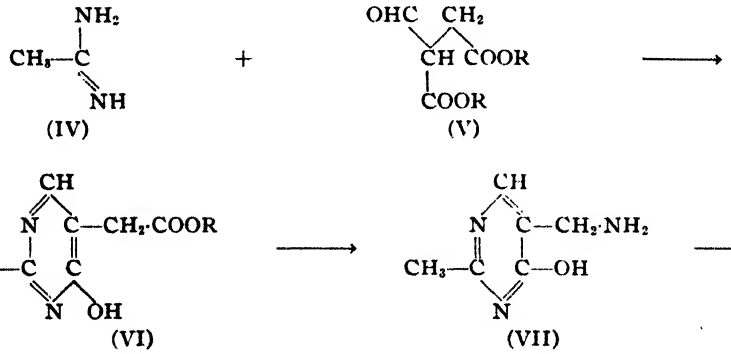
(II)



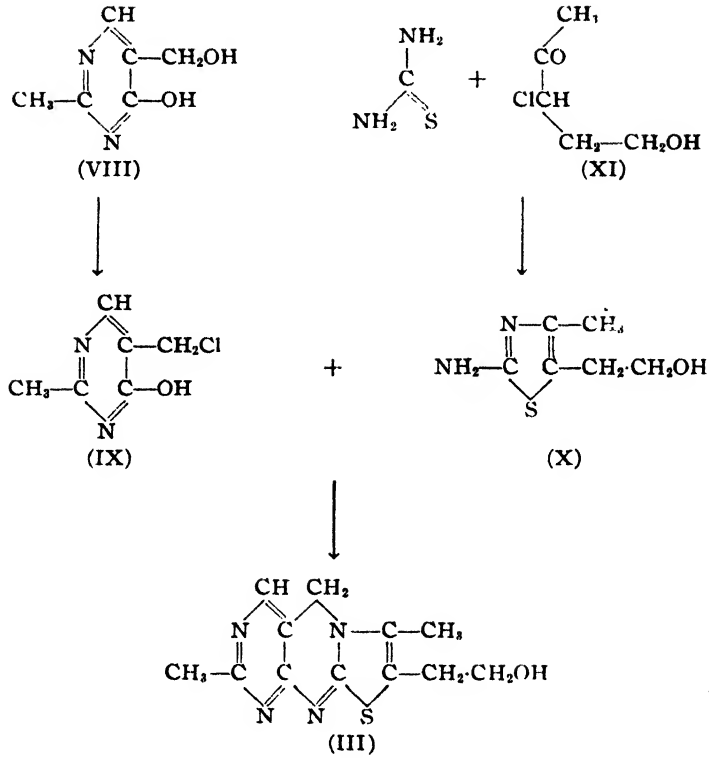
(III)

Thiochrome

Thiochrome has been prepared synthetically in the following manner:[120]



[120] A. R. Todd, F. Bergel, H. L. Fraenkel-Conrat and A. Jacob, *J. Chem. Soc.*, 1936, 1601.



Condensation of acetamidine (IV) with formyl-succinate (V) yielded 4-hydroxy-2-methyl-pyrimidine-5-ethyl-acetate (VI), from which by Curtius degradation 4-hydroxy-5-amino-methyl-2-methyl-pyrimidine (VII) was obtained. Replacement of the amino-group by hydroxyl was effected by means of nitrous acid and the resulting 4-hydroxy-5-hydroxy-methyl-2-methyl-pyrimidine (VIII) on boiling with phosphorus chloride, yielded the chloro-compound (IX). 2-Amino-4-methyl-5-β-hydroxy-ethyl-thiazole (X) was obtained by condensing methyl-α-chloro-γ-hydroxy-propyl-ketone (XI) with thiourea. The pyrimidine and the thiazole compound form thiochrome upon condensation.

Compounds with the same ring-skeleton as thiochrome are called quinochromes.

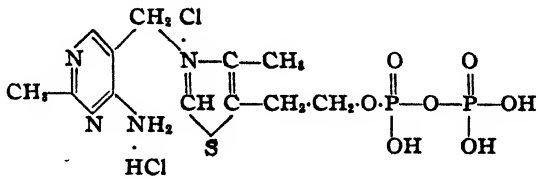
10. Vitamin B_1-Pyrophosphate

The biological action of vitamin B_1 is partly or mainly due to the action of its pyrophosphoric acid ester. Vitamin B_1 apparently acts as an ac-

ceptor of phosphoric acid, for example, from adenosin-triphosphoric acid.[121] What probably represents the reaction product of this ester interchange reaction has been isolated[122] from bottom yeast in the form of its crystallized hydrochloride of the formula $C_{12}H_{19}O_7N_4SP_2Cl$, and has been identified as a coenzyme, cocarboxylase,[123] by its biological action. The carboxylase system consists of the apoenzyme, which is a specific protein, and the coenzyme, the cocarboxylase, and metal ions. Of these, Mn, Mg[124] and Fe are stimulants, whereas Zn, Ca, Ni and Co retard in small concentrations (5×10^{-5} to 5×10^{-3} millimols in 1.7 cc. reaction mixture) and stimulate in higher concentrations,[125] (5×10^{-3} to 2×10^{-3}), but stop the reaction entirely and irreversibly at still higher concentrations. This is probably quite important since most cells contain an appreciable amount of Zn, whereas during times of vitamin B_1 deficiency a low Zn content has been observed.[126]

Carboxylase has been isolated from top brewer's yeast in highly purified form and is a diphosphothiamin-magnesium-protein.[127] The molecular ratio of protein to diphosphothiamin is 1:1 and of diphosphothiamin to magnesium is 1:5. One mg. of this enzyme catalyzes the formation of 12,000 microliters of carbon dioxide per hour at 30°C. and one mol of diphosphothiamin catalyzes the breakdown of 840 mols of pyruvic acid per min. at 30° C. The metal in the enzyme acts apparently as a "cement" substance binding the protein to the prosthetic group. The magnesium can be replaced by all bivalent metals tested but not by monovalent or trivalent cations. Carboxylase is a firmly bound conjugated proteid in high salt concentrations, but dissociates considerably in dilute salt solutions or in alkaline (for example, ammonium-sulfate) solutions.

Chemical investigation of the crystallized water-soluble cocarboxylase (m. p. 242–244° C.) proved its constitution as the pyrophosphoric-acid-ester of vitamin B_1 of the following formula:



[121] T. W. Birch and L. W. Mapson, *Nature*, **138**, 27 (1936).
[122] K. Lohmann and P. Schuster, *Naturwissenschaften*, **25**, 26 (1937); *Angew. Chem.*, **50**, 221 (1937).
[123] E. Auhagen, *Z. physiol. Chem.*, **204**, 149 (1932).
[124] K. Lohmann and P. Schuster, *Biochem. Z.*, **294**, 188 (1937).
[125] K. Lohmann and A. J. Kossel, *Naturwissenschaften*, **27**, 595 (1939).
[126] W. G. E. Eggleton, *Biochem. J.*, **33**, 403 (1939).
[127] D. E. Green, D. Herbert and V. Subrahmanyan, *J. Biol. Chem.*, **135**, 795 (1940); **138**, 327 (1941).

By enzymatic dephosphorylation with prostata-phosphatase or with a phosphatase which is liberated from yeast cells during drying,[128] free vitamin B_1 is obtained. Only one molecule of phosphoric acid is hydrolyzed by the action of alkaline kidney-phosphatase[129, 130] or by acid hydrolysis. The second mol of phosphoric acid is much more difficult to hydrolyze. Neither the monophosphoric acid obtained nor vitamin B_1 shows cocarboxylase action.

The chemical reactions of cocarboxylase resemble closely those of the free vitamin B_1. By slight oxidation, a blue fluorescent compound of the thiochrome type is obtained. By the action of sulfite, the molecule is split as described for the vitamin itself, into the pyrimidine part $C_6H_9O_3N_3S$ and the diphosphorylated thiazole compound.

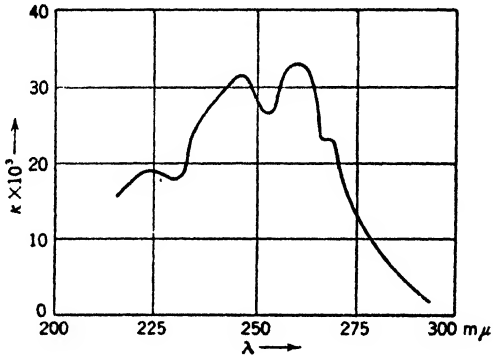


Fig. 9.—Absorption spectrum of vitamin B_1-pyrophosphate (cocarboxylase). (H. Rudy in W. Stepp, *Ernährungslehre.*)

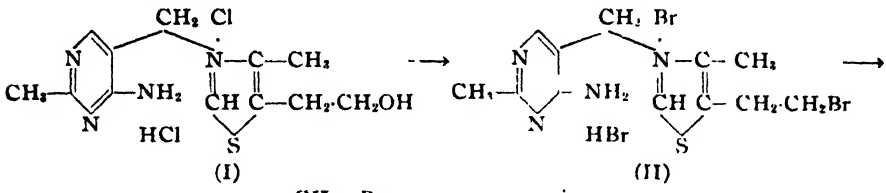
Vitamin B_1-pyrophosphate in aqueous solution forms an inner salt connecting the amino-group of the pyrimidine part with one of the hydroxyl groups of the phosphoric acid part. The absorption spectrum of vitamin B_1-pyrophosphate (see Fig. 9) resembles closely that of the free vitamin.

The synthesis of cocarboxylase has been achieved by enzymatic and by chemical methods. Phosphatase[131] of the duodenal mucosa of the pig, dried brewer's yeast and living yeast[132] were successfully employed as enzymatic agents for the phosphorylation of vitamin B_1 in the presence of

[128] D. Melnick and H. Field, *J. Biol. Chem.*, **127**, 531 (1939).
[129] K. Lohmann and P. Schuster, *Biochem. Z.*, **294**, 188 (1937).
[130] H. Tauber, *J. Biol. Chem.*, **123**, 499 (1938).
[131] H. Tauber, *Science*, **86**, 180 (1937); *Enzymologia*, **2**, 171 (1937); *J. Biol. Chem.*, **123**, 499 (1938).
[132] H. W. Kinnersley and R. A. Peters, *J. Soc. Chem. Ind.*, **56**, 447 (1937); *Biochem. J.*, **32**, 697 (1938). M A. Lipschitz, Van R. Potter and C. A. Elvehjem, *Biochem. J.*, **32**, 474 (1938).

hexose-diphosphate and adenine-triphosphate.[133] Phosphoglyceric acid and phosphopyruvic[134] acid can also act as phosphate donator in the presence of catlaytic amounts of adenylic acid or adenyl-pyrophosphate.[135] Cocarboxylase is also enzymatically prepared from vitamin B_1-monophosphate, which apparently is first hydrolyzed to the non-phosphorylated vitamin.[135] All these enzymatic processes occur only in the presence of the apocarboxylase, the specific protein of the carboxylase, and are stopped when the protein is partly or fully saturated.[134, 135] Therefore no preparative use can be made of the enzymatic synthesis of cocarboxylase. Cocarboxylase has also been obtained from vitamin B_1 by bacterial synthesis, using *Propionibacterium pentosaceum*.[136]

Chemically, cocarboxylase was synthesized from vitamin B_1 in low yields by the action of phosphorus-oxychloride,[137] with better yields by condensation with sodium-pyrophosphate in the presence of phosphoric acid[138] or by the use of pyrophosphoryl-chloride.[139] Another method consists in the conversion of vitamin B_1 (I) into the bromo-vitamin (II) by the action of hydrobromic acid. The bromo-compound in turn is reacted with a solution of silver pyrophosphate in pyrophosphoric acid to yield the cocarboxylase (III).[140] For the purification of the synthesized cocarboxylase chloride a precipitation with phosphotungstic acid has been recommended and final crystallization is achieved from alcoholic HCl.[141]



[133] H. v. Euler and R. Vestin, *Naturwissenschaften*, **25**, 416 (1937).

[134] M. A. Lipton and C. A. Elvehjem, *Cold Spring Harbor Symposia on Quant. Biol.*, **7** (1939), *Nature*, **145**, 226 (1940).

[135] H. Weil-Malherbe, *J. Soc. Chem. Ind.*, **58**, 1021 (1939).

[136] M. Silverman and C. H. Werkman, *Proc. Soc. Exptl Biol. Med.*, **40**, 369 (1939).

[137] K. G. Stern and J. W. Hofer, *Enzymologia*, **3**, 82 (1937).

[138] J. Weijlard and H. Tauber, *J. Am. Chem. Soc*, **60**, 730, 2263 (1938).

[139] K. Lohmann, quoted in C. Oppenheimer and K. G. Stern, *Biological Oxidation*, New York, 1939, p. 207.

[140] H. Weil-Malherbe, *J. Soc. Chem. Ind.*, **58**, 1021 (1939); *Biochem J.*, **34**, 980 (1940).

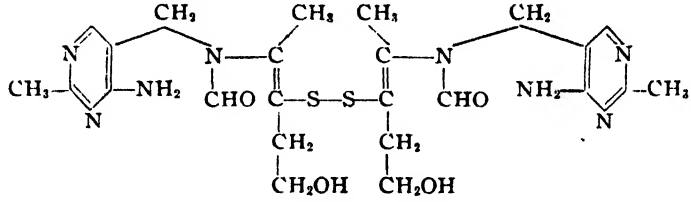
[141] J. Weijlard, *J. Am. Chem. Soc.*, **63**, 1160 (1941).

Cocarboxylase as well as the vitamin-monophosphate have the biological activity of the vitamin B_1.[142]

The chemistry of the apocarboxylase, the protein part of the carboxylase, is not known. The molecular weight is about 150,000.[143]

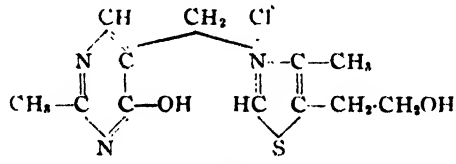
11. Specificity of the Vitamin B_1 Action

The vitamin action of vitamin B_1 seems to be connected with the specific structure of the molecule. The different vitamin salts, as the hydrochloride, hydrobromide,[144] sulfate, etc., and the pyrophosphoric acid ester, cocarboxylase, have a corresponding activity. The vitamin B_1-disulfide (I) is also as active as the vitamin.



(I)

Structural alterations, however, cause disappearance of the vitamin action. Inactive are thiochrome,[145, 146] the oxychlorothiamin[147] (II) and the products obtained from the sulfite cleavage of the vitamin.[148, 149] Dihydro-



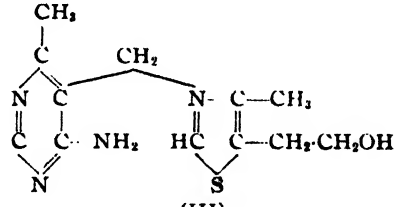
(II)

vitamin B_1 is also inactive, but the dihydro-cocarboxylase was found to be active.[150] Polyneuritis in pigeons may, however, be cured[151] by 4-amino-

[142] K. Lohmann and P. Schuster, *Biochem. Z.*, **294**, 188 (1937).
[143] C. Oppenheimer and K. G. Stern, *Biological Oxidation*, New York, 1939, p. 208.
[144] R. R. Williams and J. K. Cline, *J. Am. Chem. Soc.*, **58**, 1504 (1936).
[145] G. Barger, F. Bergel and A. R. Todd, *Nature*, **136**, 259 (1935); *Ber.*, **68**, 2257 (1935).
[146] R. Kuhn and U. Vetter, *Ber.*, **68**, 2375 (1935).
[147] E. R. Buchman, and R. R. Williams, *J. Am. Chem. Soc.*, **57**, 1751 (1935).
[148] R. R. Williams, *Ibid.*, **57**, 229 (1935).
[149] R. R. Williams, R. E. Waterman, J. C. Keresztesy and E. R. Buchman, *Ibid.*, **57**, 536 (1935).
[150] C. Oppenheimer and K. G. Stern, *Biological Oxidation*, New York, 1939, p. 204.
[151] W. J. Robbins, M. A. Bartley, A. G. Hogan and L. R. Richardson, *Proc. Natl Acad. Sci. U. S.*, **23**, 388 (1937).

2-methyl-5-bromo-methyl-pyrimidine and 4-methyl-5-β-hydroxy-ethyl-thiazole if given simultaneously. (A synthesis of vitamin B_1 probably occurs from the intermediates.)

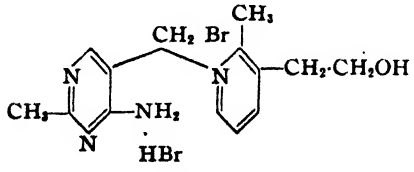
A great number of compounds have been prepared synthetically[152] with slight differences in the vitamin structure. Only one (III) has been found active and that one only in much larger doses.[153]



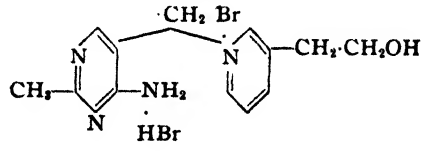
(III)

The pyrimidine ring, the thiazole ring and the methylene bridge between them, an unsubstituted amino-group in 4-position of the pyrimidine ring,[154] the 5-hydroxy-alkyl-group[155] and a free 2-position in the thiazole nucleus are all necessary for the vitamin action. It is also probable that the nature of the substituents in position 6 influences the vitamin activity.[152]

So far as is known only one substance with vitamin B_1 properties occurs naturally. Growth action of *Phycomyces* is, however, also obtained by the 2-ethyl- instead of the 2-methyl-derivative.[156] Hetero-vitamin B_1 (IV), containing instead of the thiazole ring a pyridine ring, has an activity of about $^1/_{26}$ of that of vitamin B_1.[157]



(IV)



(V)

The next lower homolog (V), devoid of the methyl group in the pyridine ring, is only $^1/_{10}$ as active as the methylated product.[158]

[152] F. Bergel and A. R. Todd, *J. Chem. Soc.*, 1937, 1504.
[153] H. Andersag and K. Westphal, *Ber.*, 70, 2035 (1937).
[154] F. Bergel and A. R. Todd, *J. Chem. Soc.*, 140, 1504 (1937).
[155] D. Price and F. D. Pickel, *J. Am. Chem. Soc.*, 63, 1067 (1941).
[156] W. J. Robbins and F. Kavanagh, *Proc. Natl. Acad. Sci. U.S.*, 24, 229 (1938).
[157] F. C. Schmelkes, *Science*, 90, 113 (1939). F. C. Schmelkes and R. R. Joiner, *J. Am. Chem. Soc.*, 61, 2562 (1939). P. Baumgarten and A. Dornow, *Ber.*, 73, 44 (1940).
[158] A. Dornow, *Ber.*, 73, 156 (1940).

It may be noted that substances of the cyclo-pentano-perhydro-phenanthrene type (for example, male and female sex hormones, vitamin D) have been reported [159] as delaying the vitamin B_1-avitaminosis (polyneuritic symptoms in pigeons). These results, however, need confirmation.

12. Determination

(*a*) *Chemical Methods*

A number of color reactions have been proposed for the determination of vitamin B_1. Reliable quantitative results can be obtained with the first three procedures when carefully controlled conditions are observed.

1. **Formaldehyde-azo-test of Kinnersley and Peters.**[160] Diazobenzene-sulfuric acid in carbonate-containing sodium hydroxide solution gives with vitamin B_1 and formaldehyde a red color. Since this reaction attacks the 4-amino-group of the pyrimidine nucleus, it is obvious that a deaminated vitamin or thiochrome does not give this reaction. Therefore, this reaction is quite valuable for the determination of the natural vitamin. Synthetic compounds of similar structure give the same reaction. Substituents in the thiazolium nucleus influence the reaction.[161] This method of vitamin B_1 determination is applicable to rather highly purified solutions only. The presence of reducing substances interferes with the color development. The use of acetone in place of vitamin B_1 also gives the same color with the reagents of this test-method.[162]

2. **Thiochrome Test by Jansen.** Vitamin B_1 in aqueous solution is oxidized by means of potassium ferricyanate to thiochrome, the fluorescence of which is determined photoelectrically after extraction of the thiochrome with isobutanol.[163, 164, 165, 166, 167] The oxidation is carried out at pH 10. The intensity of the fluorescence is a function of the alkalinity of the solution and of the amount of thiochrome present. Excess potassium ferricyanide destroys the formed thiochrome rapidly. Irradiation also destroys the thiochrome.

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[159] J. Sanchez-Rodriguez and J. M. Sarda, *Z. Vitaminforsch.*, **6**, 193 (1937).

[160] H. W. Kinnersley and R. A. Peters, *Biochem. J.*, **28**, 667 (1934); *Ibid.*, **32**, 1516 (1938).

[161] F. Bergel and A. R. Todd, *J. Chem. Soc.*, 1937, 1504.

[162] C. R. Addinall, *The Story of Vitamin B_1*, Merck & Co., 1937, p. 18.

[163] G. Barger, F. Bergel and A. R. Todd, *Nature*, **136**, 259 (1935); *Ber.*, **68**, 2257 (1935).

[164] B. C. P. Jansen, *Rec. trav. chim.*, **55**, 1046 (1936). H. G. K. Westenbrink and J. Goudsmit, *Ibid.*, **56**, 803 (1937). P. Karrer and U. Kubli, *Helv. Chim. Acta*, **20**, 369 (1937). F. Widenbauer, O. Huhn and G. Becker, *Z. ges. exptl. Med.*, **101**, 178 (1937). J. Goudsmit and H. G. K. Westenbrink, *Nature*, **139**, 1108 (1937). K. Ritsert, *Deut. med. Wochschr.*, **64**, 481 (1938). H. Otto and F. Rühmekorb, *Klin. Wochschr.*, **17**, 1246 (1938).

[165] H. G. K. Westenbrink and J. Goudsmit, *Nature*, **142**, 150 (1938).

[166] G. M. Hills, *Biochem. J.*, **33**, 1966 (1939).

[167] R. G. Booth, *J. Soc. Chem. Ind.*, **59**, 181 (1940).

The results obtained with this method are in fair agreement with those obtained by the biological bradycardia method. It must, however, be noted that only the free vitamin B_1 is determined by this method, since only the free thiochrome can be extracted with organic solvents. This difficulty can be overcome by digesting the sample with pepsin followed by digestion with taka-diastase.[168] Ambiguity arises sometimes through colored fluorescence of other compounds which obscures the effect of thiochrome.[169]

3. **Colorimetric Method by Prebluda and McCollum.**[170] Diazotized *p*-amino-acetanilide, *p*-amino-acetophenone, or methyl-*p*-amino-acetophenone gives with vitamin B_1 red dyes, specific for this vitamin, which can be extracted with organic solvents such as xylene, acetone, isobutanol, etc. The sensitivity of this reaction is increased by the presence of phenol or ethyl-alcohol, or preferably, both.[171] Willstaedt[172] suggests the use of 2,4-dichloro-benzene-diazonium-chloride, extraction of the formed yellow-red dye with ether and separation from by-products by adsorption on calcium hydroxide. This method has been modified by running the analysis with diazotized sulfanilic acid with and without the presence of potassium ferricyanide. Since vitamin B_1 does not give a color under these conditions but other amino-compounds which may accompany the vitamin do give colors, the difference between the color values gives a measure of the amount of vitamin B_1 present.[173]

The results of these methods compare favorably with the bioassay values. Vitamin C interferes with the development of the color,[174] unless it is first oxidized, for example, by titrating with iodine or by the addition of calcium ions.[175] The phosphorylated forms of vitamin B_1 give the color but the dye formed cannot be extracted by organic solvents such as xylene.[176]

4. **Gravimetric Method by Naiman.**[177] This method is based on the production of an orange-red precipitate of vitamin B_1 with bismuth potassium iodide.

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[168] M. Pyke, *J. Soc. Chem. Ind.* **58**, Trans., 338 (1939).
[169] M. Pyke, *J. Soc. Chem. Ind.*, **58**, 1021 (1939).
[170] H. J. Prebluda and E. V. McCollum, *Science*, **84**, 488 (1936); *J. Biol. Chem.*, **127**, 495 (1939). D. Melnick and H. Field, *J. Biol. Chem.*, **127**, 505, 515 (1939).
[171] D. Melnick and H. Field, *J. Biol. Chem.*, **127**, 515 (1939).
[172] H. Willstaedt, *Naturwissenschaften*, **25**, 682 (1937).
[173] P. Meunier and C. P. Blancpain, *Compt. rend.*, **208**, 768 (1939).
[174] A. D. Emmett, G. Peacock and R. A. Brown, *J. Biol. Chem.*, **135**, 131 (1940).
[175] M. E. Auerbach, *J. Am. Pharm. Assoc.*, **29**, 313 (1940).
[176] D. Melnick and H. Field, *J. Biol. Chem.*, **127**, 531 (1939).
[177] B. Naiman, *Science*, **85**, 290 (1937).

5. **Method of Spruyt.**[178] Vitamin B_1 is separated as the phosphotungstate and reduced with nascent hydrogen. The resulting brown color is believed to be proportional to the amount of vitamin B_1 present.

6. **Raybin Reaction.**[179] Vitamin B_1 in a borax solution of pH 9.6 forms with 2,6-dibromo-quinone-chloroimide an orange color which gradually decreases in intensity. The color formed can be extracted with chloroform and measured in a photometer.

7. **Tauber Reaction.**[180] Vitamin B_1 and *p*-dimethyl-amino-benzaldehyde in the presence of acetic acid produce upon evaporation of the acid and addition of fresh acid an intense brick-red color. This reaction should not be carried out in the presence of proteins or amino-acids which interfere.

Vitamin B_1 in the presence of vitamin B_1-pyrophosphate can be determined by either the thiochrome method (thiochrome-pyrophosphate cannot be extracted from an alkaline solution with isobutanol)[181] or by condensation with diazo-compounds according to Prebluda and McCollum. Vitamin B_1-pyrophosphate gives the same color reactions as does vitamin B_1. The color developed cannot, however, be extracted by organic solvents. For the determination of the *total vitamin B_1* present, the material might be hydrolyzed enzymatically, followed by the determination of the then free vitamin B_1.[181, 182]

(*b*) *Biological Methods*

More accurate than any of the described chemical methods for the determination of vitamin B_1 are the biological methods. Either birds, especially the pigeon, or rats are used.

The curative pigeon test by Kinnersley, Peters and Reader uses the disappearance of tonic spasms and convulsions.[183] Also a prophylactic pigeon test has been worked out. Rats are recommended by the U. S. Pharmacopoeia as test animals in the curative method.[184, 185] Vitamin B_1 deficiency in rats causes convulsions and paralysis of the lower extremities

[178] J. P. Spruyt, *Chem. Weekblad*, **27**, 298 (1930). H. W. Acton, S. Ghosh and A. Dutt, *Ind. J. Med. Research*, **1933**, 103.

[179] H. W. Raybin, *Science*, **88**, 35 (1938).

[180] H. Tauber, *Ibid.*, **86**, 594 (1937).

[181] H. G. K. Westenbrink and B. C. P. Jansen, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **8**, 119 (1938). H. W. Kinnersley and R. A. Peters, *Biochem. J.*, **28**, 667 (1934); **29**, 2369 (1935); **32**, 1516 (1938).

[182] J. Houston and S. K. Kon, *Nature*, **143**, 558 (1939).

[183] H. W. Kinnersley, R. A. Peters and V. Reader, *Biochem. J.*, **19**, 820 (1928).

[184] F. Hofmeister, *Biochem. Z.*, **129**, 477 (1922). M. I. Smith, *U. S. Pub. Health Service Pub. Health Repts.*, **24**, 116 (1930). E. F. Cook, *J. Am. Pharm. Assoc.*, **28**, 267 (1939).

[185] O. L. Kline, C. D. Tolle and E. M. Nelson, *J. Assoc. Official Agr. Chem.*, **21**, 305 (1938).

which are cured by the administration of the vitamin. The rat-growth test involves the determination of the minimum amount of the vitamin required for growth and maintenance.[186] The electrocardiographic "bradycardia" test according to Birch and Harris is based on the decline of the heart rate of rats during avitaminosis[187] and its cure by the administration of the vitamin. Vitamin B_1 may also be determined with chicks by a method[188] in which the degree of postponement of fatal polyneuritic symptoms afforded by the test material is compared with that afforded by various levels of synthetic thiamin-chloride-hydrochloride. Another method, using chicks as assay animals, determines the rate of growth produced by vitamin B_1.[189]

(c) *Biochemical Methods*

1. **The "Catatorulin"[189a] test**[190] which is often used measures *in vitro* the uptake of oxygen by brain tissue from avitaminotic pigeons. Addition of vitamin B_1 increases proportionately the amount of oxygen consumed.

2. **Yeast Fermentation Test.** The stimulating effect of vitamin B_1 on alcoholic fermentation has been developed into a quantitative test.[191, 192] As little as 1 γ may be detected ($^1/_3$ International Unit).

3. **Mold Growth Method.** Within certain limits, the growth of the mold *Phycomyces blakesleanus* is proportional to the concentration of vitamin B_1 present in a synthetic nutritive medium, and has been used for the determination of vitamin B_1 in blood; 0.01 γ can be detected according to this procedure.[193] A modification of this method[194] measures the increase of carbon dioxide formed by the addition of vitamin B_1 to growing *Phycomyces* (sensitivity: 0.1 γ).

4. **Cocarboxylase** can be determined quantitatively by the amount of CO_2 produced by decarboxylation of pyruvic acid by yeast, which has

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[186] H. C. Sherman and A. Spohn, *J. Am. Chem. Soc.*, **45**, 2719 (1923). E. F. Chase, *Dissertation*, Columbia, 1928. H. C. Sherman and S. L. Smith, *The Vitamins*, Chemical Catalog Co., 1931.

[187] T. W. Birch and L. J. Harris, *Biochem. J.*, **28**, 602 (1934).

[188] T. H. Jukes and H. Heitman, *J. Nutrition*, **19**, 21 (1940).

[189] A. Arnold and C. A. Elvehjem, *Ibid.*, **15**, 403 (1938).

[189a] Torulin = vitamin B_1.

[190] R. Passmore, R. A. Peters and H. M. Sinclair, *Biochem. J.*, **27**, 842 (1933). H. W. Kinnersley, J. R. O'Brien and R. A. Peters, *Ibid.*, **29**, 701 (1935). R. A. Peters, *Ibid.*, **32**, 2031 (1938).

[191] A. S. Schultz, L. Atkin and C. N. Frey, *J. Am. Chem. Soc.*, **59**, 948, 2457 (1937); **60**, 1514 (1938).

[192] A. S. Schultz, L. Atkin and C. N. Frey, *Ibid.*, **60**, 490 (1938).

[193] W. H. Schopfer, *Z. Vitaminforsch.* **4**, 67, 187 (1935). W. H. Schopfer and A. Jung, *Compt. rend. soc. biol.*, **122**, 249 (1936). A. P. Meiklejohn, *Biochem J.*, **31**, 1441 (1937). H. M. Sinclair, *Ibid.*, **32**, 2185 (1938).

[194] K. Heyns, *Z. physiol. Chem.*, **258** 219 (1939).

been freed from cocarboxylase, in the presence of maximum amounts of vitamin B_1.[195]

13. Standards

One International Unit vitamin B_1 = 3 γ of vitamin B_1-hydrochloride, colorless monoclinic plates m. p. 246–247° (decomp.) = 1 U. S. Pharmacopoeia Unit.

This unit replaces the earlier one of 1934, according to which 10 mg. of a special adsorbate of vitamin B_1 on fuller's earth represented 1 International Unit. The new Unit is the biological equivalent of the old Unit. The adsorption product, which represented the old standard, was prepared as follows: 100 kg. rice polishings are extracted with water, sufficient sulfuric acid being added to obtain a pH of 4.5. Salicylic acid (0.2%) and toluene are added to prevent bactericidal decomposition. After two days' extraction, the solution is filtered and shaken for 24 hours with especially adsorptive fuller's earth. After filtration, the fuller's earth is dried.

In order to standardize vitamin B_1 preparations of unknown activity, the U. S. Pharmacopoeia recommends the rat curative method (see above).[196]

1 I. U. vitamin B_1 corresponds about to:[197] 0.5 Smith Curative Unit,[198] 2.0 Chase-Sherman Units,[199] 1.0 Roscoe Unit,[200] and 20.0 Milligram Equivalents (Cowgill).[201] Units other than I. U. should no longer be used. Actually it is impossible to translate one unit system into another by factors or by mathematical computation. The above figures are given only as a guide to indicate the approximate order of magnitude for the purpose of facilitating the reading of some of the original literature.

14. Physiology of Plants and Microorganisms[202]

Thiamin is a vitamin with respect to animal nutrition. Its occurrence in plants and microorganisms has raised the question as to the physiological significance of this compound in plants and microorganisms. This question can partly be answered. Vitamin B_1 is a true growth-promoting substance for such organisms and is needed in small amounts for normal development.

Plants and microorganisms can be classified into two groups, namely, those which need an external supply of the growth-promoting substance

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[195] S. Ochoa and R. A. Peters, *Biochem. J.*, **32**, 1501 (1938).
[196] E. F. Cook, *J. Am. Pharm. Assoc.*, **28**, 267 (1939).
[197] C. R. Addinall, *The Story of Vitamin B_1*, Merck Co., 1937.
[198] M. I. Smith, *U. S. Pub. Health Service Pub. Health Repts.*, **24**, 116 (1930).
[199] H. C. Sherman and A. Spohn, *J. Am. Chem. Soc.*, **45**, 2719 (1923). E. F. Chase, *Dissertation*, Columbia, 1928. H. C. Sherman and S. L. Smith, *The Vitamins*, Chemical Catalog Co., 1931.
[200] H. Chick and M. H. Roscoe, *Biochem. J.*, **23**, 498 (1929).
[201] G. R. Cowgill, *The Vitamin B Requirement of Man*, Yale University Press, 1934.
[202] W. J. Robbins, *Science*, **89**, 303 (1939).

and those which are able to synthesize it. Among the lower plants and microorganisms which have the power of synthesizing vitamin B_1 are yeasts, bacteria, fungi, etc. Special examples are *Aspergillus niger, Agaricus campestris, Absidia glauca, Bacterium coli, Bacillus pyocyaneus, Chlamydomonas, Chlorogonium* and *Polytoma obtusum* (see also page 101). Since many of these organisms contain no chlorophyll, it must be concluded that chlorophyll is not an essential factor for the synthesis of the vitamin. It seems significant, however, that plants (peas) raised in the dark contain very little thiamin, whereas the content of this compound in plants increases rapidly in light.[203] All higher plants are able to synthesize thiamin. It has been demonstrated, for example, for tomatoes, that the vitamin is synthesized in the shoots. In leaves the concentration of vitamin B_1 amounts to a constant value of about 25 International Units per 100 g. regardless of the botanical family.[204] Roots do not have the power of synthesizing vitamin B_1; however, they need thiamin for continued cell division in the embryonic region. Vitamin B_1 is essential for the growth of all species of roots investigated.[205] Thus, thiamin appears to be a true plant hormone. On the other hand, tomato roots are able to synthesize the pyrimidine portion of thiamin, but require an external supply of the thiazole part. (Experiments done with excised tomato roots.) Pea roots require an external supply of both parts.[206] Vitamin B_1 is stored in seeds and more specifically in the outer integuments.

Some plant species (for example, cosmos, camellia, etc.) are said to grow more luxuriantly when the roots obtain, besides the self-synthesized vitamin B_1, some of this vitamin from an outside source. Under very carefully conducted experiments it has not, however, been possible to detect any beneficial effects from added vitamin B_1 when plants were raised from their seeds.[207] It has been shown that in cuttings and after transplanting, vitamin B_1 promotes root growth.[208]

Higher plants not only synthesize vitamin B_1 but excrete it through the roots. Therefore the soil in the immediate vicinity of plant roots supports a much higher microbial population than that existing outside the plant's

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[203] J. Bonner and J. Greene, *Botan. Gaz.*, 100, 226 (1938).

[204] M. Pyke, *Biochem. J.*, 34, 330 (1940).

[205] J. Bonner, *Am. Chem. Soc., Div. Agr. Food Chem. Meeting*, Sept., 1939, Abst. 13. F. W. Went J. Bonner and G. C. Warner, *Science*, 87, 170 (1938).

[206] F. Kögl and A. J. Haagen-Smit, *Z. physiol. Chem.*, 243, 209 (1936). J. Bonner, *Science*, 85, 183 (1937). W. J. Robbins and M. A. Bartley, *Ibid.*, 85, 246 (1937). W. J. Robbins, M. A. Bartley, A. G. Hogan and L. R. Richardson, *Proc. Natl. Acad. Sci. U. S.*, 23, 388 (1937).

[207] D. I. Arnon, *Science*, 92, 264 (1940).

[208] J. Bonner, *Am. Chem. Soc., Div. Agr. Food Chem., Meeting*, Sept., 1939, Abst. 13. F. W. Went J. Bonner and G. C. Warner, *Science*, 87, 170 (1938).

zone of influence.[209] A great number of bacteria, yeasts and fungi[210] are known to require an external supply of thiamin, for example, *Staphylococcus aureus*,[211] *Phycomyces blakesleanus*[212] and *Polytomella caeca*.[213] Most of them are parasites, some are saprophytes. Some organisms which need an external supply of thiamin are able to synthesize it from the thiazole and the pyrimidine intermediate, for example, *Phycomyces blakesleanus*. *Phytophthoras*, on the other hand, is unable to do so and resembles a higher animal in this respect.

A few organisms are known to be able to synthesize only one part of the thiamin molecule, either the pyrimidine part, for example, *Polytoma caudatum* and *Chilomonas paramaecium*[214] or the thiazole part. These organisms, however, can combine the portion synthesized with the portion obtained from outside, when properly administered.

The mechanism of the action of thiamin in plants is not known. It is, however, reasonable to assume that thiamin functions similarly in plants as it does in animals (see page 135).

It should finally be noted that vitamin B_1 also promotes alcoholic fermentation by yeast,[215] which effect can be used for the determination of the vitamin (see page 131). The growth of acetic acid bacteria is also stimulated by the vitamin.[216]

15. Animal Physiology

(*a*) *Metabolism of Vitamin* B_1

Vitamin B_1 is completely absorbed in the small gut and partly secreted in the gastric juice probably by means of diffusion.[217] It has already been mentioned that vitamin B_1 is not stored in the organism, but relatively higher concentrations are found in the liver, kidneys, heart, muscles and brain.[218] The amount actually present is enough to maintain proper life only for a few days. A daily intake of vitamin B_1 is, therefore, necessary.

[209] P. M. West, *Nature*, **144**, 1050 (1939).

[210] R. J. Williams and R. R. Roehm, *J. Biol. Chem.*, **87**, 581 (1930). H. Burgeff, *Ber. deut. botan. Ges.*, **52**, 384 (1934). W. H. Schopfer, *Arch. Mikrobiol.*, **6**, 510 (1935). F. Kögl and N. Fries, *Z. physiol. Chem.*, **249**, 93 (1937).

[211] B. C. J. G. Knight, *Nature*, **139**, 628 (1937); *J. Soc. Chem. Ind.*, **56**, 445 (1937); *Biochem. J.*, **31**, 731 (1937).

[212] W. H. Schopfer, *Compt. rend.*, **200**, 1965 (1935); *Z. Vitaminforsch.*, **4**, 187 (1937). W. H. Schopfer and A. Jung, *Compt. rend.*, **204**, 1500 (1937). H. M. Sinclaire, *Nature*, **140**, 361 (1937).

[213] A. Lwoff and H. Dusi, *Compt. rend.*, **205**, 630 (1937).

[214] A. Lwoff and H. Dusi, *Ibid.*, **205**, 756 (1937).

[215] A. Schultz, L. Atkin and C. N. Frey, *J. Am. Chem. Soc.*, **59**, 948 (1937).

[216] T. J. Palei, *Mikrobiol.*, **7**, 843 (1938).

[217] M. Stockholm, T. L. Althausen and H. J. Borson, *Proc. Soc. Exptl. Biol. Med.*, **46**, 387 (1941).

[218] H. G. K. Westenbrink, *Arch. néerland. physiol.*, **17**, 560 (1932); **19**, 116 (1932).

The organism absorbs only as much vitamin B_1 as is needed for the time being. All excess is excreted and to a small extent destroyed. Even intramuscular injection causes immediate excretion in the urine. A vitamin B_1 deficiency can be detected from the amount of vitamin excreted in the urine: a normal person should excrete 20–80 International Units per day. Vitamin B_1 is also secreted in milk and eggs.

Vitamin B_1 is present in the organism both in the free and esterified forms. The pyrophosphoric acid ester has been identified as cocarboxylase. The presence of vitamin B_1-monophosphate in the animal organism is suspected. Both vitamin B_1 and cocarboxylase occur also in combination with proteins.[219]

The vitamin B_1 may be absorbed in the intestines in the free or in the phosphorylated form. Phosphatase from the duodenum (pigs) can phosphorylate vitamin B_1 *in vitro*.[220] But it seems that vitamin B_1 circulates in blood plasma and in cerebrospinal fluid[221] in the free form (concentration about 1 γ per 100 cc.) which diffuses readily and passes into tissue fluid, cerebrospinal fluid, urine and cells of the body. A constant phosphorylation and dephosphorylation take place inside the cells. Liver, kidney[222] and to a lesser extent muscle and brain[223] can convert the vitamin into cocarboxylase. Nucleated blood cells and probably all nucleated animal cells can phosphorylate vitamin B_1.[224] The theory has been advanced that the mammalian non-nucleated erythrocytes obtain their cocarboxylase while in the nucleated form within the bone marrow.[224]

In blood the cocarboxylase is entirely confined to the blood cells, whereas the free vitamin occurs only in the serum. Of the total vitamin B_1 in blood nearly 90% is in the phosphorylated form. There seems to be a constant level of about 0.5 γ of free vitamin B_1 per 100 cc. of blood.

(*b*) *Physiological Action of Vitamin* B_1

Vitamin B_1 in the form of its pyrophosphoric acid ester is intimately concerned with the carbohydrate metabolism. Specifically this vitamin is involved in the utilization of pyruvic acid,[225] an intermediary degradation product of carbohydrates both in alcoholic fermentation and in tissue me-

[219] R. S. Goodhart and H. M. Sinclair, *Biochem. J.*, 33, 1099 (1939).
[220] H. Tauber, *Science*, 86, 180 (1937); *Enzymologia*, 2, 171 (1937); *J. Biol. Chem.*, 123, 499 (1938).
[221] H. M. Sinclair, *Biochem. J.*, 33, 1816 (1939).
[222] H. G. K. Westenbrink and J. Goudsmit, *Enzymologia*, 5, 307 (1938).
[223] S. Ochoa and R. A. Peters, *Biochem. J.*, 32, 1501 (1938).
[224] R. S. Goodhart and H. M. Sinclair, *Ibid.*, 33, 1099 (1939).
[225] See the review by R. A. Peters, *Current Science*, 5, 207 (1936).

tabolism. Thus, pyruvic acid is accumulated[226, 227, 228] in various tissues and body fluids of the animal organism deprived of vitamin B_1 and can be easily detected. Under normal physiological conditions, pyruvic acid is further metabolized in a number of different ways according to the type of cells in which the carbohydrate breakdown occurs. All observations concerned with ultimate or intermediary reaction products of pyruvic acid can be explained on the basis of the concept that vitamin B_1 catalyzes principally two closely analogous reactions, namely, the decarboxylation and the carboxylation of pyruvic acid. While the former reaction prevails in yeast, the latter reaction occurs principally in animal tissues. The possibility, however, that the vitamin takes part in reactions other than these cannot be excluded entirely although no experimental evidence for the occurrence of other reactions has been obtained.

The Decarboxylation Reaction. In the anaerobic fermentation of carbohydrates pyruvic acid is decarboxylated and yields acetaldehyde and carbon dioxide:

$$CH_3{\cdot}CO{\cdot}COOH \xrightarrow{\text{Vitamin } B_1\text{-pyrophosphate}} CH_3{\cdot}CHO + CO_2 \qquad (1)$$

It has been shown experimentally that in a properly adjusted system the rate of carbon dioxide evolution is dependent upon the concentration of the vitamin B_1-pyrophosphate.[229] This reaction of yeast is not confined to pyruvic acid. It seems that, generally speaking, alpha-oxo-carboxylic acids are decarboxylated by the enzyme system of yeast as has been shown experimentally with keto-glutaric, keto-valeric, keto-butyric acids and others.[230]

The Carboxylation Reaction. In animal tissues, in plants, molds and certain bacteria pyruvic acid is carboxylated[231, 232, 233] and yields oxaloacetic acid:

$$CO_2 + CH_3{\cdot}CO{\cdot}COOH \longrightarrow COOH{\cdot}CH_2{\cdot}CO{\cdot}COOH \qquad (2)$$

The actual occurrence of this reaction has not been proved directly but a considerable amount of indirect evidence has accumulated. The utilization of carbon dioxide is indicated, for example, in *B. coli* since the formation of succinic acid from pyruvic acid depends upon the pressure of carbon

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[226] B. S. Platt and G. D. Lu, *Biochem. J.*, **33**, 1525 (1939).

[227] G. D. Lu and B. S. Platt, *Ibid.*, **33**, 1538 (1939).

[228] Pyruvate in the blood of animals with experimental vitamin B_1 deficiency: R. H. S. Thompson and R. E. Johnson, *Biochem. J.*, **29**, 694 (1935). Pyruvate in the blood of men with beri-beri.[226]

[229] K. Lohmann and P. Schuster, *Biochem. Z.*, **294**, 188 (1937).

[230] C. Long and R. A. Peters, *Biochem. J.*, **33**, 759 (1939).

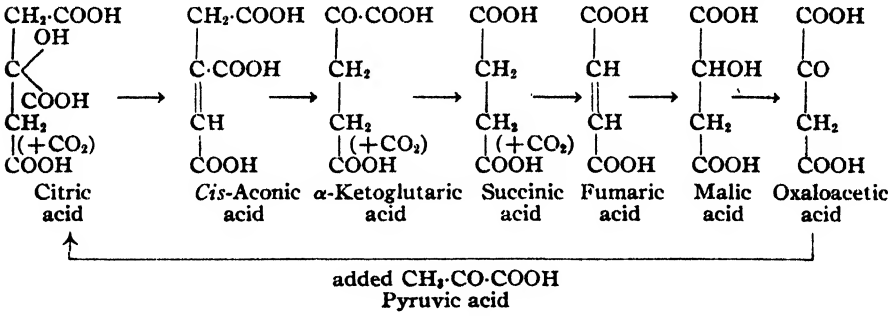
[231] H. G. Wood and C. H. Werkman, *Ibid.*, **32**, 1262 (1938).

[232] H. G. Wood and C. H. Werkman, *Ibid.*, **34**, 7 (1940).

[233] H. A. Krebs and L. V. Eggleston, *Ibid.*, **34**, 1383 (1940).

dioxide.[234] Furthermore, by means of radioactive carbon the assimilation of carbon dioxide by the rat liver and by other heterotrophic cells has been demonstrated,[235] and the presence of carboxyl groups in the reaction product has been shown.[236] Radioactive α-keto-glutarate has been isolated, formed by liver from pyruvate and radioactive sodium bicarbonate.[237] Oxaloacetic acid, the product of reaction (2), cannot be isolated since it undergoes a rapid transformation. Thus oxaloacetic acid when added to animal tissues yields fumarate, malate, α-keto-glutarate, citrate, succinate and carbon dioxide.[238] Actually, the carboxylated product is metabolized differently in various tissues. The available evidence indicates that at least two systems, the citric acid cycle and the succinic acid cycle, are involved in the utilization of oxaloacetic acid. Although vitamin B_1 is not involved in these cycles, they must be briefly presented in order to explain the ultimate reaction products of pyruvic acid as catalyzed initially by this vitamin.

(*a*) *The Citric Acid Cycle.* According to the citric acid cycle[239] pyruvic acid condenses with oxaloacetic acid in the presence of oxygen to citric



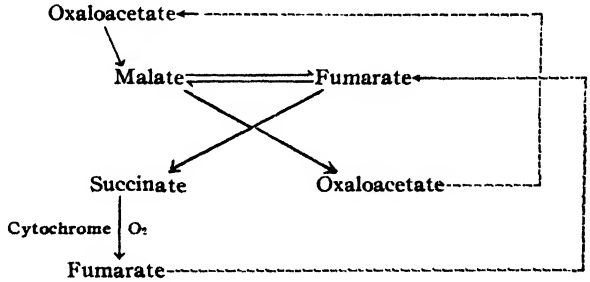
acid which is broken down by a series of enzymatic reactions to oxaloacetic acid. The complete cycle effects a total oxidation of pyruvic acid (to carbon dioxide and water) but the rate of pyruvic acid utilization is dependent upon the presence of vitamin B_1. Thus it was shown with liver suspensions from avitaminotic pigeons that when the deficiency symptoms of the animals become gradually more severe the utilization rate of pyruvic acid is correspondingly decreased. On the other hand, in minced livers from healthy pigeons, citrate, α-keto-glutarate, succinate, fumarate and malate

[234] S. R. Elsden, *Biochem. J.*, **32**, 187 (1938).
[235] S. Ruben and M. D. Kamen, *Proc. Natl. Acad. Sci. U. S.*, **26**, 418 (1940).
[236] S. Ruben, M. D. Kamen, W. Z. Hassid and D. C. de Vault, *Science*, **90**, 570 (1939).
[237] E. A. Evans and L. Slotin, *J. Biol. Chem.*, **136**, 301 (1940).
[238] H. A. Krebs, L. V. Eggleston, A. Kleinzeller and D. H. Smyth, *Biochem. J.*, **34**, 1234 (1940).
[239] H. A. Krebs and W. A. Johnson, *Enzymologia*, **4**, 148 (1937).

can be obtained by the addition of oxaloacetic acid or of pyruvic acid in the presence of carbon dioxide. The rate of pyruvate utilization in these experiments depended upon the concentration of carbon dioxide used, thus indicating the formation of oxaloacetic acid according to reaction (2). This pyruvic acid metabolism through the citric acid cycle occurs apparently in many animal tissues, such as liver, brain and several others, in plants, molds and in those types of bacteria[240] in which citrate, succinate, malate or oxaloacetate[241] is formed, but seems to be of minor importance in skeletal[242] and heart muscles.[243]

The concept that vitamin B_1 is concerned in reaction (2) explains a number of other observations. Thus, in contrast to the apparent universal decarboxylation of α-oxo-carboxylic acids by yeast, animal tissues have been found to utilize pyruvic acid exclusively.[244] In *in vitro* experiments with rat kidney slices the synthesis of citric acid from pyruvic acid was found to be markedly accelerated by vitamin B_1.[245] *In vivo* a deficiency of vitamin B_1 in the diet of rats results in decreased excretion of citric acid through the urine,[246] but on the basis of the results obtained from paired feeding experiments it is indicated that the decrease in citric acid secretion is correlated with a diminished intake of food rather than with absence of vitamin B_1 *per se*.[247]

(*b*) *The Succinic Acid Cycle.* The succinic acid cycle[248] may be considered as a variation of the citric acid cycle in that citric acid is not in-



volved and in that there is a difference in the nature of the oxidative and reductive reactions. The common principle of both cycles is the reduction

[240] H. G. Wood and C. H. Werkman, *Biochem. J.*, **34**, 129 (1940).
[241] A. I. Virtanen and T. Laine, *Nature*, **141**, 748 (1938).
[242] H. A. Krebs and L. V. Eggleston, *Biochem. J.*, **34**, 442 (1940). H. A. Krebs, *Ibid.*, **34**, 460 (1940).
[243] H. A. Krebs and L. V. Eggleston, *Ibid.*, **34**, 1383 (1940).
[244] G. K. McGowan and R. A. Peters, *Ibid.*, **31**, 1637 (1937).
[245] E. S. G. Barron and C. M. Lyman, *Science*, **92**, 337 (1940).
[246] H. A. Sober, M. A. Lipton and C. A. Elvehjem, *J. Biol. Chem.*, **134**, 605 (1940).
[247] A. H. Smith and C. E. Meyer, *Ibid.*, **139**, 227 (1941).
[248] K. Laki, F. B. Straub and A. Szent-Györgyi, *Z. physiol. Chem.*, **247**, 1 (1937).

of one part of the oxaloacetate (or pyruvate) at the expense of the oxidation of another part.[249]

According to the succinic acid cycle oxaloacetate is reduced to malate at the expense of an oxidation of pyruvate. Thus this reaction may be formulated:[249]

$$\text{Pyruvate} + \text{oxaloacetate} + \text{water} \longrightarrow \text{acetate} + CO_2 + \text{malate} \qquad (3)$$

The malate formed according to (3) may react further in two different ways. It may react with another molecule of pyruvate (4).

$$\text{Malate} + \text{pyruvate} \longrightarrow \text{oxaloacetate} + \text{lactate} \qquad (4)$$

Or malate may according to the succinic acid cycle be converted into fumarate and finally into succinate.

It is postulated that all these reactions occur at considerably different rates in various tissues. They serve well to explain the experimental findings. The succinic acid cycle appears to be involved in the metabolism of pyruvic acid through reaction (2) in various bacteria and to a certain extent also in animal tissues.

In certain bacteria anaerobic fermentation of pyruvate yields[249] malate, fumarate and succinate and results in an accumulation of the latter, as postulated by the succinic cycle concept. This reaction can then be written:[250]

$$\begin{array}{l} CH_3 \\ | \\ CO\cdot COOH \end{array} + CO_2 \longrightarrow \begin{array}{l} CH_2\cdot COOH \\ | \\ CO\cdot COOH \end{array} \longrightarrow \begin{array}{l} CH_2\cdot COOH \\ | \\ CHOH\cdot COOH \end{array} \longrightarrow \begin{array}{l} CH\cdot COOH \\ \| \\ CH\cdot COOH \end{array} \longrightarrow \begin{array}{l} CH_2\cdot COOH \\ | \\ CH_2\cdot COOH \end{array}$$

and occurs in *Propionibacterium*, *B. coli* and other species. In the case of *B. coli* it was shown[251] that the yield of succinate depends upon the pressure of CO_2. Succinic acid formation from pyruvic acid has also been demonstrated in certain animal tissues, such as, for example, in the kidney of rats.[252] In experiments in which the fixation of radioactive carbon dioxide by bacteria was studied, the radioactive element was actually found in the succinic acid isolated from the reaction products[253] and by degradation of the succinic acid it was shown that the radioactive carbon is exclusively in the carboxyl groups of the acid.[254]

[249] H. A. Krebs and L. V. Eggleston, *Biochem. J.*, **34**, 1383 (1940).
[250] H. G. Wood and C. H. Werkman, *Ibid.*, **34**, 7 (1940).
[251] S. R. Elsden, *Ibid.*, **32**, 187 (1938).
[252] E. S. G. Barron, C. M. Lyman, M. A. Lipton and J. Goldinger, *Proc. Am. Soc. Biol. Chem.*, **1941**, XI.
[253] H. G. Wood, C. H. Werkman, A. Hemingway and A. O. Nier, *J. Biol. Chem.*, **139**, 265 (1941).
[254] H. G. Wood, C. H. Werkman, A. Hemingway and A. O. Nier, *Ibid.*, **139**, 277 (1941).

The reaction mechanism in certain other bacteria, such as in *Staphylococci*[255] and *Lactobacillus delbrückii* is somewhat different.[256, 257] In these organisms the reaction proceeds mainly through reaction (3) and results in an accumulation of acetic acid. The other reaction products are totally metabolized.

In animal tissues, for example in the brain, and in certain bacteria, lactic acid is accumulated,[255, 258, 259] which originated from the succinic cycle according to reaction (4).[249]

In most animal tissues and in bacteria, all these reactions occur at the same time but at different rates. Thus, in quantitative experiments with brain tissue,[260] pyruvic acid was utilized in the following manner: 67% was completely metabolized according to the citric acid cycle yielding only CO_2, 29.5% was carried through the succinic acid cycle yielding, besides CO_2, acetic acid and lactic acid.

Certain other explanations for the reaction mechanism of the pyruvic acid metabolism in the presence of vitamin B_1 have been postulated at various times. They all differ from the mechanism as presented in the previous paragraphs in that reaction (2), the initial formation of oxaloacetic acid, is not postulated. As the result an involvement of the citric and succinic acid cycles was not considered. Thus, the formation of acetic acid has been explained as decarboxylation (according to reaction (1)) with simultaneous dehydrogenation.[261]

$$CH_3{\cdot}CO{\cdot}COOH + H_2O \longrightarrow CH_3{\cdot}COOH + CO_2 + H_2 \cdot$$

or

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

The occurrence of lactic acid has been explained as a dismutation:[262, 263, 264]

$$2CH_3{\cdot}CO{\cdot}COOH \rightarrow CH_3{\cdot}CHOH{\cdot}COOH + CH_3{\cdot}COOH + CO_2$$

It has been suggested that the dehydrogenations and dismutations are accomplished through some flavin-adenine-enzyme systems[265] while only the decarboxylations are carried out by the vitamin B_1-pyrophosphate-enzyme system. These explanations do not appear attractive in the light of the previously discussed hypothesis and the evidence for an intermediary formation of oxaloacetic acid by means of the vitamin B_1 enzyme system.

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[255] E. S. G. Barron and C. M. Lyman, *J. Biol. Chem.*, **127**, 143 (1939).
[256] F. Lipmann, *Enzymologia*, **4**, 64 (1937); *Nature*, **140**, 25 (1937); **143**, 436 (1939).
[257] G. M. Hills, *Biochem. J.*, **32**, 383 (1938).
[258] H. A. Krebs, *Nature*, **138**, 288 (1936).
[259] H. A. Krebs, *Biochem. J.*, **31**, 661 (1937).
[260] C. Long, *Ibid.*, **32**, 1711 (1938).
[261] F. Lipmann, *Enzymologia*, **4**, 64 (1937); *Nature*, **140**, 25 (1937); **143**, 436 (1939).
[262] H. A. Krebs, *Nature*, **138**, 288 (1936).
[263] E. S. G. Barron and C. M. Lyman, *J. Biol. Chem.*, **127**, 143 (1939).
[264] H. A. Krebs, *Biochem. J.*, **31**, 661 (1937).
[265] F. Lipmann, *Nature*, **143**, 281 (1939).

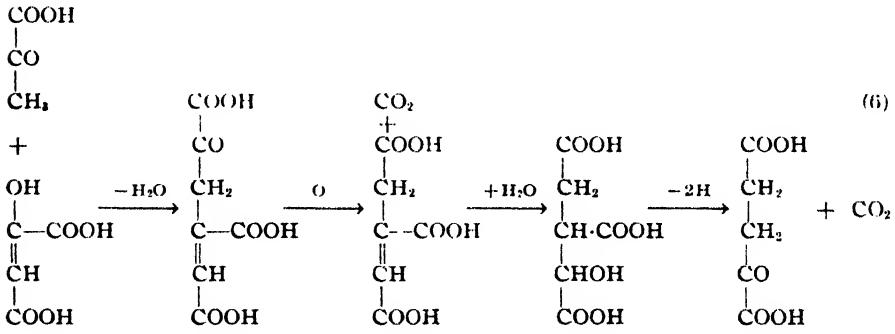
The initial formation of oxaloacetate from pyruvate under the influence of diphospho-vitamin B_1 also makes a condensation of pyruvic acid with other acids plausible. Thus it might be possible [266] that acetic acid condenses with pyruvic acid (reaction (5)) in strict analogy to the condensation of CO_2 with pyruvic acid (reaction (1)).

$$CH_3\cdot COOH + CH_3\cdot CO\cdot COOH \xrightarrow{-H_2O} CH_3\cdot CO\cdot CH_2\cdot CO\cdot COOH \qquad (5)$$

$$HO\cdot COOH + CH_3\cdot CO\cdot COOH \xrightarrow{-H_2O} HO\cdot CO\cdot CH_2\cdot CO\cdot COOH \qquad (1)$$

Actually, the formation of acetoacetate in pigeon[266] and chicken[267] liver has been demonstrated to be a function of vitamin B_1-pyrophosphate and might be explained by an initial formation of acetopyruvate according to reaction (5).

The citric and the succinic acid cycles are probably not the only reactions involved in the utilization of pyruvic and oxaloacetic acids. At least one other mechanism must be assumed, since in radioactive α-keto-glutaric acid obtained in rat liver from pyruvic acid and radioactive sodium bicarbonate, the radioactive carbon atom is fixed solely in the carboxyl group which is in α-position to the keto group as evident from degradation reactions.[268] Thus, this α-keto-glutaric acid cannot be formed according to the citric acid cycle which would require an equal distribution of the radioactive carbon in both carboxyl groups. Another series of reactions (6) has therefore been postulated[268] for the formation of this acid from oxaloacetic and pyruvic acids.



So far the action of vitamin B_1 in the carbohydrate metabolism has been discussed only in its relation to the utilization of pyruvic acid. While this is apparently the most important action, other observations may be men-

[266] H. A. Krebs and L. V. Eggleston, *Biochem. J.*, 34, 1383 (1940).
[267] E. S. G. Barron, C. M. Lyman, M. A. Lipton and J. Goldinger, *Proc. Am. Soc. Biol. Chem.* 1941, XI.
[268] H. G. Wood, C. H. Werkman, A. Hemingway and A. O. Nier, *J. Biol. Chem.*, 139, 483 (1941).

tioned concerning a disturbed carbohydrate metabolism during vitamin B_1 deficiency. Thus, glycogen, the specific animal carbohydrate, requires the presence of this vitamin to be properly metabolized. An increase of the glycogen content in liver and in heart muscles has been observed in pigeons during avitaminosis.[269] In the urine of children suffering from vitamin B_1 deficiency, methyl-glyoxal is found.[270] In rabbits, intramuscular injection of vitamin B_1 causes hypoglycemia in amounts of 0.75 mg., while 2 mg. or more bring about marked hyperglycemia.[271] In man it has generally been observed that an increased carbohydrate metabolism, for example, after an intake of large amounts of carbohydrates, during physical labor or during fever periods, requires an increased vitamin B_1 utilization.

The fat metabolism is influenced by vitamin B_1 only in so far as the synthesis of fat from carbohydrates is concerned, that is, the metabolism of ingested fats apparently does not need any vitamin B_1. On the other hand, the utilization of acetic acid, as an intermediate in the carbohydrate breakdown, appears to be accelerated in bacteria by the presence of vitamin B_1.[272] Fat synthesis in experimental animals, for example, in pigeons and in rats, on a pure carbohydrate diet, is dependent upon the presence of vitamin B_1.[273, 274]

The water metabolism in the organism also seems connected with the action of vitamin B_1, probably through the carbohydrate metabolism. Edema and water imbibitions in the heart and in other organs are symptoms of B_1-avitaminosis. Vitamin B_1 also takes part in the regulation of the nervous system. During nerve excitement, two compounds are liberated from the nerve: acetylcholine and vitamin B_1.[275] It has been shown experimentally that vitamin B_1 cannot bring about contractions of the gut, whereas the acetylated vitamin B_1 has the power to do so,[276] as does acetylcholine. Acetyl-thiamin, like acetylcholine, can be hydrolyzed enzymatically, perhaps even by the same acetylcholine-esterase.[277] It has, therefore, been suggested that actually not vitamin B_1 but an ester is

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[269] E. Abderhalden and W. Wertheimer, *Arch. ges. Physiol (Pflügers)*, **233**, 395 (1933).

[270] A. Geiger and A. Rosenberg, *Klin. Wochschr.*, **12**, 1258 (1933).

[271] G. Ortoleva, *Biochim, terap. sper.*, **25**, 511 (1938).

[272] J. H. Quastel and D. M. Webley, *Nature*, **144**, 633 (1929).

[273] E. W. McHenry, *Science*, **86**, 200 (1937). E. W. McHenry and G. Gavin, *J. Biol. Chem.*, **125**, 653 (1938). E. W. McHenry and G. Gavin, *Ibid.*, **128**, 45 (1939).

[274] E. W. McHenry, *J. Physiol.*, **89**, 287 (1937). H. E. Longenecker, G. Gavin and E. W. McHenry, *J. Biol. Chem.*, **134**, 693 (1940).

[275] B. Minz and R. Agid, *Compt. rend.*, **205**, 576 (1937). L. Binet and B. Minz, *Arch. intern. physiol.*, **42**, 281 (1936). B. Minz, *Compt. rend. soc. biol.*, **127**, 1251 (1938).

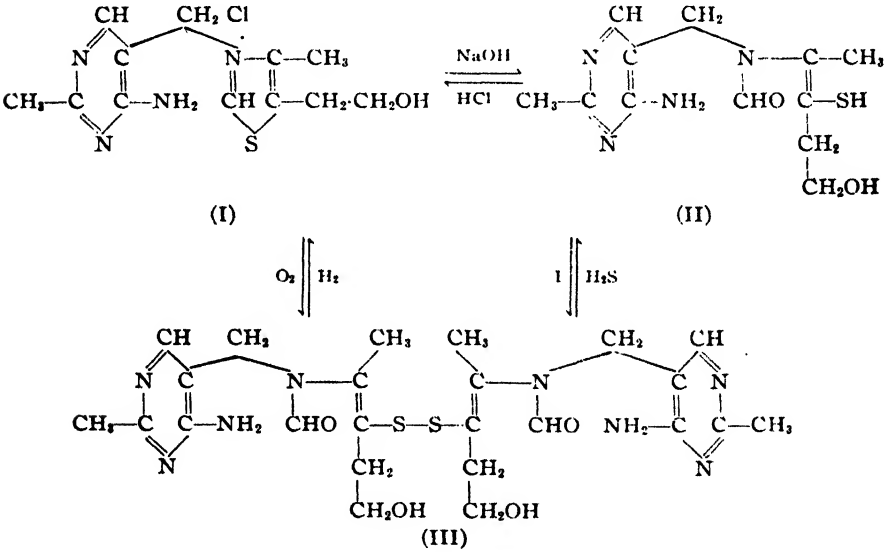
[276] R. Kuhn, T. Wieland and H. Huebschmann, *Z. physiol. Chem.*, **259**, 48 (1939).

[277] L. Massart and R. Dufait, *Naturwissenschaften*, **27**, 567 (1939). H. Süllmann and H. Birkhäuser, *Schweiz. med. Wochschr.*, **69**, 648 (1939). L. Massart and R. Dufait, *Enzymologia*, **7**, 385 (1939).

liberated from the nerves.[278] It should, however, be noted that one would expect rapid removal of a mediator of nerve impulses from the site of action. Thus, acetylcholine is made inactive hydrolytically by cholinesterase. The enzymatic hydrolysis of acetyl-thiamin by horse serum and by brain extracts proceeds, however, very slowly.[279]

(c) *Mechanism of the Vitamin B_1 Action*

Vitamin B_1 takes part in tissue oxidations of carbohydrates. It might, therefore, be expected that vitamin B_1 acts as a compound capable of reversible oxidation and reduction. This is apparently the case. Vitamin B_1 (I) represents the reduced form and can be oxidized to a disulfide (III).[280] This oxidation occurs under physiological conditions, for example, at pH 7.5 with hydrogen peroxide or oxygen from air,[281] but can also be brought about by oxidation in alkaline solution with iodine. On the other hand, the disulfide can be reduced to the thiol form (II) of vitamin B_1 by hydrogen, hydrogen sulfide, glutathione or cysteine[281] and the thiol form is converted into the vitamin by acids such as hydrochloric acid. In



[278] H. U. Graf and A. v. Muralt, *Angew. Chem.*, **52**, 465 (1939).

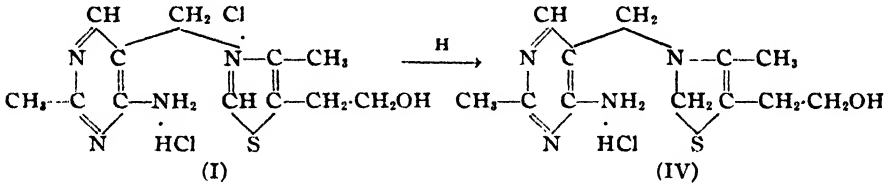
[279] H. Süllmann and H. Birkhäuser, *Schweiz. med. Wochschr.*, **69**, 648 (1939). L. Massart and R. Dufait, *Naturwissenschaften*, **27**, 567 (1939). D. Glick and W. Autopol, *Proc. Soc. Exptl. Biol. Med.*, **42**, 396 (1939).

[280] O. Zima and R. R. Williams, *Ber.*, **73**, 941 (1940).

[281] O. Zima, K. Ritsert and T. Moll, *Z. physiol. Chem.*, **267**, 210 (1941).

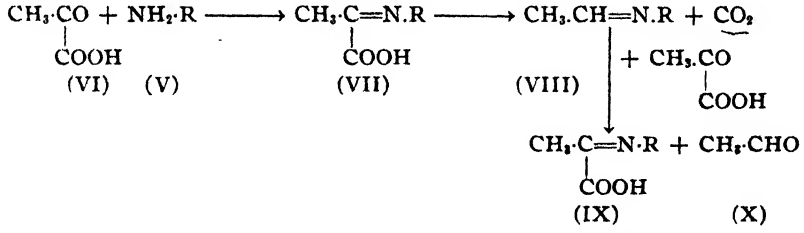
body fluids and in tissues, the reduction apparently does not stop at the thiol stage. The disulfide shows the full biological activity of the vitamin itself and is about five times less toxic than the vitamin.

The possibility that vitamin B_1 represents not the reduced but the oxidized form has also been investigated. Actually, by mild reducing agents, for example, by the action of hydrosulfite in sodium carbonate solution, one atom of hydrogen is absorbed[282] (I → IV). The reduced form is a reducing agent but is non-autoxidizable. It is also devoid of vitamin activity. Furthermore, upon pyrophosphorylation the molecule becomes more resistant to the action of reducing agents.[283] The possibility, therefore, that vitamin B_1 acts according to this scheme can be discarded.



(I) (IV)

It has also been suggested that vitamin B_1 may act through its amino-group according to the Langenbeck cycle.[284] In this system all primary amines (V) which have been investigated, decarboxylate α-keto-carboxylic acids (VI) to the corresponding lower aldehyde through the formation of an imino-acid (VII) which decomposes into carbon dioxide and an aldimine (VIII). The latter reacts with another molecule of the keto-acid, thus yielding again one mol of imino-acid (IX) and one mol of aldehyde (X). This reaction mechanism is, however, very improbable since in the actual test system for the Langenbeck cycle vitamin B_1 was found to be completely inactive.[285]



(d) *Relation of Vitamin B_1 to Other Vitamins, to Hormones, and Minerals*

The close relation of vitamin B_1 to the carbohydrate metabolism becomes apparent from the relation of the vitamin to the hormone of the thyroid gland, thyroxine. Thyroxine secretion increases the intensity of the general metabolism and hence necessitates an increased amount of vita-

[282] F. Lipmann, *Nature*, **138**, 1097 (1937).
[283] E. S. G. Barron and C. M. Lyman, *Science*, **92**, 337 (1940).
[284] *Ergeb. Enzymforsch.*, **2**, 314 (1933).
[285] K. G. Stern and J. L. Melnick, *J. Biol. Chem.*, **131**, 597 (1939).

min B_1.[286, 287, 288, 289, 290] A similar relationship can be demonstrated for vitamin B_1 and insulin. Vitamin B_1 increased the sugar tolerance of rats in insulin hypoglucemia experiments.[291]

A close relationship exists between one of the hormones of the adrenal cortex and vitamin B_1. During vitamin B_1-avitaminosis a hypertrophy of the adrenal cortex occurs. Since one of the hormones of the adrenal cortex regulates the lipoid content of blood, hypertrophy of the adrenal gland causes increase of the cholesterol content of blood. If during B_1-avitaminosis extracts of the adrenal cortex are injected, hypertrophy of the gland does not occur and the cholesterol content of the blood does not increase. Hypertrophy of the gland, on the other hand, can apparently be cured by vitamin B_1 intake.

It has been reported that substances of the cyclo-pentano-perhydrophenanthrene type, for example, female and male sex hormones and vitamins D,[295] delay symptoms of vitamin B_1 deficiency. These results, however, need confirmation.

The relation of vitamin B_1 to the other vitamins also needs further study. Vitamin A is said to act antagonistically, since increase of the vitamin A intake increases the symptoms of vitamin B_1 deficiency. On the other hand, vitamin A deficiency in rats on a diet containing normal amounts of B_1 causes the appearance of symptoms of a B_1 deficiency (pyruvic acid in the blood) which can be cured by increased B_1 intake.[294]

Interesting is the relationship of vitamin B_1 to the zinc metabolism. During avitaminosis the zinc contents of the blood,[292] the toenails, fingernails and skin are reduced to half their normal values.[293] In natural food-stuffs there seems to be a correlation of the amount of zinc and vitamin B_1.[293]

A possible relation of vitamin B_1 to manganese has been observed. When rats were fed 50 International Units per day, there resulted, after one generation, interference with lactation, loss of maternal instinct, cannibalism and progressive loss of fertility. When small amounts of manganese chloride (2 mg. per day) were added to the diet, all these effects disap-

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[286] H. E. Himwich, W. Goldfarb and G. R. Cowgill, *Am. J. Physiol.*, **99**, 689 (1938).
[287] G. R. Cowgill and M. L. Palmieri, *Ibid.*, **105**, 146 (1933).
[288] B. Sure and K. S. Buchanan, *J. Nutrition*, **13**, 513 (1937).
[289] V. A. Drill and C. R. Sherwood, *Am. J. Physiol.*, **124**, 683 (1938).
[290] R. A. Peters and R. J. Rossiter, *Biochem. J.*, **33**, 1140 (1939).
[291] J. C. Burke and A. R. McIntyre, *J. Pharmacol.*, **64**, 465 (1938).
[292] W. G. E. Eggleton, *Chinese J. Physiol.*, **15**, 33 (1940).
[293] W. G. E. Eggleton, *Biochem. J.*, **33**, 403 (1939).
[294] H. v. Euler and B. Högberg, *Naturwissenschaften*, **27**, 769 (1939).
[295] J. Sanchez-Rodriguez and J. M. Sarda, *Z. Vitaminforsch.*, **6**, 193 (1937).

peared.[296] It has also been noted[297, 298] that Mn^{++} greatly stimulates the carboxylase system if present in appropriate concentrations.

16. Avitaminosis and Hypovitaminosis

Severe cases of vitamin B_1 deficiency in man have been and still are very common in the tropics, especially in the Philippines and India and in Japan and are due to poor nourishment, the food consisting mainly of polished rice. In America and Europe, severe cases are only occasionally observed and are caused, for example, by chronic alcoholic addiction, by pregnancy, by toxic agents such as nicotine, lead, thallium, arsenic and mercury, etc. Cases of more or less severe hypovitaminosis are quite common in the Western countries due to inadequate nutrition.

The symptoms generally ascribed to vitamin B_1 deficiency are mostly symptoms of combined deficiencies of several vitamins of the B-group, especially B_2, nicotinic amide, B_6, etc. Vitamin B_1 deficiency in man affects first of all the emotions and the tonus of the nervous system. Further symptoms are loss of appetite (anorexia), unusual susceptibility to fatigue combined with lower physical endurance, gastrointestinal disturbances, muscular weakness, pains and paraesthesia in arms and legs, edema in ankles and face and decrease of the blood pressure. In more severe cases, the entire nervous system is affected and symptoms of polyneuritis and neuralgia occur. The specific polyneuritis due to vitamin B_1 deficiency is bilateral, symmetrical and involves predominantly the lower extremities.[299] Muscle cramps of the calf are often noted and, at later stages, calf muscle atrophy. The position sense in the toes is disturbed and foot drop is manifested. The typical form of beriberi, the severe disease of the tropics, is accompanied by symptoms such as lameness, ataxia, disturbance of the motor and sensory nerves followed by labored breathing and hypertrophy of the right heart and finally death from heart failure. Cardiovascular dysfunction may also arise from mild but continued deficiency of vitamin B_1.[300]

The symptoms of vitamin B_1 deficiency in rats resemble very closely the symptoms of human beings. In addition to these, there is a marked influence upon the growth of young animals. More severe avitaminosis causes convulsions and paralysis of the lower extremities.

[296] D. Perla, *Proc. Soc. Exptl. Biol. Med.*, **37**, 169 (1937); *Science*, **89**, 132 (1939).
[297] K. Lohmann and P. Schuster, *Biochem. Z.*, **294**, 188 (1937).
[298] S. Ochoa and R. A. Peters, *Biochem. J.*, **32**, 1501 (1938).
[299] N. Jolliffe, *Minnesota Med.*, **23**, 542 (1940).
[300] S. Weiss and R. W. Wilkins, *Tr. A. Am. Physicians*, **51**, 341 (1936); *Ann. Internal Med.*, **11**, 104 (1937). W. A. Jones and B. Sure, *J. Lab. Clin. Med.*, **22**, 991 (1937).

The symptoms in pigeons, which have been used for the biological detection of the vitamin are quite characteristic. These birds first show lassitude and eat little. They sit with ruffled feathers and draw their heads far back and upside down (head retraction: opisthotonus). Convulsions may follow. Two or three days after these symptoms appear the bird dies.

(*a*) *Clinical Test Methods*

A diagnosis for vitamin B_1 deficiency can be carried out by a number of different procedures. The amount of this vitamin present in blood and in urine can be determined. The cocarboxylase content in blood can also be used as an assay procedure. Finally, a determination of the amount of pyruvic acid present in blood or in urine can serve as an indication for a vitamin B_1 deficiency. In addition to these direct methods, determinations of the tissue saturation with vitamin B_1 may be carried out in which the effects of defined doses of vitamin B_1 are measured by any of the indicated methods before and after administration.

Urine Tests. The urinary output of vitamin B_1 is closely related to the state of nutrition of the body. Healthy humans on an adequate diet excrete between 50 and 150 γ vitamin B_1 per day.[301, 302, 303, 304] Lower values are found during vitamin B_1 deficiency,[305] pregnancy[304] and a variety of diseases.[303, 306] Vitamin B_1 pyrophosphate (cocarboxylase) does not appear normally in the urine.

For the actual *determination of vitamin* B_1 a number of different methods have been recommended. The *bradycardia assay*,[307] for example, has given most satisfactory results. The *thiochrome method* must be modified since urine contains fluorescent compounds which interfere with the thiochrome determination. Various modifications of the basic method have been recommended[306, 308, 309, 310, 311] and have been applied successfully as

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[301] J. Houston, S. K. Kon and S. Y. Thompson, *J. Soc. Chem. Ind.*, **58**, 651 (1939). J. Houston and S. K. Kon, *Nature*, **143**, 558 (1939).

[302] K. Ritsert, *Klin. Wochschr.*, **17**, 1397 (1938).

[303] H. Schroeder, *Ibid.*, **18**, 148 (1939).

[304] H. G. K. Westenbrink and J. Goudsmit, *Arch. néerland. Physiol.*, **23**, 79 (1938).

[305] L. J. Harris, P. C. Leong and C. C. Ungley, *Lancet*, **234**, 539 (1938). L. J. Harris and P. C. Leong, *Ibid.*, **1**, 886 (1936).

[306] Y. L. Wang and L. J. Harris, *Biochem. J.*, **33**, 1356 (1939). Y. L. Wang and J. Yudkin, *Ibid.*, **34**, 343 (1940).

[307] T. W. Birch and L. J. Harris, *Ibid.*, **28**, 602 (1934).

[308] J. Marrack and H. F. Höllering, *Lancet*, **236**, 325 (1939).

[309] G. M. Hills, *Biochem. J.*, **33**, 1966 (1939).

[310] H. G. K. Westenbrink, J. Goudsmit, and B. C. P. Jansen, *Nature*, **139**, 1108 (1937); *Rec. trav. chim.*, **56**, 803 (1937); *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **8**, 21,119 (1938).

[311] M. Jowett, *J. Soc. Chem. Ind.*, **58**, 556 (1939); *Biochem. J.*, **34**, 1348 (1940).

clinical test methods, especially when larger quantities of the vitamin are being assayed such as in saturation tests. The *colorimetric method by Prebluda and McCollum* has also been applied successfully to the assay of vitamin B_1 in urine.[312] The *yeast fermentation method* offers promising results for urine tests.[313, 314] Since urine contains, however, other substances which stimulate the fermentation, it has been recommended[315] to carry out a determination before and after an oxidative inactivation of the vitamin. In a parallel test in which vitamin B_1 has been added to the urine, the efficiency of the inactivation procedure is evaluated.

Pyruvic acid determinations[316, 317, 318, 319, 320, 321, 322, 323, 324, 325] can be made to detect a state of vitamin B_1 deficiency. One assay procedure is based on the isolation of sodium pyruvic acid-2,4-dinitrobenzoate and the red color developed by the latter with strong alkali. In practice, the urine (or the blood) is mixed with trichloroacetic acid (or in the case of blood, better with tungstic acid) and a solution of 2,4-dinitro-phenyl-hydrazine in diluted hydrochloric acid solution is added. The hydrazones formed are extracted with ethyl acetate followed by an extraction with a sodium carbonate solution. Finally a sodium hydroxide solution is added and the color developed is measured. This method is, of course, not specific for the determination of pyruvic acid since other α-keto-carboxylic acids, such as acetoacetic acid, oxaloacetic acid and α-keto-glutaric acid, give the same reaction. Pyruvic acid can also be estimated by determination of bisulfite-binding substances. According to this procedure urine is mixed with solutions of oxalic acid and of sodium bisulfite. After an appropriate length of time, the excess of bisulfite is removed and the bisulfite-bound substances are titrated iodometrically.

The amount of pyruvic acid in the urine of the normal and vitamin B_1-deficient man has not been studied systematically. In rats the amount excreted increases during vitamin B_1 deficiency 200–400%, but is also a

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[312] D. Melnick and H. Field, *J. Biol. Chem.*, **123**, 83 (1938); **130**, 97 (1939); *Proc. Soc. Exptl. Biol. Med.*, **38**, 723 (1938).
[313] A. S. Schultz, L. Atkin and C. N. Frey, *J. Am. Chem. Soc.*, **59**, 948, 2457 (1937); **60**, 1514 (1938).
[314] A. S. Schultz, L. Atkin and C. N. Frey, *Proc. Soc. Exptl. Biol. Med.*, **38**, 404 (1938).
[315] A. S. Schultz, L. Atkin and C. N. Frey, *J. Biol. Chem.*, **136**, 713 (1940).
[316] B. S. Platt and G. D. Lu, *Quart. J. Med.*, **5**, 355 (1936). G. D. Lu, *Biochem. J.*, **33**, 249 (1939).
[317] F. P. Clift and R. R. Cook, *Biochem. J.*, **26**, 1788 (1932).
[318] G. G. Banerji and L. J. Harris, *Ibid.*, **33**, 1346 (1939).
[319] M. Shils, H. G. Day and E. V. McCollum, *Science*, **91**, 341 (1940).
[320] H. A. Harper and H. J. Denel, *J. Biol. Chem.*, **137**, 233 (1941).
[321] D. Klein, *Ibid.*, **137**, 311 (1941).
[322] E. M. Case, *Biochem. J.*, **26**, 753 (1932).
[323] R. A. Peters and R. H. S. Thompson, *Ibid.*, **28**, 916 (1934).
[324] E. Bueding and H. Wortis, *J. Biol. Chem.*, **133**, 585 (1940).
[325] M. Shils, H. G. Day and E. V. McCollum, *Ibid.*, **139**, 145 (1941).

function of the type and quantity of food taken in. The urinary output of pyruvic acid appears to be higher in the male than in the female.

Blood Tests. The *determination of vitamin B_1 in plasma* is rather difficult since the concentration is normally very small, about 1 γ in 100 ml. Furthermore, slight hemolysis of the blood affects the results considerably.[326] The only method, therefore, which has been found useful is the determination of the growth rate of *Phycomyces blakesleanus*.[327] Blood contains, however, other factors which increase the growth rate of this fungus. Nevertheless, the method is valuable for comparing the apparent vitamin B_1 content in different samples of blood.[328] The difficulties can partly be overcome by evaluation of the growth rate by blood with and without the addition of vitamin B_1.

The *determination of vitamin B_1-pyrophosphate (cocarboxylase)*[326, 329, 330, 331] can be carried out much more easily. The pyrophosphate occurs exclusively in the blood cells. Therefore in cases in which the blood cell count is disturbed, for example, in cases of polycythemia or of myeloid leukemia, the cocarboxylase determination should not be used. The method consists in the determination of the carbon dioxide production from pyruvic acid in the presence of yeast as a source of carboxylase and in the presence of excess amounts of vitamin B_1. Instead of using whole blood for the determination, the use of washed blood cells has been recommended. Blood of healthy adults contains an average of about 7 γ of cocarboxylase per 100 ml. A value below 3 γ is considered as indicative of a state of vitamin B_1 deficiency.

The *determination of pyruvic acid* is carried out as described for its determination in urine. A marked increase in pyruvic acid has been found in man and in experimental animals, such as in pigeons[332] and in rats[333] during times of vitamin B_1 deficiency. The normal pyruvic acid content of human blood averages 2.8 mg. per 100 cc.

17. Hypervitaminosis

Vitamin B_1 has no specific pharmacological action, as far as is known today. The vitamin is non-toxic, even in doses which are several thousand times larger than a normal daily dose. No cases of human vitamin B_1

[326] R. Goodhart and H. M. Sinclair, *Biochem. J.*, 33, 1099 (1939).
[327] H. M. Sinclair, *Ibid.*, 33, 2027 (1939).
[328] H. M. Sinclair, *Ibid.*, 32, 2185 (1938).
[329] S. Ochoa and R. A. Peters, *Ibid.*, 32, 1501 (1938).
[330] R. Goodhart and H. M. Sinclair, *J. Biol. Chem.*, 132, 11 (1940).
[331] R. Goodhart, *Ibid.*, 135, 77 (1940).
[332] R. H. S. Thompson and R. E. Johnson, *Biochem. J.*, 29, 694 (1935).
[333] G. D. Lu. *Ibid.*, 33, 774 (1939).

hypervitaminosis have ever been reported. The therapeutic index, that is, the ratio of the therapeutic dose to the minimum lethal dose, is extremely high: 600 for mice, 5000 for rats and 70,000 for dogs. Doses of 125 mg. per kg. body weight for mice, 250 mg. per kg. for rats, 300 mg. per kg. for rabbits, or 350 mg. per kg. for dogs are lethal when injected intravenously.[334]

18. Requirements

It is impossible to define accurately the requirements of vitamin B_1. The amount needed is not a constant, but a function of the supplied food, of the intensity of the metabolism, of the outside temperature and of other factors. The consumption of carbohydrates increases and of fat decreases the amount of vitamin B_1 needed by the organism, while proteins do not have any influence on the vitamin requirement. The intensity of the total metabolism is again a function of many single factors, among which might be mentioned the secretion of thyroxin, the physical labor performed, pregnancy, etc.

The ratio of the minimum amount of vitamin B_1 needed to support life to the optimal amount for general development of the body is very high and can vary from 3 up to 100.

The vitamin B_1 requirement of man, therefore, cannot be defined exactly. The allowances as recommended by the Food and Nutrition Board of the National Research Council (for details see page 613) call for a daily intake varying from 1.2–2.3 mg. of thiamin for adults depending upon sex and relative activity. The requirements for children are correspondingly lower. A six to eight months old baby should receive 0.4 mg. of thiamin.[335]

Vitamin B_1 is, as far as is known, needed by all animals, even by insects and microorganisms. Sheep[336] and cattle[337, 338, 339] apparently need no external supply of this vitamin, since it is synthesized by bacteria in the rumen of these animals.

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[334] H. Molitor, *Merck's Jahresberichte*, 1936, 51.

[335] E. M. Knott, *Proc. Soc. Exptl. Biol. Med.*, 45, 765 (1940).

[336] L. W. McElroy and H. Goss, *J. Biol. Chem.*, 130, 437 (1939).

[337] L. W. McElroy and H. Goss, *Ibid.*, 133, LXV (1940).

[338] M. I. Wegner, A. N. Booth, C. A. Elvehjem and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.*, 45, 769 (1940).

[339] S. I. Bechdel, H. E. Honeywell, R. A. Dutcher and M. H. Knutsen. *J. Biol. Chem.*, 80, 231 (1928).

VITAMIN B_2—
RIBOFLAVIN

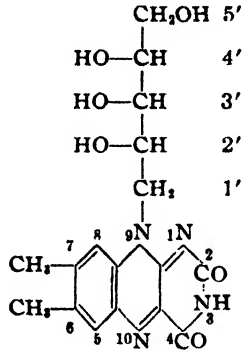
VITAMIN B_2—RIBOFLAVIN

1. Nomenclature and Survey

Names:

Riboflavin: name used in America.[1]
Lactoflavin: name used in Europe.
Ovoflavin and hepatoflavin: historical names, indicating the origin.
Vitamin G: historical designation by Sherman.

Chemical formula:



Chemical name:

6,7-Dimethyl-9-(*d*-1′-ribityl-)iso-alloxazine.
6,7-Dimethyl-9-*d*-riboflavin.

Empirical formula:

• $C_{17}H_{20}N_4O_6$.

Efficacy:

1 g. riboflavin = 400,000 Bourquin-Sherman Units.

Classification:

Vitamin B_2 belongs to the class of colored, water-soluble, naturally occurring substances, which carry the group name, lyochromes. In distinction from the lyochromes, the colored, fat- or organic solvent-soluble, naturally occurring substances

[1] The term *d*-riboflavin or just riboflavin was suggested by the Council on Pharmacy and Chemistry, *J. Am. Med. Assoc.*, **108**, 1340 (1937), and approved by the Committee on Vitamin Standards, American Society of Biological Chemists, and also by the Committee on Vitamin Nomenclature, American Institute of Nutrition.

are called lipochromes. The lyochromes are not as yet systematically subclassified. Arbitrarily, vitamin B_2 is a member of the subdivision flavins. The members of this group carry names indicating the origin of their occurrence. Thus lactoflavin comes from milk, ovoflavin from eggs, hepatoflavin from liver, uroflavin from urine, etc. All naturally occurring flavins which have been investigated, with the exception of uroflavin, proved to be identical with riboflavin.

2. Chronology

1879 BLYTH[2] isolated an impure flavin from whey.

1913 OSBORNE and MENDEL recognized in milk the presence of a water-soluble substance which promotes growth.[3]

1917 EMMETT and MCKIM differentiated the physiological action of vitamin B_1 from vitamin B_2.[4]

1925 BLEYER and KALLMANN obtained a yellow pigment called "lactochrome" in crude form from milk.[5]

1932 WARBURG and CHRISTIAN isolated the "yellow enzyme" from yeast.[6] BANGA and SZENT-GYÖRGYI[7] recognized a respiration co-ferment in yeast, the colored part of which was called "cytoflav."

1933 ELLINGER and KOSCHARA, and KUHN, GYÖRGY and WAGNER-JAUREGG isolated pure riboflavin[8]. The latter authors recognized the identity of vitamin B_2 and riboflavin.

1935 KUHN and KARRER and their groups established the constitution of riboflavin by total synthesis.

3. Occurrence

Riboflavin is very widely distributed over the entire animal and plant kingdom.[8] It seems that each animal and plant cell contains small amounts. The amount in plant seeds is small but increases rapidly during germination. The richest source of riboflavin is anaerobic growing fermentation bacteria; for example, butyric acid bacteria in the dried state contain up to 15 mg. %. Also various yeasts contain fair amounts of riboflavin. Liver, kidney and heart of vertebrata and fish livers contain about 10–30 mg. % or about 10–30 times more than muscles. The retina

[2] A. W. Blyth, *J. Chem. Soc.*, **35**, 530 (1879).

[3] T. B. Osborne and L. B. Mendel, *J. Biol. Chem.*, **15**, 311 (1913).

[4] A. D. Emmett and L. H. McKin, *Ibid.*, **32**, 409 (1917).

[5] B. Bleyer and O. Kallmann, *Biochem. Z.*, **155**, 54 (1925).

[6] O. Warburg and W. Christian, *Naturwissenschaften*, **20**, 688, 980 (1932); *Biochem. Z.*, **254**, 438 (1932); **257**, 492 (1933); **266**, 377 (1933).

[7] I. Banga and A. Szent-Györgyi, *Biochem. Z.*, **246**, 203 (1932). I. Banga, A. Szent-Györgyi and L. Vargha, *Z. physiol. Chem.*, **210**, 228 (1932).

[8] P. György, R. Kuhn and T. Wagner-Jauregg, *Z. physiol. Chem.*, **223**, 21 (1934). H. v. Euler and E. Adler, *Ibid.*, **223**, 105 (1934).

of the eyes of many species of animals contains considerable quantities of riboflavin.[9]

Vitamin B_2 occurs in the animal organism in a number of different forms. In milk, in urine[10] and in the retina,[11] or, generally speaking, in places where no respiration or fermentation takes place, the free riboflavin is found. In tissues, riboflavin occurs as such, as riboflavin-phosphoric acid and in the form of riboflavin-phosphoric-acid-adenine-dinucleotide. Each of these forms occurs in the free state as well as combined with specific proteins (see page 171). For a combination of riboflavin with protein in muscles, see[12].

4. Isolation

The isolation of riboflavin is carried out by extraction of flavin-containing materials with aqueous acid solutions, with water-alcohol mixtures, with alcohol or with acetone. Direct extraction of natural materials, especially of plant origin, yields only part of the total riboflavin present. The remaining vitamin is chemically bound but can be liberated by heating.[13] The extraction is followed by a combination of different precipitation and adsorption procedures. The following precipitation methods have been used:[14, 15] lead acetate, phosphotungstic acid in normal sulfuric acid followed by extraction of the precipitate with amyl alcohol; silver nitrate or mercuric sulfate in acid solution precipitates other substances, leaving the vitamin B_2 in solution, but silver nitrate in neutral solution precipitates the vitamin. By the addition of alcohol to concentrated aqueous riboflavin solutions, salts and glycogen are eliminated.

Riboflavin is adsorbed in acid solution by fuller's earth[16] or in neutral solution by frankonit. Charcoal can also be used successfully. Talc, aluminum oxide, calcium carbonate, kaolin and kieselguhr do not absorb vitamin B_2. The elution is carried out by basic solvents such as pyridine-

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[9] O. Brunner and E. Baroni, *Monatsh.*, **68**, 264 (1936).

[10] A. Emmerie, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **8**, 116 (1938).

[11] H. v. Euler and E. Adler, *Arkiv Kemi, Mineral. Geol.*, **B11**, No. 28 (1934). R. Kuhn and H. Kaltschmitt, *Ber.*, **68**, 386 (1935). E. Adler and H. v. Euler, *Nature*, **141**, 790 (1938). H. v. Euler and E. Adler, *Z. physiol. Chem.*, **223**, 105 (1934).

[12] J. Schormüller, *Z. Untersuch. Lebensm.*, **77**, 1 (1939).

[13] E. M. Lantz, *Agr. Exptl. Sta. New Mexico Coll. Agr. Mech. Arts, Bull.* **268** (1939).

[14] B. C. Guha, *Biochem. J.*, **25**, 945 (1931).

[15] P. György, R. Kuhn and T. Wagner-Jauregg, *Z. physiol. Chem.*, **223**, 21, 27, 236, 241 (1934).

[16] A. Seidell, *U. S. Pub. Health Service Pub. Health Repts.*, **31**, 464 (1916). W. D. Salmon, N. B. Guerrant and I. M. Hays, *J. Biol. Chem.*, **80**, 91 (1928). B. T. Narayanan and J. C. Drummond, *Biochem. J.*, **24**, 19 (1930).

methanol-water mixtures,[17, 18] ammonia, caustic solutions, triethanolamine or by neutral organic solvents, such as 80% acetone. After precipitation, together with lead sulfide, riboflavin is extracted with hot water.[17, 19]

From concentrates obtained from the elution of adsorbates, riboflavin usually crystallizes out in clusters of fine orange-yellow needles. They can be purified further by precipitation with thallium ions or by one of the previously described precipitation methods. Final recrystallizations are carried out from water, aqueous alcohol or diluted acetic acid.

By such methods, the pure vitamin B_2 has been obtained by many workers,[20-29] and the identity of the flavins from different sources has been established chemically and biologically. Ovoflavin from egg white, lactoflavin from milk, hepatoflavin from liver, etc., are identical with riboflavin. One gram of riboflavin was obtained from 5400 liters of milk.

Methods for the separation of vitamin B_1 from vitamin B_2 are discussed on page 102. It might be added here that vitamin B_1 can be destroyed by heating to 120° C. for six hours, leaving the vitamin B_2 mainly intact.[30]

For methods of separating riboflavin, riboflavin-phosphoric acid and the flavin-enzymes from each other see page 183.

5. Properties

Riboflavin crystallizes in fine orange-yellow needles which melt at 282° C. under decomposition (darkening at about 240°). The pure compound is slightly soluble in water (12 mg. in 100 cc. at 27.5°, 19 mg. at 40°) and in ethyl alcohol (4.5 mg. at 27.5°), amyl-alcohol, cyclo-hexanol, phenol, amyl-acetate, etc., and is very soluble in alkali solutions. It is insoluble in acetone, ether, benzene and chloroform. The impure material is much

[17] P. Ellinger and W. Koshara, *Ber.*, **66**, 315, 808, 1411 (1933).

[18] R. Kuhn, P. György and T. Wagner-Jauregg, *Ibid.*, **66**, 317, 576, 1034, 1577 (1933); *Naturwissenschaften*, **21**, 560 (1933); *Klin. Wochschr.*, **12**, 1241 (1933).

[19] P. Karrer, H. Salomon and K. Schöpp, *Helv. Chim. Acta*, **17**, 419, 735 (1934).

[20] P. Ellinger and W. Koschara, *Ber.*, **66**, 315, 808, 1411 (1933).

[21] R. Kuhn, P. György and T. Wagner-Jauregg, *Ibid.*, **66**, 317, 576, 1034, 1577 (1933); *Naturwissenschaften*, **21**, 560 (1933); *Klin. Wochschr.*, **12**, 1241 (1933).

[22] P. Karrer, H. Salomon and K. Schöpp, *Helv. Chim. Acta*, **17**, 419, 735 (1934).

[23] L. E. Booher, *J. Biol. Chem.*, **102**, 39 (1933); **107**, 591 (1934).

[24] S. Lepkovsky, W. Popper and H. M. Evans, *Ibid.*, **108**, 257 (1935); **109**, Proc. 54 (1935).

[25] S. Itter, E. R. Orent and E. V. McCollum, *Ibid.*, **108**, 579 (1935).

[26] C. A. Elvehjem and C. J. Koehn, *Ibid.*, **108**, 709 (1935).

[27] F. J. Stare, *Ibid.*, **111**, 567 (1935).

[28] S. Ansbacher, G. C. Supplee and R. C. Bender, *J. Nutrition*, **11**, 401 (1936).

[29] R. D. Greene and A. Black, *J. Am. Chem. Soc.*, **59**, 1820 (1937).

[30] M. I. Smith and E. G. Hendrick, *U. S. Pub. Health Service Pub. Health Repts.*, **41**, 201 (1926). A. Seidell, *Bull. soc. chim. biol.*, **8**, 746 (1926). A. Hassan and J. C. Drummond, *Biochem. J.*, **21**, 653 (1927). H. Chick and M. H. Roscoe, *Ibid.*, **21**, 698 (1927). H. C. Sherman and J. H. Astmayer, *J. Biol. Chem.*, **75**, 207 (1927).

more soluble than the pure material. The water solution is of greenish yellow color and displays an intense yellow-green fluorescence, which vanishes on the addition of acids. Alkali also causes disappearance of the fluorescence by shifting the hydrogen from the imino-group in 3-position to the neighboring oxo-group, thus forming an enol. The fluorescence (maximum at 565 mμ) is used for the quantitative determination of riboflavin (see page 182). Optimum fluorescence occurs at pH 3 to 9. The isoelectric point of vitamin B_2 is at pH 6. Riboflavin is thus

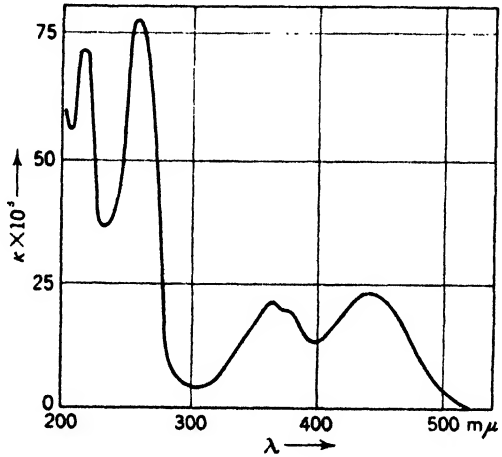

Fig. 10.—Absorption spectrum of vitamin B_2 (riboflavin). (R. Kuhn.)

amphoteric in character. The isoelectric constants for acid and base are calculated to be:[31] $K_{acid} = 63 \times 10^{-12}$ and $K_{base} = 0.5 \times 10^{-5}$. Riboflavin shows optical rotation: $[\alpha]_D^{20} = -114°$ in 0.1 normal sodium hydroxide.[32] In neutral and in acid solution, the optical activity is exceedingly small. Riboflavin has a characteristic absorption spectrum with maxima at 445, 372, 269 and 225 mμ (Fig. 10).

Crystalline riboflavin, when protected against light, is stable at ordinary temperatures. Light slowly destroys the vitamin activity. In solution, vitamin B_2 is essentially unstable. The decomposition is greatly influenced by light, temperature and pH of the solution. Under alkaline

[31] R. Kuhn and G. Moruzzi, *Ber.*, 67, 888 (1934).

[32] R. Kuhn and H. Rudy, *Ibid.*, 68, 169 (1935). P. Karrer and H. Fritzsche, *Helv. Chim. Acta*, 18, 1026 (1935).

conditions riboflavin decomposes rapidly. Riboflavin possesses a relatively high degree of thermostability, thus only slight destruction occurs by heating to 120° C. for six hours.

6. Chemical Constitution of Vitamin B_2: Degradation Reactions

Riboflavin has the empirical formula $C_{17}H_{20}N_4O_6$. It is resistant against acids, bromine and oxidizing agents, such as hydrogen peroxide and concentrated nitric acid. Chromic acid oxidizes the molecule to ammonia, carbon dioxide and a nitrogen-free residue of unknown constitution. Acetylation of riboflavin yields a tetra-acetate, indicating the presence of four hydroxyl groups.[33, 34] A diacetone compound can be formed, which indicates the close position of each two hydroxyl groups.[35] Oxidation of riboflavin with lead tetra-acetate yields 0.8 mol of formaldehyde. A primary hydroxyl group is, therefore, in α-position to a secondary hydroxyl group.[34] Primary amino-groups are not present since nitrous acid does not affect riboflavin. Alkaline hydrolysis yields urea,[33] indicating the presence of the configuration —NH—CO—NH—. The other two nitrogen atoms are tertiary. Irradiation destroys vitamin B_2. Irradiation in alkaline solution yields a new compound, called lumiflavin (lumi-lactoflavin) or photoflavin, and a sugar compound, $C_4H_8O_4$, which could not be isolated as such, probably because of decomposition. Lumiflavin cannot be acetylated further and does not give formaldehyde by oxidation with lead tetra-acetate. Vitamin B_2, therefore, contains a side chain of the constitution of a tetrahydroxy-butyl group,

$$R—CHOH—CHOH—CHOH—CH_2OH$$

the configuration of which could only be determined by total synthesis of riboflavin. It proved to be *d*-ribose.

Photolysis of riboflavin in neutral solution *in vacuo* causes the disappearance of the yellow color with the formation of "deutero-leuco-riboflavin" which can be dehydrogenated by oxygen. Alkali causes the dehydrogenated compound to be converted into the already mentioned lumiflavin.[36]

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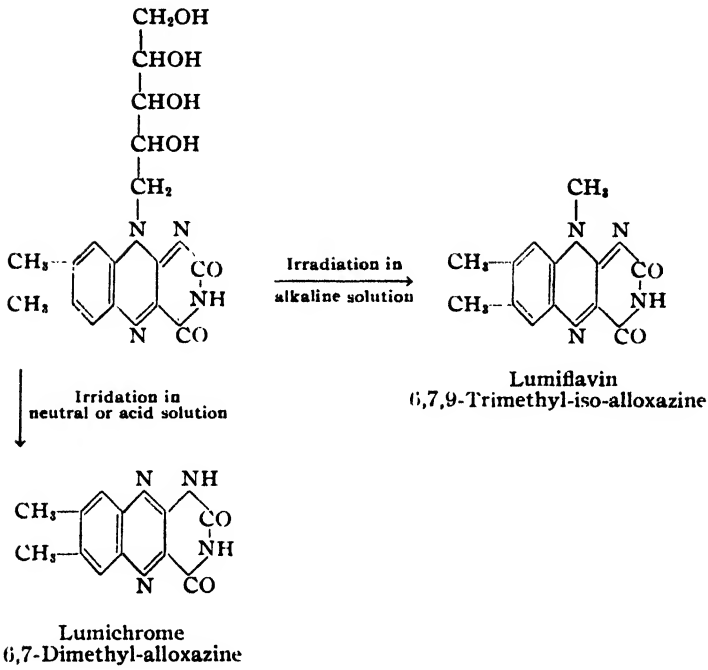
[33] R. Kuhn and T. Wagner-Jauregg, *Ber.*, 66, 1577 (1933).

[34] R. Kuhn, H. Rudy and T. Wagner-Jauregg, *Ibid.*, 66, 1950 (1933).

[35] R. Kuhn, H. Rudy and F. Weygand, *Ibid.*, 68, 625 (1935).

[36] R. Kuhn, H. Rudy and T. Wagner-Jauregg, *Ibid.*, 66, 1950 (1933).

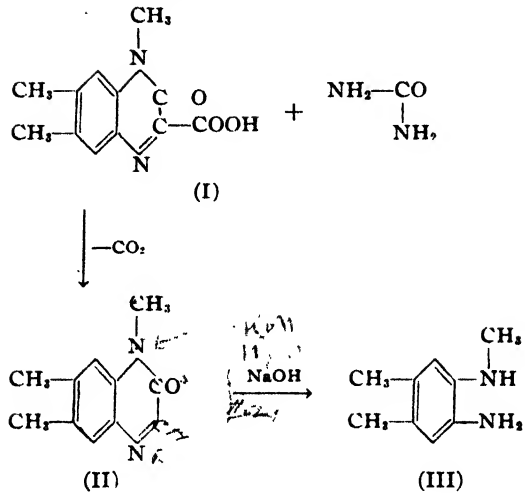


Lumiflavin
6,7,9-Trimethyl-iso-alloxazine

Lumichrome
6,7-Dimethyl-alloxazine

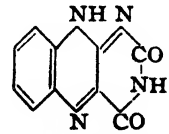
Lumiflavin contains a methyl-imid-group[37] which was not present in riboflavin, and which, therefore, must have replaced the missing sugar side chain. The hydroxyl groups of the side chain are therefore connected with the flavin ring system by a methylene group. Alkaline hydrolysis of lumiflavin yields urea and an oxo-carboxylic acid, $C_{12}H_{12}N_2O_3$[38, 39] (I), which contains one active hydrogen atom and exhibits the properties of a monobasic acid upon titration. Since two molecules of water are required for the alkaline hydrolysis of lumiflavin, the urea must come from a ring system and not from a side chain ureide or guanidino-group, which would require only one molecule of water for hydrolysis. The oxo-acid (I) is decarboxylated upon heating, yielding the lactam (II). By heating the lactam with sodium hydroxide, 1,2-dimethyl-4-amino-5-methyl-aminobenzene (III) is formed. This *o*-phenylene-diamine gives a bluish green color reaction with ferric chloride, which is, according to Noelting, characteristic for *p,p*-disubstituted *o*-phenylene-diamines.

[37] R. Kuhn and H. Rudy, *Ber.*, **67**, 1298 (1934).
[38] R. Kuhn and T. Wagner-Jauregg, *Ibid.*, **66**, 1577 (1933).
[39] R. Kuhn and H. Rudy, *Ibid.*, **67**, 892 (1934).



Irradiation of riboflavin yields lumiflavin in alkaline solution and lumichrome, in neutral or in acid solution.[40] Lumichrome has the constitution of 6,7-dimethyl-alloxazine, and is an intensely fluorescent compound. It is a derivative of alloxazine as proved by synthesis, whereas lumiflavin and riboflavin are derivatives of the hypothetical iso-alloxazine. Lumiflavin and riboflavin have one active hydrogen atom in 3-position; lumichrome contains active hydrogens in both 1- and 3-positions.

Alloxazine



Iso-alloxazine

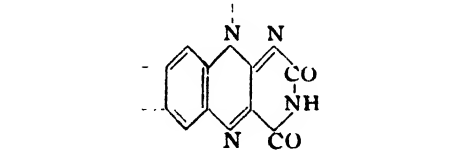
Riboflavin readily takes up two hydrogen atoms[41]. It is reversibly reduced by hydrogen in the presence of a catalyst, by zinc in acid solution, by sodium thiosulfate, by hydrogen sulfide in alkaline solution and by titanous chloride. The dihydro-compound is colorless, shows no fluorescence and is called leuco-riboflavin (leuco-lactoflavin). The leuco-compound is easily oxidized to riboflavin by air. The redoxy-potential of an equimolecular mixture of riboflavin and its leuco-compound is -0.21 volts.[42]

[40] P. Karrer, H. Salomon, K. Schöpp, E. Schlitter and H. Fritzsche, *Helv. Chim. Acta*, **17**, 1010 (1934).

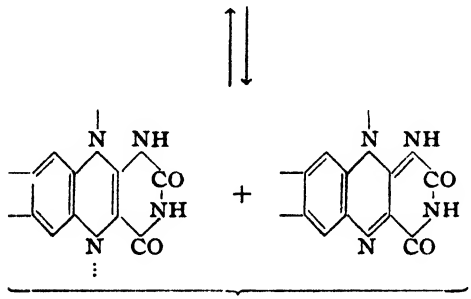
[41] R. Kuhn, P. György and T. Wagner-Jauregg, *Ber.*, **66**, 576 (1933).

[42] E. S. G. Barron and A. B. Hastings, *J. Biol. Chem.*, **105**, VII (1934). R. Bierich and A. Lang, *Z. physiol. Chem.*, **223**, 180 (1934). K. G. Stern, *Biochem. J.*, **28**, 949 (1934). R. Kuhn and G. Moruzzi, *Ber.*, **67**, 1220 (1934). R. Kuhn and P. Boulanger, *Ibid.*, **69**, 1557 (1936). F. J. Stare, *J. Biol. Chem.*, **112**, 223 (1935).

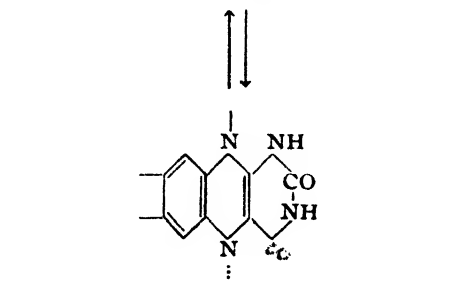
It has been postulated that by reduction of riboflavin to the leuco-compound, three intermediate compounds result, which consist of molecular compounds of reduced and unreduced molecules.[43] *Verdoflavin* consists of riboflavin and monohydro-riboflavin, *chloroflavin* is a quinhydrone of riboflavin and leuco-riboflavin, and *rhodoflavin*-hydrochloride contains the hydrochlorides of leuco-riboflavin and monohydro-riboflavin:



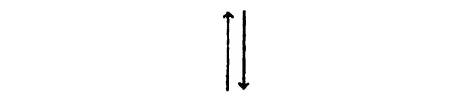
Riboflavin (yellow)



Verdoflavin (green)

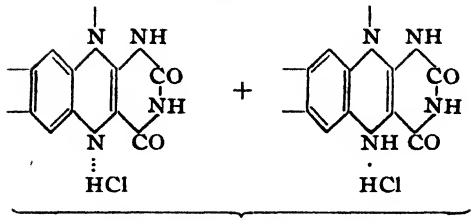


Chloroflavin (green)

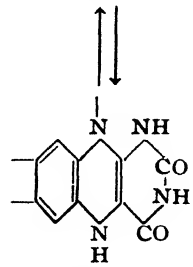


(*Formula continued on following page.*)

[43] R. Kuhn and R. Ströbele, *Ber.*, **70**, 753 (1937).



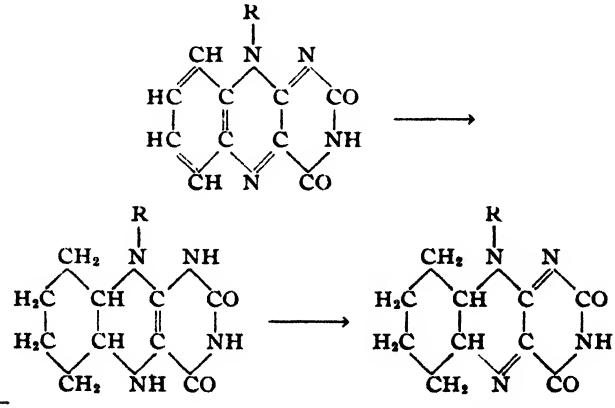
Rhodoflavin (carmine red)



Leucoflavin (colorless)

The existence of these intermediate stages is somewhat dubious, since titration curves of the reduction process indicate the presence of only one intermediate.[44] Since the quinhydrone is the only form that exists in *diluted* aqueous solution, it seems reasonable to assume that only this form occurs under physiological conditions.

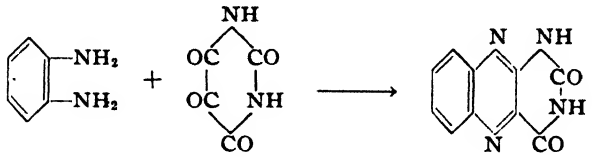
By energetic catalytic hydrogenation of flavins, octahydro-flavins are obtained[45] which are easily oxidized in alkaline solution by air to the corresponding hexahydro-flavins.



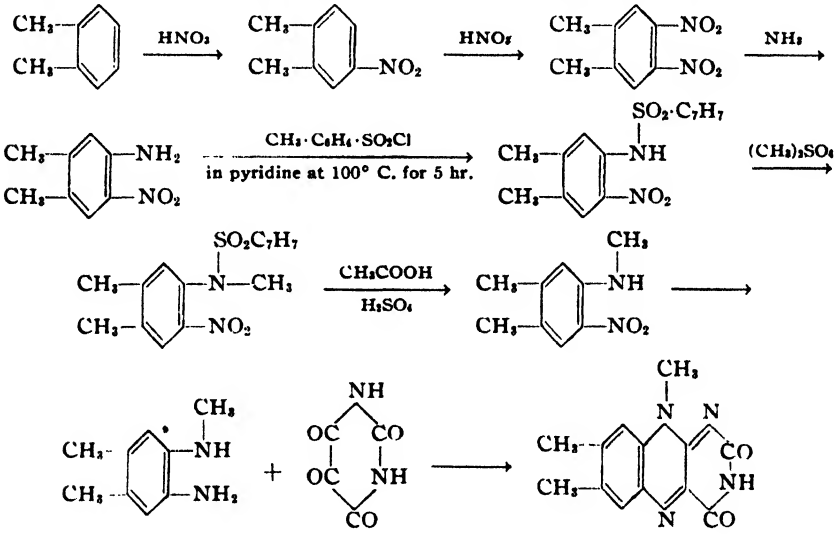
[44] L. Michaelis and G. Schwarzenbach, *J. Biol. Chem.*, **123**, 527 (1938).
[45] P. Karrer and R. Ostwald, *Rec. trav. chim.*, **57**, 500 (1938).

7. Synthesis of Vitamin B_2 and Other Flavins

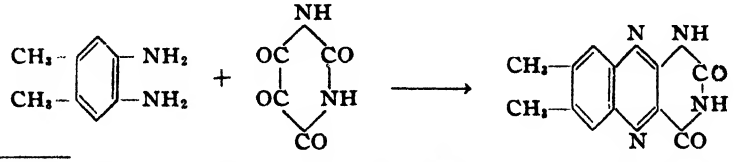
Alloxazines were first synthesized by Kühling[46] by condensation of the hydrochloride of *o*-phenylene-diamine with alloxan:



Kuhn and co-workers[47] synthesized lumiflavin from alloxan and 1,2-dimethyl-4-amino-5-methyl-amino-benzene in acid solution. The *N*-methyl-*o*-xylylene-diamine was obtained according to the following scheme:



Karrer and co-workers[48] synthesized lumichrome similarly:

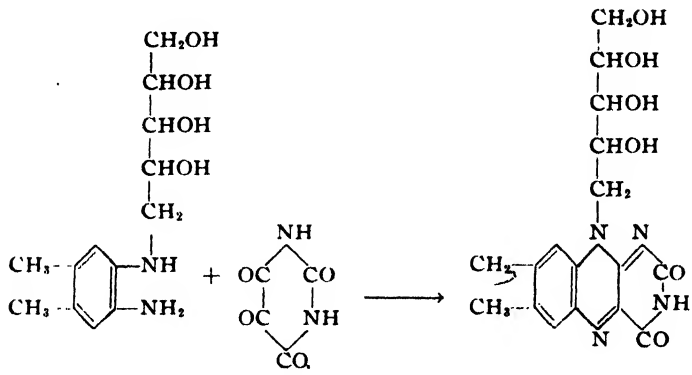


[46] O. Kühling, *Ber.*, **24**, 2363 (1891); **27**, 2116 (1894); **28**, 1968 (1895). O. Kühling and O. Kaselitz, *Ibid.*, **39**, 1314 (1906).

[47] R. Kuhn, K. Reinemund and F. Weygand, *Ibid.*, **67**, 1460 (1934). R. Kuhn and K. Reinemund, *Ibid.*, **67**, 1932 (1934). R. Kuhn, H. Rudy and K. Reinemund, *Ibid.*, **68**, 170 (1935).

[48] P. Karrer, H. Salomon, K. Schöpp, E. Schlitter and H. Fritzsche, *Helv. Chim. Acta*, **17**, 1010 (1934).

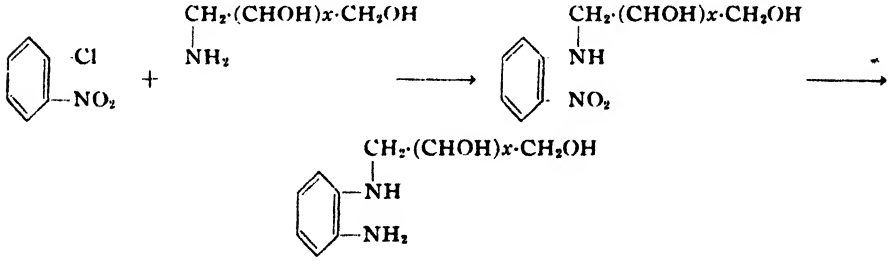
According to the same principle, riboflavin is obtained from alloxan and dimethyl-amino-phenyl-ribamine:



The condensation of the 4,5-dimethyl-2-amino-phenyl-ribamine with alloxan is carried out in acid solution. When boric acid is used as catalyst in acetic acid solution the yield of the condensation product increases considerably.[49]

The synthesis of the dimethyl-amino-phenyl-ribamine has been achieved by the following methods:

1. *o*-Nitro-chloro-benzenes are condensed with amino-sugars and the reaction product hydrogenated to the diamine.[50] This method gives satisfactory yields when the sugar compound contains two or three hydroxyl groups, but poor yields with sugars containing four and five hydroxyl groups.



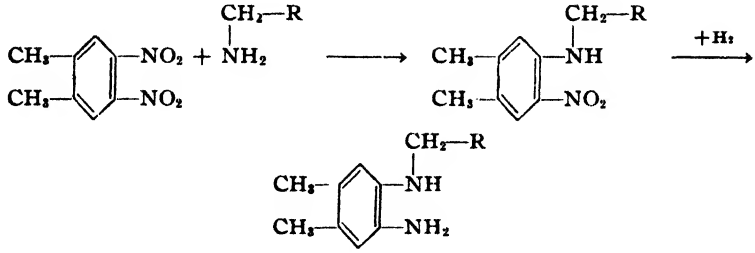
2. *o*-Dinitro-xylene is condensed with ribamine in aqueous alcoholic solution and catalytically reduced to the corresponding diamine.[51] The

[49] R. Kuhn and F. Weygand, *Ber.*, **68**, 1282 (1935). See also R. Kuhn and A. H. Cook, *Angew Chem.*, **49**, 6 (1936).

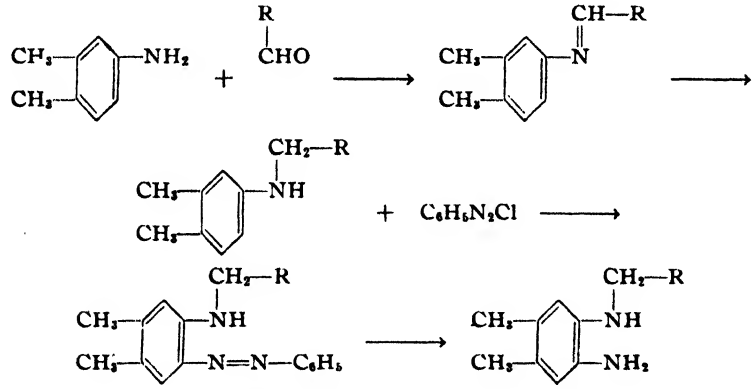
[50] P. Karrer, H. Salomon, K. Schöpp and E. Schlitter, *Helv. Chim. Acta*, **17**, 1165 (1934). P. Karrer, E. Schlitter, K. Pfaehler and F. Benz, *Ibid.*, **17**, 1516 (1934).

[51] R. Kuhn and F. Weygand, *Ber.*, **68**, 1001 (1935).

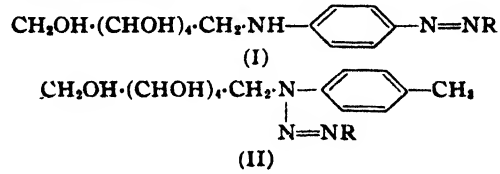
over-all yield of riboflavin according to this process is 4.5% of the ribose used.



3. 3,4-Xylidene[52] is condensed with ribose and the formed riboside is catalytically reduced. The second amino-group is introduced by coupling with diazonium salts to form azo-dyes, which by reduction yield the desired diamine:[53]



This method gives very high yields on riboflavin (38%) calculated from the required ribose, but cannot be used for the preparation of any other flavin. Only *m*-*p*-disubstituted aniline derivatives couple with diazonium salts in *o*-position. Phenyl-glucamine, for example, couples in *p*-position, yielding (I); *p*-toluene-glucamine yields (II).



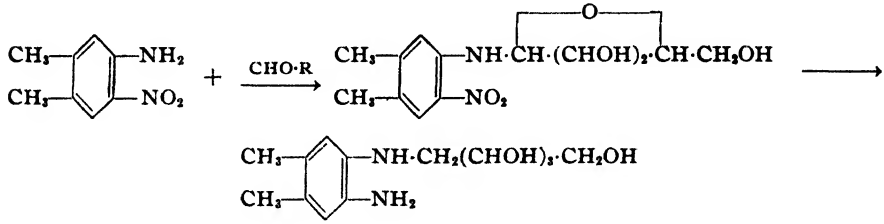
(I)

(II)

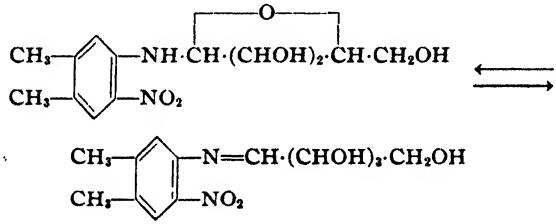
[52] P. Karrer, B. Becker, F. Benz, P. Frei, H. Salomon and K. Schöpp, *Helv. Chim. Acta*, **18**, 1435 (1935). W. A. Wisansky and S. Ansbacher, *J. Am. Chem. Soc.*, **63**, 2532 (1941).

[53] P. Karrer and H. Meerwein, *Helv. Chim. Acta*, **18**, 1130 (1935); **19**, 264 (1936).

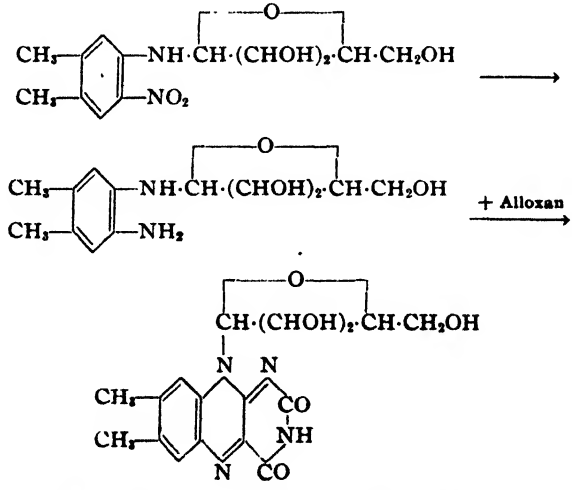
4. *o*-Nitro-xylidine is condensed with ribose and the reaction product is catalytically reduced to the diamine.[54] This method yields 16% riboflavin calculated on the amount of ribose used.



The intermediate is an amino-glucoside which exists in an equilibrium with the tautomeric Schiff base:



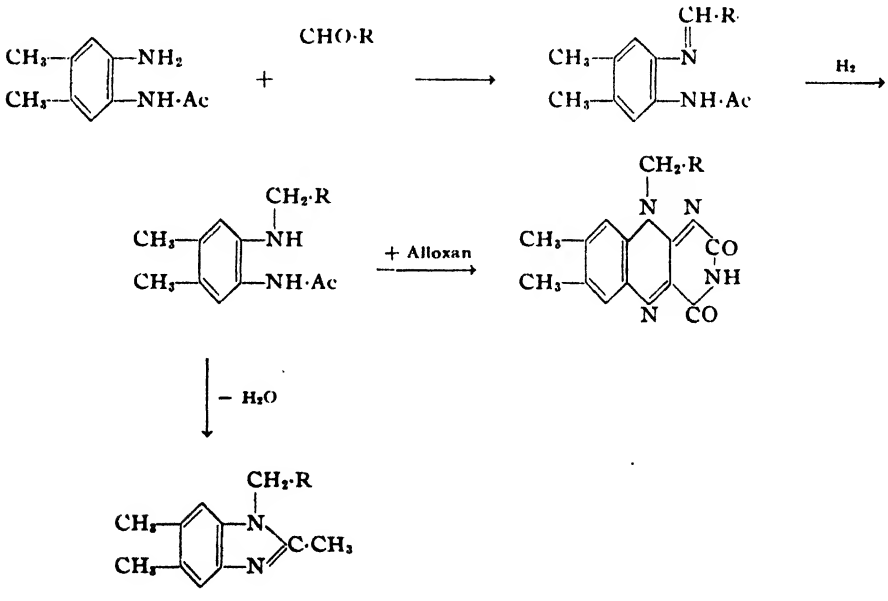
The Schiff base can also be reduced partially to the *o*-phenylene-diamine-glucoside which after acetylation of the free hydroxyl groups may be con-



[54] R. Kuhn and R. Ströbele, *Ber.*, **70**, 773 (1937). R. Kuhn, K. Reinemund, F. Weygand and R. Ströbele, *Ibid.*, **68**, 1765 (1935).

densed with alloxan to the corresponding acetylated flavin-glucoside.[55] The latter upon saponification yields the free glucoside. Flavin-glucosides do not exhibit the physiological properties of vitamin B_2.

5. *N*-Mono-acyl-*o*-phenylene-diamines condense with sugars to the Schiff bases which are reduced simultaneously to the *N*-(*o*-acyl-amino-phenyl)-amino-sugars.[56] These are condensed in acid solution with alloxan to the corresponding flavins. The acyl groups are automatically saponified during the reaction. The yields on flavins according to this process are extremely low, since the main reaction is an intermolecular dehydration which yields benzimidazole derivatives.

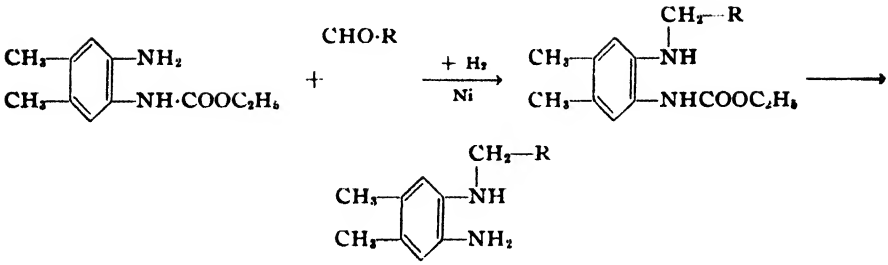


6. In the preceding method, one amino-group is temporarily protected by acetylation while the other amino-group is condensed with ribose. A better method is the protection of the amino-group by the carbethoxy-group:[57]

[55] R. Kuhn and R. Ströbele, *Ber.*, **70**, 747 (1937).

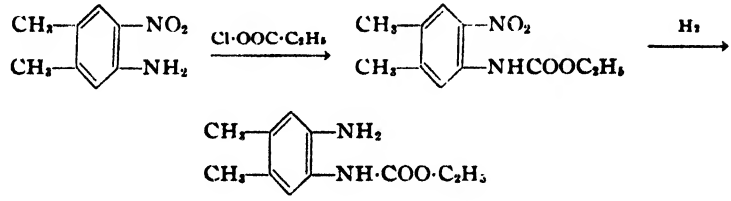
[56] P. Karrer, K. Schöpp, F. Benz and K. Pfaehler, *Helv. Chim. Acta*, **18**, 69 (1935); *Ber.*, **68**, 216 (1935).

[57] P. Karrer, K. Schöpp, F. Benz and K. Pfaehler, *Helv. Chim. Acta*, **18**, 69 (1935). P. Karrer, K. Schöpp and F. Benz, *Ibid.*, **18**, 426 (1935). H. v. Euler, P. Karrer, H. Malmberg, K. Schöpp, F. Benz, B. Becker and P. Frei, *Ibid.*, **18**, 522 (1935). P. Karrer, H. Salomon, K. Schöpp, F. Benz and B. Becker, *Ibid.*, **18**, 908, 1143, 1435 (1935). P. Karrer and F. M. Strong, *Ibid.*, **18**, 1343 (1935).

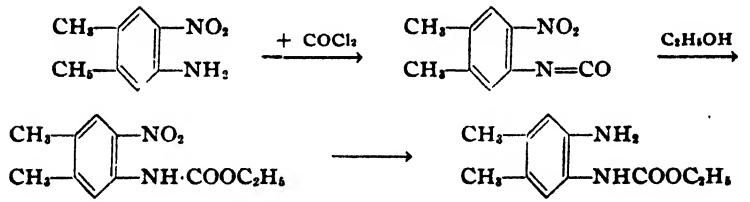


The urethane can be prepared by one of the following procedures:

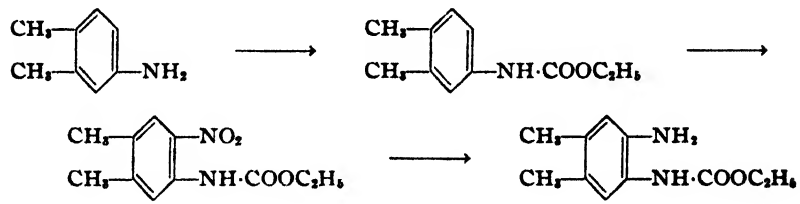
(*a*)



(*b*)



(*c*)

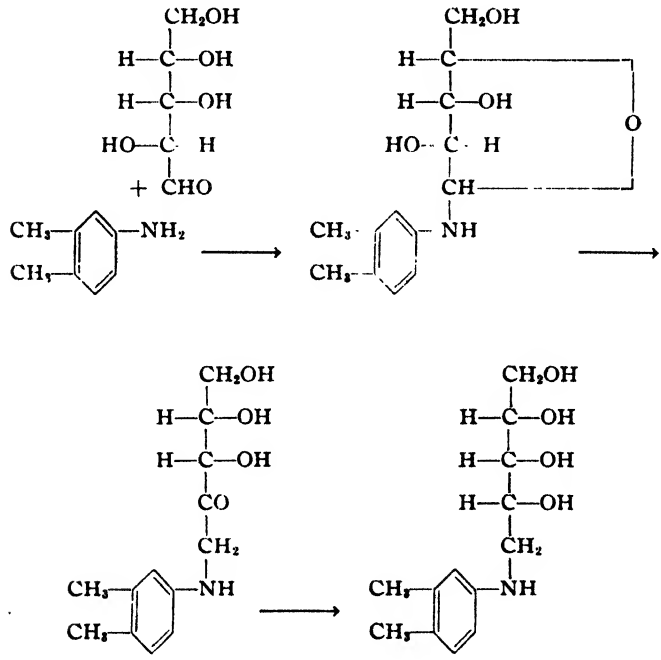


The yield of riboflavin according to this procedure is approximately 14–15% of the ribose used.

7. While in the procedures described under (1 to 6) *d*-ribose has been used as an intermediate, it is also possible to use some other sugar derivatives. A very elegant method[56a] for the preparation of dimethyl-aminophenyl-ribamine is to condense 3,4-xylidine with *d*-arabinose in the presence of small amounts of acid to form the *d*-arabinoside which upon heat-

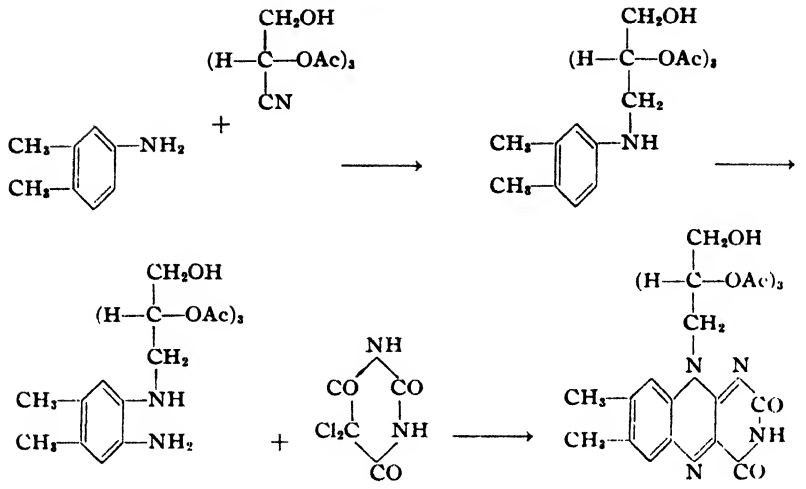
[56a] F. Weygand, *Ber.* **73**, 1259 (1940).

ing to 75° C. undergoes an Amadori-rearrangement to the *d*-iso-arabinosamine. Upon catalytic hydrogenation in alkaline solution the *d*-iso-arabinosamine is converted into 3,4-dimethyl-phenyl-*d*-ribamine. This compound is obtained in about 13% of theory from the arabinose originally used. The arabamine is then coupled with a diazonium salt and reduced to the desired diamine as described under (3).



8. 3,4-Xylidine is condensed[56b] reductively with tetra-acetyl-ribononitrile which is obtained from ribonic acid via the amide. The dimethyl-phenyl-ribamine is then coupled with para-nitrophenyl-diazonium chloride and reduced to N-tetra-acetyl-ribitylamino-2-amino-4,5-dimethyl benzene according to the procedure described under (3). It has been recommended to condense the last mentioned compound with 5,5′-dichloro-barbituric acid to obtain vitamin B_2 instead of using alloxan as has been described previously.

[56b] M. Tishler and J. W. Wellman, U. S. P. 2 261,608.



8. Industrial Methods of Preparation

The relative technical importance of natural *versus* synthetic vitamin B_2 is dependent on the use for which the vitamin is intended. For animal foods, various riboflavin preparations from natural sources such as yeast, whey or anaerobic growing fermentation bacteria, for example, from butyl alcohol fermentations, are used. The riboflavin content of these preparations varies considerably and must be standardized. For human therapy, the use of pure riboflavin is preferred. Since the isolation of pure riboflavin is more expensive than the synthesis thereof, synthetic vitamin B_2 is generally used clinically, especially in cases of an exclusive vitamin B_2 deficiency.

The synthesis of vitamin B_2 is carried out according to the methods described in the preceding section. The starting materials are *o*-xylene, *d*-ribose and alloxan. *o*-Xylene is available as a by-product from the petroleum industry. *d*-Ribose is obtained either from natural sources or by synthesis. A convenient method for the preparation of small amounts of *d*-ribose is the hydrolysis of yeast-nucleic acid.[58] Synthetic *d*-ribose is prepared from *d*-glucose.[59, 60] Alloxan is usually obtained by oxidation of uric acid or barbituric acid.

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[58] H. Bredereck, *Ber.*, **71**, 408 (1938). H. Bredereck, M. Köthnig and E. Berger, *Ibid.*, **73**, 956 (1940).

[59] W. C. Austin and F. L. Humoller, *J. Am. Chem. Soc.*, **56**, 1152 (1934).

[60] R. Kuhn, K. Reinemund, F. Weygand and R. Ströbele, *Ber.*, **68**, 1765 (1935).

9. Flavin-Enzymes

(*a*) *Enzyme Systems Containing Riboflavin*

Riboflavin takes part in a number of different enzyme systems in tissues. These systems consist of an apoenzyme and a coenzyme. The apoenzyme is a specific protein and is also called pheron, bearer protein, or "Zwischenferment." The coenzyme constitutes the prosthetic group of the enzyme system and contains riboflavin as part of its constitution. There are two different riboflavin-containing coenzymes, namely, a mononucleotide and a dinucleotide. The mononucleotide has the constitution of a riboflavin-phosphate while the dinucleotide is a riboflavin-adenine-dinucleotide. (For details see page 177.) These coenzymes combine with different apoenzymes to carry out specific reactions. The same coenzyme can serve as the prosthetic group of a number of different apoenzymes. The function of all enzyme systems is to transfer hydrogen, for example, in the carbohydrate and amino-acid metabolism. In the carbohydrate metabolism, for example, the substrate is oxidized by dehydrogenation through the nicotinamide-containing enzymes, which are reduced in this process to the dihydro-compounds which in turn are oxidized by the riboflavin-containing enzymes. Thus the dihydro-nicotinamide enzymes serve as substrates for the riboflavin enzymes which are converted into dihydro-derivatives (see page 180). The dihydro-compounds are then reoxidized to the corresponding riboflavin-coenzymes by a number of specific

TABLE I

Enzyme	Source	Prosthetic group	Hydrogen donor	Hydrogen acceptor
"Old" yellow enzyme	Bottom yeast	Mononucleotide	Dihydro-coenzymes I and II	Molecular oxygen
Cytochrome *c* reductase	Top yeast	Mononucleotide	Dihydro-coenzyme II	Cytochrome *c*
Diaphorase I	Heart	Dinucleotide	Dihydro-coenzyme I	Cytochrome (*a* and) *b*
Diaphorase II	Bottom yeast, adrenal gland	Dinucleotide	Dihydro-coenzyme II	?
Diaphorase	Milk	Dinucleotide	Dihydro-coenzyme I (and II?)	?
Aldehyde oxidase			Aldehydes	?
Xanthine oxidase			Xanthine	?
Aldehyde oxidase	Liver	Dinucleotide	Aldehydes	?
d-Amino-acid oxidase	Kidney, liver	Dinucleotide	*d*-Amino-acids	?
Glucose oxidase	Yeast	?	Glucose	?
?	Bottom yeast	Dinucleotide	?	Fumaric acid
Diamine oxidase	Kidney	?	Di- and polyamines	?
?	Top yeast	Dinucleotide[61]	?	?

[61] D. E. Green, W. E. Knox and P. K. Stumpf, *J. Biol. Chem.*, **138**, 775 (1941).

reactions. Thus, the hydrogen may be transferred to fumaric acid or may react directly with oxygen or indirectly through the cytochromes *a*, *b* or *c*. The different reactions which are catalyzed by the riboflavin-containing coenzymes and which have been elucidated are summarized in Table I.

Some of the enzymes listed in Table I apparently have a second prosthetic group in addition to the riboflavin-coenzyme. This other compound is characterized by a brownish color but has not been identified. It has been observed in the flavin-enzymes isolated from milk (diaphorase, aldehyde- and xanthine-oxidase), from liver (aldehyde-oxidase) and from top yeast (action not characterized). Whether or not the group with the brownish color is the same in these three flavin-enzymes has not been established.

The reactions catalyzed by the flavin-enzymes as indicated in Table I are discussed more fully below.

Oxidation of the Codehydrogenases I and II. This reaction can be accomplished by enzymes containing either the riboflavin mono- or dinucleotide in the presence of specific apoenzymes. The mononucleotide-containing flavin-enzyme is the "old" yellow enzyme of Warburg and Christian.[62] It is purified by adsorption methods[63, 64] or by cataphoresis,[65] has a molecular weight of about 70,000[66] and a redoxy-potential of -0.06 volts at pH 7.0 and 20° C. It is split into the apoenzyme and the coenzyme by denaturation of the apoenzyme with methanol or by dialysis against dilute hydrochloric acid. A resynthesis of the enzyme from the apoenzyme and the coenzyme can be accomplished. Riboflavin, if added to the apoenzyme in large excess, forms an active flavin-enzyme[67] although the union is not quite as firm as with the mononucleotide. The riboflavin-adenine-dinucleotide is also able to combine with the specific apoenzyme and to react like the "old" yellow enzyme. The chemistry of the apoenzyme is largely unknown, but by hydrolysis the following amino-acids have been obtained in a total yield of 65%: arginine, histidine, lysine, proline, tyrosine, phenyl-alanine, tryptophane, cystine and glutamic acid.[68] The "old" yellow enzyme dehydrogenates codehydrogenases I and II and

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[62] O. Warburg and W. Christian. *Naturwissenschaften*, **20**, 688, 980 (1932); *Biochem. Z.*, **254**, 438 (1932); **257**, 492 (1933); **266**, 377 (1933).

[63] F. Weygand and H. Stocker, *Z. physiol Chem.*, **247**, 167 (1937).

[64] F. Weygand and L. Birkofer, *Ibid.*, **261**, 172 (1939).

[65] H. Theorell, *Biochem. Z.*, **275**, 37, 344 (1934); **278**, 263 (1935).

[66] H. Theorell, *Ibid.*, **278**, 279 (1935). R. A. Kekwick and K. O. Pederson, *Biochem. J.*, **30**, 2201 (1936).

[67] R. Kuhn and H. Rudy, *Ber.*, **69**, 2557 (1936).

[68] R. Kuhn and P. Desnuelle, *Ibid.*, **70**, 1907 (1937).

the dihydro-form is dehydrogenated by oxygen. The rate of the last reaction is a function of the partial pressure of the oxygen present. In animal tissues the oxygen tension is so low that the oxidation can hardly be accomplished. Thus this reaction of the reduced riboflavin-coenzyme with oxygen demonstrates a chemical function of the molecule but not its real physiological action. The reaction product of oxygen with the reduced riboflavin-coenzyme is hydrogen peroxide which destroys the life of the cells. The latter has been demonstrated in the case of anaerobic growing lactic acid bacteria. Thus, if the "old" yellow enzyme plays an essential function in the living organism, an oxygen transporting system must take care of the dehydrogenation of the dihydro-riboflavin-enzyme. It has been observed that cytochrome *c* reacts with the reduced "old" yellow enzyme, but this reaction is too slow to be considered of physiological importance.[69, 70] Fumaric acid[71, 72] and glyoxal[73] have been suggested as substances capable of oxidizing the dihydro-riboflavin system but convincing experimental evidences to substantiate these conceptions have not been reported.

An enzyme containing the riboflavin mononucleotide but an apoenzyme different from that of the "old" yellow enzyme has been found in the cytochrome *c* oxidase. This enzyme catalyzes the dehydrogenation of the codehydrogenase II and the reduction of the oxidized form of cytochrome *c*[74] under physiological conditions.

Besides the enzyme systems which contain riboflavin mononucleotide and react with the dehydrogenases, there, are enzyme systems which contain the dinucleotide and which are capable of carrying out the same reactions. They are called the "diaphorases,"[75] "coenzyme factor"[76] or "pyridine nucleotide oxidase," and occur in animal and in plant tissues.[77] The enzymes from heart, skeletal muscle and yeast are apparently identical.[78, 79, 80] The same enzyme or enzymes also occur in bacteria.[81] There are two dif-

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[69] H. Theorell, *Angew. Chem.*, **51**, 738 (1938).

[70] H. Theorell, *Nature*, **138**, 687 (1936); *Biochem. Z.*, **288**, 317 (1936); **279**, 463 (1935).

[71] E. Adler and H. v. Euler, *Arkiv. Kemi, Mineral. Geol.*, **B12**, No. 36 (1937).

[72] A. Szent-Györgyi, *Z. physiol. Chem.*, **244**, 105 (1936).

[73] T. Bersin, *S. B. Ges. Bef. ges. Naturw. Marburg*, **71**, 56 (1936).

[74] E. Haas, B. L. Horecker and T. R. Hogness, *J Biol. Chem.*, **136**, 747 (1940).

[75] H. v. Euler and H. Hellström, *Z. physiol. Chem.*, **252**, 31 (1938). H. v. Euler and K. Hasse, *Naturwissenschaften*, **26**, 187 (1938). H. v. Euler and G. Günther, *Ibid.*, **26**, 676 (1938).

[76] J. G. Dewan and D. E. Green, *Biochem. J.*, **32**, 626 (1938). F. B. Straub, H. S. Corran and D. E. Green, *Nature*, **143**, 76, 119 (1939).

[77] E. E. Lockhart, *Biochem. J.*, **33**, 613 (1939).

[78] F. B. Straub, *Ibid.*, **33**, 787 (1939).

[79] H. S. Corran, D. E. Green and F. B. Straub, *Ibid.*, **33**, 793 (1939).

[80] E. Haas, *Biochem. Z.*, **298**, 378 (1938).

[81] D. E. Green and J. G. Dewan, *Biochem. J.*, **32**, 626 (1938).

ferent diaphorases, namely, diaphorase I which dehydrogenates codehydrogenase I, and diaphorase II, which dehydrogenates codehydrogenase II.[82] The reversed reaction, the dehydrogenation of the reduced diaphorases, is probably carried out by cytochromes *a* and *b*. It has been shown experimentally that diaphorase I reacts with cyctochrome *b*[83] and some evidence has been obtained for the participation of cyctochromes *a* and *b* in the dehydrogenation of the diaphorases.[84]

The apoenzyme of the diaphorase I has a molecular weight of about 70,000. It appears that the combination of the apoenzyme with the coenzyme is rather loose and that the coenzyme may change from one apoenzyme molecule to another with considerable speed. On the other hand, the same specific protein may possibly serve as apoenzyme for both the coenzymes of the riboflavin and the nicotinamide type.[85] One mol of the diaphorase catalyzes the oxidation of about 8000 mols of codehydrogenase I under optimum conditions in a synthetic system containing methylene blue as an oxygen carrier.[86]

Through the codehydrogenases the riboflavin-enzyme systems take part[87] in the reactions carried out by the nicotinamide-containing enzymes[88] (see page 227), such as in the dehydrogenation of hexose-monophosphate in yeast[89] and muscle,[90] of alcohol in yeast,[91] of glucose in liver,[92] of malic acid in muscle,[93] of lactic acid in muscle,[94] of citric acid in muscle and in plant cells,[95] of dioxy-acetone-phosphate in yeast,[96] of glycerin-aldehyde-phosphate in yeast[97] and of glycerin-phosphoric acid in seeds.[98]

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[82] E. Adler, H. v. Euler, G. Günther and E. D. Plass, *Skand. Arch. Physiol.*, **82**, 61 (1939). E. Adler, H. v. Euler and G. Günther, *Nature*, **143**, 641 (1939). E. P. Abraham and E. Adler, *Biochem. J.*, **34**, 119 (1940).

[83] K. Okunuki and E. Yakusizi, *Proc. Imp. Acad.* (*Tokyo*), **16**, 144 (1940).

[84] J. G. Dewan and D. E. Green, *Biochem. J.*, **32**, 626 (1938).

[85] E. Haas, *Biochem. Z.*, **290**, 291 (1937).

[86] E. Haas, *Ibid.*, **298**, 378 (1938). H. S. Corran, D. E. Green and F. B. Straub, *Biochem. J.*, **33**, 793 (1939).

[87] H. Theorell, *Ergeb. Enzymforsch.*, **6**, 132 (1937). O. Warburg, *Ibid.*, **7**, 210 (1938).

[88] O. Warburg, W. Christian and H. Griese, *Biochem. Z.*, **282**, 157 (1935).

[89] O. Warburg and W. Christian, *Ibid.*, **254**, 438 (1932); **257**, 492 (1933); **266**, 377 (1933).

[90] T. Wagner-Jauregg, *Z. physiol. Chem.*, **231**, 55 (1935).

[91] H. v. Euler and E. Adler, *Ibid.*, **226**, 195 (1934).

[92] E. Adler and H. v. Euler, *Ibid.*, **232**, 6 (1935).

[93] T. Wagner-Jauregg, *Ibid.*, **228**, 273 (1934); **231**, 55 (1935). E. Adler and M. Michaelis, *Ibid.*, **238**, 261 (1935).

[94] E. Adler and M. Michaelis, *Ibid.*, **235**, 154 (1935); **238**, 261 (1936). T. Wagner-Jauregg and E. F. Möller, *Ibid.*, **236**, 216 (1935).

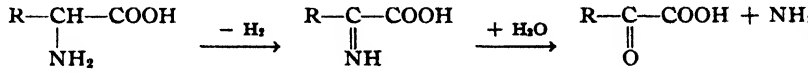
[95] T. Wagner-Jauregg, *Ibid.*, **233**, 215 (1935).

[96] H. v. Euler, E. Adler and H. Hellström, *Ibid.*, **241**, 239 (1936).

[97] H. v. Euler, E. Adler and H. Hellström, *Ibid.*, **241**, 239 (1936).

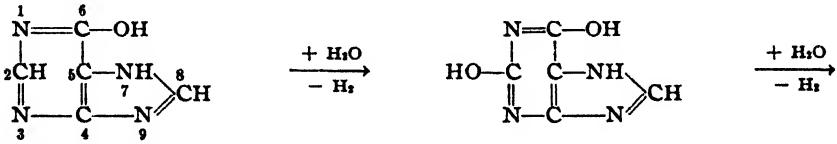
[98] T. Wagner-Jauregg and H. Rauen, *Ibid.*, **237**, 233 (1935).

Oxidation of *d*-Amino-acids. The oxidation of the antipodes of the naturally occurring amino-acids into oxo-acids is carried out by an enzyme system containing riboflavin-adenine-dinucleotide[99–101] according to the following reaction scheme:[102]



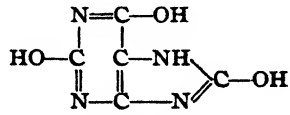
One mol of the riboflavin-adenine-dinucleotide transports in the presence of an excess of the apoenzyme approximately 2000 mols of oxygen per minute at 38° C. The apoenzyme has a molecular weight of about 65,000. The coenzyme is highly dissociated from the apoenzyme in solution, but the reduced coenzyme apparently is not dissociated.

Oxidation of Xanthine. By means of an enzyme system containing riboflavin-adenine-dinucleotide plus an unknown colored compound, several purines, primarily xanthine and hypoxanthine, are dehydrogenated to uric acid:[103–106]



Hypoxanthine
6-Oxy-purine

Xanthine
2,6-Dioxy-purine



Uric acid
2,6,8-Trioxy-purine

The conversion of hypoxanthine to xanthine and the conversion of xanthine to uric acid require the presence of the same riboflavin-enzyme. One mol

[99] O. Warburg and W. Christian, *Biochem. Z.*, **295**, 261 (1938); **298**, 150 (1938).
[100] E. Negelein and H. Brömel, *Ibid.*, **300**, 225 (1939).
[101] F. B. Straub, *Nature*, **141**, 603 (1938).
[102] H. A. Krebs, *Z. physiol. Chem.*, **217**, 191 (1933); **218**, 157 (1933); *Biochem. J.*, **29**, 1620 (1935).
[103] E. G. Ball, *Science*, **88**, 131 (1938); *Angew .Chem.*, **51**, 738 (1938); *J. Biol. Chem.*, **128**, 51 (1939).
[104] H. S. Corran and D. E. Green, *Biochem. J.*, **32**, 2231 (1938).
[105] D. E. Green and H. S. Corran, *Angew. Chem.*, **51**, 738 (1938).
[106] H. S. Corran, J. G. Dewan, A. H. Gordon and D. E. Green, *Biochem. J.*, **34**, 1694 (1939).

of the coenzyme catalyzes the oxidation of about 300 mols of hypoxanthine or xanthine per minute. The oxidation rate of other purines is considerably slower.[107]

The same enzyme system is also able to effect an anaerobic dismutation of xanthine to hypoxanthine and uric acid:

$$2 \text{ xanthine} \rightleftharpoons \text{hypoxanthine} + \text{uric acid}$$

This is an equilibrium reaction and xanthine can therefore be formed from uric acid and hypoxanthine.[108, 109]

This riboflavin-containing enzyme, xanthine oxidase, occurs, for example, in milk and liver. The apoenzyme has a molecular weight of about 280,000[110] and probably binds two riboflavin groups per molecule in addition to some other unknown colored group which may act as coenzyme. The apoenzyme is different from those of the other riboflavin-containing enzyme systems.

Oxidation of Aldehydes. The enzyme which catalyzes the oxidation of aldehydes to carboxylic acids, for example, propionic aldehyde to propionic acid, was originally observed in milk and is called aldehyde oxidase or Schardinger enzyme.[111] The aldehyde oxidase from liver[112, 113] contains the riboflavin-adenine-dinucleotide and is specific for the oxidation of aldehydes. The aldehyde oxidase from milk also acts as xanthine oxidase and as dihydro-codehydrogenase I oxidase. Whether or not these enzymes are identical has not been decided. There is also a discrepancy of opinion as to whether or not the aldehyde oxidase and xanthine oxidase from liver are a single compound or a mixture of compounds. Much evidence has accumulated which favors the view that a single compound acts as an enzyme in milk for the oxidation of both xanthine and aldehydes.[114, 115] If that is the case, the aldehyde oxidase from liver must be a different compound.

Oxidation of Diamines. A riboflavin-containing coenzyme of unknown composition takes part in the enzymatic conversion of di- and poly-

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[107] M. Dixon, *Enzymologia*, **5**, 198 (1938). L. Reichel and W. Burkart, *Z. physiol. Chem.*, **260**, 135 (1939).

[108] D. E. Green, *Biochem. J.*, **28**, 1550 (1934).

[109] S. Fillitti, *J. chim. phys.*, **32**, 1 (1935).

[110] J. S. L. Philpot, *Biochem. J.*, **32**, 2240 (1938).

[111] F. Schardinger, *Z. Untersuch. Nahr. u. Genussm.*, **5**, 1113 (1902).

[112] V. Subrahmanyan, D. E. Green and A. H. Gordon, *Nature*, **144**, 1016 (1939).

[113] A. H. Gordon, D. E. Green and V. Subrahmanyan, *Biochem. J.*, **34**, 764 (1940).

[114] V. H. Booth, *Ibid.*, **32**, 494 (1938).

[115] M. Dixon, *Enzymologia*, **5**, 198 (1938). L. Reichel and W. Burkart, *Z. physiol. Chem.*, **260**, 135 (1939).

amines into amino-aldehydes.[116] This action is similar to the oxidation of *d*-amino-acids, but the enzymes (at least the apoenzymes) involved appear to be different. The amines studied in this reaction are primarily histamine, cadaverin and spermin.

Oxidation of Glucose. The oxidation of glucose to gluconic acid in yeast is carried out with the aid of an enzyme which contains riboflavin.[117, 118] The manner in which riboflavin is bound in this enzyme system is not known.

Reduction of Fumaric Acid. In yeast, fumaric acid is reduced to succinic acid by an enzyme system which contains riboflavin-adenine-dinucleotide.[119] Per mol of dinucleotide 2000–3000 mols of hydrogen are transported per minute. This enzyme is also able to reduce other compounds, such as maleic acid, crotyl-alcohol, phenyl-crotyl-alcohol and geraniol, but the rate of reduction is less than $^1/_{50}$ that of the rate of fumarate reduction. The specificity of the apoenzyme has not been determined, but it appears probable that the apoenzyme of at least one of the oxidases previously discussed is identical with that of the fumaric hydrogenase.

Reduction of Cytochrome *a*, *b* and *c*. The reduction of the oxidized form of the cytochromes is linked with the oxidation of the dihydrocodehydrogenases (see page 174).

(*b*) *Coenzymes Containing Riboflavin*

Riboflavin-5′-phosphoric Acid (Riboflavin-mononucleotide). Riboflavin-5′-phosphoric acid was first obtained from heart muscle and was characterized as a yellow water-soluble dye. This compound was given the name "cytoflav."[120] The position of the phosphoric acid in the riboflavin molecule was established by oxidation with periodic acid.[121] Formaldehyde was not obtained as a reaction product as would be expected in case a free primary hydroxyl group should be present in the 5′-position. This result was confirmed by synthesis which was carried out according to the following scheme:[122]

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[116] E. A. Zeller, R. Stern and N. Wenk, *Helv. Chim. Acta*, **23**, 1 (1940).

[117] W. Franke and M. Deffner, *Ann.*, **541**, 117 (1939).

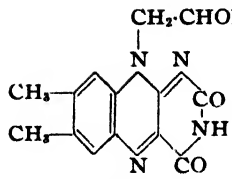
[118] D. Mueller, *Biochem. Z.*, **199**, 136 (1928); **205**, 111 (1929); **213**, 211 (1929); **232**, 423 (1931). W. Franke and M. Deffner, *Ann.*, **532**, 1 (1937).

[119] F. G. Fisher, A. Roedig and K. Rauch, *Naturwissenschaften*, **27**, 197 (1939). F. G. Fisher and H. Eysenbach, *Ann.*, **529**, 87 (1937); **530**, 99 (1937).

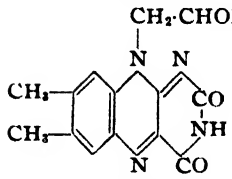
[120] I. Banga and A. Szent-Györgyi, *Biochem. Z.*, **246**, 203 (1932).

[121] P. Karrer, P. Frei and H. Meerwein, *Helv. Chim. Acta*, **20**, 79 (1937).

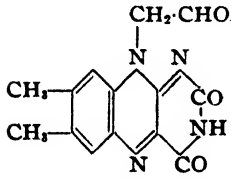
[122] R. Kuhn, H. Rudy and F. Weygand, *Ber.*, **69**, 1543 (1936). R. Kuhn and H. Rudy, *Ibid.*, **69**, 1974 (1936).



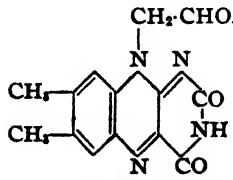
Riboflavin



5′-Trityl-riboflavin

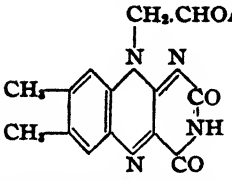


2′ 3′,4′-Triacetyl-5′-trityl-riboflavin

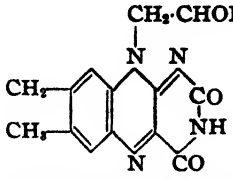


$POCl_3$

2′,3′,4′-Triacetyl-riboflavin



2′,3′,4′-Triacetyl-riboflavin-5′-phosphate



Riboflavin-5′-phosphate

The phosphorylation of riboflavin *in vitro* has also been carried out by a phosphatase concentrate prepared as a dry powder from the intestinal mucosa of rats, cats and pigs,[123] and by a glycerol extract of the small intestine of rats in 0.01 molar phosphate solution.[124]

The properties of riboflavin-5′-phosphoric acid correspond closely to those of the free riboflavin. The ester is considerably more soluble in water than the free riboflavin, and can be precipitated in the form of various salts. Lumiflavin and lumichrome, respectively, are formed upon irradiation similar to the formation of these compounds from riboflavin. In cataphoresis experiments, riboflavin does not move; the phosphoric acid ester, however, migrates to the anode.

In neutral solution, the ester is quite stable. Hydrolysis occurs rapidly in acid solution, but considerably more slowly in alkaline medium. The ester is also hydrolyzed by phosphatases, such as the α-glycero-phosphatase.

Riboflavin-5′-phosphoric acid combines with specific proteins, the apoenzymes, by attachment at two points, namely, at the phosphoric acid group and at the slightly acidic imino-group in 3-position of the riboflavin molecule. In accordance with this conception, flavins which are substituted in the 3-position do not form enzyme systems[125] and are also devoid of vitamin activity. Furthermore, the typical fluorescence of riboflavin is dependent upon the presence of a free 3-imino-group and neither 3-substituted riboflavins nor the enzyme systems exhibit fluorescence.

Riboflavin-adenine-dinucleotide. The riboflavin-adenine-dinucleotide has the composition $C_{27}H_{32}O_{15}N_9P_2$ and forms salts such as a monobarium and mono-sodium salt. The constitution of the dinucleotide has not been well defined. By enzymatic[126] or by acid[127] hydrolysis, the dinucleotide is split into two mononucleotides, namely, into riboflavin-5′-phosphoric acid and adenylic acid (adenosine-5′-monophosphoric acid). (See formula on following page.)

The dinucleotide is widely distributed in animal tissues and in microorganisms. It is also believed to occur in plants. It has been isolated from liver, kidney, heart, muscles, Jensen-sarcoma (a special type of rat tumor) and yeast.[128, 129] The isolation involves a denaturation of the apoen-

[123] H.-Hübner and F. Verzár, *Helv. Chim. Acta*, **21**, 1006 (1938). R. Pulver and F. Verzár, *Enzymologia*, **6**, 333 (1939).

[124] H. Rudy, *Naturwissenschaften*, **23**, 286 (1935).

[125] R. Kuhn and H. Rudy, *Ber.*, **69**, 2557 (1936).

[126] O. Warburg and W. Christian, *Biochem. Z.*, **298**, 150, 368 (1938).

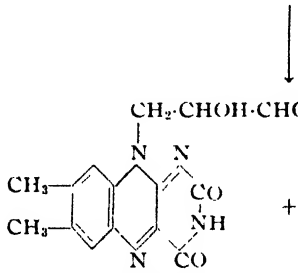
[127] E. P. Abraham, *Biochem. J.*, **33**, 543 (1939).

[128] P. Karrer, P. Frei and M. Meerwein, *Helv. Chim. Acta*, **20**, 79 (1937). P. Karrer, P. Frei, B. H. Ringier and H. Bendas, *Ibid.*, **21**, 826 (1938).

[129] O. Warburg, W. Christian and A. Griese, *Biochem. Z.*, **295**, 261 (1938); **297**, 417 (1938); **298**, 150 (1938).

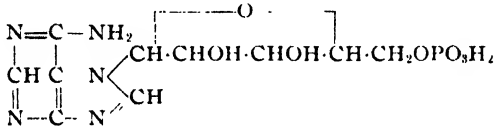
zyme by heat treatment (75° C.) followed by extraction with phenol. From the phenol solution the dinucleotide is extracted with water in the presence of ether. From the acidified water solution the dinucleotide is precipitated as the silver salt and after conversion into the barium salt the latter is recrystallized from water.

Riboflavin-adenine-dinucleotide



+

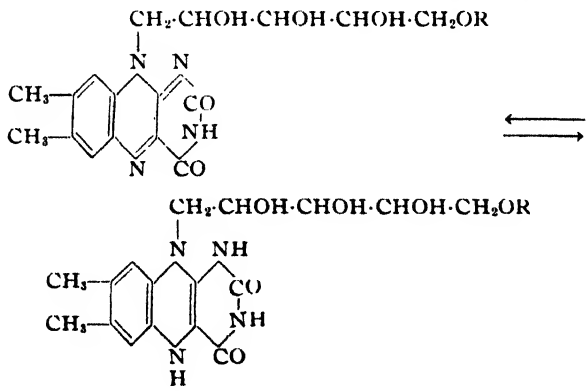
Riboflavin-5′-phosphoric-acid



Adenylic acid

(*c*) *Mechanism of the Coenzyme Action*

Riboflavin acts in various enzyme systems by reversibly accepting and donating two atoms of hydrogen. This is accomplished by the addition of hydrogen to the 1- and 10-positions of riboflavin, thus forming the previously discussed dihydro- or leuco-riboflavin (see page 160).



10. Specificity

Riboflavin is the only naturally occurring flavin with vitamin B_2 activity. A great number of flavin-compounds have been prepared synthetically and have been tested for vitamin activity. Besides riboflavin, the following compounds were found to exhibit vitamin B_2 activity:

7-Methyl-9-(*d*,1′-ribityl)-isoalloxazine.[130]
6-Methyl-9-(*d*,1′-ribityl)-isoalloxazine.[130]
6-Ethyl-7-methyl-9-(*d*,1′-ribityl)-isoalloxazine.[131]

Whereas rats need about 5 γ of riboflavin per day, they require about twice as much of these three compounds. Similar results are obtained when lactic acid bacteria are used for the evaluation of the vitamin activity.[132]

The following compounds possess some stimulating activity for rats and lactic acid bacteria in the presence of suboptimal amounts of riboflavin:

6,7-Dimethyl-9-(*l*,1′-arabityl)-isoalloxazine.[133]
6,7-Dimethyl-9-(*d*,1′-arabityl)-isoalloxazine.[134, 135]
7-Ethyl-9-(*d*,1′-ribityl)-isoalloxazine.[136]
6,7-Trimethylene-9-(*l*,1′-arabityl)-isoalloxazine.[137]
6,7-Tetramethylene-9-(*l*,1′-arabityl)-isoalloxazine.[137]

Riboflavin-tetra-acetate and diacetone-riboflavin are active on rats, probably due to hydrolysis in the organism. The tetra-acetate is, however, inactive on lactic acid bacteria. The 5′-phosphoric acid ester of riboflavin[138] and the flavin-adenine-dinucleotide show full vitamin activity. The flavin-glucosides are inactive.

Generally speaking, substitution in 6- or 7-position is necessary for the vitamin activity. Absence of substituents in both positions is accompanied by high toxicity.[139] An unsubstituted imino-group in 3-position is also necessary. Riboflavins which are alkylated in 3-position are totally devoid of vitamin and coenzyme activity.

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[130] P. Karrer, H. v. Euler, M. Malmberg and K. Schopp, *Svensk Kem. Tid.*, **47**, 153 (1935). P. Karrer, H. Salomon, K. Schöpp, F. Benz and B. Becker, *Helv. Chim. Acta*, **18**, 908 (1935).
[131] P. Karrer and T. H. Quibell, *Helv. Chim. Acta*, **19**, 1034 (1936).
[132] P. Karrer, H. Salomon, K. Schöpp, E. Schlitter and H. Fritzsche, *Ibid.*, **17**, 1010 (1934).
[133] H. v. Euler, P. Karrer, M. Malmberg, K. Schöpp, F. Benz, B. Becker and P. Frei, *Ibid.*, **18**, 522 (1935). P. Karrer, H. v. Euler, M. Malmberg, K. Schöpp and F. Benz, *Svensk. Kem. Tid.*, **47**, 99 (1935). P. György, *Z. Vitaminforsch.*, **4**, 223 (1935).
[134] H. v. Euler, P. Karrer and M. Malmberg, *Helv. Chim. Acta*, **18**, 1336 (1935).
[135] Vitamin activity questionable.
[136] P. Karrer and T. H. Quibell, *Helv. Chim. Acta*, **19**, 1034 (1936).
[137] R. Kuhn, H. Vetter and H. W. Rzeppa, *Ber.*, **70**, 1307 (1937).
[138] P. György, *Proc. Soc. Exptl. Biol. Med.*, **35**, 207 (1936).
[139] R. Kuhn and P. Boulanger, *Z. physiol. Chem.*, **241**, 233 (1936).

11. Determination

(a) *Physical Methods*

1. Determination of the Absorption Spectrum. The determination of vitamin B_2 by its characteristic ultraviolet absorption spectrum is suitable only for solutions of the pure compound, and this method has the additional disadvantage that the vitamin is sensitive to light. Thus during the determination the vitamin is at least partly destroyed, and the material destroyed before the beginning of the determination adds to the apparent value of the vitamin.

2. Determination of the Fluorescence Spectrum. Vitamin B_2 can be determined by its characteristic fluorescence spectrum[140] which shows a maximum at 565 mμ at pH 6. It is best to compare the intensity of the fluorescence with some standard such as pure riboflavin,[141] potassium dichromate,[142] fluorescein[143] or uranium glass.[144] The fluorescence is directly proportional to the vitamin content. Relatively reliable results have been obtained with this method when the vitamin B_2 content of animal tissues or milk is assayed. The method becomes inaccurate, however, in cases when other fluorescent substances are present as in the case of urine and yeast extracts. Another source of error often encountered is a non-quantitative extraction of the vitamin. Such extractions are often necessary to separate riboflavin from interfering substances. A method which has given results in agreement with biological assays consists in enzymatic hydrolysis of, for example, tissues, followed by extraction with water and determination of the fluorescence of the extract.[145]

Several modifications of this basic assay procedure have been recommended. Thus, vitamin B_2 has been determined indirectly by the fluorescence of the vitamin before and after reduction with sodium hydrosulfite.[146] This has been possible since the reduced vitamin does not show the typical fluorescence of the unreduced vitamin. Another modification is to determine, first, the total fluorescence of a given solution and, second, the fluorescence of the interfering substances by destroying the vitamin through alkali.[147]

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[140] H. v. Euler and E. Adler, *Svensk. Kem. Tid.*, **45**, 276 (1933); *Z. physiol. Chem.*, **223**, 105 (1934). G. C. Supplee, S. Ansbacher, G. E. Flanigan and Z. M. Hanford, *J. Dairy Sci.*, **19**, 215 (1936).

[141] A. Z. Hodson and L. C. Norris, *J. Biol. Chem.*, **131**, 621 (1939).

[142] A. J. Charite and N. W. Khaustov, *Biochem. J.*, **29**, 34 (1935). G. N. Murthy, *Indian J. Med. Research*, **24**, 1083 (1937).

[143] F. H. Cohen, *Rec. trav. chim.*, **54**, 133 (1935). S. M. Weisberg and I. Levin, *Ind. Eng. Chem. Anal. Ed.*, **9**, 523 (1937).

[144] D. B. Hand, *Ind. Eng. Chem. Anal. Ed.*, **11**, 306 (1939).

[145] F. O. Van Duyne, *J. Biol. Chem.*, **139**, 207 (1941).

[146] A. Z. Hodson and L. C. Norris, *Ibid.*, **131**, 621 (1939).

[147] H. Kahler and E. P. Davis, *Proc. Soc. Exptl. Biol. Med.*, **44**, 604 (1940).

3. **Polarographic Determination.** It has been proposed to determine vitamin B_2 by the polarographic technique.[148] Assays have been carried out so far only on relatively pure vitamin B_2 preparations and the method needs further study to determine whether or not this procedure can be applied for the determination of riboflavin in natural products.

4. **Lumi-Lactoflavin Method.** This method is based on the conversion of riboflavin into lumi-lactoflavin by irradiation in alkaline solution. The so-formed lumi-lactoflavin is extracted from the acidified solution with chloroform and determined by its absorption in the fluorescence spectrometer.[149] The accuracy of this method is limited, since the conversion of riboflavin into lumi-lactoflavin is by no means quantitative. Nevertheless, this method proved to be effective, if the conversion rate of pure riboflavin in a solution of about the same concentration as in the solution to be examined is determined separately, taking care that the same pH, temperature and amount of air are present. In another experiment a known amount of pure riboflavin is added to the unknown solution and the conversion is determined. By proper correlation of the three figures obtained an accurate determination of the vitamin B_2 content of almost any solution can be achieved.

The separation of riboflavin from its phosphoric acid ester is carried out by extraction of the aqueous solution with benzyl-alcohol, in which only the free riboflavin is soluble.[150]

The separation of riboflavin and riboflavin-5'-phosphoric acid from flavin combined with proteins is achieved by dialysis in cellophane tubes at 3° C. for 16 hours.

(*b*) *Biological Methods*

1. **Rat Growth Test.** The most accurate and generally accepted biological method for the determination of riboflavin is the curative growth test on rats.[151, 152, 153] Young rats kept on a riboflavin-free diet stop

[148] J. J. Lingane and O. L. Davis, *J. Biol. Chem.*, **137**, 567 (1941).

[149] R. Kuhn, T. Wagner-Jauregg and H. Kaltschmitt, *Ber.*, **67**, 1452 (1934). H. v. Euler, E. Adler and A. Schlötzer, *Z. physiol. Chem.*, **226**, 87 (1934). F. Vivanco, *Naturwissenschaften*, **23**, 306 (1935).

[150] A. Emmerie, *Nature*, **141**, 416 (1938); *Rec. trav. chim.*, **58**, 290 (1939).

[151] A. Bourquin and H. C. Sherman, *J. Am. Chem. Soc.*, **53**, 3501 (1931).

[152] P. György, F. W. van Klaveren, R. Kuhn and T. Wagner-Jauregg, *Z. physiol. Chem.*, **223**, 236 (1934).

[153] A. Bourquin and H. C. Sherman, *J. Am. Chem. Soc.*, **53**, 3501 (1931). H. E. Munsell, *J. Nutrition*, **4**, 203 (1931). P. György, *Biochem. J.*, **29**, 741 (1935). P. L. Day and W. C. Langston, *J. Nutrition*, **7**, 97 (1934). G. C. Supplee, R. C. Bender and O. G. Jensen, *Ind. Eng. Chem., Anal. Ed.*, **11**, 495 (1939). T. S. Hamilton and H. H. Mitchell, *J. Nutrition*, **10**, 117 (1935). L. A. Randoin, A. Raffy and J. Aquirrezabla, *Compt. rend. soc. biol.*, **126**, 872 (1937). H. Lindholm, *Biochem. J.*, **32**, 314 (1938). E. V. Carlsson and H. C. Sherman, *J. Nutrition*, **15**, 57 (1938). M. M. El-Sadr, T. F. Macrae and C. E. Work, *Biochem. J.*, **34**, 601 (1940). J. R. Wagner, A. E. Axelrod, M. A. Lipton and C. A. Elvehjem, *J. Biol. Chem.* **136**, 357 (1940).

growing within a few days. Addition of riboflavin to the diet causes continuation of growth. The same principle has also been used in a prophylactic method.

2. **Chick Growth Test.** An apparently very reliable method is the growth response of chicks, which is within certain limits a linear function of the amount of riboflavin administered.[154]

3. **Lactic Acid Bacteria Test.** This method[155] is based on the essential nature of riboflavin for the growth of *Lactobacillus casei*. The evaluation is carried out by measurement of the turbidity produced by the growth of the organism or by titration of the lactic acid formed. This method determines not only riboflavin in the free form, but also in the combined forms (coenzymes and enzymes).[156, 157]

(c) *Biochemical Methods*

1. **Yellow Enzyme Test.**[158] The oxygen uptake of the following system is measured: Hexose-phosphoric acid, codehydrogenase II (page 235), apodehydrogenase and the specific protein of the yellow enyzme. The rate of the oxygen uptake per minute is a function of the amount of the riboflavin added to this system.

2. **Determination of Riboflavin-adenine-dinucleotide.**[159] The dinucleotide is determined by its ability to act in combination with a specific protein as *d*-amino-acid oxidase.[160, 161, 162] Actually, the rate of oxygen uptake is measured during the oxidation and is compared with the rate of oxygen uptake catalyzed by known amounts of the dinucleotide in the same system.

12. Standards

One B. S. Rat Unit (Bourquin-Sherman Unit) is defined as the daily amount of riboflavin required by rats, to insure proper growth (10 g. increase of body weight per week for two to four weeks).

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[154] T. H. Jukes, *J. Nutrition*, **14**, 223 (1937).

[155] E. E. Snell and F. M. Strong, *Ind. Eng. Chem., Anal. Ed.*, **11**, 346 (1939); *Enzymologia*, **6**, 186 (1939). E. E. Snell, F. M. Strong and W. H. Peterson, *Biochem. J.*, **31**, 1789 (1937); *J. Am. Chem. Soc.*, **60**, 2825 (1938).

[156] R. E. Feeney and F. M. Strong, *Proc. Am. Soc. Biol. Chem.*, **1940**, XXXI.

[157] F. M. Strong, R. E. Feeney, B. Moore and H. T. Parsons, *J. Biol. Chem.*, **137**, 363 (1941).

[158] R. Kuhn and H. Rudy, *Ber.*, **69**, 2557 (1936).

[159] O. Warburg and W. Christian, *Biochem. Z.*, **298**, 150, 368 (1938).

[160] E. Negelein and H. Brömel, *Ibid.*, **300**, 225 (1939).

[161] J. R. Klein and H. I. Kohn, *J. Biol. Chem.*, **136**, 177 (1940).

[162] S. Ochoa and R. J. Rossiter, *Biochem. J.*, **33**, 2008 (1939).

An International Unit of riboflavin has not been established. Von Euler[163] proposed as International Unit 5 γ of pure crystallized riboflavin, which amount produces an increase in weight of 0.8–1.0 g. per day in young rats.

1 Cornell Unit of riboflavin = 1 γ of riboflavin, defined by the growth effect on chicks.[164]

1 B. S. Unit = 2.0–3.0 γ of riboflavin.[165, 166]

1 g. riboflavin = 400,000–500,000 B. S. Units.

13. Physiology of Plants and Microorganisms

The physiology of plants and microorganisms with respect to the action of riboflavin in their cells has not been studied sufficiently. It is, however, reasonable to assume that principally the same reactions are carried out by riboflavin in plants and in microorganisms as in animals, especially as part of enzyme systems.

Most microorganisms, bacteria,[167] molds,[168] fungi,[169] etc., synthesize riboflavin, just as the higher plants do. *Microbacillus tuberculosis*, for example, produces 0.5 to 2.9 γ of riboflavin per day.[170] A few, however, have been found, for example, special strains of lactic acid bacteria[171] and of the streptococci[172] which require an outside supply of riboflavin and are thus parasitic in character (see page 193).

In anaerobic cells, for example, in yeast, in lactic and butyric acid bacteria, the riboflavin-containing enzyme systems maintain the respiration[173] or that part of the respiration which cannot be disturbed by either hydrocyanic acid or by carbon monoxide and which, therefore, is not a function of a porphyrin enzyme system which contains iron and is easily poisoned. Aerobic living bacteria contain and apparently need considerably less riboflavin than do the anaerobic living organisms.

Riboflavin is, as far as is known, synthesized by all higher plants. It is apparently formed in the green leaves, where it is found predomi-

[163] H. v. Euler, Institut international de Chimie Solvay, Sixieme Conseil de Chimie, rapport et discussions sur *Les Vitamines et les Hormones*, Paris, 1938, p. 198.

[164] L. C. Norris, H. S. Wilgus, A. T. Ringrose, V. Heiman and G. F. Heuser, *Cornell Univ. Agr. Exptl. Sta. Bull.*, **660**, 3 (1936).

[165] O. A. Bessey, *J. Nutrition*, **15**, 11 (1938).

[166] H. v. Euler, P. Karrer, E. Adler and M. Malmberg, *Helv. Chim. Acta*, **17**, 1157 (1934).

[167] O. Warburg and W. Christian, *Biochem. Z.*, **266**, 377 (1933).

[168] J. Lavollay and F. Laborey, *Compt. rend.*, **204**, 1686 (1937); **205**, 179 (1937); **206**, 1055 (1938).

[169] A. Guillermond, M. Fontaine and A. Raffy, *Ibid.*, **201**, 1077 (1935). A. Raffy, *Ibid.*, **209**, 900 (1939).

[170] C. H. Boissevain, W. F. Drea and H. W. Schultz, *Proc. Soc. Exptl. Biol. Med.*, **39**, 481 (1938).

[171] E. E. Snell and F. M. Strong, *Enzymologia*, **6**, 186 (1939).

[172] E. J. Krauskopf, E. E. Snell and E. McCoy, *Ibid.*, **7**, 327 (1939).

[173] M. Dondroff, *Ibid.*, **5**, 239 (1938).

nantly. In broccoli, the flower buds contain only a little over half as much as the leaves, and the twigs contain even less.[174] In carrots, the riboflavin content of the roots is only one-fourth of the content of the tops. It has been shown that excised cosmos roots need a supply of vitamin B_2[175] which apparently is furnished in the intact plant from some other part, probably the leaves. A beneficial effect on the growth of eggplants in synthetic nutrient solution has been observed upon the addition of 2.5% of riboflavin.[176] There seems to exist a definite species difference in the response of various plants to riboflavin administration.

As leaves become older, the riboflavin content diminishes. Younger parts of plants always seem to contain more than older parts. Ungerminated seeds generally contain little riboflavin (peas are an exception). During germination, the riboflavin content increases many times.

It has been reported that riboflavin increases the phototropic action of plants.[177]

14. Animal Physiology

(a) *General Physiology, Metabolism and Mechanism of the Vitamin B_2 Action*

The chemically bound vitamin B_2, as it occurs, for example, in vegetables and in seeds, cannot be absorbed in the intestines of animals as has been shown in the case of the rat.[178] After cooking, however, the vitamin is liberated and completely absorbed.

The three naturally occurring free riboflavin compounds, namely, riboflavin, riboflavin-5′-phosphoric acid and riboflavin-adenine-dinucleotide, are easily absorbed by the intestines in the small gut. The free riboflavin is phosphorylated in the intestines as can be shown *in vitro* with dried powdered intestinal mucosa or with glycerol extracts of intestines. When the phosphorylation mechanism is disturbed, for example, experimentally with iodo-acetic acid or by extirpation of the adrenals,[179] riboflavin is not absorbed and the organism (rats were used for these experiments) ceases growing. Only by the addition of riboflavin-phosphoric acid (and possibly also of the dinucleotide) to the diet does further growth occur.

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[174] Mentioned in the review by H. C. Sherman and C. S. Lanford in *The Vitamins*, Am. Med. Assoc., 1939, p. 292.

[175] J. Bonner, *Am. Chem. Soc. Div. Agr. Food Chem., Meeting*, Sept. 1939, Abst. 13–14.

[176] R. Dennison, *Science*, 92, 17 (1940).

[177] M. Heiman, *Wien. klin. Wochschr.*, 49, 398 (1936).

[178] E. M. Lantz, *Agr. Exptl. Sta. New Mexico Coll. Agr. Mech. Arts, Bull.* 268 (1939).

[179] F. Verzár and L. Laszt, *Arch. ges. Physiol. (Pflügers)*, 236, 693 (1935): 237, 476, 483 (1936); *Verhandl. Schweiz. Physiol.*, I, VI (1936); *Enzymologia*, 3, 16 (1937).

The transformation of riboflavin to its phosphoric acid ester and the dinucleotide is also a general cellular reaction. Human blood cells, for example, but not the plasma, can synthesize the dinucleotide from riboflavin both *in vitro* and *in vivo*.[180] Therefore, riboflavin can also be administered parenterally.

Riboflavin-phosphoric acid is built into various enzyme systems, which have been discussed previously. The liver and kidney seem to undertake these reactions to a greater extent than the other organs.

Riboflavin is excreted predominantly in the feces, and to a smaller extent in urine, During vitamin B_2 avitaminosis, no riboflavin is excreted in the urine; small amounts, however, are found in the feces. Vitamin B_2 is excreted mainly in the free form, but in varying amounts, up to 50%, also as phosphoric acid ester. An increase in the riboflavin intake of humans increases the urinary output of riboflavin.[180, 181] The intake of riboflavin-phosphoric acid increases to a small extent the excretion of the phosphorylated compound besides increasing to an appreciable extent the excretion of free riboflavin.[182] Normal human beings on a balanced diet excrete about 500–800 γ per day.[183]

Besides riboflavin and its phosphoric acid ester, another flavin called aquoflavin or uroflavin is found in urine. The chemical constitution of this compound is not quite clear; it seems to contain more oxygen than does riboflavin and is more water-soluble. It is also sensitive to illumination and appears to be similarly converted into a lumiflavin compound.[184] Uroflavin is apparently a degradation product of riboflavin.

The animal organism has no special storage organs for riboflavin, although a certain level is maintained in the various tissues (0.5 γ per gram of blood[183]). The flavin-dinucleotide level in blood cells and plasma is fairly constant.[180] Relatively large amounts are found, for example, in liver and in kidney. Intake of large amounts of riboflavin does not increase the riboflavin content of the liver to any appreciable extent[185] but increases the dinucleotide concentration in the blood cells.[180] On the other hand, the organs of animals which die of vitamin B_2-avitaminosis still contain considerable amounts of this vitamin, approximately one-third of the normal level.[185, 186] In man during times of clinical B_2-avitaminosis

[180] J. R. Klein and H. I. Kohn, *J. Biol. Chem.*, **136**, 177 (1940).
[181] A. Emmerie and M. van Eekelen, *Acta Brevia Neerland Physiol. Pharmacol. Microbiol.*, **7**, 169 (1937).
[182] A. Emmerie, *Ibid.*, **8**, 116 (1938).
[183] F. M. Strong, R. E. Feeney, B. Moore and H. T. Parsons, *J. Biol. Chem.*, **137**, 363 (1941).
[184] W. Koschara, *Z. physiol. Chem.*, **229**, 103 (1934); **232**, 101 (1935).
[185] R. Kuhn, H. Kaltschmitt and T. Wagner-Jauregg, *Ibid.*, **232**, 36 (1935).
[186] F. Vivanco, *Arkiv Kemi, Mineral. Geol.*, **A12**, No. 3 (1935).

no substantial decrease of the riboflavin content in blood and in muscles could be observed.[187] In rats[188] and in dogs,[189] riboflavin deficiency causes a decrease of the tissue level of this vitamin. The amount of the riboflavin-adenine-dinucleotide also decreases during times of low riboflavin intake and increases again upon administration of the vitamin (experiments with rats[190]). Thus, the xanthine-oxidase activity in the livers of riboflavin-depleted rats is only a small percentage of the activity in rats on an adequate riboflavin intake, as measured by the rate of oxygen consumption.[191]

Riboflavin is, like all the other vitamins, secreted in the milk, where it is found predominantly in the free form. In human milk, riboflavin is said to occur also to a small extent in combination with a protein.[192]

The fundamental action of riboflavin in living tissue is to take part in enzyme systems which regulate cellular oxidations. These enzyme systems have already been described. They take part in the general carbohydrate metabolism (fermentation and glycolysis). Riboflavin is also involved in the absorption of carbohydrates from the intestines by phosphorylation. Thus, glucose and galactose are rapidly absorbed only in the presence of riboflavin.[193] Riboflavin is connected to a certain extent with the fat metabolism:[194] increased fat content of the livers has been observed in hens and dogs which died of riboflavin insufficiency.[195, 196] Furthermore, it has been demonstrated[197] that the symptoms of a vitamin B_2 deficiency in rats increase in severity upon administration of increased amounts of fats. On the other hand, when increased doses of riboflavin are given together with increased amounts of fat no deleterious effect is observed. Riboflavin bears an important relation to the amino-acid metabolism, since *d*-amino-acids are deaminated by an enzyme system, which contains the flavin-adenine-dinucleotide.

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[187] A. E. Axelrod, T. D. Spies and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.*, **46**, 146 (1941).

[188] F. Vivanco, *Naturwissenschaften*, **23**, 306 (1935). R. Kuhn, H. Kaltschmitt and T. Wagner-Jauregg, *Z. physiol. Chem.*, **232**, 36 (1935). J. Groen and J. W. Schuyl, *Arch. néerland. physiol.*, **23**, 271 (1938).

[189] H. F. Frazer, N. H. Topping and H. Isbell, *U. S. Pub. Health Service Pub. Health Repts.*, **55**, 280 (1940).

[190] S. Ochoa and R. J. Rossiter, *Biochem. J.*, **33**, 2008 (1939).

[191] A. E. Axelrod and C. A. Elvehjem, *Proc. Am. Soc. Biol. Chem.*, **1941**, VI.

[192] P. Ellinger and W. Koschara, *Nature*, **133**, 553 (1934).

[193] L. Laszt and F. Verzár, *Biochem. Z.*, **292**, 159 (1937). M. Judowitz and F. Verzár, *Biochem. J.*, **292**, 182 (1937).

[194] E. W. McHenry and G. Gavin, *J. Biol. Chem.*, **125**, 653 (1938).

[195] S. Lepkovsky, L. W. Taylor, T. H. Jukes and H. J. Almquist, *Hilgardia*, **11**, 559 (1938).

[196] W. H. Sebrell and R. H. Onstott, *U. S. Pub. Health Service Pub. Health Repts.*, **53**, 83 (1938). H. R. Street and G. R. Cowgill, *Am. J. Physiol.*, **125**, 323 (1939).

[197] G. J. Mannering, M. A. Lipton and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.*, **46**, 100 (1941).

Riboflavin in the free form plays an important role in the vision mechanism in the retina.[198] Light converts riboflavin into a "photo-compound" of unknown structure, which process seems to have some bearing on the stimulation mechanism of the optical nerve. The primary "photo-compound" is extremely sensitive. In the absence of oxygen it is destroyed, in the presence of oxygen it is reconverted into riboflavin (Theorell). The mechanism of the riboflavin action in the retina is especially well understood for dim light, since light of short wave length is converted into light of longer (yellow-green) waves by the fluorescent activity of riboflavin.[199] The human eye has a maximum sensitivity for greenish light.

The theory has also been advanced that riboflavin takes part in an oxidation system in the cornea.[200] Since the cornea is avascular, the cornea cells are nourished according to this hypothesis by a specific riboflavin-containing enzyme system. It is thought that during riboflavin deficiency the body attempts to counteract the missing oxygenation by vascularization.

An interesting fact about riboflavin is that the phosphorescence of the glow worm (*Lampyris*) is caused by riboflavin in combination with a special protein.[201] The phosphorescence consists of light of the wave lengths 562–570 mμ. It must be assumed that riboflavin plays an important role in some unknown biochemical process in the luminous organs.

It is also interesting that the mold *Aspergillus niger*, which is free of riboflavin when cultivated under optimum conditions, becomes tinted with riboflavin on a medium deficient in magnesium. The formation of the pigment is not obtained by restricting the other elements, but is increased if a deficiency in magnesium and iron exists simultaneously.[202]

(*b*) *Relation of Vitamin B_2 to Other Vitamins, Hormones and Minerals*

Riboflavin bears an obvious relation to other members of the vitamin B-group. It seems that most of these vitamins act as part of enzyme systems, which regulate the carbohydrate, fat and amino-acid metabolism. For example, the close relation of riboflavin to the enzyme systems containing nicotinic amide has already been discussed. The relation of vita-

[198] A. M. Chase, *Science*, **85**, 484 (1937).

[199] H. v. Euler and E. Adler, *Arkiv Kemi, Mineral. Geol.*, **B11**, No. 28 (1934). R. Kuhn and H. Kaltschmitt, *Ber.*, **68**, 386 (1935). E. Adler and H. v. Euler, *Nature*, **141**, 790 (1938). H. v. Euler and E. Adler, *Z. physiol. Chem.*, **223**, 105 (1934).

[200] O. A. Bessey and S. B. Wolbach, *J. Exptl. Med.*, **69**, 1 (1939). R. E. Eckardt and L. V. Johnson, *Arch. Ophthalmol.*, **21**, 315 (1939).

[201] G. Brooks, *Compt. rend.*, **210**, 228 (1940).

[202] J. Lavollay and F. Laborey, *Ibid.*, **208**, 1056 (1939).

min B_2 to vitamin B_1 may be demonstrated by the fact that riboflavin has an apparent sparing action on thiamin.[203]

An important relation exists between riboflavin and the adrenal cortex hormone: the phosphorylation of riboflavin is carried out with the aid of the adrenal cortex hormone. Adrenalectomized animals lose their power of phosphorylating riboflavin. Thus, riboflavin-phosphoric acid, but not riboflavin, maintains life and growth of adrenalectomized rats.[204]

A number of observations have been made which demonstrate the close relationship of riboflavin to other hormones concerned in the carbohydrate metabolism. Rats lose their liver glycogen after injections of thyroxine unless increased amounts of thiamine and riboflavin are given simultaneously.[205] Depancreatized dogs respond to insulin only in the presence of thiamin and riboflavin, but not in the presence of one of these vitamins alone.[206]

A certain relation of riboflavin to the minimum amount of indispensable magnesium is noted. A minimum riboflavin intake increases the minimum magnesium requirements.[207]

15. Avitaminosis and Hypovitaminosis

The basic but unspecific symptoms of a riboflavin deficiency are the same throughout the entire animal kingdom—cessation in growth of young organisms and sudden death of adult organisms.

In rats, riboflavin deficiency, even in a relatively early stage, considerably decreases the resistance forces of the organism against infectious diseases, for example, endemic typhus.[208] Growth of young rats is impaired when fed a vitamin B_2-deficient diet and when no riboflavin is administered the animals die. Riboflavin-deficient animals sometimes show an abnormal intracellular metabolism.[208] In riboflavin-deficient rats an early atrophy of the testis and an involution of the thymus have been observed.[209] Increase of the diaphragm metabolism in rats is also reported.[210] The development of alopecia (baldness) and cataract (loss of transparency of the

[203] L. N. Ellis and A. Zmachinsky, *Science*, **86**, 245 (1937).

[204] F. Verzár and L. Laszt, *Arch. ges. Physiol. (Pflügers)*, **236**, 693 (1935); **237**, 476, 483 (1936); *Verhandl. Schweiz. Physiol.*, I, VI (1936); *Enzymologia*, **3**, 16 (1937).

[205] V. A. Drill, *J. Nutrition*, **14**, 355 (1937).

[206] R. W. Martin, *Z. physiol. Chem.*, **248**, 242 (1937); *Verhandl. deut. Ges. inn. Med.*, **50**, 420 (1938).

[207] E. V. Tufs and D. M. Greenberg, *J. Biol. Chem.*, **122**, 715 (1938); *Am. J. Physiol.*, **121**, 416 (1938).

[208] H. Pinkerton and O. A. Bessey, *Science*, **89**, 369 (1939).

[209] J. W. Schuyl and J. Groen, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **8**, 195 (1938). J. H. Shaw and P. H. Phillips, *J. Nutrition*, **22**, 345 (1941).

[210] A. B. Hastings, J. Muus and O. A. Bessey, *J. Biol. Chem.*, **129**, 295 (1939).

lens of eyes) in rats on riboflavin-deficient diets is of special interest.[211, 212] Certain eye lesions, namely, diffuse corneal opacity and vascularization of the cornea, are specific for riboflavin deficiency in rats.[213, 214]

Pigs also need riboflavin for normal growth and physical well-being.[215] Dogs usually collapse after about 100 days on a riboflavin-deficient diet.[216] For chicks and fowls, riboflavin is of importance in the production of eggs and for normal hatchability of eggs.[217, 218, 219, 220] Chicks hatched from eggs on a partially riboflavin-deficient ration exhibit a characteristic curled toe ("nutritional leg paralysis").[220] Besides this slowly developing paralysis, an acute paralysis, characterized as neuromalacia, was observed.[221] The main peripheral nerve trunks are especially involved and characteristic changes in the myelin have been noticed. These severe nerve degenerations occur also in rats.[222] Turkey poults develop a typical dermatitis as the result of a riboflavin deficiency.[223] In addition to the specific symptoms which are caused by a riboflavin deficiency, a premature aging has been observed in all animals studied.[224]

Riboflavin deficiency in humans of all ages[225] causes the occurrence of specific symptoms. Primarily cheilosis, an eversion of the lips and in the corners of the mouth, has been observed.[226, 227] There is also a seborrheic syndrome, a scaly, slightly greasy desquamation on a mildly erythematous base in the nasobial fold, on the alae nasi, in the vestibule of the nose and on the ears. A special type of glossitis may develop which is characterized by a purplish red or magenta-colored inflammation of the tongue.[228] The entire condition is called "ariboflavinosis."[226]

Riboflavin deficiency in man also causes characteristic ocular symptoms similar to those already described for rats. In mild cases a sensation of

[211] P. L. Day, W. C. Langston and C. S. O'Brien, *Am. J. Opthalmol.*, **14**, 1005 (1931).
[212] P. L. Day, W. J. Darby and W. C. Langston, *J. Nutrition*, **13**, 389 (1937).
[213] M. M. El-Sadr, *J. Soc. Chem. Ind.*, **58**, 1020 (1939).
[214] O. A. Bessey and S. B. Wolbach, *J. Exptl. Med.*, **69**, 1 (1939). R. E. Eckardt and L. V. Johnson, *Arch. Opthalmol.*, **21**, 315 (1939).
[215] E. H. Hughes, *J. Nutrition*, **17**, 527 (1939).
[216] H. R. Street and G. R. Cowgill, *Am. J. Physiol.*, **125**, 323 (1939).
[217] S. Lepkovsky, L. W. Taylor, T. H. Jukes and H. J. Almquist, *Hilgardia*, **11**, 559 (1938).
[218] H. J. Davis, L. C. Norris and G. F. Heuser, *Poultry Sci.*, **17**, 81, 87 (1938)
[219] G. F. Heuser, H. S. Wilgus and L. C. Norris, *Ibid.*, **17**, 105 (1938).
[220] A. E. Schumacher and G. F. Heuser, *Ibid.*, **18**, 369 (1939).
[221] P. H. Phillips and R. W. Engel, *J. Nutrition*, **16**, 451 (1938); *Poultry Sci.*, **17**, 463 (1938). E. L. R. Stokstad and P. D. V. Manning, *J. Nutrition*, **16**, 279 (1938).
[222] J. H. Shaw and P. H. Phillips, *J. Nutrition*, **22**, 345 (1941).
[223] S. Lepkovsky and T. H. Jukes, *J. Nutrition*, **12**, 515 (1938).
[224] P. L. Day and W. J. Darby, *J. Biol. Chem.*, **123**, 28 (1938).
[225] T. D. Spies, W. B. Bean, R. W. Vilter and N. E. Huff, *Am. J. Med. Sci.*, **200**, 697 (1940).
[226] W. H. Sebrell and R. E. Butler, *U. S. Pub. Health Service Pub. Health Repts.*, **53**, 2282 (1938).
[227] J. W. Oden, L. H. Oden and W. H. Sebrell, *Ibid.*, **54**, 790 (1939).
[228] H. D. Kruse, V. P. Sydenstricker, W. H. Sebrell and H. M. Cleckley, *Ibid.*, **55**, 157 (1940).

roughness in the eyes and itching and burning are observed[229] which are accompanied by a mild photophobia. In more severe cases a corneal opacity has been noted.[228] Interstitial keratitis (of the cornea) in cases of syphilis improves markedly upon treatment with riboflavin.[228] Persons showing subnormal dark adaptation which does not respond to vitamin A treatment may be relieved by riboflavin administration.[230, 231]

Riboflavin treatment is also of importance in diseases caused by multiple vitamin deficiencies, such as pellagra,[232] black tongue[233] and beriberi. Beneficial results from riboflavin have also been observed in a case of pemphigus.[234]

(*a*) *Clinical Test Methods*

Vitamin B_2 can be determined in blood, in muscles and in urine. The value of these tests for a detection of a state of a hypovitaminosis or avitaminosis is, however, very limited. While the riboflavin content of tissues from experimental animals, especially from rats, is lowered during times of inadequate vitamin B_2 intake [235, 236, 237, 238, 239, 240] this is not the case in human beings as is evident from blood and muscle analysis.[241] The most promising test is the tissue saturation test but not enough experimental data have accumulated to determine the actual value of this procedure. *Blood determinations* for riboflavin are best carried out by the Lactobacillus test,[242] the reliability of which has been established.[243, 244] The riboflavin-adenine-dinucleotide content of blood can be determined by the *d*-amino-acid-oxidase test.[245] In *muscle studies* the quadriceps femoris muscle[241] and the Lactobacillus test[242] are used for the determination of the riboflavin

[229] T. D. Spies, R. W. Vilter and W. F. Ashe, *J. Am. Med. Assoc.*, **113**, 931 (1939).
[230] M. S. Kimble and E. S. Gordon, *J. Biol. Chem.*, **128**, lii (1939).
[231] P. H. Pock-Steen, *Aknephascopia Geneesk. tijschr. v. Nederl.-Indie*, **79**, 1986 (1939).
[232] R. W. Vilter, S. Vilter and T. D. Spies, *J. Am. Med. Assoc.*, **112**, 420 (1939).
[233] L. H. Margolis, G. Margolis and S. G. Smith, *J. Nutrition*, **17**, 63 (1939).
[234] M. C. Topping and A. F. Knoefel, *J. Am. Med. Assoc.*, **114**, 2102 (1940).
[235] S. Ochoa and R. J. Rossiter, *Biochem. J.*, **33**, 2008 (1939).
[236] A. E. Axelrod, H. A. Sober and C. A. Elvehjem, *J. Biol. Chem.*, **134**, 749 (1940).
[237] R. Kuhn, H. Kaltschmitt and T. Wagner-Jauregg, *Z. physiol. Chem.*, **232**, 36 (1935).
[238] J. Groen and J. W. Schuyl, *Arch. néerland. physiol.*, **23**, 271 (1938).
[239] E. V. Carlsson and H. C. Sherman, *J. Nutrition*, **15**, 57 (1938).
[240] H. F. Frazer, N. H. Topping and H. Isbell, *U. S. Pub. Health Service Pub. Health Repts.*, **55**, 280 (1940).
[241] A. E. Axelrod, T. D. Spies and C. A. Elvehjem, *Proc. Soc. Expll. Biol. Med.*, **46**, 146 (1941).
[242] E. E. Snell and F. M. Strong, *Ind. Eng. Chem., Anal. Ed.*, **11**, 346 (1939); *Enzymologia*, **6**, 186 (1939). E. E. Snell, F. M. Strong and W. H. Peterson, *Biochem. J.*, **31**, 1789 (1937); *J. Am. Chem. Soc.*, **60**, 2825 (1938).
[243] R. E. Feeney and F. M. Strong, *Proc. Am. Soc. Biol. Chem.*, **1940**, XXXI.
[244] F. M. Strong, R. E. Feeney, B. Moore and H. T. Parsons, *J. Biol. Chem.*, **137**, 363 (1941).
[245] J. R. Klein and H. I. Kohn, *Ibid.*, **136**, 177 (1940).

present. The amount of riboflavin present *in urine* can be assayed by the Lactobacillus test[244] or by the fluorescence method. Urine containing 0.0001 mg. or less of riboflavin per cc. cannot be used in the latter procedure.[245] In the *tissue saturation test* the amount of riboflavin excreted through the urine is measured after oral administration of excess doses of the vitamin.[245]

16. Hypervitaminosis

Riboflavin administered in excessive amounts by mouth was found to be non-toxic in dogs and rats. Following intraperitoneal injection, however, riboflavin produced death due to kidney concretions.[246, 247]

17. Requirements

A regular dietary intake of riboflavin is necessary for all members of the animal kingdom and for some microorganisms. From eleven species of lactic acid bacteria investigated, four were found to require riboflavin for growth and acid production. The remaining seven did not require an external supply of this vitamin and it has been established that four of these seven species synthesize vitamin B_2 when cultured on a riboflavin-free medium.[248, 249] Riboflavin also stimulates the growth of propionic acid-[250] and of luminous[251] bacteria.

The only organism so far reported as actually needing the riboflavin-adenine-dinucleotide is the larva of mosquitoes. Riboflavin itself is not effective.[252]

In general, the vitamin B_2 requirements of animals are related to body size and weight, to the amount of food ingested, to the ambient temperature, etc. Man needs about 2–3 mg. of riboflavin daily.[253, 254] The riboflavin allowances recommended by the Food and Nutrition Board, National Research Council, will be found on page 613.

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[244] R. Kuhn and P. Boulanger, *Z. physiol. Chem.*, **241**, 233 (1936) R. Kuhn, *Klin Wochschr.*, **17**, 222 (1938). V. Demole, *Z. Vitaminforsch.*, **7**, 138 (1938).

[245] K. Unna and J. G. Greslin, *J. Pharmacol.*, **76**, 75 (1942).

[246] E. E. Snell and F. M. Strong, *Enzymologia*, **6**, 186 (1939).

[247] E. E. Snell, F. M. Strong and W. H. Peterson, *Biochem. J.*, **31**, 1789 (1937). S. Orla-Jensen, N. C. Otte and A. Snog-Kjaer, *Zentr. Bakt. Parasitenk.*, **II, 94**, 434, 447 (1936). H. G. Wood, A. A. Anderson and C. H. Werkman, *J. Bact.*, **34**, 132 (1937); *Proc Soc Exptl Biol. Med.*, **36**, 217 (1937).

[248] H. G. Wood, A. A Anderson and C. H. Werkman, *J. Bact*, **36**, 201 (1938).

[249] M. Doudoroff, *Enzymologia*, **5**, 239 (1938).

[250] W. Trager and V Subbarow, *Biol. Bull.*, **75**, 75 (1938)

[251] W. Stepp, J. Kuhnau and H. Schroeder, *Die Vitamine*, Stuttgart, 1937, p. 75.

[252] W. H. Sebrell, R. E. Butler, J. G. Wooley and H. Isbell, *U. S. Pub. Health Service Pub. Health Repts.*, **56**, 510 (1941).

Chickens need 230–245 γ per 100 g. of diet to maintain normal hatchability.[255, 256] Baby chicks need increased amounts.[256] Turkeys require about 25% more riboflavin than chickens.

Some animals apparently need no riboflavin or only a very small amount in their food. Microorganisms which synthesize this vitamin live in the rumen of sheep[257] and of cattle[258, 259] and make the vitamin available to the animal.

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[255] H. J. Davis, L. C. Norris and G. F. Heuser, *Poultry Sci.*, **17**, 81, 87 (1938).

[256] G. F. Heuser, H. S. Wilgus and L. C. Norris, *Ibid.*, **17**, 105 (1938).

[257] L. W. McElroy and H. Goss, *J. Biol. Chem.*, **130**, 437 (1939).

[258] L. W. McElroy and H. Goss, *Ibid.*, **133**, LXV (1940).

[259] M. I. Wegner, A. N. Booth, C. A. Elvehjem and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.*, **45**, 769 (1940).

VITAMIN B_6—
PYRIDOXIN

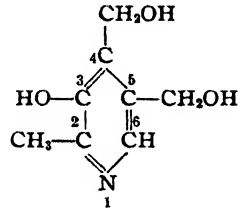
VITAMIN B_6—PYRIDOXIN

1. Nomenclature and Survey

Names:

Pyridoxin: Term suggested by György[1] and generally adopted in the United States.
Adermin: European name.
Vitamin B_6 of György.
Anti-acrodynia rat factor.
Rat antidermatitis factor.
Yeast eluate factor.[2, 3]
Factor 1 of Lepkovsky.[4]
Factor Y of Chick.[5]
Vitamin H of Richardson and Hogan[6] and of Booher.[7, 7a]
Complimentary factor.[8]

Chemical formula:



Chemical name:

3-Hydroxy-4,5-di(hydroxy-methyl)-2-methyl-pyridine.

Empirical formula:

$C_8H_{11}O_3N$.

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[1] P. György and R. E. Eckhardt, *Nature*, **144**, 512 (1939).

[2] C. E. Edgar and T. F. Macrae, *Biochem. J.*, **31**, 886 (1937).

[3] M. M. El-Sadr, T. F. Macrae and C. E. Work, *Ibid.*, **33**, 611 (1939).

[4] S. Lepkovsky, T. H. Jukes and M. E. Krause, *J. Biol. Chem.*, **115**, 557 (1936).

[5] H. Chick, A. M. Copping and M. H. Roscoe, *Biochem. J.*, **24**, 1748 (1930).

[6] L. R. Richardson and A. G. Hogan, *Missouri Agr. Exptl. Station Research Bull.* No. 241 (1936); *Science*, **83**, 17 (1936).

[7] L. E. Booher, *J. Biol. Chem.*, **119**, 223 (1937).

[7a] The term vitamin H was used in earlier days of vitamin research to designate vitamin B_6.[9] The same letter H has also been used for a trout growth factor.[10] In present-day literature, the term "vitamin H" is reserved for biotin, the curative factor for egg-white injury (see page 469).

[8] P. György, R. Kuhn and T. Wagner-Jauregg, *Naturwissenschaften*, **21**, 561 (1933); *Klin. Wochschr.*, **12**, 1241 (1933).

[9] A. G. Hogan in *The Vitamins*, Am. Med. Assoc., Chicago, 1939, p. 273.

[10] C. M. McCay, F. C. Bing and W. E. Dilley, *Science*, **67**, 249 (1928).

2. Chronology

1926 GOLDBERGER and LILLIE[11] reported the occurrence of a characteristic dermatitis, called acrodynia, on rats fed a diet deficient in vitamin B_2.

1932 OHDAKE[12] in Japan isolated a compound of the formula $C_8H_{11}O_3N \cdot HCl$ from rice polishings, but failed to recognize its vitamin character.

1934 GYÖRGY[13] established the difference of the "rat pellagra preventive factor" from vitamin B_2 (and vitamin B_4) and called the new vitamin "B_6."

1938 The isolation of the pure crystalline vitamin B_6 was announced independently by five different groups, namely, by LEPKOVSKY,[14] KERESZTESY and STEVENS,[15] GYÖRGY,[16] KUHN and WENDT,[17] and ITIBA and MITI.[18]

1939 The chemical structure was elucidated and vitamin B_6 was synthesized independently by two groups of workers—by KUHN, WESTPHAL, WENDT and WESTPHAL in Germany and by KERESZTESY, STEVENS, HARRIS, STILLER and FOLKERS in the U. S. A.

3. Occurrence

Vitamin B_6 appears to be very widely distributed over the entire animal and plant kingdom. Reliable systematic studies as to the relative quantities in various foodstuffs are scarce. Yeast and rice polishings are especially rich in vitamin B_6. Seeds and cereals, for example, wheat and maize, are good sources,[19] especially the germs and the integuments.[20] Molasses,[21] fish and fish livers[22] and mammalian livers contain moderate amounts,[21, 23] and milk, egg yolks, lettuce, spinach, etc., contain small amounts of vitamin B_6.

Vitamin B_6 occurs in animal and in plant tissues, for example, in yeast[24] and in fish muscle,[25] only to a small extent in the free form. The majority (60-80%) is chemically bound to protein[24] and to starch.[26]

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[11] J. Goldberger and R. D. Lillie, *U. S. Pub. Health Service Pub. Health Rept.*, **41**, 1025 (1926).
[12] S. Ohdake, *Bull. Agr. Chem. Soc. Japan*, **8**, 111 (1932). P. W. Wiardi, *Nature*, **142**, 1158 (1938).
[13] P. György, *Nature*, **133**, 498 (1934); *Biochem. J.*, **29**, 741, 760, 767 (1935).
[14] S. Lepkovsky, *Science*, **87**, 169 (1938).
[15] J. C. Keresztesy and J. R. Stevens, *Proc. Soc. Expll. Biol. Med.*, **38**, 64 (1938).
[16] P. György, *J. Am. Chem. Soc.*, **60**, 983 (1938).
[17] R. Kuhn and G. Wendt, *Ber.*, **71**, 780, 1118 (1938).
[18] A. Itiba and K. Miti, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **34**, 623 (1938).
[19] H. A. Schneider, J. K. Ascham, B. R. Platz and H. Steenbock, *J. Nutrition*, **18**, 99 (1939).
[20] A. M. Copping, *Biochem. J.*, **30**, 849 (1936).
[21] A. van Schoor, *Merck's Jahresberichte*, **52**, 7 (1938).
[22] G. Lunde and H. Kringstad, *Biochem. J.*, **32**, 708 (1938).
[23] C. E. Edgar, M. M. El-Sadr and T. F. Macrae, *Ibid.*, **32**, 2225 (1938).
[24] R. Kuhn and G. Wendt, *Ber.*, **71**, 780 (1938).
[25] T. W. Birch and P. György, *Biochem. J.*, **30**, 304 (1936).
[26] H. Chick, M. M. El-Sadr and A. N. Worden, *Biochem. J.*, **34**, 595 (1940).

4. Isolation

As has been pointed out in the previous section, vitamin B_6 occurs to a great extent bound to a protein. From this symplex, vitamin B_6 cannot be separated by dialysis. The symplex can, however, be split by heating[27, 28, 29] or by enzymatic hydrolysis.[30]

The free vitamin B_6 is extracted with water or with organic solvents such as ether, propanol or butanol.[31, 32] The latter solvents extract less by-products than does water, but continuous extraction is necessary due to the water solubility of the vitamin.

From neutral or acidified water solutions, vitamin B_6 can be adsorbed on charcoal and fuller's earth.[33] The adsorption on fuller's earth is greatly influenced by the pH of the solution. At pH 5–6 vitamin B_6 is not quantitatively adsorbed even after three consecutive adsorptions. At pH 1 factors other than B_6 are also adsorbed.[34] The elution is carried out with barium hydroxide[35] or with butyl alcohol.[36] Vitamin B_6 is quantitatively adsorbed on zeolite and can be subsequently eluted with 10% potassium chloride.[37] Inert material can be removed with acetone, ethyl alcohol, ethyl acetate, platinum chloride, etc. The vitamin is precipitated by a number of acids, such as by sulfuric acid, phosphotungstic acid, silicotungstic acid, Reinecke's acid, etc. By repeated precipitations, the pure vitamin B_6 can be obtained in the form of various salts, such as the hydrochloride. The free base is prepared therefrom by treatment with silver salts.[38]

5. Properties

Vitamin B_6, as a free base, is a colorless crystalline powder, has a slightly bitter taste and melts at 160° C.[38, 39] It is readily soluble in water, in acetone and in alcohol, and slightly soluble in ether and chloroform. Vitamin B_6 dialyzes easily. It crystallizes in the form of various salts,

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[27] R. Kuhn and G. Wendt, *Ber.*, **71**, 780 (1938).
[28] E. M. Lantz, *New Mexico Agr. Exptl. Station Bull.*, No. **268**
[29] H. Chick, M. M. El-Sadr and A. N. Worden, *Biochem. J.*, **34**, 595 (1940)
[30] T. W. Birch and P. György, *Ibid.*, **30**, 304 (1936).
[31] J. V. Scudi, H. F. Koones and J. C. Keresztesy, *Proc. Am. Physiol. Soc.*, **1940**, 163; *Proc. Soc. Exptl. Biol. Med.*, **43**, 118 (1940).
[32] R. D. Greene, *J. Biol. Chem.*, **130**, 513 (1939).
[33] T. W. Birch and P. György, *Biochem. J.*, **30**, 304 (1936).
[34] G. Lunde and H. Kringstad, *J. Nutrition*, **19**, 321 (1940).
[35] N. Halliday and H. M. Evans, *J. Biol. Chem.*, **118**, 255 (1937).
[36] G. A. Emerson, A. Mohammad, O. H. Emerson and H. M. Evans, *Ibid.*, **124**, 377 (1938).
[37] J. V. Scudi, H. F. Koones and J. C. Keresztesy, *Proc. Am. Physiol. Soc.*, **1940**, 163; *Proc. Soc. Exptl. Biol. Med.*, **43**, 118 (1940).
[38] A. Itiba and K. Miti, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **34**, 1014 (1938).
[39] J. C. Keresztesy and J. P. Stevens, *J. Am. Chem. Soc.*, **60**, 1267 (1938).

for example, as hydrochloride, m. p. 204–206° C.[39] (with decomposition), and as picrate. The hydrochloride is soluble in water (1 g. in 4.5 cc. water) and in alcohol (1 g. in 90 cc. alcohol) and somewhat soluble in acetone. The aqueous solution has a pH of about 3.2. Both the free vitamin B_6 and its hydrochloride sublime readily.[39] The hydrochloride is the form in which this vitamin is marketed. It is a white, odorless powder with salty taste. It is stable to heat, concentrated hydrochloric acid and alkali, but is destroyed by light.

Vitamin B_6 is optically inactive. It exhibits a typical ultraviolet absorption spectrum which changes markedly with a change of hydrogen ion concentration. The spectra of the vitamin in aqueous solution between pH 4 and 6.75 are shown in Fig. 11.

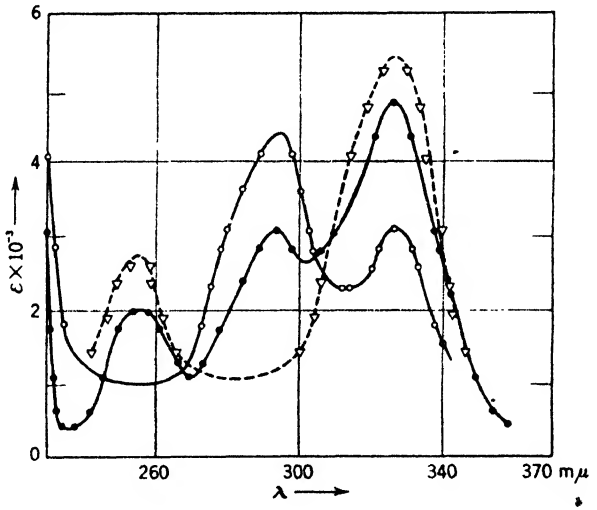


Fig. 11.- Absorption spectra of vitamin B_6 at: O, pH 4; ●, pH 5.1; ▽, pH 6.75. (E. T. Stiller, J. C. Keresztesy and J. R. Stevens.)

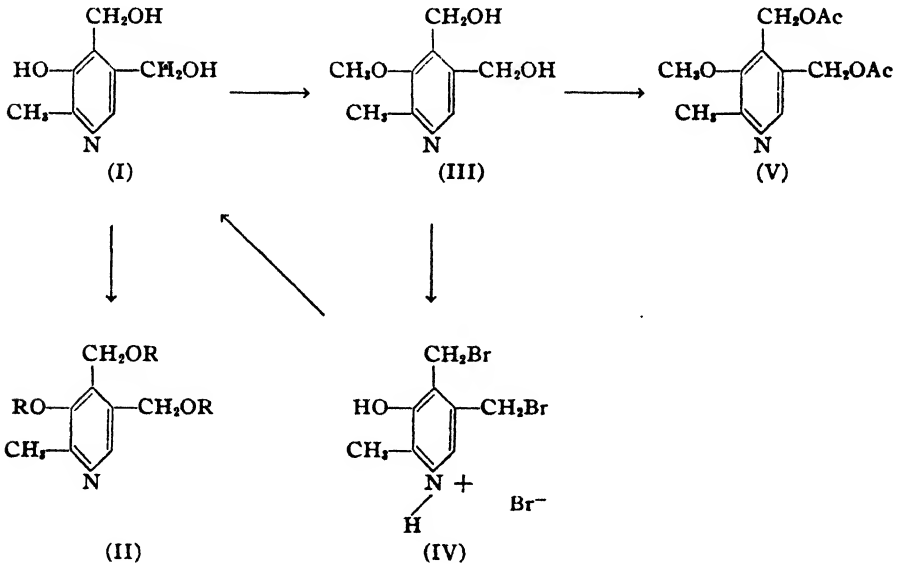
6. Constitution

Vitamin B_6 (I) has the empirical formula $C_8H_{11}O_3N$ and forms salts easily with acids, such as hydrochloric acid, picric acid, etc. The hydrochloride yields an orange-red color with ferric chloride and couples with diazonium salts, for example, with diazotized sulfanilic acid, which properties suggest the presence of a phenolic or enolic hydroxyl group.[40]

[40] R. Kuhn and G. Wendt, *Ber.*, 71, 1118 (1938).

All three oxygens of the molecule are present in the form of hydroxyl groups. A triacetate, which can be distilled,[41, 42] and a tribenzoate[43] (II) can be prepared. An estimation of active hydrogen atoms showed the presence of three in the molecule.[43]

Upon reaction with diazomethane, a compound, $C_9H_{13}O_3N$, is obtained, which does not give a color reaction with ferric chloride and which does not couple with diazotized sulfanilic acid. Treatment of this compound with hydriodic acid yields methyl-iodide. Quantitative estimation of these properties led to the conclusion that a mono-methyl-ether of vitamin B_6 (III) is produced by diazomethane.[44] Vitamin B_6-methyl-ether is split upon reaction with hydrobromic acid to a hydrobromide of a dibromide, $C_8H_{10}ONBr_3$ (IV), which yields vitamin B_6 by treatment with silver acetate.[45]



Besides the methyl-ether, diazomethane produces by reaction with vitamin B_6 the *N*-methyl-vitamin B_6,[46] which yields a color reaction with ferric chloride.

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[41] R. Kuhn and G. Wendt, *Ber.*, **71**, 780 (1938).
[42] A. Itiba and K. Miti, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **35**, 73 (1938); **36**, 1 (1939).
[43] E. T. Stiller, J. C. Keresztesy and J. R. Stevens, *J. Am. Chem. Soc.*, **61**, 1237 (1939).
[44] R. Kuhn and G. Wendt, *Ber.*, **71**, 1534 (1938).
[45] R. Kuhn and G. Wendt, *Ibid.*, **72**, 311 (1939).
[46] A. Itiba and K. Miti, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **35**, 73 (1938); **36**, 1 (1939).

The methyl-ether of vitamin B_6 is converted by the action of acetic anhydride in pyridine into a diacetyl-methyl-ether (V). Thus it is concluded that of the three hydroxyl groups one is phenolic (or enolic) and the other two are aliphatic hydroxyl groups. All active hydrogen atoms are accounted for and no further active hydrogen atoms could be detected in the diacetyl-methyl-ether. Therefore, it must be concluded that the nitrogen is present in a ring.[47]

The position of the phenolic hydroxyl group was determined, first, by application of the Folin-Denis phenol reagent[48] which produced a color with vitamin B_6 and with β-hydroxy-pyridine, but not with α- and γ-hydroxy-pyridine,[49] and second, by investigation of the ultraviolet absorption spectrum which proved to be similar to that of β-hydroxy-pyridine.[49, 50] Upon application of the color test with 2,6-dichloro-quinone-chlorimide, vitamin B_6 gives a blue color,[50] which effect proves, according to Gibbs,[51] that the *p*-position to the hydroxyl group is not substituted.

Vitamin B_6-methyl-ether does not react with lead tetra-acetate, which indicates that the two aliphatic hydroxyl groups are not in α,β-position.[49] Oxidation in neutral aqueous solution with potassium permanganate in an amount corresponding to two atoms of oxygen converts vitamin B_6-methyl-ether (III) into a lactone (VI), which indicates that the two aliphatic hydroxyl groups are either in 1,4- or in 1,5-position to each other.[49, 52] Upon further oxidation of vitamin B_6-methyl-ether with barium permanganate, four atoms of oxygen are taken up. The reaction product is a dicarboxylic acid (VII)[52, 53, 54, 55] which contains all the carbon atoms of the vitamin-methyl-ether. By treatment of this dicarboxylic acid with acetic anhydride, the dicarboxylic acid is dehydrated yielding the anhydride (VIII),[54] indicating that the two carboxyl groups are vicinal. Indication of the same fact is given by the fusion of the dicarboxylic acid with resorcinol[55] which yielded a phthalein having a greenish yellow fluorescence. Decarboxylation of the dibasic acid, by heating the disodium salt with calcium hydroxide, yielded a hydroxy-picoline (XI).[55]

Vitamin B_6-methyl-ether on oxidation with potassium permanganate in alkaline solution takes up seven atoms of oxygen and forms a tricarboxylic

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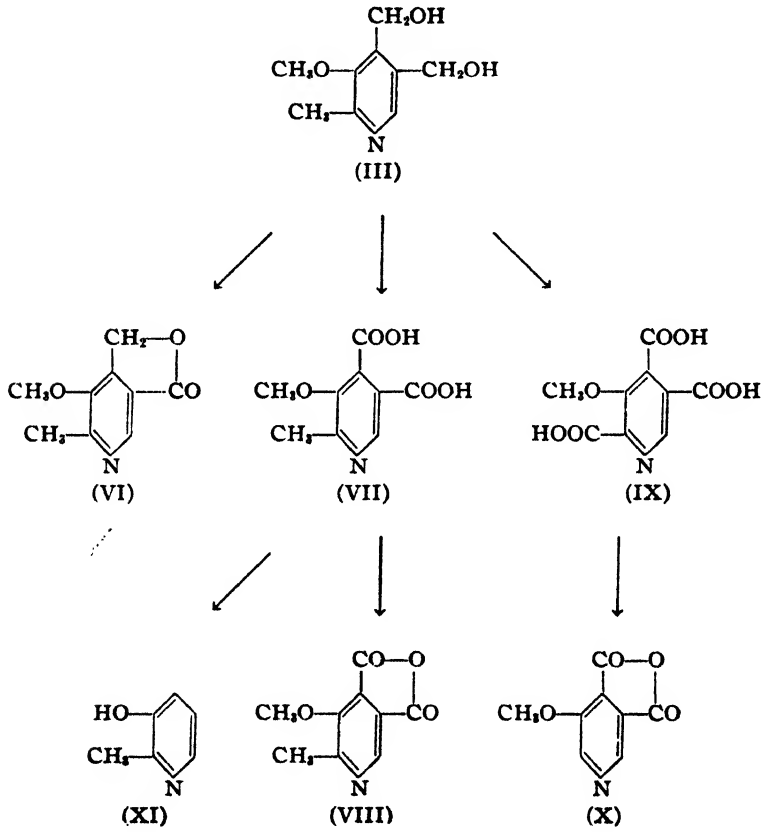
[47] R. Kuhn and G. Wendt, *Ber.*, **71**, 1534 (1938).
[48] O. Folin and W. Denis, *J. Biol. Chem.*, **12**, 239 (1912); **22**, 305 (1915).
[49] R. Kuhn and G. Wendt, *Ber.*, **72**, 305 (1939).
[50] E. T. Stiller, J. C. Keresztesy and J. R. Stevens, *J. Am. Chem. Soc.*, **61**, 1237 (1939).
[51] H. D. Gibbs, *J. Biol. Chem.*, **72**, 649 (1927). E. J. Theriault, *Ind. Eng. Chem.*, **21**, 343 (1929).
[52] A. Itiba and K. Miti, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **35**, 73 (1938); **36**, 1 (1939).
[53] R. Kuhn, H. Andersag, K. Westphal and G. Wendt, *Ber.*, **72**, 309 (1939).
[54] R. Kuhn, G. Wendt and K. Westphal, *Ibid.*, **72**, 310 (1939).
[55] E. T. Stiller, J. C. Keresztesy and J. R. Stevens, *J. Am. Chem. Soc.*, **61**, 1237 (1939).

acid (IX) without the loss of carbon atoms.[56] This tricarboxylic acid yields an anhydride of a dicarboxylic acid (X) while simultaneously losing one mol of carbon dioxide.[56] The tricarboxylic acid yields with ferrous sulfate a reddish color that is characteristic for pyridine-α-carboxylic acids.



This color reaction is not given by the dicarboxylic acids (VII) and (X). This proves that the carboxyl group lost by anhydration of the tricarboxylic acid was in α-position to the ring-nitrogen[56] and that this carboxyl group originated from a methyl group.[57] The existence of the methyl group has also been shown by oxidation of the vitamin chlorohydrate with chromic acid in sulfuric acid whereby acetic acid is obtained.

[56] R. Kuhn and G. Wendt, *Ber.*, **72**, 305 (1939).
[57] R. Kuhn, H. Andersag, K. Westphal and G. Wendt, *Ibid.*, **72**, 309 (1939).

7. Synthesis

Two different methods have been described for the synthesis of vitamin B_6, one of which is a complete synthesis building up the pyridine nucleus from small aliphatic molecules. The other synthesis is a partial degradation of a higher molecular compound to the pyridine derivative of the constitution of vitamin B_6.

(*a*) *The Complete Synthesis of Harris and Folkers*[58, 59] *and of Morii and Makino*[60]

By a series of seven reactions vitamin B_6 has been synthesized as follows:

Step 1: Cyano-acetamide (I) is condensed with ethoxy-acetyl-acetone (II) in the presence of piperidine to yield 3-cyano-4-ethoxy-methyl-6-methyl-2-pyridone (III).

Step 2: By nitration of the reaction product of step *1*, 3-cyano-4-ethoxy-methyl-5-nitro-6-methyl-2-pyridone (IV) is obtained.

Step 3: Chlorination converts the last-mentioned compound (IV) into 2-methyl-3-nitro-4-ethoxy-methyl-5-cyano-6-chloro-pyridine (V).

Step 4: Partial catalytic hydrogenation of (V) yields 2-methyl-3-amino-4-ethoxy-methyl-5-cyano-6-chloro-pyridine (VI).

By a less attractive series of reactions the amino-chloro-compound (VI) can be obtained from the nitro-pyridone (IV) by reduction of the nitro-group to the amino-pyridone (X) followed by chlorination.

Step 5: The cyano-chloro-pyridine (VI) is catalytically hydrogenated to 2-methyl-3-amino-4-ethoxy-methyl-5-amino-methyl-pyridine (VII).

A modification of this step consists in first acetylating the 3-amino-group of (VI) to give (XI) followed by hydrogenation to remove the chlorine and to convert the cyano-group in the amino-methyl group (XII). Finally the acetyl groups of the 3-amino-group are split off by hydrolysis.

Step 6: The 4-ethoxy-group is now saponified with dilute hydrochloric acid to yield the dihydrochloride of 2-methyl-3-amino-4-hydroxy-methyl-5-amino-methyl-pyridine (VIII).

If this reaction is carried out with hydrobromic acid, the dihydrobromide of a 4-bromo-methyl compound (XIII) is obtained, which must be saponified to give (VIII).

Step 7: The last step of this synthesis consists in diazotization of the diamine (VIII) to yield vitamin B_6 (IX).

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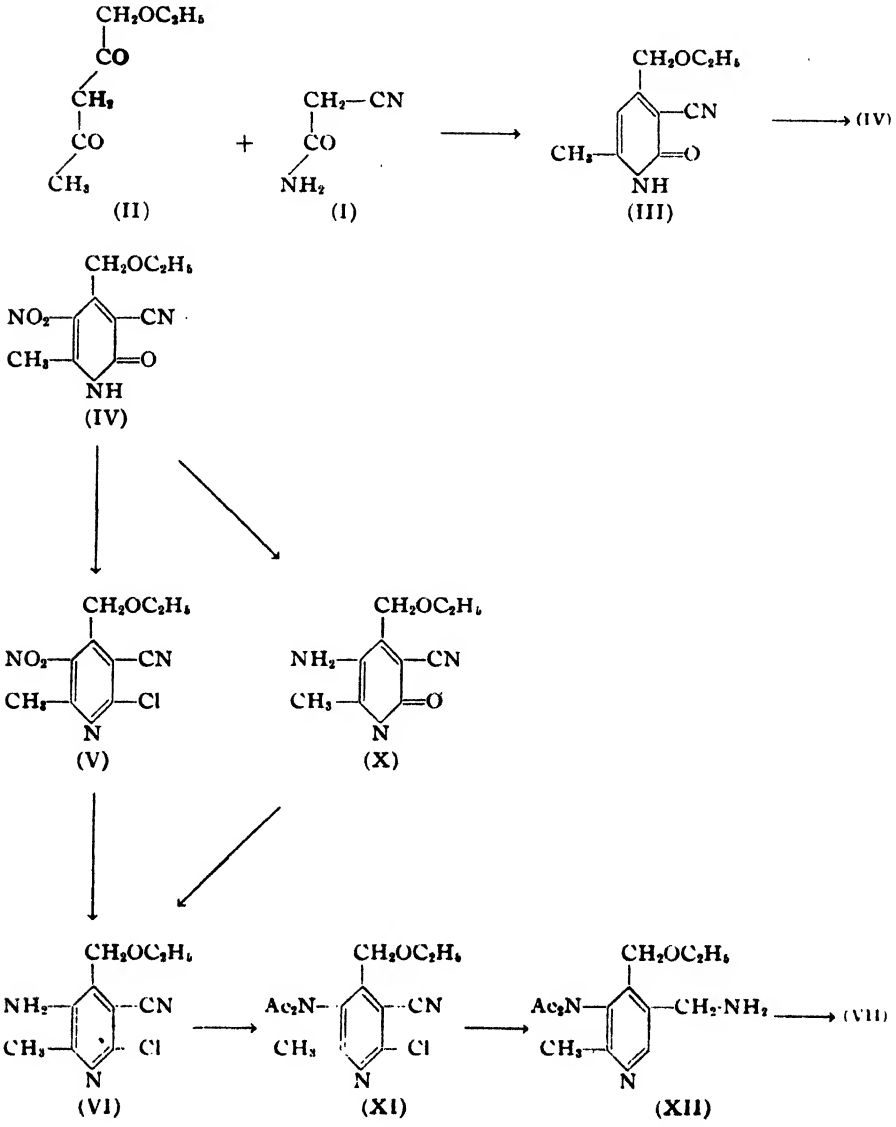
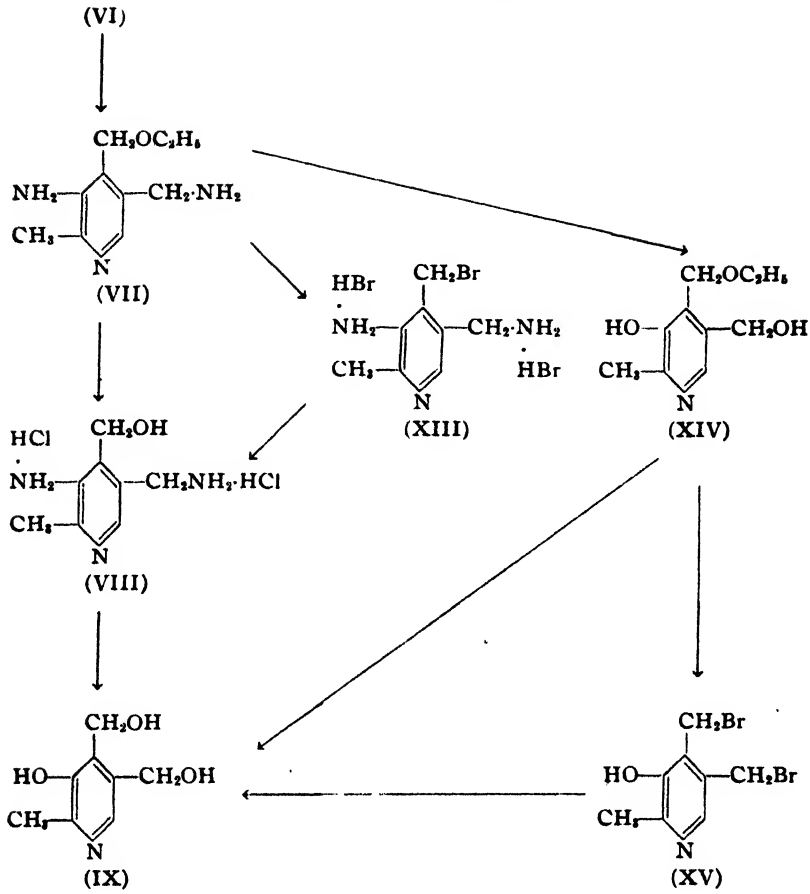
[58] S. A. Harris and K. Folkers, *J. Am Chem. Soc.*, **61**, 1245 (1939). S. A. Harris, E. T. Stiller and K Folkers, *Ibid.*, **61**, 1242 (1939).

[59] S. A. Harris and K. Folkers, *Ibid.*, **61**, 3307 (1939).

[60] S. Morii and K. Makino, *Enzymologia*, **7**, 385 (1939).

Vitamin B_6 has also been obtained by a slightly more difficult route, starting with the ethoxy-diamine (VII). By diazotization an ethoxy-dihydroxy-compound (XIV) is obtained, which upon hydrolysis with dilute hydrochloric acid gives vitamin B_6. When instead of the hydrochloric





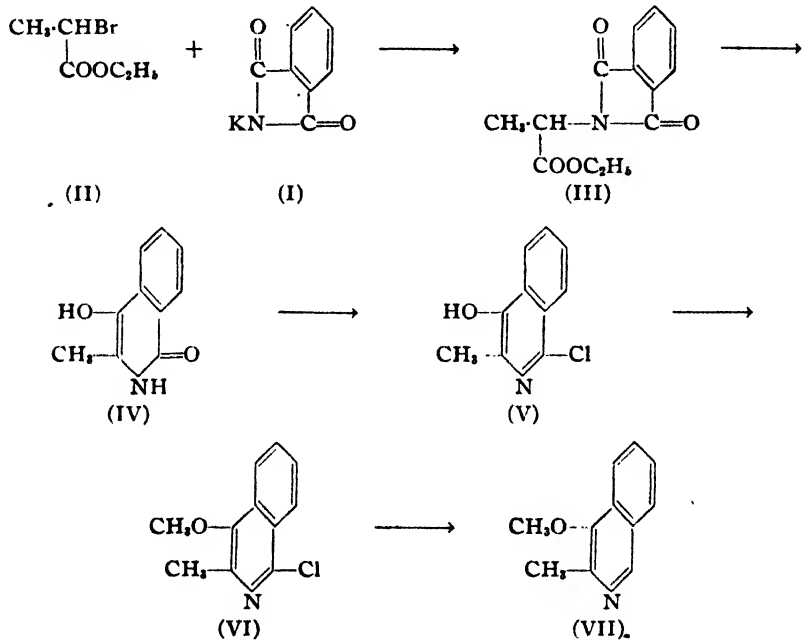
acid, hydrobromic acid is used, the dibromide (XV) is obtained, which requires another step to yield finally vitamin B_6.

(*b*) *Synthesis by Degradation of Isoquinoline*

The principle of this synthesis has independently been used by two groups of workers, by Kuhn and co-workers[61] in Germany and by Itiba and Miti[62] in Japan, and consists of the oxidative degradation of 2-methyl-3-methoxy-isoquinoline to 2-methyl-3-methoxy-pyridine-4,5-dicarboxylic acid. The isoquinoline derivative is prepared[63] by condensation of potas-

[61] R. Kuhn, K. Westphal, G. Wendt and O. Westphal, *Naturwissenschaften*, **27**, 469 (1939).
[62] A. Itiba and K. Miti, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **36**, 173 (1939).
[63] S. Gabriel and J. Colman, *Ber.*, **33**, 988 (1900).

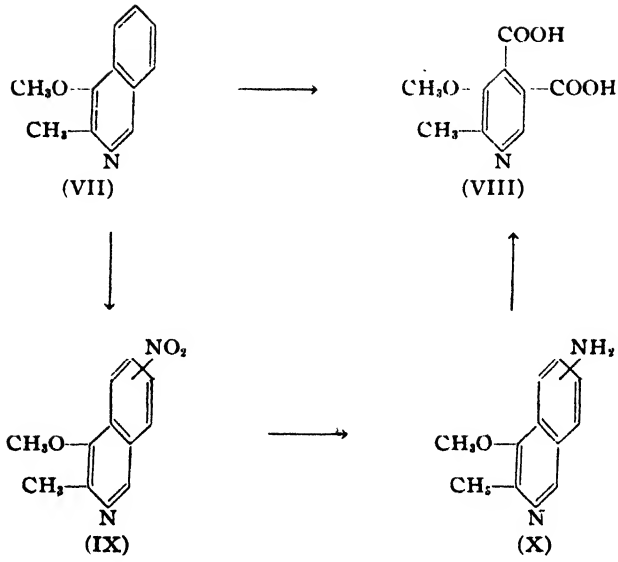
sium phthalimide (I) with α-bromo-propionic acid ester (II) to yield α-phthalimide-propionic acid ester (III), which upon saponification gives 2-methyl-3-hydroxy-isocarbostyril (IV). Chlorination yields the chloro-compound (V) which is converted into the *O*-methyl-ether (VI) by methylation with methyl-iodide. The latter compound upon treatment with tin and hydrochloric acid yields the 2-methyl-3-methoxy-isoquinoline (VII).



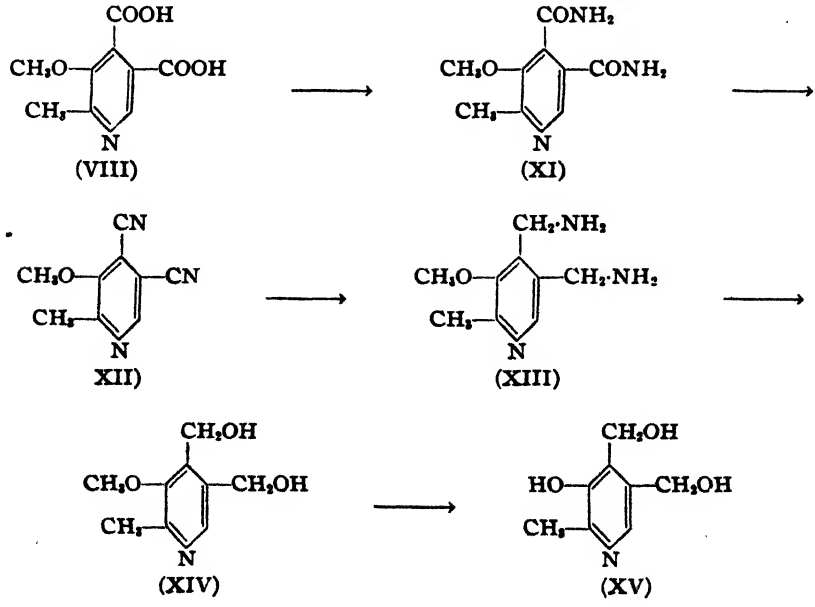
The 2-methyl-3-methoxy-isoquinoline (VII) is converted into 2-methyl-3-methoxy-pyridine-4,5-dicarboxylic acid (VIII) either by direct oxidation in alkali solution with permanganate[64] or by nitration to a Bz-nitro-compound (IX) followed by reduction to a Bz-amino-compound (X) which is then oxidized with permanganate to yield the dicarboxylic acid (VIII).[65]

The 2-methyl-3-methoxy-pyridine-4,5-dicarboxylic acid (VIII) is converted through its diamide (XI) into 2-methyl-3-methoxy-4,5-dicyano-pyridine (XII), which by catalytic hydrogenation yields the 2-methyl-3-methoxy-4,5-diamino-methyl-pyridine (XIII). The latter upon reaction with nitrite gives the vitamin B_6 *O*-methyl-ether (XIV). The methyl-

[64] A. Itiba and K. Miti, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **36**, 173 (1939).
[65] R. Kuhn, K. Westphal, G. Wendt and O. Westphal, *Naturwissenschaften*, **27**, 469 (1939).



ether can be converted into the vitamin B_6 (XV) according to one of the previously mentioned methods.



8. Industrial Methods of Preparation

Pure vitamin B_6, obtained by extraction of animal or plant material, has never appeared on the market. It has, however, been commercially available in the form of yeast or liver concentrates in mixture with several other members of the vitamin B complex. In the future, extracts of this type will probably be prepared primarily as a source of the less well-known members of the vitamin B-group.

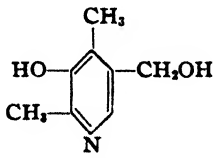
Vitamin B_6 is commercially available in the pure crystalline form, synthesized according to both methods outlined in the previous section dealing with the synthesis of vitamin B_6.

9. Biogenesis

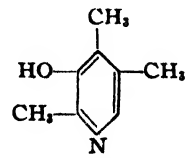
The biogenesis of vitamin B_6 is unknown. Theories pertaining to the biogenesis of this vitamin have not been suggested.

10. Specificity

Vitamin B_6 apparently owes its physiological action to the molecule as an entity. Compounds of very similar structure and simple derivatives of vitamin B_6 are inactive. The di- and triacetate are fully active,[66, 67] probably because the organism is able to hydrolyze these esters. The benzoate, however, is inactive. The same is true for the methyl-ether,[67, 68] which, however, shows some activity in a concentration that corresponds to about 500 times the concentration of the free vitamin.[69] 4-Desoxy-vitamin B_6 (2,4-dimethyl-3-hydroxy-5-hydroxy-methyl-pyridine (I)) appears to be active in 50 times the concentration of vitamin B_6[69, 70] and 4,5-bis-desoxy-vitamin B_6 (2,4,5-trimethyl-3-hydroxy-pyridine (II)) appears to be inactive.[69] A great number of other pyridine derivatives have been tested,[69] but no active compound has been found.



(I)
4-Desoxy-vitamin B_6



(II)
4,5-Bis-desoxy-vitamin B_6

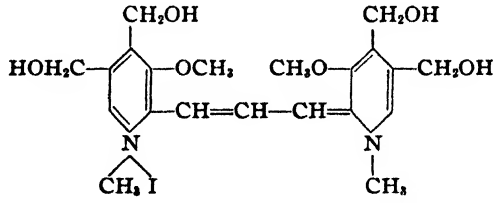
[66] R. Kuhn and G. Wendt, *Ber.*, **71**, 1118 (1938).
[67] K. Unna, *Proc. Soc. Expll. Biol. Med.*, **43**, 122 (1940).
[68] R. Kuhn and G. Wendt, *Ber.*, **71**, 1534 (1938).
[69] E. F. Möller, *Z. physiol. Chem.*, **260**, 246 (1939).
[70] E. F. Möller, O. Fima, F. Jung and T. Moll, *Naturwissenschaften*, **27**, 228 (1939).

11. Determination

(a) *Chemical Methods*

Phenol Test According to Folin and Denis.[71, 72] This test consists in a blue color given by a phenolic hydroxyl group in the presence of a mixture of sodium tungstate, phosphotungstic acid, phosphoric acid and lithium hydroxide. By the use of this method 0.02–0.08 mg. vitamin B_6 can be determined. The color is measured in a photometer. This method is not specific for vitamin B_6.

Cyanine Dye Test.[72] This test can be applied only to the methyl-ether of quaternary vitamin B_6 salt. Therefore, vitamin B_6 must, first, be converted into the methyl-ether with diazo-methane, and, second, into the iodo-methyl-pyridinium- or a similar pyridinium-compound. These two reactions can be carried out in a yield of 30–40%. The methyl-ether-pyridinium salt, upon heat treatment with chloroform and potassium hydroxide, yields a violet dye of the structure of a carbo-pyridine-cyanine dye (absorption maxima at 599 and 555 mμ in alcohol-chloroform). This test is believed to be quite specific to the vitamin since no other α-picoline derivatives are known to occur in plants or animals. By the use of this method 0.8 mg. of pyridoxin can be determined.



Gibb's Phenol Indophenol Test. This test has been adapted to the quantitative colorimetric determination of vitamin B_6.[73, 74] A blue color develops when a vitamin B_6 solution is mixed with a veronal buffer (pH 7.6) and a butanol solution of 2,6-dichloroquinone chloroimide. The color has an absorption maximum at 660 mμ and is measured in a colorimeter or spectrophotometer.

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[71] O. Folin and W. Denis, *J. Biol. Chem.*, **12**, 239 (1912); **22**, 305 (1915).

[72] R. Kuhn and I. Löw, *Ber.*, **72**, 1453 (1939).

[73] J. V. Scudi, H. F. Koones and J. C. Keresztesy, *Proc. Am. Physiol. Soc.*, **1940**, 163; *Proc. Soc. Exptl. Biol. Med.*, **43**, 118 (1940).

[74] J. V. Scudi, K. Unna and W. Antopol, *J. Biol. Chem.*, **135**, 371 (1940).

Ferric Chloride Method.[75, 76] This method has proved to be of value in the estimation of vitamin B_6 in rich sources. The red-brown color developed by the unknown material is compared with those of standards.

(*b*) *Biological Methods*

Yeast Growth Test. Since it has been observed that vitamin B_6 stimulates the growth of yeast, a method of determining this vitamin by the rate of growth of yeast has been proposed.[77]

Bacteria Test. *Streptobacterium plantarum* (*Bacterium Acetyl-Cholini Keil* 10 S) responds in growth and production of acid to the amount of vitamin B_6 present. The increase in growth is measured with a nephelometer.[78, 79]

Rat Growth Test. In this proposed test, the growth rate increase of rats, following the administration of vitamin B_6, is determined.[80, 81, 82, 83] Since the decline of the growth rate is not specific for a vitamin B_6 deficiency, this test often gives unsatisfactory results.[85]

Acrodynia Rat Test. Vitamin B_6 is assayed comparatively on the basis of the percentage incidence of the typical acrodynia type dermatitis in rats[84, 85, 86, 87, 88, 89, 90, 91, 92] in either prophylactic or curative assay procedures.

12. Standard

One rat unit vitamin $B_6 = 10\ \gamma$, is defined as the amount necessary per day to cure or prevent the typical symptoms of avitaminosis (György).[93]

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[75] J. C. Keresztesy and J. P. Stevens, *J. Am. Chem. Soc.*, **60**, 1267 (1938).
[76] R. D. Greene, *J. Biol. Chem.*, **130**, 513 (1939).
[77] A. S. Schultz, L. Atkin and C. N. Frey, *J. Am. Chem. Soc.*, **61**, 1931 (1939).
[78] E. F. Möller, *Z. physiol. Chem.*, **254**, 285 (1938).
[79] E. F. Möller, O. Fima, F. Jung and T. Moll, *Naturwissenschaften*, **27**, 228 (1939).
[80] M. K. Dimick and C. B. Schreffler, *J. Nutrition*, **17**, 23 (1939).
[81] R. Kuhn and G. Wendt, *Z. physiol. Chem.*, **256**, 127 (1938).
[82] C. E. Edgar, M. M. El-Sadr and T. F. Macrae, *Biochem. J.*, **32**, 2207 (1938).
[83] T. W. Conger and C. A. Elvehjem, *J. Biol. Chem.*, **138**, 555 (1941).
[84] P. György and R. E. Eckardt, *Biochem. J.*, **34**, 1143 (1940).
[85] R. C. Bender and G. C. Supplee, *J. Am. Chem. Soc.*, **59**, 1178 (1937).
[86] P. György, *Biochem. J.*, **29**, 760 (1935).
[87] G. Lunde and H. Kringstad, *Ibid.*, **32**, 708 (1938).
[88] H. E. C. Wilson and G. K. Roy, *Indian J. Med. Research*, **25**, 879 (1938).
[89] H. A. Schneider, J. K. Ascham, B. R. Platz and H. Steenbock, *J. Nutrition*, **18**, 99 (1939).
[90] N. Halliday and H. M. Evans, *J. Biol. Chem.*, **118**, 255 (1937).
[91] M. K. Dimick and C. B. Schreffler, *J. Nutrition*, **17**, 23 (1937).
[92] E. J. Reedman, W. L. Sampson and K. Unna, *Proc. Soc. Exptl. Biol. Med.*, **43**, 112 (1940).
[93] P. György, *Ibid.*, **35**, 204 (1936).

13. Physiology of Plants and Microorganisms

Although it can be assumed that vitamin B_6 is necessary for plants and microorganisms, only few actual facts are known. It has been demonstrated experimentally that vitamin B_6 is a nutrilite for yeast[94, 95] and *staphylococcus albus*[96] and that it is necessary for lactic acid bacteria[97] and for some hemolytic streptococci.[98] Some other bacteria are able to synthesize vitamin B_6, for example, those in the rumen of sheep.[99] Only a few observations concerning the vitamin B_6 action in higher plants are available. Vitamin B_6 acts as a growth stimulant[100] on isolated tomato roots and cosmos.[101] The isolated pea root does not require an external supply of vitamin B_6 and it must be presumed that the root is able to build up the vitamin from compounds present in the culture medium.[101] Analytical determination of the vitamin B_6 content of wheat and maize plants seems to indicate a greater concentration of this vitamin in the germs and the integuments than in the endosperm.[102]

14. Animal Physiology

Free vitamin B_6 is rapidly absorbed in the intestines.[103] Vitamin B_6 bound to protein material is not absorbed. Plant material, for example, seeds and vegetables, should be cooked to insure full utilization of the vitamin B_6 present[104] (as shown in experiments with rats).

Investigations on the behavior of vitamin B_6 in the human organism are scarce. Vitamin B_6 has not been detected in blood[105] (a reinvestigation seems advisable) but is present in urine,[105, 106] which indicates that this compound is of physiological importance for man. The vitamin is also secreted in milk. When excess amounts of vitamin B_6 are administered, they are largely destroyed in the organism. Storage of this vitamin in the organism has not been observed.

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[94] A. S. Schultz, L. Atkin and C. N. Frey, *J. Am. Chem. Soc.*, **61**, 1931 (1939).
[95] R. E. Eakin and R. J. Williams, *Ibid.*, **61**, 1932 (1939).
[96] S. P. Vilter and T. D. Spies, *Science*, **91**, 200 (1940).
[97] E. F. Möller, *Z. physiol. Chem.*, **254**, 285 (1938).
[98] B. L. Hutchings and D. W. Woolley, *Science*, **90**, 41 (1939).
[99] L. W. McElroy and H. Goss, *J. Biol. Chem.*, **130**, 437 (1939).
[100] W. J. Robbins and M. B. Schmidt, *Proc. Natl. Acad. Sci. U. S.*, **25**, 1 (1939).
[101] J. Bonner, *Am. Chem. Soc., Div. Agr. Food Chem., Meeting*, **Sept., 1939**, Abst., p. 13.
[102] A. M. Copping, *Biochem. J.*, **30**, 849 (1936).
[103] J. V. Scudi, K. Unna and W. Antopol, *J. Biol. Chem.*, **135**, 371 (1940).
[104] E. M. Lantz, *New Mexico Agr. Expll. Station Bull.*, **No. 268.**
[105] Cited from W. Stepp, J. Kühnau and H. Schroeder, *Die Vitamine*, 2nd edition, Stuttgart, 1937, p. 86.
[106] J. V. Scudi, H. F. Koones and J. C. Kereszteny, *Proc. Am. Physiol. Soc.*, **1940**, 163; *Proc. Soc. Expll. Biol. Med.*, **43**, 118 (1940).

Little is known about the physiological action of vitamin B_6. It has been suggested that vitamin B_6 is connected with the utilization of unsaturated fatty acids.[107] It has also been observed that in vitamin B_6-avitaminotic rats, the livers are significantly heavier and contain a higher percentage of total fatty acids,[108] which increase cannot be correlated to the food intake.[109] Rats maintained on a vitamin B_6-deficient diet can, according to some investigators, be protected from the symptoms of vitamin B_6 deficiency by supplementing the diet with the essential unsaturated fatty acids.[110, 111] Others were unable to obtain this effect but called attention to the very similar type of dermatitis in both vitamin B_6 and essential fatty acid deficiency which, however, can be differentiated since edema occurs only in the case of vitamin B_6 deficiency. The crux of the problem of the relation of vitamin B_6 to the fat metabolism is that the animal organism (tested on rats) needs vitamin B_6 for the synthesis of fat from protein.[112] This vitamin is apparently concerned with the metabolism of the amino-acids, as has also been shown in a determination of the protein and carbohydrate appetite in rats by the self-selection method.[113]

The mechanism of the vitamin B_6 action is still unknown. Upon the discovery[114] that vitamin B_6 occurs in tissues partly bound to proteins, it was suspected that the principal function of this vitamin is to act as part of some enzyme system, like some of the other members of the vitamin B group. The result of an approach to solve this problem was negative: the methyl-iodo-compound of vitamin B_6 is not reduced to a dihydro-compound[115] in a manner similar to the reduction of the methyl-iodo-compound of nicotinamide (see page 238).

15. Avitaminosis and Hypovitaminosis

Vitamin B_6-avitaminosis in rats causes a specific symmetrical dermatitis, which is called "acrodynia"[116] and which affects primarily the peripheral parts of the body, such as the paws, the mouth, the tail, the ears and nose,[117]

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[107] T. W. Birch, *J. Biol. Chem.*, **124**, 775 (1938).
[108] N. Halliday, *J. Nutrition*, **16**, 285 (1938).
[109] R. W. Engel, *Proc. Am. Soc. Biol. Chem.*, 1941, XXXVII.
[110] F. W. Quackenbush and H. Steenbock, *Proc. XVI Intern. Physiol. Congr. Zurich*, 1938, 108.
[111] W. D. Salmon, *Proc. Am. Soc. Biol. Chem.*, **34**, LXXXII (1940).
[112] E. W. McHenry and G. Gavin, *J. Biol. Chem.*, **138**, 471 (1941).
[113] C. P. Richter and C. D. Hawkes, *Am. J. Physiol.*, **129**, 459 (1940).
[114] R. Kuhn and G. Wendt, *Ber.*, **71**, 780 (1938).
[115] R. Kuhn and I. Löw, *Ibid.*, **72**, 1453 (1939).
[116] T. W. Birch, P. György and L. J. Harris, *Biochem. J.*, **29**, 2830 (1935).
[117] W. W. Jefremow, *Voprosy Pitaniya*, **7**, No. 3, 43 (1938). See *Chem. Zentr.*, I, 1939, 4496. *Voprosy Pitaniya*, **6**, No. 1, 55 (1937). See *Chem. Zentr.*, I, 1938, 1152.

and which is accompanied by edema and scaliness.[118] Furthermore, rats cease growing.[119, 120] In dogs,[121] rats[122, 123, 124] and pigs[125, 126] fits of an epileptiform nature were observed besides the symptoms of subnormal growth and dermatitis. The animals become abnormally excited and any extra stimulus induces fits. The striated and cardiac muscles degenerate, and pathological changes have been noted in the nervous system, especially in the columns of the spinal cord. Chicks also need vitamin B_6,[127, 128] but no characteristic dermatitis can be observed during times of vitamin B_6 deficiency. The symptoms in chicks consist of slow growth, depressed appetite and inefficient utilization of food. In some cases spastic convulsions and death were observed.[129] In dogs, vitamin B_6 deficiency causes a microcytic, hypochromic anemia[130, 131, 132, 133] and in rats a thymus atrophy has been observed.[134] In rats kept on a vitamin B_6-deficient diet the accessory organs of reproduction are reduced and the animals show defective sexual behavior.[135]

The present-day knowledge of the action of vitamin B_6 in human beings is very limited. The necessity of this vitamin for man has not been proved, but it seems reasonable to assume that vitamin B_6 is a vitamin in human nutrition. Upon administration of vitamin B_6 to pellagrins, recovery has been observed in some cases in which the vitamins B_1, B_2 and nicotinic acid failed to remove the symptoms.[136] These symptoms include nervousness, insomnia, irritability, cramping pains in the stomach, muscular weakness and muscular rigidity.[137] Patients with pseudohypertrophic muscular dystrophy, for example, respond well to vitamin B_6 treatment.[138] Atten-

[118] W. Antopol and K. Unna, *Proc. Soc. Exptl. Biol. Med.*, **42**, 126 (1939).
[119] R. Kuhn and G. Wendt, *Z. physiol. Chem.*, **256**, 127 (1938).
[120] C. E. Edgar, M. M. El-Sadr and T. F. Macrae, *Biochem. J.* **32**. 2207 (1938).
[121] P. J. Fouts, O. M. Helmer, S. Lepkovsky and T. H. Jukes, *J. Nutrition*, **16**, 197 (1938). J. M. McKibbin, R. J. Madden, S. Black and C. A. Elvehjem, *Am. J. Physiol* **128**, 102 (1939).
[122] H. Chick, A. N. Worden and M. M. El-Sadr, *J. Soc. Chem. Ind.*, **58** 1019 (1939).
[123] H. Chick, M. M. El-Sadr and A. N. Worden, *Biochem. J.*, **34**, 595 (1940).
[124] J. J. Oleson, H. R. Bird, C. A. Elvehjem and E. B. Hart, *J. Biol. Chem.*, **127**, 23 (1939).
[125] H. Chick, T. F. Macrae, A. J. P. Martin and C. J. Martin, *Biochem. J.*, **32**, 2207 (1938).
[126] M. M. Wintrobe, *Am. J. Physiol.*, **126**, 375 (1939).
[127] D. M. Hegsted, J. J. Oleson, C. A. Elvehjem and E. B. Hart, *J. Biol. Chem.*, **130**, 423 (1939).
[128] C. W. Carter and J. R. O'Brien, *Proc. 7th World's Poultry Congress and Exposition*, **1939**, 126.
[129] T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.*, **42**, 180 (1939).
[130] P. J. Fouts, O. M. Helmer, S. Lepkovsky and T. H. Jukes, *J. Nutrition*, **16**, 197 (1938). P. J. Fouts, O. M. Helmer and S. Lepkovsky, *Proc. Soc. Exptl. Biol. Med.*, **40**, 4 (1939).
[131] H. J. Borson and S. R. Mettier, *Ibid.*, **43**, 429 (1940).
[132] G. Lunde and H. Kringstad, *Biochem. J.*, **32**, 708 (1938).
[133] H. E. C. Wilson and G. K. Roy, *Indian J. Med. Research*, **25**, 879 (1938).
[134] M. K. Dimick and C. B. Schreffler, *J. Nutrition*, **17**, 23 (1939).
[135] G. A. Emerson and H. M. Evans, *Am. J. Physiol.*, **129**, 352 (1940).
[136] T. D. Spies, W. B. Bean and W. F. Ashe, *J. Am. Med. Assoc.*, **112**, 2414 (1939).
[137] T. D. Spies, D. P. Hightower and L. H. Hubbard, *Ibid.*, **115**, 292 (1940).
[138] W. Antopol and C. E. Schotland, *Ibid.*, **114**, 1058 (1940).

tion has also been called to the possibility that chilblains may afford a clinical demonstration of vitamin B_6 deficiency.[139] Promising results have also been obtained in the treatment of myasthenia.[137, 140] Improvement has furthermore been noted in patients with idiopathic epilepsy[137] and with macrocytic anemia of pellagra or pernicious anemia in relapse.[141]

(a) *Clinical Test Methods*

The Urine Test. In urine the excretion of vitamin B_6 can be demonstrated for rats and for man.[142] The methods used are either the chemical indophenol test or the biological rat test. The former can be applied to urine when it contains 1 γ per cc., but not when less material is present. Urine also contains substances which interfere with the test. In human beings, vitamin B_6 can be demonstrated in the urine only after the intake of excessive doses. The normal urinary excretion of man and dogs is less than 0.5 γ per cc., while rats excrete about 0.5–1.0 γ per cc.

The Blood Test. There is no method known by which vitamin B_6 can be determined in blood. As a matter of fact, the presence of this vitamin in blood has not been demonstrated as yet.

16. Hypervitaminosis

Vitamin B_6 is a substance of relatively low toxicity.[143, 144] Chronic toxicity was studied in rats, dogs and monkeys by daily feeding up to 10 mg. per kilogram body weight over periods extending to three months. No significant differences in weight or in the hemoglobin, erythrocytes, leucocytes, etc., were observed. Twenty mg. per kilogram body weight, injected intravenously into cats, had no effect. Single doses up to 1 g. were tolerated without untoward effects. Higher doses produced tonic convulsions and suggest involvement of certain parts of the nervous system. The lethal dose in rats is about 3 g. per kilogram body weight.[140] Vitamin B_6 has a sedative effect in man.[145]

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[139] P. György, *J. Nutrition*, **16**, 69 (1938).

[140] J. V. Scudi, H. F. Koones and J. C. Keresztesy, *Proc. Am. Physiol. Soc.*, **1940**, 163; *Proc. Soc. Exptl. Biol. Med.*, **43**, 118 (1940).

[141] R. W. Vilter, H. S. Schiro and T. D. Spies, *Nature*, **145**, 388 (1940).

[142] T. D. Spies, R. K. Ladisch and W. B. Bean, *J. Am. Med. Assoc.*, **115**, 839 (1940).

[143] K. Unna and W. Antopol, *Proc. Soc. Exptl. Biol. Med.*, **43**, 116 (1940).

[144] K. Unna, *Am. J. Physiol.*, **129**, 483 (1940).

[145] T. D. Spies, D. P. Hightower and L. H. Hubbard, *J. Am. Med. Assoc.*, **115**, 292 (1940).

17. Requirements

Since vitamin B_6 has not yet been demonstrated as necessary for human life, no data are available as to the requirements of this vitamin for human beings. In a few clinical cases daily doses of 10–100 mg. were given.[146]

The requirements for rats have been repeatedly investigated and were found to be 10 γ daily[147, 148] of the vitamin hydrochloride. Chicks also need this vitamin (about 30 γ per day).[149] Sheep, however, do not require an external supply of vitamin B_6, since it is synthesized by bacteria in the rumen.[150] The same has also been reported for cattle.[151, 152]

[146] T. D. Spies, W. B. Bean and W. F. Ashe, *J. Am. Med. Assoc.*, **112**, 2414 (1939).
[147] R. Kuhn and G. Wendt, *Ber.*, **71**, 780 (1938).
[148] M. K. Dimick and C. B. Schreffler, *J. Nutrition*, **17**, 23 (1939).
[149] D. M. Hegsted, J. J. Oleson, C. A. Elvehjem and E. B. Hart, *J. Biol. Chem.*, **130**, 423 (1939).
[150] L. W. McElroy and H. Goss, *Ibid.*, **130**, 437 (1939).
[151] L. W. McElroy and H. Goss, *Ibid.*, **133**, LXV (1940).
[152] M. I. Wegner, A. N. Booth, C. A. Elvehjem and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.*, **45**, 769 (1940).

NICOTINIC ACID—
NICOTINAMIDE

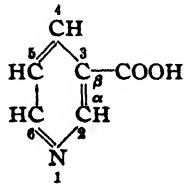
NICOTINIC ACID—NICOTINAMIDE

1. Nomenclature and Survey

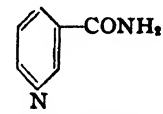
Names:

Nicotinic acid and nicotinamide.
Niacin: synonym for nicotinic acid.
Niacin amide: synonym for nicotinamide.
P. P. factor: Pellagra Preventive factor.[1]
Pellagramine.[2]
Niamid: Suggested abbreviation for nicotinamide.

Chemical formulas:



Nicotinic acid



Nicotinamide
(abbreviated formula)

Chemical names:

Pyridine-3-carboxylic acid and acid-amide.
Pyridine-β-carboxylic acid and acid-amide.

Empirical formulas:

Nicotinic acid: $C_6H_5O_2N$.
Nicotinamide: $C_6H_6ON_2$.

Nicotinic acid may possibly be identical with vitamin B_5. The term vitamin B_5 was given originally to a fuller's earth eluate fraction from yeast and was shown to be necessary for the growth of pigeons. Vitamin B_6 was later found to be present in vitamin B_5 fractions but could not fully replace the vitamin B_5 requirements of pigeons. The remaining factor, designated as vitamin B_5, appears to be identical with nicotinic acid.[3]

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[1] The term pellagra-preventive factor was applied to the substance originally postulated as necessary for the prevention of human pellagra: J. Goldberger and W. F. Tanner, *U. S. Pub. Health Service Pub. Health Repts.*, 39, 87 (1924).

[2] R. L. Jones, *Science*, 68, 480 (1928).

[3] C. W. Carter and J. R. O'Brien, *Biochem. J.*, 33, 1810 (1939).

2. Chronology

1735 First description of the human disease "pellagra" by CASAL.

1907 SEARCY reported the occurrence of pellagra in epidemic forms.

1912–1914 FUNK[4] in Europe and SUZUKI[5] in Japan isolated nicotinic acid from yeast and rice bran during their search for the antipolyneuritic vitamin (vitamin B_1), but failed to recognize the vitamin character of the isolated compound. FUNK observed, however, the beneficial effect of nicotinic acid when given in mixture with the antipolyneuritic vitamin.

1917 CHITTENDEN and UNDERHILL[6] produced a pathological condition, resembling human pellagra, in dogs, which is called canine blacktongue.

1926 GOLDBERGER and LILLIE obtained an experimental pellagra-like condition in albino rats by feeding a diet deficient in a substance called "Pellagra Preventive Factor" (P. P. Factor).[7]

1928 GOLDBERGER and associates[8] collected evidence that substances capable of curing blacktongue in dogs were equally effective in the cure of human pellagra.

1929 AYKROYD and ROSCOE recognized that the pellagra preventive factor and the anti-blacktongue factor always occur together.[9]

1930 NORRIS and RINGROSE[10] produced a pellagra-like condition in chicks.

1935 WARBURG and CHRISTIAN[11] and EULER, ALBERS and SCHLENK[12] demonstrated that nicotinamide is a part of the hydrogen transporting coenzymes.

1936 EULER and MALMBERG[13] reported that nicotinic acid prolonged the lives of rats fed vitamin B_1, vitamin B_2 and vitamin B_4.

1937 KNIGHT[14] and MUELLER[15] found nicotinamide to be essential to the growth of certain unicellular organisms. ELVEHJEM and co-workers[16] showed that nicotinic acid is effective in the cure of blacktongue in dogs and that it can be isolated from anti-blacktongue active liver extracts. FOUTS, HELMER, LEPKOVSKY and JUKES[17] reported the first successful treatment of human pellagra with nicotinamide.

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[4] C. Funk, *J. Physiol.*, **46**, 173 (1913); *Brit. Med. J.*, **1913, I**, 814. J. C. Drummond and C. Funk, *Biochem. J.*, **8**, 594 (1914).

[5] U. Suzuki and S. Matsunaga, *J. Agr. Tokyo Imp. Univ.*, **5**, 59 (1912). U. Suzuki, T. Shamimura and S. Okade, *Biochem. Z.*, **43**, 89, 99 (1912).

[6] R. H. Chittenden and F. P. Underhill, *Am. J. Physiol.*, **44**, 13 (1917).

[7] J. Goldberger and R. D. Lillie, *U. S. Pub. Health Service Pub. Health Repts.*, **41**, 1025 (1926).

[8] J. Goldberger and G. A. Wheeler, *Ibid.*, **43**, 172 (1928). J. Goldberger, G. A. Wheeler, R. D. Lillie and L. M. Rogers, *Ibid.*, **43**, 1385 (1928). J. Goldberger, G. A. Wheeler, L. M. Rogers and W. H. Sebrell, *Ibid.*, **45**, 1297 (1930).

[9] W. R. Aykroyd and M. H. Roscoe, *Biochem. J.*, **23**, 483 (1929).

[10] L. C. Norris and A. T. Ringrose, *Science*, **71**, 643 (1930).

[11] O. Warburg and W. Christian, *Biochem. Z.*, **275**, 464 (1935). O. Warburg, W. Christian and A. Griese, *Ibid.*, **279**, 143 (1935).

[12] H. v. Euler, H. Albers and F. Schlenk, *Z. physiol. Chem.*, **237**, I (1935).

[13] H. v. Euler and M. Malmberg, *Biochem. Z.*, **284**, 455 (1936).

[14] B. C. J. G. Knight, *Biochem. J.*, **31**, 731 (1937).

[15] J. H. Mueller, *J. Bact.*, **34**, 429 (1937); *J. Biol. Chem.*, **120**, 219 (1937).

[16] C. A. Elvehjem, R. J. Madden, F. M. Strong and D. W. Woolley, *J. Am. Chem. Soc.*, **59**, 1767 (1937); *J. Biol. Chem.*, **123**, 137 (1938).

[17] P. J. Fouts, O. M. Helmer, S. Lepkovsky and T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.*, **37**, 405 (1937).

3. Occurrence of Nicotinic Acid and of Nicotinamide

Nicotinic acid occurs in all living cells in small amounts. The liver,[18] the adrenal gland[19] and yeast and wheat germs are especially rich in nicotinic acid. The eye lenses contain a fair amount[19] and corn meal, corn syrup, alfalfa,[20] fatty meat and milk[21] contain small amounts.

Free nicotinic acid apparently does not occur in the living organism but is found in the urine of animals. Nicotinic acid occurs in tissues in the form of its amide. Besides the occurrence of the free nicotinamide, there exists a number of enzyme systems (see page 227) in which nicotinamide is chemically bound. Nicotinamide occurs to a much greater extent in the bound form than as the free nicotinamide. Thus, in rats, bound nicotinamide occurs, for example, in the liver, kidney and muscles, while free nicotinic acid (or its amide) has been found only in the liver.

4. Isolation of Nicotinic Acid and of Nicotinamide

The isolation of nicotinic acid from natural sources is a relatively simple matter. Tissue material is freed from fats by extraction with organic solvents. The remaining material is saponified, preferably with alkali. The acid fraction of the saponification mass contains the nicotinic acid, which can be separated as such, as the ester or as the Cu-salt. From this salt, the free acid is obtained by means of hydrogen sulfide.

The isolation of the total nicotinamide from animals or plants is carried out by water extraction of the material, followed by partial hydrolysis in 0.1 *N* sulfuric acid in order to split the nicotinamide from its chemical combination with the various enzymes. The water phase is then extracted with butanol or chloroform.[22] The chloroform solution is subjected to fractional distillation. Nicotinamide distills at 150–160° C. at 5×10^{-4} mm. Hg.[23] The distillate may be recrystallized from chloroform and benzene.

The separation of free nicotinic acid from nicotinamide is effected by solvent extraction (ether, chloroform, butanol) of a water solution. The amide is soluble in organic solvents, while the free acid remains in the water phase.

[18] W. J. Dann, *Science*, **86**, 616 (1937).
[19] E. Kodicek, *J. Soc. Chem. Ind.*, **58**, 1088 (1939).
[20] W. R. Wyatt, *Iowa State Coll. J. Sci.*, **14**, 103 (1939).
[21] E. Kodicek, *Biochem. J.*, **34**, 712, 724 (1940).
[22] H. v. Euler, F. Schlenk, L. Melzer and B. Högberg, *Z. physiol. Chem.*, **258**, 212 (1939). P. Karrer and H. Keller, *Helv. Chim. Acta*, **22**, 1292 (1939).
[23] R. Kuhn and H. Vetter, *Ber.*, **68**, 2374 (1935).

The separation of free nicotinamide from chemically bound nicotinamide (coenzymes) must be carried out immediately after death,[24, 25] and is usually achieved by an acetone extraction of the material. Acetone dissolves nicotinamide, but does not dissolve the coenzymes. Acetone, furthermore, prevents the naturally occurring enzyme systems from splitting the nicotinamide-containing coenzymes into their components.

5. Properties of Nicotinic Acid and of Nicotinamide

1. **Nicotinic acid** crystallizes in needles from water or alcohol and melts at 235.5–236.6° C.[26] It sublimes without decomposition. Nicotinic acid exhibits a typical absorption spectrum with a maximum at 385 mμ.[27]

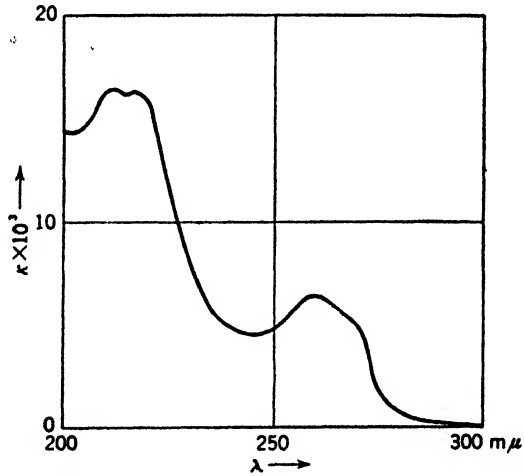

Fig. 12.—Absorption spectrum of nicotinamide in water. (R. Kuhn and H. Vetter.)

2. **Nicotinamide** crystallizes in needles from benzene and melts at 129° C. It distills at 150–160° C. and 5 × 10^{-4} mm. Hg.[28] The absorption spectrum of nicotinamide is shown in Fig. 12.

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[24] H. v. Euler, F. Schlenk, L. Melzer and B. Högberg, *Z. physiol. Chem.*, **258**, 212 (1939).

[25] H. v. Euler and K. Myrbäck, *Ibid.*, **117**, 237 (1928). H. v. Euler and G. Günther, *Ibid.*, **243**, 1 (1936). H. v. Euler, H. Heiwinkel and F. Schlenk, *Ibid.*, **247**, IV (1937).

[26] R. Gording and L. A. Flexser, *J. Am. Pharm. Assoc.*, **29**, 230 (1940).

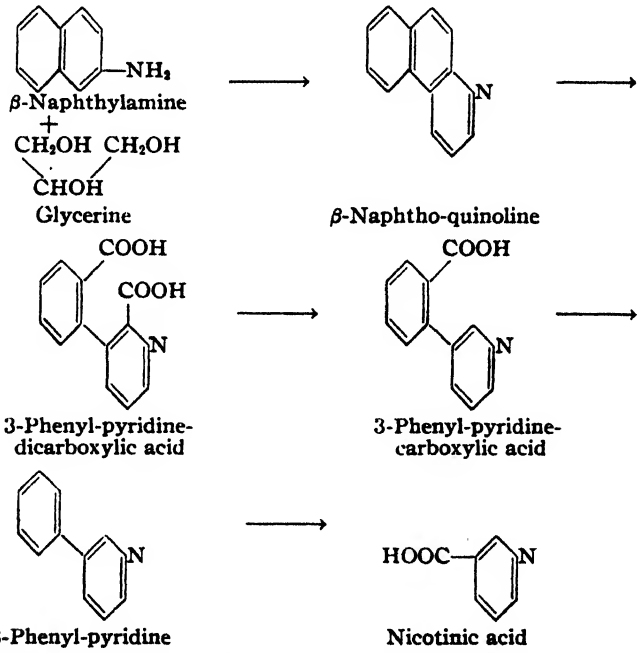
[27] H. Hünecke, *Ber.*, **60**, 1451 (1927).

[28] R. Kuhn and H. Vetter, *Ibid.*, **68**, 2374 (1935).

6. Constitution of Nicotinic Acid and of Nicotinamide

The constitution of nicotinic acid was determined when this acid was first obtained by oxidation of nicotine,[29] from which the name of this acid originates.

The acid character was established by the formation of a silver and a copper salt and by the formation of various derivatives such as esters, acid chloride, etc. The basic character was recognized by the formation of crystallized salts such as the hydrochloride, the hydrobromide, etc. By distillation of the calcium salt of the acid, the carboxylic acid group is split off and pyridine is obtained.[30]



The *m*- or 3-position of the carboxylic acid group in reference to the ring nitrogen was suspected by Skraup who investigated the physical constants and the decarboxylation of the three possible pyridine-mono-carboxylic acids: picolinic acid (*o*- or 1,2-), nicotinic acid (*m*- or 1,3-) and γ-pyridine-carboxylic acid (*p*- or 1,4-position).[31] The *m*-position was proved to be the correct one upon oxidation of the synthetically prepared 3-phenyl-

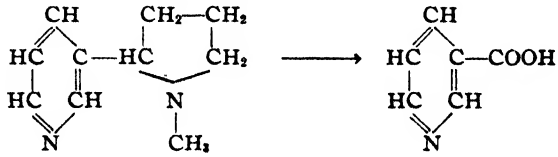
[29] C. Huber, *Ber.*, 3, 849 (1870); *Ann.*, 141, 271 (1867). H. Weidel, *Ann.*, 165, 346 (1873).
[30] H. Weidel, *Ann.*, 165, 331 (1873).
[31] Z. H. Skraup, *Monatsh.*, 1, 800 (1880).

pyridine, the constitution of which is beyond doubt. 3-Phenyl-pyridine was prepared from β-naphtho-quinoline, which in turn was synthesized from β-naphthylamine and glycerine. β-Naphtho-quinoline yields, upon oxidation with permanganate, β-phenyl-pyridine-dicarboxylic acid, which by stepwise decarboxylation yields the mono-carboxylic acid and finally 3-phenyl-pyridine.[32] A further proof for the 3-position of the carboxylic acid group in nicotinic acid is its formation from the synthetically prepared *m*-dipyridyl.[33]

7. Synthesis

(*a*) *Nicotinic Acid*

1. By Oxidation of Nicotine. This is the method by which nicotinic acid was discovered. The oxidation may be accomplished by fuming nitric acid,[34] by chromic acid[35] or by permanganate.[36]



2. By Oxidation of β-Pyridines. This method is really a generalization of the method first discussed. β-Picoline,[37] 3-ethyl-pyridine,[38] 3-phenyl-pyridine,[39] 3,3′-dipyridyl[40] and similar compounds have been converted by this method into nicotinic acid.

3. By Decomposition of Pyridine-poly-carboxylic Acids. Any pyridine-poly-carboxylic acid, which has one carboxylic acid group in 3-position can be converted into the 3-mono-carboxylic acid by thermal decomposition[41] or by acidic[42] decomposition. An exception to this rule

[32] Z. H. Skraup and A. Cobenzl, *Monatsh.*, **4**, 436 (1883).

[33] Z. H. Skraup and G. Vortmann, *Ibid.*, **4**, 594 (1883).

[34] H. Weidel, *Ann.*, **165**, 331 (1873).

[35] C. Huber, *Ber.*, **3**, 849 (1870); *Ann.*, **141**, 271 (1867). H. Weidel *Ann.*, **165**, 346 (1873).

[36] R. Laiblin, *Ber.*, **10**, 2136 (1877); *Ann.*, **196**, 135 (1879).

[37] H. Weidel, *Ber.*, **12**, 1992, 2004 (1879). H. Ost, *J. prakt. Chem.*, [2], **27**, 286 (1883). E. Seyfferth, *Ibid.*, [2], **34**, 258 (1886).

[38] H. Weidel and K. Hazura, *Monatsh.*, **3**, 783 (1882). A. Ladenburg, *Ann.*, **301**, 152 (1898). A. Wischnegradski, *Ber.*, **12**, 1480 (1879).

[39] Z. H. Skraup and A. Cobenzl, *Monatsh.*, **4**, 458 (1883).

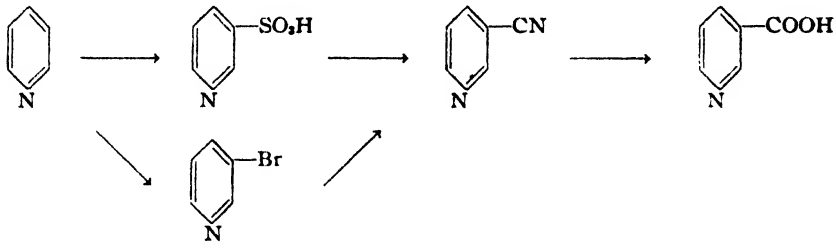
[40] Z. H. Skraup and G. Vortmann, *Ibid.*, **4**, 594 (1883).

[41] S. Hoogewerff and W. A. van Dorp, *Ann.*, **204**, 117 (1880); *Ibid.*, **207**, 219, 226 (1881); *Rec. trav. chim.*, **1**, 122 (1882). R. Camps, *Arch. Pharm.*, **240**, 353, 359 (1902). H. Weidel and J. Herzig, *Monatsh.*, **1**, 16 (1880). F. B. Ahrens and R. Gorkow, *Ber.*, **37**, 2063 (1904).

[42] H. Ost, *J. prakt. Chem.*, [2], **27**, 286 (1883). S. Hoogewerff and W. A. van Dorp, *Ber.*, **14**, 974 (1881). H. Weidel and J. Herzig, *Monatsh.*, **6**, 982 (1885).

is the 3,4-dicarboxylic acid, which decarboxylates only under very drastic conditions, yielding pyridine-4-carboxylic acid.

4. By Synthesis from Pyridine. This is a total synthesis of nicotinic acid. Pyridine is sulfonated with fuming sulfuric acid, yielding the 3-sulfonic acid. By distillation of its sodium salt with potassium cyanide, nicotinic acid nitrile is obtained.[43]



Considerably better yields are obtained by first brominating pyridine in the 3-position, followed by conversion into 3-cyano-pyridine by means of cuprous cyanide.[44] Saponification of the nicotinonitrile yields nicotinic acid.

(*b*) *Nicotinamide*

1. By Amidation of Nicotinic Acid. Nicotinamide is obtained by passing ammonia gas into nicotinic acid at 230° C.[45]

2. By Amidation of Esters of Nicotinic Acid. The methyl- or ethyl-ester of nicotinic acid yields nicotinamide on reaction with aqueous or, better, alcoholic ammonia.[46]

8. Industrial Methods of Preparing Nicotinic Acid and Nicotinamide

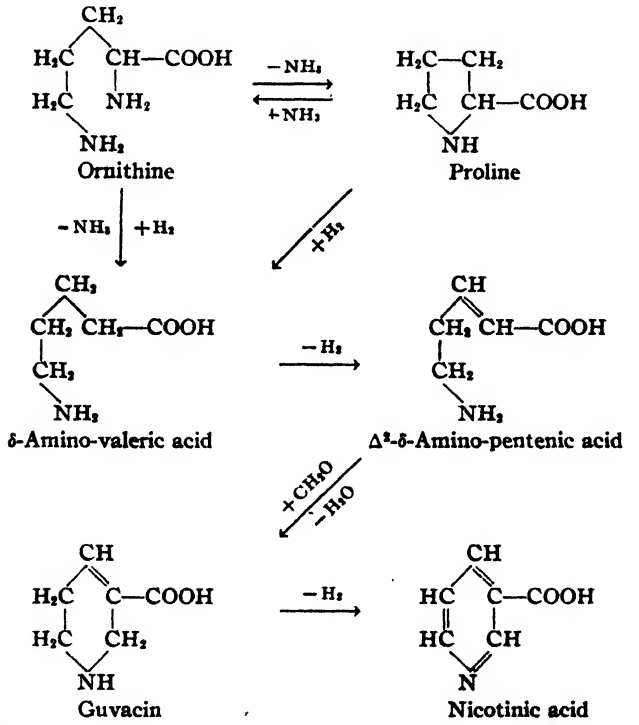
Nicotinic acid and its amide are prepared according to the methods described before. The nitric acid oxidation of nicotine, a relatively cheap alkaloid obtained as a by-product from tobacco, is of special interest.[47] Alternative procedures are permanganate oxidations of β-picoline or quinoline. For technical purposes the oxidation of the fraction of bases from coal tar or petroleum which distills between 135° and 142° C.[48] or the oxidation of the corresponding fraction of bone tar oil[49] has been considered.

[43] O. Fischer. *Ber.*, **15**, 63 (1882).
[44] S. M. McElvain and M. A. Goese, *J. Am. Chem. Soc.*, **63**, 2283 (1941).
[45] S. Keimatsu, K. Yokata and I. Satoda, *J. Pharm. Soc., Japan*, **53**, 994 (1933).
[46] C. Engler, *Ber.*, **27**, 1787 (1894). R. Camps, *Arch. Pharm.*, **240**, 354 (1902). F. Pollak, *Monatsh.*, **16**, 53 (1895). F. B. La Forge, *J. Am. Chem. Soc.*, **50**, 2477 (1928).
[47] *Org. Syntheses*, Coll. Vol. 1, 378 (1932).
[48] A. Pinner, *Ber.*, **33**, 1227 (1900).
[49] H. Weidel, *Ibid.*, **12**, 1992 (1879).

A number of *N*-substituted nicotinamide derivatives have attracted commercial interest because of their clinical use in the treatment of shock, collapse and cardiac decompensation.[50] The most useful compound of these derivatives is the *N*-diethyl-nicotinamide.

9. Biogenesis of Nicotinic Acid

The biogenesis of nicotinic acid is not known with certainty, but is believed to be linked with the amino-acid metabolism. It has been suggested[51] that nicotinic acid may originate from ornithine or proline. The first reaction product would be δ-amino-valeric acid. This reaction is common for bacteria[52] but has not been proved for plants. The further course of the suggested nicotinic acid synthesis is indicated in the following scheme:



[50] A selected number of patents for the preparation and use of these compounds appear in the patent index at the end of the book.

[51] M. Guggenheim, *Die biogenen Amine*, New York, 1940, p. 174.

[52] D. Ackermann, *Z. Biol.*, **57**, 104 (1910). C. Neuberg, *Biochem. Z.*, **37**, 490 (1911). E. Salkowski and H. Salkowski, *Ber.*, **16**, 1191 (1883).

10. Enzyme Systems Containing Nicotinamide

All plant and animal cells contain among their enzyme systems certain dehydrogenases which transport hydrogen and take care of a number of different dehydrogenation reactions. The classical conception is that each of these enzymes or, better, holoenzymes consists of an apoenzyme and a coenzyme. The apoenzyme, which is believed to have no catalytic properties of its own, is the protein bearer of the coenzyme, which is regarded as the prosthetic group of the protein.[53] Another view is that the protein is the enzyme itself and that the coenzyme merely acts as a specific substrate to accept hydrogen.[54] There are two coenzymes known of this class of dehydrogenases, namely, codehydrogenases I and II, which are also called coenzymes I and II. The number of apoenzymes which combine with these two coenzymes is considerably greater. It is believed that the two coenzymes need different apoenzymes for their action as

TABLE I

Substrate system	Apoenzyme source	Coenzyme
β-Hydroxy-butyrate ⇌ acetoacetate[55]	Cardiac muscle	I
Formate → CO_2 + H_2O[56]	Dried pea seeds	I
Lactate ⇌ pyruvate[57]	Skeletal muscle	I
Malate ⇌ oxaloacetate[57]	Skeletal muscle	I
Alcohol ⇌ acetaldehyde	Yeast[58, 59, 60]	I
	Liver[61]	I
Glucose → gluconic acid	Liver[62]	I or II[63]
Glutamic acid ⇌ α-keto-glutaric acid + NH_3[64, 65]	Plants	II[64]
	Yeast	II[64, 66]
	Liver	I or II[64]
2 Aldehydes → 1 alcohol + 1 acid[67] (aldehyde mutation)	Liver	I
α-Glycerophosphate → phosphoglycerate[68]	Skeletal, intestinal and cardiac muscle	I
Phospho-glyceraldehyde ⇌ diphospho-glycerate[69] (triose catabolism)[70]	Skeletal and cardiac muscle, brain	I
Glucose-6-phosphate → 6-phospho-gluconate[71]	Yeast, erythrocytes	II
6-Phospho-gluconate → phospho-keto-hexonate[72]	Yeast, animal tissues	II
Citrate → α-keto-glutarate[73]	Liver, heart	II

(*See following page for table footnotes.*)

[53] O. Warburg, *Ergeb. Enzymforsch.*, **7**, 210 (1937).
[54] M. Dixon and L. G. Zerfas, *Biochem. J.*, **34**, 371 (1940).

oxidizing and as reducing agents. Specific proteins are used for each substrate and a specific protein may in special cases dehydrogenate the same substrate with different coenzymes.

Table I summarizes the better known dehydrogenation reactions in which the codehydrogenases participate. It is evident that these coenzymes are involved in a wide variety of reactions. There are quite probably other such reactions, which have not been thoroughly investigated. It has, for example, been postulated that the oxidation of cysteine to cystine involves the participation of a codehydrogenase.[74]

During the course of the dehydrogenation reactions indicated in the table, the coenzymes are reduced to dihydro-compounds. The reverse reaction, the oxidation of the dihydro-codehydrogenases to the codehydrogenases, is carried out in the presence of different apoenzymes as previously stated. Principally all dehydrogenation reactions are reversible, although in living tissues usually no such equilibrium occurs due to the fact that the reaction products do not accumulate but undergo further reaction. Practically, the equilibrium can be demonstrated in many cases, such as in the system involving alcohol and acetaldehyde and is indicated in the table above for those systems for which the reversible reaction has been experimentally demonstrated. Equilibrium constants have been determined for the coenzyme I and for many of the reactions

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[56] D. E. Green, J. G. Dewan and L. F. Leloir, *Biochem. J.*, **31**, 934 (1937). D. E. Green and J. G Dewan, *Ibid.*, **31**, 1069, 1074 (1937).

[66] E. Adler and M. Sreenivasaya, *Z. physiol. Chem.*, **249**, 24 (1937).

[67] D. E. Green, D. M. Needham and J. G. Dewan, *Biochem. J.*, **31**, 2327 (1937). D. E Green and J. Brosteaux, *Ibid.*, **30**, 1489 (1936). D. E. Green, *Ibid.*, **30**, 2095 (1936).

[68] E. Negelein and H. J. Wulff, *Biochem. Z.*, **289**, 436 (1937); **293**, 351 (1937).

[69] O. Warburg and W. Christian, *Helv. Chim. Acta*, **19**, E 79 (1936).

[70] H. v. Euler, E. Adler and H. Hellström, *Z. physiol. Chem.*, **241**, 239 (1936).

[61] C. Lutwak-Mann, *Biochem. J.*, **32**, 1364 (1938).

[62] D. C. Harrison, *Ibid.*, **25**, 1016 (1931). E. Adler and H. v. Euler, *Z. physiol. Chem.*, **232**, 6 (1935).

[63] N. B. Das, *Z. physiol. Chem.*, **238**, 269 (1936).

[64] H. v. Euler, E. Adler, G. Günther and N. B. Das, *Ibid.*, **259**, 61 (1938).

[65] E. Adler, N. B. Das and E. v. Euler, *Sv. Vet. Akad. Ark. Kemi*, **12**, 1 (1937). J. G. Dewan, *Biochem. J.*, **32**, 1378 (1938). H. A. Krebs and P. P. Cohen, *Ibid.*, **33**, 1895 (1939).

[66] H. v. Euler, E. Adler and T. S. Eriksen, *Z. physiol. Chem.*, **248**, 227 (1937). E. Adler, G. Günther and J. E. Everett, *Ibid.*, **255**, 27 (1938).

[67] M. Dixon and C. Lutwak-Mann, *Biochem. J.*, **31**, 1347 (1937). M. Dixon, *Ergeb. Enzymforsch.*, **8**, 217 (1939).

[68] H. v. Euler, E. Adler and G. Günther, *Z. physiol. Chem.*, **249**, 1 (1937).

[69] O. Warburg and W. Christian, *Biochem. Z.*, **301**, 221 (1939); **303**, 40 (1939).

[70] D. E. Green, D. M. Needham and J. D. Dewan, *Biochem. J.*, **31**, 2327 (1937).

[71] O. Warburg and W. Christian, *Biochem. Z.*, **242**, 206 (1931); **254**, 438 (1932). E. Negelein and W. Gerischer, *Ibid.*, **284**, 289 (1936).

[72] F. Dickens, *Biochem. J.*, **32**, 1626 (1938). F. Lipmann, *Nature*, **138**, 588 (1936). O. Warburg and W. Christian, *Biochem. Z.*, **287**, 440 (1936); **292**, 287 (1936).

[73] E. Adler, H. v. Euler, G. Günther and M. Plass, *Biochem. J.*, **33**, 1028 (1930).

[74] E. Maschmann, *Naturwissenschaften*, **27**, 628 (1939).

catalyzed by the coenzymes and are usually expressed in the form of the oxidation-reduction potential.[75] The potential E_0' for the coenzyme I at 30° C. is approximately -0.29 volts.[76]

In tissues, the oxidation of the dihydro-coenzymes may be accomplished by coenzyme-linked reactions.[77] It is, for example, possible that β-hydroxy-butyrate is oxidized to acetoacetate (see Table I) with the formation of dihydro-cozymase I which in turn reduces an aldehyde to an alcohol. Thus, the following compounds have been shown to act as acceptors in the presence of the corresponding apoenzymes; acetaldehyde, pyruvate, oxaloacetate, triose-phosphate, imino-glutarate (or α-keto-glutarate + NH_3) and fumarate[77] (in the presence of succinic-dehydrogenase). It has also been shown that the riboflavin-containing enzyme systems (see page 171) may be linked with the oxidation of the dihydro-codehydrogenases.

11. Coenzymes Containing Nicotinamide

(a) *Codehydrogenase I*

Synonyms. Codehydrogenase I, Coenzyme I, Cozymase, Coferment I, Diphosphopyridine nucleotide, Coferment of fermentation, Coreductase, Factor V.[78, 79, 80]

Occurrence. Codehydrogenase I has been found in all animal and plant cells in which carbohydrates are metabolized. Yeast and red blood cells are especially rich sources, and some muscles, for example, heart muscles, contain relatively high amounts. In fresh yeast about 0.5 g. of codehydrogenase I is present per kilogram[81] and in the heart muscle of rabbits 0.4 g. per kilogram.[82] The same amount (0.1–0.4 g.) has been calculated to be present in muscles of man[83] and of invertebrata.[84] Codehydrogenase I occurs also in microorganisms and has, for example, been obtained from *Azotobacter chroococcum*.[85] There seems to be a fairly con-

[75] W. M. Clark, *The Determination of Hydrogen Ion Concentration*, Baltimore, 1928. L. Michaelis, *Oxidation-Reduction Potentiale*, Berlin, 1933.

[76] F. Schlenk, H. Hellström and H. v. Euler, *Ber.*, **71**, 1471 (1938). H. Borsook, *J. Biol. Chem.*, **133**, 629 (1940).

[77] J. G. Dewan and D. E. Green, *Biochem. J.*, **31**, 1074 (1937).

[78] A. Lwoff and M. Lwoff, *Proc. Roy. Soc.* (*London*), **B122**, 352, 360 (1937); *Compt. rend.*, **203**, 520 (1936).

[79] H. 1. Kohn, *Biochem. J.*, **32**, 2075 (1938).

[80] T. M. Rivers, *Bull. Johns Hopkins Hosp.*, **33**, 149, 429 (1922).

[81] O. Meyerhof and P. Ohlmeyer, *Biochem. Z.*, **290**, 334 (1937).

[82] H. v. Euler, *Angew. Chem.*, **50**, 831 (1937).

[83] H. v. Euler, *Atti 10th Congr. Intern. Chim.*, **1**, 178 (1938).

[84] S. Ochoa and C. G. Ochoa, *Nature*, **140**, 1097 (1937).

[85] R. Nilsson, *Arch. Mikrobiol.*, **7**, 598 (1937).

stant ratio of coenzyme to dihydro-coenzyme in the muscles of all animals, the reduced form being present in about 35–45% of the total amount.[84] An increased amount of the reduced form has been found in Jensen sarcoma.[86, 87]

Isolation.[88] The isolation of codehydrogenase I is carried out by water extraction of the source, for example, of yeast or red blood cells. It is necessary to destroy some of the other enzymes present prior to the extraction, by short heating to about 80° C., since otherwise the codehydrogenase is rapidly destroyed. After filtration or dialysis some of the protein impurities are removed by precipitation with lead acetate. The coenzyme itself may be extracted with phenol[89] and is precipitated by mercuric acetate or nitrate, picric acid, phosphotungstic or silicotungstic acid (and decomposed by ether-amyl-alcohol-sulfuric acid), by silver salts either in ammoniacal solution or in a solution containing barium hydroxide (and freed from silver by hydrogen sulfide), by cuprous halides in the presence of hydrochloric acid (and freed from copper by hydrogen sulfide), by ethyl-acetate from an acidified methanol solution, by acetone, and by alcohol. The latter is also used for fractional precipitation of this coenzyme. Purification may also be accomplished by fractional adsorption on aluminum oxide or charcoal from weakly acid solutions.

The methods used for the separation of nicotinamide from the coenzymes have been described in the section on the isolation of nicotinamide (page 222). The methods used for the separation of codehydrogenase I from codehydrogenase II will be found in the section on the isolation of codehydrogenase II (page 235). To separate the flavin-adenine-dinucleotides (see page 179) which occur together with the codehydrogenases I and II in the phenol extract from certain sources such as yeast, the flavin compounds are precipitated in acid solution as silver salts.[89, 90]

Properties. Codehydrogenase I is a colorless, water-soluble substance, and is insoluble in most organic solvents. It exhibits a characteristic absorption spectrum with a maximum at 260 mμ.[91] Hydrogenation to the dihydro-codehydrogenase, which occurs during the enzyme action (see page 238), changes the absorption spectrum characteristically with the

[84] H. v. Euler, F. Schlenk, H. Heiwinkel and B. Högberg, *Z. physiol. Chem.*, **256**, 208 (1938).

[87] H. v. Euler, M. Malmberg and G. Günther, *Z. Krebsforsch.*, **45**, 425 (1937).

[88] H. v. Euler, *Ergeb. Physiol.*, **38**, 1 (1936). O. Warburg and W. Christian, *Biochem. Z.*, **287**, 291 (1936). O. Meyerhof, *Ergeb. Physiol.*, **39**, 10 (1937). H. v. Euler, H. Albers and F. Schlenk, *Z. physiol. Chem.*, **260**, 113 (1936); *Fortschr. Chem. org. Naturstoffe*, **1**, 99 (1938). P. Ohlmeyer, *Biochem. Z.* **297**, 66 (1938). H. v. Euler and E. Adler, *Z. physiol. Chem.*, **238**, 233 (1936). B. J. Jandorf, *J. Biol. Chem.*, **138**, 305 (1941). K. Myrbäck and H. Larsson, *Z. physiol. Chem.*, **225**, 131 (1934).

[89] O. Warburg and W. Christian, *Biochem. Z.*, **298**, 150, 377 (1938).

[90] J. R. Klein, *J. Biol. Chem.*, **134**, 43 (1940).

[91] O. Warburg and W. Christian, *Helv. Chim. Acta*, **19**, E 79 (1936). H. v. Euler, E. Adler and H. Hellström, *Z. physiol. Chem.*, **241**, 239 (1936). K. Myrbäck, H. v. Euler and H. Hellström, *Ibid.*, **212**, 7 (1932).

appearance of an additional band at 320–360 mμ with a maximum at 340 mμ (see Fig. 13).

While the codehydrogenase I shows no fluorescence, the dihydro-compound exhibits a strong whitish fluorescence upon irradiation with ultraviolet light.

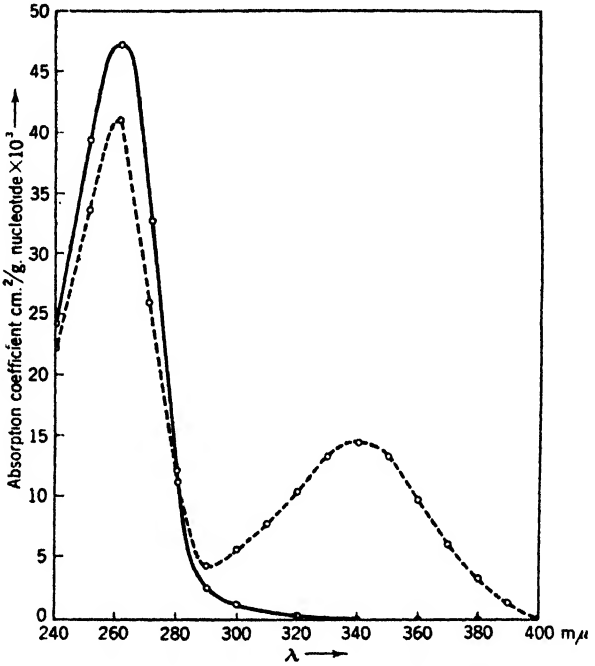


Fig. 13.—Absorption spectrum of codehydrogenase II in oxidized (——) and in reduced (– – – –) form. (O. Warburg and W. Christian.)

Codehydrogenase I is optically active, the specific rotation being approximately −20° for the red cadmium line (643.9 mμ)[92] and −70° for the green mercury line (546 mμ).

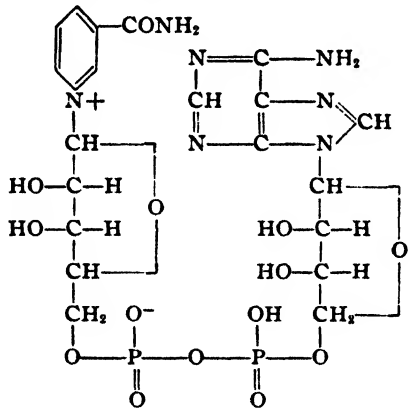
Codehydrogenase I is quite stable in acid solution at moderate temperatures,[93] whereas the dihydro-codehydrogenase is destroyed by acids. In alkaline solution, codehydrogenase is rapidly destroyed,[94] whereas the dihydro-codehydrogenase remains unchanged when heated for 30 minutes

[92] K. Myrbäck, H. v. Euler and H. Hellström, *Z. physiol. Chem.*, **212**, 7 (1932).
[93] K. Myrbäck, *Ibid.*, **234**, 259 (1935).
[94] K. Myrbäck and B. Örtenblad, *Ibid.*, **234**, 254 (1935).

to 100° C. in 0.1 *N* sodium hydroxide solution.[95] Codehydrogenase is inactivated by ultraviolet light.[96]

Constitution. Codehydrogenase I is water-soluble and relatively stable against oxidation, for example, against hydrogen peroxide, but is attacked by this oxidizing agent in the presence of various catalysts, such as iron, etc.

Codehydrogenase I has the empirical formula $C_{21}H_{27}N_7O_{14}P_2$.[97, 98] It is a dinucleotide which yields on hydrolysis adenine,[99] nicotinic acid amide[100, 101] and two mols of *d*-ribose-phosphoric acid.[102] The phosphoric acid is linked to the ribose in 5-position, since no formaldehyde is obtained by oxidation of the ribose-phosphoric acid with periodic acid.[103] Alkaline hydrolysis of codehydrogenase I yields adenosine-diphosphoric acid,[104] which proves the existence of a pyrophosphate linkage in the coenzyme molecule. Cozymase titrates as a monobasic acid,[98] the naturally occurring dihydro-cozymase as a dibasic acid. These experimental results suggest that one of the free hydroxyl groups of the phosphoric acid parts of the molecule is linked to the pyridinium nitrogen atom. Euler and Schenk suggested the following formula for codehydrogenase. I:[98, 105]



[95] E. Adler, H. Hellström and H. v. Euler, *Z. physiol. Chem.*, **242**, 225 (1936).

[96] J. Runnström and L. Michaelis, *J. Gen. Physiol.*, **18**, 717 (1935).

[97] H. v. Euler and F. Schlenk, *Svensk Kem. Tid.*, **48**, 135 (1936).

[98] F. Schlenk and H. v. Euler, *Naturwissenschaften*, **24**, 794 (1936).

[99] H. v. Euler and K. Myrbäck, *Z. physiol. Chem.*, **177**, 237 (1928); *Naturwissenschaften*, **17**, 291 (1929).

[100] O. Warburg and W. Christian, *Biochem. Z.*, **285**, 156 (1936); **287**, 291 (1936).

[101] H. Albers, F. Schlenk and H. v. Euler, *Arkiv. Kemi, Mineral. Geol.*, **B12**, No. 21 (1936); *Z. physiol. Chem.*, **237**, 1 (1935); *Biochem. Z.*, **286**, 140 (1936).

[102] F. Schlenk, *Arkiv Kemi, Mineral. Geol.*, **B12**, No. 17 (1936).

[103] H. v. Euler, P. Karrer and B. Becker, *Helv. Chim. Acta*, **19**, 1060 (1936).

[104] H. v. Euler, F. Schlenk and R. Vestin, *Naturwissenschaften*, **25**, 318 (1937).

[105] K. Myrbäck, *Tab. Biol.*, **14**, 110 (1937). H. v. Euler and F. Schlenk, *Z. physiol. Chem.*, **246**, 64 (1936).

Synthesis. No clear-cut chemical synthesis of codehydrogenase I has been reported. It has, however, been observed that certain blood constituents are able to convert *in vitro* nicotinic acid and nicotinamide into both coenzymes I and II. It was first assumed that normal erythrocytes accomplish this synthesis,[106] but it was shown subsequently[107] that nucleated cells, that is, the white cells of, for example, the lymphoid and myeloid series, must be held responsible for the synthesis of these coenzymes.

There is also a definite indication of an enzymatic synthesis of codehydrogenase I from codehydrogenase II.[108, 109]

Determination by Physical Methods. *1. Absorption spectrum:* While codehydrogenase exhibits a typical maximum at 260 mμ, which might be used for its determination, the typical band at 340 mμ which appears only when the coenzyme is in the dihydro form, is much more useful. Codehydrogenase II gives the same absorption spectra. The spectrophotometric method therefore cannot be used to differentiate between the two coenzymes.

2. Fluorescence: The fluorescence of the dihydro-codehydrogenase has successfully been used for following the oxidation-reduction of this coenzyme.[110] The fluorescence is acid sensitive.

Determination by Chemical Methods. Codehydrogenase can be determined by the same chemical methods which are used for the determination of nicotinic acid (see page 240). These methods, however, do not differentiate the nicotinic acid from the coenzymes. The state of equilibrium of codehydrogenase with its reduced form in active enzyme systems can be analyzed by acidification which destroys the dihydro-compound (probably due to addition of the mineral acid to the double bonds of the partly reduced pyridine nucleus[111]) but leaves the codehydrogenase itself untouched. The latter is then determined by any of the known methods.[112, 113] On the other hand, the dihydro-compound can be determined alone by making the solution containing the reduced and the non-reduced form alkaline whereby the codehydrogenase I is destroyed. Another chemical method which has been suggested for the determination

[106] H. J. Kohn and J. R. Klein, *J. Biol. Chem.*, 130, 1 (1939).
[107] S. P. Vilter, R. W. Vilter and T. D. Spies, *Nature*, 144, 943 (1939).
[108] H. v. Euler, E. Adler and T. S. Eriksen, *Z. physiol. Chem.*, 248, 227 (1937).
[109] H. v. Euler, F. Schlenk, H. Heiwinkel and B. Högberg, *Ibid.*, 256, 208 (1938).
[110] O. Warburg and W. Christian, *Biochem. Z.*, 286, 81 (1936).
[111] P. Karrer, B. H. Ringier, J. Büchi, H. Fritzsche and U. V. Solmssen, *Helv. Chim. Acta*, 20, 55 (1937).
[112] E. Adler and H. v. Euler, *Svensk Kem. Tid.*, 48, 221 (1936).
[113] H. v. Euler, F. Schlenk, H. Heiwinkel and B. Högberg, *Z. physiol. Chem.*, 256, 208 (1938).

of codehydrogenase is the determination of the total hydrogen consumption upon catalytic hydrogenation in the presence of sodium borate.

Determination by Biochemical Methods. Codehydrogenase I and its dihydro-form are usually determined by the degree of activation which they exert on fermentation[114, 115, 116] in the presence of an excess of the apodehydrogenase. The criterion is the amount of CO_2 evolved under specified conditions which is proportional to the codehydrogenase I concentration. This method is somewhat unreliable due to the fact that the purity of the apoenzyme is not standardized.[117] This method is specific for codehydrogenase I and codehydrogenase II. Nicotinic acid and its amide do not respond to this test.

Another biochemical assay procedure for codehydrogenase I is based upon the fact that the oxidation of lactic acid by animal tissues requires the presence of codehydrogenase I.[118]

Determination by Biological Methods. *Bacillus influenzae* can be used to measure accurately the total content of codehydrogenases in blood and of yeast (and probably of other sources), since this bacillus cannot synthesize the codehydrogenases from their constituents, but needs the coenzymes for proper development.[119, 120, 121]

Standard. One unit of codehydrogenase I is defined as that quantity which produces 1 cc. of carbon dioxide in a normal fermentation[122] under specified conditions.

(*b*) *Codehydrogenase II*

Synonyms. Triphosphopyridine nucleotide, Warburg's Coferment, Respiratory coenzyme, Growth factor V.[123, 124, 125]

Occurrence. Codehydrogenase II seems, like codehydrogenase I, to occur in practically all living cells. These apparently have the power

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[114] K. Myrbäck, *Z. physiol. Chem.*, **177**, 158 (1928).

[115] A. E. Axelrod and C. A. Elvehjem, *J. Biol. Chem.*, **131**, 77 (1939).

[116] B. J. Jandorf, F. W. Klemperer and A. B. Hastings, *Ibid.*, **138**, 311 (1941).

[117] H. v. Euler and K. Myrbäck, *Z. physiol. Chem.*, **190**, 93 (1930).

[118] D. E. Green and J. Brosteaux, *Biochem. J.*, **30**, 1489 (1936).

[119] A. Lwoff and M. Lwoff, *Proc. Roy. Soc.* (*London*), **B122**, 352, 360 (1937); *Compt. rend.*, **203**, 520 (1936).

[120] R. W. Vilter, S. P. Vilter and T. D. Spies, *J. Am. Med. Assoc.*, **112**, 420 (1939).

[121] H. I. Kohn, *Biochem. J.*, **32**, 2075 (1938).

[122] H. v. Euler, H. Albers and F. Schlenk, *Z. physiol. Chem.*, **240**, 113 (1936). H. v. Euler and K. Myrbäck, *Ibid.*, **136**, 108 (1924). H. v. Euler and S. Karlson, *Ibid.*, **123**, 93 (1922).

[123] T. M. Rivers, *Bull. Johns Hopkins Hosp.*, **33**, 149, 429 (1922).

[124] H. I. Kohn, *Biochem. J.*, **32**, 2075 (1938).

[125] A. Lwoff and M. Lwoff, *Proc. Roy. Soc.* (*London*), **B122**, 352, 360 (1937); *Compt. rend.*, **203**, 520 (1936).

of synthesizing both codehydrogenases (see under Synthesis, page 233) from nicotinic acid. It has also been postulated that the living cell is able to convert codehydrogenase I into codehydrogenase II. It seems plausible, therefore, that both coenzymes are found together. It is noteworthy that the ratio of the amounts of the two coenzymes may vary considerably in different sources. While yeast contains very little codehydrogenase II, animal tissue contains as much as 40–80 γ per gram.[126]

Isolation. The isolation of codehydrogenase II is carried out, for example, from washed red blood cells, by destruction of the cell structure, followed by a combination of various precipitation reactions.[127, 128] Codehydrogenase II is precipitated from a water solution by acetone, by ethylacetate, especially from a methanol-HCl solution, by mercuric acetate, by barium salts, by lead salts, etc.

Separation of Codehydrogenase I from Codehydrogenase II. The following methods have been recommended for the separation of the two coenzymes:

1. Codehydrogenase I is separated as the cuprous salt[129] whereby codehydrogenase II remains in solution and can be isolated separately.
2. Codehydrogenase II is precipitated by lead acetate, codehydrogenase I is not.[130]
3. Codehydrogenase II is more strongly adsorbed on aluminum oxide than codehydrogenase I.[129] The codehydrogenase II is eluted from the Al_2O_3 by KH_2PO_4 solutions.
4. The barium salts of the two codehydrogenases can be separated by fractional precipitation with alcohol.[131]

Properties. The properties of codehydrogenase II resemble very closely those of codehydrogenase I. They are colorless, water-soluble compounds which are insoluble in organic solvents. Codehydrogenase II is soluble in organic solvents in the presence of hydrochloric acid, for example, in methanol-HCl.

Codehydrogenase II exhibits the same characteristic absorption band at 260 mμ as does codehydrogenase I. The maximum of the typical dihydro-codehydrogenase absorption is at 340 mμ[131] (see Fig. 13). The latter compound also shows the characteristic fluorescence when irradiated

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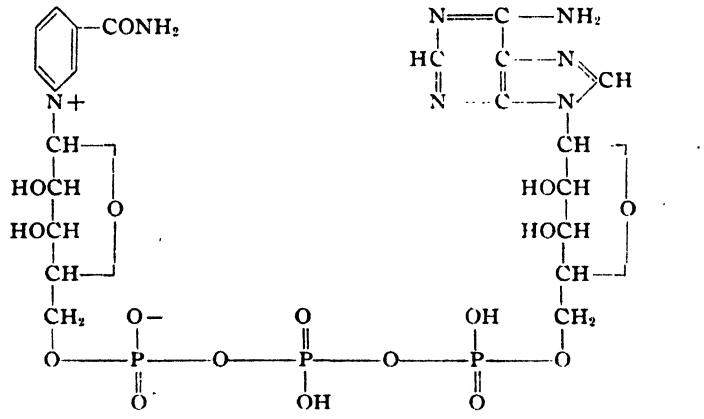
[126] H. v. Euler, F. Schlenk, H. Heiwinkel and B. Högberg, *Z. physiol. Chem.*, **256**, 208 (1938).
[127] F. Schlenk, *Tab. Biol.*, **14**, 186 (1937).
[128] O. Warburg, W. Christian and A. Griese, *Biochem. Z.*, **282**, 157 (1935).
[129] H. v. Euler and E. Adler, *Z. physiol. Chem.*, **238**, 233 (1936).
[130] H. v. Euler, *Ibid.*, **240**, 113 (1936).
[131] O. Warburg and W. Christian, *Biochem. Z.*, **287**, 291 (1936).

with ultraviolet light.[132] This destroys both codehydrogenases rapidly.[133, 134] Codehydrogenase II is unstable in alkaline solution, but stable in acid solution.[135]

In isolated muscle tissue codehydrogenase II is rapidly inactivated.[136] Codehydrogenase II is optically active: $[\alpha]_{589\ m\mu} = -24.6°$, $[\alpha]_{546\ m\mu} = -29.4°$.

Constitution. Codehydrogenase II has not yet been isolated in the pure form. The probable empirical formula is $C_{21}H_{28}N_7O_{17}P_3$.[137] This corresponds to 1 mol adenine, 1 mol nicotinamide, 2 mols pentose (probably *d*-ribose) and 3 mols phosphoric acid. Codehydrogenase II seems thus to differ from codehydrogenase I only by one additional phosphoric acid group. Adenine and nicotinamide have been isolated from the breakdown products of this coenzyme.[138, 139] Codehydrogenase II is dibasic since the results of electrophoresis determinations established two different dissociation constants: $p_{K_1} = 1.8$ and $p_{K_2} = 6.1$. The following formula for codehydrogenase II has tentatively been suggested:[140]



[132] O. Warburg and W. Christian, *Helv. Chim. Acta*, **19**, E 79 (1936). H. v. Euler, E. Adler and H Hellström, *Z. physiol. Chem.*, **241**, 239 (1936). K. Myrbäck, H. v. Euler and H. Hellström, *Ibid.*, **212**, 7 (1932).

[133] O. Warburg and W. Christian, *Biochem. Z.*, **282**, 221 (1936).

[134] J. Runnström and L. Michaelis, *J. Gen. Physiol.*, **18**, 717 (1935).

[135] O. Warburg, W. Christian and A. Griese, *Biochem. Z.*, **282**, 157 (1935).

[136] H. v. Euler, H. Heiwinkel and F. Schlenk, *Z. physiol. Chem.*, **247**, IV (1937).

[137] O. Warburg, W. Christian and A. Gri se, *Biochem. Z.*, **282**, 157 (1935).

[138] O. Warburg and W. Christian, *Helv. Chim. Acta*, **19**, E 79 (1936). H. v. Euler, E. Adler and H. Hellström, *Z. physiol. Chem.*, **241**, 239 (1936). K. Myrbäck, H. v. Euler and H. Hellström, *Ibid.*, **212**, 7 (1932).

[139] O. Warburg and W. Christian, *Biochem. Z.*, **275**, 464 (1935).

[140] H. v. Euler and F. Schlenk, *Z. physiol. Chem.*, **246**, 64 (1936).

No definite proof for this structure has as yet been obtained. As a matter of fact, this structure seems doubtful in view of the fact that an apparent synthesis of codehydrogenase II from codehydrogenase I by two different methods has been accomplished (for details see below). Since these synthetic methods consist in the addition of one mol of phosphoric acid to codehydrogenase I, it would appear that the proposed formula for the coenzyme II containing three phosphoric acid groups in one chain is rather improbable. It may be that one of the phosphoric acid radicals is attached to the molecule of codehydrogenase I as a side chain, thus forming the molecule of codehydrogenase II. It is noteworthy that codehydrogenase II has apparently no free amino-group since it does not react with nitrite.[141]

Synthesis. It has already been pointed out in the section on Synthesis of Codehydrogenase I (p. 233), that a partial synthesis of codehydrogenase II can be accomplished from nicotinic acid or nicotinamide by the action of nucleated cells *in vitro*.

Codehydrogenase II can apparently be synthesized from codehydrogenase I, since the product obtained shows the same properties as does codehydrogenase II in the test for dehydrogenating Robison ester. Ultimate proof for the accomplished conversion is still lacking. This assumed conversion of codehydrogenase I into codehydrogenase II has been carried out (1) by means of phosphorus oxychloride in ether;[142] (2) by enzymatic phosphorylation.[143]

Determination by Physical and Chemical Methods. The same methods which were described for the determination of codehydrogenase I can also be applied to codehydrogenase II. There is no physical or chemical method of differentiating between these two coenzymes.

Determination by Biochemical Methods. 1. Codehydrogenase II and its dihydro-form are usually determined according to the Warburg technic by comparison with a standard preparation of this coenzyme in a system which dehydrogenates hexose-monophosphoric acid (Robison ester). In addition to the coenzyme, the specific apoenzyme and the "yellow ferment" are also necessary.

Determination by Biological Methods. Codehydrogenase II can be determined by the growth test with *Haemophilus parainfluenzae* as described for the determination of codehydrogenase I.

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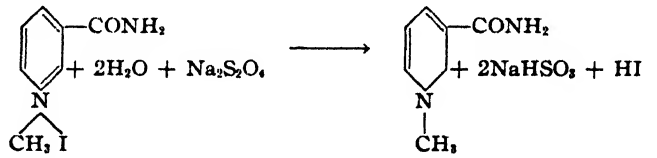
[141] O. Warburg, W. Christian and A. Griese, *Biochem. Z.*, **282**, 157 (1935).

[142] F. Schlenk, *Naturwissenschaften*, **25**, 668 (1937).

[143] R. Vestin, *Ibid.*, **25**, 667 (1937). H. v. Euler and R. Vestin, *Arkiv Kemi, Mineral. Geol.*, **B12**, No. 44 (1938). H. v. Euler and E. Bauer, *Ber.*, **71**, 411 (1938). H. v. Euler and E. Adler, *Z. physiol. Chem.*, **252**, 41 (1938).

12. Mechanism of the Nicotinamide Coenzyme Action

It has previously been stated that the action of the various nicotinamide-containing enzyme systems consists in the dehydrogenation of various substrates. During this reaction, the codehydrogenase absorbs two atoms of hydrogen, thus being reduced to a dihydro-form. The dihydro-codehydrogenase is in turn oxidized, and thus reconverted into the codehydrogenase. The nicotinamide part of the molecule is responsible for the phenomenon of the reversible reduction-oxidation reaction of the codehydrogenases.[144] *In vitro*, this reduction to the dihydro-codehydrogenases can be brought about by the action of sodium hydrosulfite, $Na_2S_2O_4$, in weakly alkaline solution. In order to study the mechanism of this reaction and the constitution of the reduced and oxidized forms of the coenzymes, the reversible reduction of a number of simple nicotinamide derivatives has been examined.[145] It was found that only those derivatives which have a pentavalent ring-nitrogen yield dihydro-compounds with the same characteristic absorption spectrum as the dihydro-coenzyme. Upon reduction, the nitrogen becomes trivalent. Nicotinamide-iodomethylate proved to be the most characteristic model substance:

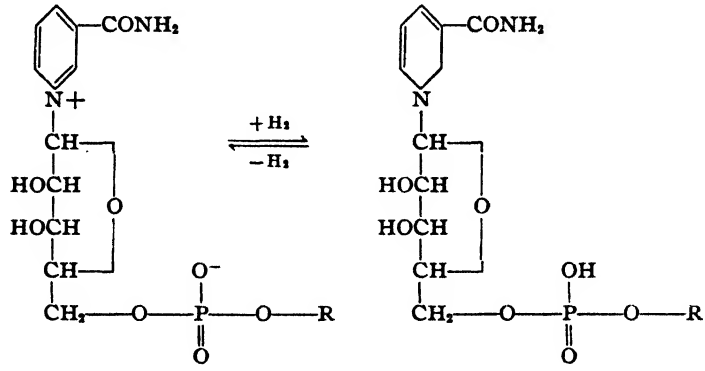


The dihydro-nicotinamide compounds, but not the corresponding unreduced molecules, exhibit a typical whitish fluorescence upon irradiation with ultraviolet light. The dihydro-compounds show an additional typical absorption band in the ultraviolet with a maximum at 340 mμ. By comparison of these characteristics with known dihydro-pyridine compounds, it has been concluded that the constitution of the dihydro-compound is that of an *o*-dihydro-compound. Additional evidence is furnished by the deep yellow color of the dihydro-compound, which would be improbable for a *p*-dihydro-compound. The results of these experiments indicate that in the natural codehydrogenases the ring-nitrogen of nicotinamide is chemically bound in such a way that upon reduction a tertiary

[144] O. Warburg, W. Christian and A. Griese, *Biochem. Z.*, **282**, 157 (1935).

[145] P. Karrer and O. Warburg, *Ibid.*, **225**, 297 (1936). P. Karrer, G. Schwarzenbach, F. Benz and U. V. Solmssen, *Helv. Chim. Acta*, **19**, 811 (1936). P. Karrer and F. Benz, *Ibid.*, **19**, 1028 (1936). P. Karrer, B. H. Ringier, J. Büchi, H. Fritzsche and U. V. Solmssen, *Ibid.*, **20**, 55 (1937). P. Karrer, G. Schwarzenbach and G. E. Utzinger, *Ibid.*, **20**, 72 (1937).

nitrogen is formed. The formula of the codehydrogenases is therefore written in the form of a quaternary pyridinium salt, which in the dihydro-form possesses an additional acidic group:



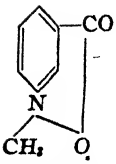
The exact position of the reduced double bond in relation to the carboxylic acid-amide group in the dihydro-nicotinamide is still unknown. It is generally assumed that the double bond in α,β-position is reduced; the γ,δ-position, however, cannot be excluded on the basis of the present knowledge.[146]

13. Specificity of Nicotinic Acid and Nicotinamide

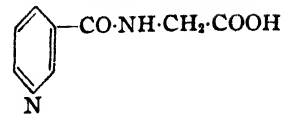
The compounds of this group which are known to be effective when given orally are nicotinic acid, its salts, for example, the sodium salt, nicotinamide, the *N*-methyl-amide and the *N*-diethyl-amide of nicotinic acid, ethyl-nicotinate, nicotinamide-glucosido-iodide and nicotinuric acid.[148] It is assumed, as would be expected on a chemical basis, that all these compounds are converted in the organism into nicotinamide. Since a number of other *N*-substituted nicotinamides have been found quite effective in the treatment of heart weakness, it is suspected that these derivatives will also prove effective as vitamins.

The ring-nitrogen apparently must be unsubstituted for the exhibition of vitamin activity, since trigonelline, the nicotinic acid-methyl-betaine, is inactive.[147] This is easily understood, since in enzyme systems the ring-nitrogen is attached to the rest of the enzyme molecule.

[146] P. Karrer, F. W. Kahnt, R. Epstein, W. Jaffé and T. Ishii, *Helv. Chim. Acta*, 21, 223 (1938).
[147] D. W. Woolley, F. M. Strong, R. J. Madden and C. A. Elvehjem, *J. Biol. Chem.*, 124, 715 (1938).
[148] R. W. Vilter and T. D. Spies, *Lancet*, II, 423 (1939).



Trigonelline



Nicotinuric acid

Quinolinic acid,[148] pyrazine-carboxylic acid and pyrazine-2,3-dicarboxylic acid are active in curing human pellagra[149, 150] (500–800 mg. daily *per os*) and are said not to produce the vasodilator symptoms which often follow the administration of nicotinic acid. Dysentery bacilli can use thiazole-5-carboxylic acid as a substitute for nicotinic acid.[151] All these compounds were found inactive in dogs.[147, 152]

14. Determination of Nicotinic Acid and Nicotinamide

(*a*) *Chemical Methods*

1. The Cyanogen Bromide Method. This method is based on the principle[153] that pyridine derivatives give specific colors with cyanogen bromide and a primary or secondary amine. In order to determine the quantity of nicotinic acid in natural products, the latter must be totally hydrolyzed to contain only the free acid. The solutions, protected from light, are warmed with CNBr in the dark and treated with an amine,[154] for example, with *p*-amino-acetophenone,[155] β-naphthylamine,[156] aniline,[157, 158, 159] *N*-methyl-*p*-amino-phenol,[160] *p*-methyl-amino-phenol-sulfate[160] or others. A yellowish green color develops which is measured in one of the usual apparatus and can be extracted with amyl-alcohol.[154] The color can be stabilized by a phosphate buffer of pH 6.1.[161]

[148] C. E. Bills, F. G. McDonald and T. D. Spies, *Southern Med. J.*, **32**, 793 (1939).
[150] T. D. Spies, A. A. Walker and A. W. Woods, *J. Am. Med. Assoc.*, **113**, 1481 (1939).
[151] F. C. Schmelkes, *Science*, **90**, 113 (1939).
[152] H. A. Waisman, O. Mickelsen, J. M. McKibbin and C. A. Elvehjem, *J. Nutrition*, **19**, 483 (1940).
[153] W. König, *J. prakt. Chem.*, **69**, 105 (1904); **70**, 19 (1904).
[154] M. Swaminathan, *Nature*, **141**, 830 (1938). H. v. Euler, F. Schlenk, H. Heiwinkel and B. Högberg, *Z. physiol. Chem.*, **256**, 208 (1938). G. E. Shaw and C. A. Macdonald, *Quart. J. Pharm. Pharmacol.*, **11**, 380 (1938). E. Bandier and J. Hald, *Biochem. J.*, **33**, 264 (1939).
[155] L. J. Harris and W. D. Raymond, *J. Soc. Chem. Ind.*, **58**, 652 (1939).
[156] H. v. Euler, F. Schlenk, H. Heiwinkel and B. Högberg, *Z. physiol. Chem.*, **256**, 208 (1938).
[157] H. Kringstad and T. Naess, *Naturwissenschaften*, **43**, 709 (1938); *Z. physiol. Chem.*, **260**, 108 (1939).
[158] M. Swaminathan, *Nature*, **141**, 830 (1938); *Indian J. Med. Research*, **26**, 427 (1938).
[159] D. Melnick and H. Field, *J. Biol. Chem.*, **134**, 1 (1940); **135**, 53 (1940).
[160] E. Bandier and J. Hald, *Biochem. J.*, **33**, 264 (1939). E. Bandier, *Ibid.*, **33**, 1130 (1939).
[161] H. Kringstad and T. Naess, *Naturwissenschaften*, **26**, 709 (1938); *Z. physiol. Chem.*, **260**, 108 (1939).

It has been stated that the color developed with *p*-amino-acetophenone is more suited for quantitative determinations of minute amounts of nicotinic acid than the color from any of the other investigated amines. About 1 mg. of nicotinic acid in a 1 g. sample can be recognized by this procedure.[162]

The cyanogen-bromide method is not specific for nicotinic acid. A number of other pyridine derivatives and especially derivatives of nicotinic acid, such as trigonelline, nicotinuric acid and nicotine, give similar color tests.[163]

2. The 2,4-Dinitro-chlorobenzene Method.[164] The material to be analyzed according to this method should contain only the free nicotinic acid or its amide. In most cases, therefore, at least a partial hydrolysis must be carried out. The dry material is then fused with 2,4-dinitro-chlorobenzene and the reaction product is dissolved in alcohol and potassium hydroxide is added. The color developed is measured colorimetrically.[165, 166]

Neither of these two methods is specific for nicotinic acid as the same colors are given by many pyridine derivatives. During systematic studies of the nicotinic acid content of various plant and animal materials it has been observed that the materials, especially cereals, give color reactions with these two methods in amounts which cannot be explained on the basis of the amount of the nicotinic acid actually present.[167] Further inaccuracies arise from procedures in which nicotinic acid is extracted from other undesirable compounds and from attempts to prepare colorless extracts as is necessary for the determination of nicotinic acid in urine and in blood (see p. 247).

For the determination of nicotinic acid in tablets and in ampules, a tentative A. O. A. C. method (Association of Official Agricultural Chemists) has been worked out[168] which consists in subliming preferentially the nicotinic acid out of the sample and by weighing or titrating[169] the sublimate.

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[162] E. Kodicek, *Biochem. J.*, **34**, 712, 724 (1940).

[163] D. Melnick, W. D. Robinson and H. Field, *J. Biol. Chem.*, **136**, 131 (1940).

[164] E. Vongerichte, *Ber.*, **32**, 2571 (1899).

[165] P. Karrer and H. Keller, *Helv. Chim. Acta*, **21**, 463, 1170 (1938). S. P. Vilter, T. D. Spies and A. P. Mathews, *J. Biol. Chem.*, **125**, 85 (1938); *J. Am. Chem. Soc.*, **60**, 731 (1938).

[166] H. v. Euler, F. Schlenk, L. Melzer and B. Högberg, *Z. physiol. Chem.*, **258**, 212 (1939). P. Karrer and H. Keller, *Helv. Chim. Acta*, **22**, 1292 (1939).

[167] E. Kodicek, *Biochem. J.*, **34**, 712, 724 (1940).

[168] *Methods of Analysis*, Assoc. Official Agricultural Chemists, 1940, p. 610. *J. Assoc. Official Agr. Chem.*, **23**, 58 (1940).

[169] P. S. Jorgensen, *J. Assoc. Official Agr. Chem.* **23** 765 (1940).

Nicotinic acid is separated from its amide by ether extraction of a water solution. Only the amide is extracted by ether.

(*b*) *Biochemical Methods*

Lactobacillus Test. In this test the amount of lactic acid produced by the *Lactobacillus arabinosus* is measured. When adequately supplied with all necessary growth factors, the quantity of lactic acid produced is directly proportional to the amount of nicotinic acid present.[170] This method has several advantages over the chemical methods of nicotinic acid assay. Turbidity and color of the sample to be tested do not interfere with the determination. Nicotinic acid and its amide have equal activities. The sensitivity of this test is greater than that of the chemical tests. One γ or less can be determined accurately.

(*c*) *Biological Methods*

None of the proposed biological methods listed below has been used extensively enough to claim quantitative determinations under all conditions. In earlier qualitative experiments, dogs proved very valuable as test animals and it seems that some of the microorganisms will serve in the future for quantitative determinations. The following methods have been suggested:

1. Dog test.[171, 172, 173, 174]
2. Chick test.[172]
3. Moth test (*Galleria melonella*).[175]
4. Microorganism growth tests on *Staphylococcus aureus*,[176, 177, 178] *Shigella paradysenteriae*[179] and *Bacterium proteus*.[180]

[170] E. E. Snell and L. D. Wright, *Proc. Am. Soc. Biol. Chem.*, **1941**, CXIX.

[171] C. A. Elvehjem, R. J. Madden, F. M. Strong and D. W. Woolley, *J. Am. Chem. Soc.*, **59**, 1767 (1937); *J. Biol. Chem.*, **123**, 137 (1938).

[172] C. J. Koehn and C. A. Elvehjem, *J. Biol. Chem.*, **118**, 693 (1937).

[173] J. Goldberger, G. A. Wheeler, R. D. Lillie and L. M. Rogers, *U. S. Pub. Health Service Pub. Health Repts.*, **43**, 1385 (1928).

[174] W. H. Sebrell, *Ibid.*, **49**, 754 (1934). W. H. Sebrell, G. A. Wheeler and D. J. Hunt, *Ibid.*, **50**, 1333 (1935).

[175] D. Rubinstein and L. Shekun, *Nature*, **143**, 1064 (1939).

[176] M. Landy, *Ibid.*, **142**, 618 (1938).

[177] B. C. J. G. Knight and H. McIlwain, *Biochem. J.*, **32**, 1241 (1938).

[178] B. C. J. G. Knight, *Ibid.*, **31**, 731 (1937).

[179] H. F. Frazer, N. H. Topping and W. H. Sebrell, *U. S. Pub. Health Service Pub. Health Repts.*, **53**, 1836 (1938).

[180] A. Lwoff and A. Querido, *Compt. rend. soc. biol.*, **129**, 1039 (1938).

15. Standard of Nicotinic Acid and Nicotinamide

Up to the present time no national or international standard has been set up for nicotinic acid or nicotinamide. There is actually little need for such a standard, since nicotinic acid and its amide are well-known crystallized compounds which are used on the weight basis.

16. Physiology of Plants and Microorganisms

Nicotinic acid amide is a normal cell constituent and growth factor for plants and microorganisms. It is generally assumed that the coenzyme function in reversible oxidation-reduction systems represents the main activity of nicotinic acid, but some evidences have been brought forward which indicate that nicotinic acid or its amide may have an additional function outside the coenzyme linkages (see page 245).

The actual need of plants for nicotinic acid has been demonstrated on isolated pea embryos and isolated pea, radish and cosmos roots.[181, 182] Among the microorganisms are certain species which synthesize their own nicotinic acid requirements while others depend upon an external supply of this essential nutrient. Among the organisms which need nicotinic acid as a regular food constituent are certain bacteria such as *B. diphtheriae*,[183] *B. dysenteriae*,[184] *B. proteus*,[185] *Staphylococcus aureus*,[186, 187, 188] lactic acid bacteria,[189] protozoa such as Protista[188] and certain fungi.[188] Still more parasitic organisms need an external supply of the codehydrogenases for proper growth which is not induced by the administration of the components of the coenzyme. The *B. influenzae* belongs to this group.[190]

17. Animal Physiology

General Physiology, Metabolism and Mechanism of the Vitamin Action

The normal dietary intake of nicotinic acid consists mostly of the coenzymes which are present in food of plant and animal origin. It is assumed that the enzyme complex is split in the intestinal tract but it is uncertain if the coenzymes can be absorbed as such or if they are hy-

[181] J. Bonner, *Plant Physiol.*, **13**, 865 (1938). F. T. Addicott and J. Bonner, *Science*, **88**, 577 (1938).
[182] J. Bonner, *Am. Chem. Soc., Div. Agr. Food Chem., Meeting*, **Sept. 1939**, Abst. 13–14.
[183] J. H. Mueller, *J. Bact.*, **34**, 429 (1937); *J. Biol. Chem.*, **120**, 219 (1937).
[184] S. A. Koser, A. Dorfman and F. Saunders, *Proc. Soc. Exptl. Biol. Med.*, **38**, 311 (1938); *Science*, **90**, 544 (1939).
[185] P. Fildes, *Brit. J. Exptl. Path.*, **19**, 239 (1938).
[186] B. C. J. G. Knight, *Biochem. J.*, **31**, 741 (1937).
[187] M. Landy, *Nature*, **142**, 618 (1938).
[188] B. C. J. G. Knight and H. McIlwain, *Biochem. J.*, **32**, 1241 (1938).
[189] E. E. Snell, F. M. Strong and W. H. Peterson, *J. Am. Chem. Soc.*, **60**, 2825 (1938).
[190] R. W. Vilter, S. P. Vilter and T. D. Spies, *J. Am. Med. Assoc.*, **112**, 420 (1939).

drolyzed prior to the absorption. Nicotinic acid and its amide are absorbed unchanged. Nicotinic acid is amidated in the organism after absorption by the blood stream. The nicotinic acid and its amide are transported in the blood serum, which maintains a certain level of this essential compound, but contains no coenzymes. The blood corpuscles, on the other hand, contain relatively high concentrations of the coenzymes but no free nicotinamide.

There are no special storage organs for nicotinic acid or any of its derivatives. In the form of the coenzymes, nicotinic acid is present in practically all cells. While nicotinic acid deficiency in the dog and pig results in a lowered coenzyme content of the liver and muscles,[191, 192, 193] no substantial effect has been noted upon the coenzyme content of the brain, kidney cortex and blood. In human beings, nicotinic acid deficiency causes a marked decrease in the coenzyme content of striated muscles but has only a slight effect upon the coenzyme content of the erythrocytes.[194]

Nicotinic acid is, like all the other vitamins, secreted in milk and is found in eggs.

Nicotinic acid and its metabolic end-products are excreted through the urine. The normal human organism excretes a certain amount in the free form. Coenzymes are not excreted.[195, 196] Ingested nicotinic acid is excreted in considerable amounts in the form of nicotinuric acid and trigonelline. There is, furthermore, apparently a combined form of nicotinic acid in the urine[197] which may be different from nicotinuric acid. The total output depends upon the intake. Normally about 4–5 mg. are excreted daily.[198] Lowered values have been observed in pellagrins and during times of anorexia. Guinea pigs deprived of nicotinic acid show a progressive decrease of nicotinic acid excretion which reaches a zero value at the time when clinical deficiency symptoms develop. Dogs excrete only trigonelline and nicotinuric acid but no nicotinic acid or its amide.[199] Rabbits, on the other hand, cannot synthesize trigonelline from nicotinic acid.[200]

Nicotinic acid appears to function mainly as a part of enzyme systems which have been discussed previously and which take part in the protein

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[191] A. E. Axelrod, R. J. Madden and C. A. Elvehjem, *J. Biol. Chem.*, **131**, 85 (1939).
[192] H. I. Kohn, J. R. Klein and W. J. Dann, *Biochem. J.*, **33**, 1432 (1939).
[193] M. Pittman and H. F. Frazer, *U. S. Pub. Health Service Pub. Health Repts.*, **55**, 915 (1940).
[194] A. E. Axelrod, T. D. Spies and C. A. Elvehjem, *J. Biol. Chem.*, **138**, 667 (1941).
[195] R. W. Vilter, S. P. Vilter and T. D. Spies, *J. Am. Med. Assoc.*, **112**, 420 (1939).
[196] H. v. Euler, F. Schlenk, H. Heiwinkel and B. Högberg, *Z. physiol. Chem.*, **256**, 208 (1938).
[197] E. Bandier, *Biochem. J.*, **33**, 1787 (1939).
[198] L. J. Harris and W. D. Raymond, *J. Soc. Chem. Ind.*, **58**, 652 (1939).
[199] D. Ackermann, *Z. Biol.*, **59**, 17 (1912).
[200] W. A. Perlzweig, H. P. Sarett and J. W. Huff, *Proc. Am. Soc. Biol. Chem.*, **1941**, C.

and the carbohydrate metabolism by transporting hydrogen. At birth the tissues of mammals contain only small amounts of the nicotinamide coenzymes I and II (about 100–150 γ per gram of liver or kidney in rats), but the amount rises rapidly and reaches the normal range of adults (about 550 γ in rats) in about seven days.[201]

There is the possibility that nicotinic acid may act in other functions than through the coenzymes I and II. Thus, nicotinic acid deficiency causes water retention and may therefore be linked with the water metabolism.[202] This effect could, however, be understood as a side reaction of the disturbance of the carbohydrate metabolism. Nicotinic acid also influences the metabolism of the heavy metals. Also in this case, as in the influence upon the water metabolism, no decision can be made on the basis of the experimental facts as to whether this is a primary action of nicotinic acid, its amide or the coenzymes, or if these disturbances are secondary reactions. A pigmentation in the skin and the mucous membranes of the mouth is often observed in a human being suffering from a nicotinic acid deficiency. The pigment is not melanin but an iron pigment.[203] As the result of a nicotinic acid deficiency a type of anemia occurs which responds favorably to iron therapy. The iron-containing porphyrins undergo some kind of decomposition and as the result of this an increased amount of porphyrin-compounds is secreted through the urine.[204] The copper- and iron-containing enzyme systems such as cytochrome, the polyphenol-oxidases, the peroxidase and catalase, appear to be affected. Thus, for example, a disturbance of the tryptophane catabolism has been observed in pellagrins which resulted in the excretion of indoxyl-ethyl-amin.[205] The disturbances of the sympathetic nervous system could perhaps be explained as a disturbed relation of the polyphenol oxidases to adrenalin.

Nicotinic acid decreases the peristaltic action of the stomach and small intestine, while inositol (see page 279) increases this action. The other members of the B-complex have no apparent action of this type. It has therefore been suggested that the balance or ratio of nicotinic acid to inositol is the nutritional factor which determines hypo- or hyper-gastrointestinal motility.[206]

There are observations which indicate functions of nicotinic acid which cannot be explained on the basis that nicotinic acid acts only through the

[201] F. Bernheim and A. v. Felsovanyi, *Science*, **91**, 76 (1940).
[202] C. Funk and I. C. Funk, *Z. Vitaminforsch.*, **8**, 330 (1938).
[203] H. Herzenberg, *Beitr. path. Anat.*, **96**, 97 (1935).
[204] T. D. Spies, W. B. Bean and R. E. Stone, *J. Am. Med. Assoc.*, **111**, 584 (1938).
[205] M. X. Sullivan, *J. Biol. Chem.*, **50**, 39 (1922).
[206] G. J. Martin, M. R. Thompson and J. de Carvajal-Forero, *Am. J. Digestive Dis.*, **8**, 290 (1941).

coenzymes. Blacktongue in dogs can be cured easily by nicotinic acid, but no significant effect could be noted when cozymase was injected intravenously in an amount which on the basis of its nicotinic acid content would have been expected to exert beneficial effects.[206] Growth and respiration of dysentery bacilli are much more favorably influenced by nicotinic acid or its amide than by the coenzymes, and the efficacy of the action of the coenzymes can be increased markedly by hydrolysis under conditions which free the nicotinamide.[207, 208]

18. Avitaminosis

There are no clinical symptoms known for slight nicotinic acid deficiency in man. Definite diagnostic evidences develop late.[209] In severe cases the blood level of nicotinic acid is decreased. This has frequently been observed[210] in pregnant women on diets low in nicotinic acid, since during pregnancy increased amounts of this vitamin are needed. Another relatively early symptom of nicotinic acid deficiency is the excretion of porphyrins in the urine.[211]

The typical symptoms of nicotinic acid deficiency in man are commonly summarized in the term pellagra. The typical pellagrin shows characteristic lesions of the mucous membranes, for example, in the mouth (glossitis), and of the skin over the nose, forehead, dorsum, hands, wrists, elbows, knees and feet. This type of dermatitis involves especially those parts of the body which are exposed to sunlight or to friction. Disturbances of the gastrointestinal tract are observed in many cases. In later stages of the disease, mental disorders and lesions of the central nervous system occur, which are characterized by clouding of consciousness, cogwheel rigidities and uncontrollable grasping and sucking reflexes.[212] A certain form of anemia has been observed in severe cases. Diagnosis of pellagra is sometimes very difficult since the symptoms described do not necessarily occur all at once.

Typical pellagra is, however, not a disease caused solely by a nicotinic acid deficiency. Usually pellagra is the result of a multiple vitamin deficiency and can be cured only by the administration of several or all

[207] F. S. Daft, H. F. Frazer, W. H. Sebrell and M. Pittman, *Science*, **88**, 128 (1938).

[208] A. Dorfman, S. A. Koser, H. R. Reames, K. F. Swingle and F. Saunders, *Proc. Soc. Exptl. Biol. Med.*, **43**, 163 (1940). F. Saunders, A. Dorfman and S. A. Koser, *J. Biol. Chem.*, **138**, 69 (1941). L. C. Norris and A. T. Ringrose, *Science*, **71**, 643 (1930).

[209] M. A. Blankenhorn and T. D. Spies, *J. Am. Med. Assoc.*, **108**, 589 (1937).

[210] A. Lwoff, A. Querido, L. Digonnet and Garmier, *Compt. rend. soc. biol.*, **131**, 900 (1939).

[211] T. D. Spies, W. B. Bean and R. E. Stone, *J. Am. Med. Assoc.*, **111**, 584 (1938).

[212] N. Jollifee, K. M. Bowman, L. A. Rosenblum and H. D. Fein, *Ibid.*, **114**, 307 (1940).

members of the vitamin B-complex. Besides nicotinic acid, riboflavin and thiamin therapy are especially necessary.[213, 214]

Nicotinic acid treatment has been observed to give beneficial results to pneumonia patients who under sulfanilamide therapy have developed a black-dotted heavy furring of the tongue which resembles the blacktongue of dogs.[215]

The lesions in the mouth caused by nicotinic acid deficiency sometimes are very similar in appearance to the lesions of Vincent's infection but are genetically different. It seems that Vincent's infection frequently sets in when the mucous membranes are weakened due to nicotinic acid deficiency and in such cases a treatment with nicotinic acid is, of course, beneficial.[216] Encouraging results have been obtained by administration of nicotinic acid for the prevention and treatment of irradiation (x-ray) sickness.[217] Furthermore, it has been suggested[218] that nicotinic acid may be of value in the treatment of eighth nerve (high tone) deafness.

In dogs, the typical syndrome of nicotinic acid deficiency is the occurrence of blacktongue. In adrenalectomized rats a special form of dermatitis has been noted which responds favorably to treatment with nicotinic acid.[219]

(*a*) *Clinical Test Methods*

The methods which have been suggested for the determination of a state of nicotinic acid deficiency comprise the determination of the amount excreted in urine or present in blood. In addition, the occurrence of porphyrins in the urine can be used as an additional test method.

Urine Tests. The determination of the excretion of nicotinic acid is difficult since urine contains this compound not only in the free form but also in the form of various derivatives, mainly as nicotinamide, nicotinuric acid and trigonelline. In order to obtain uniform results, the urine must be hydrolized, preferably with alkali.[220] The color present in urine invariably interferes with colorimetric determinations for the nicotinic acid content. Preferential adsorption of the colored materials on

[213] G. Margolis, L. H. Margolis and S. G. Smith, *J. Nutrition*, **16**, 541 (1938); **17**, 63 (1939). O. M. Helmer and P. J. Fouts, *Ibid.*, **16**, 271 (1938). W. H. Sebrell, *J. Am. Med. Assoc.*, **110**, 1665 (1938). R. W. Vilter, S. P. Vilter and T. D. Spies, *Ibid.*, **112**, 420 (1939).

[214] R. W. Vilter, S. P. Vilter and T. D. Spies, *J. Am. Med. Assoc.*, **112**, 420 (1939).

[215] E. M. Josephson and G. Klewan, *Nature*, **143**, 725 (1939).

[216] W. Sophie, *Am. J. Digestive Diseases Nutrition*, **7**, 298 (1940). J. D. King, *Lancet*, **2**, 32 (1940).

[217] J. W. Graham, *J. Am. Med. Assoc.*, **113**, 664 (1939).

[218] G. Selfridge, *Ann. Otol. Rhin. Laryng.*, **48**, 39 (1939).

[219] L. Laszt, *Z. Vitaminforsch.*, **11**, 76 (1941).

[220] E. Bandier, *Biochem. J.*, **33**, 1787 (1939).

charcoal or on zinc hydroxide[221] has been suggested. None of the color reactions can be considered reliable since urine contains substances which interfere with the determination. As the result of these difficulties, only approximate values can be expected from the determination of nicotinic acid in urine. For the actual test, the *color reaction with 2,4-dinitrochlorobenzene* has been modified for urine analysis.[222] The hydrolyzed and decolorized urine is evaporated and the dry residue is mixed with 2,4-dinitro-chlorobenzene in alcohol, and after standing for several hours the alcohol is distilled off and the residue is heated to 105° C. for 10 minutes. After cooling, a solution of KOH in alcohol is added. A purple color develops which can be determined by the usual methods. The *cyanogen-bromide method* has frequently been used for the determination of nicotinic acid in urine and a number of slight modifications of the basic technic have been recommended.[223, 224, 225, 226] Both of these methods are unreliable because other compounds, for example, nicotine and trigonelline, give similar color reactions.[227]

A somewhat better method for the determination of a deficiency is to perform an *excretion test* on the patient.[228] Excess doses of nicotinic acid or its amide are given and the amount excreted is determined before and after the ingestion. The total amount of compounds which behave like nicotinic acid in the color tests varies considerably in normal persons[229] and is dependent on many factors. Thus, smokers usually excrete increased amounts of chromogenic material. Upon oral administration of, for example, 500 mg. of nicotinic acid to normal individuals, the urinary output rises to its maximum within one hour and drops to normal in about four hours. In a series of experimental tests it was found that from the ingested 500 mg. about 110 mg. are excreted by normal persons. Of this total amount about 51% is present as trigonelline, 36% as nicotinuric acid and 13% as free nicotinic acid or nicotinamide. Coenzymes are normally not excreted.

A state of nicotinic acid deficiency can also be suspected from the *excretion of porphyrins through the urine*, but it should be remembered that

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[221] T. E. Friedemann and C. J. Barborka, *J. Biol. Chem.*, **138**, 787 (1941).

[222] P. Karrer and H. Keller, *Helv. Chim. Acta*, **21**, 463, 1170 (1938). S. P. Vilter, T. D. Spies and A. P. Mathews, *J. Biol. Chem.*, **125**, 85 (1938); *J. Am. Chem. Soc.*, **60**, 731 (1938).

[223] E. Bandier, *Biochem. J.*, **33**, 1787 (1939).

[224] L. J. Harris and W. D. Raymond, *Ibid.*, **33**, 2037 (1939).

[225] D. Melnick and H. Field, *J. Biol. Chem.*, **134**, 1 (1940).

[226] L. A. Rosenblum and N. Jolliffe, *Ibid.*, **134**, 137 (1940).

[227] D. Melnick, W. D. Robinson and H. Field, *Ibid.*, **136**, 131 (1940).

[228] D. Melnick, W. D. Robinson and H. Field, *Ibid.*, **136**, 145 (1940).

[229] D. Melnick, W. D. Robinson and H. Field, *Ibid.*, **136**, 157 (1940).

porphyruria may have other causes than a nicotinic acid deficiency. In the actual test, the urine is acidified with acetic acid and extracted with ether. The ether solution is washed with water and then with 25% hydrochloric acid. The latter causes the appearance of a color, which indicates the presence of porphyrins.[230]

Blood Tests. Blood contains both nicotinic acid (or its amide) and the codehydrogenases. The latter are almost completely confined to the blood corpuscles,[231, 232, 233] while the serum contains only the free nicotinic acid (or its amide). Of the total amount, about 90% is in the erythrocytes and this amount decreases only very slightly at times of nicotinic acid deficiency. The small amount of free nicotinic acid present is a function of the intake. Upon oral administration of nicotinic acid to fasting persons the blood level increases promptly but returns rapidly to values only slightly higher than the basal level.[233] The total nicotinic acid content of the blood of normal persons ranges between 0.52 and 0.83 mg. %.[233]

The actual determination of the nicotinic acid (or its amide) can be carried out by means of the *cyanogen-bromine method*,[234, 235, 236, 237, 238] by the *Bacillus proteus growth method*[239] and the codehydrogenase I can be determined by the *fermentation method*.[240]

19. Hypervitaminosis

The intake of about 1000 times the amount of nicotinic acid which is normally consumed in food may be considered as relatively non-toxic. Higher doses, however, exhibit typical toxicological symptoms. Dogs kept at a daily intake of 2 g. of nicotinic acid died within twenty days. In human beings, the oral administration of large quantities of nicotinic acid is followed by flushing, burning, itching and increased sensations of local heat in the skin. Nicotinic acid amide does not produce these symptoms,[241] and is, therefore, recommended for clinical use.

[230] T. D. Spies, R. W. Vilter and W. F. Ashe, *J. Am. Med. Assoc.*, **113**, 931 (1939).

[231] A. Dorfman, M. K. Horwitt, S. A. Koser and F. Saunders, *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **128**, XX (1939).

[232] H. I. Kohn and J. R. Klein, *J. Biol. Chem.*, **130**, 1 (1939).

[233] D. Melnick, W. D. Robinson and H. Field, *Ibid.*, **136**, 157 (1940).

[234] D. Melnick and H. Field, *Ibid.*, **134**, 1 (1940); **135**, 53 (1940).

[235] M. Swaminathan, *Indian J. Med. Research*, **26**, 427 (1938).

[236] P. B. Pearson, *J. Biol. Chem.*, **129**, 491 (1939).

[237] K. Ritsert, *Klin. Wochschr.*, **18**, 934 (1939).

[238] T. D. Spies, A. A. Walker and A. W. Woods, *J. Am. Med. Assoc.*, **113**, 1481 (1939).

[239] A. Querido, M. Albeaux-Fernet and A. Lwoff, *Compt. rend. soc. biol.*, **131**, 182 (1939). A. Querido, A. Lwoff and C. Lataste, *Ibid.*, **130**, 1580 (1939).

[240] A. E. Axelrod and C. A. Elvehjem, *J. Biol. Chem.*, **131**, 77 (1939).

[241] T. D. Spies, D. P. Hightower and L. H. Hubbard, *J. Am. Med. Assoc.*, **115**, 292 (1940).

20. Nicotinic Acid Requirements

It is established that all living tissues need nicotinic acid. Some organisms need an external supply of this vitamin while others are able to synthesize it.

The daily requirements of man are of the order of 12 23 mg. (Details see page 613). Therapeutically, nicotinic acid has been given orally in daily doses ranging from 50 to 500 mg. Monkeys apparently need about 5 mg. daily.[242] The necessity of a regular intake of nicotinic acid has also been proved for dogs,[243] pigs[244] and the moth, *Galleria melonella*.[245]

Certain species of microorganisms are able to synthesize nicotinic acid, such as, for example, *Chilomonas parameciuni*,[246] while other species, such as *Bacterium pneumococcus*,[247] need an external supply of nicotinic acid for proper growth.[248, 249] Some other unicellular organisms, for example, the *Bacillus influenzae*,[250] do not grow upon administration of nicotinic acid or its amide, but need codehydrogenase I or II. Some of the microorganisms which are able to synthesize nicotinic acid have been shown to live in the intestinal tract of higher animals, such as, for example, in sheep[251] and in cattle.[252]

The nicotinic acid requirement of the rat has been the subject of extensive discussion. While it is certain that the rat needs this vitamin,[253, 254, 255] the question as to whether or not the rat must have nicotinic acid in its daily food intake is not settled. It has been suggested that the rat is able to synthesize its own supply,[256] but it seems more plausible to assume that if rats do not need to have nicotinic acid in their food, they have microorganisms living in their intestinal tract which synthesize this vitamin and from which the rats obtain the necessary amount.

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[242] L. J. Harris, *Biochem. J.*, **32**, 1479 (1938).

[243] C. A. Elvehjem, R. J. Madden, F. M. Strong and D. W. Woolley, *J. Am. Chem. Soc.*, **59**, 1767 (1937); *J. Biol. Chem.*, **123**, 137 (1938).

[244] H. Chick, T. F. Macrae, A. J. P. Martin and C. J. Martin, *Biochem. J.*, **32**, 10 (1938).

[245] D. Rubinstein and L. Shekun, *Nature*, **143**, 1064 (1939).

[246] J. O. Hutchens, B. J. Jandorf and A. B. Hastings, *J. Biol. Chem.*, **138**, 321 (1941).

[247] L. Rane and Y. Subbarow, *Ibid.*, **134**, 455 (1940).

[248] B. C. J. G. Knight, *Biochem. J.*, **31**, 731 (1937).

[249] J. H. Mueller, *J. Bact.*, **34**, 429 (1937); *J. Biol. Chem.*, **120**, 219 (1937).

[250] A. Lwoff and M. Lwoff, *Proc. Roy. Soc.* (*London*), **B122**, 352, 360 (1937); *Compt. rend.*, **203**, 520 (1936).

[251] A. H. Winegar, P. B. Parson and H. Schmidt, *Science*, **91**, 508 (1940).

[252] M. I. Wegner, A. N. Booth, C. A. Elvehjem and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.*, **45**, 769 (1940).

[253] D. V. Frost and C. A. Elvehjem, *J. Biol. Chem.*, **121**, 255 (1937). P. György, *Proc. Soc. Exptl. Biol. Med.*, **37**, 732 (1938). W. R. Wyatt, *Iowa State Coll. J. Sci.*, **14**, 103 (1939).

[254] H. v. Euler, F. Schlenk, L. Melzer and B. Högberg, *Z. physiol. Chem.*, **258**, 212 (1939).

[255] L. Laszt, *Z. Vitaminforsch.*, **11**, 76 (1941).

[256] W. J. Dann and H. I. Kohn, *J. Biol. Chem.*, **136**, 435 (1940).

PANTOTHENIC ACID

PANTOTHENIC ACID

1. Nomenclature and Survey

Names:

Pantothenic acid[1] (the name is derived from the Greek meaning "from everywhere").
Pantothen: Abbreviated term.
Antidermatosis vitamin.[2]
Chick antidermatitis factor.[3]
Vitamin B_x.[4]
Filtrate factor (liver filtrate factor,[5] yeast filtrate factor[6]).
Factor 2 (from liver).[7]
Chick A-P factor (Chick Anti-pellagra factor):[8] The term "pellagra" is properly used only in describing human diseases (in compliance with the Committee on Vitamin Nomenclature).
Vitamin B_2 (G)[9] (abandoned, historical name).

Probably also identical with:

Vitamin B_3: Vitamin B_3 is a term given in 1928 to a heat-labile factor, necessary for "weight maintenance of pigeons."[10, 11] Recent findings point to the identity of this vitamin with pantothenic acid.[12]

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[1] R. J. Williams, C. M. Lyman, G. H. Goodyear, T. H. Truesdail and D. Holaday, *J. Am. Chem Soc.*, **55**, 2912 (1933).

[2] J. C. Bauernfeind, A. E. Schumacher, A. Z. Hodson, L. C. Norris and G. F. Heuser, *Proc. Soc. Exptl. Biol. Med.*, **39**, 108 (1938).

[3] O. Mickelsen, H. A. Waisman and C. A. Elvehjem, *J. Biol. Chem.*, **124**, 313 (1938). D. W. Woolley, H. A. Waisman, O. Mickelsen and C. A. Elvehjem, *Ibid.*, **125**, 715 (1938).

[4] A. Bakke, V. Aschehoug and C. Zbinden, *Compt. rend.* **191**, 1157 (1930). A. F. Morgan, B. B. Cook and H. G. Davison, *J. Nutrition*, **15**, 27 (1938). A. F. Morgan and H. D. Simms, *Ibid.*, **19**, 233 (1940). G. Lunde and H. Kringstad, *Arch. Norske Vid. Akad. Oslo*, I. Math. Naturw. Klasse, No. 1, 1 (1938); *Z. physiol. Chem.* **257**, 201 (1939); **261**, 110 (1939); *Naturwissenschaften*, **27**, 755 (1939); *Angew. Chem.* **52**, 521 (1939). G. Lunde, H. Kringstad and E. Jansen, *Naturwissenschaften*, **29**, 62 (1941).

[5] T. H. Jukes and S. Lepkovsky, *J. Biol. Chem.*, **111**, 119 (1935); **114**, 109, 117 (1936).

[6] C. E. Edgar and T. F. Macrae, *Biochem. J.*, **31**, 886, 893 (1937).

[7] S. Lepkovsky, T. H. Jukes and M. E. Krause, *J. Biol. Chem.*, **115**, 557 (1936).

[8] C. J. Koehn and C. A. Elvehjem, *Ibid.*, **118**, 693 (1937).

[9] C. A. Elvehjem and C. J. Koehn, *Nature*, **134**, 1007 (1934); *J. Biol. Chem.*, **108**, 709 (1935). These authors suggested that the chick pellagra preventative factor be called vitamin B_2 or G in differentiation from riboflavin. Since, however, this vitamin is now called vitamin B_2 or G the utilization of these letters to designate pantothenic acid has been abandoned. For a discussion of this nomenclature see also ref. 5 and C. J. Koehn and C. A. Elvehjem, *J. Nutrition*, **11**, 67 (1936).

[10] R. R. Williams and R. E. Waterman, *J. Biol. Chem.*, **78**, 311 (1928).

[11] C. W. Carter and J. R. O'Brien, *Biochem. J.*, **30**, 43 (1936).

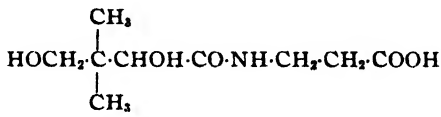
[12] C. W. Carter and J. R. O'Brien, *Ibid.*, **33**, 1810 (1939).

The "spectacled eye condition" preventive factor for rats: The "spectacled eye condition" has been described[13] as an erosion occurring around the eyes of rats, and imparting to the animals a spectacled appearance. The lids became denuded and scaly. In more severe cases the eyes are closed by a sticky exudate. Since concentrates of pantothenic acid brought about a rapid cure of the spectacled eye condition, it is supposed[14] that the two factors may be identical. (See, however, pages 280 and 477.)

Empirical formula:

$C_9H_{17}O_5N$.

Structural formula:



Chemical name:

(+)-α,γ-Dihydroxy-β,β-dimethyl-butyryl-β'-alanide.

Efficacy:

1 g. of pantothenic acid = 70,000–75,000 Chick Units.

2. Chronology

1930 NORRIS and RINGROSE[15] described a specific dermatosis of chicks.

1933 WILLIAMS and co-workers[16] found that a naturally occurring compound of unknown chemical composition, called "pantothenic acid," stimulates the growth of yeast.

1934–1935 ELVEHJEM and KOEHN[17] differentiated the chick antidermatitis factor from riboflavin and cured the chick syndrome with liver extract.

1936 LEPKOVSKY, JUKES and KRAUSE showed the need of rats for a factor distinct from thiamin, riboflavin and vitamin B_6.[18]

1937 FOUTS, LEPKOVSKY, HELMER and JUKES[19] and independently DANN[20] stated that nicotinic acid does not cure or prevent chicken dermatitis.

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[13] J. Goldberger and R. D. Lillie, *U. S. Pub. Health Service Pub. Health Repts.*, **41**, 1025 (1926). A. Bourquin and H. C. Sherman, *J. Am. Chem. Soc.*, **53**, 3501 (1931). H. E. Robinson and R. C. Newton, Abstracts, Division of Biological Chemistry, Am. Chem. Soc., Kansas City, April 13–17 (1936). S. Lepkovsky, T. H. Jukes and M. E. Krause, *J. Biol. Chem.*, **115**, 557 (1936).

[14] J. J. Oleson, H. R. Bird, C. A. Elvehjem and E. B. Hart, *J. Biol. Chem.*, **127**, 23 (1939).

[15] L. C. Norris and A. T. Ringrose, *Science*, **71**, 643 (1930). A. T. Ringrose, L. C. Norris and G. F. Heuser, *Poultry Sci.*, **10**, 166 (1931).

[16] R. J. Williams, C. M. Lyman, G. H. Goodyear, T. H. Truesdail and D. Holaday, *J. Am. Chem. Soc.*, **55**, 2912 (1933).

[17] C. A. Elvehjem and C. J. Koehn, *Nature*, **134**, 1007 (1934); *J. Biol. Chem.*, **108**, 709 (1935).

[18] S. Lepkovsky, T. H. Jukes and M. E. Krause, *J. Biol. Chem.*, **115**, 557 (1936).

[19] P. J. Fouts, S. Lepkovsky, O. M. Helmer and T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.*, **37**, 405 (1937).

[20] W. J. Dann, *Science*, **86**, 616 (1937).

1938 The chemical nature of pantothenic acid was worked out by R. J. WILLIAMS and co-workers.[21]

1939 The identity of pantothenic acid and the chick antidermatitis factor was recognized by Jukes[22] and by WOOLLEY, WAISMAN and ELVEHJEM.[23]

1940 Pantothenic acid was totally synthesized by STILLER, HARRIS, FINKELSTEIN, KERESZTESY and FOLKERS[24] and independently by REICHSTEIN and GRÜSSNER,[25] and by KUHN and WIELAND.[26]

3. Occurrence

Pantothenic acid occurs in all types of animal tissues and seems to be present universally in protoplasm. The liver and kidney are the richest known animal sources of pantothenic acid, followed by heart, spleen, brain, pancreas, tongue and lung;[27] the muscular tissue (of beef, lamb, pork and veal) contains considerably less. Pantothenic acid is produced by various molds and microorganisms[28] and by green plants after they develop their photosynthetic apparatus. The storage organs in plants, for example rice bran, appear to be especially rich in pantothenic acid.[29] Molasses[30] contains considerably more than normal plant tissue, for example, alfalfa.[30]

In animal tissue (and probably also in plant tissue) pantothenic acid occurs free only to a very small extent, but is usually found chemically bound to protein material.[31]

4. Isolation

The isolation of pantothenic acid consists, for example, when using liver as the starting material, of the following steps:[32] First the liver is allowed to autolyze in water. The water suspension is then heated and filtered from coagulated, inactive material. In place of this step alcoholic liver extracts may also be used as starting material. By treatment with fuller's earth much inactive, especially basic, material is removed from the water

[21] R. J. Williams, H. H. Weinstock, E. Rohrmann, J. H. Truesdail, H. K. Mitchell and C. E. Meyer, *J. Am. Chem. Soc.*, **61**, 454 (1939).

[22] T. H. Jukes, *Ibid.*, **61**, 975 (1939).

[23] D. W. Woolley, H. A. Waisman and C. A. Elvehjem, *Ibid.*, **61**, 977 (1939).

[24] E. T. Stiller, S. A. Harris, J. Finkelstein, J. C. Keresztesy and K. Folkers, *Ibid.*, **62**, 1785 (1940).

[25] T. Reichstein and A. Grüssner, *Helv. Chim. Acta*, **23**, 650 (1940).

[26] R. Kuhn and T. Wieland, *Ber.*, **73**, 971, 1134 (1940).

[27] H. A. Waisman, O. Mickelsen and C. A. Elvehjem, *J. Nutrition*, **18**, 247 (1939).

[28] C. H. McBurney, W. B. Bollen and R. J. Williams, *Proc. Natl. Acad. Sci. U. S.*, **21**, 301 (1935).

[29] R. J. Williams and R. Moser, *J. Am. Chem. Soc.*, **56**, 169 (1934).

[30] T. H. Jukes, *J. Biol. Chem.*, **114**, 11 (1937).

[31] R. Kuhn and G. Wendt, *Ber.*, **71**, 780 (1938).

[32] R. J. Williams, J. H. Truesdail, H. H. Weinstock, E. Rohrmann, C. M. Lyman and C. H. McBurney, *J. Am. Chem. Soc.*, **60**, 2719 (1938).

solution. Pantothenic acid is then adsorbed on Norite at a pH of approximately 3.6 and immediately afterwards eluted[32] with ammonium hydroxide solution. A second adsorption on Norite may be carried out[33] followed by elution with a mixture of pyridine and methanol.

After neutralization with oxalic acid, the brucine salt of pantothenic acid is prepared and is extracted with chloroform. The crude brucine salts are fractionated by various distributions between chloroform and water. These salts are converted into the corresponding calcium salts by treating in solution with an excess of lime water and freeing from brucine by filtration and repeated extraction with chloroform. Purification of the calcium pantothenate consists in the precipitation of the active principle with alcohol, removal of some impurities by precipitation with mercuric chloride, fractional precipitation of the calcium salt with isopropyl ether and fractional precipitation from pyridine solution with acetone.

By application of these methods, about 3 g. of crude, approximately 40% pure material were obtained from 250 kg. of liver.[32, 34] A modification[33] of this procedure is to precipitate impurities from the concentrated eluates of the charcoal adsorption with barium hydroxide until a pH of about 8 is reached and to dissolve the precipitated barium salt of pantothenic acid in absolute alcohol. After filtration, the barium salt of the vitamin is obtained by concentrating the filtrate and by adding acetone, thus precipitating the acetone-insoluble barium salts. The free acids are obtained from the barium salts by means of sulfuric acid and can be extracted with ether or amyl alcohol. Thus a concentrate containing approximately 20–25% pantothenic acid is obtained.

A somewhat different method consists[35] in freeing a liver (for example, tuna fish liver) extract from the fat-soluble material and precipitating some impurities with mercuric acetate. The active material is then adsorbed on charcoal and eluted with a pyridine-methanol-water mixture. Impurities are removed from the eluate by precipitation with phosphotungstic acid and uracil-*d*-riboside (uridine) is removed from the concentrated filtrate by crystallization from methanol. The pantothenic acid is then precipitated from the methanol solution with barium hydroxide. Further purification can be achieved by repeated precipitation (of impurities) with phosphotungstic acid. The active compound is then adsorbed on aluminum oxide, which has been activated with hydrochloric acid.

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[32] H. K. Mitchell, H. H. Weinstock, E. E. Snell, S. R. Stanbery and R. J. Williams, *J. Am. Chem. Soc.*, **62**, 1776 (1940).

[34] Y. Subbarow and G. H. Hitchings, *Ibid.*, **61**, 1615 (1939).

[35] R. Kuhn and T. Wieland, *Ber.*, **73**, 962 (1940).

5. Properties

Pantothenic acid is predominantly of acid character but shows also some basic properties.[36, 37] The vitamin is readily soluble in water, ethyl-acetate, dioxane, glacial acetic acid, etc., somewhat soluble in ether and amyl-alcohol[33] and practically insoluble in benzene, chloroform, etc.[38] The compound is highly hydrophilic and can be adsorbed on charcoal[37] but not on fuller's earth.[37, 39] The acetyl-derivative can be distilled at approximately 10^{-5} mm. Hg.[38] The vitamin is sensitive toward acids, bases and heat.

Pantothenic acid in pure form is a pale yellow viscous oil. The vitamin is dextrorotatory showing $[\alpha]_D^{25}$ +37.5°. It forms a microcrystalline calcium salt, $[\alpha]_D^{26}$ +24.3°.[40]

6. Chemical Constitution

Pantothenic acid has a molecular weight of 219 (values obtained experimentally are approximately 200[41, 42]), and has the empirical formula $C_9H_{17}O_5N$.[42] The presence of a carboxyl group in the molecule of this vitamin was proved[42] by esterification with both diazomethane and methanol and recovery of the free acid by careful saponification. Thus two of the five oxygens of the suggested empirical formula are accounted for. Two other oxygens are present in the form of free hydroxyl groups.[43] The presence and number of hydroxyl groups are suggested by the hydrophilic nature of pantothenic acid and of its methyl-ester. The ester is even more soluble in water than in ether. Pantothenic acid is esterified by various acids and acid halides and thereby loses the vitamin activity, which can be recovered by careful saponification. A determination of the active hydrogen atoms suggested the presence of two hydroxyl groups. Further evidence for the existence of at least two hydroxyl groups is based upon the observation that pantothenic acid condenses reversibly with acetaldehyde,

[36] R. J. Williams and D. H. Saunders, *Biochem. J.*, 28, 1887 (1934). O. W. Richards, *J. Biol. Chem.*, 113, 531 (1936).

[37] R. J. Williams, J. H. Truesdail, H. H. Weinstock, E. Rohrmann, C. M. Lyman and C. H. McBurney, *J. Am. Chem. Soc.*, 60, 2719 (1938).

[38] D. W. Woolley, H. A. Waisman, O. Mickelson and C. A. Elvehjem, *J. Biol. Chem.*, 125, 715 (1938).

[39] S. Lepkovsky, T. H. Jukes and M. E. Krause, *Ibid.*, 115, 557 (1936).

[40] E. T. Stiller, S. A. Harris, J. Finkelstein, J. C. Keresztesy and K. Folkers, *J. Am. Chem. Soc.*, 62, 1785 (1940).

[41] R. J. Williams, C. M. Lyman, G. H. Goodyear, T. H. Truesdail and D. Holaday, *Ibid.*, 55, 2912 (1933).

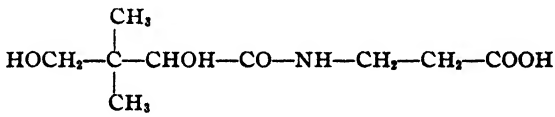
[42] R. J. Williams, H. H. Weinstock, E. Rohrmann, J. H. Truesdail, H. K. Mitchell and C. E. Meyer, *Ibid.*, 61, 454 (1939).

[43] R. J. Williams and R. Moser, *J. Am. Chem. Soc.*, 56, 169 (1934).

acetone and benzaldehyde. This type of reaction suggests the presence of an α,β-, α,γ-or α,δ-glycol. The behavior of pantothenic acid in an electrical field[43] indicated the ionization constant to be approximately 3.9×10^{-5}. This corresponds in strength approximately to that of a β- or γ-hydroxy-carboxylic acid. The absence of a hydroxyl group in α-position to the carboxyl group is also suggested by the failure of pantothenic acid to produce a color reaction with ferric chloride.

The chemical nature of the fifth oxygen atom and of the nitrogen present in pantothenic acid becomes apparent from hydrolysis experiments. Pantothenic acid is inactivated by acids and by alkali. From the alkali hydrolysis material, β-alanine has been isolated[44] and identified as β-naphthalene-sulfo-β-alanine.[45] The other part of the molecule is an aliphatic dihydroxy-acid which has not been isolated as such. In acid solution, especially upon heating, a lactone[46] is readily formed, indicating that a hydroxyl group may be in γ- or in δ-position to the carboxyl group. The former position is indicated by the previously discussed condensation reaction with acetone and other ketones or aldehydes since the condensation would cause the formation of seven-membered rings in case the second hydroxyl group was in δ-position.

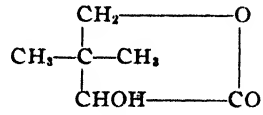
Pantothenic acid has then the following structural formula:



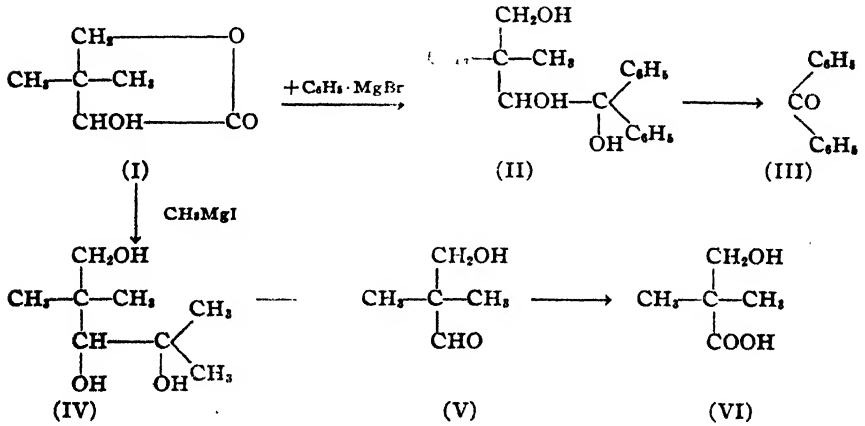
The amino-group of the β-alanine is bound to the carboxyl group of the dihydroxy-acid, forming an acid-amide group. Thus, the nitrogen has been accounted for and it has been shown that there are no amino-, imino- or tertiary amino-groups in the molecule. The other hydroxyl group is in α-position to the carbonyl group, since the acid but not the lactone from the non-β-alanine portion gives[47] a positive ferric chloride test[48] and since carbon monoxide has been obtained[47] by sulfuric acid decomposition of the dihydroxy-acid. The α,β-positions of the two hydroxyl groups are excluded by the failure[47] of pantothenic acid to react with lead tetra-acetate and with periodic acid. The lactone has been obtained by ether extraction

[43] D. W. Woolley, H. A. Waisman and C. A. Elvehjem, *J. Am. Chem. Soc.*, **61**, 977 (1939).
[44] H. H. Weinstock, H. K. Mitchell, E. F. Pratt and R. J. Williams, *Ibid.*, **61**, 1421 (1939).
[45] D. W. Woolley, H. A. Waisman and C. A. Elvehjem, *J. Biol. Chem.*, **129**, 673 (1939).
[46] H. K. Mitchell, H. H. Weinstock, E. E. Snell, S. R. Stanbery and R. J. Williams, *J. Am. Chem. Soc.*, **62**, 1776 (1940).
[47] M. A. Berg, *Bull. soc. chim.*, (3) **11**, 882 (1894).

of acid hydrolyzates as a pure crystalline material[49, 50] and proved to be α-hydroxy-β,β-dimethyl-γ-butyrolactone.[50]



Direct titration[51] of this compound showed the absence of a free carboxyl group, but on heating with alkali one mol of alkali was consumed, proving the presence of a lactone ring. The lactone contains one active hydrogen atom and one hydroxyl group since a mono-acetate was obtained upon acetylation. Oxidation[51] of the lactone with cold alkaline barium hydroxide yielded acetone, which indicates the presence of a dimethyl group. This was furthermore shown by a Kuhn-Roth determination of CH_3 side chains. Upon reaction with phenyl-magnesium-bromide the lactone yields a diphenyl-carbinol (II) which upon treatment with lead-tetra-acetate forms benzophenone (III). Methyl-magnesium-iodide, when reacted with the lactone, forms a dihydroxy-dimethyl-carbinol (IV), which upon oxidation with lead tetra-acetate forms the aldehyde (V). The latter on oxidation with alkaline silver hydroxide yields α-dimethyl-β-hydroxy-propionic acid (VI), thus proving the structure the complete carbon skeleton. By comparing the lactone with synthesized material it was shown to be the laevorotatory form.[51]



The α,γ-dihydroxy-β,β-dimethyl-butyric acid is dextrorotatory.

[49] D. W. Woolley, *Science*, 91, 245 (1940).
[50] R. J. Williams and R. T. Major, *Ibid.*, 91, 246 (1940).
[51] E. T. Stiller, J. C. Keresztesy and J. Finkelstein, *J. Am. Chem. Soc.*, 62, 1779 (1940).

7. Synthesis

At a time when the structure of pantothenic acid was unknown, a hemisynthesis was achieved[52, 53] by condensation of synthetic β-alanine-ethyl-ester with the dihydroxy-carboxylic acid isolated from the hydrolysis products of pantothenic acid. The dihydroxy-acid was acetylated and converted into the acid chloride for utilization in the condensation. After careful saponification of the condensation product pantothenic acid was obtained.

Since the utilization of an acetylated acid chloride is somewhat difficult and gives low yields, another method has been worked out. This consists in the condensation of an ester of β-alanine with the lactone of the dihydroxy-acid.[54, 55, 56] Yields up to 50% are obtained by using this procedure.

An improved method, both simple and effective, is the direct condensation[54] of β-alanine (IV) with the lactone (III). This method has the advantage of avoiding the use of esters of β-alanine which polymerize on standing[57] and, furthermore, of yielding pantothenic acid or its calcium salt directly, thus avoiding the saponification procedure after condensation. Approximately a theoretical yield can be obtained. This type of condensation has also been carried out with the dry sodium salt of β-alanine[54, 55, 58, 59] and yields directly the sodium salt of pantothenic acid. The sodium salt can also be obtained[59] from the ethyl-ester of pantothenic acid by hydrolysis with barium hydroxide followed by reacting the obtained barium salt with sodium sulfate. Pantothenic acid has also been synthesized[60] by condensation of the lactone (III) with β-alanine-benzyl-ester (VI) followed by catalytic hydrogenation of the pantothenic acid benzyl-ester (VII) to the free pantothenic acid (V). The synthetically obtained racemic pantothenic acid can be resolved into its components by crystallization of its quinine,[60, 61] quinine methohydroxide[62] or cin-

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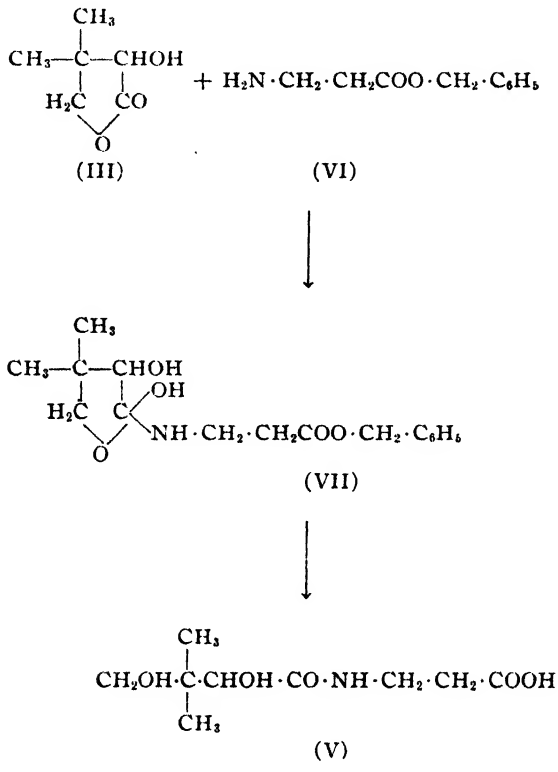
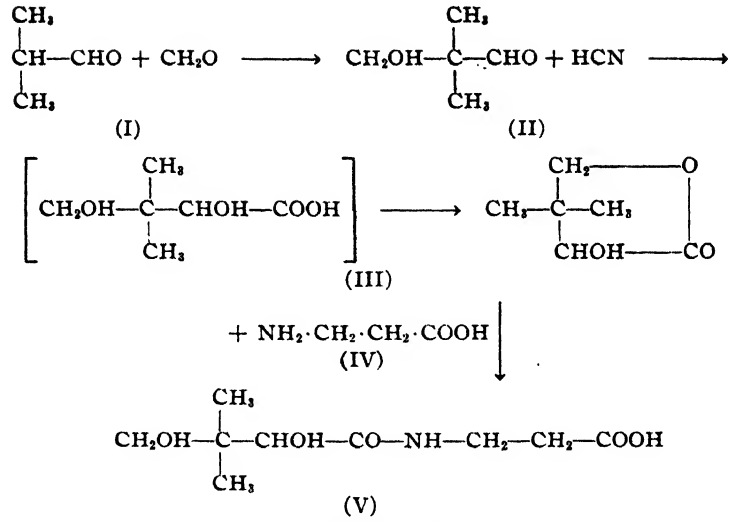
[52] D. W. Woolley, H. A. Waisman and C. A. Elvehjem, *J. Am. Chem. Soc.*, **61**, 977 (1939).
[53] D. W. Woolley, H. A. Waisman and C. A. Elvehjem, *J. Biol. Chem.*, **129**, 673 (1939).
[54] R. J. Williams, H. K. Mitchell, H. H. Weinstock and E. E. Snell, *J. Am. Chem. Soc.*, **62**, 1784 (1940).
[55] T. Reichstein and A. Grüssner, *Helv. Chim. Acta*, **23**, 650 (1940).
[56] R. J. Williams, *Science*, **89**, 486 (1939).
[57] E. Abderhalden, and A. Fodor, *Z. physiol. Chem.*, **85**, 118 (1913).
[58] S. H. Babcock and T. H. Jukes, *J. Am. Chem. Soc.*, **62**, 1628 (1940).
[59] M. Gätzi-Fichter, H. Reich and T. Reichstein, *Helv. Chim. Acta*, **24**, 185 (1941).
[60] R. Kuhn and T. Wieland, *Ber.*, **73**, 971, 1134 (1940).
[61] A. Grüssner, M. Gätzi-Fichter and T. Reichstein, *Helv. Chim. Acta*, **23**, 1276 (1940).
[62] **E. T. Stiller and P. F. Wiley, *J. Am. Chem. Soc.*, 63, 1237 (1941).**

chonidine methohydroxide[61] salts. The (+)-compound is identical with the naturally occurring pantothenic acid.



The synthesis of the α-hydroxy-β,β-dimethyl-γ-butyrolactone has been carried out as follows:[63, 64, 65, 66] isobutyro-aldehyde (I) is condensed[67] with formaldehyde to give α,α-dimethyl-β-hydroxy-propionaldehyde (II) which upon condensation with hydrocyanic acid, or, better, by condensation with potassium cyanide in the presence of calcium chloride[68] or by reaction of the bisulfite compound of the aldehyde (III) with potassium cyanide, yields racemic α-hydroxy-β,β-dimethyl-γ-butyrolactone (III).

[63] E. Glaser, *Monatsh.*, **25**, 46 (1904).
[64] M. Kohn and V. Neustädter, *Ibid.*, **39**, 293 (1918).
[65] E. T. Stiller, S. A. Harris, J. Finkelstein, J. C. Keresztesy and K. Folkers, *J. Am. Chem. Soc.*, **62**, 1785 (1940).
[66] T. Reichstein and A. Grüssner, *Helv. Chim. Acta*, **23**, 650 (1940).
[67] L. Wessely, *Monatsh.*, **21**, 231 (1900).
[68] H. E. Carter and L. F. Ney, *J. Am. Chem. Soc.*, **63**, 312 (1941).



Pantothenic acid

The natural dextrorotatory pantothenic acid yields upon hydrolysis the (+)α,γ-dihydroxy-β,β-dimethyl-butyric acid. The lactone corresponding to this (+)dihydroxy-acid is the (−) form.

The racemic, synthetically obtained α-hydroxy-β,β-dimethyl-γ-butyrolactone can be resolved by conversion into the quinine,[65, 66] quinine methohydroxide, quinidine methohydroxide or cinchonine methohydroxide[69] salt. Thus, the pure (−)α,γ-dihydroxy-β,β-dimethyl-butyro-lactone can be obtained. The isomeric (+) lactone can be racemized by heating the sodium salt.

β-Alanine can be prepared from β-halogenated propionic acids and ammonia,[70] or from succinimide by means of hypobromide and potassium hydroxide.[71] Commercially, β-alanine is produced by adding ammonia to the double bond of acrylic esters[72] followed by saponification of the ester with barium hydroxide. An excellent alternative method is the catalytic hydrogenation of ethyl-cyano-acetate.[73]

8. Industrial Methods of Preparation

Pantothenic acid is commercially available in the form of its crystalline, synthetic calcium or sodium salt, prepared according to the previously

[69] R. T. Major and J. Finkelstein, *J. Am. Chem. Soc.*, **63**, 1368 (1941).

[70] W. Heintz, *Ann.*, **156**, 36 (1870). E. Mulder, *Ber.*, **9**, 1903 (1876).

[71] S. Hoogewerff and W. A. van Dorp, *Rec. trav. chim.*, **10**, 4 (1891). H. T. Clarke and L. D. Behr, *Org. Syntheses*, **16**, 1 (1936).

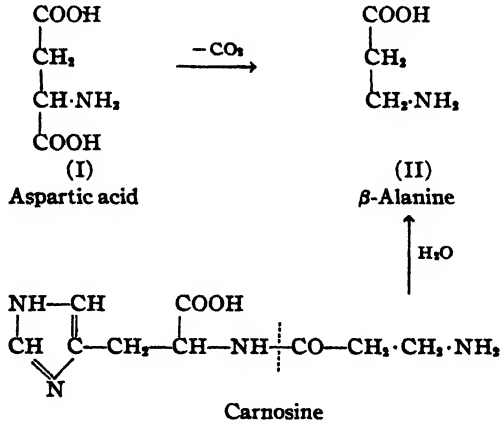
[72] V. Wender, *Gazz. Chim. Ital.*, **19**, 438 (1889). K. Morsch, *Monatsh.*, **63**, 220 (1937).

[73] F. Weygand, *Ber.*, **74**, 256 (1941).

described methods. Pantothenic acid concentrates, for example, from rice brans, from which most of the other vitamins of the B-complex are removed by treatment with fuller's earth, are also marketed. Extracts from certain strains of yeast and bacteria are being prepared which contain most members of the vitamin B-complex including pantothenic acid. The residues of liver preparations which have been worked up for the anti-pernicious anemia factor contain considerable amounts of pantothenic acid and are used commercially for the extraction of this vitamin.

9. Biogenesis

The biogenesis of pantothenic acid is not known, but it is reasonable to assume that those organisms which are able to synthesize this vitamin accomplish this by condensation of β-alanine with the dihydroxy-dimethylbutyric acid or its lactone. The biogenesis of this acid has not been elucidated. β-Alanine may arise from a number of different reactions. Thus, it has been shown that β-alanine (II) is obtained by various bacteria upon decarboxylation of aspartic acid (I).[74] Certain bacteria are able to hydrolyze carnosine (III) to β-alanine.[75]



10. Specificity

Natural pantothenic acid appears to be a single substance[76] of considerable specificity. Even the optical isomer, the (−) pantothenic acid, is

[74] D. Ackermann, *Z. physiol. Chem.*, **54**, 1 (1907); **60**, 482 (1909); *Z. Biol.*, **56**, 87 (1908). A. I. Virtanen and T. Laine, *Suomen Kemistilehti*, B10, 2 (1937); *Enzymologia*, **3**, 266 (1937); *Nature*, **142**, 674 (1938).

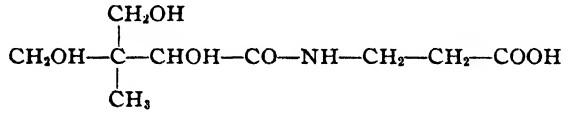
[75] J. H. Mueller, *J. Biol. Chem.*, **123**, 421 (1938).

[76] H. K. Mitchell, E. E. Snell and R. J. Williams, *J. Am. Chem. Soc.*, **62**, 1791 (1940).

inactive in bacterial[77] and rat assays.[61, 78] Both the carboxyl group and the hydroxyl groups are necessary for the physiological action. The acetate,[79] benzoate and diphosphate[80] of the acid are inactive.[79] The salts of pantothenic acid and certain esters, such as the ethyl-ester, are active.[61] β-Alanine alone may act as a growth stimulant for yeast,[81] certain strains of diphtheria bacillus[82, 83] and to a certain extent for rats,[84, 85] but not for chicks. It has been shown that organisms which depend upon an external supply of β-alanine alone convert this compound into pantothenic acid.[81, 82] Other microorganisms, for example, lactic acid bacteria,[86] are unable to utilize only β-alanine. Some hemolytic bacteria have been found[87] which require only an external supply of the dihydroxy-dimethyl-butyric acid part of the pantothenic acid, but not the β-alanine part.

Chondroitin-sulfuric acid has some growth-promoting action on rats,[85, 88] but its effect, even in large doses, is slight compared with that of pantothenic acid.

A number of compounds, similar in structure to pantothenic acid, have been prepared. Of these, the most interesting one is "hydroxy-pantothenic acid" (I) prepared[89] from β-alanine and α-hydroxy-β-methyl-β-hydroxymethyl-butyrolactone. This compound possesses striking biological activity which varies, according to the organism used for testing and the assay conditions, between 1.5% and 25% of pantothenic acid.



(I)

Hydroxy-pantothenic acid

[77] E. T. Stiller, S. A. Harris, J. Finkelstein, J. C. Keresztesy and K. Folkers, *J. Am. Chem. Soc.*, **62**, 1785 (1940).

[78] R. Kuhn and T. Wieland, *Ber.*, **73**, 971, 1134 (1940).

[79] D. W. Woolley, H. A. Waisman, O. Mickelsen and C. A. Elvehjem, *J. Biol. Chem.*, **125**, 715 (1938).

[80] D. W. Woolley, *Ibid.*, **134**, 461 (1940).

[81] H. H. Weinstock, H. K. Mitchell, E. F. Pratt and R. J. Williams, *J. Am. Chem. Soc.*, **61**, 1421 (1939).

[82] J. H. Mueller, *Proc. Soc. Exptl. Biol. Med.*, **36**, 706 (1937). J. H. Mueller and A. W. Klotz, *J. Am. Chem. Soc.*, **60**, 3086 (1938).

[83] W. C. Evans, W. R. C. Handley and F. C. Happold, *Brit. J. Exptl. Path.*, **20**, 396 (1939).

[84] M. Hoffer and T. Reichstein, *Nature*, **144**, 72 (1939).

[85] M. M. El-Sadr, H. G. Hind, T. F. Macrae, C. E. Work, B. Lythgoe and A. R. Todd, *Ibid.*, **144**, 73 (1939).

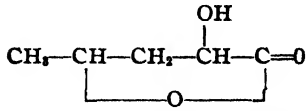
[86] E. E. Snell, F. M. Strong and W. H. Peterson, *J. Am. Chem. Soc.*, **60**, 2825 (1938); *Biochem. J.*, **31**, 1789 (1937).

[87] D. W. Woolley, *J. Biol. Chem.*, **130**, 417 (1939).

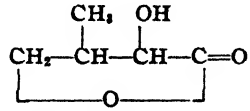
[88] H. E. Robinson, R. E. Gray, F. F. Chesley and L. A. Crandall, *J. Nutrition*, **17**, 227 (1939).

[89] H. K. Mitchell, E. E. Snell and R. J. Williams, *J. Am. Chem. Soc.*, **62**, 1791 (1940).

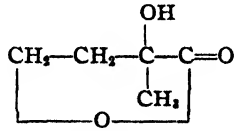
The other compounds tested possess definite, but small activity (less than a fraction of 1% of the activity of pure pantothenic acid). These compounds have been prepared by condensation of β-alanine with the following acids or lactones:



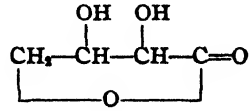
α-Hydroxy-γ-valerolactone[90, 91]



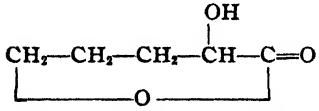
α-Hydroxy-β-methyl-γ-butyrolactone[90]



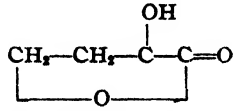
α-Hydroxy-α-methyl-γ-butyrolactone[90]



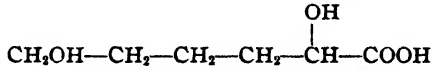
α,β-Dihydroxy-γ-butyrolactone[90]



α-Hydroxy-δ-valerolactone[89, 91, 92]



α-Hydroxy-γ-butyrolactone[90]



α,ε-Dihydroxy-caproic acid[89]

The high structural specificity of pantothenic acid is also evident from the failure to obtain active compounds by replacing the β-alanine part of the molecule with other amino-acids. Thus the analogous compounds obtained from α-alanine, from the methyl homolog β-amino-butyric acid, from carboxy-β-alanine (aspartic acid)[93] and from *l*-leucine[94] proved to be inactive.

11. Determination

There are no specific physical or chemical tests known for pantothenic acid. The following biological tests have been proposed and have been used for the determination of various samples of crude preparations:

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[90] H. K. Mitchell, H. H. Weinstock, E. E. Snell, S. R. Stanbery and R. J. Williams, *J. Am. Chem. Soc.*, **62**, 1776 (1940).

[91] T. Reichstein and A. Grüssner, *Helv. Chim. Acta*, **23**, 650 (1940).

[92] Y. Subbarow and L. Rane, *J. Am. Chem. Soc.*, **61**, 1616 (1939).

[93] H. H. Weinstock, E. L. May, A. Arnold and D. Price, *J. Biol. Chem.*, **135**, 348 (1940).

[94] R. Kuhn and T. Wieland, *Ber.*, **73**, 962 (1940).

1. **Chick test** according to Jukes and Lepkovsky [95, 96] In this test, the growth of chicks and the prevention or cure of the specific chick dermatitis is measured.

2. **Rat test.**[97, 98, 99] The increase in weight over a five-week period is measured.

3. **Bacteria tests.** This is a growth test for Proteus morganii,[100, 101] lactic acid organisms[102, 103, 104, 105] and hemolytic streptococci.[106] The potency of unknown material is judged by quantitative estimation of the turbidity caused by the growth of the bacteria and by titration of the acid produced during growth.

4. **Yeast test.**[107] According to this test, the increase in growth of yeast following the administration of pantothenic acid is determined. Using this method, five parts in ten billion parts of culture medium (0.0005 γ per ml.) can be determined quantitatively.[108]

12. Standards

No International Unit of pantothenic acid has been established so far. The sodium salt of pantothenic acid has been recommended as standard.[109]

1 g. pantothenic acid = 71,000 Chick Units.[110]
= 50,000,000 Streptobacterium Units.[111]

Yeast Unit. One Yeast Unit[108] of pantothenic acid is that amount which when tested by the yeast growth method[107, 108] is equivalent to one g. of a standard dry rice bran extract. This extract is prepared from rice bran with 60% methanol.

Ratio Yeast Unit to Chick Unit = 5:1.[112]

[95] T. Jukes and S. Lepkovsky, *J. Biol. Chem.*, **111**, 119 (1935); **114**, 109, 117 (1936).
[96] T. H. Jukes, *Ibid.*, **117**, 11 (1937).
[97] C. E. Edgar, M. M. El-Sadr and T. F. Macrae, *Biochem. J.*, **32**, 2200 (1938).
[98] C. E. Edgar and T. F. Macrae, *Ibid.*, **31**, 886, 893 (1937).
[99] G. H. Hitchings and Y. Subbarow, *J. Nutrition*, **18**, 265 (1939).
[100] M. J. Pelczar and J. R. Porter, *Proc. Exptl. Biol. Med.*, **43**, 151 (1940).
[101] M. J. Pelczar and J. R. Porter, *J. Biol. Chem.*, **139**, 111 (1941).
[102] E. E. Snell, D. Pennington and R. J. Williams, *Proc., Am. Soc. Biol. Chem., J. Biol. Chem.*, **133**, XCII (1940).
[103] E. E. Snell, F. M. Strong and W. H. Peterson, *Biochem. J.*, **31**, 1789 (1937); *J. Bact.*, **38**, 293 (1939).
[104] D. Pennington, E. E. Snell and R. J. Williams, *J. Biol. Chem.*, **135**, 213 (1940).
[105] E. E. Snell and L. D. Wright, *Proc. Am. Soc. Biol. Chem.*, **1941**, CXIX.
[106] Y. Subbarow and L. Rane, *J. Am. Chem. Soc.*, **61**, 1616 (1939).
[107] R. J. Williams and D. H. Saunders, *Biochem. J.*, **28**, 1887 (1934). R. J. Williams, E. D. McAlister and R. R. Roehm, *J. Biol. Chem.*, **83**, 315 (1929).
[108] R. J. Williams, J. H. Truesdail, H. H. Weinstock, E. Rohrmann, C. M. Lyman and C. H. McBurney, *J. Am. Chem. Soc.*, **60**, 2719 (1938).
[109] M. Gätzi-Fichter, H. Reich and T. Reichstein, *Helv. Chim. Acta*, **24**, 185 (1941).
[110] L. W. McElroy and H. Goss, *J. Biol. Chem.*, **130**, 437 (1939).
[111] R. Kuhn and T. Wieland, *Ber.*, **73**, 971, 1134 (1940).
[112] T. H. Jukes, *J. Am. Chem. Soc.*, **61**, 975 (1939).

Chick Unit. One Chick Unit[113] of pantothenic acid 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 conjugation with a diet free of this vitamin.

1 Chick Unit = 14 γ pantothenic acid.[110]

Sbm. Unit (Streptobacterium Unit). One Sbm. Unit of pantothenic acid has been defined as that amount of the acid which under standardized conditions must be present in one cc. of a culture medium in order to achieve maximum cell growth of *Streptobacterium plantarum*.[114]

13. Physiology of Plants and Microorganisms

Plants and microorganisms may be classified with respect to pantothenic acid into those which synthesize this vitamin and those which need an external supply.[115] The latter group may be subclassified into those which need the entire pantothenic acid molecule, for example, lactic acid-[116] and propionic acid-[117] bacteria, streptococci and pneumococci,[118] those which need only the β-alanine part and those which need only the aliphatic dihydroxy-carboxylic acid part (for examples see page 264).

The fact that pantothenic acid plays a role in the growth of plants and microorganisms is undeniable, but the extent of that role is undetermined. At the present time only a stimulating action of pantothenic acid has been proved, for example, for pea embryos.[119] The actual necessity of this compound for plant life, especially green plant life, has not been convincingly demonstrated.[120]

Green plants synthesize pantothenic acid after they develop their photosynthetic apparatus. Yeast synthesizes small amounts, but only in the absence of an external supply,[121,122] that is, yeast is a parasitoid. On the other hand, yeast synthesizes pantothenic acid *ad libitum* from administered β-alanine.[121]

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[113] T. H. Jukes, *J. Biol. Chem.*, 117, 11 (1937).
[114] R. Kuhn and T. Wieland, *Ibid.*, 73, 962 (1940).
[115] E. J. Krauskopf, E. E. Snell and E. McCoy, *Enzymologia*, 7, 327 (1939).
[116] E. E. Snell, F. M. Strong and W. H. Peterson, *J. Am. Chem. Soc.*, 60, 2825 (1938); *J. Bact.*, 38, 293 (1939).
[117] E. J. Krauskopf, E. E. Snell and E. McCoy, *Enzymologia*, 7, 327 (1939).
[118] L. Rane and Y. Subbarow, *J. Biol. Chem.*, 134, 455 (1940).
[119] J. Bonner and G. Axtman, *Proc. Natl. Acad. Sci. U. S.*, 23, 453 (1937).
[120] R. J. Williams and E. Rohrmann, *Plant Physiol.*, 10, 559 (1935).
[121] H. H. Weinstock, H. K. Mitchell, E. F. Pratt and R. J. Williams, *J. Am. Chem. Soc.*, 61, 1421 (1939).
[122] R. J. Williams, W. A. Mosher and E. Rohrmann, *Biochem. J.*, 30, 2036 (1936).

14. Animal Physiology

Very little is known about the physiology of pantothenic acid and its metabolism in man and animals. Since pantothenic acid is a vitamin, it must be concluded that it is adsorbed from the intestinal tract, but it is not known if it is adsorbed as such or after partial or total hydrolysis into the two chemically different parts. It may be assumed that in the intestines pantothenic acid is freed from the protein material to which it is bound in animal tissues although some evidence exists that the bound vitamin is considerably less well utilized than the free vitamin. Blood contains a constant amount of this vitamin and the liver and kidneys are apparently able to store this compound to a certain extent. In tissues from chicks fed a pantothenic acid-deficient diet, the content of this vitamin was found decidedly lower than normal.[123] In the spinal cord, brain, muscle and blood the differences exceeded 50%, in the liver and kidneys 65%.[124] The blood of men having symptoms of deficiencies of the vitamins B_1, B_2 or nicotinic acid showed also a decrease of 25–50% of the normal level of pantothenic acid.[125] In man, the pantothenic acid content in the serum is increased after injection, but returns to the normal level within one day.[126] Pantothenic acid is partly destroyed in the organism and is constantly excreted through the urine.[127]

In weanling rats kept on a diet deficient in pantothenic acid, the amount of liver fat is markedly less than in control animals kept on a non-deficient diet. Addition of pantothenic acid increases the liver fat.[128] The low levels of liver fat can, however, to a certain extent be related to low food intake during the vitamin deficiency.

15. Avitaminosis and Hypovitaminosis

The symptoms of a pantothenic acid deficiency in man are largely unknown and the human need for this vitamin has not been demonstrated. However, since this compound is necessary for rats and chicks, it seems reasonable to assume that it will prove of necessity to man. Indeed a lowering of the pantothenic acid level in blood has been observed in pellagra, beriberi and riboflavin deficiency.[129]

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[123] E. E. Snell, D. Pennington and R. J. Williams, *J. Biol. Chem.*, **133**, 559 (1940).
[124] E. E. Snell, D. Pennington and R. J. Williams, *Proc. Am. Soc. Biol. Chem.*, **34**, XCII (1940).
[125] S. R. Stanbery, E. E. Snell and T. D. Spies, *J. Biol. Chem.*, **135**, 353 (1940).
[126] T. D. Spies, S. R. Stanbery, R. J. Williams, T. H. Jukes and S. H. Babcock, *J. Am. Med. Assoc.*, **115**, 523 (1940).
[127] D. Pennington, E. E. Snell and R. J. Williams, *J. Biol. Chem.*, **135**, 213 (1940).
[128] R. W. Engel, *Proc. Am. Soc. Biol. Chem.*, **1941**, XXXVII.
[129] S. R. Stanbery, E. E. Snell and T. D. Spies, *J. Biol. Chem.*, **135**, 353 (1940).

Chicks on a pantothenic acid-deficient diet suffer from a specific dermatosis. Incrustations occur about the eyes, the corners of the mouth and the areas between the toes.[130] The skin epithelium becomes keratinized and a dry sloughing sets in. Feathering is retarded and the feathers produced are rough. Besides the dermatitis, certain lesions occur in the spinal cord, characterized by degeneration of the myelinated fibers.[131] Chicks suffering from the specific dermatitis often show thymus involution and liver damage (fatty livers).[131]

Domestic fowls need pantothenic acid for hatchability and reproduction,[132] not, however, for egg production.[133] The rate of hatching eggs has been shown to drop from 70% to 3% when the vitamin was removed from the hen's diet.

Pantothenic acid deficiency in young rats[134, 135] and mice[136] causes retardation of growth. A specific symmetrical depigmentation of the fur (nutritional achromotrichia) sets in in rats and mice[137, 138, 139, 140, 141] which has also been observed in foxes, especially silver foxes. As the result of this deficiency the fur turns gray.[139] In albino rats a rustiness of the fur develops[142] and blood-caked whiskers have been observed regularly.[140]

In rats on a pantothenic acid-deficient diet a marked adrenal hemorrhage, atrophy and necrosis have been observed.[143, 144, 145] No such adrenal changes were found in mice.[131] Hemorrhages under the skin have also been observed in rats.[140]

Another symptom in rats which might be cured by pantothenic acid is the so-called "spectacled eye condition."[146, 147] (See pages 280 and 477.)

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[130] L. C. Norris and A. T. Ringrose, *Science*, **71**, 643 (1930). A. T. Ringrose, L. C. Norris and G. F. Heuser, *Poultry Sci.*, **10**, 166 (1931).

[131] P. H. Phillips and R. W. Engel, *J. Nutrition*, **18**, 227 (1939).

[132] T. H. Jukes, *J. Biol. Chem.*, **129**, 225 (1939)

[133] J. C. Bauernfeind and L. C. Norris, *Science*, **89**, 416 (1939).

[134] C. E. Edgar, M. M. El-Sadr and T. F. Macrae, *Biochem. J.*, **32**, 2200 (1938).

[135] G. H. Hitchings and Y. Subbarow, *J. Nutrition*, **18**, 265 (1939).

[136] H. P. Morris and S. W. Lippincott, *Proc. Am. Soc. Biol. Chem.*, **1941**, XCIII.

[137] P. György, C. E. Poling and Y. Subbarow, *J. Biol. Chem.*, **132**, 789 (1940).

[138] P. György and C. E. Poling, *Science*, **92**, 202 (1940).

[139] P. György and C. E. Poling, *Proc. Soc. Exptl. Biol. Med.*, **45**, 773 (1940); K. Unna, G. V. Richards and W. L. Sampson, *J. Nutrition*, **22**, 553 (1941).

[140] K. Unna, *Proc. Am. Physiol. Soc.*, **1941**, 285; *J. Nutrition*, **20**, 565 (1940).

[141] M. K. Dimick and A. Lepp, *J. Nutrition*, **20**, 413 (1940).

[142] H. S. Owens, M. Trautman and E. Woods, *Science*, **93**, 406 (1941).

[143] F. S. Daft and W.-H. Sebrell, *U. S. Pub. Health Service Pub. Health Repts.*, **54**, 2247 (1939).

[144] F. S. Daft, W. H. Sebrell, S. H. Babcock and T. H. Jukes, *Ibid.*, **55**, 1333 (1940).

[145] L. L. Ashburn, *Ibid.*, **55**, 1337 (1940).

[146] J. J. Oleson, H. R. Bird, C. A. Elvehjem and E. B. Hart, *J. Biol. Chem.*, **127**, 23 (1939).

[147] J. Goldberger and R. D. Lillie, *U. S. Pub. Health Service Pub. Health Repts.*, **41**, 1025 (1926). A. Bourquin and H. C. Sherman, *J. Am. Chem. Soc.*, **53**, 3501 (1931). H. E. Robinson and R. C. Newton, Abstracts, Divisions of Biological Chemistry, Am. Chem. Soc., Kansas City, April 13–17 (1936). S. Lepkovsky, T. H. Jukes and M. E. Krause, *J. Biol. Chem.*, **115**, 557 (1936).

It has also been postulated that pantothenic acid deficiency causes an earlier aging.

(a) *Clinical Test Methods*

In man and in animals, pantothenic acid can be determined by bacteriological methods either in blood or in urine. For *blood determinations*[148] fresh venous blood is citrated to prevent clotting. The diluted material is then tested according to the bacteria tests as previously described. In normal human beings, the pantothenic acid content of blood ranges from 0.19 to 0.32 γ per cc. and an average of 0.225 γ per cc. has been noted. In patients with vitamin B-complex deficiency a lowered value of 0.05 to 0.09 γ per cc. has been observed. In *urine determinations*,[149] which are best carried out according to the bacteria tests, it is advisable to run check determinations with samples in which the pantothenic acid has been destroyed chemically or to which a known amount of pantothenic acid has been added.

16. Hypervitaminosis

Pantothenic acid is essentially non-toxic. At least 100 mg. may be injected intravenously into man without producing any toxic reactions.[150]

17. Requirements

Pantothenic acid is probably required by all living matter. It has been shown to stimulate the growth of bacteria (lactic acid bacteria,[151] pneumococcus,[152] propionic acid bacteria,[153] streptococci,[153] diphtheria bacillus,[154] molds (*aspergillus niger*)), protozoa, fungi, seed plants (alfalfa[155]), higher animals (mice, rats,[156, 157] fox, pigs, dogs,[158] birds[159, 160]) and is

[148] S. R. Stanbery, E. E. Snell and T. D. Spies, *J. Biol. Chem.*, **135**, 353 (1940).

[149] D. Pennington, E. E. Snell and R. J. Williams, *Ibid.*, **135**, 213 (1940).

[150] K. Unna and J. Greslin, *Proc. Soc. Exptl. Biol. Med.*, **45**, 311 (1940); T. D. Spies, D. P. Hightower and L. H. Hubbard, *J. Am. Med. Assoc.*, **115**, 292 (1940).

[151] S. Orla-Jensen, N. C. Otte and A. Snog-Kjaer, *Kgl. Danske Videnskab. Selskab Skrifter Naturvidenskab. math. Afdel.*, **6**, No. 5, 52 pp. (1936); *Zentr. Bakt. Parasitenk*, (II), **94**, 434 (1936).

[152] L. Rane and Y. Subbarow, *J. Biol. Chem.*, **134**, 455 (1940).

[153] E. J. Krauskopf, E. E. Snell and E. McCoy, *Enzymologia*, **7**, 327 (1939).

[154] Y. Subbarow and L. Rane, *J. Am. Chem. Soc.*, **61**, 1616 (1939).

[155] C. H. McBurney, W. B. Bollen and R. J. Williams, *Proc. Natl. Acad. Sci. U. S.*, **21**, 301 (1935).

[156] S. Lepkovsky, T. H. Jukes and M. E. Krause, *J. Biol. Chem.*, **115**, 557 (1936).

[157] Y. Subbarow and G. H. Hitchings, *J. Am. Chem. Soc.*, **61**, 1615 (1939).

[158] O. Mickelsen, H. A. Waisman and C. A. Elvehjem, *J. Biol. Chem.*, **124**, 313 (1938). D. W. Woolley, H. A. Waisman, O. Mickelsen and C. A. Elvehjem, *Ibid.*, **125**, 715 (1938). J. M. McKibbin, S. Black and C. A. Elvehjem, *Am. J. Physiol.*, **130**, 365 (1940).

[159] J. C. Bauernfeind and L. C. Norris, *Science*, **89**, 416 (1939).

[160] T. H. Jukes, *J. Biol. Chem.*, **129**, 225 (1939).

probably essential also for human beings. While sheep[161] and cattle[162, 163, 164] require pantothenic acid, they do not need an external supply since it is synthesized by microorganisms in the rumen.

The amounts required by the various organisms are largely unknown. Yeast needs as growth stimulant 0.008 γ of a purified pantothenic acid per cc. of culture medium. In rats a single dose of 800 γ given to depleted animals produced a rapid and marked gain in weight.[165] The optimal daily dose for rats is above 10 γ[166] and about 100 γ are needed for the cure or prevention of achromotrichia[167] and hemorrhagic adrenal necrosis.[168, 169] For chicks about 500 γ per 100 g. of diet appear to be necessary to prevent the typical dermatitis and about 600 γ to insure optimal growth.

[161] L. W. McElroy and H. Goss, *J. Biol. Chem.*, **130**, 437 (1939).

[162] L. W. McElroy and H. Goss, *Ibid.*, **133**, LXV (1940).

[163] M. I. Wegner, A. N. Booth, C. A. Elvehjem and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.*, **45**, 769 (1940).

[164] L. W. McElroy and H. Goss, *J. Nutrition*, **21**, 405 (1941).

[165] E. T. Stiller, S. A. Harris, J. Finkelstein, J. C. Keresztesy and K. Folkers, *J. Am. Chem. Soc.*, **62**, 1785 (1940).

[166] A. Grüssner, M. Gätzi-Fichter and T. Reichstein, *Helv. Chim. Acta*, **23**, 1276 (1940).

[167] P. György and C. E. Poling, *Science*, **92**, 202 (1940).

[168] F. S. Daft, W. H. Sebrell, S. H. Babcock and T. H. Jukes, *U. S. Pub. Health Service Pub. Health Repts.*, **55**, 1333 (1940).

[169] L. L. Ashburn, *Ibid.*, **55**, 1337 (1940).

INOSITOL

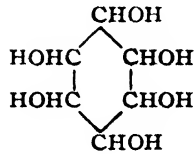
INOSITOL

1. Nomenclature

Names:

Inositol, or, more accurately, meso- or inactive inositol (*i*-inositol.)
Inosite.
Cyclohexanehexol.
Bios I.
Mouse antialopecia factor.[1]
Rat anti-spectacled eye factor.[2] (See also pages 269 and 477).

Chemical formula:



Empirical formula:

$C_6H_{12}O_6$.

2. Chronology

1901 WILDIERS[3] recognized that yeast needs for optimal growth a special material, "bios," in addition to the known nutrients. This had previously been postulated by LIEBIG in 1869.

1924 LUCAS[4] separated "bios" into two components, bios I and II.

1928 EASTCOTT[5] isolated bios I in the pure form and identified it with the long-known inactive inositol.

1940 WOOLLEY[6] produced an alimentary deficiency, alopecia, in mice and identified the missing factor with inositol.

3. Occurrence

Inositol occurs as a normal cell constituent in practically all plant and animal tissues. In plants, the highest amounts are found in the leaves.

[1] D. W. Woolley, *J. Biol. Chem.*, **136**, 113 (1940).
[2] P. L. Pavcek and H. M. Baum, *Science*, **93**, 502 (1941).
[3] E. Wildiers, *La cellulé*, **18**, 313 (1901).
[4] G. H. W. Lucas, *J. Phys. Chem.*, **28**, 1180 (1924).
[5] E. V. Eastcott, *Ibid.*, **32**, 1094 (1928).
[6] D. W. Woolley, *Science*, **92**, 384 (1940).

The concentration varies according to the season and reaches its maximum shortly before the time that the fruit ripens. Fruits, especially citrus fruit (lemon, orange, grapefruit), are good sources of inositol.[7] Cereal grains are also rich sources. Yeast, molds and bacteria, at least certain species, contain large amounts of inositol.[8] Inositol occurs in the animal organism both in the tissues and in the body fluids. Thus, inositol has been isolated from skeletal and heart muscle, lungs, kidneys, liver, brain, blood, milk, urine, eggs, etc. Inositol is also found in the eye lens and the optical nerve.[9]

Inositol occurs naturally in a number of different forms. In liver it exists in an alkali-labile combination with a large molecule, probably a protein. A combined form also exists in the heart muscle[10, 11] (for example, that of dogs) and perhaps other types of muscles. Cardiac muscles of sheep, pigs and oxen[12] contain, if any, only very small amounts in the combined form. Some inositol appears to occur in the free state. In plants, the majority is present in the form of the hexa-phosphate (phytic acid). In the tubercle bacillus[13] and the soybean, inositol is bound in the phosphatide fraction as a glucoside.[14]

4. Isolation

Inositol is obtained, for example, from muscles, liver or leaves by hydrolysis with aqueous potassium hydroxide or calcium hydroxide or with concentrated hydrochloric acid,[15] followed by precipitation of large amounts of by-products with normal lead acetate. The inositol is then precipitated with basic lead acetate and, after removal of the lead, is precipitated by baryta in alcoholic solution. The free inositol is obtained from the barium precipitate by means of carbon dioxide, and is recrystallized, for example, from a minimum amount of water to which alcohol and ether are added to complete the crystallization.[16] Inositol may be prepared from phytin by hydrolysis with formic acid[17] or with calcium hydroxide.

i-Inositol can be identified as the hexa-acetate, m. p. 210° C.

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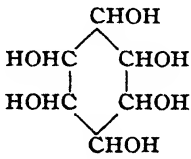
[7] E. K. Nelson and G. L. Keenan, *Science*, **77**, 561 (1933).
[8] F. Kögl and W. van Hasselt, *Z. physiol. Chem.*, **242**, 43 (1936).
[9] A. C. Krause and R. Weekers, *Arch. Ophthalmol.*, **20**, 299 (1938).
[10] L. B. Winter, *Biochem. J.*, **28**, 6 (1934).
[11] F. Rosenberger, *Z. physiol. Chem.*, **64**, 341 (1910).
[12] L. B. Winter, *Biochem. J.*, **34**, 249 (1940).
[13] R. J. Anderson and E. G. Roberts, *J. Biol. Chem.*, **89**, 599, 611 (1930).
[14] R. J. Anderson, W. C. Lothrop and M. M. Creighton, *Ibid.*, **125**, 299 (1938).
[15] D. W. Woolley, *Ibid.*, **139**, 29 (1941).
[16] Maquenne, *Compt. rend.*, **104**, 225 (1886); *Ann. chim. phys.*, [6], **12**, 89 (1887). A. Cloetta, *Ann.*, **99**, 289 (1856).
[17] K. Lindenfeld, *Biochem. Z.*, **272**, 284 (1934).

5. Properties

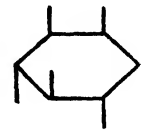
Inositol has a sweet taste. The dihydrate melts at 215–216° C., while the anhydrous compound melts at 225–226° C. Inositol is soluble in water (one part in 5.7 parts of water at 24° C.), but is insoluble in absolute alcohol and in ether. It crystallizes from acetic acid or from water above 50° C. as the water-free compound, but below 50° C. as the dihydrate.

6. Chemistry

Inositol has the formula of a hexahydroxy-cyclohexane (I). Theoretically, the latter can exist in nine different cis-trans isomers.



(I)



(II)

Inositol is optically inactive and is often called meso-inositol. It has the configuration (II) and forms various esters, such as a hexa-acetate, a hexa-phosphate, etc.

7. Industrial Methods of Preparation

Inositol is not a commercially important product. However, due to the abundant occurrence of this compound in all sorts of natural materials it should not be difficult to find a cheap raw material which contains relatively high amounts. The recovery of inositol from the steep water of starch factories, for example, from corn starch plants, is of potential commercial value.[18] Inositol can also be prepared synthetically by hydrogenation of hexa-hydroxy-benzene.[19]

8. Biogenesis

The mechanism of the inositol formation in plants is not definitely known, but it is generally assumed that inositol is synthesized by aldol-type condensations from carbon dioxide and water under the influence of light[20] or

[18] E. Bartow, W. W. Walker and F. A. Hoglan, *Atti X° congr. intern. chim.*, 4, 561 (1939).
[19] H. Wieland and R. S. Wishart, *Ber.*, 47, 2082 (1914).
[20] R. Kögel, *Biochem. Z.*, 95, 313 (1919); 97, 21 (1919).

from hexoses by ring-closure.[21] Inositol is apparently also synthesized by the chick embryo. After about the seventh day of incubation the inositol concentration starts to increase and eventually becomes six times that present in the original egg.[22]

9. Specificity

Little is known about the specificity of inositol. The optical isomers of *i*-inositol are inactive. The compounds reported to be active in mice are, besides inositol, the naturally occurring phosphoric acid ester, tested as the calcium-magnesium salt phytin,[23] inositol hexa-acetate and soybean cephalin.[24] Mytilitol (methyl-inositol) is also active. Only inositol, and not its esters, is active as a growth factor for yeast.[24]

10. Determination

Specific methods have not been worked out for the determination of inositol especially in natural materials. A *chemical method* based on an oxidation with potassium-iodo-mercurate[25, 26] can be used for determination of about 1–5 mg. but is not specific since many polyhydroxy compounds including glycol, mannitol, etc., can be oxidized similarly. A *biological method* based on the ability of inositol to cure or prevent alopecia in mice has occasionally been used.[27, 28, 29] The best method is undoubtedly the *microbiological method* in which the growth of, for example, yeast is measured as a function of the amount of inositol present. This procedure has, however, not been perfected.

11. Physiology of Plants and Microorganisms

A number of different strains of yeast have been investigated for the growth-promoting effect of inositol. Some require an external supply of inositol for optimum growth while others show no beneficial effect upon the addition of inositol to the culture medium.[30,31] Similarly, some fung

[21] F. Micheel, H. Ruhkopf and F. Suckfüll, *Ber.*, 68, 1523 (1935).
[22] E. E. Snell and E. Quarles, *J. Nutrition*, 22, 483 (1941).
[23] D. W. Woolley, *Science*, 92, 384 (1940).
[24] D. W. Woolley, *J. Nutrition*, 21, Supplement, 17 (1941).
[25] L. Young, *Biochem. J.*, 28, 1428, 1435 (1934).
[26] R. A. Gregory, *Ibid.*, 29, 2798 (1935).
[27] D. W. Woolley, *J. Biol. Chem.*, 136, 113 (1940).
[28] D. W. Woolley, *Ibid.*, 139, 29 (1941).
[29] D. W. Woolley, *J. Nutrition*, 21, Supplement, 17 (1941).
[30] H. Stantial, *Trans. Roy. Soc. Can.*, III, 26, 163 (1932).
[31] J. B. Lesh, L. A. Underkofler and E. I. Fulmer, *J. Am. Chem. Soc.*, 60, 2505 (1938).

require inositol while others are apparently able to synthesize this compound.[32]

In higher plants, for example, in the tobacco plant, the inositol content increases during growth until maturity is reached. Thereafter the inositol content decreases slowly. Tobacco seeds contain little inositol.[33] Some plants or microorganisms apparently excrete considerable amounts of inositol into the soil since this compound has been isolated in appreciable quantities from agricultural soils.[34]

There is also the possibility that inositol is used, at least by some species, not only as a growth stimulant but also as a building unit. Thus in caoutchouc a mono- and a dimethyl-ether of inositol have been found, the physiological significance of which is not known.

12. Animal Physiology

While little is known about the physiological action of inositol, a few pertinent observations have been made. Inositol occurs in animal tissues to a certain extent in the free form, but also as the phosphoric acid ester, for example, in the blood of chicken.[35] It is thus possible that inositol is phosphorylated in the organism. The possibility, however, cannot be excluded that the naturally occurring phosphate is directly absorbed.

The mechanism of the inositol action is not known. It has been shown that certain bacteria oxidize one of the hydroxyl groups of inositol to a keto group, but it is not known whether or not such an oxidation occurs in the animal organism. The physiological significance of this oxidation has not been elucidated. It has been claimed that inositol can be oxidized to compounds of unknown composition in muscle, liver, kidney and brain, but in other laboratories no significant effect upon the respiration of the brain tissue of the rat or the rabbit could be observed.[36]

Inositol markedly increases the peristalsis of the stomach and the small intestine.[37] It has been suggested that inositol is the nutritional factor which determines gastrointestinal motility.[37]

Inositol appears to act as a lipotropic factor. In rats fed a synthetic diet supplemented with the vitamins B_1, B_2, B_6, pantothenic acid, choline and biotin, fatty livers containing large amounts of cholesterol are pro-

[32] F. Kögl and N. Fries, *Z. physiol. Chem.*, **249**, 93 (1937).
[33] A. P. Smirnov, *State S. R. Inst. Tobacco Makhorka Ind. (U. S. S. R.)*, **No. 140**, 115 (1939).
[34] R. K. Yoshida, *Soil Sci.*, **50**, 81 (1940).
[35] S. Rapopert, *J. Biol. Chem.*, **135**, 403 (1940).
[36] B. C. Guha and N. Das, *Current Sci.*, **3**, 157 (1934); *Z. physiol. Chem.*, **231**, 157 (1935). L. Young, *Proc. Soc. Exptl. Biol. Med.*, **35**, 507 (1936).
[37] G. J. Martin, M. R. Thompson and J. de Carvajal-Forero, *Am. J. Digestive Dis.*, **8**, 290 (1941).

duced. The development of this special type of fatty liver is prevented by feeding inositol.[38]

13. Avitaminosis

Inositol-deficiency symptoms have been noted, especially in young mice[39, 40] and in rats.[41] These rodents require inositol for normal growth and maintenance of hair. White mice on an inositol-deficient diet become completely hairless over the trunk and severe dermatitis follows. In rats, the denudation sets in around the eyes and results in the so-called "spectacled eye" syndrome.[42] Inositol deficiency in rats causes also the development of a special type of fatty liver containing large amounts of cholesterol.[43]

14. Hypervitaminosis

No ill effects have been observed from an intake of inositol but the true toxicity threshold of inositol has not been determined.

15. Requirements

The optimum requirements of inositol are largely unknown. Deficiency symptoms have been cured in white mice by feeding 10 mg. of inositol per 100 g. of food[40] and in rats by feeding a daily dose of 20 mg.[41] The requirements of other animals are not known. The human requirements have also not been established.

[38] G. Gavin and E. W. McHenry, *J. Biol. Chem.*, **139**, 485 (1941).
[39] D. W. Woolley, *Ibid.*, **136**, 113 (1940). *Science*, **92**, 384 (1940).
[40] G. J. Martin and S. Ansbacher, *Proc. Soc. Exptl. Biol. Med.*, **48**, 118 (1941).
[41] P. L. Pavcek and H. M. Baum, *Am. Chem. Soc., St. Louis Meeting*, April, **1941**, Div. Biol. Chem. Abstr., p. 2.
[42] P. L. Pavcek and H. M. Baum, *Science*, **93**, 502 (1941). (See, however, pages 269 and 477.)
[43] G. Gavin and E. W. McHenry, *J. Biol. Chem.*, **139**, 485 (1941).

PARA-AMINO-
BENZOIC ACID

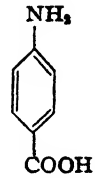
PARA-AMINO-BENZOIC ACID

1. Nomenclature and Survey

Names:

p-Amino-benzoic acid.
Vitamin B_x[1a].
B_x-Factor.[1]
Chromotrichia factor.[2]
Anti-gray-hair-factor.
Anticanitic vitamin.[2a]
Trichochromogenic factor.[2b]
Growth factor "P" for bacteria.[3]

Chemical formula:



Empirical formula:

$C_7H_7O_2N$.

2. Chronology

1940 WOODS and FILDES[4] observed in *in vitro* experiments an anti-sulfanilamide action of *p*-amino-benzoic acid. WOODS[5] found that yeast extracts contain a factor which counteracts the sulfanilamide activity and suggested that this factor is identical with *p*-amino-benzoic acid. SELBIE[6] was able to inhibit by means of *p*-amino-benzoic acid the action of sulfanilamide in mice infected with *Streptococcus haemolyticus*. RUBBO and GILLESPIE[7] isolated *p*-amino-benzoic acid from yeast. FILDES[8] concluded that *p*-amino-benzoic acid is an essential metabolite for bacteria.

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[1] B. Sure, *Proc. Am. Soc. Biol. Chem.*, 1941, CXXX; *Science*, 94, 167 (1941).
[1a] G. Lunde and H. Kringstad, *Norsk Pelsdyrblad*, 13, 500 (1939).
[2] S. Ansbacher, *Science*, 93, 164 (1941).
[2a] L. L. Lachat, *Science*, 93, 452 (1941). V. H. Kugel, *Am. J. Med. Sci.*, 202, 629 (1941).
[2b] A. L. Bacharach, *Food*, 10, 219 (1941).
[3] H. N. Green, *Brit. J. Exptl. Path.*, 21, 38 (1940)
[4] D. D. Woods and P. Fildes, *J. Soc. Chem. Ind.*, 59, 133 (1940)
[5] D. D. Woods, *Brit. J. Exptl. Path.*, 21, 74 (1940).
[6] F. R. Selbie, *Ibid.*, 21, 90 (1940).
[7] S. D. Rubbo and J. M. Gillespie, *Nature*, 146, 838 (1940).
[8] P. Fildes, *Lancet*, 238, 955 (1940).

1941 ANSBACHER[9] found that *p*-amino-benzoic acid is a vitamin, namely, a chromotrichia factor for the rat and a growth-promoting factor for the chick. SIEVE[10] demonstrated that nutritional achromotrichia in man is favorably influenced by *p*-amino-benzoic acid.

3. Occurrence, Isolation and Properties

p-Amino-benzoic acid is apparently widely distributed[10a] over the entire plant and animal kingdom but no quantitative data are available. *p*-Amino-benzoic acid is a natural constituent of yeast and has been isolated therefrom as the benzoyl derivative.[11] *p*-Amino-benzoic acid occurs both in a free and combined form.[11a]

p-Amino-benzoic acid is a colorless substance which melts at 186–187° C.

4. Determination

A number of chemical and biological methods have been used or suggested for the detection of *p*-amino-benzoic acid. None of these, however, has been sufficiently investigated to determine its degree of specificity.

(*a*) *Chemical Method*

p-Amino-benzoic acid in glacial acetic acid yields upon addition of *p*-dimethyl-amino-benzaldehyde in glacial acetic acid a deep yellow color, which can be measured colorimetrically.[12] It has been stated that this method is quite specific and only the *o*- and *m*-isomers and their derivatives, and aniline and its derivatives, give this test. The other vitamins, aliphatic amino-acids and their aromatic derivatives do not respond to this reagent.

(*b*) *Biological Methods*

Biological methods for the determination of *p*-amino-benzoic acid have not been worked out very well. Principally, either the cure or prevention of achromotrichia in rats[13] or the growth of chickens[13] can be used as a criterion for biological determinations. A more simple method would be a microbiological test which makes use of the inhibitory action of *p*-amino-benzoic acid toward the bactericidal effect of sulfonamides. It would seem possible also to develop an assay procedure employing a

[9] S. Ansbacher, *Science*, **93**, 164 (1941).
[10] B. F. Sieve, *Ibid.*, **94**, 257 (1941).
[10a] N. S. Dimond, *Science*, **94**, 420 (1941). S. Wiedling, *Science*, **94**, 389 (1941).
[11] S. D Rubbo and J. M. Gillespie, *Nature*, **146**, 838 (1940).
[11a] K. C. Blanchard, *J. Biol. Chem.*, **140**, 919 (1941).
[12] H. Tauber and S. Laufer, *J. Am. Chem. Soc.*, **63**, 1488 (1941).
[13] S. Ansbacher, *Science*, **93**, 164 (1941).

bacterium which needs *p*-amino-benzoic acid for growth such as *Brucella abortus*[14] or *Streptococcus haemolyticus*.[15] Besides growth the production of acid could probably be used as a measure of the *p*-amino-benzoic acid present when organisms like *Clostridium acetobutylicum*[16] are used as test objects.

5. Physiology

Little is known about the physiological action of *p*-amino-benzoic acid. Excess amounts given either orally or parenterally are converted in the human and animal organism[17] into the acetate which is secreted through the urine.

In the organism *p*-amino-benzoic acid counteracts the action of hydroquinone[18] which, for example, in the cat[19] and in mice[20] causes a graying of the fur. The action of sulfanilamide,[21, 22] for example, in mice infected with *Streptococcus haemolyticus*,[23] and the action of sulfapyridine in mice infected with *Pneumococcus*[24] are also inhibited by *p*-amino-benzoic acid.

In vitro, para-amino-benzoic acid modifies melanin formation[24a] and its possible role in pigmentation processes was suggested.[24b] It has a ronounced influence on tyrosinase activity,[24c] inhibits the oxidative destruction of adrenaline[24d] and appears to act by blocking enzymes.[24e] Experimental evidence for Woods' hypothesis[24f] that para-amino-benzoic acid and sulfonamides have a common point of attack on some enzyme system or systems is important.[24g] The sulfonamide inhibition as influenced by para-amino-benzoic acid was mathematically analyzed[24h] and found to be of the competitive type as was already experimentally demonstrated.[24i]

[14] H. N. Green, *Brit. J. Exptl. Path.*, **21**, 38 (1940).
[15] F. R. Selbie, *Ibid.*, **21**, 90 (1940).
[16] S. D. Rubbo and J. M. Gillespie, *Nature*, **146**, 838 (1940).
[17] A. Ellinger and M. Henzel, *Z. physiol. Chem.*, **91**, 37 (1914). B. Harrow, F. W. Power and C. P. Sherwin, *Proc. Soc. Exptl. Biol. Med.*, **24**, 422 (1926–27). K. Bernhard, *Z. physiol. Chem.*, **267**, 91 (1940).
[18] G. J. Martin and S. Ansbacher, *J. Biol. Chem.*, **138**, 441 (1941).
[19] H. Oettel, *Arch. exptl. Path. Pharmakol.*, **183**, 319 (1936).
[20] G. J. Martin, *Proc. Am. Assoc. Advance Sci., Pharm. Section*, January, **1941**.
[21] D. D. Woods and P. Fildes, *J. Soc. Chem. Ind.*, **59**, 133 (1940).
[22] M. Landy and J. Wyeno, *Proc. Soc. Exptl. Biol. Med.*, **46**, 59 (1941).
[23] F. R. Selbie, *Brit. J. Exptl. Path.*, **21**, 90 (1940).
[24] M. McCarty, *Proc. Soc. Exptl. Biol. Med.*, **46**, 133 (1941).
[24a] G. J. Martin, W. A. Wisansky and S. Ansbacher, *Proc. Soc. Exptl. Biol. Med.*, **47**, 26 (1941).
[24b] F. Lipmann, *J. Biol. Chem.*, **139**, 977 (1941).
[24c] W. A. Wisansky, G. J. Martin and S. Ansbacher, *J. Am. Chem. Soc.*, **63**, 1771 (1941).
[24d] W. A. Wisansky, G. J. Martin, C. T. Tchiowski and S. Ansbacher, *J. Am. Chem. Soc.*, **64**, in press (1942).
[24e] G. J. Martin, C. T. Tchiowski, W. A. Wisansky and S. Ansbacher, *Am. J. Physiol.*, **127**, in press (1942).
[24f] D. D. Woods, *Brit. J. Exptl. Path.*, **21**, 74 (1940).
[24g] J. Kimmig, *Klin. Wochschr.*, **20**, 204 (1941).
[24h] O. Wyss, *Proc. Soc. Exptl. Biol. Med.*, **48**, 122 (1941).
[24i] W. A. Wisansky, G. J. Martin and S. Ansbacher, *J. Am. Chem. Soc.*, **63**, 1771 (1941).

6. Avitaminosis

A deficiency in the *p*-amino-benzoic acid intake causes a graying of the fur in the black or piebald rat, a syndrome which is called nutritional achromotrichia.[25] A retardation of growth has been observed in chicks on a *p*-amino-benzoic-acid-deficient diet.[25] Nutritional achromotrichia in man has also been cured with *p*-amino-benzoic acid.[26] In the female albino rat also a disturbance in the lactation has been noted.[27] *p*-Amino-benzoic acid seems to have a therapeutic effect in certain types of asthma,[27a] possibly because of its protecting or sparing action on adrenaline.[27b]

7. Hypervitaminosis

p-Amino-benzoic acid is essentially non-toxic.[28]

8. Requirements

The requirements of *p*-amino-benzoic acid by various species have not been determined. In rats, 0.75 mg. of the acid per day cures nutritional or hydroquinone achromotrichia.[29,30] In mice, 0.25 mg. daily prevents depigmentation of the fur.[30a] Chicks have been given[29] 30 mg. per 100 g. of ration, but the lowest optimum amount has not been established. In human therapy a dose of 100 mg. twice daily has been used successfully.[31]

p-Amino-benzoic acid is required as a growth factor by many bacteria[32] such as *Clostridium acetobutylicum*,[33] *Brucella abortus*[34] and *Streptococcus haemolyticus*,[35] and the addition of this vitamin to all routine culture media has been recommended.[36]

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[25] S. Ansbacher, *Science*, **93**, 164 (1941).
[26] B. F. Sieve, *Ibid.*, **94**, 257 (1941).
[27] B. Sure, *Proc. Am. Soc. Biol. Chem.*, **1941**, CXXX; *Science*, **94**, 167 (1941).
[27a] G. J. Martin and S. Ansbacher, *Proc. Soc. Exptl. Biol. Med.*, **48**, 118 (1941).
[27b] W. A. Wisansky, G. J. Martin, C. T. Tchiowski and S. Ansbacher, *J. Am. Chem. Soc.*, **64**, in press (1942).
[28] E. Strauss, F. C. Lowell and M. Finland, *J. Clin. Investigation*, **20**, 189 (1941).
[29] S. Ansbacher, *Science*, **93**, 164 (1941); S. Ansbacher and M. Landy, *Proc. Soc. Exptl. Biol. Med.*, **48**, 3 (1941); R. Richardson and A. G. Hogan, *Proc. Soc. Exptl. Biol. Med.*, **48**, 459 (1941).
[30] G. J. Martin and S. Ansbacher, *J. Biol. Chem.*, **138**, 441 (1941).
[30a] G. J. Martin and S. Ansbacher, *Proc. Soc. Exptl. Biol. Med.*, **48**, 118 (1941).
[31] B. F. Sieve, *Science*, **94**, 257 (1941).
[32] P. Fildes, *Lancet*, **238**, 955 (1940).
[33] S. D. Rubbo and J. M. Gillespie, *Nature*, **146**, 838 (1940). J. O. Lampen and W. H. Peterson, *J. Am. Chem. Soc.*, **63**, 2283 (1941).
[34] H. N. Green, *Brit. J. Exptl. Path.*, **21**, 38 (1940).
[35] F. R. Selbie, *Ibid.*, **21**, 90 (1940).
[36] C. A. Janeway, *J. Am. Med. Assoc.*, **116**, 941 (1941).

VITAMIN C—
ASCORBIC ACID

VITAMIN C—ASCORBIC ACID

1. Nomenclature and Survey

Names:

Vitamin C.[1]
Ascorbic acid.[2]
Cevitamic acid.[3]

Historical names:

Hexuronic acid.[4]
Antiskorbutin (Holst 1912).
Antiscorbutic vitamin.
Scorbutamin.[5]

Chemical names:

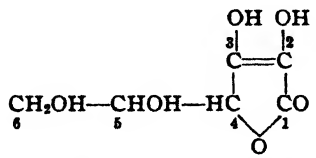
l-Ascorbic acid.
l-Threo-3-keto-hexuronic acid lactone.
l-Xylo-ascorbic acid.
3-Keto-*l*-gulo-furano-lactone.
l,3-Keto-threo-hexuronic acid lactone.
l-Threo-2,3,4,5,6-pentoxy-hexen-2-carboxylic acid lactone.

For the purpose of convenience, a system of nomenclature has been adopted for compounds related to ascorbic acid in which the term "ascorbic acid" is preceded by the name of the osone used or theoretically feasible in a synthesis by the osone-hydrogen cyanide method (example: *d*-gluco-ascorbic acid).

Empirical formula:

$C_6H_8O_6$.

Structural formula:



[1] J. C. Drummond proposed in 1920 that the compound responsible for the prevention of scurvy be called "vitamin C," *Biochem. J.*, 14, 660 (1920).

[2] A. Szent-Györgyi and W. N. Haworth, *Nature*, 131, 23 (1933).

[3] Name introduced by the Council on Pharmacy and Chemistry of the American Medical Association. This organization has now changed the name to "ascorbic acid."

[4] A. Szent-Györgyi, *Biochem. J.*, 22, 1387 (1928).

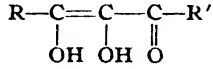
[5] R. L. Jones, *Science*, 68, 480 (1928).

Potency:

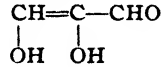
1 g. = 20,000 I. U.

Classification:

Ascorbic acid belongs, according to its structure and properties, to the class of "reductones,"[6] originally called "glucide X" and later "redoxin" (R. Wurmser, 1927–1934), which are characterized by the constitution



The simplest representative of this class is the enol-tartronic-dialdehyde[7, 8]



All compounds of this class form reversible oxidation-reduction systems.

2. Chronology

Scurvy, the typical syndrome of a vitamin C deficiency, has been known for many centuries and occurred epidemically during times of war, crusades, voyages, famines, pestilences, etc. (described, for example, by Euricius Cordus in 1534). Fresh vegetables have been known to provide a potent remedy for over three hundred years.

1903–1913 BOLLE,[9] BARTENSTEIN[10] and later HOLST and FRÖLICH[11] observed that guinea pigs could acquire scurvy just as men could, and that the scurvy syndrome is a condition caused by an avitaminosis.

1918–1925 ZILVA attempted the concentration of the antiscorbutic substance from lemon, obtained almost pure ascorbic acid and established the important basic properties of the vitamin, such as the molecular composition and size, the resemblance to hexoses, the instability toward oxygen, especially in alkaline solution, etc.[12]

1920–1925 AGOPIAN[13] described methods for the isolation of practically pure ascorbic acid from cabbage.

1927 On the basis of the observation that vitamin C solutions immediately after mild oxidation retained their antiscorbutic properties, ZILVA[14] concluded that the

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[6] H. v. Euler and E. Klussmann, *Arkiv. Kemi, Mineral. Geol.*, **B11**, No. 12, 6 pp. (1933).

[7] H. v. Euler and C. Martius, *Svensk. Kem. Tid.*, **45**, 73 (1933); *Arkiv. Kemi, Mineral. Geol.*, **B11**, No. 14, 4 pp. (1933); *Ann.*, **505**, 73 (1933).

[8] T. Reichstein and R. Oppenauer, *Helv. Chim. Acta*, **16**, 988 (1933).

[9] Bolle, *Z. diät. physik. Therap.*, **6**, 354 (1903).

[10] L. Bartenstein, *Jahrb. Kinderheilk.*, **61**, 6 (1905).

[11] A. Holst, *J. Hyg.*, **7**, 619 (1907). A. Holst and T. Frölich, *Ibid.*, **7**, 634 (1907). A. Holst and T. Frölich, *Z. Hyg.*, **72**, 1 (1912).

[12] A. Harden and S. S. Zilva, *Biochem. J.*, **12**, 259 (1918). S. S. Zilva, *Ibid.*, **17**, 416 (1923); **18**, 186 (1924); **19**, 589 (1925). S. J. B. Connell and S. S. Zilva, *Ibid.*, **18**, 641 (1924).

[13] L. A. Agopian, F. P., 595,537, 532,398, 26,034 and 27,271.

[14] S. S. Zilva, *Biochem. J.*, **21**, 689 (1927).

antiscorbutic compound and the "reducing factor" were closely related although not necessarily identical.

1928 SZENT-GYÖRGYI[15] isolated from adrenal glands, from oranges and from cabbage a strongly reducing compound, the "hexuronic acid."

1932 The identity of vitamin C with SZENT-GYÖRGYI's hexuronic acid and with ZILVA's "reducing factor" was discovered by various groups of workers, namely, by SVIRBELY and SZENT-GYÖRGYI,[16] by WAUGH and KING,[17] and by TILLMANS[18] who deduced that vitamin C can be reversibly oxidized and reduced without loss of antiscorbutic efficacy.

1933 The constitution of vitamin C was established by the combined work of HAWORTH, HIRST and co-workers[19] and of MICHEEL and KRAFT.[20] REICHSTEIN[21] and HAWORTH[22] announced the first successful synthesis of ascorbic acid.

3. Occurrence

Vitamin C is widely distributed over the animal and plant kingdoms. It is present in all living plant cells and augmented amounts are found in all actively growing parts of higher plants. Leaves and flowers, for example of gladiolus, nettle, hip (hull and shell) and paprika, are especially rich in vitamin C. Excellent sources are the citrus fruits, berries, green vegetables, apples, etc. (see page 324).

Animal tissues contain small but definite amounts of ascorbic acid. Increased amounts are found in various glands, organs, etc., especially those of endocrine functions, for example, in liver, hypophysis, corpus luteum, adrenal gland, thymus, etc. Human milk contains considerable amounts of ascorbic acid, blood contains small amounts (for details see pages 325 and 334).

Ascorbic acid occurs in plants predominantly as such. In animal and probably also in plant tissues, ascorbic acid occurs apparently in an equilibrium with its oxidized form, the "dehydro-ascorbic acid." The percentage present of the oxidized and the reduced forms varies considerably according to the tissue and various other physiological factors. In a rabbit, for example, the liver was found to contain 27% and muscles

[15] A. Szent-Györgyi, *Biochem. J.*, **22**, 1387 (1928).

[16] J. L. Svirbely and A. Szent-Györgyi, *Nature*, **129**, 576 (1932); *Biochem. J.*, **26**, 865 (1932).

[17] W. A. Waugh and C. G. King, *Science*, **75**, 357 (1932); *J. Biol. Chem.*, **97**, 325 (1932).

[18] J. Tillmans and P. Hirsch, *Biochem. Z.*, **250**, 312 (1932).

[19] R. W. Herbert, E. G. V. Percival, R. J. W. Reynolds, F. Smith and E. L. Hirst, *J. Soc. Chem. Ind.*, **52**, 221, 481 (1933); E. G. Cox and T. H. Goodwin, *J. Chem. Soc.*, **1936**, 769.

[20] F. Micheel and K. Kraft, *Z. physiol. Chem.*, **222**, 235 (1933).

[21] T. Reichstein, *Nature*, **132**, 280 (1933). T. Reichstein, A. Grüssner and R. Oppenauer, *Helv. Chim. Acta*, **16**, 561, 1019 (1933).

[22] R. G. Ault, D. K. Baird, H. C. Carrington, W. N. Haworth, R. W. Herbert, E. L. Hirst, E. G. V. Percival, F. Smith and M. Stacey, *J. Chem. Soc.*, **1933**, 1419. D. K. Baird, W. N. Haworth, R. W. Herbert, E. L. Hirst, F. Smith and M. Stacey, *Ibid.*, **1934**, 62.

50% of the oxidized form. Fresh blood and fr[redacted][24, 25] contain no dehydro-ascorbic acid, but only the reduced form[redacted].

Besides free ascorbic acid and dehydro-ascorbic acid, vitamin C occurs both in animal and in plant tissues in "combined form."[26, 27, 28] The composition of this combined form, which is called "ascorbigen," is unknown (see page 301). It has been shown[29] that, for example, livers of fresh water fish contain from 0.5–20% of the total vitamin C content in the combined form, whereas muscle tissue of the same fish contains only 0.1–0.5%. Cow's milk apparently does not contain any combined ascorbic acid, but this form has been found in human milk.[30] (There is considerable controversy as to whether or not the combined form actually exists,[30, 31] depending upon the technic used for the detection of the combined form.)

4. Isolation

The isolation of vitamin C, which was achieved a number of years before the compound was recognized as a vitamin, is largely dependent upon a quick removal from its solutions. Vitamin C in solution is easily oxidized. This oxidation does not occur to the same extent as long as the vitamin is in its natural environment, which contains protective antioxidants (for details see page 327). All isolation procedures must, therefore, be carried out in the absence of oxygen, in the absence of copper,[32] which catalyzes the oxidation, and in the absence of light,[33] especially when riboflavin,[34] or other fluorescent compounds are present in the raw materials.

Practically, the isolation consists in first preparing a water extract of the starting material (for example, from hips) or pressing the juice out of water-containing materials (for example, of citrus fruits, plant leaves,

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[23] E. J. Reedman, *Can. Pub. Health J.*, 29, 339 (1937).

[24] A. Cimmino, *Quaderni nutriz.*, 5, 239 (1938).

[25] C. A. Knight, R. A. Dutcher and N. B. Guerrant, *Science*, 89, 183 (1939).

[26] B. Ahmad, *Nature*, 136, 797 (1935). E. W. McHenry and M. L. Graham, *Ibid.*, 135, 871 (1935). E. J. Reedman and E. W. McHenry, *Biochem. J.*, 32, 85 (1938). H. Scarborough and C. P. Stewart, *Ibid.*, 31, 2232 (1937); *Nature*, 142, 40 (1938).

[27] K. C. Saha, *J. Indian Chem. Soc.*, 16, 511 (1939). B. C. Guha and P. N. Sen-Gupta, *Nature*, 141, 974 (1938). J. C. Pal and B. C. Guha, *J. Indian Chem. Soc.*, 16, 481 (1939). P. N. Sen-Gupta and B. C. Guha, *Ibid.*, 16, 496 (1939). B. Ghosh and B. C. Guha, *Ibid.*, 16, 505 (1939).

[28] K. Wachholder and A. Okrent, *Z. physiol. Chem.*, 264, 254 (1940).

[29] K. C. Saha, *J. Indian Chem. Soc.*, 16, 511 (1939).

[30] K. Wachholder and A. Okrent, *Z. physiol. Chem.*, 264, 254 (1940).

[31] A. Fujita and T. Ebihara, *Biochem. Z.*, 301, 229 (1939).

[32] A. F. Hess and L. J. Unger, *Proc. Soc. Expll. Biol. Med.*, 19, 119 (1921).

[33] C. L. Arcus and S. S. Zilva, *Biochem. J.*, 34, 61 (1940).

[34] D. B. Hand, E. S. Guthrie and P. F. Sharp, *Science*, 87, 439 (1938). F. G. Hopkins, *J. Soc. Chem. Ind.*, 56, 93d (1937).

etc.). From these aqueous solutions, impurities are precipitated with barium acetate or with neutral lead acetate and after filtration, and if necessary after the further addition of lead acetate, the vitamin is precipitated as the lead salt by bringing the solution to a pH of about 7.6 (indicator, for example, bromo-thymol-blue) with ammonia. The precipitate is filtered or centrifuged and decomposed in water solution with sulfuric or hydrochloric acid by bringing the solution to a pH of about 2. The sulfates or chlorides precipitated thereby are filtered off and washed, and coloring matter may now be extracted, for example, with *n*-butyl-alcohol. The combined water solutions are concentrated under vacuum without heating or with only slight heating, since temperatures above 60° C. decompose the vitamin. The concentrate is purified further by fractional precipitation with organic solvents, such as acetone, ether, methanol, ethanol, propanol, butanol, etc. The pure ascorbic acid is then obtained from the filtered solution by distillation of the solvent followed by crystallization of the residue. The vitamin may now be precipitated with acetone or with petroleum ether.[35, 36] Final crystallization is carried out from methanol.

A modification of this method consists in transforming the ascorbic acid into its acetone derivative (see page 295) and isolating this compound which is then decomposed by water to yield the pure vitamin C.[37]

By these methods, only the ascorbic acid which occurs in the free state is isolated. In order to achieve a better yield, the ascorbic acid present in the "combined form" must first be liberated. This is achieved by gentle heating of a water suspension of the tissue material or of the pressed juice preferably under nitrogen, followed by a reduction by means of hydrogen sulfide of the dehydro-ascorbic acid originally present and formed during the operations.[38]

Ascorbigen alone may be isolated from plant material by extraction of the dry material with chloroform, anhydrous ether, etc., followed by water extraction. From juices, for example, from cabbage juice, ascorbigen is isolated by adsorption on charcoal followed by elution with a mixture of 30% chloroform and 70% absolute alcohol. Further concentration may be achieved by precipitation of impurities with tungstic acid.[38]

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[35] W. A. Waugh, O. A. Bessey and C. G. King, *Proc. Soc. Exptl. Biol. Med.*, **30**, 1281 (1933).

[36] J. L. Svirbely and A. Szent-Györgyi, *Biochem. J.*, **27**, 279 (1933).

[37] E. J. Baumann, *J. Biol. Chem.*, **89**, 213 (1930). E. J. Baumann and N. Metzger, *Proc. Soc. Exptl. Biol. Med.*, **30**, 1268 (1933).

[38] B. Ghosh and B. C. Guha, *J. Indian Chem. Soc.*, **16**, 505 (1939).

5. Properties

Vitamin C crystallizes in white, odorless and colorless plates melting at 190–192° C. The vitamin has a somewhat acid taste. Crystallo-

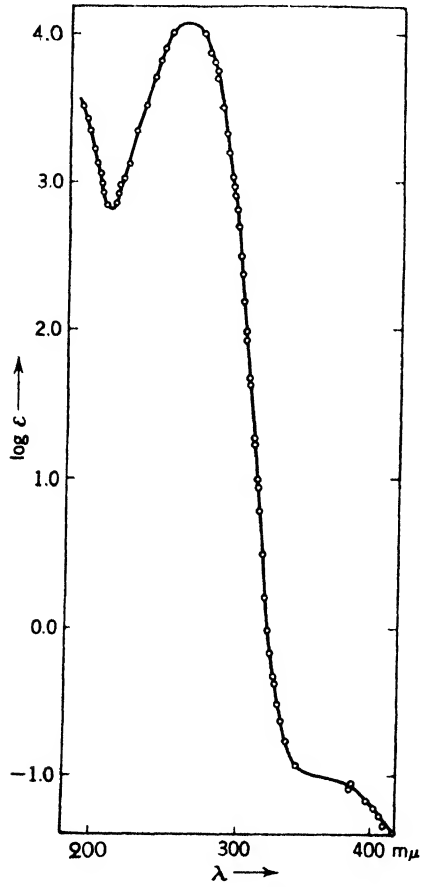


Fig. 14.—Absorption spectrum of ascorbic acid in water solution (with an equimolecular amount of KCN). (H. Mohler and H. Lohr.)

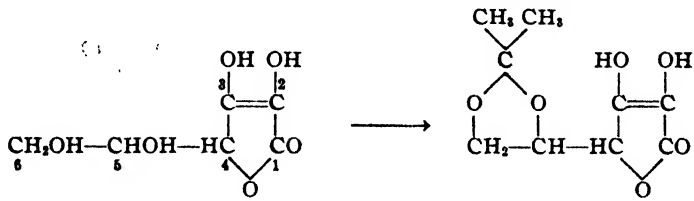
graphic and x-ray measurements of the crystallized substance show that the molecule is almost completely flat.[39]

[39] E. G. Cox, *Nature*, 130, 205 (1932). E. G. Cox and T. H. Goodwin, *J. Chem. Soc.*, 1936, 769. E. G. Cox and E. L. Hirst, *Nature*, 131, 402 (1933).

Ascorbic acid is quite soluble in water (one gram dissolves in 3 cc. of water), less in alcohols (one gram in 50 cc. of absolute alcohol or in about 100 cc. of glycerine) and is insoluble in benzene, ether, chloroform, fats, etc. Whereas vitamin C is quite stable in crystalline form, it is easily deteriorated in solution, especially in the presence of air, traces of metals such as copper and iron, and light,[40] especially in the presence of riboflavin.[41] The most characteristic property of vitamin C is its strong reducing action in solution and the ease of its oxidation which is catalyzed by some metals, especially by copper[42] and by silver.[43] The oxidation-reduction potential of ascorbic acid at pH 4 and at 35° C.: $E_0' = +0.166$ V.[44, 45] In solution, vitamin C exhibits acidic properties, the dissociation constants being $pK_1 = 4.17$ and $pK_2 = 11.57$. Vitamin C has an optical activity of $[\alpha]_D^{20} = +23°$ in water or $[\alpha]_D^{23} = +48°$ in methanol. Ascorbic acid has a typical ultraviolet absorption spectrum with a maximum at 265 mμ[46] ($\log \epsilon = 3.98$) and a small band between 350 and 400 mμ[47] ($\log \epsilon = \sim 1$) (Fig. 14) (see also under Determination of vitamin C: Physical methods, page 315). The infra-red spectrum of vitamin C has also been investigated.[48]

6. Constitution

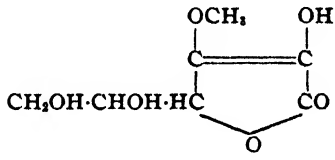
Ascorbic acid has the empirical formula $C_6H_8O_6$. It is a monobasic acid, giving well-defined salts of the type $C_6H_7O_6M$. Four of the six oxygen atoms belong to four hydroxyl groups as indicated by a determination of active hydrogen atoms. Acetone condenses[49] with ascorbic acid (I) with the formation of a mono-isopropylidene derivative (II),



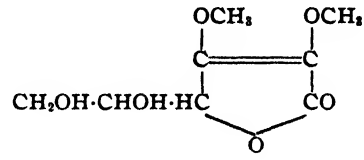
(I) (II)

[40] C. L. Arcus and S. S. Zilva, *Biochem. J.*, **34**, 61 (1940).
[41] F. G. Hopkins, *Compt. rend. trav. lab. Carlsberg*, **22**, 226 (1938).
[42] A. F. Hess and L. J. Unger, *Proc. Soc. Exptl. Biol. Med.*, **19**, 119 (1921).
[43] F. Schlemmer, B. Bleyer and H. Cahnmann, *Biochem. Z.*, **254**, 187 (1932).
[44] E. G. Ball, *J. Biol. Chem.*, **118**, 219 (1937).
[45] H. Borsook, H. W. Davenport, C. E. P. Jeffreys and R. C. Warner, *Ibid.*, **117**, 237 (1937).
[46] F. P. Bowden and C. P. Snow, *Nature*, **129**, 720 (1932).
[47] H. Mohler and H. Lohr, *Helv. Chim. Acta*, **21**, 485 (1938).
[48] E. Heintz, *Compt. rend.*, **208**, 1813 (1939).
[49] L. v. Vargha, *Nature*, **130**, 847 (1932).

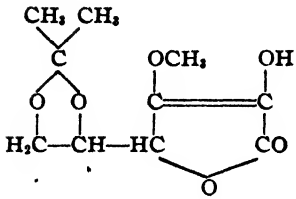
which proves the presence of two vicinal hydroxyl groups. Ascorbic acid, as well as this derivative, shows strongly reducing properties. By the action of diazomethane on ascorbic acid two methyl groups are introduced stepwise. The first methoxy-group is formed almost immediately in the cold giving rise to a 3-mono-methyl-ether (III) which compound no longer has reducing properties. It is only slightly acidic, but gives a strong and permanent violet-blue color reaction with ferric chloride. The constitution of this compound as the 3-mono-methyl-ether and not the 2-mono-methyl-ether is shown by the fact that cyclic 1,3-enols are acids, whereas cyclic 1,2-enols behave like phenols.[50] At room temperature, two methoxyl-groups are introduced by diazomethane,[51] namely, in 2- and 3-position of the ascorbic acid molecule (IV) indicating the enolic character of these two hydroxyl groups which is also suspected from the color reaction with ferric chloride. The dimethyl-ether does not show any reducing



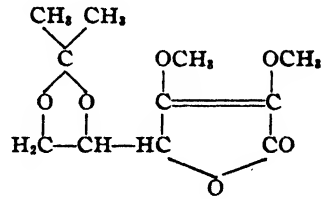
(III)



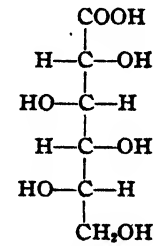
(IV)



(V)



(VI)



(VII)

properties whatsoever. Also the isopropylidene derivative of vitamin C (II) reacts with diazomethane giving rise to a mono-methoxy-[52] and a dimethoxy-isopropylidene derivative[53] (V and VI). The enolic character of the two hydroxyl groups requires the presence of a double bond, which can be demonstrated by color reaction with tetra-nitro-methane, by addition

[50] T. Reichstein and R. Oppenauer, *Helv. Chim. Acta*, 17, 390 (1934).

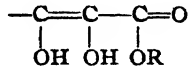
[51] P. Karrer, H. Salomon, K. Schöpp and R. Morf, *Ibid.*, 16, 181 (1933). P. Karrer, H. Salomon, R. Morf and K. Schöpp, *Biochem. Z.*, 258, 4 (1933). P. Karrer, G. Schwarzenbach and K. Schöpp, *Helv. Chim. Acta*, 16, 302 (1933).

[52] T. Reichstein, A. Grüssner and R. Oppenauer, *Helv. Chim. Acta*, 17, 510 (1934).

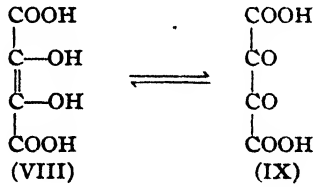
[53] P. Karrer, H. Salomon, K. Schöpp and R. Morf, *Ibid.*, 16, 181 (1933). P. Karrer, H. Salomon, R. Morf and K. Schöpp, *Biochem. Z.*, 258, 4 (1933).

of the one mol of iodine in acid solution or by catalytic hydrogenation[53] whereby among other reaction products *l*-idonic acid is obtained[54] (VII).

Further information was obtained by the formation of keto-derivatives of ascorbic acid. Thus phenyl-hydrazine and various substituted phenyl-hydrazines combine readily with vitamin C yielding products of the composition of osazones,[55] whereas the dimethyl-ether of ascorbic acid does not react with keto-reagents. This proves the presence of at least one keto-group capable of undergoing enolization. In addition, the absorption spectrum with a single band at 245 mμ in acid solution or at 265 mμ in neutral solution suggests the presence of a system of conjugated double bonds. All these evidences lead to the conclusion of the presence of a group —CHOH—CO—COOR which upon enolization yields a system of conjugated double bonds:



An analogy to this type of structure is present in dihydroxy-maleic acid (VIII)[56] the absorption spectrum of which is quite similar to that of ascorbic acid. It also shares with the latter compound the reducing properties, thus being oxidized to a product (IX) which may be reduced to the original compound by means of hydrogen sulfide. This reversible



(VIII) (IX)

oxidation is the most characteristic feature of ascorbic acid and various phases of this property will be discussed later.

Triphenyl-methyl-chloride reacts with vitamin C thereby producing a mono-trityl-ether[57] which contains the two enolic hydroxyl groups in free state, which hydroxyl groups can be etherified with diazomethane.[58] This and the fact that formaldehyde is produced[59] by lead tetra-acetate oxidation of the dimethyl-ether of ascorbic acid proves that one of the

[54] F. Micheel and K. Kraft, *Z. physiol. Chem.*, 222, 235 (1933).
[55] E. G. Cox, E. L. Hirst and R. J. W. Reynolds, *Nature*, 130, 888 (1932).
[56] R. W. Herbert, E. L. Hirst, E. G. V. Percival, R. J. W. Reynolds and F. Smith, *J. Chem. Soc.*, 1933, 1270.
[57] L. v. Vargha, *Nature*, 131, 363 (1933).
[58] P. Karrer, G. Schwarzenbach and K. Schöpp, *Helv. Chim. Acta*, 16, 302 (1933).
[59] F. Micheel and K. Kraft, *Z. physiol. Chem.*, 222, 235 (1933).

hydroxyl groups is primary and that another hydroxyl group must be in α-position to that primary hydroxyl group.

Further insight into the structure of vitamin C was obtained by a number of oxidation reactions. Oxidation of ascorbic acid (I) with one oxygen atom, for example, by means of air,[60] hydrogen peroxide, ferric chloride, quinones, [redacted]tate,[61] dichloro-phenol-indophenol in acid solution, iodine in [redacted] neutral solution, and even by irradiation with ultraviolet ligh[redacted]lds dehydro-ascorbic acid (X) which can be reconverted to ascorbic acid by reducing agents such as hydrogen sulfide and hydroiodic acid. The dehydro-ascorbic acid, at the moment of its formation, is not an acid but a neutral compound, a lactone. The lactone ring, in contrast to that of ascorbic acid, readily hydrolyzes in water solution with the formation of a free carboxylic acid group (XI). This behavior shows that ascorbic acid is not a true acid in the sense that the acidic properties are caused by a carboxylic acid group but that the acidity is caused by an enolic hydroxyl group.[62] The open chain dehydro-ascorbic acid cannot be reduced to ascorbic acid by means of hydrogen sulfide alone. If, however, simultaneously with the H_2S treatment a lactone formation is achieved, for example, by evaporation in the presence of hydriodic acid, the vitamin is formed. The opening of the lactone ring is followed by complex reactions or rearrangements of the molecule, as indicated by changes of the absorption spectrum, optical rotation, etc.[64, 65] Further oxidation of ascorbic acid with hydrogen peroxide yields oxalic acid[66] (XII), which suggests that a keto-group is in α-position to the carboxyl group. Upon oxidation of the vitamin with sodium hypo-iodite in alkaline solution, oxalic acid (XII) and *l*-threonic acid (XIII) were obtained almost quantitatively.[67] The latter acid was identified by methylation (XIV) followed by amidation as trimethyl-*l*-threonamide (XV) and by oxidation with nitric acid as *d*-tartaric acid (XVI) the constitution of which was proved by the formation of dimethoxy-*d*-succinamide (XVII). This sequence of oxidation reactions can only be explained when the constitution of the first oxidation product, of dehydro-ascorbic acid, is that of an

[60] I. Antener, *Helv. Chim. Acta*, 20, 742 (1937).

[61] P. Karrer, H. Salomon, K. Schöpp and R. Morf, *Ibid.*, 16, 181 (1933). P. Karrer, H. Salomon, R. Morf and K. Schöpp, *Biochem. Z.*, 258, 4 (1933). P. Karrer, G. Schwarzenbach and K. Schöpp, *Helv. Chim. Acta*, 16, 302 (1933).

[62] C. L. Arcus and S. S. Zilva, *Biochem. J.*, 34, 61 (1940).

[63] E. L. Hirst, *J. Soc. Chem. Ind.*, 52, 221 (1933).

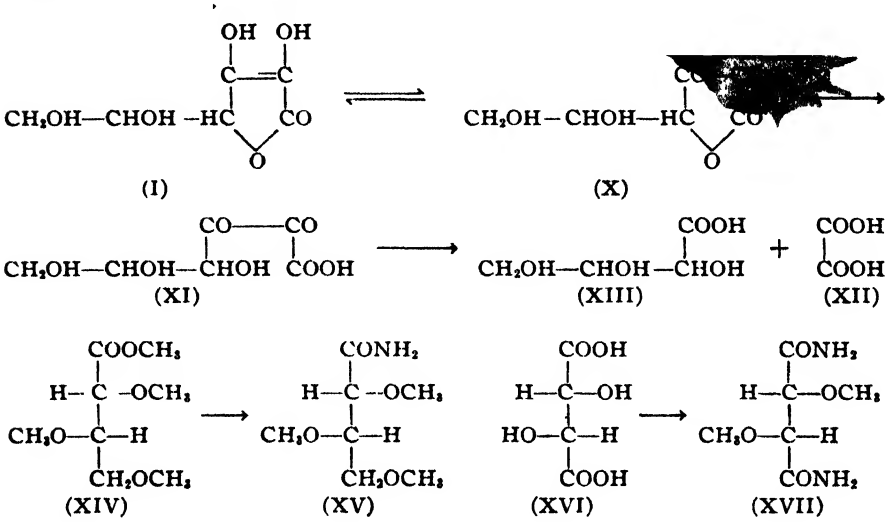
[64] E. L. Hirst, E. G. V. Percival and F. Smith, *Nature*, 131, 617 (1933). E. L. Hirst, E. G. V. Percival, R. W. Herbert, R. J. W. Reynolds and F. Smith, *J. Chem. Soc.*, 1933, 1270.

[65] H. Borsook, H. W. Davenport, C. E. P. Jeffreys and R. C. Warner, *J. Biol. Chem.*, 117, 1 (1937).

[66] P. Karrer, G. Schwarzenbach and K. Schöpp, *Helv. Chim. Acta*, 16, 302 (1933).

[67] E. L. Hirst, *J. Soc. Chem. Ind.*, 52, 221 (1933).

α,β-diketo-compound. The isolation of *l*-threonic acid as a degradation product of ascorbic acid shows that the latter substance is a derivative of *l*-gulose. This is quite remarkable since practically all naturally occurring hexoses belong to the *d*-series.



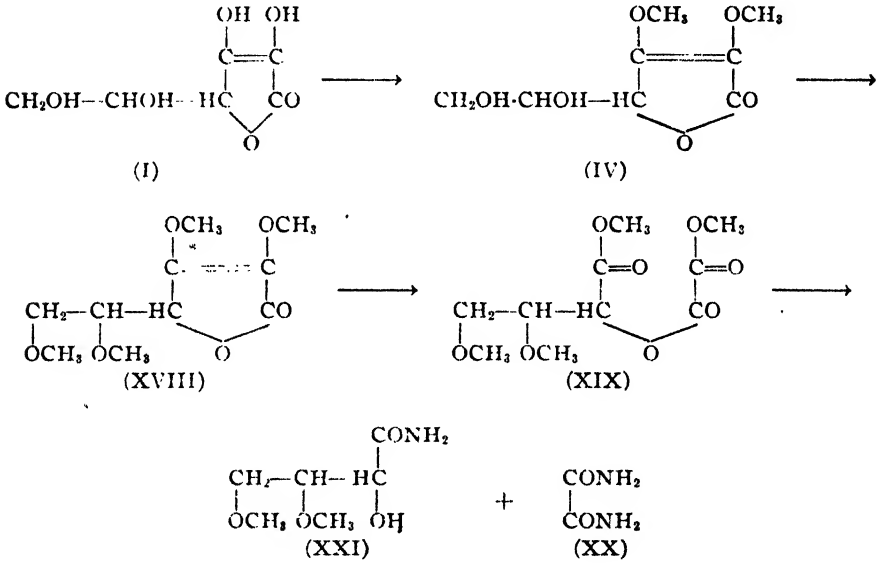
Further degradation reactions were carried out after protecting the four hydroxyl groups. Thus the dimethyl-ether of ascorbic acid was esterified with *p*-nitro-benzoyl-chloride yielding a di-*p*-nitro-benzoate which upon oxidation of the double bond gave rise to a neutral ester containing the same number of carbon atoms as the starting material.[68] This proves that the molecule contains a straight chain of six carbon atoms and that the double bond is situated in a ring, or, more specifically, in a lactone ring. By saponification of the reaction product, oxalic acid and *l*-threonic acid are obtained which shows that the position of the double bond is between carbon atoms 2 and 3 since from the six carbon atom-containing molecule a two- and a four-carbon atom-containing substance are obtained.

The nature of the ring present in the vitamin C molecule was elucidated by a degradation reaction[69] very similar to the one just described. Ascorbic acid was methylated with diazomethane to the dimethyl-ether (IV), which was further methylated by means of methyl-iodide and silver oxide. The tetramethyl-ether (XVIII) obtained yielded upon ozonization a

[68] F. Micheel and K. Kraft, *Z. physiol. Chem.*, 215, 215 (1933).

[69] E. L. Hirst, E. G. V. Percival and F. Smith, *Nature*, 131, 617 (1933). R. W. Herbert, E. L. Hirst E. G. V. Percival, R. J. W. Reynolds and F. Smith, *J. Chem. Soc.*, 1933, 1270.

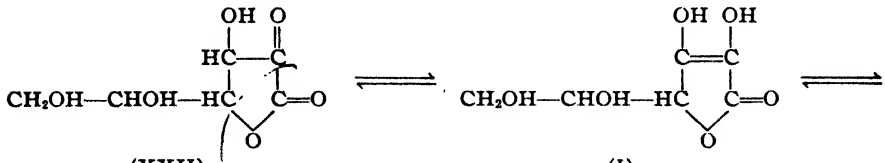
neutral ester (XIX). The latter upon treatment with ammonia yielded oxamide (XX) and the amide of 3,4-dimethyl-*l*-threonic acid (XXI) the constitution of which was proved by the formation of sodium cyanate by the action of sodium hypochlorite on the amide (Weerman reaction[70]). Thus only the hydroxyl group in α-position to the carboxyl group of the *l*-threonic acid is not etherified and the carboxyl of the original ascorbic acid must have been attached to this free hydroxyl group in lactone formation. Other evidences for the presence of a furane ring were mentioned previously: the formation of formaldehyde upon lead tetra-acetate oxidation of the dimethyl-ether of ascorbic acid[71] and the formation of *l*-idonic acid upon catalytic hydrogenation of ascorbic acid.[71]



Since ascorbic acid has the constitution of an unsaturated α-glycol or "enediol-" configuration the hydroxyl groups of which are enolic in character, the existence of two different keto-forms (XXII and XXIII) appears theoretically possible. Practically only the enolized form (I) has been observed and the ultraviolet absorption spectrum does not contradict this observation. Nevertheless there seems to be a true equilibrium between the enediol-form (I) and the 3-keto-form (XXIII) which is quickly reached

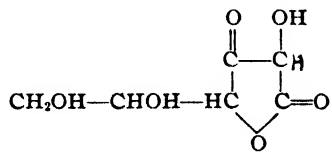
[70] R. A. Weermann, *Rec. trav. chim.*, **37**, 16 (1918).
[71] F. Micheel and K. Kraft, *Z. physiol. Chem.*, **222**, 235 (1933).

and which is almost completely on the side of the enediol-form. It has therefore been impossible to isolate the 3-keto-form. The 2-keto-form (XXII), on the other hand, is stable and can be isolated as such and does not seem to be in an equilibrium with the enediol or the 3-keto-form. Or, if there is a state of equilibrium, it is accomplished only extremely slowly or under drastic conditions.[72]



(XXII)
2-Keto-form

(I)
Enediol-form



(XXIII)
3-Keto-form

The formula of ascorbic acid (I) shows the presence of two asymmetric carbon atoms, namely carbon atoms 4 and 5. Therefore, two pairs of optically active isomeric compounds are possible. Ascorbic acid belongs to the *l*-series, as has been previously deduced. The corresponding *d*-form and the other pair called *d*- and *l*-isoascorbic acid have been prepared synthetically.

The chemical constitution of "ascorbigen," the naturally occurring combined form of ascorbic acid, is unknown.[73, 74, 75]

7. Synthesis

The synthesis of vitamin C has been achieved by four principally different methods, namely:

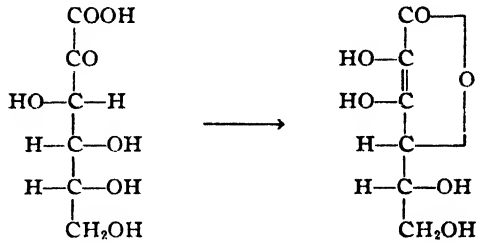
[72] T. Reichstein and A. Grüssner, *Helv. Chim. Acta*, **17**, 311 (1934).

[73] E. Ott, K. Krämer and W. Faust, *Z. physiol. Chem.*, **243**, 199 (1936).

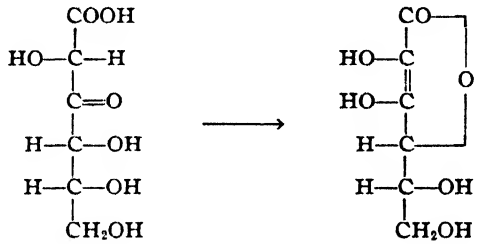
[74] B. Ahmad, *Nature*, **136**, 797 (1935). E. W. McHenry and M. L. Graham, *Ibid.*, **135**, 871 (1935). E. J. Reedman and E. W. McHenry, *Biochem. J.*, **32**, 85 (1938). H. Scarborough and C. P. Stewart, *Ibid.*, **31**, 2232 (1937); *Nature*, **142**, 40 (1938).

[75] K. C. Saha, *J. Indian Chem. Soc.*, **16**, 511 (1939). B. C. Guha and P. N Sen-Gupta, *Nature*, **141**, 9, 974 (1938). J. C. Pal and B. C. Guha, *J. Indian Chem. Soc.*, **16**, 481 (1939). P. N. Sen-Gupta and B. C. Guha, *Ibid.*, **16**, 496 (1939). B. Ghosh and B. C. Guha, *Ibid.*, **16**, 505 (1939).

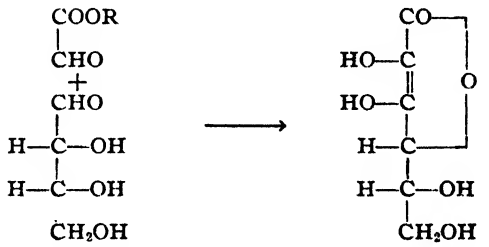
1. **By Isomerization and Lactonization of 2-Keto-hexonic Acids.**



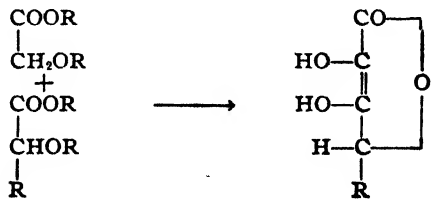
2. **By Isomerization and Lactonization of 3-Keto-hexonic Acids.**



3. **By a Benzoin Type Condensation of Two Aldehydes of Lower Molecular Weight.**



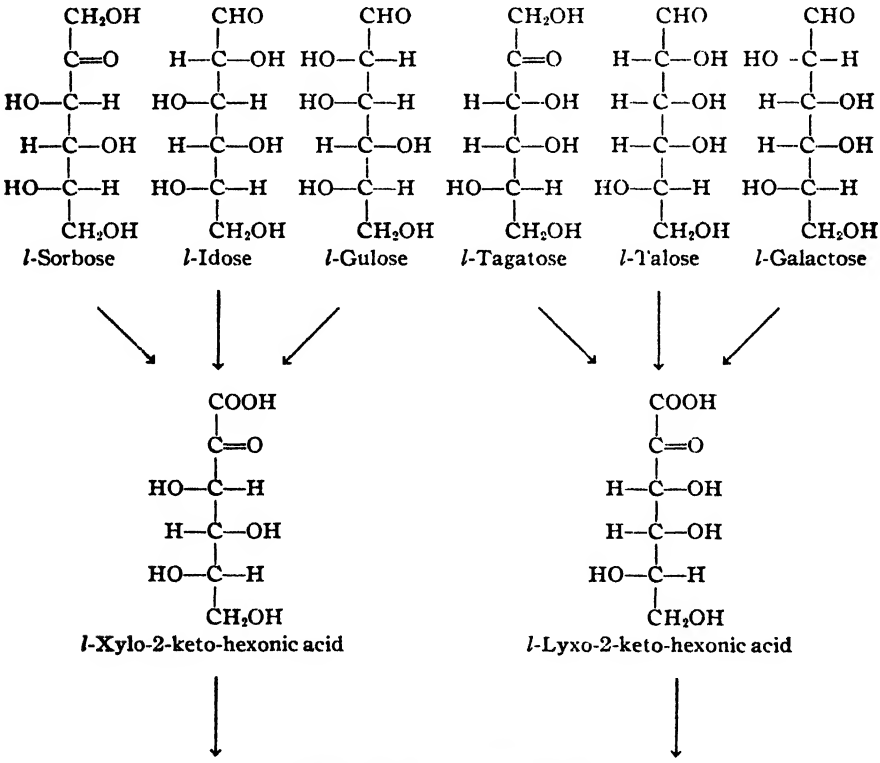
4. **By Ester Condensation of α-Oxy-acids.**



The various methods applicable for the synthesis of ascorbic acid according to the four outlined schemes will be shown in the following paragraphs.

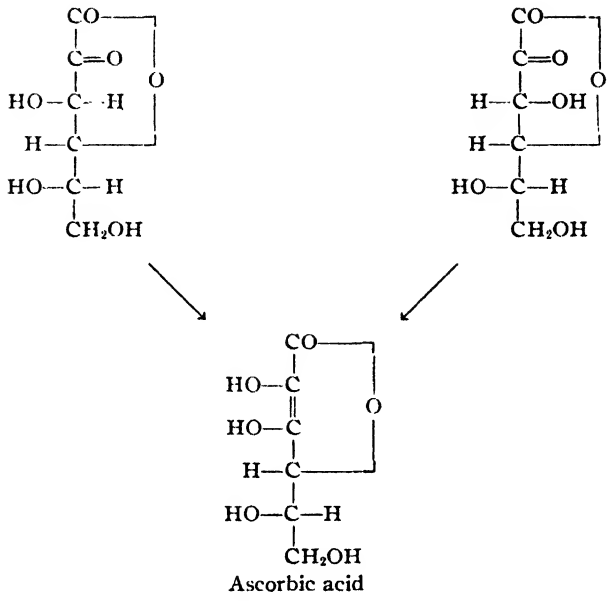
(*a*) *Synthesis of Ascorbic Acid by Isomerization and Lactonization of 2-Keto-hexonic Acids*

The synthesis of ascorbic acid according to this method[76] is characterized by using a 2-keto-hexonic acid as intermediate, which may also be called 5-keto-hexuronic acid or hexusonic acid. Since such an acid contains one more asymmetrical carbon atom than ascorbic acid, namely, the carbon atom 3, there are two different 2-keto-hexonic acids which might be converted into ascorbic acid and which have in common the stereochemical configuration at carbon atoms 4 and 5. These two compounds are called



(*Formula continued on following page.*)

[76] T. Reichstein and A. Grüssner, *Helv. Chim. Acta*, 17, 311 (1934).



Ascorbic acid

l-xylo-2-keto-hexonic acid and *l*-lyxo-2-keto-hexonic acid according to the specific configuration of the pentoses to which the hexonic acids correspond. Each of these two 2-keto-hexonic acids may in turn be derived from three different hexoses, namely, two aldoses and one ketose. Thus, *l*-idose, *l*-gulose and *l*-sorbose may yield *l*-xylo-2-keto-hexonic acid which is also called 2-keto-*l*-gulonic acid, and *l*-talose, *l*-galactose and *l*-tagatose may yield *l*-lyxo-2-keto-hexonic acid. Of these six hexoses, only *l*-sorbose is readily accessible. Besides this aldose also *l*-gulose has been used for synthesizing vitamin C.

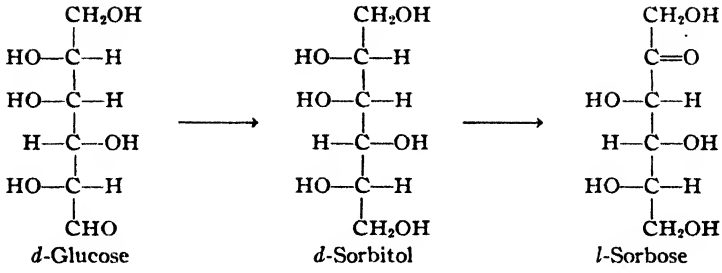
l-Sorbose is obtained from *d*-sorbitol which occurs in nature and can, for example, be isolated from the fruits of mountain ash (*Sorbus aucuparia*). This hexitol is also obtained by catalytic hydrogenation of *d*-glucose,[77] a process which is carried out technically on a large scale.

The *d*-sorbitol is converted into *l*-sorbose by bacterial oxidation according to Bertrand.[78] A number of bacteria are able to accomplish this reaction. In practice, acetic acid bacteria, are used.[79]

[77] W. Ipatiev, *Ber.*, **45**, 3225 (1912). W. E. Cake, *J. Am. Chem. Soc.*, **44**, 859 (1922). L. W. Floyd, R. Connor and H. Adkins, *Ibid.*, **54**, 1651 (1932). F. P. 694,424. I. G. Farbenindustrie, G. P., 544,666.

[78] G. Bertrand, *Bull. soc. chim.*, [3], **15**, 627 (1896); *Ann. chim.*, [8], **3**, 183, 227 (1904). H. Schlubach and J. Vorwerk, *Ber.*, **66**, 1251 (1933).

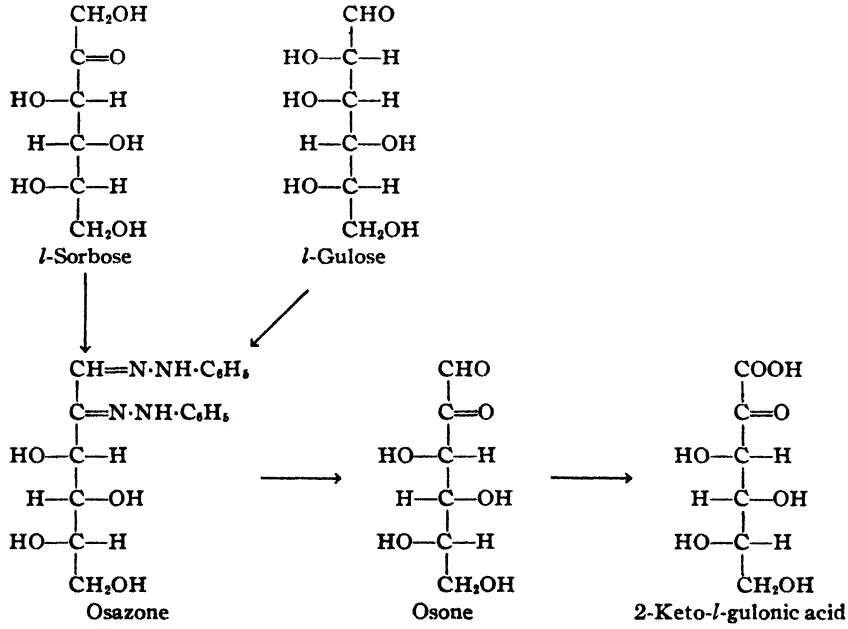
[79] P. A. Wells, L. B. Lockwood, J. J. Stubbs, N. Porges and E. A. Gastrock, *Ind. Eng. Chem.*, **31**, 1425 (1939).



l-Gulose, which can also be used as starting material for the synthesis of ascorbic acid, is obtained by oxidation of starch or of *d*-glucose to *d*-saccharic acid, the lactone of which is reduced to *l*-gulonic acid. The lactone of the latter compound yields *l*-gulose upon reduction.[80]

The oxidation of the hexoses to the 2-keto-hexonic acid, namely, 2-keto-*l*-gulonic acid, can be carried out by a number of different methods:

(*1*) *Via the Osone:* *l*-Sorbose[81] or *l*-gulose[82] is converted into the phenyl-hydrazone which in turn is reacted with benzaldehyde to yield the



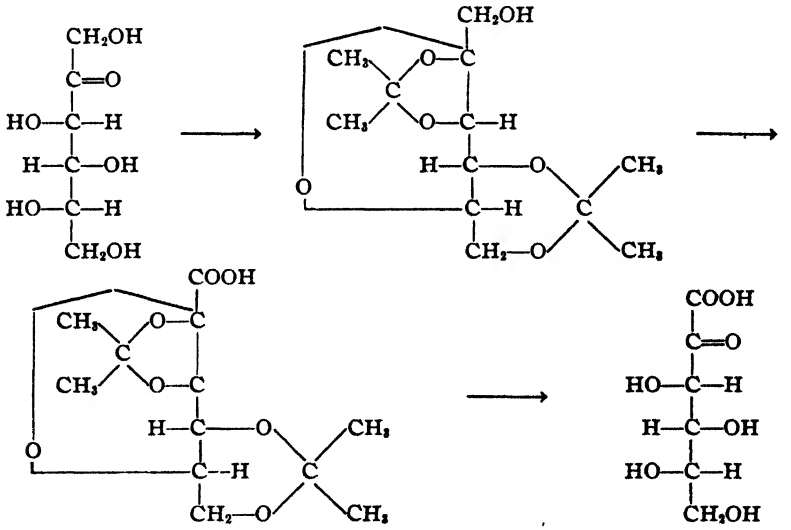
[80] P. P. T. Sah, *Ber.*, 69, 158 (1936).

[81] F. Micheel and K. Kraft, *Naturwissenschaften*, 22, 205 (1934). F. Micheel and W. Lohmann *Z. physiol. Chem.*, 225, 13 (1934).

[82] P. P. T. Sah, *Ber.*, 69, 158 (1936).

corresponding osone. This compound must be carefully purified in order to give finally an ascorbic acid which crystallizes well. By careful oxidation with bromine[83] in aqueous solution, the osone is converted into the 2-keto-*l*-gulonic acid.

(*2*) *Via the Diacetone-sorbose.*[84] *l*-Sorbose is condensed with acetone in order to protect all groups with the exception of the primary hydroxyl group in 1-position. The main reaction product is sorbose-diacetone of the structure indicated below and as a by-product a mono-acetone-sorbose is obtained. The latter compound is easily separated from the diacetone derivative since only the di-isopropylidene compound is soluble in typical organic solvents, such as ether. The mono-acetone derivative can be converted into the diacetone compound by condensation with acetone. The diacetone-sorbose is oxidized by means of permanganate in alkali to the 2:3,4:6-diacetone derivative of 2-keto-*l*-gulonic acid. The free keto-acid is obtained by warming the acetone compound in water. This last reaction can be carried out in a yield of 82%. As a by-product small amounts of ascorbic acid are formed.



Instead of acetone, other ketones or aldehydes, for example, methyl-ethyl-ketone, benzaldehyde, etc., and especially cyclic ketones, for example, cyclo-hexanone, may be used to protect the sorbose.

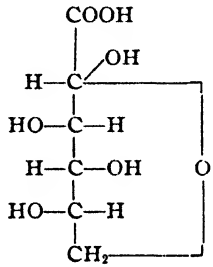
(*3*) *By direct Oxidation:* Sorbose can be oxidized directly to 2-keto-

[83] C. Neuberg and T. Kitasato, *Biochem. Z.*, **183**, 485 (1927).
[84] T. Reichstein and A. Grüssner, *Helv. Chim. Acta*, **17**, 311 (1934).

gulonic acid since the 1-hydroxyl group is especially sensitive to oxidation. This oxidation may be carried out by means of nitric acid[85] or by catalytic oxidation.[86]

The 2-keto-hexonic acids may also be obtained from the corresponding aldonic acids. Thus, *l*-gulonic acid can be selectively oxidized to 2-keto-*l*-gulonic by means of chromic acid[87] or by chlorates in the presence of a vanadium catalyst.[88]

The last step of the synthesis of vitamin C is the conversion of the 2-keto-gulonic acid into ascorbic acid by lactonization. This reaction does not occur voluntarily, probably because the keto-acid exists mainly in the form of the stable lactol:

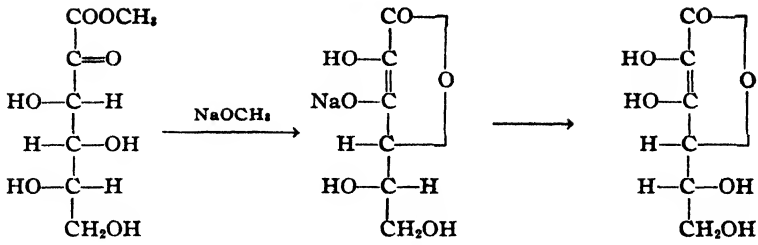


This lactonization, can, however, be accomplished by a number of different reaction conditions:

1. From the free keto-acid by heating in neutral, acid or alkaline solution.[89] The best yields are obtained in acid solution.[90] The double bond produced by enolization of the keto-group gives rise to a *cis*- and a *trans*-compound. Only the *cis*-form is able to undergo lactonization, the *trans*-form is either reconverted into the keto-form or undergoes conversion into compounds different from vitamin C.

2. From esters of the keto-acid by the action of sodium alkoxides,[91] sodium bicarbonate,[92] sodium acetate, [92] calcium carbonate,[93] etc. The reaction is said to occur also in acid solution.[94] The esters of the keto-acid are obtained by esterification of the free acid, for example, with alcohols and mineral acids or with diazo-alkanes.

[85] W. N. Haworth, *Nature*, **134**, 724 (1934). B. P. 443,901.
[86] O. Dalmer and K. Heyns, U. S. P., 2,189,778.
[87] R. Pasternack and P. P. Regna, U. S. P., 2,153,311.
[88] R. Pasternack and P. P. Regna, U. S. P., 2,188,777.
[89] Swiss P., 187,933, 187,934, 180,810 and 188,804.
[90] T. Reichstein and A. Grüssner, *Helv. Chim. Acta*, **17**, 311 (1934).
[91] Swiss P., 174,208.
[92] Swiss P., 187,932.
[93] Swiss P., 180,810.
[94] Swiss P., 188,800, 188,802 and 188,803.



3. From the diacetone-keto-acid by heating in the presence of diluted mineral acids.[95]

(b) Synthesis of Ascorbic Acid by Isomerization and Lactonization of 3-Keto-hexonic Acids Obtained by the Osone-Hydrogen Cyanide Method[96, 97, 98]

The synthesis of ascorbic acid according to this method involves the utilization of a 3-keto-hexonic acid, which also may be called 4-keto-hexuronic acid. This acid can practically only be obtained by building up the molecule from a compound of five carbon atoms. This is achieved by the addition of hydrogen cyanide to the osone (IV). Since specific stereochemical configurations are required for the carbon atoms 4 and 5 of ascorbic acid, there is only one pentosone (IV) with the same configuration. Theoretically, this pentosone can be obtained from three pentoses, namely, two aldoses, *l*-lyxose and *l*-xylose, and one ketose. The latter one is still unknown.

Actually, the osone (IV) has been prepared from both aldoses, *l*-lyxose and *l*-xylose. *l*-Xylose occurs in nature as part of hemicellulose and can be obtained from soft wood materials, for example, from corncobs, elder-pith, beeches, sawdust, shells of coconuts, etc., by acid hydrolysis. It can also be prepared from rice starch or from glucose by oxidation to *d*-saccharic acid, reduction to *l*-gulonic acid lactone and degradation of the latter compound to *l*-xylose. Perhaps the easiest method is to convert *d*-sorbitol, which is technically prepared from *d*-glucose, into di-ethylidene-sorbitol[99] by means of paraldehyde or into mono-benzal-sorbitol[100] by

[95] G. P., 641,639 recommends the use of hydrochloric acid, whereas Swed. P., 88,094 prefers the use of sulfuric acid.

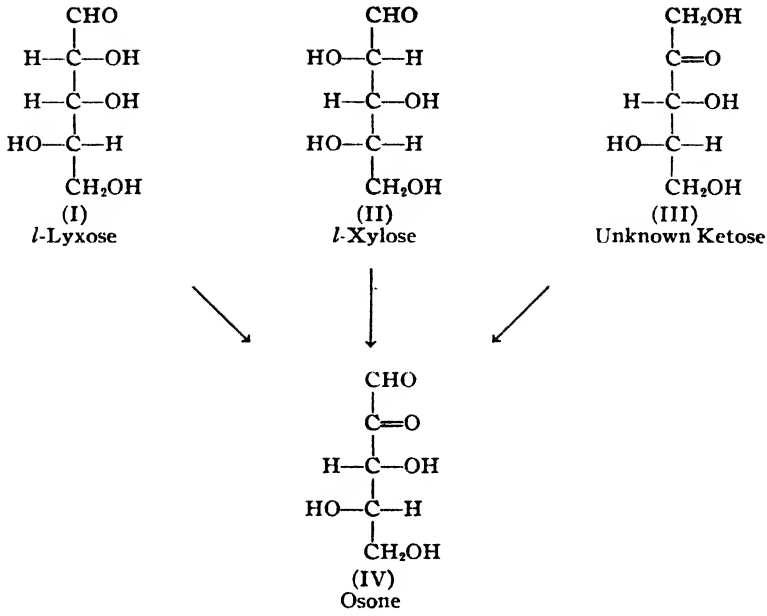
[96] T. Reichstein, *Nature*, **132**, 280 (1933). T. Reichstein, A. Grüssner and R. Oppenauer, *Helv. Chim. Acta*, **16**, 561, 1019 (1933).

[97] T. Reichstein, A. Grüssner and R. Oppenauer, *Helv. Chim. Acta*, **17**, 510 (1934).

[98] R. G. Ault, D. K. Baird, H. C. Carrington, W. N. Haworth, R. W. Herbert, E. L. Hirst, E. G. V. Percival, F. Smith and M. Stacey, *J. Chem. Soc.*, 1933, 1419. D. K. Baird, W. N. Haworth, R. W. Herbert, E. L. Hirst, F. Smith and M. Stacey, *Ibid.*, 1934, 62.

[99] Hoffman-La Roche, G. P., 627,249. H. Appel, *J. Chem. Soc.*, 1935, 425.

[100] L. v. Vargha, *Ber.*, **68**, 18 (1935).



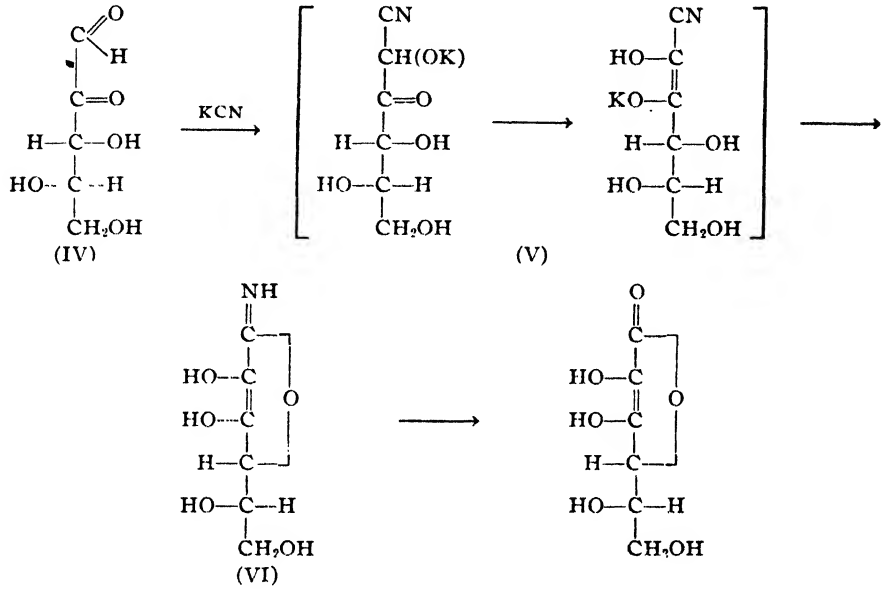
means of benzaldehyde, followed by oxidation to di-ethylidene-*l*-xylose or benzal-*l*-xylose, respectively, and hydrolysis to the free *l*-xylose.

The preparation of *l*-lyxose is more complicated. *d*-Galactose (from pectins) is converted into the 1:2,3:4-diacetone-derivative and oxidized to 1:2,3:4-diacetone *d*-galacturonic acid and hydrolyzed to *d*-galacturonic acid. By reduction of the aldehyde group of the latter compound, *l*-galactonic acid is obtained which is converted into the acid-amide, *l*-galactonamide and finally into *l*-lyxose.

The oxidation of the pentoses to the osone, which is called *l*-xylosone, is accomplished by either preparing first the osazone which is decomposed by the aid of benzaldehyde, or by direct oxidation with hydrogen peroxide and ferrous sulfate as catalyst. If the osazone is prepared as an intermediate, it is not necessary to start with pure crystalline pentoses.

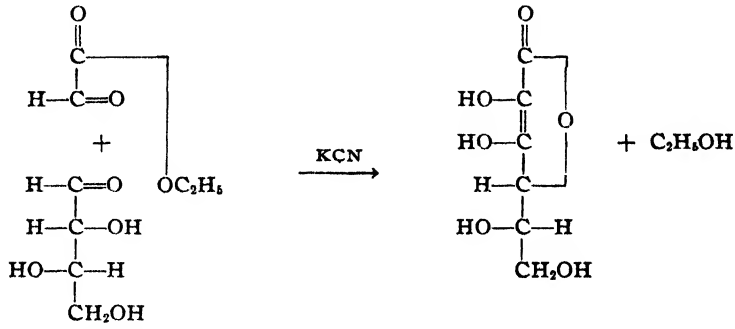
For the conversion of *l*-xylosone into ascorbic acid, hydrogen cyanide is added to the osone yielding the nitrile (V) which apparently enolizes and lactonizes immediately to form the cyclic imino-compound (VI) since it gives none of the reactions for a nitrile group. The use of potassium cyanide in the presence of calcium chloride instead of hydrogen cyanide shortens the reaction time from days to hours. The cyclic imino-compound (VI) can be isolated. The isolation, however, is not necessary.

By acid hydrolysis *l*-ascorbic acid is obtained, the imino group being eliminated as an ammonium salt.



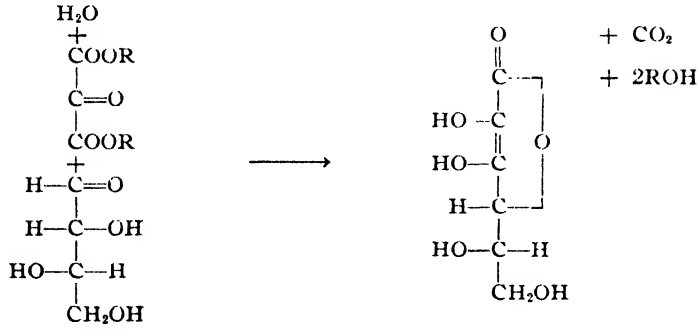
(c) *Synthesis of Ascorbic Acid by Condensation of Ethyl-glyoxylate with l-Threose*[101]

According to this method ascorbic acid is synthesized by a benzoin type condensation from ethyl-glyoxylate and *l*-threose in alkaline solution. Practically, the acetylated cyano-hydrine of *l*-threose is used which in one operation yields ascorbic acid.



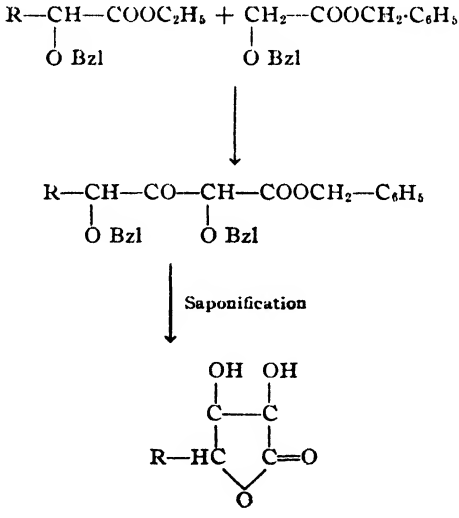
[101] B. Helferich and O. Peters, *Ber.*, 70, 465 (1937).

Instead of the ethyl-glyoxylate ethyl-mesoxylate may also be used with the same effect:[102]



(d) Synthesis of Ascorbic Acid by Ester Condensation of Benzoyl-glycolic Acid with α-Oxy-acids[103]

This method is best illustrated by the following formulas:



8. Industrial Methods of Preparation

Vitamin C is commercially available in the crystalline form, made either by synthesis or by extraction from natural sources. Since very

[102] B. Helferich, G. P., 683,954.
[103] F. Micheel and H. Haarhoff, *Ann.*, **545**, 28 (1940).

economic methods have been worked out for the synthetic route, using the 2-keto-hexonic acid as intermediate, the isolation procedure from natural sources becomes less and less attractive. The purity of commercial ascorbic acid is about 99%.

In the canning industry special methods have been developed for essentially maintaining the natural vitamin C content of foods. For example, the water used to cook vegetables is boiled before the plant material is added in order to avoid oxidation by the air dissolved in the water.[104] The boiling process itself is carried out anaerobically.[104] Vegetables are stored in refrigerators where the loss of vitamin C is slow, whereas at room temperature most plant materials lose their vitamin C content rapidly. The freezing method for preserving food causes no essential loss of vitamin C provided the material is consumed immediately after defrosting.

9. Biogenesis

The mechanism of the ascorbic acid formation in plants and in animals is largely unknown. It seems conceivable that ascorbic acid may be produced by transformation of sugar acids of related structure such as glucuronic acid or galacturonic acid, or by total synthesis. In favor of the latter hypothesis is the observation that volatile constituents of plant and animal unsaponifiable matter, lipid in nature, can serve as the precursor of ascorbic acid in the body of the rat.[105] Furthermore, it has been found that the vitamin C content of livers and intestines of rats which had been subjected to extreme periods of inanition did not change significantly. This suggests that the precursor of ascorbic acid was probably of endogenous origin and independent of carbohydrate intake.[106]

In favor of the hypothesis that vitamin C is formed by chemical transformation of compounds of related structure is the observation that dextrose apparently increases the ascorbic acid content of slices of intestinal tissue,[107] but not of tissue slices from liver, spleen, stomach and brain, when the vitamin is determined by iodine titration. Among many sugars investigated, mannose causes a greater rise of ascorbic acid than any other sugar investigated, in plant[108] and animal[109] tissue in *in vitro* and *in vivo* experiments. Also *l*-sorbose has been found active as precursor of vita-

[104] E. F. Kohman, W. H. Eddy and C. Z. Gurin, *Ind. Eng. Chem.*, 23, 1064 (1931); 25, 682 (1933).
[105] R. R. Musulin, R. H. Tully, H. E. Longenecker and C. G. King, *J. Biol. Chem.*, 129, 437 (1939).
[106] C. Mentzer and G. Urbain, *Compt. rend. soc. biol.*, 128, 270 (1938).
[107] F. Widenbauer and K. Koschorreck, *Biochem. Z.*, 291, 209 (1937).
[108] S. N. Ray, *Biochem. J.*, 28, 996 (1934).
[109] B. C. Guha and A. R. Ghosh, *Nature*, 134, 739 (1934); 135, 234, 871 (1935); 138, 844 (1936).

min C.[110] These findings, however, could not be confirmed.[111] It has recently been postulated[112] that the presence of traces of manganese is necessary for the successful synthesis of ascorbic acid from mannose and to a lesser extent from galactose and glucose in plant and in animal tissues, especially in the liver. Also the inability of man and guinea pigs to produce vitamin C has been discussed in view of the low Mn concentration of their tissues.[113] It has furthermore been shown that whereas the optimum Mn concentration in *in vitro* and *in vivo* experiments with rat liver tissue is about 0.001–0.005%, the concentration of manganese necessary for a successful synthesis of ascorbic acid in *in vitro* and *in vivo* experiments with guinea-pig liver tissue is much higher.[113]

A correlation of available manganese with ascorbic acid production has also been found in plants. The ascorbic acid content of tomatoes from plants grown on soils low in manganese is considerably lower than from plants grown on soils with higher manganese content. In pot cultures the application of one gram of $MnSO_4 \cdot 4H_2O$, in a localized area, to 15,000 g. of Sassafras sandy loam soil increased the ascorbic acid content in tomato pulp from 142 to 243 mg. per liter.[114]

The various observations of increased ascorbic acid excretion following the administration of various chemicals appear to result from a slightly different chemical mechanism.[115] Particularly effective are terpene-like cyclic ketones, for example, *l*- and *d*-carvone, *d,l*-piperitone, isophorone, α- and β-ionone, pulegone, thujone, camphor and neridol and somewhat less active are di-isobutyl-ketone, dipropyl-ketone and dimethyl-acetyl-carbinol. These results indicate that a stimulation of the normal ascorbic acid synthesis occurs in order to detoxify compounds which are foreign to the tissues. The increase of ascorbic acid content of rat adrenals after feeding 3-hydroxy-acetonyl-acetone may probably be explained similarly.[116]

For the site of ascorbic acid formation in plants and for the influence of light upon the synthesis see page 324.

10. Specificity

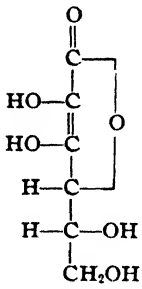
The antiscorbutic acitivity of vitamin C is quite specific. A number of salts are active, for example, the sodium, copper, manganese and iron

[110] G. v. Sztareczy, *Biochem. Z.*, **295**, 369 (1938).
[111] A. Scheunert and M. Schieblich, *Z. physiol. Chem.*, **246**, 272 (1937). J. R. Hawthorne and D. C. Harrison, *Biochem. J.*, **31**, 1061 (1937).
[112] M. N. Rudra, *Nature*, **141**, 203 (1938); **143**, 811 (1939); *Biochem. Z.*, **301**, 238 (1939).
[113] M. N. Rudra, *Nature*, **144**, 868 (1939).
[114] J. B. Hester, *Science*, **93**, 401 (1941).
[115] H. E. Longenecker, R. R. Musulin, R. H. Tully and C. G. King, *J. Biol. Chem.*, **129**, 445 (1939).
[116] J. Mosonyi, *Z. physiol. Chem.*, **230**, 240 (1934).

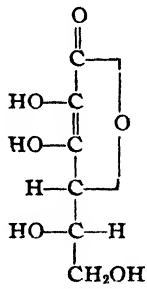
salts, and salts of organic amines, for example, of mono-ethanol-amine and of quinine. None of the simple derivatives of ascorbic acid, for example, the acetone derivative,[117] the dimethyl-ether, the dihydro-compound or the imino-ascorbic acid, are active. It is important to note, however, that the oxidized form, the dehydro-ascorbic acid, in lactone form has the same activity as the non-oxidized form. The open chain dehydro-ascorbic acid, on the other hand, is completely inactive, whereas the methyl-2-keto-*l*-gulonate, but not the free acid, is active,[118] presumably because the organism is able to convert this compound into ascorbic acid.

Practically all the theoretically possible stereoisomers and simple homologs of ascorbic acid have been prepared and tested for antiscorbutic activity. From the results of these experiments it is concluded that for antiscorbutic activity a *d*-configuration is necessary at carbon atom 4, that a side chain must be attached to this carbon atom, and that in the side chain a hydroxyl group is necessary in 5-position. All hydroxyl groups must be free.

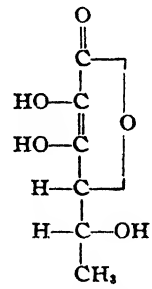
The number of active compounds is, therefore, quite limited. They are: *d*-arabo-ascorbic acid[119, 120, 121, 122] (iso-ascorbic acid) (about $^1/_{20}$ of the activity of ascorbic acid), 6-desoxy-*l*-ascorbic acid[123] (about $^1/_3$ of the activity of ascorbic acid), *l*-rhamno-ascorbic acid[124] (about $^1/_5$), *l*-gluco-ascorbic acid[125] (about $^1/_{40}$), *l*-fuco-ascorbic acid[125] (about $^1/_{50}$) and *d*-gluco-



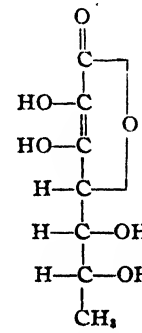
l-Ascorbic acid



d-Arabo-ascorbic acid

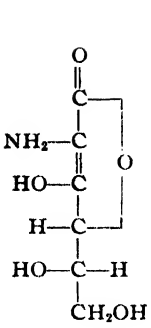


6-Desoxy-*l*-ascorbic acid

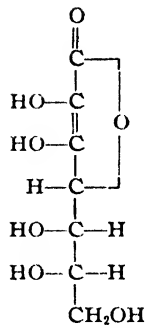


l-Rhamno-ascorbic acid

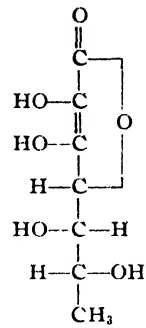
[117] F. Micheel and T. Moll, *Z. physiol. Chem.*, **219**, 253 (1933).
[118] T. Reichstein and V. Demole, *Festschrift für E. C. Barell*, Basel, 1936.
[119] T. Reichstein, A. Grüssner and R. Oppenauer, *Helv. Chim. Acta*, **17**, 510 (1934).
[120] H. Ohle, H. Erbach and H. Carls, *Ber.*, **67**, 324 (1934).
[121] K. Maurer and B. Schiedt, *Ibid.*, **66**, 1054 (1933); **67**, 1239 (1934).
[122] O. Dalmer and T. Moll, *Z. physiol. Chem.*, **222**, 116 (1933).
[123] H. Müller and T. Reichstein, *Helv. Chim. Acta*, **21**, 273 (1938).
[124] T. Reichstein, L. Schwarz and A. Grüssner, *Ibid.*, **18**, 353 (1935).
[125] T. Reichstein and V. Demole, *Festschrift für E. C. Barell*, Basel, 1936.



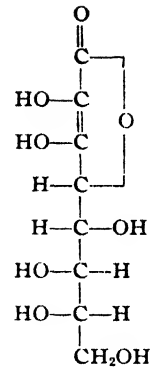
l-Scorbamic acid



l-Gluco-ascorbic acid



l-Fuco-ascorbic acid



d-Gluco-hepto-ascorbic acid

hepto-ascorbic acid[125] (about $^1/_{100}$). *l*-Scorbamic acid has also been reported[126] to show antiscorbutic activity. Of all the compounds, *l*-ascorbic is the most potent.

Vitamin C is the only naturally occurring antiscorbutic compound. However, another substance has been isolated from adrenal cortex[127] of beef, which is said to show faint antiscorbutic activity and which possesses the reducing and general chemical properties of ascorbic acid. The composition was found to be: C 25.86%, H 5.97%, N 5.97%, P 0.55%. The chemical and physical data of this compound do not permit any conclusion to be drawn as to the purity or the constitution of the compound. It might well be that a form of ascorbigen, a combined ascorbic acid, was isolated. Another possibility would be that ascorbic acid can be chemically bound in a way similar to the vitamins of the B-group to complex molecules containing phosphoric acid, thus forming part of enzyme systems. Any definite opinion about the existence of a second naturally occurring antiscorbutic substance must be withheld until further experimental data become available.

11. Determination

(*a*) *Physical Methods*

Spectroscopical Method. The determination of the intensity of the characteristic absorption spectrum in water solution at 265 mμ, for ex-

[126] F. Micheel and R. Mittag, *Naturwissenschaften*, **25**, 158 (1937); *Z. physiol. Chem.*, **247**, 34 (1937).
[127] E. Ott, K. Krämer and W. Faust, *Z. physiol. Chem.*, **243**, 199 (1936).

ample, before and after destruction of the vitamin, has been advocated.[128] Since vitamin C in solution is quickly destroyed, the determination must be carried out rapidly. An addition of reducing agents has been recommended. The position of the absorption band is somewhat different in various solvents. In alcohol, for example, the maximum is at 245 mμ. The position of the band is, furthermore, a function of the pH. With decreasing pH the maximum shifts toward shorter wave lengths.[129] The best method of determining ascorbic acid is to use a water solution of the vitamin to which an equimolecular amount of potassium cyanide as stabilizer has been added (Fig. 14 on page 294).[130, 131]

Polarographic Method. Determination of the oxidation potential of ascorbic acid in acid solution, for example, in extracts from fruits or vegetables, has given quite satisfactory results.[132]

(*b*) *Chemical Methods*

Chemical methods of determining vitamin C are generally used today replacing almost entirely the biological methods of earlier days. The chemical methods are mostly based on the great reducing ability of ascorbic acid. Since besides ascorbic acid the naturally occurring dehydro-ascorbic acid also exhibits vitamin C activity, care must be taken to include the non-reducing dehydro-ascorbic acid in determinations of vitamin C in natural products. It should, furthermore, be observed that apparently varying amounts of vitamin C are chemically bound to protein materials, in which combination the ascorbic acid shows no reducing action.

Titration with Iodine. The vitamin C content of pure solutions can be determined by titration with 0.01 *N* iodine solution. This method proved to be inadequate for the determination of ascorbic acid in natural products, since they contain other reducing substances besides vitamin C and since the color of such products interferes with the determination of the end-point of the iodine titration.

Titration with 2,6-Dichloro-phenol-indophenol. The determination of vitamin C by titration with 2,6-dichloro-phenol-indophenol (see formula p. 317), originally suggested by Tillmans,[133] is the most widely used method today. A great number of different modifications have been

[128] E. B. Robertson, *J. Soc. Chem. Ind.*, **53**, 277 (1934). A. Chevallier and Y. Choron *Compt. rend. soc. biol.*, **124**, 453 (1937).

[129] B. Starzynski, *Bull. Acad. Pol. Sci. Letter*, **A 1937**, 462.

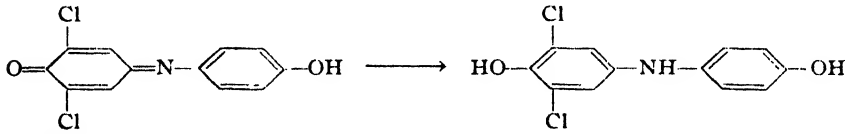
[130] R. A. Morton, *The Application of Absorption Spectra to the Study of Vitamins and Hormones*, London, 1935.

[131] H. Mohler and H. Lohr, *Helv. Chim. Acta*, **21**, 485 (1938).

[132] K. Schwartz, *Z. anal. Chem.*, **115**, 161 (1939). T. Osterud, *Tek. Ukeblad*, **86**, 216 (1939).

[133] J. Tillmans, *Z. Untersuch. Lebensm.*, **54**, 33 (1927).

proposed to overcome the various insufficiencies resulting from the determination of vitamin C in the presence of other plant or animal materials. The indophenol dye oxidizes besides ascorbic acid, for example, sulfhydryl-compounds,[134] thiosulfate,[135] pyridinium compounds[136] (medication), the reduced forms of nicotinic acid derivatives and of riboflavin,[137] etc. Inorganic and organic ferrous and ferric compounds also interfere with the determination.[138] In beer, yeast, malt, mold, etc., organic reducing substances are found which react exactly like ascorbic acid toward the indophenol indicator. These compounds are sugar derivatives, formed by alkali. Known examples of these are reductic acid[139] and reductone.[140]



2,6-Dichloro-phenol-indophenol

A certain amount of vitamin C in solution is often present in the dehydro-form, which is not determined by the indophenol titration. In order to include the dehydro-form, it is necessary to convert this form first into ascorbic acid. This is carried out by hydrogen sulfide at **pH** 4–7, followed by an elimination of the excess hydrogen sulfide by blowing nitrogen through the solution. The results obtained by this method, however, are not always trustworthy. The following method has therefore been suggested: (1) to convert the total vitamin C content into the dehydro-form, for example, by passing through Norite or by ascorbic acid oxidase; (2) to determine the amount of reducing substances left; (3) to reduce the dehydro-compound with hydrogen sulfide, and (4) to titrate the vitamin C with indophenol.[141] The difference between the value from the second titration and the value of the first titration gives fairly reliable results of the total amount of ascorbic acid present, unless, as in urine, substances are present which are capable of slow reduction with hydrogen sulfide to indophenol-reducing substances.[142] This difficulty can, how-

[134] A. Emmerie, *Biochem. J.*, **28**, 268 (1934).

[135] M. van Eekelen, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **4**, 137 (1934). M. Heinemann, *Ibid.*, **6**, 67 (1936).

[136] C. F. Gannon and T. McGovern, *Proc Soc. Exptl. Biol. Med.*, **38**, 267 (1938).

[137] W. W. Woessner, C. A. Elvehjem and H. A. Schuette, *J. Nutrition*, **18**, 619 (1939).

[138] K. P. Basu and M. C. Nath., *J. Indian Chem. Soc.*, **15**, 133 (1938).

[139] T. Reichstein and R. Oppenauer, *Helv. Chim. Acta*, **17**, 390 (1934).

[140] E. K. Nelson and C. A. Browne, *J. Am. Chem. Soc.*, **51**, 830 (1929). H. v. Euler and C. Martius, *Ann.*, **505**, 73 (1933).

[141] A. Emmerie and M. van Eekelen, *Biochem. J.*, **28**, 1151 (1934); **30**, 25 (1936).

[142] H. Scarborough and C. P. Stewart, *Ibid.*, **31**, 2282 (1937).

ever, be overcome by appropriate use of a photoelectric colorimeter, by following the progress of the titration as a function of time and by extrapolation of the results obtained.[143, 144, 145] The determination of vitamin C in fruits and vegetables may be carried out by any of these methods with a fair degree of accuracy. Since the amount of the dehydro-form in the living plant is generally insignificant (it is significant, however, in stored materials), approximate values can be obtained by direct titration if the fresh material is extracted sufficiently rapidly with a strong acid, for example, acetic acid,[146] trichloro-acetic acid,[147] metaphosphoric acid,[148] oxalic acid,[149] etc., to diminish the oxidation of vitamin C by the oxidase present in the plant cells and to exclude the titration of glutathione present, and if the titration is performed immediately thereafter.

Some other modifications have also been proposed. Thus, it has been suggested[150] to extract the tissues in the cold with sulfuric acid or with phosphoric acid, to reduce the extract, for example, with hydrogen sulfide, cadmium, zinc, aluminum, palladium, chromium or titanium at a pH of about 4.5, and to titrate with indophenol before and after an addition of copper sulfate. Since the copper ions oxidize preferentially the ascorbic acid, the difference of the two titrations corresponds to the ascorbic acid content.

The accuracy of the determination of vitamin C in extracts of animal origin is somewhat limited. Liver, for example, may give values up to 20% too high. Metaphosphoric acid is particularly useful for the extraction of animal tissues, since it also deproteinizes the extracts.[151] Foreign reducing materials may be removed with lead acetate[152] or with mercuric acetate.[153] The latter, however, also oxidizes ascorbic acid to a considerable extent to dehydro-ascorbic acid.

Special methods have been worked out to determine the ascorbic acid concentration in blood plasma[154] and in milk,[155] which involve the use of oxalic acid either alone or in mixture with metaphosphoric acid. Dehydro-ascorbic acid is determined by reduction with hydrogen sulfide.

[143] R. L. Mindlin and A. M. Butler, *J. Biol. Chem.*, **122**, 673 (1938).
[144] O. A. Bessey, *Ibid.*, **126**, 771 (1938).
[145] W. W. Woessner, C. A. Elvehjem and H. A. Schuette, *J. Nutrition*, **18**, 619 (1939).
[146] R. Strohecker and R. Vaubel, *Angew. Chem.*, **49**, 666 (1936).
[147] T. W. Birch, L. J. Harris and S. Ray, *Biochem. J.*, **27**, 590 (1933).
[148] W. W. Woessner, C. A. Elvehjem and H. A. Schuette, *J. Nutrition*, **18**, 619 (1939).
[149] B. Willberg, *Z. Untersuch. Lebensm.*, **76**, 128 (1938).
[150] M. Ott, *Angew. Chem.*, **54**, 170 (1941).
[151] A. Fujita and T. Ebihara, *Biochem. Z.*, **290**, 172 (1936).
[152] V. A. Deviatnin and V. M. Doroshenko, *Ibid.*, **280**, 118 (1935).
[153] A. Emmerie and M. Eekelen, *Biochem. J.*, **28**, 1151 (1934); **30**, 25 (1936).
[154] R. L. Mindlin and A. M. Butler, *J. Biol. Chem.*, **122**, 673 (1938).
[155] W. W. Woessner, C. A. Elvehjem and H. A. Schuette, *J. Nutrition*, **18**, 619 (1939).

The sensitivity of the determination of ascorbic acid with indophenol is quite satisfactory and in questionable cases, for example, in cases of prevalent turbidity, the results obtained are checked by adding a known amount of ascorbic acid to the solution.

The end point of the titration with the indophenol dye may be determined by visual observation or by the aid of a colorimeter[156] or a photoelectrometer.[157, 158, 159] The latter method is especially accurate since a decrease in concentration of the dye, produced by the addition of a vitamin C-containing extract of insufficient concentration to cause complete reduction of the indicator,[160] can be measured. The titration of very dark solutions may be carried out by extracting the excess dye after the complete oxidation of the vitamin.[161]

All these methods, as described, include only the determination of ascorbic acid and of dehydro-ascorbic acid, but not the determination of the combined form, ascorbigen, although experimental evidence has been presented that the ascorbic acid is liberated from ascorbigen by extraction with metaphosphoric or sulfo-salicylic acid.[162, 163] In order to determine the total true ascorbic acid content of plant or animal material, the vitamin must be liberated from its carrier. An aqueous suspension of the material is heated while passing hydrogen sulfide through the solution, and after removal of the excess hydrogen sulfide by carbon dioxide or nitrogen the solution is titrated, for example, before and after a treatment with ascorbic acid oxidase. The transformation into the dehydro-compound by means of the specific enzyme or by some other means such as copper ions appears to be necessary, since during the heating process in the presence of hydrogen sulfide an appreciable amount of reducing compounds other than vitamin C is formed which are capable of being oxidized by the indophenol dye.[164]

Titration with Methylene-blue. Ascorbic acid reduces methylene-blue in the presence of light to the leuco-compound. This reaction has repeatedly been used[165, 166] and advocated for the quantitative deter-

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[156] M. Ott, *Angew. Chem.*, **51**, 537 (1938).
[157] K. A. Evelyn, *J. Biol. Chem.*, **115**, 163 (1936). K. A. Evelyn, H. T. Malloy and C. Rosen, *Ibid.*, **126**, 645 (1938).
[158] T. Guthe and K. K. Nygaard, *J. Soc. Chem. Ind.*, **57**, 1195 (1938).
[159] R. L. Mindlin and A. M. Butler, *J. Biol. Chem.*, **122**, 673 (1938).
[160] O. A. Bessey, *Ibid.*, **126**, 771 (1938),
[161] F. Siebert, *Dissertation*, Frankfurt a. Main, 1931.
[162] K. Wachholder and A. Okrent, *Z. physiol. Chem.*, **264**, 254 (1940).
[163] A. Fujita and T. Ebihara, *Biochem. Z.*, **301**, 229 (1939).
[164] P. N. Sen-Gupta and B. C. Guha, *J. Indian Chem. Soc.*, **16**, 549 (1939).
[165] H. Lund and H. Lieck, *Nature*, **137**, 784 (1936).
[166] I. Gál, *Ibid.*, **138**, 799 (1936).

mination of vitamin C. The vitamin is extracted from plant or animal material by means of trichloro-acetic acid[167] or sulfo-salicylic acid.[168]

Quantitative Determination by Means of Folin's Reagent.[169] Vitamin C, extracted from animal or plant material by metaphosphoric acid, has been claimed to be oxidized specifically by mono-iodo-acetic acid and Folin's reagent,[170] which consists of a solution of molybdenum-free sodium tungstate in dilute orthophosphoric acid to which a small amount of bromine water is added. A blue color is produced which is measured in a colorimeter. This reaction is not very specific and is given, for example, by other dihydroxy-compounds and by phenols.

A similar color is obtained by the addition of sodium tungstate in sulfuric acid to a solution of the vitamin in metaphosphoric acid followed by the addition of alkali.[171]

Determination by Means of Molybdenum-phosphotungstic acid.[172] This reagent produces with vitamin C a violet color which is measured colorimetrically.

Test According to Giri Using Ferricyanide and Ammonium Molybdate.[173] Ascorbic acid in trichloro-acetic acid solution reduces potassium ferricyanide which upon further addition of ammonium molybdate yields a red-brown precipitate. Vitamin C solutions from natural sources are purified by the addition of mercuric acetate which precipitates pigments, tannins, glutathione, cysteine, etc.

Phosphomolybdic Acid Test.[174, 175] Phosphomolybdic acid produces a blue color in acid solution with ascorbic acid.

Bachstez-Cavallini Reaction with Uranyl-acetate.[176, 177] Uranyl-acetate in slightly alkaline solution produces with ascorbic acid a brown color discharged by the alkali followed by precipitation of sodium uranate. This reaction serves as a test for differentiating vitamin C from isoascorbic acid, since with the latter compound only a brownish color develops but no precipitation occurs.

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[167] E. Martini and A. Bonsignore, *Biochem. Z.*, **273**, 170 (1934); *Boll. soc. ital. biol. sper.*, **9**, 388 (1934).

[168] W. Quensel and K. Wachholder, *Z. physiol. Chem.*, **231**, 65 (1935). K. Wachholder and H. H. Podesta, *Ibid.*, **239**, 149 (1936).

[169] A. Fujita and T. Ebihara, *Biochem. Z.*, **290**, 182 (1936).

[170] O. Folin, *J. Biol. Chem.*, **106**, 311 (1934).

[171] A. Fujita, D. Iwatake and T. Mijata, *Biochem. Z.*, **277**, 296 (1935).

[172] N. Bezssonoff, *Z. Vitaminforsch.*, **5**, 193 (1936).

[173] K. V. Giri, *Microchemie*, **23**, 283 (1938).

[174] H. Tillmans and P. Hirsch, *Z. Untersuch. Lebensm.*, **63**, 2, 13 (1922).

[175] N. Bezssonoff, *Bull. soc. chim. biol.*, **16**, 1107, 1133, 1160 (1934). H. v. Euler and D. Burström, *Biochem. Z.*, **283**, 153 (1936).

[176] M. Bachstez and G. Cavallini, *Z. physiol. Chem.*, **228**, 25 (1934).

[177] A. Emmerie, *Acta Brevia Neerland. Physiol. Pharmocol. Microbiol.*, **4**, 141 (1934).

Vanadium Test.[178] Vitamin C yields a blue color, which later changes to green, with a reagent prepared from vanadium pentoxide and sulfuric acid.

Barac's Azo Test.[179] Diazotized sulfanilic acid is reduced by the action of ascorbic acid and forms the orange compound:

$$HO_3S—C_6H_4N{=}NC_6H_4—SO_3H$$

Sulfanilamide Test.[180] A solution of sulfanilamide, sodium nitrate, sulfo-salicylic acid and urea is mixed with a solution of ascorbic acid, and α-dimethyl-naphthyl-amine is added. The color developed is compared with the color of the same mixture without the vitamin.

Selenous Acid Test.[181] Selenous acid solutions produce an orange-red color with ascorbic acid in solution.

Gold-trichloride test.[181] The ability of ascorbic acid to reduce gold trichloride has been used for the determination of vitamin C.

Mercuric Chloride Test.[182] Mercuric chloride is precipitated from a solution of mercurous chloride by the addition of ascorbic acid.

Pitarelli's Test with Cupric Sulfate and Ammonium Thiocyanate.[182] A white precipitation occurs upon the addition of cupric sulfate and ammonium thiocyanate and a green color develops upon the addition of further amounts of ammonium thiocyanate to ascorbic acid solutions.

Szent-Györgyi's Reaction with Ferrous Sulfate. A dark violet color develops upon addition of a ferrous sulfate solution to an alkali solution of vitamin C. The color is bleached upon reduction with hyposulfite and can be restored by air oxidation.

Test According to Tauber.[183] An acetic acid solution of ascorbic acid yields a blue color when first a solution of ferric cyanide and then a solution of ferric sulfate and phosphoric acid is added.

Furfural Test.[184] Vitamin C upon boiling with hydrochloric acid forms furfural, which is determined with the known aniline phloroglucinol or resorcinol test. Pentoses are included in the values obtained, whereas hexoses and glucuronic acid have a comparatively small furfural-producing capacity.[185]

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[178] A. T. Freses, *Bol. soc. quim. Peru*, **4**, 22 (1938).
[179] G. Barac, *Compt. rend. soc. biol.*, **126**, 61 (1937).
[180] J. V. Scudi and H. D. Ratish, *Ind. Eng. Chem., Anal. Ed.*, **10**, 420 (1938).
[181] A. Emmerie, *Acta Brevia Neerland. Physiol. Pharmocol. Microbiol.*, **4**, 141 (1934).
[182] E. Pittarelli, *Biochim. terap. sper.*, **22**, 100 (1936).
[183] H. Tauber, *Microchemie*, **17**, 111 (1935).
[184] J. H. Roe, *Science*, **80**, 561 (1934).
[185] G. E. Youngberg, *J. Biol. Chem.*, **73**, 599 (1927).

A modification of the furfural test is the *osazone-furfural method.* This has especially been adapted for the quantitative determination of vitamin C in urine. The vitamin is oxidized to dehydro-ascorbic acid by passing through Norite, and separated as a dinitro-phenyl-osazone. The nitro-groups are reduced with stannous chloride followed by hydrolysis of the osazone and the dehydro-ascorbic acid obtained is determined by conversion into furfural, etc. Another possibility is to titrate the dinitro-phenyl-osazone with titanium chloride in acid solution.[186]

Cacotheline Test.[187] Cacotheline (nitro-brucin) produces in hydrochloric acid solution a lilac color with vitamin C and other reducing agents.

Determination of Vitamin C by Its Oxidation to Oxalic Acid. Vitamin C is oxidized in acid solution with permanganate. After destroying the excess oxidizing agent with hydrogen peroxide, the oxalic acid produced is determined.[188]

Prussian Blue Method. An acid ferricyanide solution is easily reduced by vitamin C and converted into Prussian blue by known methods. The amount of blue is measured with a colorimeter.

The *determination of dehydro-ascorbic acid in the presence of ascorbic acid can be accomplished* by reaction with 2,4-dinitro-phenyl-hydrazine. The osazone of the dehydro-ascorbic acid precipitates and can be determined gravimetrically or by titration with titanium chloride.

The rough *determination of vitamin C* in cells is carried out by the silver nitrate staining technic of Bourne, Giroud, Leblond and associates.

(*c*) *Biochemical Methods*

Ascorbic Acid Oxidase Method.[189] This method is based on the ability of ascorbic acid oxidase to oxidize, preferentially, ascorbic acid. This oxidase, however, is not specific enough to give trustworthy results in the presence of other natural products,[190] especially in the presence of extracts from animal tissues which contain, besides ascorbic acid, other compounds capable of reducing methylene blue.[191] It has, furthermore, been observed that a special preparation of oxidase from pumpkin did not react with vitamin C in human urine, spinal fluid and cow's milk.[192]

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[186] L. Espil and L. Genevois, *Bull. soc. chim.*, **5**, 1532 (1938).
[187] L. Rosenthaler, *Z. Vitaminforsch.*, **7**, 126 (1938).
[188] M. Paget and R. Berger, *Compt. rend. soc. biol.*, **129**, 960 (1938)
[189] H. Tauber and I. S. Kleiner, *J. Biol. Chem.*, **110**, 559 (1935).
[190] G. A. Snow and S. S. Zilva, *Biochem. J.*, **32**, 1926 (1938).
[191] K. Wachholder and A. Okrent, *Z. physiol. Chem.*, **264**, 254 (1940).
[192] N. Bezssonoff and H. Vertruyen, *Compt. rend. soc. biol.*, **128**, 407 (1938).

(*d*) *Biological Methods*

Although chemical and to a small extent also physical methods are replacing more and more the biological determinations of vitamin C, the biological tests maintain their place as the ultimate and most correct method of determining vitamin C.

Guinea pigs are the only animals which can be used. When they are kept on a vitamin C-free diet, they develop scurvy within two to three weeks. Vitamin C determination tests are carried out using either the principle of the preventive method, that is, determining the smallest amount which prevents macroscopic signs of scurvy, for example, decrease in weight, or the principle of the curative method,[193] that is, determining the smallest amount which will cure guinea pigs showing definite signs of scurvy. Another method is the histological examination of the teeth of guinea pigs.[194] In a scorbutic tooth the odontoblast layer is disorganized, the predentine is calcified and an irregular layer of bone is formed between the odontoblasts and the predentine.

12. Standards

By definition:

1 I. U. (International Unit) vitamin C = 0.05 mg. of pure ascorbic acid[195]
= 1 U. S. Pharmacopoeia Unit.
1 Sherman Unit = 0.5-0.6 mg. vitamin C
= 1 minimum protective dose (guinea pig).

The minimum protective unit for guinea pigs has been defined as the smallest amount of ascorbic acid which when ingested daily prevents in guinea pigs of about 200 g. weight at least for 60 days the occurrence of the syndromes of a vitamin C deficiency.

The Standard Reference as recommended by the Permanent Commission on Biological Standardization of the League of Nations in 1931 was 0.1 ml. of freshly squeezed lemon juice, which was defined as 1 International Unit. This standard was changed in 1934 following the availability of the pure material. One Unit of the new standard corresponds in activity approximately to one Unit of the older Standard.

13. Physiology of Plants and Microorganisms

Vitamin C is a necessary growth factor for plants and microorganisms. This has been demonstrated[196] on pea embryos which have been isolated

[193] L. J. Harris, J. I. Mills and J. R. M. Innes, *Lancet*, **2**, 235 (1935). L. J. Harris and S. N. Ray, *Biochem. J.*, **27**, 2006 (1933).

[194] J. A. Höjer, *Brit. J. Expil. Path.*, **7**, 356 (1926). K. M. Key and G. K. Elphick, *Biochem. J.*, **25**, 888 (1931).

[195] *Quart. Bull. Health Organisation League Nations*, **3**, 428 (1934).

[196] J. Bonner and D. Bonner, *Proc. Natl. Acad. Sci. U. S.*, **24**, 70 (1938). D. Glick, *Z. physiol. Chem.*, **245**, 211 (1937). S. von Hausen, *Biochem. Z.*, **288**, 378 (1936).

from their seeds and thereby from their natural nutritional resources. Dry seeds do not contain any demonstrable amounts of this vitamin during their inactive rest period, but contain some unknown precursor which is converted into ascorbic acid immediately upon the beginning of germination. Tubers, such as the potato tuber, on the other hand, contain appreciable amounts of vitamin C.

The beneficial effect of ascorbic acid on higher plants can also be demonstrated in other cases. There is a considerable species difference, for example, eggplant shows no response; tobacco plants, however, gain considerably in growth upon administration of ascorbic acid solutions.[197]

It is not known where vitamin C is formed in higher plants but it is found regularly in high amounts in all growing parts. Adult parts contain some vitamin C, but parts which have turned into wood do not contain any ascorbic acid at all. Haw contains the vitamin in the hull and shell, but not in the seeds.[198] The highest amount is found usually in flowers and in leaves. Petals contain more ascorbic acid than pistils, stamens and calyces.[199]

Light seems to have a beneficial effect upon the production of ascorbic acid in plants.[200] This finding might probably be linked with the observation that red and violet flowers generally contain more active material than white and yellow flowers.[201] The concentration of ascorbic acid in plant leaves is also a function of the light received and fluctuates considerably during the day, the maximum being reached, for example, in potato leaves in the forenoon.[202]

The mechanism of the ascorbic acid action in plants is largely unknown. The conception of a participation in the oxidation-reduction systems of the living plant will be discussed with the corresponding action in animal tissues. (See page 326.)

The physiology of vitamin C in microorganisms differs considerably with the species. Thus some bacteria need vitamin C and are apparently able to synthesize it.[203] This question has especially been studied on lactic acid bacteria and it has been found that some strains show a definite growth response to vitamin C added to the culture medium,[204] while others are re-

[197] R. Dennison, *Science*, **92**, 17 (1940).
[198] H. Winckelmann, *Hippokrates*, **9**, 714 (1938).
[199] H. Mituda, *J. Agar. Chem. Soç. Japan*, **14**, 1228 (1938).
[200] M. E. Reid, *Am. J. Botany*, **25**, 702 (1938).
[201] H. Mituda, *J. Agr. Chem. Soc. Japan*, **14**, 1228 (1938).
[202] A. M. Smith and J. Gillies, *Biochem. J.*, **34**, 1312 (1940).
[203] G. Bourne and R. Allen, *Australian J. Exptl. Biol. Med. Sci.*, **13**, 165 (1935).
[204] O. Rahn and C. P. Hegarty, *Proc. Soc. Exptl. Biol. Med.*, **38**, 218 (1938). O. Rahn, C. P. Hegarty, and R. E. Deuel, *J. Bact.*, **35**, 547 (1938). A. Sartory, R. Sartory and J. Meyer, *Compt. rend.*, **206**, 1414 (1938). J. G. Davis and J. McClemont, *J. Dairy Research*, **10**, 94 (1939).

tarded and some apparently do not respond either way. Vitamin C is synthesized, for example, by *Bacillus prodigiosus*[205] as proved by chemical and biological determination. It has also been demonstrated that some protozoa need an external supply of ascorbic acid for optimal growth.[206]

14. Animal Physiology

Of all the vitamins, vitamin C has received the widest attention in a large number of various investigations relating to different phases of physiological interest. Nevertheless, the knowledge about the general physiology, the metabolism and the mechanism of the vitamin C action is still fragmentary and the fundamentals of these subjects are still unknown.

(*a*) *Metabolism*

Ascorbic acid is absorbed by the tissues of the intestinal tract,[207] principally of the small intestine. After oral ingestion of vitamin C the vitamin level in blood plasma rises to a maximum within about 1.5 hours, but occasionally, for example, after an intake of strawberries or cauliflower a considerably longer period of time elapses before the vitamin level in the blood is increased.[208] In any event, the increase is only temporary. The vitamin is transported with the blood throughout the entire organism and excess amounts are secreted in the urine[209] where it appears mostly in the free form, but to a small extent also in a combined form.[210] Ascorbic acid given intravenously or subcutaneously raises temporarily the vitamin C content of the blood, but is excreted within one to three hours.[211, 212] More prolonged effects are obtained if the vitamin is injected intramuscularly. This, however, causes sloughing due to the acidity of the vitamin. The preferred method in cases of necessary parenteral administration is to inject salts, for example, sodium salt, or salts of organic amines, for example, of monoethanol-amine.[213]

[205] K. H. Busing and F. Peters, *Biochem. Z.*, **304**, 134 (1940).

[206] M. Lwoff, *Compt. rend.*, **206**, 540 (1938); *Compt. rend. soc. biol.*, **130**, 406 (1939). R. Cailleau, *Compt. rend. soc. biol.*, **127**, 861 (1938); **131**, 964 (1939); **138**, 319 (1939).

[207] S. S. Zilva, *Biochem. J.*, **29**, 100 (1935).

[208] E. N. Todhunter, *Am. Inst. Nutrition, Abstracts J. Nutrition*, **21**, 12 (1941).

[209] L. J. Harris and S. N. Ray, *Lancet*, **1**, 71 (1935). H. v. Euler and M. Malmberg, *Biochem. Z.*, **279**, 338 (1935).

[210] B. C. Guha and P. N. Sen-Gupta, *Nature*, **141**, 874 (1938). H. Scarborough and C. P. Stewart, *Biochem. J.*, **31**, 2232 (1937); *Nature*, **142**, 40 (1938).

[211] E. E. Hawley and D. J. Stephens, *Proc. Soc. Exptl. Biol. Med.*, **34**, 854 (1937). M. van Eekelen and M. Heinemann, *J. Clin. Investigation*, **17**, 293 (1938).

[212] E. P. Ralli, G. J. Friedman and M. Kaslow, *Proc. Soc. Exptl. Biol. Med.*, **36**, 52 (1937). I. S. Wright, A. Lilienfeld and E. MacLenathen, *Arch. Internal Med.*, **60**, 264 (1937). J. M. Faulkner and F. H. L. Taylor, *J. Clin. Investigation*, **17**, 69 (1938).

[213] E. L. Lozner, F. J. Pohle and F. H. L. Taylor, *New England J. Med.*, **220**, 987 (1939).

Tissues and body fluids contain various amounts of this vitamin. Normal human blood plasma contains about 1.2 mg. ascorbic acid in 100 cc.[214] There are no real storage organs for this vitamin, although some organs contain increased amounts. Among these the adrenal gland contains the most. It has been shown, however, that, for example, in guinea pigs the amount of ascorbic acid in this gland cannot be increased, even by feeding excess amounts of vitamin C.[215] Generally, tissues of high metabolic activity have the highest vitamin C content. Thus, young tissues contain more vitamin C than older tissues, as has been shown on the thymus[216] and the corpus luteum.[217] Elderly people generally have less vitamin C reserves than young people. Vitamin C is secreted in the milk. Cow's milk contains on the average about 22 mg./l. Human milk contains several times more vitamin C than cow's milk (about 75 mg./l.), since babies, but not calves, need an external supply of this vitamin. Colostrum contains somewhat more vitamin C.[218] The ascorbic acid secretion in cow's milk varies somewhat with the season. The highest amount has been found in the late summer or early fall.[219] Ascorbic acid is also at times excreted in the sweat.[220] The main excretion is through the urine, as stated before. The excretion occurs within four to six hours following ingestion.[221] Some ascorbic acid is also excreted in the feces.[222]

(*b*) *Physiological Action*

The most obvious property of ascorbic acid is the reversible oxidation and reduction capacity, and much speculation and experimental work arose from the idea of correlating this behavior with the mechanism of the vitamin action. It seems established that under physiological conditions the reversibility of the reducing capacity of ascorbic acid exists, although this reversibility is only partial in *in vitro* experiments. The instability of the oxidized form, dehydro-ascorbic acid, even in the intact cell, is probably the reason for the fact that the organism needs a relatively greater amount of this vitamin than of the other vitamins.

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[214] A. F. Abt, C. J. Farmer and I. M. Epstein, *J. Pediatrics*, **8**, 1 (1936). D. J. Stephens and E. E. Hawley, *J. Biol. Chem.*, **115**, 653 (1936).

[215] G. Mouriquand, M. Dauvergne and V. Edel, *Compt. rend.*, **209**, 1023 (1939).

[216] D. Glick and G. R. Biskind, *J. Biol. Chem.*, **113**, 27 (1936); **114**, 1 (1936).

[217] B. C. Guha and P. N. Sen-Gupta, *Nature*, **141**, 974 (1938).

[218] F. Schlemmer, B. Bleyer and H. Cahnmann, *Biochem. Z.*, **254**, 187 (1932).

[219] A. D. Holmes, F. Tripp, E. A. Woelffer and G. H. Satterfield, *J. Nutrition*, **17**, 187 (1937).

[220] A. Lilienfeld, I. S. Wright and E. MacLenathen, *Proc. Soc. Exptl. Biol. Med.*, **35**, 184 (1936). R. E. Bernstein, *Nature*, **140**, 684 (1937).

[221] E. E. Hawley and D. J. Stephens, *Proc. Soc. Exptl. Biol. Med.*, **34**, 854 (1936). M. van Eekelen and M. Heinemann, *J. Clin. Investigation*, **17**, 293 (1938).

[222] H. Chinn and C. J. Farmer, *Proc. Soc. Exptl. Biol. Med.*, **41**, 561 (1939).

It has been postulated that there exist special enzyme systems which take care of both the oxidation and the reduction of ascorbic acid. Thus, an enzyme has been found in blood[223] and in plant juices[224] which reduces dehydro-ascorbic acid. On the other hand, glutathione has been shown to be the most effective reductant and protective agent for vitamin C in the living animal and in some plant cells.[225, 226] Besides glutathione, other compounds with fixed sulfhydryl-groups exert reducing capacities upon the oxidized form of vitamin C. Also certain purines, such as xanthine, uric acid and theophylline, but not caffeine and theobromine, and creatinine, but not creatine, have been shown experimentally to protect the vitamin against oxidation.[227] The opposite reaction, the oxidation of ascorbic acid, is much easier to demonstrate and is carried out by a special enzyme, "ascorbic acid oxidase." The existence of this enzyme in plants is established,[228, 229, 230] but its occurrence in animal tissues is questionable. Ascorbic acid oxidase has the constitution of a copper protein[230] and is specific for the stereochemical configuration of *l*-ascorbic acid.[231] Also, various polyphenylases are able to oxidize ascorbic acid in plants.[232] The search for the nature of the ascorbic acid-oxidizing enzyme in animal tissues has brought forward many experimental evidences which partly support and partly contradict the conception of the constitution of the enzyme as a copper protein. It seems plausible to assume that a number of different oxidation-reduction systems act on ascorbic acid in the living organism. Thus, conclusive evidence has been presented that ascorbic acid is rapidly oxidized by cytochrome oxidase plus cytochrome-*c*.[233] The question of the nature of the mechanism of the ascorbic acid action should therefore be separated from any discussion of the reversible oxidation-reduction action of this vitamin until specific reactions of physiological importance in various organs, cells, body fluids, etc., have been established.

A number of observations along these lines have been made, but it is impossible at the moment to correlate these findings and to decide which ones

[223] J. H. Roe and G. L. Barnum, *J. Nutrition*, **11**, 359 (1936).
[224] E. M. Crook and F. G. Hopkins, *Biochem. J.*, **32**, 1356 (1938).
[225] H. Borsook, H. W. Davenport, C. E. P. Jeffreys and R. C. Warner, *J. Biol. Chem.*, **117**, 237 (1937).
[226] F. G. Hopkins and E. J. Morgan, *Biochem. J.*, **30**, 1446 (1936).
[227] K. V. Giri and P. V. Krishnamurthy, *Nature*, **147**, 59 (1941).
[228] A. Szent-Györgyi, *J. Biol. Chem.*, **90**, 385 (1931).
[229] Z. I. Kertesz, R. B. Dearborn and G. L. Mack, *Ibid.*, **116**, 717 (1936).
[230] P. L. Lovett-Janison and J. M. Nelson, *J. Am. Chem. Soc.*, **62**, 1409 (1940).
[231] H. Rosenberg, *Skand. Arch. Physiol.*, **76**, 119 (1937).
[232] D. Keilin and T. Mann, *Proc. Roy. Soc.* (*London*), **B125**, 187 (1938). F. Kubowitz, *Biochem. Z.*, **299**, 32 (1938).
[233] D. Keilin and E. F. Hartree, *Proc. Roy. Soc.* (*London*), **B125**, 171 (1938). E. Stotz, C. J. Harrer, M. O. Schultze and C. G. King, *J. Biol. Chem.*, **122**, 407 (1938).

are of actual reality or physiological possibility in the organism. Some of these findings are noteworthy, for example, guinea pigs on a vitamin C-depleted diet show a parallel decrease in total oxygen consumption.[234]

Peroxide-peroxidase destroys the physiologically toxic peroxides and is reduced by phenols which thereby become quinones, which in turn are reconverted to phenols by ascorbic acid.[235] The participation of ascorbic acid in hydrolytic or respiratory systems of the quinone and quinonimid type appears also probable from various other studies.[236]

On the basis of the known behavior of ascorbic acid, Szent-Györgyi[237] suggested that this vitamin takes part in a respiratory system, involving the following reactions:

$$\text{Ascorbic acid} + O_2 \xrightarrow[\text{hexoxidase}]{\text{Cu ions}} \text{dehydro-ascorbic acid} + H_2O_2 \qquad (1)$$

$$\text{Flavone} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{oxidized flavone} + H_2O \qquad (2)$$

$$\text{Flavone-oxide} + \text{ascorbic acid} \rightarrow \text{dehydro-ascorbic acid} + \text{flavone} \qquad (3)$$

$$\text{Dehydro-ascorbic acid} + \text{glutathione} \rightarrow \text{ascorbic acid} + \text{oxidized glutathione} \qquad (4)$$

$$\text{Oxidized glutathione} + \text{glucose phosphate} \rightarrow \text{glutathione} + CO_2 + H_2O \qquad (5)$$

This hypothetical system contains "flavones," for example, vitamin P (see page 513) as components. In the absence of this component the reactions (2) and (3) are substituted by

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + {}^1/_2\, O_2 \qquad (6)$$

Another process involving the consumption of oxygen and the possible participation of ascorbic acid is the reversible or irreversible oxidation of hemoglobin or of hemochromogen to the green verdo-hemo-chromogen.[238]

A number of enzymes have been recorded which are supposedly activated by the addition of ascorbic acid, for example, cathepsin, arginase,[239] papain,[240] amylase,[241] catalase, urease,[242] tyrosinase, nuclease,[243] phos-

[234] J. Mosonyi and L. Rigo, *Z. physiol. Chem.*, **222**, 100 (1933).
[235] H. Tauber, *Enzymologia*, **1**, 209 (1936).
[236] C. G. King, *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 137 (1940).
[237] A. Szent-Györgyi, *Studies on Biological Oxidation*, Leipzig, 1937, p. 76.
[238] H. Fischer and F. Lindner, *Z. physiol. Chem.*, **153**, 54 (1926). P. Karrer, H. v. Euler and H. Hellström, *Arkiv. Kemi, Mineral. Geol.*, **B11** No. 6, 6 pp. (1933). R. Lemberg, B. Cortis-Jones and M. Norrie, *Biochem. J.*, **32**, 149, 171 (1938).
[239] H. Tauber, *Ergeb. Enzymforsch.*, **4**, 42 (1935).
[240] E. Maschmann, *Z. physiol. Chem.*, **228**, 141 (1934).
[241] A. Purr, *Biochem. J.*, **28**, 1141 (1934).
[242] F. Lenthardt and F. Koller, *Helv. Chim. Acta*, **17**, 1030 (1934). C. A. Badimond, *Compt. rend soc. biol.*, **125**, 283 (1937).
[243] M. E. Maver and C. Voegtlin, *Am. J. Cancer*, **25**, 780 (1935).

phatase,[244, 245] succinic dehydrogenase[245] and cytochrome oxidase.[245] Observations which seem to indicate that ascorbic acid functions as a coenzyme or as part of a coenzyme are quite interesting. In patients suffering from vitamin C depletion, the amount of blood esterase was found greatly decreased[246] and again increased upon administration of ascorbic acid.[247] Furthermore, the amount of liver esterase found in guinea pigs fed with vitamin C was considerably higher than that found in avitaminotic animals.[248, 249] Liver esterase loses its activity upon dialysis with diluted hydrochloric acid and can be reactivated by addition of ascorbic acid.[248] The amount required for this reactivation was about a thousand times the amount calculated to be present in the original preparation and it took considerable, unphysiological time before the reactivation occurred. It might, therefore, be considered that the active principle is ascorbic acid, but that this compound is chemically bound to some other dialyzable material to make up the coenzyme, similar to the mechanism of riboflavin in various enzyme systems. The material of faint antiscorbutic activity, isolated from adrenal cortex of beef[250] and containing both nitrogen and phosphorus, might perhaps constitute such a coenzyme or part of it. (For a discussion of this compound see also under Specificity of Vitamin C, page 315) Experimental proof for this assumption is lacking, but it should be recalled that "combined ascorbic acid," ascorbigen, has been shown to occur in both animal and plant material.[251]

Explanations other than the coenzyme concept for the activating ability of vitamin C for various enzymes also exist. It has, for example, been postulated that ascorbic acid merely protects some active group of the enzyme system, which is easily oxidized.[252, 253]

The function of vitamin C in the body, discussed so far, is that of a hydrogen transporter in cellular respiration. Another experimentally well-founded function is the participation, in a still unknown manner, in the formation of colloidal intercellular substances, which comprise those of cartilage, dentine, and the matrical of bone and, furthermore, the collagen

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[244] H. v. Euler and T. Svensson, *Svensk. Kem. Tid.*, **47**, 16 (1936).
[245] C. J. Harrer and C. G. King, *J. Biol. Chem.*, **138**, 111 (1941).
[246] O. W. Palladin, *Biochem. Z.*, **152**, 420 (1924).
[247] J. Moster, *Klin. Wochschr.*, **15**, 1558 (1936).
[248] W. v. Pantschenko-Jurewicz and H. Kraut, *Biochem. Z.*, **285**, 407 (1936).
[249] C. J. Harrer and C. G. King, *J. Biol. Chem.*, **138**, 111 (1941).
[250] E. Ott, K. Krämer and W. Faust, *Z. physiol. Chem.*, **243**, 199 (1936).
[251] B. Ahmad, *Nature*, **136**, 797 (1935). E. W. McHenry and M. L. Graham, *Ibid.*, **135**, 871 (1935). E. J. Reedman and E. W. McHenry, *Biochem. J.*, **32**, 85 (1938). H. Scarborough and C. P. Stewart, *Ibid.*, **31**, 2232 (1937); *Nature*, **142**, 40 (1938).
[252] S. Raabe, *Biochem. Z.*, **299**, 141 (1938).
[253] C. J. Harrer and C. G. King, *J. Biol. Chem.*, **138**, 111 (1941).

of all fibrous tissues and non-epithelial cement substances.[254, 255] During avitaminosis, these fibrils or collagen are not formed. These phenomena are in close relationship to the disturbance of the calcium metabolism during times of vitamin C depletion, which affects growth and maintenance of bones and teeth and which sometimes appears similar to that observed during vitamin D deficiency. Also the phenomena of hemorrhagic lesions associated with vitamin C depletion appear to be connected with this basic function of the ascorbic acid. Experimental studies have also shown the particular presence of vitamin C in Golgi cells in the cerebral cortex and the cord, the function of which cells is to bring neighboring cells into relation to each other, and in the mitochondria, which form an essential part of the cytoplasm.[256]

Of particular interest is the reaction of the human organism toward ascorbic acid administration in cases of various poisonings. It has already been pointed out in the section on the Biogenesis of vitamin C (page 312) that the organism of ascorbic acid-producing animals reacts to the administration of chemicals, especially of ketones, by increasing the ascorbic acid excretion. In human beings beneficial effects of large doses of vitamin C have been recorded in cases in which the vitamin may act as a detoxicant. Cases have become known in which toxic doses of drugs, for example, *l*-tyrosine,[257] chemicals,[258] for example, lead[259] and arsenic[260] compounds, benzene,[261] etc., toxins,[262] virus and substances producing anaphylaxis,[263] etc., have had no toxic effect when administered simultaneously with vitamin C. It seems that ascorbic acid combines with some of these substances and is excreted in such combination. Similar detoxification effects are observed toward diphtheria toxins,[264, 265] tuberculosis,[266] and

[254] S. B. Wohlbach and P. R. Howe, *Arch. Path.*, **1**, 1 (1926). S. B. Wohlbach, *Am. J. Path.*, **9**, 689 (1933); *J. Am. Med. Assoc.*, **108**, 7 (1937).

[255] A. v. Jeney and E. Torö, *Arch. path. Anat. (Virchow's)*, **298**, 87 (1936).

[256] A. Giroud and C. P. Leblond, *Anat. Record*, **68**, 113 (1937). G. Bourne and R. Allen, *Australian J. Exptl. Biol. Med. Sci.*, **13**, 165 (1935).

[257] R. R. Sealock and H. E. Silberstein, *Science*, **90**, 517 (1939).

[258] M. Vauthey, *Ann. maladies vénériennes*, **32**, 98 (1937); *Ann. dermatol. syphilol.*, **8**, 568 (1937).

[259] H. N. Holmes, E. J. Amberg and K. Campbell, *Science*, **89**, 322 (1939); *J. Lab. Clin. Med.*, **24**, 1119 (1939).

[260] M. B. Sulzberger and B. L. Oser, *Proc. Soc. Exptl. Biol. Med.*, **32**, 716 (1935). F. E. Cormia, *Can. Med. Assoc. J.*, **36**, 392 (1937). S. Landfisch, *Polska gaz. lek.*, **16**, 575 (1937). I. Dainow, *Presse méd.*, **45**, 1670 (1937).

[261] A. Meyer, *Z. Vitaminforsch.*, **6**, 83 (1937).

[262] C. W. Jungeblut, *J. Immunol.*, **33**, 203 (1937).

[263] F. Diehl, *Klin. Wochschr.*, **18**, 956 (1939).

[264] A. Sigal and C. G. King, *J. Pharmacol. Exptl. Therapy*, **59**, 468 (1937); **61**, 1 (1937).

[265] C. G. King and M. L. Menten, *J. Nutrition*, **10**, 129, 141 (1935). C. K. Greenwald and E. Harde, *Proc. Soc. Exptl. Biol. Med.*, **32**, 1157 (1935).

[266] W. W. Jetter and T. S. Bumbalo, *Am. J. Med. Sci.*, **195**, 362 (1938). M. N. Rudra, *Current Sci.*, **8**, 210 (1939).

many other infectious diseases.[267] During such times the amount of ascorbigen excreted in the urine is increased.[268] Guinea pigs which died from diphtheria intoxications showed reduced vitamin C content of the suprarenals,[269] whereas no significant differences were found between the vitamin C content of the suprarenals of animals injected with a sublethal dose of diphtheria toxins and those of normal animals.[270] Bacterial toxins can cause a decrease of as much as 50 to 85% of the normal vitamin C content of the adrenals.[271]

In this connection the correlation of vitamin C to the complement is of importance. The complement is a thermolabile protein substance in the blood serum which destroys bacteria and other cells. The complement exhibits a reversible oxidation-reduction potential the maintenance of which is to a great extent a function of the ascorbic acid content of the blood serum.[272]

Vitamin C has also been linked with the amino-acid metabolism. It has, for example, been shown *in vitro* that dehydro-ascorbic acid dehydrates amino-acids, for example, leucine, with the formation of ammonia and strongly reducing acidic compounds, probably keto-acids.[273]

A close relationship of vitamin C to the carbohydrate metabolism has, for example, been demonstrated in the case of guinea pigs in which the capacity for metabolizing glucose[274] or dextrose[275] is moderately lowered in the prescorbutic and scorbutic stage of vitamin C deficiency. Vitamin C increases blood sugar in cases of hypoglycemia and in prolonged usage tends to prevent hypoglycemia. In schizophrenics given insulin shock treatment, the vitamin raises the blood sugar and enables the patient to be revived more quickly than by sugar administration. Vitamin C is thus a factor in carbohydrate metabolism. This has also been shown in a disease in which there is a disturbance of muscle-glycogen metabolism.[276]

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[267] D. Perla and J. Marmorston, *Arch. Path.*, **23**, 683 (1937).

[268] B. Ghosh, *J. Indian Chem. Soc.*, **16**, 241 (1939).

[269] E. Harde, *Compt. rend.*, **199**, 618 (1934). C. W. Jungeblut and R. L. Zwemer, *Proc. Soc. Exptl. Biol. Med.*, **32**, 1229 (1934–35).

[270] C. C. Torrance, *J. Biol. Chem.*, **132**, 575 (1940).

[271] C. M. Lyman and C. G. King, *J. Pharmacol. Exptl. Therapy*, **56**, 209 (1936). L. J. Harris, R. Passmore and W. Pagel, *Lancet*, **2**, 183 (1937). C. C. Torrance, *J. Bact.*, **33**, 645 (1937).

[272] E. E. Ecker, L. Pillemer, D. Wertheimer and H. Gradis, *J. Immunol.*, **34**, 19 (1938). E. E. Ecker, L. Pillemer, J. J. Griffiths and W. P. Schwartz, *J. Am. Med. Assoc.*, **112**, 1449 (1939).

[273] H. v. Euler, P. Karrer and F. Zehnder, *Helv. Chim. Acta*, **17**, 157 (1934).

[274] A. Sigal and C. G. King, *J. Biol. Chem.*, **116**, 489 (1936).

[275] A. Sigal and C. G. King, *J. Pharmacol. Exptl. Therapy*, **59**, 468 (1937); **61**, 1 (1937).

[276] E. Wille, *Deut. med. Wochschr.*, **65**, 1117 (1937).

It is also suggested[277] that vitamin C, copper and protective substances, like glutathione, which occur together in all tissues, play an important role in the regulation of the activity of tissue phosphatases.

(c) *Relation of Vitamin C to Other Vitamins, Hormones, Etc.*

As stated previously in the general chapter on vitamins, there is no synergistic or antagonistic action of one vitamin to another.[278] Vitamin C has the special faculty of detoxifying a large variety of different compounds including toxic doses of other vitamins. In animals which have the power of synthesizing their own supply of ascorbic acid the observation has generally been made that the amount produced is reduced at times of low vitality. Therefore, in rats fed a vitamin A-free diet the ascorbic acid content of the heart and probably also of the kidney and the thymus is significantly reduced.[279] Similar reductions in the ascorbic acid concentration in various tissues and endocrines have been observed in vitamin B_1 and riboflavin deficiencies, but no noteworthy changes occurred as a result of B_6-avitaminosis.[279]

A close relationship of vitamin C to various hormones is noted, since during avitaminosis a decreased hormone secretion is observed from those glands which normally contain high concentrations of vitamin C, for example, pituitary, pancreas, adrenal, thyroid, liver, intestinal wall.[280] In particular, it has been observed that vitamin C is necessary for the utilization of the adrenal cortex hormones, especially for the salt metabolism controlled by these hormones.[281] There seems to exist an antagonism between vitamin C and thyroxine. Administration of this hormone reduces the vitamin C content of liver, adrenals, etc. This effect can probably be linked with the property of ascorbic acid of detoxifying harmful compounds.

The relationships of vitamin C to various enzymes and to traces of metals, especially to manganese and copper, have already been discussed.

15. Avitaminosis and Hypovitaminosis

The state of hypovitaminosis which is quite common among human beings is characterized by impairment of physiological functions. Guinea

[277] K. V. Giri, *Biochem. J.*, 33, 309 (1939).

[278] P. E. Simola, *Acta Soc. Med. Fenn. Duodecim.*, 16, No. 3, 1 (1933); *Ber. ges. Physiol. exptl. Pharmakol.*, 83, 312. A. Scheunert, *Naturwissenschaften*, 28, 297 (1940).

[279] B. Sure, R. M. Theis and R. T. Harrelson, *J. Biol. Chem.*, 129, 245 (1939).

[280] A. Sigal and C. G. King, *Ibid.*, 116, 489 (1936).

[281] J. L. Svirbely, *Ibid.*, 116, 543 (1936).

pigs, during hypovitaminosis, are very sensitive to injury from diphtheria and other toxins[282, 283] and toward infectious diseases.[284] In humans, a depletion of the vitamin C reserves occurs during many diseases, for example, during fever,[285] tuberculosis,[286] etc. During avitaminosis the vitamin C level in blood serum and in the urine is lowered and the normal metabolic activity is decreased. More severe deficiency causes sore and swollen joints, edema, shortness of breath and a decline in weight. The clinical symptoms of vitamin C-avitaminosis are summarized under the term scurvy and are mainly characterized by hemorrhagic conditions. The actual place of these is largely influenced by growth and stress.[287] Thus in the growing human, hematomas, steoporosis, bone pains, etc., occur. The site of hemorrhages in adults is determined mainly by physical stress. During avitaminosis bones cease to grow and the normal junctions are replaced by connective tissue which contains calcified cartilages, but is devoid of osteoid tissue. This phenomenon can be seen in roentgenograms. The enamel, cementum and, most predominantly, the dentine change in structure, become resorbed and porotic and the newly formed material is of inferior strength (osteodentine). The gingiva, the gum of the jaws and surrounding the teeth, swells up, becomes spongy and bleeds easily. In severe cases hemorrhagic lesions are also observed in muscles, eyes (cataract[288]) and skin (lesions of the acne type[289]). Furthermore, a typical anemia of scurvy develops. Concomitant signs of a vitamin C depletion are atrophy of the glands of internal secretion and of the lymphatic tissues. The clinical symptoms of vitamin C deficiency in infants are known under the name Möller-Barlow's disease.

Vitamin C shortage in the organism is most dangerous in cases of bone fractures[290] and wound healing,[291] which are not cured rapidly and properly unless enough vitamin C is available. Cases have also been reported[292] showing that subnormal dark adaptation of the eye may be caused by vita-

[282] A. Sigal and C. G. King, *J. Pharmacol. Exptl. Therapy*, **59**, 468 (1937); **61**, 1 (1937).

[283] C. G. King and M. L. Menten, *J. Nutrition*, **10**, 129, 141 (1935). C. K. Greenwald and E. Harde, *Proc. Soc. Exptl. Biol. Med.*, **32**, 1157 (1935).

[284] D. Perla and J. Marmorston, *Arch. Path.*, **23**, 683 (1937).

[285] K. Daum, K. Boyd and W. D. Paul, *Proc. Soc. Exptl. Biol. Med.*, **40**, 129 (1939). Falke, *Klin. Wochschr.*, **18**, 818 (1939).

[286] W. W. Jetter and T. S. Bumbalo, *Am. J. Med. Sci.*, **195**, 362 (1938). M. N. Rudra, *Current Sci.*, **8**, 210 (1939).

[287] G. Dalldorf, *J. Exptl. Med.*, **50**, 293 (1929).

[288] H. Scarborough and C. P. Stewart, *Biochem. J.*, **31**, 2232 (1937).

[289] M. Lozza, *Minerva Medica*, **30**, 235 (1939).

[290] H. Hanke, *Deut. Z. Chirurgie*, **245**, 530 (1935).

[291] W. Aron, *Die Nährschäden des Kindes*, Berlin, 1928. T. H. Lanman and T. H. Ingalls, *Ann. Surg.*, **105**, 616 (1937). M. Taffel and S. C. Harvey, *Proc. Soc. Exptl. Biol. Med.*, **38**, 418 (1938). A. W. Allen, *Intern. Abstract Surg.*, **69**, 111 (1939).

[292] M. S. Kimble and E. S. Gordon, *J. Biol. Chem.*, **128**, LII (1939).

min C depletion at times when enough vitamin A and riboflavin were ingested. However a possible relationship of vitamin C to certain forms of cataract has been denied.[293]

Administration of vitamin C in human therapy is also indicated in cases of excessive bleedings (with the exception of hemophilia),[294] rheumatic fever[295] and arthritis, anaphylaxis, drug hypersensitivity, certain forms of allergy, lead and arsenic poisoning,[296] and Addison's disease in order to decrease the degree of pigmentation of the skin.[297] In cases of disturbances of the gastrointestinal tract and especially in gastric and duodenal ulcers,[298] vitamin C deficiencies occur quite often due to intertestinal destruction of the vitamin or to poor absorption. Thus, chronic scurvy is often diagnosed as pyorrhea.[299] In such cases the vitamin is administered parenterally, preferably by intramuscular injection.[300] Since vitamin C is also a factor in carbohydrate metabolism, an administration of this vitamin has been suggested in those illnesses in which there is a disturbance of the glycogen metabolism, in hypoglycemia, etc.[301]

In guinea pigs, typical changes of the female sex organs occur. The development of the follicles becomes greatly retarded and no corpora lutea develop at all. If the state of avitaminosis endures over a prolonged length of time, these changes cannot be repaired by the administration of vitamin C.

(*a*) *Clinical Test Methods*

Clinically the detection of a vitamin C deficiency is of importance especially when a state of hypovitaminosis is suspected. The following methods are now generally used:

Blood Test. The ascorbic acid content of normal human blood plasma is about 1.2 mg. per 100 cc., but this value decreases considerably upon a

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[293] J. Urbanek, *Klin. Monatsbl. f. Augenh.*, 101, 671 (1938).

[294] E. Vogt, *Münch. med. Wochschr.*, 82, 263 (1935). H. O. Neumann, *Klin. Wochschr.*, 15, 368 (1936). D. K. Miller and C. P. Rhoades, *J. Clin. Investigation*, 15, 462 (1936).

[295] J. F. Rinehart, *J. Lab. Clin. Med.*, 21, 597 (1936). J. F. Rinehart, L. D. Greenberg, M. B. Olney and F. Choy, *Arch. Internal Med.*, 61, 552 (1938). M. A. Abbasy, N. G. Hill and L. J. Harris, *Lancet*, 2, 1413 (1936). M. A. Abbasy, L. J. Harris and P. Ellman, *Ibid.*, 2, 181 (1937).

[296] H. N. Holmes, E. J. Amberg and K. Campbell, *Science*, 89, 322 (1939); *J. Lab. Clin. Med.*, 24, 1119 (1939).

[297] T. Cornbleet, *Arch. Dermatol. Syphilol.*, 35, 471 (1937). J. F. Wilkinson and C. A. Ashford, *Lancet*, 2, 967 (1936). A. F. Abt and C. J. Farmer, *J. Am. Med. Assoc.*, 111, 1555 (1938).

[298] A. B. Rivers and L. A. Carlson, *Proc. Staff Meetings Mayo Clinic*, 12, 383 (1937). G. Bourne, *Brit. Med. J.*, 1, 560 (1938). B. Portnoy and J. F. Wilkinson, *Ibid.*, 1, 554 (1938).

[299] D. Weisberger, *J. Conn. State Med. Soc.*, 1, 492 (1937).

[300] M. Vauthey, *Rev. Gastroenterol.*, 6, 337 (1939).

[301] E. Wille, *Deut. med. Wochschr.*, 65, 1117 (1937).

vitamin C-deficient diet. In the "prescorbutic state" the content is about 0.8 mg. and by the time clinical symptoms of scurvy become evident the vitamin C content of blood plasma is around 0.5 mg.[302, 303] When vitamin C is administered in excess amounts, the blood level may approach a value of 2.00 mg. per 100 cc.

The actual determination of vitamin C in blood is carried out, for example, by titration with the indophenol indicator.[304] It is necessary to deproteinize the blood by means of a strong acid, for example, trichloro-acetic acid, tungstic acid[305] or metaphosphoric acid,[306] and thereafter to separate the clear blood plasma. This method has also been adapted for microwork.[307] The Prussian blue method,[308] the methylene blue[309] and some of the other methods described under "chemical methods of determining vitamin C" (page 316) have occasionally been used. Since the precipitation of protein material sometimes carries ascorbic acid along, it has also been suggested to titrate blood serum directly with indophenol in the presence of hydrochloric acid.[310] It is necessary to determine the vitamin C content of blood serum immediately after the separation of the red blood cells since the vitamin C content decreases rapidly.[311] On the other hand, these determinations of vitamin C in the blood plasma are of fair accuracy if carried out properly, since no dehydro-ascorbic acid is present in fresh blood, but is found only as an artefact.

Urine Test. The daily urinary output of vitamin C in avitaminotic humans is greatly diminished in comparison to the normal output. Clinical determinations are made on the urine of patients before and after an oral or preferably after an intramuscular administration of moderate doses of vitamin C. Since a normal human being needs about 25–50 mg. of ascorbic acid daily, the response of the urinary excretion to the administration of this amount is determined.[312, 313] The urinary output of ascorbic acid of

[302] J. M. Faulkner and F. H. L. Taylor, *J. Clin. Investigation*, 17, 69 (1938). I. S. Wright, *Ann. Internal Med.*, 12, 516 (1938). H. Lund, *Klin. Wochschr.*, 16, 1085 (1937). G. A. Goldsmith and G. F. Ellinger, *Arch. Internal Med.*, 63, 531 (1939). E. N. Todhunter and R. C. Robbins, *J. Nutrition*, 19, 263 (1940).

[303] C. J. Farmer and I. M. Epstein, *J. Pediatrics*, 8, 1 (1936).

[304] R. L. Mindlin and A. M. Butler, *J. Biol. Chem.*, 122, 673 (1938).

[305] F. H. L. Taylor, D. Chase and J. M. Faulkner, *Biochem. J.*, 30, 1119 (1936).

[306] C. J. Farmer and A. F. Abt, *J. Pediatrics*, 8, 1 (1936); *Proc. Soc. Exptl. Biol. Med.*, 38, 399 (1938).

[307] C. J. Farmer and A. F. Abt, *Proc. Soc. Exptl. Biol. Med.*, 34, 146 (1936).

[308] H. Tauber and I. S. Kleiner, *J. Biol. Chem.*, 110, 559 (1935).

[309] H. Lund and H. Lieck, *Klin. Wochschr.*, 16, 555 (1937). H. Wahren, *Ibid.*, 16, 1496 (1937). A. Elmby and T. Wirth, *Ibid.*, 16, 746 (1937).

[310] N. Berend and M. Fischer, *Biochem. Z.*, 291, 221 (1937).

[311] R. J. Kassan and J. H. Roe, *J. Biol. Chem.*, 133, 579 (1940).

[312] W. B. Belser, H. M. Hauck and C. A. Storvick, *J. Nutrition*, 17, 513 (1939).

[313] L. J. Harris and S. N. Ray, *Lancet*, 1, 71 (1935). H. E. Archer and G. Graham, *Ibid.*, 1, 710 (1936). R. Sloan, *J. Lab. Clin. Med.*, 23, 1015 (1938).

healthy people varies considerably and can be influenced by a change in the acid-base balance of the food consumed.[314]

The exact actual determination of the total urinary excretion of vitamin C, comprising ascorbic acid, dehydro-ascorbic acid and ascorbic acid bound to protein material, is rather difficult. It has, for example, been suggested to use the titration with indophenol in various modifications[315] or with methylene blue,[316] the colorimetric determination with molybdo-phospho-tungstic acid[317] or to use the osazone-furfural method.[318, 319]

A certain small amount of ascorbic acid is said to be excreted in combined form.[320] This amount is not included in all the results of ascorbic acid determination, unless this part of the total ascorbic acid content is liberated from the combined form. During many diseases, particularly during times of fever, diphtheria, etc., the amount of the combined form is considerably increased.[321]

Skin Capillary Fragility Test. One of the first signs of a state of subclinical scurvy (and of a vitamin P deficiency) is the considerably lowered capillary resistance.[322, 323] A relative quantitative picture of the vitamin C depletion of a patient can be obtained by measuring the fragility. This is done, for example, in the compression test, by pinching the skin with the finger for one minute and investigating the number and severity of the petechiae (hemorrhagic spots) produced. A more exact effect is obtained by applying pressure with a sphygmomanometer[324] which is inflated to a pressure below the diastolic pressure of the pulse. A suction method can also be used, applying negative pressure. The average resistance of human skin is about 30 cm. Hg but varies considerably with different parts of the skin.

Roentgenographic Examination of Bones.[325] Since a slight hypovitaminosis causes no changes in the bone structure, only more severe cases can be detected by roentgenographic studies. On the other hand, scurvy may be manifest in the skeleton without other clinical symptoms. The patho-

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[314] E. E. Hawley, J. P. Frazer, L. L. Button and D. L. Stevens, *J. Nutrition*, 12, 215 (1936).
[315] A. Jezler and W. Niederberger, *Klin. Wochschr.*, 15, 710 (1936). J. Gander and W. Niederberger, *Münch. Med. Wochschr.*, 83, 1386, 2074 (1936). H. Kaiser, *Süddeut. Apoth.-Ztg.*, 1936, No. 85.
[316] H. Lund, *Klin. Wochschr.*, 16, 1085 (1937).
[317] N. Bezssonoff and E. Stoerr, *Z. Vitaminforsch.*, 5, 193 (1936).
[318] J. H. Roe and J. M. Hall, *J. Biol. Chem.*, 128, 329 (1939).
[319] L. Espil and L. Genevois, *Bull. soc. chim.*, 5, 1532 (1938).
[320] S. Banerjee, *J. Indian Chem. Soc.*, 17, 463 (1940).
[321] B. Ghosh, *Ibid.*, 16, 241 (1939).
[322] G. Dalldorf, *J. Exptl. Med.*, 58, 289 (1931); *Am. J. Diseases Children*, 46, 794 (1933).
[323] G. F. Göthlin, *Skand. Arch. Physiol.*, 61, 225 (1931).
[324] I. S. Wright and A. Lilienfeld, *Arch. Internal Med.*, 57, 241 (1936).
[325] E. A. Park, H. G. Guild, D. Jackson and M. Bord, *Arch. Disease Childhood*, 10, 265 (1935).

logical symptoms are encountered in the peripheral region and the end of the shaft of the long bones.

Adrenal Cortex Examination. For autopsy purposes a rough determination of the vitamin C content is made by soaking the opened gland in a silver nitrate solution. In cases of deaths caused by vitamin C depletion, very little silver precipitation occurs.

The Intradermal Test. This method is based on the observation that a solution of dichlor-phenol-indophenol injected under the epithelium decolorizes.[326] It has been estimated that a discoloration time of a given amount of the dye of five minutes indicates saturation with vitamin C and ten minutes or more indicates hypovitaminosis.[327] This test, however, proved to lack sufficient specificity for clinical work.[328]

16. Hypervitaminosis

A state of vitamin C hypervitaminosis is unknown. It has been impossible to produce any toxic symptoms with guinea pigs by feeding excess amounts of this vitamin and no increase in the vitamin C content of the organs over their normal levels could be detected.[329] No toxic signs were observed in human beings who were given doses of from 1 to 6 g. orally or intravenously.[330] Occasional vagotonic symptoms are attributed to idiosyncrasy or drug sensitivity.[331] Ingestion of ascorbic acid has a slight diuretic effect,[332] less than that caused by theobromine, but greater than the diuresis induced by digitalis.[333] In animals, the blood pressure rises somewhat upon injections of ascorbic acid.[334]

17. Requirements[335]

Of the entire living world only man, the other primates, the guinea pig and a few microorganisms (see under Physiology, page 324) are known to require an external supply of vitamin C. All other animals and plants also need vitamin C but are able to synthesize it, that is, ascorbic acid is a hormone for all these organisms. Guinea pigs need from 1 to 2 mg. of

[326] H. Rotter, *Nature*, 139, 717 (1937).
[327] B. Portnoy and J. F. Wilkinson, *Lancet*, 1, 328 (1938).
[328] H. G. Poucher and C. H. Stubenrauch, *J. Am. Med. Assoc.*, 111, 302 (1938).
[329] G. Mouriquand, M. Dauvergne and V. Edel, *Compt. rend.*, 209, 1023 (1939).
[330] A. F. Abt and C. J. Farmer, *J. Am. Med. Assoc.*, 111, 1555 (1938).
[331] F. Widenbauer, *Klin. Wochschr.*, 15, 1158 (1936).
[332] M. A. Abbasy, *Biochem. J.*, 31, 339 (1937).
[333] W. Evans, *Lancet*, 1, 308 (1938).
[334] M. Kasahara and R. Kawamura, *Klin. Wochschr.*, 16, 1543 (1937).
[335] S. L. Smith, *J. Am. Med. Assoc.*, 111, 1753 (1938).

ascorbic acid daily. The average optimal intake for human beings is about 50–100 mg. The fact that infants require vitamin C should be especially emphasized, since cow's milk does not contain sufficient amounts and human milk is given usually only over a relatively short period of time and its vitamin C content decreases after a few weeks. Infants need 3 to 8 mg. per kilogram of body weight per day, children about 5 to 7.5 mg., adults 0.7 to 1.6 mg., and aged people need about 3 to 5 mg. of ascorbic acid per kilogram of body weight per day. Pregnant and nursing women need 5 to 10 mg. per kilogram of body weight per day. The recommended daily allowances for ascorbic acid as established by the Food and Nutrition Board of the National Research Council will be found on page 613.

It is interesting to note the relatively high requirements of this vitamin on the weight basis compared to the daily needs of man and animals for the other vitamins. The order of magnitude is about 1000 times the weight of some of the other vitamins required. (Compare, however, the high requirements of choline.) A normal diet contains, however, adequate amounts of vitamin C.

THE GROUP OF VITAMINS D

THE GROUP OF VITAMINS D[1]

1. Nomenclature and Survey

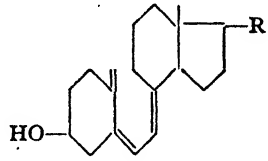
Vitamins D are usually called simply "vitamin D." The individual members of this group have not been properly named as yet. Provisionally they are called vitamin D_2, D_3, D_4, etc. Vitamin D_2 is called "calciferol" in England and has been given the name "viosterol" by the Council on Pharmacy and Chemistry (United States).

Historical Names, now abandoned:

Rachitamin.
Rachitasterol[2].
Antirachitic vitamin.

Chemical formulas:

General formula for vitamins D:*

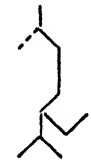


Vitamin D_2 (activated ergosterol, calciferol, viosterol): R =

Vitamin D_3 (activated 7-dehydrocholesterol): R =

Vitamin D_4 (activated 22-dihydroergosterol): R =

Vitamin D_5 (activated 7-dehydrositosterol): R =



(* *See page 342 for footnote.*)

[1] See also C. I. Read, H. C. Struck and I. E. Steck, *Vitamin D*, Chicago, 1939.
[2] R. L. Jones, *Science*, 68, 480 (1928).

2. Chronology

1822 TROUSSEAU, in his book on *Clinical Medicine*, recommended cod liver oil as a remedy for the cure of rickets,[3] and J. SNIADECKI inferred in his book, *On the Physical Education of Children*, the curative effect of sunlight,[4] but their viewpoints did not become widely known.

1890 PALM[5] associated through geographical studies the incidence of rickets with deficiency of sunlight.

1905 BUCHHOLZ[6] apparently cured cases of human rickets with artificial light.

1906 HOPKINS[7] suggested that rickets is caused by the absence of an "accessory foodstuff." FUNK[8] (1914) corroborated this concept of the origin of rickets.

1913 RACZYNSKI[9] established the beneficial influence of sunlight on the calcium assimilation of puppies.

1919 MELLANBY[10] discovered the nutritional importance of animal fats for the normal calcification of bones by raising dogs affected with rickets through special diet and by curing the animals with animal fats. He concluded that the "antirachitic factor" is either identical with "fat-soluble A" (vitamin A) or has a natural distribution somewhat similar to fat-soluble A.

HULDSCHINSKY[11] proved on the basis of x-ray studies that severe rickets in children can be cured by the light of a mercury vapor quartz lamp. Not until this report was published was the importance of sunlight appreciated.

1920–1921 SHERMAN and PAPPENHEIMER[12] and MCCOLLUM and SIMMONDS[13] succeeded in inducing rickets in rats by special diet.

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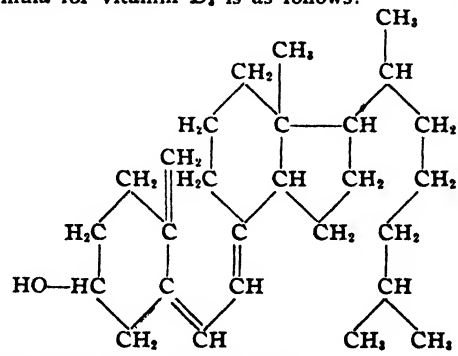
* The formulas of the vitamins D, as indicated on p. 341, are abbreviated. The complete chemical formula for vitamin D_3 is as follows:



[3] A. Trousseau, *Clinical Medicine*, Philadelphia, 1882.

[4] W. Mozolowski, *Nature*, 143, 121 (1939).

[5] T. A. Palm, *Practitioner*, 45, 271, 321 (1890).

[6] E. A. Park, *Physiol. Rev.*, 3, 106 (1923).

[7] F. G. Hopkins, *Analyst*, 31, 385 (1906); *J. Physiol.*, 44, 425 (1912).

[8] C. Funk, *Die Vitamine*, Wiesbaden, 1914.

[9] J. Raczynski, *Compt. rend. assoc. intern. pediat.*, 1913, 308.

[10] E. Mellanby, *J. Physiol.*, 52, LIII (1919); *Lancet*, I, 407 (1919).

[11] K. Huldschinsky, *Deut. med. Wochschr.*, 45, 712 (1919); *Z. orthop. Chir.*, 39, 426 (1919–20).

[12] H. C. Sherman and A. M. Pappenheimer, *Proc. Soc. Exptl. Biol. Med.*, 18, 193 (1920–21); *J. Exptl. Med.*, 34, 189 (1921).

[13] E. V. McCollum and N. Simmonds, *J. Biol. Chem.*, 47, 111, 139, 175, 207, 235, 507 (1921). See also V. Korenchevsky, *Brit. Med. J.*, 547 (1921); *Special Rept. Sci. Med. Research Council*, No. 71 (1922).

1921 HESS and UNGER[14] proved that the earlier observations concerning the curative effect of sunlight on patients with rickets were correct. They also reported the rickets-preventive effect of sunlight for rats on a rickets-producing diet.

1922 ZUCKER, PAPPENHEIMER and BARNETT recognized that the unsaponifiable fraction of fish liver oils contained the antirachitic factor.[15]

1922 MCCOLLUM and his co-workers[16] established experimentally the distinctive properties of vitamin A and the antirachitic factor. At the same time HUME[17] and independently SHEETS and FUNK[18] found that light would not cure the effects brought about by an insufficiency of vitamin A.

HESS and GUTMAN[19] expressed the opinion that the curative effect brought about on patients with rickets by either cod liver oil or light was fundamentally the same.

1924 STEENBOCK and associates[20] and independently HESS[21] found that antirachitic potency could be induced in foods by ultraviolet irradiation.

1925 The work of HESS, WEINSTOCK and HELMAN,[22] of STEENBOCK and BLACK,[23] and of ROSENHEIM and WEBSTER[24] indicated that the sterol fraction of foodstuffs could be made antirachitically active by irradiation although it was not active in itself.

MCCOLLUM named the antirachitic material "vitamin D."

1925–1926 SCHLUTZ and MORSE[25] postulated the possibility, and ROSENHEIM and WEBSTER,[26] HEILBRON, KAMM and MORTON[27] and POHL[28] proved that an impurity present in ordinary cholesterol and phytosterols is responsible for the antirachitic efficacy after irradiation.

1927 POHL,[29] WINDAUS and HESS[30] and ROSENHEIM and WEBSTER[31] concluded from physical, chemical and biological studies that the impurity in sterols, or the "provitamin D," is ergosterol, or a sterol of similar constitution, such as a hypothetical dehydro-cholesterol. Ergosterol had first been isolated by BRACONNOT in 1811 and was rediscovered in 1889 by TANRET.[32]

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[14] A. F. Hess and L. J. Unger, *Proc. Soc. Exptl. Biol. Med.*, 18, 298 (1921).

[15] T. F. Zucker, A. M. Pappenheimer and M. Barnett, *Ibid.*, 19, 167 (1922).

[16] E. V. McCollum, N. Simmonds, P. G. Shipley and E. A. Park, *Ibid.*, 18, 275 (1921); *J. Biol. Chem.*, 50, 5 (1922).

[17] E. M. Hume, *Lancet*, II, 1318 (1922).

[18] O. Sheets and C. Funk, *Proc. Soc. Exptl. Biol. Med.*, 20, 80 (1922).

[19] A. F. Hess and M. G. Gutman, *J. Am. Med. Assoc.*, 78, 29 (1922).

[20] H. Steenbock, *Science*, 60, 224 (1924). H. Steenbock and A. Black, *J. Biol. Chem.*, 61, 405 (1924). H. Steenbock and M. T. Nelson, *Ibid.*, 62, 209 (1924).

[21] A. F. Hess, *Am. J. Diseases Children*, 28, 517 (1924). A. F. Hess and M. Weinstock, *J. Biol. Chem.*, 62, 301 (1924).

[22] A. F. Hess, M. Weinstock and F. D. Helman, *Ibid.*, 63, 305 (1925).

[23] H. Steenbock and A. Black, *Ibid.*, 64, 263 (1925).

[24] O. Rosenheim and T. A. Webster, *Lancet*, I, 1025 (1925).

[25] F. W. Schlutz and M. Morse, *Am. J. Diseases Children*, 30, 199 (1925).

[26] O. Rosenheim and T. A. Webster, *J. Soc. Chem. Ind.*, 45, 932 (1926); *Biochem. J.*, 21, 127 (1927).

[27] I. M. Heilbron, E. D. Kamm and R. A. Morton, *J. Soc. Chem. Ind.*, 45, 932 (1926); *Biochem. J.*, 21, 78 (1927).

[28] R. Pohl, *Nachr. Ges. Wiss. Göttingen, Math. physik. Klasse*, III, 142 (1926).

[29] R. Pohl, *Ibid.*, III, 185 (1927).

[30] A. Windaus and A. Hess, *Ibid.*, III, 175 (1927). A. Windaus, *Ibid.*, III, 183 (1926).

[31] O. Rosenheim and T. A. Webster, *Lancet*, I, 306 (1927); *Biochem. J.*, 21, 389 (1927).

[32] The name "ergosterol" originates from ergot, a black fungus which grows on the rye plant and from which ergosterol was first isolated. C. Tanret, *Compt. rend.*, 108, 98 (1889); *Ann. chim. phys.*, (VI), 20, 289 (1890); *Compt. rend.*, 147, 75 (1908); *Ann. chim. phys.*, (VIII), 15, 313 (1908).

1929–1931 REERINK and VAN WIJK[33] isolated for the first time a crystallized vitamin D preparation made from activated ergosterol. LINSERT[34] isolated the pure compound.

1930 MUSSEHL and ACKERSON[35] and independently MASSENGALE and NUSSMEIER[36] showed that vitamin D obtained from ergosterol was not active for chicks when fed on the basis of Rat Units in amounts equal to those effective for chicks from cod liver oil.

1933 WINDAUS and LANGER[37] prepared a new synthetic provitamin D, 22-dihydroergosterol, from ergosterol.

1934 BILLS, MASSENGALE and IMBODEN[38] showed that the vitamin D of fish oils is not a single substance since tuna liver oil was less antirachitic than cod liver oil, Rat Unit for Rat Unit, in chickens.

WADDELL[39] found that crude cholesterol after activation yielded a vitamin D which is as effective for chicks as cod liver oil, fed in equivalent Rat Units. Thus, the provitamin present in cholesterol must be different from ergosterol.

1935 WINDAUS, LETTRÉ and SCHENCK[40] synthesized the hypothetical natural provitamin D, 7-dehydro-cholesterol, from cholesterol.

1936 BOER, REERINK, VAN WIJK and VAN NIEKERK[41] and later (1937) also WINDAUS and BOCK[42] isolated 7-dehydro-cholesterol from cholesterol (obtained from hog skin). BROCKMANN,[43] SIMONS and ZUCKER[44] and HASLEWOOD and DRUMMOND[45] isolated the vitamin D from tuna and from halibut liver oils in the form of crystallized esters and proved that the vitamin itself is mainly or entirely activated 7-dehydro-cholesterol (vitamin D_3).

1937 SCHENCK[46] obtained crystallized vitamin D_3 prepared by activation of 7-dehydrocholesterol.

1938 BILLS, MASSENGALE, HICKMAN and GRAY[47] isolated a new vitamin D of low biological activity by molecular distillation of cod liver oil.

THE CONCEPT OF PROVITAMINS D AND OF VITAMINS D

Compounds of the physiological efficacy of vitamins D occur only in the animal organism. Plants contain materials which can be converted

[33] E. H. Reerink and A. van Wijk, *Biochem. J.*, **23**, 1294 (1929); **25**, 1001 (1931).

[34] O. Linsert, Annotation in *Ann.*, **489**, 269 (1931). A. Windaus, O. Linsert, A. Lüttringhaus and G. Weidlich, *Ibid.*, **492**, 226 (1932).

[35] F. E. Mussehl and C. W. Ackerson, *Poultry Sci.*, **9**, 334 (1930).

[36] O. N. Massengale and M. Nussmeier, *J. Biol. Chem.*, **87**, 423 (1930).

[37] A. Windaus and R. Langer, *Ann.*, **508**, 105 (1933).

[38] C. E. Bills, O. N. Massengale and M. Imboden, *Science*, **80**, 596 (1934).

[39] J. Waddell, *J. Biol. Chem.*, **105**, 711 (1934).

[40] A. Windaus, H. Lettré and F. Schenck, *Ann.*, **520**, 98 (1935).

[41] A. G. Boer, E. H. Reerink, A. van Wijk and J. van Niekerk, *Proc. Acad. Sci. Amsterdam*, **39**, 622 (1936).

[42] A. Windaus and F. Bock, *Z. physiol. Chem.*, **245**, 168 (1937).

[43] H. Brockmann, *Ibid.*, **241**, 104 (1936); *Ibid.*, **245**, 96 (1937). H. Brockmann and A. Busse, *Ibid.*, **249**, 176 (1937).

[44] E. J. H. Simons and T. F. Zucker, *J. Am. Chem. Soc.*, **58**, 265 (1936).

[45] G. A. D. Haslewood and J. C. Drummond, *J. Soc. Chem. Ind.*, **55**, 598 (1936).

[46] F. Schenck, *Naturwissenschaften*, **25**, 159 (1937).

[47] C. E. Bills, O. N. Massengale, K. C. D. Hickman and E. L. Gray, *J. Biol. Chem.*, **126**, 241 (1938).

into vitamins D. These are called "provitamins D." There occur in nature a number of provitamins D and of vitamins D. The vitamins D differ in their antirachitic effectiveness in various animals. The number of known naturally occurring provitamins D and vitamins D is small: the provitamins ergosterol and 7-dehydro-cholesterol and the corresponding vitamins, namely, vitamin D_2, which is also known as "activated ergosterol," viosterol, or calciferol, and vitamin D_3 which is activated 7-dehydro-cholesterol. Two additional provitamins D have been claimed patentwise to occur in invertebrata, but very little is known about them. A number of other provitamins D and vitamins D have been prepared in the laboratory, and it is suspected that some of these compounds may also occur in nature. Furthermore, the existence of two more naturally occurring vitamins D is indicated on the basis of their outstanding physiological properties.

PROVITAMINS D

A provitamin D is defined as a compound that can be activated to a vitamin D. Provitamins D are compounds of the cyclo-pentano-perhydrophenanthrene skeleton and belong to the sterol family. They are specifically characterized by a hydroxyl group in the 3-position and a system of conjugated double bonds in ring B of the steroid nucleus, namely, in the 5,6- and 7,8-positions. Provitamins D cannot be defined physiologically but it is suspected that it will be shown eventually that those steroids of the above classification which after activation are potent vitamins D for a specific species are absorbed in the intestinal tract of an animal of that species.

According to this definition, several compounds have been tentatively classified as provitamins D. They will be discussed in the following sections. A number of other compounds, which fulfill only partly the definition of provitamins as given above, are discussed under "Specificity of Vitamin D" (page 406).

3. Occurrence

Provitamins D are widely distributed over the animal and plant kingdom. While it is impossible to make definite statements as to what provitamin D occurs in various specific natural sources, certain generalizations can be made. It appears that the most prevalent provitamin D in higher animals and in human beings is 7-dehydro-cholesterol. Plants, molds and

yeast contain predominantly ergosterol.[48] Considerable uncertainty exists about the kind of provitamins D in lower animals. Thus, ergosterol occurs in the snail *Arion empiricorium* and in the earthworm, and 7-dehydrocholesterol in the snail *Buccinum undatum*.[49] Mussels[50] are said to contain a different provitamin D and the same has been claimed for periwinkles.[51]

The provitamin D content in different sources varies considerably. Thus, in higher animals, the provitamin D content is the greatest in the skin, namely, about 4% of the total sterol content, whereas the sterols from the inner organs contain only from 0.1 to 0.5% provitamin D. (This is due to the activation mechanism of provitamins D to vitamins D as will be explained later.) The best sources of ergosterol are yeast and certain molds, some of which contain this provitamin as practically the only sterol. The highest concentration of 7-dehydro-cholesterol has been found in a species of snails (*Buccinum undatum*) and is 27% of the total sterol fraction. The following tables indicate, as far as is known, the content of ergosterol and of 7-dehydro-cholesterol in the sterol fraction of various sources. Since in most naturally occurring materials the type of provitamin is not known, another table shows the general provitamin D content in sterols from various materials.

TABLE I

ERGOSTEROL CONTENT OF VARIOUS MATERIALS

Source	Provitamin D in sterols, %
Dried yeast	90–100
Snail, *Arion empiricorium*	19–25
Earthworm	22
Cottonseed oil	5
Scopolia root	1.4

TABLE II

7-DEHYDRO-CHOLESTEROL CONTENT OF VARIOUS MATERIALS

Source	Provitamin D in sterols, %
Pigskin	3–6
Snail, *Buccinum undatum*	17–27

[48] C. Tanret, *Compt. rend.*, 108, 98 (1889); *Ann. chim. phys.*, (VI), 20, 289 (1890); *Compt. rend.*, 147, 75 (1908); *Ann. chim. phys.* (VIII), 15, 313 (1908).

[49] A. Windaus, *Nachr. Ges. Wiss. Göttingen, Math. physik. Klasse*, III, 185 (1936). F. Bock and F. Wetter, *Z. physiol. Chem.*, 256, 33 (1938).

[50] A. G. Boer, J. van Niekerk, E. H. Reerink and A. van Wijk, U. S. P. 2,163,659.

[51] A. G. Boer, J. van Niekerk, E. H. Reerink and A. van Wijk, U. S. P. 2,216,719.

TABLE III

PROVITAMIN D CONTENT OF VARIOUS MATERIALS

Source	Provitamin D in sterols, %
Vertebrata	
Skin from man	0.15–0.43
cattle	0.18
calf	0.68
mice	0.87
chicken feet[51a]	1.0–4.0
chicken trunk[51a]	0.001–0.01
Blood serum (cow)	0.15
Brain (cow)	0.01
Lung (calf)	0.025
Heart (calf)	0.032
Spleen (cow)	0.045
Placenta (cow)	0.18
Pancreas (cattle)	0.18
Invertebrata	
Lugworm (*Arenicola marina*)	4–12
Mussels (*Mytilus edulis*)	9–10
Oysters	5–6
Leech	4
Crabs (*Cancer pagurus*)	0.32
Sea anemones	2–10
Plants	
Cocksfoot grass	0.80
Rye grass	1.5
Wheat germ oil	0.8
Seaweed	0.008
Cabbage	0.05
Spinach	1.0
String beans	0.1

4. Isolation

The isolation of provitamins D from natural sources involves two different steps, namely, the isolation of the total sterols and the separation of the provitamins from other sterols present. The isolation of the total sterols is usually a simple process and consists in either first extracting

[51a] H. R. Rosenberg, unpublished data.

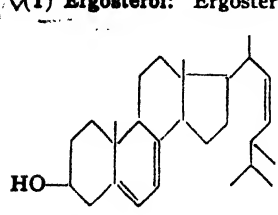
the total fat, followed by saponification of the fatty material and isolation of the unsaponifiable fraction, or in saponifying directly the total material followed by isolation of the non-saponifiable fraction. The isolation of the sterols from the non-saponifiable fraction is usually carried out by crystallization from a suitable solvent, such as alcohol. Special methods have been recommended for special cases. For example, after saponification, the fatty acids may be precipitated as calcium salts which adsorb the sterols. These are then recovered by solvent extraction from the filtered precipitate. Another modification has been used in those cases where the total amount of fatty acids is very low. Sodium benzoate is added to the saponification mass and the entire mixture is acidified. Benzoic acid precipitates and adsorbs the sterols present which can then easily be isolated by alkaline extraction of the benzoic acid.

The separation of the provitamins from other sterols is usually a difficult problem and success depends largely upon the type and amount of provitamin D present. Thus no method has been found by which the provitamin D present in cholesterol from the spinal cord of cattle can be isolated satisfactorily, since the provitamin D content is only 0.1%. Better chances for a successful isolation exist when the provitamin D is present in a concentration of at least 4–5% or more. The usual method is fractional adsorption of the sterols or of their esters, for example, on aluminum oxide, which in many cases permits an almost quantitative separation. If these methods fail, a condensation product with maleic or citraconic acid anhydride may be formed which can be split by thermal decomposition into the provitamin D and the acid anhydride.

5. Properties

The following provitamins D are known:

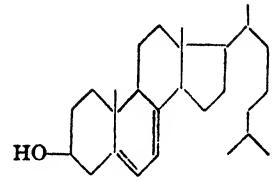
(1) **Ergosterol:** Ergosterol crystallizes in small colorless crystals with water of crystallization. The melting point varies according to the degree of hydration. The best crystallized preparation contains $1^1/_2$ mols of water and melts at 168° C.[51] Complete dehydration is very difficult to achieve and results in a product with a melting range from 166 to 183° C. Ergosterol distills in high vacuum at 250° C. without decomposition. $[\alpha]_D^{20} = -130°$ (−135°) and $[\alpha]_{5461}^{20} = -171°$ (in chloroform).



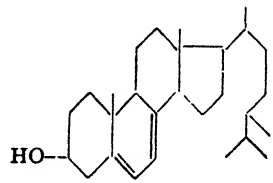
(2) **Epi-ergosterol:** Physical constants unknown.

[51] C. E. Bills and E. M. Honeywell, *J. Biol. Chem.*, **80**, 15 (1928).

(3) **7-Dehydro-cholesterol:** M. p. 150–151° C.[53] $[\alpha]_D^{20} = -113.6°$ in chloroform.



(4) **Epi-7-dehydro-cholesterol:**[54] M. p. 124–126° C. $[\alpha]_D^{20} = -70.5°$ in chloroform.
(5) **22-Dihydro-ergosterol:**[55] M. p. 152–153° C. $[\alpha]_D^{20} = -109°$ in chloroform.



(6) **22-23-Oxido-ergosterol:**[56] Physical constants unknown.
(7) **"Mussel provitamin:"**[57] Constitution unknown. M. p. 150–151° C. $[\alpha]_D^{20} = -118°$ in benzene.
(8) **"Periwinkle provitamin:"**[58] Constitution unknown. M. p. 137–137.5° C. $[\alpha]_D^{20} = -124°$ in benzene.
(9) **7-Dehydro-sitosterol:** M. p. 144–145° C. $[\alpha]_D^{20} = -116°$ in chloroform.
(10) **7-Dehydro-stigmasterol:** M. p. 154° C. $[\alpha]_D^{20} = -113.15°$ in benzene.

All these provitamins have similar solubility characteristics. They are insoluble in water but soluble in the typical organic solvents, such as ether, hydrocarbons, chlorinated hydrocarbons, alcohols, etc. The lower alcohols are usually used for recrystallization. The provitamins separate from the alcohols with water (or solvent) of crystallization.

All provitamins have the same characteristic absorption spectrum in the ultraviolet which is characterized by maxima at 260, 270, 281 and 293.5 mμ (Fig. 15). The molecular absorption coefficient K = about 30×10^3 for the band at 281 mμ.

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[53] A. G. Boer, E. H. Reerink, A. van Wijk and J. van Niekerk, *Proc. Acad. Sci. Amsterdam*, **39**, 622 (1936).
[54] A. Windaus and J. Naggatz, *Ann.*, **542**, 204 (1939).
[55] A. Windaus and R. Langer, *Ibid.*, **508**, 105 (1933).
[56] A. Windaus, Linsal and K. Buchholz, quoted by K. Dimroth and J. Paland, *Ber.*, **72**, 187 (1939).
[57] A. G. Boer, J. van Niekerk, E. H. Reerink and A. van Wijk, U. S. P. 2,163,659.
[58] A. G. Boer, J. van Niekerk, E. H. Reerink and A. van Wijk, U. S. P. 2,216,719.

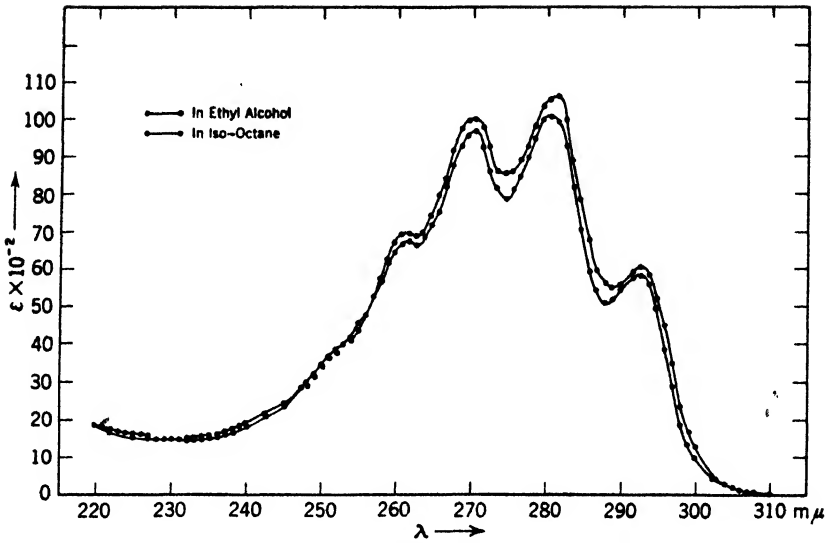


Fig. 15.—Absorption spectrum of ergosterol in ethanol and isooctane. (T. R. Hogness, A. E. Sidwell and F. P. Zscheile.)

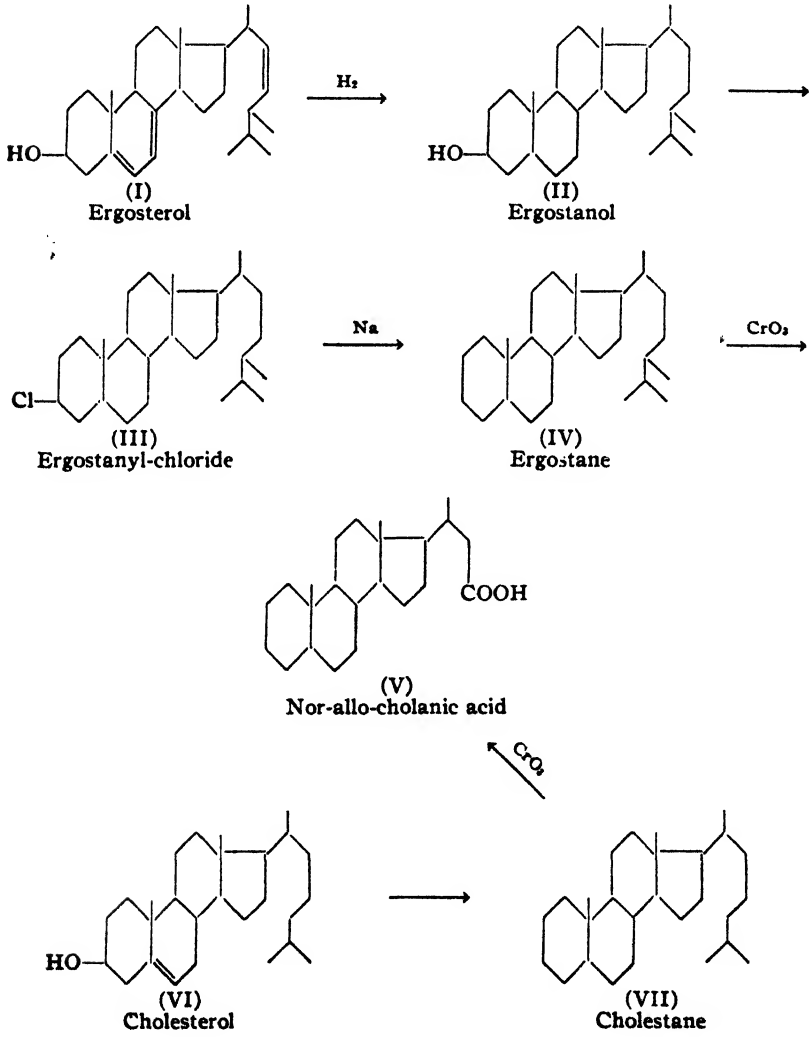
6. Chemical Constitution

(*a*) *Ergosterol*

Ergosterol (I) has the empirical formula $C_{28}H_{44}O$,[59] which was established by careful analysis of derivatives containing hetero atoms, such as the 3,5-dinitro-benzoate, halogeno-nitro-benzoates, etc. The oxygen is present in a hydroxyl group, since esters can be obtained with acid anhydrides or acid chlorides in the presence of an amine. Ergosterol contains three double bonds, since upon catalytic hydrogenation six atoms of hydrogen are absorbed.[60] The totally saturated compound is called ergostanol (II) and has the formula $C_{28}H_{50}O$. It follows that four ring systems are present. From ergostanol the corresponding hydrocarbon ergostane (IV) can be obtained by conversion into ergostanyl-chloride (III) followed by reduction with sodium and amyl alcohol.[61] Ergostane, upon oxidation with chromic acid, yields a mono-carboxylic acid, $C_{28}H_{48}O_2$,[62] which is identical with the nor-allo-cholanic acid (V) obtained from cholesterol

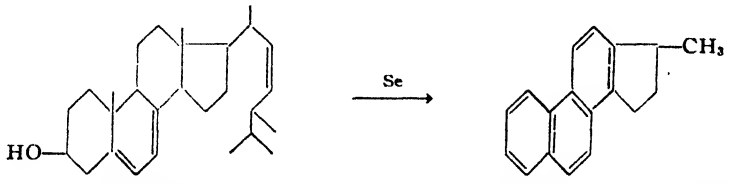
[59] A. Windaus and A. Lüttringhaus, *Nachr. Ges. Wiss. Göttingen, Math. physik. Klasse*, III, 4 (1932). A. Windaus, F. v. Werder and B. Gschaider, *Ber.*, 65, 1006 (1932).
[60] A Windaus and O. Linsert, *Ann.*, 465, 154 (1928).
[61] F. Reindel and E. Walter, *Ibid.*, 460, 222 (1928).
[62] C. K. Chuang, *Ibid.*, 500, 270 (1933).

(VI) *via* cholestane (VII). This degradation reaction proves that ergosterol belongs to the class of sterols, which are characterized by the cyclopentano-perhydro-phenanthrene skeleton. Furthermore, it follows that the steric configuration of cholestane and that of ergostane are the same.



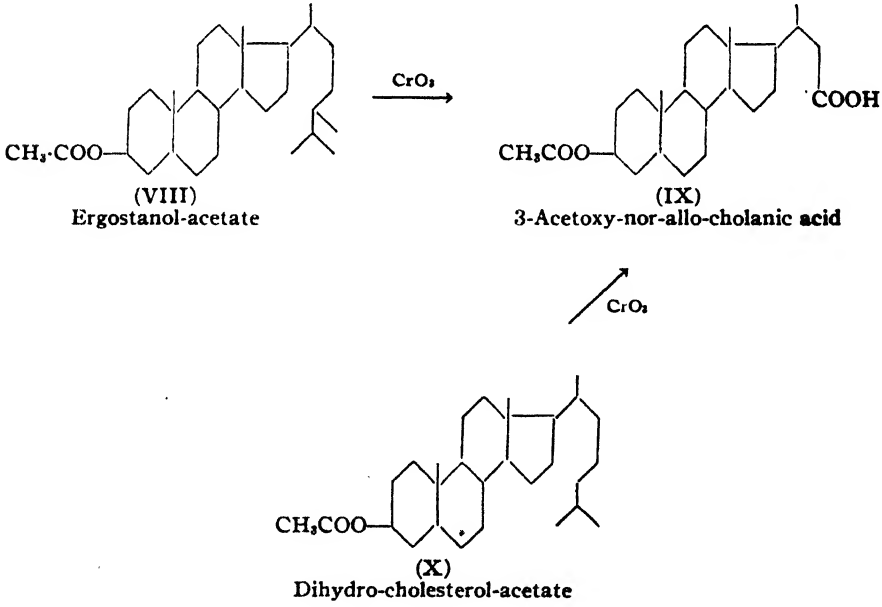
That ergosterol belongs to the sterols has furthermore been demonstrated since it yields, upon total dehydrogenation with selenium,

γ-methyl-cyclo-penteno-phenanthrene,[63] which is the typical dehydrogenation product of all sterols. No other class of compounds yields this particular hydrocarbon upon dehydrogenation.



Ergosterol — γ-Methyl-cyclo-penteno-phenanthrene

The position of the hydroxyl group in the ring system was shown to be at carbon atom 3,[64] since acetylated ergostanol (VIII) yielded upon chromic acid oxidation, 3-acetoxy-nor-allo-cholanic acid (IX) which was also obtained from dihydro-cholesterol-acetate (X). This result also proves that the steric configuration of the hydroxyl group is of the β-type which was suspected since ergosterol can be precipitated with digitonin.



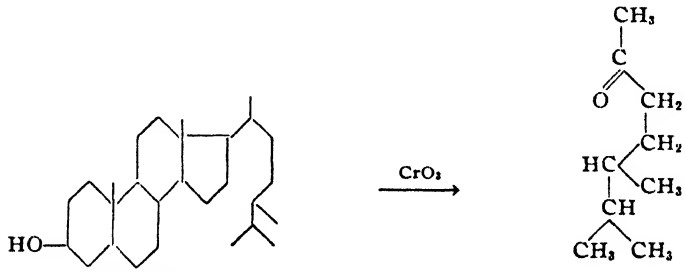
(VIII) Ergostanol-acetate

(IX) 3-Acetoxy-nor-allo-cholanic acid

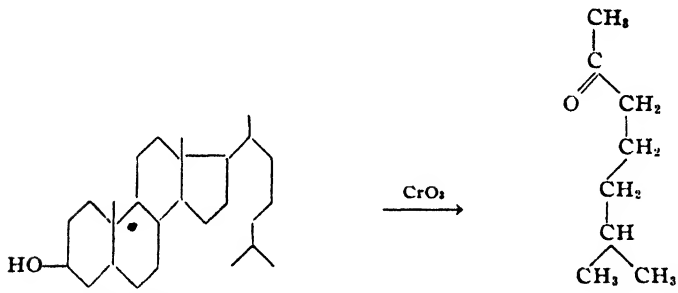
(X) Dihydro-cholesterol-acetate

[63] O. Diels and A. Karstens, *Ann.*, **478**, 129 (1930).
[64] E. Fernholz and P. N. Chakravorty, *Ber.*, **67**, 2021 (1934).

The chromic acid oxidations indicate, furthermore, that the basic structure of the ring system and of the first five carbon atoms of the side chain is identical in both cholesterol and ergosterol. Since ergosterol contains one carbon atom more than cholesterol, this must be located in that part of the side chain which is removed during the oxidation. This can be proved, since by energetic oxidation of ergostanol a ketone containing nine carbons ($C_9H_{18}O$) is obtained,[65] whereas cholestanol under the same conditions yields a ketone of only eight carbon atoms.



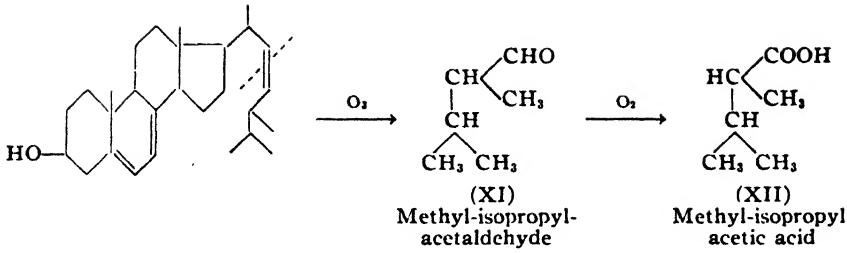
Ergostanol



Cholestanol

Ergosterol upon ozonization yields methyl-isopropyl-acetaldehyde[66, 67] (XI), which was identified by the formation of its semicarbazone and dinitro-phenyl-hydrazone and by oxidation to methyl-isopropyl-acetic acid (XII). This result indicates that a double bond is in the side chain between carbon atoms 22 and 23.

[65] A. Guiteras, *Ann.*, **494**, 116 (1932).
[66] F. Reindel and H. Kipphan, *Ibid.*, **493**, 181 (1932).
[67] A. Guiteras, *Ibid.*, **494**, 116 (1932).

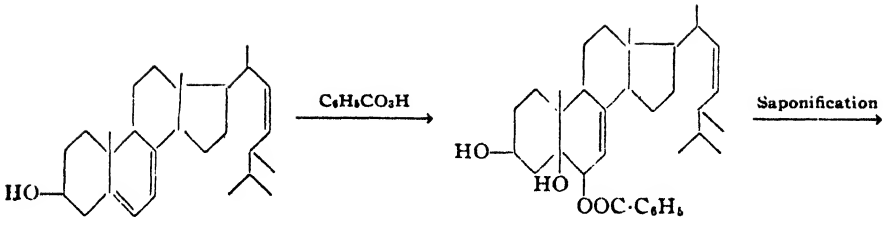


(XI) Methyl-isopropyl-acetaldehyde

(XII) Methyl-isopropyl-acetic acid

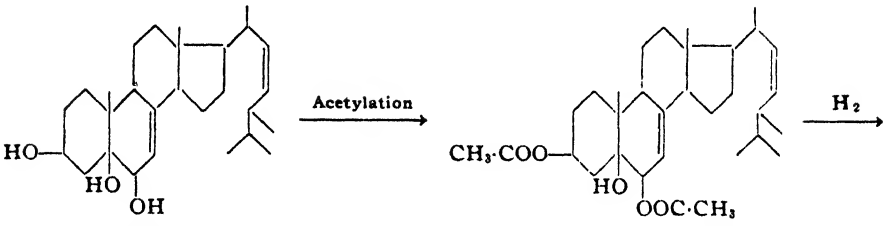
Another double bond is present in the 5,6-position, which is the position of the double linkage in cholesterol. This has been proved by the following series of reactions:[68] Ergosterol (I) upon addition of perbenzoic acid to one of its double bonds yields an ergostadiene-triol-monobenzoate (II)[69] which contains one free secondary and one free tertiary hydroxyl group since only one of these can be readily esterified. By saponification of this product, followed by acetylation, an ergostadiene-triol-diacetate (IV) is thus formed. The diacetate upon catalytic hydrogenation takes up only one mol of hydrogen (forming V), and leaving one other double bond in the molecule. This is rearranged by the action of hydrochloric acid. The newly formed compound (VI) takes up hydrogen easily,[70] yielding the totally hydrogenated ergostane-triol-diacetate (VII), which upon saponification gives the free ergostane-triol (VIII). Two of the hydroxyl groups in this compound are in α,β-position to each other since upon oxidation with lead tetra-acetate according to Criegée one atom of oxygen is consumed. By chromic acid oxidation of the triol, a hydroxy-diketone (IX) is formed which splits out water by the action of hydrochloric acid. The unsaturated diketone, ergostene-dione (X), can be transformed into a saturated diketone, ergostadione (XI), by means of zinc and acetic acid. Hydrazine condenses readily with the diketone with the formation of a pyridazine derivative (XII). The hydroxyl group originally present in ergosterol is in 3-position and therefore one of the keto-groups in ergostadione is in 3-position. Since the other keto-group according to its reactions must be three carbon atoms removed from the keto-group at carbon atom 3, it can only be located at carbon atom 6. The diketone is, therefore, ergostadione-3,6. It follows, furthermore, that the tertiary hydroxyl group in the ergostane-triol is in 5-position, the triol thus having the constitution of a 3,5,6-triol.

[68] A. Windaus, H. H. Inhoffen and S. v. Reichel, *Ann.*, **510**, 248 (1934).
[69] A. Windaus and A. Lüttringhaus, *Ibid.*, **481**, 127 (1930).
[70] I. M. Heilbron, A. L. Morrison and J. C. E. Simpson, *J. Chem. Soc.*, **1933**, 302.



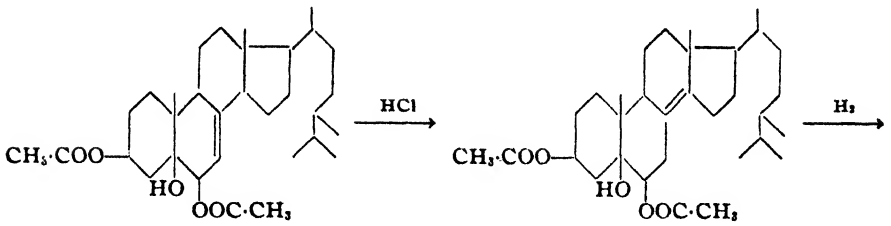
(I)
Ergosterol

(II)
Ergostadiene-triol-monobenzoate



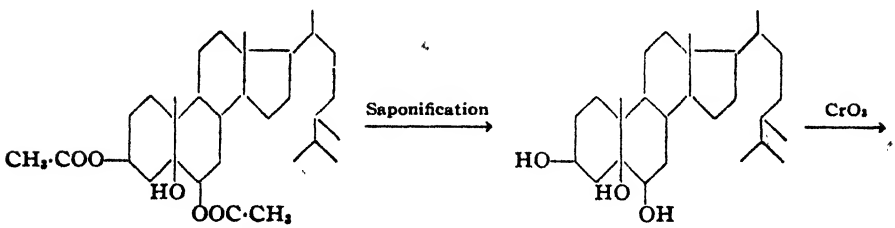
(III)
Ergostadiene-triol

(IV)
Ergostadiene-triol-diacetate



(V)
Ergostene-triol-diacetate A

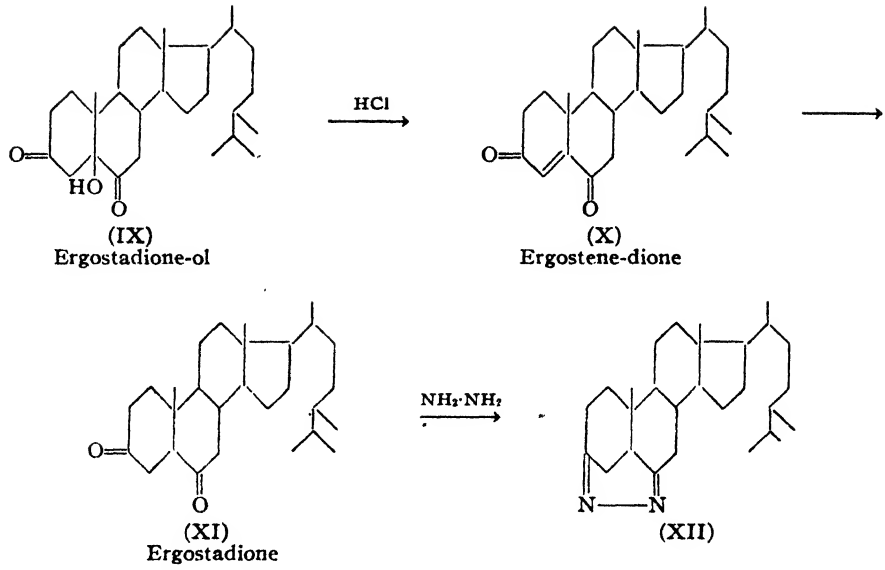
(VI)
Ergostene-triol-diacetate B



(VI')
Ergostane-triol-diacetate

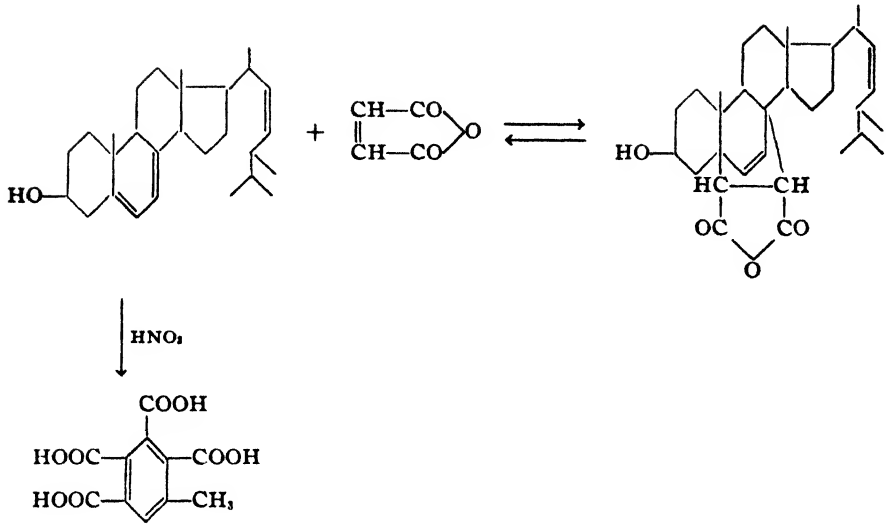
(VIII)
Ergostane-triol

(Formula continued on following page.)



The position of the third double bond is established by the refractive index,[71] by x-ray measurements,[72] and by the ultraviolet absorption spectrum, all of which exhibit characteristics of a system of two conjugated double bonds. The last double linkage cannot be located in the side chain, due to the degradation reactions discussed above, but must be in conjugation to the double bond in the 5,6-position. Thus the only possible position is the 7,8-position. The conjugation of the double bonds is furthermore suspected by the fact that ergosterol can be reduced with sodium and amyl-alcohol[73] and by the fact that ergosterol forms a characteristic addition product with maleic anhydride[74] from which ergosterol can be recovered by thermal decomposition.[75] Since it is known that one ring double bond is in the 5,6-position, this maleic anhydride condensation is only possible when the other double bond, conjugated with the one in the 5,6-position, is located in the same ring in which the first double bond is located. Further proof is indicated by nitric acid oxidation of ergosterol, which yields toluene-2,3,4,5-tetracarboxylic acid.[76] While the value of this reaction by itself is limited since a migration of a methyl group is

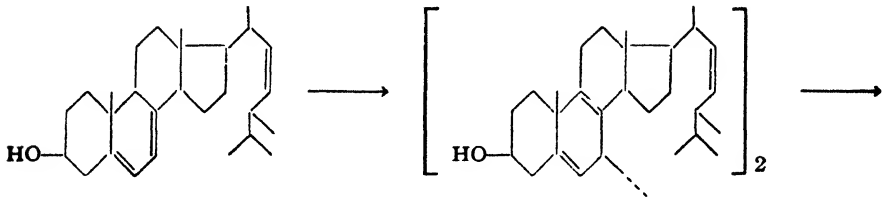
[71] K. v. Auwers and E. Wolter, *Nachr. Ges. Wiss. Göttingen, Math. physik. Klasse*, III, 101 (1931).
[72] G. E. R. Schulze, *Z. physik. Chem.*, A171, 436 (1934).
[73] A. Windaus and J. Brunken, *Ann.*, 460, 225 (1928).
[74] A. Windaus and A. Lüttringhaus, *Ber.*, 64, 850 (1931).
[75] H. H. Inhoffen, *Ann.*, 508, 81 (1933).
[76] F. Reindel and K. Niederländer, *Ibid.*, 482, 264 (1930). H. H. Inhoffen, *Ibid.*, 494, 122 (1932).



involved, the result is corroborated by another series of reactions. Ergosterol is dehydrogenated by eosin in the absence of oxygen when exposed to visible light with the formation of a bimolecular compound,[77] in which two molecules are linked together through the 7,7′-position with a shift of the 7,8-double bond into the 8,9-position[78] (II). Upon thermal decomposition of this compound methane is evolved[79] and neoergosterol (III) is formed,[77, 80] which contains an aromatic ring as indicated by the absorption spectrum and by the fact that only one double bond can be detected by perbenzoic acid or by catalytic hydrogenation. That this double bond is in the side chain is proved by ozonization of neoergosterol which yields methyl-isopropyl-acetaldehyde.[81] The reaction of nitric acid on neoergosterol yields mellophanic acid (benzene-1,2,3,4-tetracarboxylic acid (IV)) which differs from the nitric acid oxidation product of ergosterol by the absence of a methyl group. While these results can be explained only by the assumption that ring B is aromatic, further proof has been brought forward. The hydroxyl group of neoergosterol is alicyclic in character, not phenolic, thus excluding the possibility that ring A has become aromatic. On the other hand, upon catalytic dehydrogenation with platinum a β-naphthol derivative, dehydro-neoergosterol (V), is

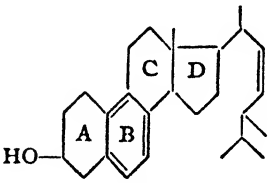
[77] A. Windaus and P. Borgeaud, *Ann.*, **460**, 235 (1928).
[78] H. H. Inhoffen, *Naturwissenschaften*, **25**, 125 (1937).
[79] H. H. Inhoffen, *Ann.*, **497**, 130 (1932).
[80] K. Bonsted, *Z. physiol. Chem.*, **185**, 165 (1929).
[81] H. H. Inhoffen, *Ann.*, **497**, 130 (1932).

obtained.[82] This result excludes the possibility that ring C in neoergosterol is aromatic in character.

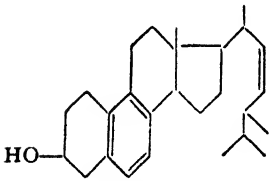


(I)
Ergosterol

(II)

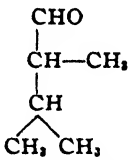


(III)
Neoergosterol



(III)
Neoergosterol

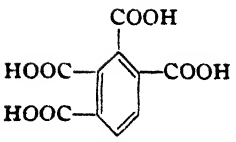
O_3



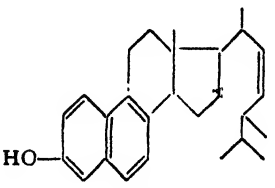
Methyl-isopropyl-acetaldehyde

HNO_3

Pt



(IV)
Mellophanic acid



(V)
Dehydro-neoergosterol

[82] H. Honigmann, *Ann.*, 511, 292 (1934).

Ergosterol is a very sensitive compound. Acids cause rearrangements of the double bonds, oxygen brings about the formation of peroxides and hydrogen causes the formation of a number of different di- and polyhydro compounds.

(*b*) *The Other Provitamins D*

The proof for the chemical constitution of the other provitamins D is mainly based upon the determination of the characteristic absorption spectrum which is common to all provitamins D since they all have the same system of conjugated double bonds in ring B. The constitution of the provitamins 7-dehydro-cholesterol and 22-dihydro-ergosterol is furthermore established since these compounds have been obtained by synthetic methods from cholesterol and from ergosterol, respectively. The constitution of the epi-compounds, namely, of epi-ergosterol and of epi-7-dehydro-cholesterol is established since these provitamins do not precipitate with digitonin whereas the parent provitamins, ergosterol and 7-dehydro-cholesterol, form addition compounds with digitonin. This reaction in combination with their absorption spectrum and the method of synthesis of the epi-compounds proves that they differ from their parent provitamins D only in the stereochemical configuration of the hydroxyl group in 3-position. This position of the 3-hydroxyl group must be related to some other group in the sterol ring system. Ruzicka chose the relationship between the substituents on C_3 and on C_5 as reference for the *cis-trans*-isomerism of the 3-hydroxyl group. Since the provitamins D have no hydrogen at C_5, this system applies by implication. Ergosterol, 7-dehydro-cholesterol, etc., belong according to this nomenclature to the *trans*-derivatives, whereas the compounds of the epi- family, which do not precipitate with digitonin, belong to the *cis*-derivatives. Another system of nomenclature suggested by Schoenheimer uses the relation of the hydroxyl group in 3-position to the methyl group on carbon atom 10. According to this system ergosterol and 7-dehydro-cholesterol belong to the *cis*-C_3-C_{10} compounds and the epi-derivatives belong to the *trans*-C_3-C_{10} compounds.

The constitution of the "mussel provitamin D" and of the "periwinkle provitamin D" are unknown. It has tentatively been assumed that the "mussel provitamin D" has 29 carbon atoms,[83] that is, it has one carbon atom more than ergosterol or two carbon atoms more than 7-dehydro-cholesterol.

[83] A. G. Boer, J. van Niekerk, E. H. Reerink and A. van Wijk, U. S. P. 2,163,659.

7. Synthesis

No provitamin D has been obtained by total synthesis. By partial synthesis, that is, by chemical conversion of sterols into provitamins D, the following provitamins D have been prepared: 7-dehydro-cholesterol and epi-7-dehydro-cholesterol, epi-ergosterol, 22-dihydro-ergosterol, 22,23-oxido-ergosterol, 7-dehydro-sitosterol and 7-dehydro-stigmasterol. The methods used will be discussed in detail in the following paragraphs.

(*a*) *The Synthesis of 7-Dehydro-cholesterol*

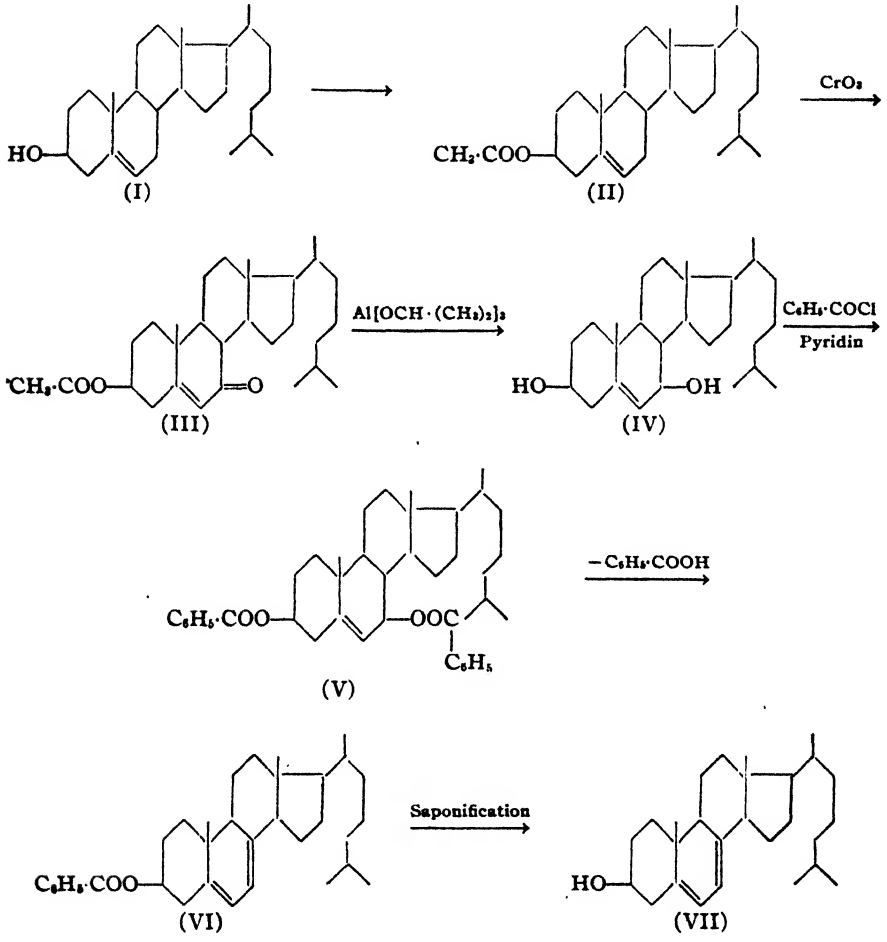
The classical synthesis of 7-dehydro-cholesterol from cholesterol has been accomplished by Windaus and his co-workers.[84] Cholesterol-acetate (II) is oxidized with chromic acid to 7-oxo-cholesterol-acetate (III). The latter upon reduction with aluminum-isopropoxide yields 7-hydroxy-cholesterol (IV) since the acetyl group undergoes an ester-interchange reaction with the isopropanol used as solvent. The 7-hydroxy-compound is benzoylated and the dibenzoate (V) is thermally decomposed into 7-dehydro-cholesterol-benzoate (VI) and benzoic acid. The former, upon saponification, yields 7-dehydro-cholesterol (VII).

This series of reactions takes a different course, when instead of the acetate of cholesterol the benzoate is used.[85] Upon reduction of the 7-oxo-cholesterol-benzoate, obtained by chromic acid oxidation of cholesterol-benzoate, the 3-mono-benzoate of 7-hydroxy-cholesterol is formed. (The latter can also be obtained by monobenzoylation of 7-hydroxy-cholesterol.[86])

The 7-hydroxy-cholesterol compound obtained by reduction of the 7-oxo-derivative represents one of the two possible geometrical isomers. The other isomer has been obtained by direct oxidation of the mono-cholesterol-ester of phthalic acid by means of permanganate, and has been called β-7-hydroxy-cholesterol.[87] The α-form has also been obtained from ox[88] and from hog[89] livers, and the β-form from the serum of pregnant mares.[90] Whether or not these 7-hydroxy-compounds are normal body constituents is unknown. They may be artefacts since cholesterol is easily oxidized to these 7-hydroxy-derivatives by molecular oxygen.[91]

[84] A. Windaus, H. Lettré and F. Schenck, *Ann.*, 520, 98 (1935).
[85] H. R. Rosenberg and J. M. Tinker, U. S. P. 2,215,727 and B. P. 537,030.
[86] H. J. Eckhardt, *Ber.*, 71, 461 (1938).
[87] T. Barr, I. M. Heilbron, E. G. Parry and F. S. Spring, *J. Chem. Soc.*, 1936, 1437.
[88] G. A. D. Haslewood, *Biochem. J.*, 33, 709 (1939).
[89] H. B. MacPhillamy, *J. Am. Chem. Soc.*, 62, 3518 (1940).
[90] O. Wintersteiner and J. R. Ritzmann, *J. Biol. Chem.*, 136, 697 (1940).
[91] O. Wintersteiner and S. Bergström, *Ibid.*, 137, 785 (1941).

Either of the two isomers can be converted into 7-dehydro-cholesterol. The method used for this reaction originally was the thermal decomposition of the dibenzoate, but this has been considerably improved by utilization of amines.[92] As a by-product in this reaction, the benzoate of

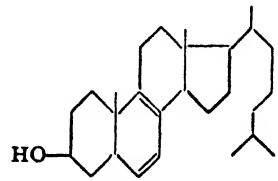


isodehydro-cholesterol is obtained.[93] The latter compound can be separated from 7-dehydro-cholesterol by fractional crystallization of their

[92] H. R. Rosenberg, U. S. P. 2,209,934, B. P. 537,036. G. A. D. Haslewood, *J. Chem. Soc.* **1938**, 224.

[93] A. Windaus, O. Linsert and H. J. Eckhardt, *Ann.*, **534**, 22 (1938).

benzoates[94] or of substituted benzoates.[95] Isodehydro-cholesterol has the probable formula (VIII),[95] that is, it differs from 7-dehydro-cholesterol only in the position of the double bonds, which are believed to be in the 6,7- and 8,9-positions.



(VIII)
Isodehydro-cholesterol

Besides this clear-cut synthesis a number of other methods have been found to yield 7-dehydro-cholesterol in small amounts. Thus by direct oxidation of cholesterol under mild conditions,[96] for example, with peroxides, a provitamin D is obtained which is probably 7-dehydro-cholesterol. Somewhat higher yields are apparently obtained by the utilization of quinones as dehydrogenating agents.[97, 98] Also methylene blue in the presence of light, and succino-dehydrogenase have been claimed to convert cholesterol into provitamin D.[99]

A number of other provitamins D are claimed to have been obtained from cholesterol or its derivatives. The following methods used for their preparation indicate that the provitamins obtained may ultimately prove to be 7-dehydro-cholesterol, although some investigators believe them to be new and different provitamins D.[100]

(*a*) Cholesterol, upon heating, is converted in very small amounts into a provitamin D.[101]

(*b*) Cholesterol, freed essentially from its naturally adherent provitamin D content and subjected to ultraviolet light, yields a vitamin D.[102]

[94] A. G. Boer, E. H. Reerink, A. van Wijk and J. van Niekerk, *Proc. Acad. Sci. Amsterdam*, **39**, 622 (1936).

[95] A. Windaus, O. Linsert and H. J. Eckhardt, *Ann.*, **534**, 22 (1938).

[96] J. Waddell, U. S. P. 2,028,364, U. S. P. 2,056,992.

[97] N. A. Milas and R. Heggie, *J. Am. Chem. Soc.*, **60**, 984 (1938).

[98] P. P. T. Sah, *Rec. trav. chim.*, **59**, 454 (1940).

[99] N. A. Milas and R. Heggie, *J. Am. Chem. Soc.*, **60**, 984 (1938).

[100] C. E. Bills, *Cold Spring Harbor Symposia Quant. Biol.*, **3**, 328 (1935).

[101] E. M. Koch and F. C. Koch, *Science*, **82**, 394 (1935); *J. Biol. Chem.*, **116**, 756 (1936). M. L. Hathaway and D. E. Lobb, *Ibid.*, **113**, 105 (1936). R. W. Hamann and H. Steenbock, *Ibid.*, **114**, 505 (1936).

[102] C. E. Bills, *J. Biol. Chem.*, **66**, 451 (1925). A. Jendrassik and A. G. Keményffi, *Biochem. Z.*, **189**, 180 (1927). S. K. Kon, F. Daniels and H. Steenbock, *J. Am. Chem. Soc.*, **50**, 2573 (1928). F. C. Koch, E. M. Koch and J. K. Ragins, *J. Biol. Chem.*, **85**, 141 (1929). E. M. Koch and H. B. Lemon, *Ibid.*, **85**, 159 (1929).

This vitamin D is alleged to be different from that obtained from 7-dehydro-cholesterol since it was found to be less effective for chicks.

(*c*) 7-Hydroxy-cholesterol upon irradiation develops a slight anti-rachitic potency.[103] The work does not indicate whether or not pure 7-hydroxy-cholesterol was employed. There exists the possibility that 7-hydroxy-cholesterol can lose one mol of water to form 7-dehydro-cholesterol.

(*d*) 7-Oxo-cholesterol-acetate upon Grignard reaction with isobutyl-magnesium-bromide followed by heating to 200° C. is said[104] to produce small amounts of a new provitamin D. While this statement has not been proved, it appears possible that the Grignard compound led to a partial reduction to a 7-hydroxy-cholesterol compound. Such reductions have been observed to occur in sterols.[105] 7-Hydroxy-cholesterol may then upon heating be transformed partially into 7-dehydro-cholesterol.

(*b*) *The Synthesis of Epi-7-dehydro-cholesterol*

The synthesis of epi-7-dehydro-cholesterol has been carried out (1) by using epi-cholesterol as starting material[106] and employing the methods outlined for the synthesis of 7-dehydro-cholesterol and (2) by epimerization of the 3-hydroxyl group of 7-dehydro-cholesterol.[107] The latter compound upon oxidation with aluminum-*tert*-butyroxide yields a dehydro-cholestenone which upon reduction with aluminum-isopropoxide is converted into epi-7-dehydro-cholesterol in a yield of 1.25%.

(*c*) *The Synthesis of Epi-ergosterol*

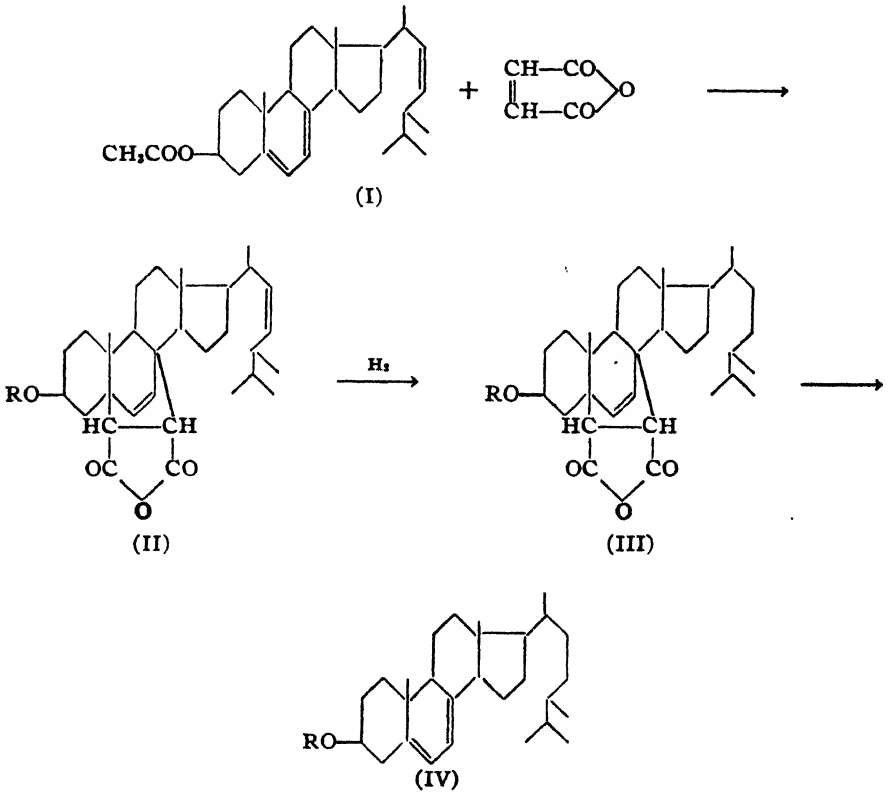
Epi-ergosterol has been obtained in about 1.3% yield by reduction of ergosterone with aluminum-isopropoxide, but has not been isolated in a high state of purity.[108]

(*d*) *The Synthesis of 22-Dihydro-ergosterol*

22-Dihydro-ergosterol has been obtained by side chain hydrogenation of ergosterol.[109] This is carried out by acetylating ergosterol, followed by formation of an addition product of maleic anhydride with ergosterol

[103] C. E. Bills, *Cold Spring Harbor Symposia Quant. Biol.*, **3**, 328 (1935).
[104] S. Weinhouse and M. S. Kharasch, *J. Org. Chem.*, **1**, 490 (1936).
[105] L. Ruzicka and H. R. Rosenberg, *Helv. Chim. Acta*, **19**, 357 (1936).
[106] A. Windaus and J. Naggatz, *Ann.*, **542**, 204 (1939).
[107] A. Windaus and O. Kaufmann, *Ibid.*, **542**, 218 (1939).
[108] A. Windaus and K. Buchholz, *Ber.*, **71**, 576 (1938); **72**, 597 (1939).
[109] A. Windaus and R. Langer, *Ann.*, **508**, 105 (1933).

acetate (II). This, upon catalytic hydrogenation, is selectively reduced in the side chain (III). By thermal decomposition, 22-dihydro-ergosterol-acetate (IV) is obtained. This yields 22-dihydro-ergosterol upon saponification.



(e) *Synthesis of 22-23-Oxido-ergosterol*

22-23-Oxido-ergosterol has been mentioned in the literature[110] but the method of synthesis has not been reported as yet. Probably the maleic anhydride addition product of an ergosterol ester is treated with a mild oxidizing agent to form the 22-23-oxido-compound, which upon thermal decomposition yields the 22-23-oxido-ergosterol-ester.

[110] A. Windaus, Linsal and K. Buchholz, quoted by K. Dimroth and J. Paland, *Ber.*, **72**, 187 (1939).

(*f*) *Synthesis of 7-Dehydro-sitosterol*

The synthesis of 7-dehydro-sitosterol has been carried out[111] according to the Windaus method for the synthesis of 7-dehydro-cholesterol. As starting material, the sitosterol mixture from soybean oil was used. This contains various isomers which are extremely difficult to separate.

(*g*) *Synthesis of 7-Dehydro-stigmasterol*

7-Dehydro-stigmasterol has been synthesized[112] from stigmasterol according to the previously outlined method for the synthesis of 7-dehydro-cholesterol from cholesterol.

8. Industrial Methods of Preparation

The industrial preparation of the provitamins ergosterol and 7-dehydro-cholesterol is of technical importance. The provitamin D from certain marine animals is also used commercially. Ergosterol is manufactured mainly by extraction from yeast and to a small extent also from the mycelium of aspergillus niger. Commercial ergosterol contains as much as 5% of α-dihydro-ergosterol,[113] which accompanies ergosterol in fungi and which has about the same solubility characteristics as ergosterol. Extensive investigations on the ergosterol content of yeast revealed that the yields are more dependent upon the conditions under which the yeast is grown than upon the strain of yeast.

7-Dehydro-cholesterol is synthesized from cholesterol according to the methods described previously.

9. Biogenesis

Little is known about the biogenesis of the provitamins D. Ergosterol is synthesized by yeast and by a number of fungi and other lower organisms. This synthesis is apparently more closely related to the metabolism of carbohydrates than to that of fats.[114] On the other hand, aspergillus niger can synthesize ergosterol when sodium acetate is the sole source of carbon.[115, 116] No connection could be demonstrated between sterol

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[111] W. Wunderlich, *Z. physiol. Chem.*, **241**, 116 (1936).

[112] O. Linsert, *Ibid.*, **241**, 125 (1936).

[113] R. K. Callow, *Biochem. J.*, **31**, 87 (1931).

[114] C. E. Bills, *Physiol. Rev.*, **15**, 1 (1935). A. Heiduschka and H. Lindner, *Z. physiol. Chem.*, **181**, 15 (1929).

[115] W. H. Maguigan and E. Walker, *Biochem. J.*, **34**, 804 (1940).

[116] I. Smedley-MacLean and D. Hoffert, *Ibid.*, **17**, 720 (1923). R. Sonderhoff and H. Thomas, *Ann.*, **530**, 195 (1937).

synthesis and the nitrogen metabolism.[117, 118] 7-Dehydro-cholesterol is apparently synthesized by higher animals and by human beings. The course of this synthesis is not known, but it is perhaps significant that both forms of 7-hydroxy-cholesterol, the α- and the β-isomer, apparently occur in animal tissues. Thus, the formation of 7-dehydro-cholesterol by dehydration of the 7-hydroxyl compounds appears possible. It has also been postulated that 7-dehydro-cholesterol is synthesized from cholesterol by enzymatic dehydrogenation. No proof for this hypothesis can be offered other than the observation that small amounts of 7-dehydro-cholesterol and of dihydro-cholesterol always are present with cholesterol.[119] It has been predicated therefore that while one molecule of cholesterol is dehydrogenated, another molecule is hydrogenated. On the other hand, it seems plausible to assume that in certain cells 7-dehydro-cholesterol is totally synthesized in a manner similar to the total synthesis of cholesterol, which has been reported as being built up from compounds of only two or three carbon atoms.[120]

10. Determination

(a) *Physical Methods*

The most accurate method for the determination of provitamins D is the spectroscopical analysis. The characteristic absorption spectrum of the provitamins in the ultraviolet region is the same for all provitamins on a molecular basis. This method, while quite accurate for the determination of provitamins D in sterol mixtures isolated from natural sources, cannot distinguish between the individual provitamins D. This method can also be used for the determination of provitamins D in preparations obtained by chemical synthesis with the restriction that the actual absorption maxima must be determined as such and in relation to each other and that general absorption must be observed and properly considered. In dubious cases, the addition of known amounts of a provitamin D may increase the accuracy of the determination of the original provitamin D content.

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[117] W. H. Maguigan and E. Walker, *Biochem. J.*, **34**, 804 (1940).

[118] O. N. Massengale, C. E. Bills and P. S. Prickett, *J. Biol. Chem.*, **94**, 213 (1931). M. Sobotka, W. Halden and F. Bilger, *Z. physiol. Chem.*, **234**, 1 (1935). F. Reindel, K. Niederländer and R. Pfundt, *Biochem. Z.*, **291**, 1 (1937).

[119] R. Schönheimer, H. v. Behring, R. Hummel and L. Schindel, *Z. physiol. Chem.*, **192**, 73 (1930); *Naturwissenschaften*, **18**, 156 (1930).

[120] D. Rittenberg, *J. Biol. Chem.*, **119**, LXXXIII (1937).

(*b*) *Chemical Methods*

No chemical method has been developed which is specific enough to determine the provitamins D accurately. Since all methods known are based on the characteristic conjugated unsaturation of the provitamin D molecule, no distinction can be made between the various provitamins. The chemical color tests based on this unsaturation are the following:

1. **The Reversed Salkowski Reaction.**[121] The provitamin D is dissolved in chloroform and concentrated sulfuric acid is added. The acid becomes deep red while the chloroform layer remains colorless. Sterols, such as cholesterol and sitosterol, give the opposite color reactions: the chloroform becomes red, while the acid exhibits a green fluorescence.

2. **The Liebermann-Burchard Reaction.**[122] The provitamin D is dissolved in chloroform, and acetic anhydride and concentrated sulfuric acid are added dropwise. A red color develops which progressively changes from blue-violet to green. Cholesterol gives a similar change of color but the initial red color remains unchanged for a longer period of time.

3. **The Tortelli-Jaffé Reaction.**[123] A solution of provitamin D in acetic acid is mixed with a 2% solution of bromine in chloroform. A green color develops. This reaction is given by steroids with a di-tertiary ethenoid linkage[124] and by vitamins D.

4. **The Rosenheim Reaction.**[125] To a solution of provitamin D in chloroform a solution of trichloro-acetic acid in water is added. A red color develops which changes slowly into a light blue. This color reaction becomes considerably more sensitive when, prior to the addition of the trichloro-acetic acid, lead tetra-acetate in glacial acetic acid is added to the chloroform solution of the provitamin. An intense green fluorescence occurs which is, however, not given by provitamin D esters. Thus, provitamins can be differentiated from their esters by this method. This modified color reaction can be employed for the detection of provitamins D in an amount of the order of 0.1 γ.[126]

5. **Chloralhydrate Reaction.** Crystals of provitamin D, when heated slowly with crystals of chloralhydrate, melt above 50° C. and the

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[121] E. Gérard, *Chem. Zentr.*, (1895) II, 229; *Arch. ges. Physiol. Pflügers*, 6, 207 (1872).

[122] C. Liebermann, *Ber.*, 18, 1804 (1885). A. Heiduschka and H. Lindner, *Z. physiol. Chem.*, 181, 15 (1929).

[123] E. P. Häussler and E. Brauchli, *Helv. Chim. Acta*, 12, 187 (1929). I. M. Heilbron and F. S. Spring, *Biochem. J.*, 24, 133 (1929).

[124] U. Westphal, *Ber.*, 72, 1243 (1939).

[125] O. Rosenheim, *Biochem. J.*, 23, 47 (1929); 25, 74 (1931).

[126] A. von Christiani and V. Anger, *Ber.*, 72, 1124, 1482 (1939).

mixture becomes first red, then green and finally deep blue. Sterols, such as cholesterol, do not give any color reaction with chloralhydrate.

6. The Antimony-Trichloride Reactions.[127] Provitamin D dissolved in chloroform and mixed with a solution of antimony trichloride in chloroform yields a red color.

7. The Tschugajeff Reaction.[130] A solution of provitamin D in glacial acetic acid with an excess of acetyl chloride and zinc chloride yields upon heating to the boiling point an eosin-red color with a greenish yellow fluorescence. The sensitivity is reported to be 1:80,000.

While none of these reactions is absolutely quantitative, the Liebermann-Burchard reaction and the Rosenheim reaction[128, 129] have been recommended and developed for quantitative assays.

CONVERSION OF PROVITAMINS D TO VITAMINS D

11. Process of Activation

Provitamins D can be activated to vitamins D by a number of different processes. They all involve in principle the input of energy into the provitamin D molecule. Thus, ultraviolet light, cathode rays, radium emanation, etc., effect activation.

(*a*) *Ultraviolet Light Activation*

Provitamins D are activated to vitamins D by ultraviolet light of the same wave lengths as those which are absorbed by the provitamins as evident from the absorption spectrum. The energy required to produce one U. S. Pharmacopoeia unit of vitamin D from ergosterol has repeatedly been investigated.[131] The data obtained under the most careful conditions indicate that 7.5×10^{13} quanta will produce one U. S. Pharmacopoeia unit of vitamin D.[132] In the active region, the energy necessary depends upon the wave length. The most effective activation of ergosterol is obtained from light of the wave length 281 mμ, which is the line that shows maximum absorption of ergosterol. On the other hand, the activation

[127] E. P. Häussler and E. Brauchli, *Helv. Chim. Acta*, **12**, 187 (1929). I. M. Heilbron and F. S. Spring, *Biochem. J.*, **24**, 133 (1929).

[128] R. K. Callow, *Biochem. J.*, **25**, 87 (1931).

[129] A. von Christiani and V. Anger, *Ber.*, **72**, 1124, 1482 (1939).

[130] L. Tschugajeff, *Chem. Ztg.*, **24**, 542 (1900); *Z. angew. Chem.*, **13**, 618 (1900).

[131] S. Kon, F. Daniels and H. Steenbock, *J. Am. Chem. Soc.*, **50**, 2573 (1928). A. L. Marshall and A Knudson, *Ibid.*, **52**, 2304 (1930). T. A. Webster and R. B. Bourdillon, *Biochem. J.*, **22**, 1223 (1928). R. W. Haman and H. Steenbock, *Ind. Eng. Chem., Anal. Ed.*, **8**, 291 (1936).

[132] R. S. Harris, J. W. M. Bunker and L. M. Mosher, *J. Am. Chem. Soc.*, **60**, 2579 (1938).

of 7-dehydro-cholesterol has been reported[133] to be significantly greater by monochromatic light of 296.7 mμ than by any other wave length. Conflicting results have been obtained from studies on the production of vitamin D in rats. While in one laboratory light of 281 mμ was found most effective,[134] in another laboratory a superior effectiveness of light of 296.7 mμ was found.[135]

There are different types of ultraviolet light sources used for the activation of provitamins D, namely, the light of the magnesium arc and of the carbon arc and of the mercury vapor lamp. The light emitted by a bismuth vapor lamp has also been recommended.[136] Cored carbon electrodes impregnated with various metals are also used.

Provitamins D can be activated in the dry state,[137] in vapor form[138] and in solutions. Irradiation of provitamin D in the dry state gives poor yields because the vitamin D is produced only on the surfaces of the crystals and further irradiation destroys the vitamin D formed before the provitamin D present in the middle of the crystals has been affected. Irradiation in the vapor phase has not been investigated thoroughly. The best method of irradiation is to expose solutions of provitamins D to the action of the ultraviolet light. Agitation of the solution[139] was found to enhance the vitamin D yield. The best technical method known today involves the utilization of a special quartz irradiation chamber which is built concentrically around the mercury vapor lamp and through which the provitamin D solution passes continuously in turbulent flow.[140]

As pointed out before, the yield of vitamin D is influenced by the wave length of the light employed. It is well established that light of wave length between 275 and 300 mμ produces the best yields of vitamin D[141] with the smallest amount of by-products. The desired light is obtained by special light filters. Light below 275 mμ is filtered out by aromatic compounds such as benzene[142] or xylene[142] or by compounds such as diphenyl[143] in benzene solution, or by a 5% lead-acetate solution.[144] Car-

[133] J. W. M. Bunker, R. S. Harris and L. M. Mosher, *J. Am. Chem. Soc.*, 62, 508 (1940).
[134] A. Knudson and F. Benford, *J. Biol. Chem.*, 124, 287 (1938).
[135] J. W. M. Bunker, R. S. Harris and L. M. Mosher, *J. Am. Chem. Soc.*, 62, 508 (1940).
[136] N.-V. Philips' Gloeilampenfabrieken, Holland, Dutch P. 35,579; U. S. P. 1,904,751.
[137] H. H. Beard, R. E. Burk, H. E. Thompson and H. Goldblatt, *J. Biol. Chem.*, 96, 307 (1932).
[138] F. A. Askew, R. B. Bourdillon and T. A. Webster, *Biochem. J.*, 26, 814 (1932).
[139] A. Windaus, K. Westphal, F. v. Werder and O. Rygh, *Nachr. Ges. Wiss. Göttingen, Math. physik. Klasse*, III, 45 (1929).
[140] F. Seitz, *Darstellung von Vitaminpräparaten*, Leipzig, 1939, 50.
[141] E. H. Reerink and A. van Wijk, *Strahlentherapie*, 40, 728 (1931). T. H. Rider, G. Sperti, G. P. Goode and H. G. Cassidy, *J. Am. Med. Assoc.*, 106, 452 (1936).
[142] N.-V. Philips' Gloeilampenfabrieken, G. P. 634,146.
[143] I. G. Farbenindustrie, G. P. 565,900.
[144] General Development Lab., Inc., U. S. P. 1,982,029.

bon tetrachloride is used to filter out light of the wave lengths 312 and 313 mμ.[145] Special types of glass which selectively allow light of 270–300 mμ to penetrate can also be made.

The presence of oxygen during the irradiation should be carefully avoided.[146] The intermediates and by-products formed during the irradiation are much more susceptible to oxidation by molecular oxygen than either the provitamins or the vitamins D. The presence of oxygenated materials makes it difficult to isolate crystalline vitamin D, but the actual yield is not essentially affected.[147]

There is apparently also a specific solvent effect involved in the irradiation procedure. The activation takes place more rapidly in ether than in alcohol.[148]

Other materials such as cyclohexane and the diether, dioxane, have also been recommended[149] as solvents either alone or in mixture with ethyl acetate, benzene or triethanolamine. Provitamins D can also be irradiated in oil solution. Of further interest are some special methods of irradiation. Thus, it is claimed that enhanced yields of vitamin D are obtained when compounds are added which protect the vitamin D after its formation. Ethylene or alkalies have been used in this manner.[150] The irradiation can also be carried out in the presence of photosensitizers, for example, of eosin, erythrosin or dibromo-dinitro-fluorescein.[151]

The temperature coefficient of activation, if it exists, is very small.[152] The enhanced effect observed when irradiating at the boiling point of the solvent[153] is probably due to more uniform activation of all molecules present.

The best yields are obtained when only 40 to 60% of the total provitamin D is converted.[154] In such cases, the yields are between 30% and 60% of theory. This calculation includes the recovery of unchanged provitamin D.

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[145] N.-V. Philips' Gloeilampenfabrieken, Holland, B. P. 385,626.

[146] A. Smakula, *Nachr. Ges. Wiss. Göttingen, Math. physik. Klasse*, **III**, 49 (1928). C. E. Bills, E. M. Honeywell and W. M. Cox, *J. Biol. Chem.*, **80**, 557 (1928). E. H. Reerink and A. van Wijk, *Biochem. J.*, **23**, 1294 (1929).

[147] T. C. Angus, F. A. Askew, R. B. Bourdillon, H. M. Bruce, R. K. Callow, C. Fischermann, J. S. L. Philpot and T. A. Webster, *Proc. Roy. Soc. (London)*, **B108**, 340 (1931).

[148] C. E. Bills, E. M. Honeywell and W. M. Cox, *J. Biol. Chem.*, **92**, 601 (1931).

[149] Standard Brands, Inc., U. S. P. 1,955,554.

[150] I. G. Farbenindustrie, B. P. 321,992.

[151] E. Merck Co., B. P. 286,665.

[152] C. E. Bills and F. G. Brickwedde, *Nature*, **121**, 452 (1928). T. A. Webster and R. B. Bourdillon, *Biochem. J.*, **22**, 1223 (1928).

[153] I. G. Farbenindustrie, U. S. P. 1,896,191. Société Usines Chimiques du Rhône-Poulenc, B. P. 335,277.

[154] N.-V. Philips' Gloeilampenfabrieken, G. P. 634,146.

Direct irradiation of foods has attracted special attention. Thus, cereals and flour have been commercially irradiated. Of considerable practical importance is the irradiation of milk, which is carried out in special equipment since ultraviolet light penetrates milk only slightly and since the odor of milk is easily influenced by ultraviolet light. Yeast and dried milk are also irradiated commercially.

(*b*) *Activation by Other Means*

Although activation by ultraviolet light has been the only method thoroughly investigated, a number of other methods are known for the conversion of provitamins D into vitamins D.

Cathode rays as such[155] or in the presence of catalysts[156] such as iron-uranium salts, canal rays,[157] α-, β- and γ-rays of radioactive elements,[157] radium emanation,[158] x-rays,[157, 159] corpuscular rays, electrons of high frequency[160] and finally alternating current of high frequency[161] have been claimed to effect activation of provitamins D. Most of these claims must be further investigated before they can be accepted. The conversion of provitamins to vitamins D by mitogenetic radiation[162] has also been postulated and will be discussed in the section on the Biogenesis of Vitamin D (page 404).

12. Mechanism of Activation

The conversion of provitamins D to vitamins D is not a simple process. During the course of this reaction, several substances are formed[163, 164, 165] before the vitamin D is obtained and the latter is not stable to the activating energy but is transformed into other compounds.

The mechanism of the provitamin D conversion to vitamin D has been studied extensively in the case of ergosterol. The activating energy was

[155] A. Knudson and C. N. Moore, *J. Biol. Chem.*, **81**, 49 (1929). R. M. Hoffman and F. Daniels, *Ibid.*, **115**, 119 (1936).

[156] American Research Prod., Inc., U. S. P. 1,983,944.

[157] K. Hembd and Vitam Fabrik, G. P. 577,170.

[158] R. B. Moore and T. DeVries, *J. Am. Chem. Soc.*, **53**, 2676 (1931).

[159] See, however, H. Goldblatt, *Ergeb. Allg. Path. and Path. Anat.*, **2**. *Abt.*, **25**, 58 (1931).

[160] Brit. Thomson-Houston Co., B. P. 292,926.

[161] I. G. Farbenindustrie, Austrian P. 119,210.

[162] H. Mai, *Abhandl. aus der Kinderheilkunde u. ihren Grenzgeb.*, 1937, H. 45.

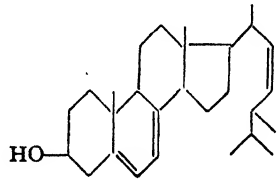
[163] C. E. Bills and F. G. Brickwedde, *Nature*, **121**, 452 (1928). T. A. Webster and R. B. Bourdillon, *Biochem. J.*, **22**, 1223 (1928).

[164] A. Smakula, *Nachr. Ges. Wiss. Göttingen, Math. physik. Klasse*, **III**, 49 (1928). C. E. Bills, E. M. Honeywell and W. M. Cox, *J. Biol. Chem.*, **80**, 557 (1928). E. H. Reerink and A. van Wijk, *Biochem. J.*, **23**, 1294 (1929).

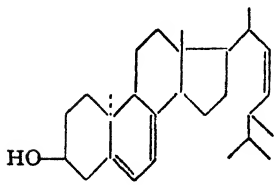
[165] O. Rosenheim and T. A. Webster, *Lancet*, **II**, 622 (1927).

supplied in these studies by ultraviolet light. It is not certain that the same intermediate products, vitamin D and final products are obtained by processes other than the ultraviolet irradiation. It seems relatively certain, however, that at least the vitamin is identical from all forms of activation.[166] The mechanism, which will be described for ergosterol in the following paragraphs, is believed to be identical for all provitamins D and certain indications are available for this assumption other than analogy, especially in the case of the activation of 7-dehydro-cholesterol[167] and 22-dihydro-ergosterol.[168]

The photochemical process is irreversible, that is, there is no equilibrium between the irradiation products. The number of the irradiation products and the sequence of these compounds during the course of the process were revealed by actual isolation of the pure intermediates and by a determination of all the products obtained from each isolated intermediate upon further irradiation. The result of these investigations is pictured in the following scheme for the reaction mechanism.[169]



Ergosterol, m. p. 166° C. $[\alpha]_D^{20} = -132°$ in chloroform. Absorption maxima at 260, 270, 282 and 293.5 mμ.



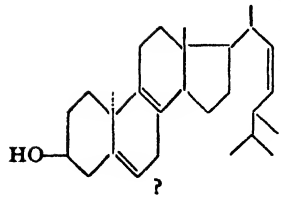
Lumisterol$_2$, m. p. 118° C. $[\alpha]_D^{19} = +192°$ in acetone. Absorption maxima at 265 and 280 mμ.

[166] For the vitamin made by activation of ergosterol by low velocity electrons, see I. McQuarrie, W. H. Thompson, A. V. Stoesser and L. G. Rigler, *J. Pediat.*, 10, 295 (1937).

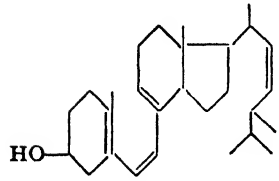
[167] A. Windaus, M. Deppe and W. Wunderlich, *Ann.*, 533, 118 (1937).

[168] A. Windaus and B. Güntzel, *Ibid.*, 538, 120 (1939).

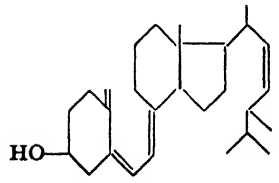
[169] P. Setz, *Z. physiol. Chem.*, 215, 183 (1933).



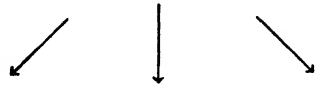
Pro-tachysterol$_2$.



Tachysterol$_2$. $[\alpha]_D^{18} = -70°$ in benzene. Absorption maxima at 268, 280 and 294 mμ.



Vitamin D$_2$ (Calciferol). M. p. 116° C. $[\alpha]_D^{20} = +106°$ in alcohol. Absorption maximum at 265 mμ.



Toxisterol$_2$ Absorption maximum at 248 mμ.

Suprasterol$_2$ I M. p. 104° C. $[\alpha]_D^{18} = -76°$ in chloroform. Absorption only in far ultraviolet.

Suprasterol$_2$ II M. p. 110° C. $[\alpha]_D^{18} = +63°$ in chloroform. Absorption only in far ultraviolet.

While there is no assurance that all intermediate products of the irradiation of ergosterol have been recognized, there is no evidence that other products are formed. During irradiation, the ultraviolet absorption characteristics change with the compounds obtained. This change is illustrated by the following curves of the irradiation products (Fig. 16).

All reaction products of the above scheme are isomers of the provitamins.

The constitutions of lumisterol, tachysterol and vitamin D are well established; the formula for pro-tachysterol is hypothetical.

The nomenclature of the irradiation products of the various provitamins D has been proposed by Windaus.[170] Accordingly, the intermediates will be called "lumisterol," "pro-tachysterol," "tachysterol," "vitamin D," etc., and the special structure due to their derivation from specific provitamins D will be designated by small index numbers such as "vitamin D_1," "vitamin D_2," "lumisterol$_3$," etc. The index numbers for all irra-

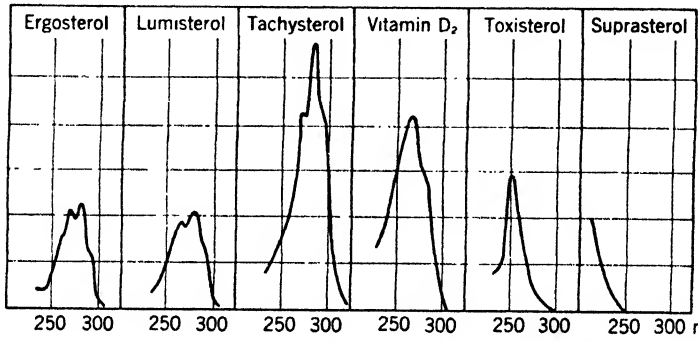


Fig. 16.—Absorption curves of ergosterol and its irradiation products in 0.2% etherial solution. (A. Windaus.)

diation products from one provitamin are the same, for example, all products derived from ergosterol are called products$_2$; from 7-dehydro-cholesterol, products$_3$; from 22-dihydro-ergosterol, products$_4$. There are no products$_1$. The term vitamin D_1 was a misnomer given originally to the first isolated crystalline material of vitamin D activity,[171] which later proved to be a molecular addition product of lumisterol$_2$ and vitamin D_2.[172]

All photochemical reaction products formed upon irradiation of any given provitamin D are subject to further photochemical attack. The irradiation can, however, be directed so that one or the other product is obtained predominantly according to the wave length used for the reaction.

Of all the irradiation products formed and isolated, only the one which is called "vitamin D" exerts antirachitic action. The others are physiologically inert or exhibit physiological properties entirely different from those of the natural vitamins D.

[170] A. Windaus, M. Deppe and W. Wunderlich, *Ann.*, **533**, 118 (1937).

[171] A. Windaus, *Proc. Roy. Soc.* (*London*), **B108**, 568 (1931). A. Windaus, A. Lüttringhaus and M. Deppe, *Ann.*, **489**, 252 (1931).

[172] A. Windaus and A. Lüttringhaus, *Z. physiol. Chem.*, **203**, 70 (1931). A. Windaus, O. Linsert, A. Lüttringhaus and G. Weidlich, *Ann.*, **492**, 226 (1932).

13. Chemistry of Activation Products

(a) **Lumisterol (Sterol X).** The first irradiation product of ergosterol which can be detected is lumisterol$_2$. Lumisterol$_2$ can be prepared predominantly by irradiation of ergosterol with light of the wave length 290–300 mμ.[173] Upon conversion of about 40% of the ergosterol used, the unchanged ergosterol is separated by crystallization from methanol. The mother liquors are evaporated to dryness and upon crystallization from acetone the molecular addition product of vitamin D_2 and lumisterol$_2$, which was called "vitamin D_1," separates. The addition product is broken up by acetylation, followed by fractional crystallization from acetic acid, whereby the lumisterol$_2$-acetate crystallizes first. Lumisterol$_2$ itself is obtained by saponification of its acetate.

Experimental investigations[174] have been made to ascertain if lumisterol$_2$ is a necessary intermediate or if ergosterol can be converted directly into the next intermediate, tachysterol$_2$. From the changes involved in the absorption spectrum of ergosterol and of lumisterol$_2$ upon short irradiation with ultraviolet light, it is concluded[175] that lumisterol$_2$ must be formed before tachysterol$_2$ can be obtained.

Analysis and molecular weight determinations indicate that lumisterol$_2$ is an isomer of ergosterol. The hydroxyl is present since esters can be formed. Lumisterol$_2$ contains three double bonds as evident from titrations with perbenzoic acid and from catalytic hydrogenation.[176, 177] One of these is in the side chain in 22,23-position as in ergosterol, since upon ozonolysis methyl-isopropyl-acetaldehyde is obtained.[178] The other two double linkages are conjugated as evident from the absorption spectrum (Fig. 17). Since upon total dehydrogenation of lumisterol$_2$ with selenium, the same hydrocarbon, $C_{18}H_{16}$ (γ-methyl-cyclo-penteno-phenanthrene), is obtained as from ergosterol[179] and since upon nitric acid oxidation of lumisterol$_2$ and of ergosterol the same toluene-tetracarboxylic acid is obtained[180] (see page 356) it must be concluded that the original ring system of ergosterol is also the ring system of lumisterol$_2$ and that the system of conjugated double bonds is present in one ring, which can be only ring B or ring C of the sterol ring skeleton. Lumisterol$_2$ upon treat-

[173] A. Windaus, K. Dithmar and E. Fernholz, *Ann.*, **493**, 265 (1932).
[174] H. Lettré, *Ibid.*, **511**, 280 (1934).
[175] K. Dimroth, *Ber.*, **70**, 1631 (1937).
[176] K. Dimroth, *Ibid.*, **68**, 539 (1935).
[177] A. Windaus, K. Dithmar and E. Fernholz, *Ann.*, **493**, 265 (1932).
[178] A. Guiteras, Z. Nakamiya and H. H. Inhoffen, *Ibid.*, **494**, 116 (1932).
[179] H. H. Inhoffen, *Ibid.*, **494**, 122 (1932).
[180] G. Ahrens, E. Fernholz and W. Stoll, *Ibid.*, **500**, 109 (1932).

ment with perbenzoic acid yields a triol, which gives only a diacetate, and upon treatment with mercuric acetate yields dehydro-lumisterol$_2$.[181] All these reactions are in strict analogy to the behavior of ergosterol, so that there is little doubt that the double bonds are in the same position as they are in ergosterol, namely, in the 5,6- and in 7,8-positions. On the other hand, lumisterol$_2$ does not form an addition product with digitonin and does not form a bimolecular compound upon irradiation in the presence of eosin. Furthermore, upon total hydrogenation a hexahydro-compound is formed which is different from that obtained from ergosterol. The difference cannot be due to isomerization of the 3-hydroxyl group

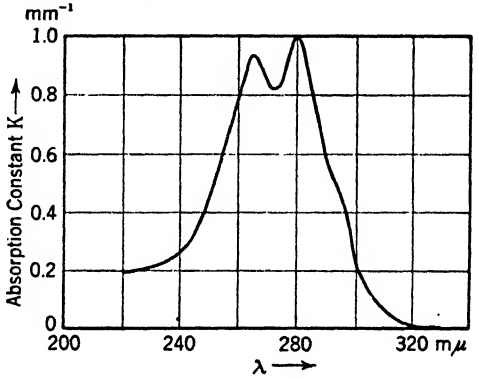


Fig. 17.—Absorption spectrum of lumisterol$_2$.
(H. Brockmann.)

since pyro-calciferol, which will be discussed later, also yields a bimolecular compound upon irradiation with eosin. It must, therefore, be concluded that the difference between ergosterol and lumisterol is only in the steric position of one substituent on one of the asymmetrical carbon atoms 9 or 10. Actually an isomerization on carbon atom 10 is involved as will be discussed in the section on the pyro-calciferols (page 399).

Lumisterol$_2$ can be converted into vitamin D$_2$ by irradiation, but is itself devoid of antirachitic efficacy.

Lumisterol$_3$[182] and lumisterol$_4$[183] have been isolated from the irradiation products of 7-dehydro-cholesterol and 22-dihydro-ergosterol. It is interesting to note that these lumisterols in contradistinction to lumisterol$_2$ do not form molecular addition compounds with their corresponding vitamins D.

[181] I. M. Heilbron, F. S. Spring and P. A. Stewart, *J. Chem. Soc.*, **1935**, 1221. I. M. Heilbron and F. S. Spring, *Chem. and Ind.*, **54**, 795 (1935).
[182] A. Windaus, M. Deppe and W. Wunderlich, *Ann.*, **533**, 118 (1937).
[183] A. Windaus and B. Güntzel, *Ibid.*, **538**, 120 (1939).

The formation of lumisterol compounds from three different provitamins D by ultraviolet irradiation allows the prediction that similar lumisterol compounds are formed by all steroids which have the system of conjugated double bonds and the steric configuration of the three provitamins ergosterol, 7-dehydro-cholesterol and 22-dihydro-ergosterol. The changes in the absorption spectrum of $\Delta^{5,7}$-androstadiene-diol-3,17 upon irradiation make the existence of a lumisterol compound certain. That the exact position of the double bonds in the starting material is necessary for the lumisterol formation is evident from the fact that isodehydro-cholesterol upon irradiation does not form a corresponding lumisterol compound. The steric specificity for the lumisterol formation is apparent, since pyro-calciferol and isopyro-calciferol (see page 399) do not yield lumisterol derivatives.

(b) **Pro-tachysterol.** Pro-tachysterol is obtained from lumisterol upon irradiation. It has not been isolated in the pure form and is apparently not stable, but undergoes rearrangement into tachysterol as evident from spectroscopical studies.[184] Thus, crude irradiation products of ergosterol kept sealed in the absence of oxygen change their absorption spectrum. While this dark reaction is slow at room temperature, it can be brought about in a few hours by heating to 55° C. The spectral changes involve the appearance of the typical absorption spectrum of tachysterol at 280 mμ.

(c) **Tachysterol.**[185] Tachysterol follows pro-tachysterol and precedes the vitamin in the sequence of irradiation products.

Tachysterol$_2$ can be obtained [186] from ergosterol by irradiation with the shorter wave lengths of the ultraviolet light until about 60% of the ergosterol is transformed. The separation from unchanged ergosterol is effected by crystallization from methanol. Tachysterol$_2$ is isolated from the other irradiation products by means of citraconic anhydride, which forms an addition compound with tachysterol$_2$. Tachysterol$_2$ itself is obtained from the adduct by thermal decomposition. While tachysterol$_2$ could not be obtained in crystalline form, the 3,5-dinitro-4-methyl-benzoic-acid-ester forms well-shaped crystals.

The constitution of tachysterol appears to be definitely established. The outstanding property of tachysterol is the ease with which it is autoxidized. This is considerably greater than the oxidation of ergosterol or of any of the other irradiation products. Analysis of the crystallized ester established that tachysterol$_2$ is an isomer of ergosterol. Tachysterol$_2$

[184] A. Windaus and E. Auhagen, *Z. physiol. Chem.*, **196**, 108 (1931).

[185] The name "tachysterol" has been given to this compound in recognition of its outstanding property, namely, the speed with which it reacts (ταχυς, Gr. = fast).

[186] A. Windaus, F. v. Werder and A. Lüttringhaus, *Ann.*, **499**, 188 (1932).

contains four double bonds, that is, one double bond more than ergosterol or lumisterol$_2$. This was established by the following series of reactions:[187] Tachysterol-acetate forms an addition product with citraconic anhydride, as mentioned before. The formation of this adduct requires that one double linkage disappears. Catalytic hydrogenation of the adduct re-

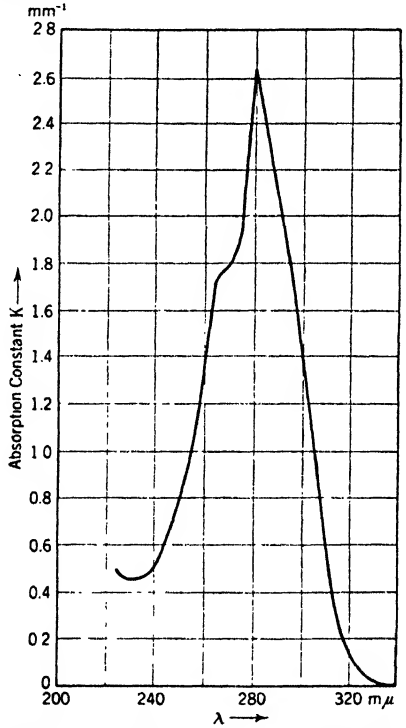


Fig. 18.—Absorption spectrum of tachysterol$_2$. (H. Brockmann.)

vealed that two more double bonds are present. The tetrahydro-tachysterol$_2$-acetate-citraconic anhydride is, however, not a saturated compound, since the presence of one more double bond can be demonstrated upon titration with perbenzoic acid. The presence of four double linkages requires the presence of only three rings instead of the four rings present in ergosterol and in lumisterol$_2$. The fourth additional double bond which is formed by ring-opening must be in conjugation to the two double bonds in ring B as evident from the absorption spectrum which shows maxima at 268, 280 and 294 mμ (Fig. 18).

[187] H. Lettré, *Ann.*, 511, 280 (1934).

The ring-opening during the formation of tachysterol$_2$ from lumisterol$_2$ occurs between carbon atoms 9 and 10. This has been assumed in view of the fact that in vitamin D_2, which follows tachysterol$_2$ in the sequence of irradiation products, ring B is open between carbon atoms 9 and 10 as has been proved by oxidation experiments (see page 392). The close relationship of tachysterol$_2$ to vitamin D_2 has also been demonstrated by hydrogenation with sodium in alcohol, both compounds yielding the identical dihydro-derivative[188] (dihydro-vitamin D_2I).

The position of the three conjugated double bonds has been established by oxidation experiments.[189] Vitamin D_2 yields upon oxidation a ketone, $C_{19}H_{32}O$, which will be discussed in the section on the Constitution of the Vitamin (see page 396). This ketone is obtained upon cleavage of the double bond between carbon atoms 7 and 8. Since the same ketone could not be obtained under similar oxidation conditions from tachysterol$_2$, it must be concluded that the latter has no double bond in the 7,8-position. The positions of the three conjugated double bonds must therefore be assumed to be in the 10,5-, 6,7- and 8,9-positions.

Tachysterol$_2$ has no antirachitic action and was found to be about half as toxic as vitamin D_2. Tachysterol$_3$ is formed from 7-dehydro-cholesterol[190] and tachysterol$_4$ from 22-dihydro-ergosterol[191] in a manner similar to the formation of tachysterol$_2$ from ergosterol. The isolation of these compounds is carried out by means of the condensation compounds with citraconic anhydride as described. None of the known tachysterols is a crystallized compound, but crystalline esters have been obtained.

Dihydro-tachysterol$_2$: A.T.10 (Anti-tetany compound No. 10.) Dihydro-tachysterol$_2$ is of considerable theoretical and practical importance. It is prepared[192] by sodium and alcohol reduction of the 3,5-dinitro-4-methyl-benzoic-acid-ester of tachysterol followed by saponification. (Dihydro-vitamin D_2I is obtained in about 30% yield as a by-product in the reaction.) While tachysterol$_2$ has not been obtained in the pure crystalline form, the dihydro-derivative$_2$ can easily be obtained in such a state.

Dihydro-tachysterol$_2$ is slightly active antirachitically.[193] It causes an increase of the calcium concentration in the blood. Tachysterol itself

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[188] M. Müller, *Z. physiol. Chem.*, **233**, 223 (1935).

[189] W. Grundmann, *Ibid.*, **252**, 151 (1936).

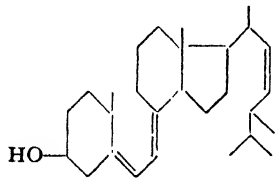
[190] A. Windaus, M. Deppe and W. Wunderlich, *Ann.*, **533**, 118 (1937).

[191] A. Windaus and B. Güntzel, *Ibid.*, **538**, 120 (1939).

[192] I. G. Farbenindustrie, U. S. P. 2,070,117.

[193] F. v. Werder, *Z. physiol. Chem.*, **260**, 119 (1939).

has the same property but is only $^1/_{10}$ as active. The dihydro-compound is used clinically under the name "A.T.10" for the treatment of idiopathic and postoperative (hypoparathyroid) tetany.[194, 195, 196] Dihydro-tachysterol has the following formula, as is evident from absorption spectrum, catalytic hydrogenation and oxidative degradation reaction:[197]



M. p. 125–127° C.
Absorption maxima at 242, 251 and 261 mμ.

(d) **Vitamin D.** The vitamins D follow the tachysterols in the sequence of photochemical reaction products from provitamins D. The isolation, properties and chemical constitution of the vitamins D will be described later.

The vitamins D are the only photoisomers of the provitamins D with antirachitic efficacy.

(e) **Toxisterol (Substance 248).** Upon further irradiation of vitamin D a toxic compound is formed, which has not yet been isolated in the pure state. It is characterized by its absorption spectrum which shows a maximum at 248 mμ. It has no antirachitic activity[198] but is quite toxic.[199, 200] There is considerable evidence that this compound is formed more readily when the irradiation is carried out in alcohol than in ether.[201]

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[194] F. Holtz, *Merck's Jahresber.*, **47**, 20 (1934).

[195] F. Holtz, *Klin. Wochschr.*, **13**, 104 (1934); *Deut. med. Wochschr.*, **I**, 560 (1934); **II**, 1830 (1934).

[196] M. MacBryde, *J. Am. Med. Assoc.*, **111**, 304 (1938). J. A. Greene and L. W. Swanson, *J. Iowa Med. Soc.*, **29**, 275 (1939). O. C. Pickhardt and A. Bernhard, *Ann. Surg.*, **108**, 362 (1938). E. Rose and F. W. Sunderman, *Arch. Intern. Med.*, **64**, 217 (1939). L. M. Hurxthal and T. S. Claiborne, *New England J. Med.*, **220**, 911 (1939).

[197] F. v. Werder, *Z. physiol. Chem.*, **260**, 119 (1939).

[198] A. Smakula, *Nachr. Ges. Wiss. Göttingen, Math. physik. Klasse*, **III**, 49 (1928). C. E. Bills, E. M. Honeywell and W. M. Cox, *J. Biol. Chem.*, **80**, 557 (1928). E. H. Reerink and A. van Wijk, *Biochem. J.*, **23**, 1294 (1929).

[199] A. Windaus, A. Lüttringhaus and P. Busse, *Nachr. Ges. Wiss. Göttingen, Math. physik. Klasse*, **III**, 150 (1932).

[200] F. Laquer and O. Linsert, *Klin. Wochschr.*, **12**, 753 (1933).

[201] A. van Wijk and E. H. Reerink, *Nature*, **122**, 648 (1928). W. E. Dixon and J. C. Hoyle, *Brit. Med. J.*, **II**, 832 (1928). L. J. Harris and T. Moore, *Biochem. J.*, **23**, 261 (1929). J. C. Hoyle and H. Buckland, *Ibid.*, **23**, 558 (1929). J. C. Hoyle, *J. Pharm.*, **40**, 351 (1930). R. Kern, M. F. Montgomery and E. W. Still, *J. Biol. Chem.*, **93**, 365 (1931).

The compound is called toxisterol or substance 248 because of its outstanding ultraviolet absorption at 248 mμ (Fig. 19).

(f) **Suprasterols I and II.** The information available concerning the formation of the irradiation products of vitamin D is scarce. It seems[202] that toxisterol and the two suprasterols I and II are formed simultaneously, but it has also been assumed[203] that toxisterol precedes the suprasterols.

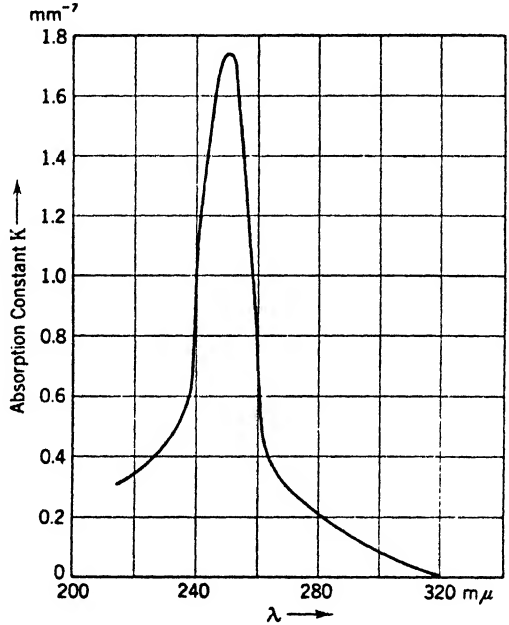

Fig. 19.—Absorption spectrum of toxisterol$_2$. (H. Brockmann.)

The two suprasterols appear to be the photochemical end-products from the irradiation of the provitamins.

The suprasterols$_2$ I and II have been obtained[204] from over-irradiated ergosterol. They have been isolated by fractional crystallization of their allophanates, whereby the suprasterol$_2$ I is obtained first. The suprasterols are isomers of the provitamins from which they have been obtained. In the case of the suprasterols$_2$ I and II the presence of the hydroxyl group and the side chain as present in ergosterol have been proved.

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[202] P. Setz, *Z. physiol. Chem.*, **215**, 183 (1933).
[203] C. E. Bills, *Physiol. Rev.*, **15**, 1 (1935).
[204] A. Windaus, J. Gaede, J. Köser and G. Stein, *Ann.*, **483**, 17 (1930).

Suprasterol$_2$ I has three double bonds according to the results obtained from catalytic hydrogenation and from titration with perbenzoic acid.[205] Since neither of the suprasterols shows any absorption in the ultraviolet region above 240 mμ (Fig. 20), it must be concluded that the double bonds are not conjugated. The presence of only three double bonds suggests that ring-closure occurred upon irradiation of vitamin D. The typical

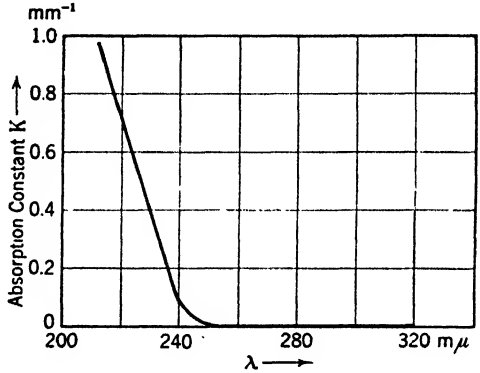
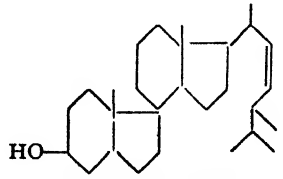


Fig. 20.—Absorption spectrum of the suprasterols$_2$. (H. Brockmann.)

hydrocarbon, γ-methyl-cyclo-penteno-phenanthrene, which is obtained from all sterol compounds upon dehydrogenation with selenium, was not obtained from either of the suprasterols$_2$. The ring-closure has, therefore, not resulted in the formation of the sterol skeleton.

Even less is known about suprasterol$_2$ II. There appear to be three double bonds, but there is no conclusive evidence to substantiate this.

As long as the structure is not certain, no definite formulas can be given for the suprasterols. The following spiro-cyclo-pentane formula has been tentatively suggested:[206]



+ 2 Double bonds

[205] M. Müller, *Z. physiol. Chem.*, **233**, 223 (1935).
[206] M. Müller, *Ibid.*, **233**, 223 (1935).

By over-irradiation of 7-dehydro-cholesterol[207] and of 22-dihydro-ergosterol[208] the suprasterols$_{3 \text{ and } 4}$, respectively, are formed.

VITAMINS D

The number of naturally occurring vitamins D is unknown, and it is an extremely difficult task to isolate and to identify the pure vitamin D from any source. Prior to the isolation of any pure antirachitic substance from natural material, various forms of vitamin D were obtained by irradiation of different provitamins. Numbers were assigned to these various vitamins D in order to simplify the nomenclature. Thus today we have the following series of five vitamins D with their corresponding provitamins:

TABLE IV

Vitamin D	Provitamin D	Remarks
Vitamin D_1	Ergosterol	Vitamin D_1 is a molecular compound consisting of vitamin D_2 and lumisterol$_2$
Vitamin D_2	Ergosterol	Vitamin D_2 is also called calciferol or viosterol
Vitamin D_3	7-Dehydro-cholesterol	Vitamin D_3 is also referred to as dimethyl-dihydro-calciferol
Vitamin D_4	22-Dihydro-ergosterol	
Vitamin D_5	7-Dehydro-sitosterol	

Actually, since at least ten different provitamins D are known, ten different vitamins D also should be listed. But only the four vitamins, designated vitamins D_{2-5}, have been prepared in essentially pure form. Whether or not the four vitamins D listed above occur in nature is not known. Only vitamin D_2 and vitamin D_3[209] have been isolated in the pure form from fish liver oils. Furthermore, another vitamin D of unknown constitution has been separated by molecular distillation from fish liver oils. From the properties of this vitamin it is inferred that it is not identical with any of the above listed vitamins and it will therefore be referred to in this monograph provisionally as vitamin $D_{6(?)}$. Besides vitamin $D_{6(?)}$ five other vitamins D occur in cod liver oil as evident from

[207] A. Windaus, M. Deppe and W. Wunderlich, *Ann.*, **533**, 118 (1937).
[208] A. Windaus and B. Guntzel, *Ibid.*, **538**, 120 (1939).
[209] H. Brockmann and A. Busse, *Z. physiol. Chem.*, **256**, 252 (1938).

molecular distillation experiments. Of these six vitamins D, two are present as the major constituents, two are present in lesser quantities and the last two are present only in traces.[210] The distillation curves for the vitamins D from spearfish and from "white sea bass" are different from each other and from those of cod liver oil. This suggests that these fish contain different vitamins D.

The complexity of the vitamins D which occur in nature can also be demonstrated by biological experiments, using different species of animals as test objects. All vitamins D are primarily standardized on rats. However, when tested on chicks, the naturally occurring vitamins D show quite inconsistent responses, Rat Unit for Rat Unit. In order to have a standardized vitamin D with which other vitamin D preparations can be compared, a special preparation of cod liver oil has been selected in the United States as a reference. This material is carefully tested on rats and the response of this preparation on chicks is designated arbitrarily as a 100% activity, that is, one Rat Unit vitamin D from the U. S. Reference Cod Liver Oil is arbitrarily taken as one Chick Unit of vitamin D. Vitamin D_3 also proved to be 100% chick-active. On the other hand, vitamin $D_{6(?)}$ has only $^1/_2$ to $^1/_4$ the chicken activity of the U. S. Reference Cod Liver Oil. Vitamin D_2 has practically no chicken activity. The livers of some fish contain vitamins D which are much more than 100% chick-active. Thus vitamin D from the "white sea bass" (*Cynoscion nobilis*) has been reported to be over 300% chick-active and the vitamin D from the dogfish (*Squalus suckleyi*) about 230% active.[211] Careful analysis of these results lead the investigators to the belief that these high chicken potencies of special fish oils are due to a real vitamin D, that is, due to at least one new, so far unknown form, and that these potencies cannot be explained on the basis of some hypothetical synergistic factor.

The species specificity has also been demonstrated with turkeys. A Chick Unit is not necessarily a Turkey Unit of vitamin D.[212] Furthermore, some evidence exists that human beings react preferentially to certain forms of naturally occurring vitamin D. Thus, it has been observed that seal oil is a much more effective antirachitic agent for humans than for rats.[213] There is also ample evidence that vitamin D_3 is somewhat more effective in man than vitamin D_2.

[210] K. C. D. Hickman and E. L. Gray, *Ind. Eng. Chem.*, **30**, 796 (1938).

[211] C. E. Bills, O. N. Massengale, M. Imboden and H. Hall, *J. Nutrition*, **13**, 435 (1937).

[212] T. H. Jukes and T. D. Sanford, *Ibid.*, **18**, 71 (1939).

[213] E. J. Mikhlina, M. J. Leizerovskaya and N. N. Milovanova, *Kazan. Med. Zhur.*, **33**, 64 (1937); *Chem. Zentr.*, **1938**, II, 3108.

14. Occurrence

Vitamin D occurs in nature only in small amounts. The living plant tissue and fresh green vegetables contain no detectable amount of this vitamin. The occurrence of very small amounts of vitamin D in some species, however, is possible due to the presence of significant amounts of provitamin D. Thus, by irradiation from the sunlight a certain amount of vitamin D should be formed.

From time to time it has been reported that certain plant materials contain considerable amounts of vitamin D. It has not been possible, however, to verify these claims. On the other hand, it has been found that yeasts and molds grow readily on many non-living plants. These organisms contain significant amounts of provitamins D as discussed previously (page 345). A transition of these provitamins into vitamins D under the influence of sunlight has been proved in many cases, for example, in the alleged antirachitic activity of cacao shells[214] and of hay.[215]

Vitamin D occurs only in small quantities in most members of the animal kingdom.[216] Only a few classes of animals and of animal products contain significant amounts.

Abundant quantities of vitamin D are present in the livers and also to a certain extent in the viscera of fish. The actual amount of vitamin D per gram of liver oil varies considerably with the species, the season and a number of other biological factors such as age, climate, food supply, condition of living, etc. Thus, for example, halibut livers give in the summer months a high yield in oil of low potency while in the winter months less oil with more vitamin D is obtained. As a general rule it appears that fish with much body oil are the richest natural source of vitamin D. The distribution of vitamin D in various fish oils may be illustrated by Table V.

While the fat from fish contains relatively large amounts of vitamin D, the fat of other animals contains little or none.[216] Exceptions to this general rule are certain fats from animals, especially from certain birds, that live upon fish. Furthermore, the nutritive animal material for the initial growth of those species that require vitamin D contains small but significant amounts. Thus, the eggs of birds are a relatively good source. More specifically, the yolks of hen and duck eggs contain definite amounts, which are usually fairly constant (see page 418). The milk of mammals contains vitamin D. Furthermore, inasmuch as milk contains the opti-

[214] A. W. Knapp and K. H. Coward, *Analyst*, **59**, 474 (1934).
[215] H. Steenbock, E. B. Hart, C. A. Elvehjem and S. W. F. Kletzien, *J. Biol. Chem.*, **66**, 425 (1925).
[216] P. A. Coppens and G. A. Metz, *Arch. neerland. physiol.*, **18**, 407 (1933).

TABLE V

DISTRIBUTION OF VITAMIN D IN VARIOUS FISH OILS

Source of oil	Zoological name	Potency, I. U. per G.
Bluefin tuna, liver	*Thunnus thynnus*	40,000
Swordfish, liver	*Xiphias gladius*	10,000
Yellowfin tuna, liver	*Neothunnus macropterus*	10,000
Black sea bass, liver	*Stereolepis gigas*	5,000
Bocaccio, liver	*Sebastodes paucispinis*	2,100
Red rockfish, liver	*Sebastodes ruberrimus*	1,500
Black rockfish, liver	*Sebastodes mystinus*	1,500
China rockfish, liver	*Sebastodes nebulosus*	1,400
"Ling cod" (not codfish), liver	*Ophiodon elongatus*	1,300
Chinook salmon, liver	*Oncorhynchus tschawytscha*	1,300
Halibut, liver	*Hippoglossus hippoglossus*	1,200
Rabbitfish, liver	*Chilomycterus schoepfi* (?)	1,100
Striped rockfish, liver	*Sebastodes elongatus*	1,000
Starry flounder, liver	*Platichthys stellatus*	1,000
Boston mackerel, liver	*Scomber scombrus*	750
"Black cod" (not codfish), liver	*Anoplopoma fimbria*	600
Pufferfish, liver	*Sphoeroides maculatus*	570
Dog salmon, liver	*Oncorhynchus keta*	400
Black horse, mesentery	*Cycleptus elongatus*	400
Turbot, liver	*Reinhardtius hippoglossoides*	260
Rex sole, liver	*Errex zachirus*	150
California sand dab, liver	*Orthopsetta sordida*	120
Cod, liver	*Gadus morrhua*	100
Herring, entire body	*Clupea harengus*	100
Yellow sole, liver	*Pseudopleuronectes dignabilis*	90
Sardine, entire body	*Sardinia caerulea*	80
Goosefish, liver	*Lophius piscatorius*	70
Pollack, liver	*Pollachius virens*	50
Menhaden, entire body	*Brevoortia tyrannus*	50
Shark, liver	(Sp.)	50
Salmon, trimmings	*Oncorhynchus* (Sp.)	40
Turbot, body minus liver	*Reinhardtius hippoglossoides*	30
Skate, liver	*Raja binoculata*	25
Dogfish (Pacific), liver	*Squalus suckleyi*	20
Muddy catfish, body	*Leptops olivaris*	20
Ohio perch, mesentery	*Aplodinotus grunniens*	11
Buffalo, mesentery	*Ictiobus cyprinella*	10
Haddock, liver	*Melanogrammus aeglefinus*	10
Channel catfish, mesentery	*Ictalurus punctatus*	5
Dogfish (Atlantic), liver	*Squalus acanthias*	3
Capelin, entire body	*Mallotus villosus*	3
Ratfish, liver	*Chimaera colliei*	2
Gray sole, liver	*Glyptocephalus cynoglossus*	<1
Sturgeon, liver	*Acipenser rubicundus*	Nil

From: C. E. Bills, *Physiol. Rev.*, **15**, 13 (1935).

mum amounts of calcium and phosphorus, it is used extensively for supplementing human needs (see page 403). Milk products, especially dried milk and butter also contain the vitamin originally present in milk.

15. Isolation

The isolation of vitamin D from naturally occurring fish liver oils is carried out by first isolating the unsaponifiable fraction. For this purpose, either the entire oil or the alcoholic extract therefrom[217] is hydrolyzed. This is done under the most careful conditions, for example, by means of potassium hydroxide in methanol in an atmosphere of nitrogen.[218] The next step consists in separating the vitamin D from the vitamin A present. This can be achieved by condensing the vitamin A with maleic anhydride[219] or, better, with citraconic anhydride.[220] Another separation method consists in the extraction of the mixture of vitamins A and D in hydrocarbon solution, such as benzene[221] or pentane;[222] with aqueous methanol (90–95%), whereby the vitamin D remains in the hydrocarbon, whereas the vitamin A goes into the methanol solution. The best method for further purification is apparently a selective adsorption on aluminum oxide according to the principle of chromatographic adsorption. The position of the vitamin in the adsorption column can be detected by adding to the vitamin solution an indicator, for example, indicator-red 33,[223] which has the same adsorption characteristics as the vitamin D. The vitamin D is separated from sterols, for example, from cholesterol, by freezing in methanol solution[224] or by precipitation with digitonin.[225, 226, 227, 228] Another method for the purification of the vitamin D is its separation from non-alcoholic constituents by esterification with phthalic acid anhydride, followed by fractionation of the esters.[229, 230, 231] A certain purification can also be obtained by high-vacuum distillation.[232, 233] Final purification is achieved by esterification, for example, with 3,5-dinitro-

[217] T. F. Zucker, *Proc. Soc. Exptl. Biol. Med.*, **19**, 167 (1922); **20**, 136 (1922).
[218] O. Neracher and T. Reichstein, *Helv. Chim. Acta*, **19**, 1382 (1936).
[219] O. Dalmer, F. v. Werder and T. Moll, *Z. physiol. Chem.*, **224**, 86 (1934).
[220] A. Windaus, O. Linsert, A. Lüttringhaus and G. Weidlich, *Ann.*, **492**, 226 (1931).
[221] H. Brockmann, *Z. physiol. Chem.*, **241**, 104 (1936); *Ibid.*, **245**, 96 (1937). H. Brockmann and A. Busse, *Ibid.*, **249**, 176 (1937).
[222] O. Neracher and T. Reichstein, *Helv. Chim. Acta*, **19**, 1382 (1936).
[223] H. Brockmann, *Z. physiol. Chem.*, **241**, 104 (1936); *Ibid.*, **245**, 96 (1937). H. Brockmann and A. Busse, *Ibid.*, **249**, 176 (1937).
[224] O. Neracher and T. Reichstein, *Helv. Chim. Acta*, **19**, 1382 (1936).
[225] H. Brockmann, *Z. physiol. Chem.*, **241**, 104 (1936); *Ibid.*, **245**, 96 (1937). H. Brockmann and A. Busse, *Ibid.*, **249**, 176 (1937).
[226] E. J. H. Simons and T. F. Zucker, *J. Am. Chem. Soc.*, **58**, 2655 (1936).
[227] G. A. D. Haslewood and J. C. Drummond, *J. Soc. Chem. Ind.*, **55**, 598 (1936).
[228] O. Neracher and T. Reichstein, *Helv. Chim. Acta*, **19**, 1382 (1936).
[229] E. J. H. Simons and T. F. Zucker, *J. Am. Chem. Soc.*, **58**, 2655 (1936).
[230] F. Ender, *Z. Vitaminforsch.*, **2**, 241 (1933); **3**, 161 (1934).
[231] O. Neracher and T. Reichstein, *Helv. Chim. Acta*, **19**, 1382 (1936).
[232] F. Ender, *Z. Vitaminforsch.*, **2**, 241 (1933); **3**, 161 (1934).
[233] O. Neracher and T. Reichstein, *Helv. Chim. Acta*, **19**, 1382 (1936).

benzoyl-chloride and purification of the dinitro-benzoate by fractional crystallization[234, 235] or by adsorption on aluminum oxide.[236] Esterification may also be carried out with isocyanic acid, followed by fractional crystallizations of the allophanates obtained.[237] The free vitamin D is then obtained by saponification of the esters. By utilization of these methods, vitamin D_3 and vitamin D_2 have been obtained[238] from tuna liver oil and from halibut liver oil.

The method of separating naturally occurring vitamins D by molecular distillation is also of considerable interest.[239] In order to facilitate the distillation process, a constant-yield oil is added to the fish liver oils. (For details of this method see page 61.) Upon distillation of such a mixture, free and esterified vitamins D can be separated. While the former distill around 160° C., the latter pass over at 230–250° C. The utilization of this method has resulted in the separation of a new vitamin D, which is characterized by the lowest boiling point of the entire vitamin D fraction and which will be provisionally referred to in this monograph as vitamin $D_{6\,(?)}$.

The methods of isolating vitamins D from the irradiation products of the provitamins are somewhat different from the methods used for the isolation of the naturally occurring vitamins D, since a different type of by-products must be eliminated. The best yield of vitamins D from the irradiation of provitamins D is obtained when about 40 to 60% of the provitamin has been transformed. The percentage converted can be determined approximately by crystallization of the unchanged provitamin D from alcohols or somewhat more accurately by precipitation with digitonin. Either of these operations, or a combination of both, is carried out as the first step in the actual isolation procedure to obtain the pure vitamin D. The isolation of the formed vitamin D is carried out in the absence of oxygen. Although vitamin D is only moderately sensitive to molecular oxygen at room temperature, the intermediate, tachysterol, which is always present in the crude irradiation products, at least in small amounts, adsorbs oxygen readily with the formation of peroxides, which in turn have the tendency to destroy the vitamin D. Tachysterol is separated from the irradiation mixture by condensation with citraconic anhydride. The crude reaction product is saponified at room temperature

[234] H. Brockmann and A. Busse, *Naturwissenschaften*, **26**, 122 (1938).
[235] E. J. H. Simons and T. F. Zucker, *J. Am. Chem. Soc.*, **58**, 2655 (1936).
[236] H. Brockmann, *Z. physiol. Chem.*, **241**, 104 (1936); *Ibid.*, **245**, 96 (1937). H. Brockmann and A. Busse, *Ibid.*, **249**, 176 (1937).
[237] G. A. D. Haslewood and J. C. Drummond, *J. Soc. Chem. Ind.*, **55**, 508 (1936).
[238] H. Brockmann and A. Busse, *Z. physiol. Chem.*, **256**, 252 (1938).
[239] K. C. D. Hickman, *Ind. Eng. Chem.*, **29**, 1107 (1937).

and extracted with petroleum ether and water, whereby the tachysterol-citraconic acid adduct remains in the water phase, while the vitamin D goes into the petroleum ether. The vitamin D is then crystallized from acetone at low temperature and recrystallized from acetone-methanol.

In another method used for the isolation of vitamin D after irradiation of its provitamin, a characteristic, sparingly soluble ester of the vitamin, such as the 3,5-dinitro-benzoate,[240] is prepared. This can be purified by fractional crystallization and the pure vitamin D is obtained by saponification of the ester.

The vitamins D form addition products very easily with other compounds, especially with substances that contain hydroxyl groups. This behavior is, of course, not characteristic for the vitamins D, for it is found in the case of many chemicals and especially of sterols. In the case of the vitamins D, this problem is, however, of practical importance. Thus, the vitamins D crystallize with the solvent of crystallization, and especially with water. The molecular compounds which the vitamins D form with other sterols are even more annoying. Thus, vitamin D_2 forms an addition product with lumisterol$_2$ (to give the so-called vitamin D_1), and vitamin D_3 forms an addition compound with the irradiation product of iso-dehydro-cholesterol,[241] which occurs as an impurity in the synthetically obtained 7-dehydro-cholesterol.

16. Properties

All the known vitamins D, in the pure state, are white, odorless crystals. They are soluble in fats and in the usual organic solvents, for example, in ether, chloroform, acetone, alcohol and are insoluble in water. They exhibit a characteristic absorption spectrum with one maximum at 265 $m\mu$ (in hexane and in ether), the molecular extinction coefficient $\epsilon = 1.82 \times 10^4$. [$K = 45 - 46 \times 10^3$] (Fig. 21).

Vitamin D_2:

M. p. 115–117° C. $[\alpha]_D^{20} = +103°$ in abs. alcohol, +82.6° in acetone, +33.3° in petroleum ether, +91.2° in ether.

3,5-Dinitro-benzoate: M. p. 148–149°. $[\alpha]_D^{22} = +55°$ in benzene.

p-Nitro-benzoate: M. p. 93°. $[\alpha]_D^{20} = +104°$ in chloroform.

Stability: Crystallized vitamin D_2, sealed in the absence of oxygen and stored in the absence of light at 2° C., is stable over a period of many months. When dissolved in olive oil and kept under similar conditions, more than half of the original amount is still present after five years. Vitamin D_2 is thermolabile. Although it can be

[240] F. A. Askew, R. B. Bourdillon, H. M. Bruce, R. K. Callow, J. S. L. Philpot and T. A. Webster, *Proc. Roy. Soc.* (*London*), **B109**, 488 (1932).

[241] H. Brockmann and A. Busse, *Naturwissenschaften*, **26**, 122 (1938).

sublimed at 125° C. in high vacuum, decomposition occurs at this temperature. The two thermal decomposition products, pyro-calciferol and isopyro-calciferol, are usually obtained by heating to 160–190° C.

Efficacy: 1 g. contains 40 million International Units vitamin D.

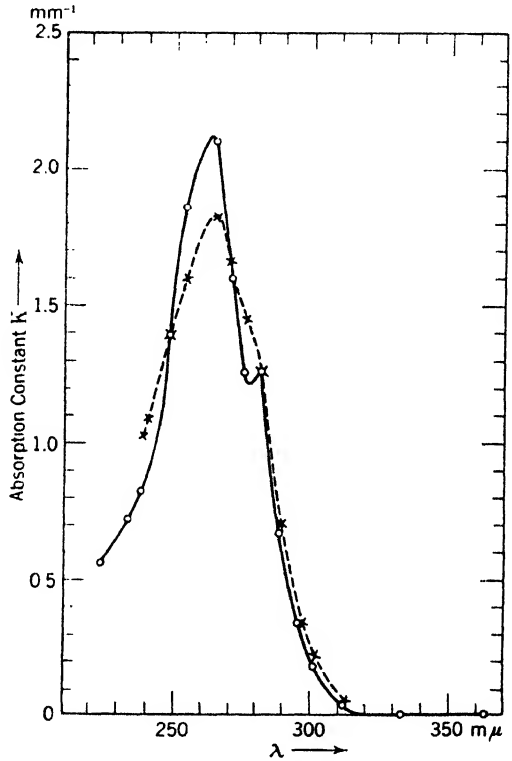


Fig. 21.—Absorption spectrum of vitamin D_2 (O—O) and of vitamin D_3 (×—×) in 0.02% solution in hexane. (H. Brockmann.)

Vitamin D_3:

M. p. 82–83° C. $[\alpha]_D^{20} = +83.3°$ in acetone.

3,5-Dinitro-benzoate: M. p. 129° and 140° C. (polymorphic). $[\alpha]_D^{20} = +98°$ in chloroform.

p-Nitro-benzoate: M. p. 127°. $[\alpha]_D^{20} = +114°$ in chloroform.

Vitamin D_4:[242]

M. p. 107–108°. $[\alpha]_D^{20} = +89.3°$ in acetone.

3,5-Dinitro-benzoate: M. p. 135–136°. $[\alpha]_D^{20} = +95.4°$ in acetone.

[242] A. Windaus, and G. Trautmann, *Z. physiol. Chem.*, **247**, 185 (1937)

17. Chemical Constitution

The chemical constitution of the vitamins D is closely related to the constitution of the provitamins, from which they can be derived. As pointed out on page 350, the provitamins differ from each other only in the number of carbon atoms in the side chain and the degree of unsaturation. The sterol skeleton is the same for all provitamins. This is also true for the vitamins D derived from the provitamins D. All chemically investigated vitamins D have the same constitution with the exception of different structures of the side chain.

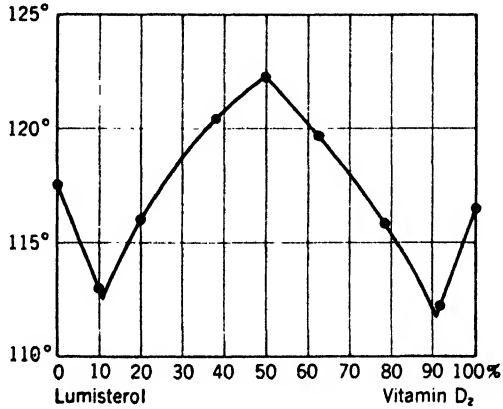


Fig. 22.—Melting point diagram of lumisterol and vitamin D_2. (A. Windaus, K. Dithmar and E. Fernholz.)

While the details of the chemical constitution of all the vitamins D have not been investigated, the constitution of vitamin D_2 or calciferol, which is derived from ergosterol, has been totally elucidated. The constitution of the other vitamins D can then be deduced by analogy and in the case of vitamin D_3, which is obtained from 7-dehydro-cholesterol, this deduction has been proved to be correct.

(*a*) *The Constitution of Vitamin* D_1

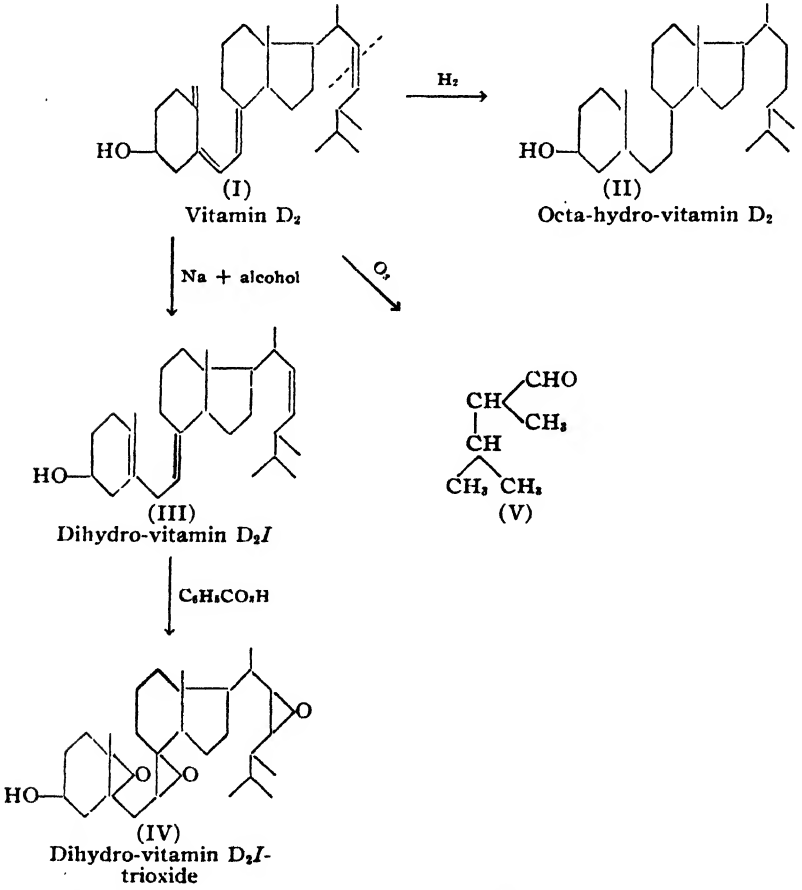
Vitamin D_1 has the constitution of a molecular addition product of vitamin D_2 with lumisterol$_2$ (see page 375). This has been shown by determination of the melting point diagram (Fig. 22) obtained by mixing various amounts of the two components.

(*b*) *The Constitution of Vitamin D_2*

Vitamin D_2 has the empirical formula $C_{28}H_{44}O$.[243] It is therefore an isomer of ergosterol, from which it is derived. The constitution of vitamin D_2 can consequently be linked with the structure of ergosterol (see page 350).

The oxygen present is in a hydroxyl group in the vitamin D_2 molecule, as is evident from the formation of esters.

Vitamin D_2 (I) contains four double bonds, since upon catalytic hydrogenation four mols of hydrogen are absorbed (II).[244] On the other hand,



[243] A. Windaus, F. v. Werder and B. Gschaider, *Ber.*, **65**, 1106 (1932).
[244] R. Kuhn and K. F. Müller, *Angew. Chem.*, **47**, 145 (1934).

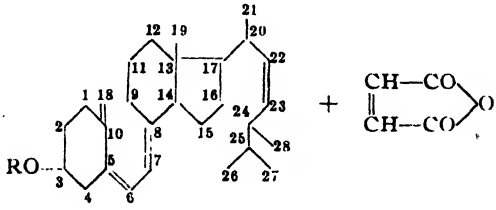
titration with perbenzoic acid indicates only three of the four double linkages. But the presence of the fourth double bond can be demonstrated as follows: Upon reduction with sodium and alcohol two different dihydro-compounds are formed—dihydro-vitamin D_2 *I* and *II* (III). Compound *I* has also been obtained by sodium reduction of tachysterol$_2$, as previously mentioned. This derivative contains three double bonds,[245] all of which react with perbenzoic acid, to form a crystallized trioxide (IV).[246] These results indicate that vitamin D_2 contains one double bond more than ergosterol, which in turn means that one of the four rings present in ergosterol has been opened, since vitamin D_2 contains only three rings according to its empirical formula and the number of double bonds present.

All sterols yield upon dehydrogenation with selenium the same characteristic hydrocarbon, γ-methyl-cyclo-penteno-phenanthrene. In accordance with the conception that vitamin D_2 does not contain the typical four-ring system of ergosterol, the typical dehydrogenation hydrocarbon could not be obtained upon reaction with selenium.[247] Vitamin D_2 has this property in common with tachysterol$_2$ as previously described.

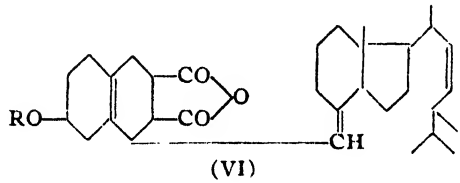
Of the four double bonds, one is in the side chain as in ergosterol, since methyl-isopropyl-acetaldehyde (V) is formed upon ozonolysis of the vitamin.[248] The other three double bonds are in conjugation to each other as must be concluded from the typical absorption spectrum and its extinction coefficient. The fact that at least two of these linkages are in conjugation is shown by the formation of an addition compound of the acetate of vitamin D_2 with maleic anhydride (VI).[249] Upon saponification, the latter yields a dicarboxylic acid which by reaction with diazomethane is converted into a dimethyl-ester (VII). This ester has been obtained in two isomeric forms. Upon catalytic hydrogenation of the mixture of the two esters, a dihydro-compound is obtained (VIII). This compound, upon oxidation with ozone, does not yield the methyl-isopropyl-acetaldehyde, which proves that the double bond in the side chain has been reduced. The reaction product of the ozonolysis is, however, a saturated ketone of the empirical formula $C_{19}H_{34}O$ (IX), according to the analysis of its semicarbazone and its oxime. The ketone contains, therefore, two rings and the side chain of the vitamin D, as evident from the number of hydrogen atoms present. The number of carbon atoms present reveals that the two rings contain only the original side chain and the

[245] A. Windaus and C. Roosen-Runge, *Z. physiol. Chem.*, **260**, 181 (1939).
[246] S. v. Reichel and M. Deppe, *Ibid.*, **239**, 143 (1936).
[247] H. Lettré, *Ann.*, **511**, 280 (1934).
[248] A. Guiteras, Z. Nakamiya and H. H. Inhoffen, *Ibid.*, **494**, 116 (1932).
[249] A. Windaus and W. Thiele, *Ibid.*, **521**, 160 (1935).

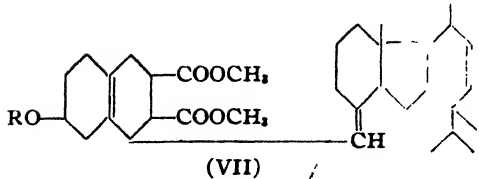
original angular methyl group between the rings C and D. The keto-group is therefore situated in what was originally ring C. In ergosterol, ring C is connected with ring B through carbon atoms 8 and 9. The



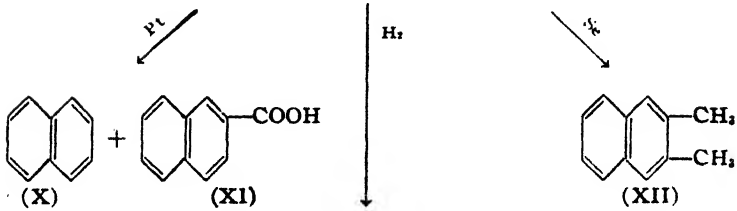
(I)
Vitamin D_2



(VI)

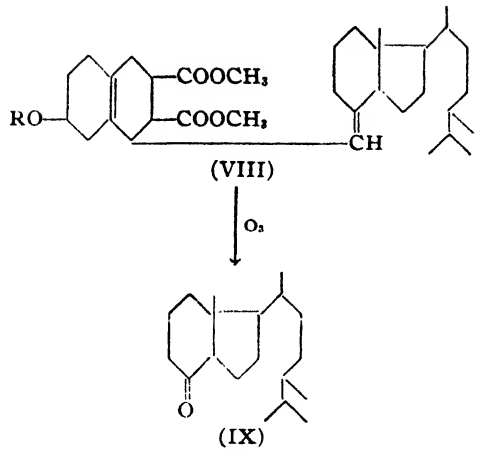


(VII)



(X) (XI) (XII)

(Formula continued on following page.)

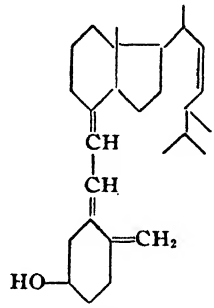


keto-group in the ozonolysis reaction product from the vitamin derivative indicates that in the vitamin molecule only one linkage exists between ring C and what was ring B in the ergosterol molecule. In addition, the formation of a ketone shows that ring C is connected with the rest of the molecule by a double bond. It follows that in vitamin D_2 ring B exists no longer, but has been opened. Ring cleavage has occurred between carbon atoms 9 and 10, for a keto-group at carbon atom 9 in the ozonolysis reaction product is impossible since a double bond cannot exist between carbon atoms 9 and 10 due to the fact that carbon atom 10 is quaternary in ergosterol. It is then concluded that one double bond in the vitamin D_2 molecule is in the 7,8-position. The constitution of rings C and D and of the side chain is thereby established.

The constitution of ring A and its connection with ring C through what was ring B in the ergosterol molecule has been elucidated as follows: The dicarboxylic acid (VII), obtained by maleic anhydride addition to vitamin D_2 followed by hydrolysis, yields upon a platinum dehydrogenation, naphthalene (X) and naphthoic acid (XI). Upon dehydrogenation of the diester of the dicarboxylic acid with selenium, 2,3-dimethyl-naphthalene (XII) is obtained. The methyl groups in the latter compound are derived from the carboxyl groups as can be shown by dehydrogenation of similar dicarboxylic acid esters.[250] The results of the dehydrogenation experiments must be interpreted as meaning that by addition of the maleic anhydride to the vitamin, a hydronaphthalene derivative is formed. This is possible only when the two additional unaccounted-for double bonds, which

[250] W. Thiele and G. Trautmann, *Ber.*, **68**, 2245 (1935).

are in conjugation with the double bond at the 7,8-position, are in the 5,6- and 10,18-positions. The addition of the maleic anhydride occurs then on carbon atoms 18 and 6. Therefore, the constitution of vitamin D_2 is that indicated in formula (I). The formula may also be written in the form (IA).

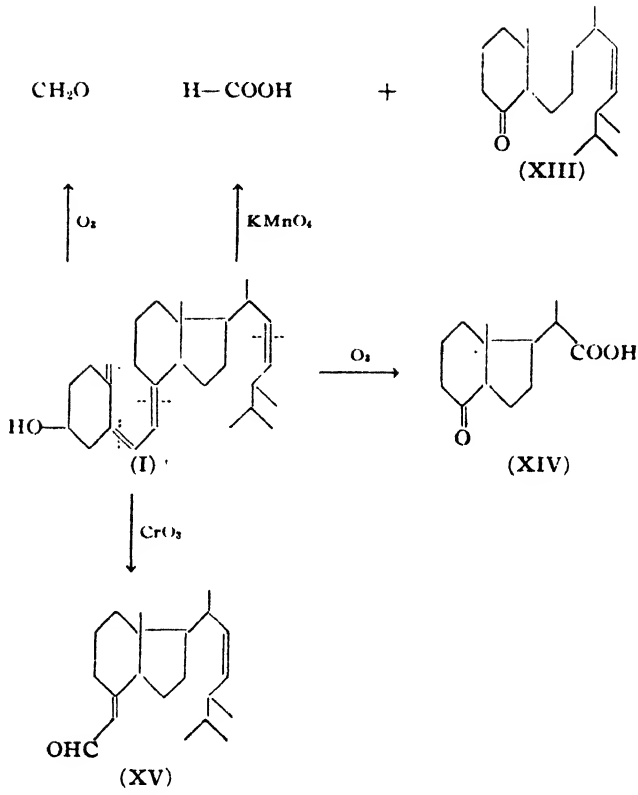


(IA)
Vitamin D_2

The structure of vitamin D_2 as deduced indicates the presence of a methylene group on carbon atom 10 instead of the methyl group present in ergosterol. This was, furthermore, proved by oxidation experiments: permanganate in acid solution yields formic acid, and ozone yields formaldehyde.[251]

These oxidation experiments give further evidence on the constitution of vitamin D_2. Permanganate oxidation yields, besides formic acid, the unsaturated ketone (XIII), which can be characterized as the semicarbazone and oxime. By selective hydrogenation of the double bond there is obtained the same saturated ketone (IX) that was found in the direct oxidation of a 22-dihydro-derivative as previously described. Oxidation of the vitamin D_2 with ozone yields, besides formaldehyde, the keto-acid (XIV), which is obtained by cleavage at the double bonds between the carbon atoms at the 7,8- and 22,23-positions. By careful oxidation of vitamin D_2 with chromic acid, rupture occurs at the double bond in the 5,6-position and the doubly unsaturated aldehyde (XV) can be isolated.[252] The constitution assigned to this aldehyde is in agreement with the result of its ultraviolet spectrum, which indicates the presence of an α,β-unsaturated oxo-group.

[251] A. Windaus and W. Grundmann, *Ann.*, **521**, 160 (1935).
[252] I. M. Heilbron, R. N. Jones, K. M. Samant and F. S. Spring, *J. Chem. Soc.*, **1936**, 905.

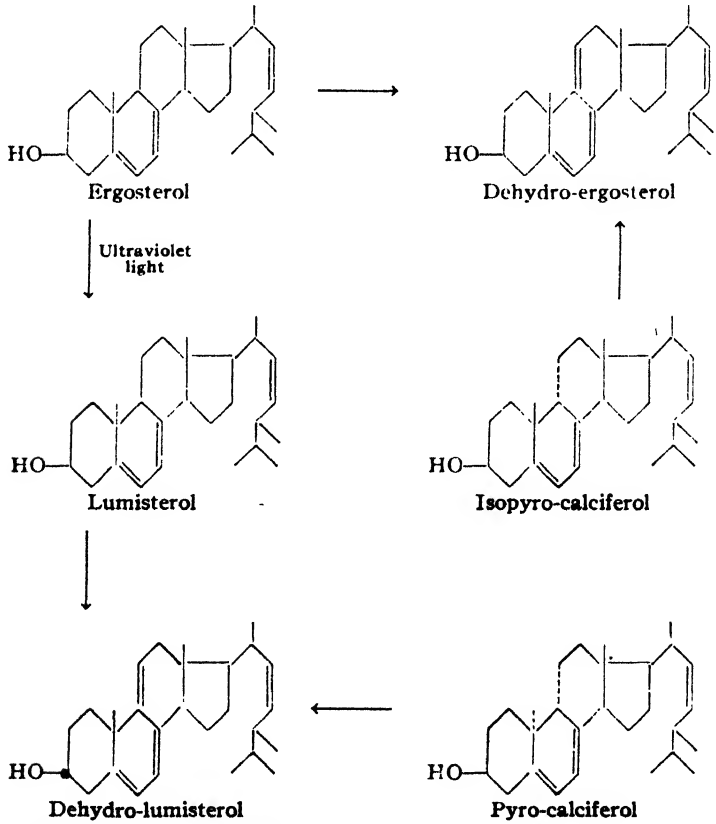


Upon heating vitamin D_2 (calciferol) in the absence of air at 160–190°, pyro-calciferol and isopyro-calciferol are obtained. These products are of interest in the determination of the structure of vitamin D_2 and also in the isomerisms involved in the photochemical activation of ergosterol and the destruction of the formed vitamin D. Pyro-calciferol and isopyro-calciferol crystallize as molecular addition products and can be separated after acetylation by fractional crystallization. Neither of the two pyro-compounds shows antirachitic activity.

Analysis and molecular weight determinations indicate that the two compounds are isomers of ergosterol and of vitamin D_2. According to the results obtained upon catalytic hydrogenation[253] and according to titrations with perbenzoic acid,[254] the pyro-compounds contain three double bonds. Hence, a ring-closure is involved in the formation of the pyro-compounds

[253] M. Müller, *Z. physiol. Chem.*, **233**, 223 (1935).
[254] P. Busse, *Ibid.*, **214**, 211 (1933).

from vitamins D. Upon dehydrogenation with selenium, γ-methyl-cyclo-penteno-phenanthrene is obtained,[253] showing that the basic sterol skeleton is present. This indicates that the ring-closure occurred between the same carbon atoms that were involved in the ring cleavage during the activation process of ergosterol. Of the three double bonds, one is inferred to be in the original 22,23-position. The other two are in conjugation with each other since both pyro- and isopyro-calciferol react with maleic anhydride to yield addition compounds. The double bonds are furthermore situated in the same ring, since upon nitric acid oxidation, toluene-2,3,4,5-tetracarboxylic acid is obtained.[255] The difference between pyro- and isopyro-calciferol is evident from the fact that isopyro-calciferol forms an addition compound with digitonin whereas pyro-calciferol does not. Furthermore, pyro-calciferol, but not the isopyro-



[255] H. H. Inhoffen, *Ann.*, **494**, 116 (1932).

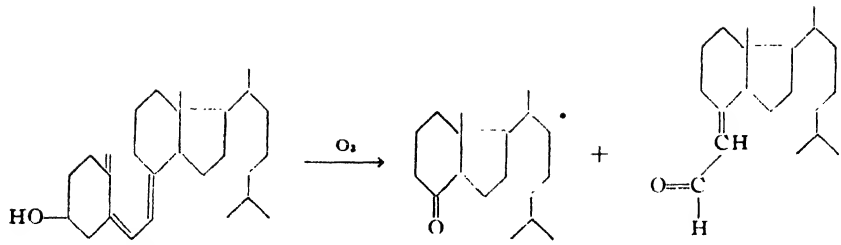
isomer, forms upon dehydrogenation with eosin in the presence of visible light a bimolecular compound,[256] as does ergosterol. Isopyro-calciferol has properties very similar to those of ergosterol in contrast to the other products in the irradiation series from ergosterol. Moreover, both ergosterol and isopyro-calciferol form upon dehydrogenation with mercuric acetate the identical compound, dehydro-ergosterol,[257] which differs from ergosterol in an additional double bond at the 9,11-position.[258] On the other hand, pyro-calciferol when subjected to the same treatment yields a dehydro-derivative which is identical with that obtained by dehydrogenation of lumisterol.[259] Thus it is evident that ergosterol and isopyro-calciferol differ only in the spatial arrangement at carbon atom 9, while lumisterol and pyro-calciferol differ only in the spatial arrangement at carbon atom 10. This series of reactions proves, furthermore, that the only change involved upon conversion of ergosterol into lumisterol is an epimerization of the substituents at carbon atom 10.

After the pyro-calciferols were shown to be stereoisomers of ergosterol and of lumisterol, it became interesting to study the changes which occur upon ultraviolet light irradiation of the pyro-compounds. The results[260] indicate that an entirely different reaction mechanism occurs. While the pyro-compounds are not stable to irradiation, no indication of the existence of intermediate products is obtained by following spectroscopically the changes occurring during the irradiation. No antirachitic substance is obtained. The end-products of the photochemical process are obtained in crystallized form, but these compounds show no absorption spectrum in the critical region between 240 and 310 mμ. On heating, the irradiation end-products are reconverted into the two pyro-compounds. This suggests that a ring cleavage similar to that obtained on irradiation of ergosterol does not occur upon irradiation of pyro-calciferol and of isopyro-calciferol. The changes involved appear to indicate only that a rearrangement of the two double bonds in ring B occurs so that they are no longer conjugated. Upon heating, the conjugation of the double bonds is restored.

(*c*) *The Constitution of Vitamin D_3*

The constitution of vitamin D_3, which can be derived from 7-dehydrocholesterol, has been inferred by analogy with the proved constitution of vitamin D_2 to be as follows (I):

[256] T. Kennedy and F. S. Spring, *J. Chem. Soc.*, 1939, 250.
[257] A. Windaus and K. Dimroth, *Ber.*, 70, 376 (1937).
[258] M. Müller, *Z. physiol. Chem.*, 231, 75 (1935).
[259] I. M. Heilbron, F. S. Spring and P. A. Stewart, *J. Chem. Soc.*, 1935, 1221.
[260] K. Dimroth, *Ber.*, 70, 1631 (1937).



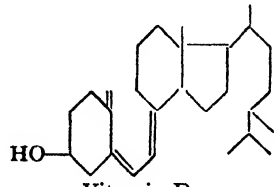
(I) Vitamin D_3 (II) (III)

Upon oxidation of vitamin D_3 with ozone formaldehyde,[261, 262] a saturated ketone (II) and an unsaturated aldehyde (III) are obtained. These have been characterized by semicarbazone and by 2,4-dinitro-phenyl-hydrazone formation. These oxo-compounds are formed by cleavage of the 10,18-, the 7,8- and the 5,6-double bonds, exactly as ozonolysis of vitamin D_2 splits these double linkages. The formula of vitamin D_3 is thereby indicated.

Vitamin D_3 upon pyrolysis yields[263] pyro-vitamin D_3 and isopyro-vitamin D_3 in strict analogy with the previously discussed formation of the pyro-compounds from vitamin D_2. The D_3 compounds yield, as do the D_2 compounds, upon dehydrogenation with mercuric acetate two dehydro-derivatives which are identical with those obtained from lumisterol$_3$ and from 7-dehydro-cholesterol, correspondingly.

(*d*) *The Constitution of Vitamin* D_4

Vitamin D_4 is obtained by activation of 22-dihydro-ergosterol. In view of the structure of the vitamins D_2 and D_3, the constitution of vitamin D_4 is believed to be the following:[264]

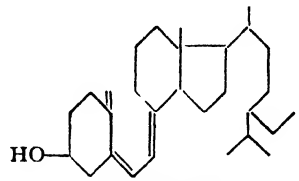


Vitamin D_4

[261] A. Windaus, M. Deppe and W. Wunderlich, *Ann.*, **533**, 118 (1937).
[262] H. Brockmann and A. Busse, *Z. physiol. Chem.*, **256**, 252 (1938).
[263] A. Windaus, M. Deppe and C. Roosen-Runge, *Ann.*, **537**, 1 (1938).
[264] A. Windaus and G. Trautmann, *Z. physiol. Chem.*, **247**, 185 (1937).

(e) *The Constitution of Vitamin D_5*

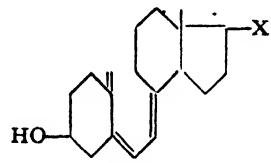
Vitamin D_5 is antirachitically activated 7-dehydro-sitosterol and should, therefore, have the following constitution:



Vitamin D_5

(f) *The Constitution of Other Vitamins D*

On the basis of the known constitutions of the vitamins D_2 and D_3, the general formula of any vitamin D is believed to be represented by (I), wherein X stands for the side chain, the constitution of which varies with each member of the vitamin D group:



(I)

It has been previously stated that not all of the members of the naturally occurring group of vitamins D have been isolated and therefore their chemical constitution is still obscure.

A new vitamin D has been isolated from cod liver oil (vitamin $D_{6\,(?)}$), the chemical constitution of which is not known. Since it has a boiling point which is considerably lower than that of the rest of the vitamins D occurring in cod liver oil, it has been suggested that it may not have a side chain at all or only a very small one.[265] It is, of course, possible that this vitamin differs from the other known ones in stereochemical arrangement at one or more of the asymmetric carbon atoms. The establishment of the structure of this new vitamin must await its isolation in crystallized form.

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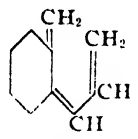
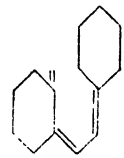
[265] C. E. Bills, O. N. Massengale, K. C. D. Hickman and E. L. Gray, *J. Biol. Chem.*, 126, 241 (1938).

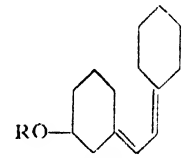
18. Synthesis

Aside from the previously discussed partial syntheses of vitamins D by activation from provitamins, no vitamin D has been obtained synthetically. The total synthesis of one of the vitamins D would be of considerable theoretical interest, mainly in order to study the exact stereochemical relations of the asymmetrical carbon atoms. Attempts have been made to synthesize part of the molecule, that is, the system of three conjugated double bonds with the attached cyclo-hexane rings. The following com-



(I)


(II)



(III)

pounds, I,[266] II[267] and III,[268] have been obtained by total synthesis. The antirachitic efficacy of these compounds has not been investigated.

19. Industrial Methods of Preparation

There are a number of different vitamin D preparations on the market. Some of these originate from fish liver oils which have been processed according to the methods described for the preparation of vitamin A from fish liver oils. Percomorph liver oil, cod liver oil and halibut liver oil are offered for human consumption, while the oils of other fish are used for animal food.

Besides the sale of the natural product, vitamin D prepared by activation of provitamins D is also marketed. Many of the commercial products are combinations of several forms of vitamin D. The activation is carried out according to the various methods described, but especially by ultraviolet irradiation and by bombardment with low velocity electrons. Quite a number of modifications of these two processes have been patented and are actually employed. The provitamins which are used commercially have been discussed on page 365.

Commercially important are the food products which are fortified with vitamin D, such as bread (containing 460 International Units to the 24-

[266] N. A. Milas and W. L. Alderson, *J. Am. Chem. Soc.*, **61**, 2534 (1939).
[267] K. Dimroth, *Ber.*, 71, 1333, 1346 (1936). K. Dimroth and H. Johnson, *Ibid.*, **71**, 2658 (1938).
[268] J. B. Aldersley and G. N. Burkhardt, *J. Chem. Soc.*, **1938**, 545.

ounce loaf), breakfast cereals, margarine, etc. Besides the actual addition of vitamin D, some foodstuffs are themselves irradiated, thus gaining antirachitic efficacy.

One of the most suitable carriers for vitamin D, as far as human nutrition is concerned, is milk. Milk can be fortified in its natural vitamin D content by the following methods:

1. A vitamin D concentrate in oil is added to yield milk of a potency of at least 400 International Units per quart.[269]

2. A solution of vitamin D in a suitable solvent, such as propylene glycol, is added.

3. Cows, upon irradiation, for example, with the sun or with artificial ultraviolet light, secrete a milk of increased vitamin D potency,[270] that has up to 40–50 International Units per quart.

4. Cows are fed with vitamin D, usually with irradiated yeast. The milk secreted by such cows contains "metabolized" vitamin D and is standardized to contain not less than 400 International Units per quart.

5. The milk itself is irradiated, thereby converting the provitamin D content into vitamin D. This procedure, which is one of the cheapest, is also one of the most difficult since an unpleasant taste and odor are easily produced by ultraviolet irradiation.[271] Quite a number of process modifications are being used and have been patented.[272] The irradiated milk is standardized at 135 International Units per quart, but up to 200 International Units can sometimes be produced. Irradiated milk is therefore of obviously low potency.

For clinical use vitamin D is marketed in oil solution or in solution with a suitable organic solvent, such as propylene glycol, ethers of polyhydroxy-alcohols, for example, glycerol-diethyl-ether and dimethoxy-trioxy-hexane, oxalkyl-ether and alkoxyethyl-ether of polyhydroxy-compounds and fatty acid amides, for example, ethyl-acetamide. Colloidal solutions of vitamin D in water can be prepared that are sterilized by the application of heat.

For general use by the public, vitamin D is available also in the form of emulsions, tablets, capsules, tonics and malt preparations.

20. Biogenesis

The biogenesis of the large amounts of *vitamin D found in fish* is a much discussed problem. It seems impossible that these great quantities

[269] T. F. Zucker, *Am. J. Pub. Health*, 13, 10 (1923).

[270] J. E. Campion, K. M. Henry, S. K. Kon and J. Mackintosh, *Biochem. J.*, 31, 81 (1937). H. E. Bechtel and C. A. Hoppert, *J. Nutrition*, 11, 537 (1936).

[271] K. G. Weckel and H. C. Jackson, *Food Research*, 1, 419 (1936).

[272] See the review by W. Diemair, *Chem. Fabrik*, 14, 51 (1941).

originate from provitamins in the skin of fish, since not enough ultraviolet radiation is available for their conversion. Furthermore, as has been proved for the catfish at least,[273] fish are very sensitive to ultraviolet irradiation, and the amount of vitamin D produced under the influence of light is negligible. Since, however, ultraviolet light penetrates both fresh water and sea water for about three feet,[274] the possibility has often been considered that vitamin D is produced in the plankton and in algae which in turn constitute the normal food supply of fish.[275] Actually small amounts of vitamin D have occasionally been found in marine microorganisms.[276, 277] It seems questionable, however, that this is a logical explanation of the presence of vitamin D in fish oil. The type of vitamin D in microorganisms is, at least as far as is known, not the type of vitamin D found in fish, since the vitamin D from microorganisms is relatively inactive when fed to chickens, whereas the vitamin D from fish oil is generally highly active. If, then, the vitamin D present in fish actually originates from the vitamin D in microorganisms, the hypothesis must be accepted that the chemical structure of the vitamin is altered in the animal organism, which is very unlikely.

Another explanation offered is that the fish organism may contain an enzyme system which takes care of the conversion of provitamin D to vitamin D. Whereas it is certain that no such enzyme system exists in humans or in chickens, the actual presence or absence of such an enzyme system in fish has not been demonstrated.

Instead of the existence of an enzyme system, the so-called mitogenetic radiation (Gurwitsch-rays) has been postulated to cause the transition of provitamins D to vitamins D.[278] It has been claimed that energy metabolism in the growing cell causes the production of ultraviolet rays of about 190–250 mμ which play a special role in the growing tissue. These rays may cause the production of vitamin D in fish and in man. Since only preliminary results have been published, it seems too early to pass any judgment on this subject. Some investigators deny the existence of the mitogenetic radiation.[279, 280]

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[273] C. E. Bills, *J. Biol. Chem.*, **72**, 751 (1927).

[274] H. H. Darby and H. T. Clarke, *Science*, **85**, 318 (1937)

[275] H. Steenbock and A. Black, *J. Biol. Chem.*, **64**, 263 (1925).

[276] H. H. Darby and H. T. Clarke, *Science*, **85**, 318 (1937).

[277] A. M. Copping, *Biochem. Z.*, **28**, 1516 (1934). J. C. Drummond and E. R. Gunther, *J. Exptl. Biol.*, **11**, 203 (1934).

[278] H. Mai, *Abhandl. aus der Kinderheilkunde u. ihren Grenzgeb.*, 1937, H. 45.

[279] K. H. Kreuchen, *Angew. Chem.*, **47**, 185 (1934). E. N. Harvey, *Naturwissenschaften*, **12**, 165 (1924).

[280] J. Levine and A. H. Steinhaus, *Proc. Am. Physiol. Soc.*, **1941**, 173.

Finally, the theory of a total vitamin D synthesis in fish has been brought forward. While this theory appears plausible and possible, the experimental evidences[281] offered to prove this thesis are not conclusive.

The *origin of vitamin D in higher animals* is an entirely different problem. Higher animals are not able to synthesize vitamin D, at least not in sufficient quantities, as is evident by the incidence of rickets. The previously discussed mitogenetic radiation may produce extremely small amounts of this vitamin from provitamin present in the tissue. If this is the case, then the susceptibility for rickets sometimes observed in infants may be explained[282, 283] on the basis of a failure to produce enough radiation or on the basis of an absence of provitamins.

There is no question but that normally a considerable amount of vitamin D is gained by exposure of the animal and human body to the sun. It is assumed that the vitamin D thus formed is absorbed into the blood stream. However, the mechanism of this reaction is not clear. The skin of various organisms—of humans,[284, 285] of cattle,[286] of pigs,[287] etc.—has been shown experimentally to contain provitamin D in an amount which is at least 10 and probably 100 times greater than the amount found in the inner parts of the body. Attempts have repeatedly been made to determine how deep ultraviolet radiation from the sun is able to penetrate through the epidermis, but no conclusive data have been obtained. While most workers believe that ultraviolet rays penetrate human skin only slightly, that is, 0.1 mm.,[288] it has been argued that the depth of penetrations depends on viability and may go through living tissue for 1.2 mm.[289] It has also been stated that provitamin D may be secreted by the sebaceous glands and that the vitamin D after its formation is absorbed by the skin. That the human skin is able to absorb vitamin D has repeatedly been shown[290] but it is unlikely that the sebaceous glands excrete provitamin D. It has, however, been reported[291] that thorough washing of human skin and subsequent irradiation failed to provide enough activation to prevent serious deficiency. This observation requires confirmation, however.

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[281] C. E. Bills, *J. Biol. Chem.*, **72**, 751 (1927).
[282] H. Mai, *Abhandl. aus der Kinderheilkunde u. ihren Grenzgeb.*, 1937, H. 45.
[283] E. Glanzmann, *Ergeb. Vitamin Hormonforsch.*, **1**, 8 (1938).
[284] A. F. Hess and M. Weinstock, *J. Biol. Chem.*, **64**, 181 (1925).
[285] H. Hentschel and L. Schindel, *Klin. Wochschr.*, **9**, 262 (1930).
[286] A. F. Hess and M. Weinstock, *J. Biol. Chem.*, **64**, 181 (1925).
[287] A. Windaus and F. Bock, *Z. physiol. Chem.*, **245**, 168 (1936).
[288] J. Clark, *Physiol. Rev.*, **2**, 277 (1922).
[289] W. T. Anderson and D. I. Macht, *Am. J. Physiol.*, **86**, 320 (1928).
[290] M. E. Foder, *Z. Vitaminforsch.*, **3**, 241 (1934). A. C. Helmer and C. Jansen, *Studies Inst. Divi. Thomae*, **1**, 83, 99 (1937).
[291] A. C. Helmer and C. Jansen, *Studies Inst. Divi. Thomae*, **1**, 207 (1937).

In birds, the production of vitamin D by sunlight is apparently still more complicated. It has been suggested[292, 293] that the preen gland excretes a provitamin D-containing oil which is distributed over the feathers and after exposure to sunlight is either ingested or absorbed through the skin. This could, however, not be proved experimentally.[294] Furthermore, no provitamin D could be detected spectroscopically in the sterol fraction from preen glands.[295] It has furthermore been shown that irradiation of the feet of chickens without preen glands cured rickets,[296] and the presence of provitamin D in the feet has been proved.[295]

The site of vitamin D formation in fur-bearing animals is also largely unknown. It has been claimed[297] that rats which are prevented from licking their fur develop rickets. On the other hand, the skin of rabbits was shown to be antirachitic, the dorsal skin more so than the ventral,[298] but only in normal animals, not in those suffering from rickets.

The formation of vitamin D in the organism is subject to seasonal variation due to the seasonal change of sunlight. Thus the incidence of rickets in the northern hemisphere is the greatest from January to March. Similar seasonal changes can also be demonstrated in the vitamin D content of milk[299] and of eggs,[300] which contain maximum amounts during the summer months and minimum amounts during the late winter months.

21. Specificity

In discussing the specificity of vitamins D a differentiation is made between "compound specificity" and "species specificity." Compound specificity is the response to various forms of vitamin D by one given species. Species specificity is the efficacy of one given form of vitamin D for various species.

The basis for all statements regarding specificity or efficacy of vitamin D is the rat test. Each form of vitamin D has a certain number of rat units per gram of the pure product. These rat units are expressed in International Units or in U. S. Pharmacopoeia Units which are based on the efficacy of vitamin D_2. This method of referring to the International

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[292] H. C. Hou, *Chinese J. Physiol.*, 2, 345 (1928); 3, 171 (1929); 4, 79 (1930); 5, 11 (1931).
[293] W. Rowan, *Nature*, 121, 323 (1928).
[294] H. R. Knowles, E. B. Hart and J. G. Halpin, *Poultry Sci.*, 14, 33 (1935).
[295] H. R. Rosenberg, unpublished experiments.
[296] H. C. Hou, *Chinese J. Physiol.*, 2, 345 (1928); 3, 171 (1929); 4, 79 (1930); 5, 11 (1931).
[297] E. Reckling, *Strahlentherapie*, 25, 568 (1927).
[298] H. C. Hou and E. Tso, *Chinese J. Physiol.*, 4, 93 (1930).
[299] J. E. Campion, K. M. Henry, S. K. Kon and J. Mackintosh, *Biochem. J.*, 31, 81 (1937). H. E. Bechtel and C. A. Hoppert, *J. Nutrition*, 11, 537 (1936).
[300] G. M. DeVaney, H. E. Munsell and H. W. Titus, *Poultry Sci.*, 12, 215 (1933).

Unit of vitamin D is, however, only justified in a discussion regarding the "compound specificity." For the determination of the "species specificity" the International Units proved to be inadequate, since vitamin D_2 has a very low efficacy on chicks. There appears to exist a very definite species specificity at least for the organisms which have been investigated. Thus, one rat unit is not necessarily one chick unit or turkey unit or man unit. The difference between rat units and man units appears to be only slight. The difference between rat units and chick units is, however, very significant. In the following table the compound and species specificity are summarized for the vitamins which have been investigated.

TABLE VI

COMPOUND AND SPECIES SPECIFICITY OF KNOWN VITAMINS D

Provitamin D	Vitamin D	Rat efficacy of pure vitamin D per gram in Standard Units (1 S. U. = 1 million I. U.)	Chick efficacy in % of rat activity
Ergosterol	Vitamin D_2	40 S. U.	1–3
Epi-ergosterol	.....	Unknown, but active	?
7-Dehydro-cholesterol	Vitamin D_3	40 S. U.	100
Epi-7-dehydro-cholesterol	.....	4 S. U.	?
22-Dihydro-ergosterol	Vitamin D_4	20–30 S. U.	20[301]
7-Dehydro-sitosterol	Vitamin D_5	1.3 S. U.	Less than 100[302]
?	Vitamin D_6(?)	?	20–50
7-Dehydro-stigmasterol	.....	0.1 S. U.	?
22,23-Oxido-ergosterol	.....	Only "feebly" active[303]	?
Mussel provitamin D	.....	40 S. U.[304]	100[304, 305]
$\Delta^{5,7}$-Androsta-diene-diol-3,17	.....	0.1 S. U.[306]	.....
3-Hydroxy-$\Delta^{5,7}$-choladienic acid	.....	0.1 S. U.[307]	.....
.....	Dihydro-tachysterol	0.2 S. U.[308]	.....

[301] F. G. McDonald, *J. Biol. Chem.*, **114**, LXV (1936).
[302] W. Grab, *Z. physiol. Chem.*, **243**, 63 (1936).
[303] A. Windaus, Linsal and K. Buchholz, quoted by K. Dimroth and J. Paland, *Ber.*, **72**, 187 (1939).
[304] A. G. Boer, J. van Niekerk, E. H. Keerink and A. van Wijk, U. S. P. 2,163,659.
[305] J. van Niekerk and F. Franken, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **8**, 13 (1938).
[306] A. Butenandt, E. Hausmann, J. Paland, D. von Dresler and U. Meinerts, *Ber.*, **71**, 1316 (1938). K. Dimroth and J. Paland, *Ibid.*, **72**, 187 (1939).
[307] G. A. D. Haslewood, *Biochem. J.*, **33**, 454 (1939); *J. Chem. Soc.*, **1938**, 224.
[308] F. v. Werder, *Z. physiol. Chem.*, **260**, 119 (1939).

Compound Specificity

The results of the compound specificity tests need further interpretation. It will be recalled that none of the activation intermediates is antirachitically effective at least in the amounts investigated. Thus, in order to obtain vitamin D activity ring B of the molecule must be opened between C_9 and C_{10} and the double bonds must be in the correct positions. The latter is evident from the inactivity of tachysterol$_2$. The most revealing result is the relatively great specificity of rats toward small differences in the vitamin D molecule. Whereas vitamins D_2 and D_3 are equally active in rats, hydrogenation of the side chain double bond of vitamin D_2, which results in the formation of vitamin D_4, decreases the activity considerably. On the other hand, activated 7-dehydro-stigmasterol, which differs from activated ergosterol by one additional methyl group in the side chain, is practically devoid of activity. Activated 7-dehydro-sitosterol, which differs from activated 7-dehydro-stigmasterol only in having a saturated side chain and which differs, therefore, from activated 22-dihydro-ergosterol in one additional methyl group in the side chain, is somewhat more active. 22,23-Oxido-ergosterol shows only feeble activity, while the total absence of the side chain as in activated $\Delta^{5,7}$-androsta-diene-diol-3,17[309] or the presence of a four-membered, carboxyl group-containing side chain as in the bile acid analog of vitamins D (activated 3-hydroxy-$\Delta^{5,7}$-choladienic acid)[310] makes the compounds practically inactive. It is thus evident that considerable compound specificity rests in the structure of the side chain. An epimerization of the hydroxyl group in vitamin D causes a considerable decrease in efficacy, although the activity is not entirely lost ($^1/_{10}$ in the case of activated epi-7-dehydro-cholesterol). Vitamins D are furthermore only active when the hydroxyl group is free Esters and ethers of vitamins D are inactive.[311] Those esters which can be hydrolyzed in the organism are active.

It has repeatedly been observed that vitamin D given in milk exerts a greater antirachitic response than when the same amount of vitamin D is given in oil solution. While it is now believed[312] that this effect is brought about by an optimum simultaneous intake of phosphorus and calcium as present in milk, it was stated that a synergistic factor may

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[309] A. Butenandt, E. Hausmann, J. Paland, D. von Dresler and U. Meinerts, *Ber.*, **71**, 1316 (1938). K. Dimroth and J. Paland, *Ibid.*, **72**, 187 (1939).

[310] G. A. D. Haslewood, *Biochem. J.*, **33**, 454 (1939); *J. Chem. Soc.*, **1938**, 224.

[311] A. Windaus and O. Rygh, *Nachr. Ges. Wiss. Göttingen, Math. physik. Klasse*, **III**, 202 (1928).

[312] B .O'Brien and K. Morgareidge, *J. Nutrition*, **16**, 91, 395 (1938); *J. Biol. Chem.*, **128**, LXXV (1939).

be involved. A symplex formed from vitamin D and lactalbumin was held responsible for the alleged enhanced antirachitic activity.[313]

A few compounds, which evidently have not the basic structure of the vitamins D, have also been found to exhibit antirachitic activity. Thus, in rats, the addition of iodine or iodides to the rachitogenic diet prevents or cures rickets.[314] Certain organic acids, especially tartaric and citric acid,[315] are able to prevent or cure rickets in rats, but not in chicks.[316] These effects are closely related to the acid-base properties of the diet and its influence upon the development of rickets (see page 425). It has been repeatedly shown that upon treatment of cholesterol with various chemicals, a compound or compounds are obtained which (without further activation) exhibit slight but definite antirachitic activity when tested on rats and which are reported to show a chicken effectiveness of over 100%. The chemistry of these products is entirely unknown. The activation is confined to sterols, is somewhat specific to the constitution of the sterol employed[317] and does not materially activate the provitamins D. The reaction is brought about by fuller's earth[318] and by sulfuric acid.[319] The active compound was believed to be a monosulfonation product of cholesterol or cholesterilene. However cholesterilene-sulfonic acid was subsequently found to be inactive.[320] Active products were obtained by the treatment of sterols with $KHSO_4$, $CuSO_4$, $ZnCl_2$, $AlCl_3$, P_2O_5, trichloro-acetic acid, phosphoric acid, etc.[321]

Species Specificity

The *chick* is considerably more sensitive than the rat, as is evident from Table VI. Only a few vitamins D have been tested on chicks. Vitamin D_3 and the vitamin derived from "mussel provitamin"[322] are highly active, while vitamin D_2 is only slightly active. The vitamins D_4 and $D_{6\,(?)}$ also have a definite, but slight chicken activity. As has been pointed out before

[313] G. C. Supplee, S. Ansbacher, R. C. Bender and G. E. Flanigan, *J. Biol. Chem.*, **114**, 95 (1936).

[314] R. Lecoq, *Compt. rend.*, **204**, 1891 (1937). R. Lecoq and R. Duffau, *Compt. rend. soc. biol.*, **128**, 619 (1938).

[315] B. Hamilton and C. Schwartz, *Am. J. Diseases Children*, **46**, 669 (1933). B. Hamilton and M. M. Dewar, *Ibid.*, **54**, 548 (1937). A. T. Shohl, *J. Nutrition*, **14**, 69 (1937).

[316] J. T. Correll, *J. Nutrition*, **21**, 515 (1941).

[317] J. C. Eck and B. H. Thomas, *J. Biol. Chem.*, **128**, 257 (1939).

[318] C. E. Bills and F. G. McDonald, *Ibid.*, **68**, 821 (1926). S. K. Kon, F. Daniels and H. Steenbock, *J. Am. Chem. Soc.*, **50**, 2573 (1928).

[319] L. Yoder, *Science*, **80**, 385 (1934). L. Yoder, B. H. Thomas and M. Lyons, *J. Nutrition*, **9**, Suppl. 6 (1935). L. Yoder, *J. Biol. Chem.*, **116**, 71 (1936). J. C. Eck, B. H. Thomas and L. Yoder, *Ibid.*, **117**, 655 (1937). J. C. Eck and B. H. Thomas, *Ibid.*, **119**, 621 (1937).

[320] A. Windaus and E. Kuhr, *Ann.*, **532**, 52 (1937).

[321] J. C. Eck and B. H. Thomas, *J. Biol. Chem.*, **119**, 631 (1937); **128**, 267 (1939).

[322] J. van Niekerk and F. Franken, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **8**, 13 (1938).

in the general discussion on vitamins D (page 384), the "chicken activity" is a relative figure indicating the efficiency of a given vitamin D when fed on the basis of rat units and compared with the same number of rat units of a "U. S. Reference Cod Liver Oil," the chicken activity of which has arbitrarily been chosen to indicate 100% activity. In this connection it is also interesting to compare the chicken activity of the vitamin D in various fish liver oils with the cod liver oil (C. L. O.) as is done in Table VII. The probable experimental error (PE) involved in the determinations is also given.

From Table VII it is evident that the natural fish vitamin D is a mixture of vitamins D and the conclusion has been drawn that at least one more vitamin D exists than is known today, since chicken activities as high as 200% and 300% have been observed.

The *turkey* appears to have a species specificity which differs from that of chicks. Although the difference between the chicken and turkey activity is considerably less than the difference between chicken and rat activity, it must be concluded that a chick unit of vitamin D is not necessarily identical with a turkey unit of vitamin D.[323, 324] The term "turkey unit" is used in analogy with the term "chick unit" and means the biological response of one International Unit of vitamin D from U. S. Reference Cod Liver Oil.

The efficiency with which *man* utilizes various forms of vitamin D is much more difficult to establish. There does not seem to be any question but that man can utilize vitamin D from fish liver oil, vitamin D_2 and vitamin D_3 when administered *per os*. None of the other forms of vitamin D has been investigated. On the basis of the available experimental data it is believed that when given by mouth vitamin D_3 is more active, perhaps 1.5 times, than vitamin D_2.[325] This is especially well demonstrated with babies in the shock therapy,[326, 327, 328] and has also been repeatedly observed by feeding infants daily amounts of vitamin D prophylactically[329, 330, 331] and curatively,[332] although some investigators believe

[323] T. H. Jukes and T. D. Sanford, *J. Nutrition*, 18, 71 (1939).

[324] H. M. Scott, J. S. Hughes and H. W. Loy, *Poultry Sci.*, 11, 177 (1932). F. D. Baird and D. J. Greene, *Ibid.*, 14, 70 (1935).

[325] P. C. Jeans, *J. Am. Med. Assoc.*, 106, 2066, 2150 (1936).

[326] E. Graser, *Z. Kinderheilk.*, 61, 716 (1940).

[327] H. J. Hartenstein, *Monatsschr. Kinderheilk.*, 76, Nos. 3 and 4 (1937).

[328] G. O. Harnapp, *Klin. Wochschr.*, 17, 390 (1938).

[329] A. F. Hess, J. M. Lewis and H. Rivkin, *J. Am. Med. Assoc.*, 94, 1885 (1930). A. F. Hess and J. M. Lewis, *Ibid.*, 99, 647 (1932); 101, 181 (1933).

[330] H. Brockmann, *Klin. Wochschr.*, 16, 1383 (1937).

[331] T. G. H. Drake, F. F. Tisdall and A. Brown, *J. Pediatrics*, 9, 421 (1936).

[332] J. M. Lewis, *J. Pediatrics*, 10, 155 (1937). J. S. Hood and I. Ravitch, *Ibid.*, 11, 521 (1937).

Table VII

Relative Effectiveness of Vitamin D from Different Sources for Rats and Chickens

Oil. No.	Name of fish (or sterol)	Vitamins given per 100 gm. diet			Response obtained			Efficacy ratio	
		A	D		Femur ash, %	I. U. of D/100 gm.	PE, %	C. L. O. = 100	PE, %
		I. U.	I. U.	PE, %					
1	Halibut	510	17.5	±7	45.42	15.1	+9; −9	86	+11; −11
2	Round-nosed sole	1900	18.9	6	45.57	15.4	9; 9	81	11; 11
3	Tuna, bluefin (Cal.)	13	7.2	7	35.98	..[a]	....[a]	..[a]	.....[a]
3	Tuna, bluefin (Cal.)	110	57.6	7	42.55	9.3	8; 8	16	11; 1
3-*x*	Tuna, bluefin (Cal.)	41	42.4	7	41.03	7.4	9; 8	17	11; 11
3-*x*	Tuna, bluefin (Cal.)	81	84.9	7	45.92	16.5	8; 8	19	11; 11
7-*a*	Tuna, New England	39	7.9	7	39.99	6.4	10; 10	81	12; 12
7-*a*	Tuna, New England	46	9.2	7	41.45	8.0	9; 9	87	11; 11
12	Albacore	9	20.5	5	44.42	12.6	9; 9	61	10; 10
12	Albacore	27	61.4	5	46.80	19.7	10; 9	32	11; 10
13	Tuna, yellowfin	40	10.7	7	45.23	14.6	9; 9	136	11; 11
14	Tuna, striped	7	9.6	5	35.62	..[a]	....[a]	..[a]	.....[a]
14	Tuna, striped	43	56.5	5	45.12	14.3	9; 9	25	10; 10
15	California bonito	20	8.4	6	34.31	..[a]	....[a]	..[a]	.....[a]
15	California bonito	140	58.0	6	45.93	16.5	10; 9	28	12; 11
16	California mackerel	630	9.8	7	44.01	11.8	10; 9	120	12; 11
17	Swordfish	180	10.1	6	45.53	15.3	9; 9	151	11; 11
18	Black sea bass	930	7.8	7	41.98	8.5	9; 8	109	11; 11
19	Cabrilla	8600	11.0	7	40 83	7.2	9; 9	65	11; 11
20	White sea bass	25	2.8	7	42.19	8.8	9; 8	314	11; 11
20	White sea bass	55	6.0	7	45 74	15.9	9; 9	265	11; 11
21	Totuava	470	10.0	7	34.90	..[a]	....[a]	..[a]	.....[a]
21	Totuava	2800	59.8	7	44.47	12.7	9; 9	21	11; 11
22	Sablefish	1600	6.8	7	43.59	11.0	10; 9	162	12; 11
22	Sablefish	2300	9.8	7	45.64	15.6	9; 9	159	11; 11
23-a	"Lingcod"	3000	15.8	6	45.66	15.7	9; 9	99	11; 11
24	Bocaccio	330	8.7	6	44.46	12.7	9; 9	146	11; 11
25	Chili-pepper	4200	7.2	7	40.01	6.4	10; 10	89	12; 12
26	Wolffish	680	9.9	7	43 08	10.1	10; 9	102	12; 11
27	Basking shark	Nil	5.2	7	41.94	8.4	9; 8	162	11; 11
28	Dogfish	5800	4.0	7	42.50	9.2	9; 9	230	11; 11
29	Pollack	210	8.5	7	38.12	4.3	13; 13	51	15; 15
30	Hake	160	8.7	6	43.91	11.6	10; 9	133	12; 11
31	Sardine	270	10.7	7	43.96	11.7	10; 9	109	12; 11
Control	Cod	310	10.0	2	42 97	9.9	10; 9	99	10; 9
Control	Cod	250	8.0	2	42.08	8.6	9; 8	108	9; 8
Control	Cod	250	8.0		40 89	7.3	9; 9	91	9; 9
Control	Cod	250	8 0	2	41.53	8.0	9; 9	100	9; 9
Control	Cod	130	4.0	2	38.81	5.1	11; 11	128	11; 11
Control	Maize oil	0	0.0	0	35.12	0.0	....[a]	..[a]	.....[a]
Sterol	Irr. ergosterol	0	200.0	...	40.00	6.4	....	3.2	.....
Sterol	Irr. ergosterol	0	403.0	...	43.30	10.4	....	2.6	.....
Sterol	Irr. ergosterol	0	1800.0	...	46.50	18.5	....	1.0	.....
Sterol	Irr. cholesterol (ordinary, from spinal cord)	0	8.0	7	41.72	8.2	9; 8	103	11; 11
Sterol	Irr. 7-dehydro-cholesterol	0	13.0	6	43.93	11.7	10; 9	90	12; 11

[a] Response too low for significant interpretation.

From: C. E. Bills, O. N. Massengale, M. Imboden and H. Hall, *J. Nutrition*, **13**, 442 (1937).

that vitamin D_2 may be equally effective.[333, 334] There are also observations to the effect that in the treatment of infantile rickets vitamin D_2 is not effective when administered intramuscularly, whereas vitamin D_3 is effective when applied in this manner.[335]

22. Determination

(*a*) *Physical Methods*

There is only one physical method which has been recommended for the determination of vitamins D, namely, the *measurement of the characteristic absorption spectrum* in the ultraviolet.[336] The vitamins D have a maximum at 265 mμ. This method is, of course, accurate only in the absence of compounds which have a similar absorption spectrum and, therefore, cannot be applied for the determination of vitamins D in fish oils or in crude irradiation products of provitamins. In the case of almost pure, crystalline vitamins D the spectroscopical method can be used. To determine vitamins D in fish oils, it has been recommended[337] to first saponify the material and to isolate the unsaponifiable part, then to separate the vitamins D from inactive sterols and from the vitamin A present by selective adsorption on aluminum oxide. The vitamins D present in the residual material is then determined spectroscopically. While this method may give approximate values, the accuracy is not too great due to the losses which are unavoidable during the processing.

(*b*) *Chemical Methods*

There is no chemical method by which vitamins D can be determined accurately or at least as accurately as by biological methods. A number of color reactions for vitamins D have been proposed and have been used advantageously from time to time:

1. **Aluminum-Chloride Color Reaction.**[338] Vitamins D in mixture with pyrogallol in benzene solution develop a deep violet color upon heating with aluminum chloride in alcohol. This method is applicable to solutions of pure vitamin D. Tachysterol and suprasterol I also give this reaction,

[333] M. M. Eliot, E. M. Nelson, D. J. Barnes, F. A. Browne and R. M. Jenss, *J. Pediatrics*, **9**, 357 (1936).

[334] N. Morris and M. M. Stevenson, *Lancet*, **237**, 876 (1939).

[335] A. Nitschke, *Z. Kinderheilk.*, **61**, 385 (1940).

[336] E. H. Reerink and A. van Wijk, *Chem. Weekblad*, **1932**, 645. H. Töpelmann and W. Schuhknecht, *Z. Vitaminforsch.*, **4**, 11 (1935).

[337] E. Marcussen, *Dansk. Tids. Farm.*, **13**, 141 (1939).

[338] W. Halden, *Naturwissenschaften*, **24**, 296 (1936). W. Halden and H. Tzoni, *Nature*, **137**, 909 (1936). H. Tzoni, *Biochem. Z.*, **287**, 18 (1936).

but the color developed is somewhat weaker. Vitamins D in oil solution cannot be determined by this method.

2. **Antimony-Trichloride Color Reaction.**[339] Vitamins D give with a saturated solution of antimony trichloride in chloroform a yellow color with a characteristic absorption maximum at 500 mμ. The color developed is determined spectroscopically. This method can be used for vitamin D preparations in oil and is also fairly accurate in the presence of vitamin A. Tachysterol gives the same color and some other sterols give similar reactions but the color developed is weaker, and the absorption maximum is somewhat different. This method has been used successfully for the determination of vitamin D preparations of natural origin and is applicable for amounts as little as 0.02–0.4 mg.[340, 341, 342, 343] A modification of this method, in which acetyl chloride is used in addition to the other reagents employed, is said to be more accurate.[344] It has also been recommended to free the solutions from sterols present by precipitation with digitonin and to absorb selectively the vitamin A present on Montana earth.[345]

3. **Aniline-Color Test.**[346] Liver oils and irradiated provitamins D give a red color with a mixture of aniline and hydrochloric acid.

4. **Fuchsine-Sulfurous Acid Color Reaction.**[347] Crystalline vitamin D acquires a violet color when added to fuchsin-sulfurous acid.

5. **Phosphorus Pentachloride Color Reaction.**[348] Vitamin D in oil solution develops a reddish brown color with PCl_5. The color gradually becomes darker and is finally almost black. This reaction is non-specific and has even been claimed to indicate all vitamins and hormones.[349]

6. **Tortelli-Jaffé Reaction.**[350, 351, 352] See description under Determination of Provitamin D (page 367).

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[339] H. Brockmann and Y. H. Chen, *Z. physiol. Chem.*, **241**, 129 (1936).
[340] H. Brockmann and Y. H. Chen, *Ibid.*, **241**, 129 (1936).
[341] L. K. Wolff, *Z. Vitaminforsch.*, **7**, 277 (1938).
[342] A. Emmerie and M. van Eekelen, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **6**, 133 (1936).
[343] K. Ritsert, *E. Merck's Jahresberichte*, **52**, 27 (1938).
[344] C. H. Nield, W. C. Russell and A. Zimmerli, *J. Biol. Chem.*, **136**, 73 (1940).
[345] L. K. Wolff, *Z. Vitaminforsch.*, **7**, 277 (1938).
[346] M. J. Shear, *Proc. Soc. Exptl. Biol. Med.*, **23**, 546 (1925).
[347] A. Steigmann, *Kolloid. Z.*, **45**, 165 (1928).
[348] W. Stoeltzner, *Münch. Med. Wochschr.*, **75**, 1584 (1928).
[349] E. Christensen, *Ibid.*, **75**, 1883 (1928).
[350] M. Tortelli and E. Jaffé, *Ann. chim. applicata*, **2**, 80 (1914).
[351] E. P. Häussler and E. Brauchli, *Helv. Chim. Acta*, **12**, 187 (1929). J. M. Heilbron and F. S. Spring, *Biochem. J.*, **24**, 133 (1930). V. A. Petrow, O. Rosenheim and W. W. Starling, *J. Chem. Soc.*, **1938**, 677.
[352] U. Westphal, *Ber.*, **72**, 1243 (1939).

(c) *Biological Methods*

The biological methods for the determination of vitamins D are reliable, when properly conducted, and are much superior to all physical and chemical assay methods developed so far. Furthermore, the amount of vitamin D needed for biological determinations is relatively small, whereas the amount needed for chemical determinations is of an entirely different order of magnitude.

Rats and chicks are used as test animals for vitamin D determinations. All preparations to be standardized are usually first assayed on rats. The chick test takes longer time and is more costly. On the other hand, as pointed out previously, the rat efficacy is no measure of the chicken efficacy of any vitamin D preparation of unknown chemical composition. It is, however, possible to determine once and for all the rat-chicken efficacy ratio of vitamins D prepared by activation of known provitamins. The ratio of such vitamins D is constant when tested under standardized conditions.

The rat, as such, is not an ideal animal for vitamin D assays. Its normal needs for this vitamin are extremely small. In order to induce rickets in rats, they must be fed a ration which contains calcium and phosphorus in an abnormal proportion. Usually a high calcium and low phosphorus diet is used, for example, a ratio of about 5:1. It is impossible to produce rickets with a calcium-phosphorus ratio of 1:1 in the diet, but a high phosphorus and low calcium diet may also be employed. The absolute amounts of these minerals in the diet are of equal importance[353] (see page 421).

The chick needs considerable amounts of vitamin D even if the calcium-phosphorus ratio may be normal—about 1.5:1. In conducting chick experiments it is important to avoid exposure of the birds to ultraviolet light since even small amounts of these rays cause the disappearance of the deficiency symptoms.

1. **The Rat Assay Method.** This method is the basis of all determinations of vitamin D and is accepted by all the leading national[353a] and international organizations concerned with vitamin D assays. Young, growing rats are placed on a suitable rachitogenic diet (for example, Steenbock and Black diet 2965[354] or McCollum diet 3143[355]) and groups of rats

[353] H. B. Brown, A. T. Shohl, E. E. Chapman, C. S. Rose and E. M. Sauerwein, *J. Biol. Chem.*, **98**, 207 (1932). A. Querido, *Arch. Neerland. physiol.*, **20**, 487 (1935). A. T. Sohl and S. B. Wolbach, *J. Nutrition*, **11**, 275 (1936).

[353a] The United States Pharmacopoeia XI–1939 Supplement specifies a rat test for vitamin D assays. The assay period of this procedure is eight days, the animals receiving the vitamin D only during the first six days. The effect is evaluated according to the "line test".

[354] H. Steenbock and A. Black, *J. Biol. Chem.*, **64**, 263 (1925).

[355] E. V. McCollum, N. Simmonds, P. G. Shipley and E. A. Parks, *Ibid.*, **51**, 41 (1922); **47**, 507 (1921); *Proc. Soc. Exptl. Biol. Med.*, **18**, 275 (1921); *Am. J. Hyg.*, **1**, 492 (1921).

(7 to 10) are fed various amounts of the vitamin D to be tested while one group is used as reference group and is fed with U. S. Standard Cod Liver Oil. After a period of from 6 to 10 days the animals are killed and the degree of rickets is determined by any one of the following methods:

(*a*) *The "Line Test."* This is a curative method and for this purpose either the proximal end of the tibia[356] or the distal end of the radii or ulnae[357] is used. The bones are removed from the animal and cleaned from adherent tissue. A longitudinal median section is made and the section is immersed in a 2% aqueous solution of silver nitrate for one minute, whereby the calcium phosphate present is converted into silver phosphate. After cleaning with distilled water, the sectioned surface of the bone is exposed in water to actinic light until the calcified areas have developed a clearly defined black stain. The criterion of healing is the development of a line of new calcification through the rachitic metaphysis. This is determined either by visual or by photographic[358] examination of the stained section.

(*b*) *Ash Content of the Bone.*[359] This method utilizes the estimation of the ash content of the bones of the hind limbs of rats maintained on prophylactic levels of vitamin D.

(*c*) *Radiographic Method.*[360] In this test the bones of rats are examined radiographically. This test is used for curative and prophylactic assays.

(*d*) *Test for Increase of Body Weight.*[361] This method is based on the increase of body weight observed on young rats on a rachitogenic diet supplemented with vitamin D (originally irradiated ergosterol). This method has not been used widely, since it is not sufficiently accurate.

2. The Chick Assay Method. The best method developed so far for the determination of vitamin D in chicks is the one recommended by the Association of Official Agricultural Chemists and is based upon the use of the percentage ash content of the tibia determined under standard conditions.[362, 363] A special radiographic method has also been sug-

[356] C. E. Bills, *J. Biol. Chem.*, **90**, 619 (1931).

[357] F. J. Dyer, *Quart. J. Pharm. Pharmacol.*, **4**, 503 (1931).

[358] P. F. Bech, *Dansk Tids. Farm.*, **13**, 253 (1939).

[359] H. Steenbock and A. Black, *J. Biol. Chem.*, **61**, 405 (1924).

[360] R. B. Bourdillon, H. M. Bruce, C. Fischmann and T. A. Webster, *Med. Research Council Brit. Special Rept. Series*, **1931**, No. 158.

[361] K. H. Coward, K. M. Key and G. E. Morgan, *Biochem. J.*, **26**, 1585 (1932).

[362] *J. Assoc. Official Agr. Chem.*, **20**, 72 (1937). W. B. Grien, *J. Assoc. Official Agr. Chem.*, **21**, 607 (1938). Methods of Analysis, A.O.A.C., 371 (1940).

[363] O. N. Massengale and C. E. Bills, *J. Nutrition*, **12**, 429 (1936). W. Grab, *Z. physiol. Chem.*, **243**, 63 (1936).

gested[364, 365] which makes use of the differences in the tarso-metatarsal distances in the joints of the legs.

The relative chicken efficacy of vitamin D preparations is determined[366] by assaying the material on chicks on the basis of its rat unit content. The efficacy ratio for rats and chicks varies with the degree of calcification produced and is not a constant.

23. Standards

The International Unit of Vitamin D was defined in 1934 by an International Vitamin Conference held by the League of Nations.[367] One International Unit is defined as 0.025 γ of pure crystalline vitamin D_2 dissolved in one milligram of olive oil. The properties of crystalline vitamin D_2 are defined:

Calciferol or vitamin D_2, $C_{28}H_{43}OH$.

(*a*) Colorless acicular crystals, odorless. M. p. 114.5–117° C. (open capillary).

(*b*) Specific rotation:

in alcohol $[\alpha]_D^{20} = +101°$ to $+102.5°$
$[\alpha]_{5461}^{20} = +119°$ to $+122°$
in chloroform $[\alpha]_D^{20} = +52°$
$[\alpha]_{5461}^{20} = +62°$.

(*c*) Absorption spectrum: in alcohol or other suitable non-absorbing solvent, a smooth curve with a maximum at 265 mμ $E_{1cm.}^{1\%} = 470$–485.

This standard was recommended for adoption when the International Standard solution prepared according to the recommendation of the International Vitamin Conference in 1931 becomes either exhausted or unsatisfactory. The 1931 Standard, although still available, should no longer be used, since it gives different results on evaluation on rats by the line test method and by the bone ash method.[368]

The International Unit is also the basis for the U. S. Pharmacopoeia Unit and in England for the M. R. C. Unit (Medical Research Council Unit). For general use in the United States, a cod liver oil preparation has been set up to serve under the term "*U. S. P. Reference Cod Liver Oil*" for comparative studies. The vitamin D content of this U. S. Pharmacopoeia Reference Cod Liver Oil has been tested carefully against

[364] N. Olsson, *Arch. Geflügelkunde*, 10, 11 (1936); *Kl. Fyriografiska Sällskapets i Lund Forhandlingar*, 9, 1 (1936).

[365] A. Z. Baker and M. D. Wright, *Analyst*, 65, 326 (1940).

[366] O. N. Massengale and C. E. Bills, *J. Nutrition*, 12, 429 (1936).

[367] League of Nations: Health Organization. Memorandum on the International Standard for Vitamin D and Its Application. March, 1935, No. 30.

[368] N. T. Gridgeman, H. Lees and H. Wilkinson, *Analyst*, 65, 493 (1940).

the International Standard. The latter measures properly only the activity of vitamin D_2. It has been pointed out before that birds are not able to utilize vitamin D_2, but require fish liver oil or vitamin D_3. The Association of Official Agricultural Chemists has therefore introduced the "*A.O.A.C. Chick Unit,*" which according to its definition[369] is equal in biological activity for the chick to one unit of vitamin D in the U. S. Pharmacopoeia Reference Cod Liver Oil when determined according to specified conditions (see page 415).

A standard such as the U. S. Pharmacopoeia Reference Cod Liver Oil, while it serves its purpose satisfactorily, can of course only be recognized temporarily. There are a number of indications that the chicken activity of cod liver oil is not the same for various batches of the oil. Ultimately this oil which contains an unknown mixture of vitamins D should be replaced by a pure, crystalline vitamin D, preferably vitamin D_3. One rat unit of this vitamin is equal to one chick unit when determined according to the method specified by the A.O.A.C.

A number of other units of vitamin D have been used or are still in use. They may be summarized in the following table:

1 International Unit	=	1 U. S. P. Unit
	=	1 M. R. C. Unit
	=	1 Coward Unit
	=	0.025 γ Vitamin D_2
	=	5–6 Poulsson Units
	=	1.66 Oslo Units
	=	6–8 Laquer Units
	=	2.6 Prophylactic Units
	=	3.25 ADMA Units
1 Clinical Unit	=	12.5–17 I. U.
1 Steenbock Unit	=	3.2 I. U.
1 "Standard Unit"	=	1,000,000 I. U.

24. Metabolism

Vitamin D can be successfully administered in various ways, for example, by oral ingestion, by parenteral injections and by absorption through the skin.[370] (See page 410 for the reported quantitative differences in the efficacy of the vitamins D_2 and D_3 when administered intramuscularly or *per os.*) The environmental temperature seems to be an additional determining factor in the response of the organism to vitamin D.[371]

[369] Assoc. Official Agr. Chem., *Methods of Analysis*, Fifth Ed., 1940, p. 371.

[370] L. Krenn, *Münch. Med. Wochschr.*, **86**, 1317 (1939). E. M. Hume, N. S. Lucas and H. H. Smith, *Biochem. J.*, **21**, 362 (1927).

[371] D. Tourtellotte and W. E. Bacon, *J. Nutrition*, **10**, 683 (1935).

Vitamin D is absorbed from the intestines, especially in the small gut. The absorption is facilitated by the presence of fat, but excess doses of fat give less favorable results.[372] Vitamin D is not absorbed from mineral oil.[373] Effective absorption of vitamin D is related to the presence of bile.[374] Thus, animals with biliary fistula cannot utilize vitamin D when given by mouth unless administered simultaneously with a bile acid, such as taurocholic acid or desoxy-cholic acid.[375] Vitamin D is not well absorbed in obstructive jaundice. Vitamin D esters of acids which can be hydrolyzed in the intestines are antirachitically effective while esters which cannot be hydrolyzed also cannot be utilized.[376]

From the intestines vitamin D is absorbed into the blood[377] and distributed all over the organism. The healthy body contains definite amounts in the lymphs and tissue fluids. Normal human blood contains from about 50 to 135 International Units per 100 cc. of serum (average about 100) while rabbits were found to have a mean of approximately 50.[378] The human body and also all animals investigated, with the exception of fish, have no special storage organ for this vitamin, although substantial amounts can be found[379] in various organs such as lung, liver, spleen, brain and wherever fat is accumulated as long as there is no shortage of this vitamin in the organism. The heart has consistently been found to be devoid of any stored amounts of this vitamin. The liver and, to a certain extent, also the viscera are special storage organs only for fish.

Vitamin D is readily metabolized as is evident from the transfer of ingested vitamin D into milk of all mammals and into the eggs of birds. The type of vitamin D fed is also secreted. Thus, vitamin D_2 or D_3 is found in milk[380] or in eggs[381, 382] according to which form has been fed to the animal. There is apparently no principal difference in the utilization of the various forms of vitamins D, since vitamin D from fish liver oils,[383]

[372] A. Knudson and R. J. Floody, *J. Nutrition*, **20**, 317 (1940).

[373] M. C. Smith and H. Spector, *Proc. Am. Soc. Biol. Chem.*, **134**, XC (1940); *J. Nutrition*, **20**, 19 (1940).

[374] W. Heymann, *J. Biol. Chem.*, **122**, 249 (1937).

[375] J. D. Greaves and C. L. A. Schmidt, *Ibid.*, **102**, 101 (1933). *Univ. Calif. Pub. Physiol.*, **8**, 49 (1934).

[376] A. Windaus and O. Rygh, *Nachr. Ges. Wiss. Göttingen, Math. physik. Klasse*, **III**, 202 (1928).

[377] A. F. Hess, R. F. Light, C. N. Frey and J. Gross, *J. Biol. Chem.*, **97**, 369 (1932). A. F. Hess, M. Weinstock and J. Gross, *Proc. Soc. Exptl. Biol. Med.*, **30**, 1357 (1933).

[378] J. Warkany, *Z. Kinderheilk.*, **49**, 191, 259 (1930); *Am. J. Diseases Children*, **49**, 318 (1935); **52**, 831 (1936); *Biochem. Z.*, **293**, 415 (1937).

[379] H. Goldblatt and K. M. Soames, *Biochem. J.*, **17**, 446 (1923). I. H. Page, *Biochem. Z.*, **220**, 420 (1930). W. Heymann, *J. Biol. Chem.*, **118**, 371 (1937).

[380] R. M. Bethke, W. E. Krauss, P. R. Record and O. H. M. Wilder, *J. Nutrition*, **11**, 21 (1936).

[381] R. M. Bethke, P. R. Record, C. H. Kirk and D. C. Kennard, *Poultry Sci.*, **15**, 326 (1936).

[382] R. M. Bethke, P. R. Record, O. H. M. Wilder and C. H. Kirk, *Ibid.*, **15**, 336 (1936).

[383] For example, R. M. Bethke, D. C. Kennard and H. L. Sassaman, *J. Biol. Chem.*, **72**, 695 (1927).

from activated ergosterol,[384] from activated 7-dehydro-cholesterol and activated "mussel provitamin"[385] are all metabolized as described, although a small difference in the efficiency of this process for different vitamins D has been observed, that is, vitamin D_2 is somewhat less effectively utilized than the other forms of vitamin D.[385, 386, 387] Colostrum (of cows[388]) contains from six to ten times the amount of vitamin D found in normal milk. Also of special interest is the fact that vitamin D apparently can pass only in limited but definite amounts through the placental walls. Newborn animals and babies have practically no vitamin D in their tissues,[389] even though their mothers had an abundant supply. On the other hand, when the mother's diet was deficient, the bones and skull of the infant were found softer than normal and the teeth when erupted showed defective formation.[390] All these observations suggest that a special regulatory mechanism exists which takes care of the vitamin D requirements of the embryo.

Some of the vitamin D is destroyed in the organism, some is excreted.[391] No quantitative balance studies are available which would indicate to what extent destruction occurs. Excretion occurs only through the intestinal tract and mainly through the bile, but not through the kidneys. The extent to which vitamin D is secreted through the skin is unknown.

25. Physiological Action

An enormous amount of work has been done in various studies to define the action and the mode of action of vitamin D. The result of all these investigations has not yet developed into a well-rounded picture. Most work has been done either with vitamin D_2 or with fish liver oils and it seems quite certain that at least some differences may be found in the specific reactions of these forms of vitamin D and of vitamin D_3, which is considered to be the natural vitamin D of man and higher animals.

[384] R. F. Light, L. T. Wilson and C. N. Frey, *J. Nutrition*, **8**, 105 (1934).

[385] J. van Niekerk and Hofstra, *Tijdschr. Diergeneesk.*, **1939**, 66.

[386] G. M. DeVaney, H. E. Munsell and H. W. Titus, *Poultry Sci.*, **12**, 215 (1933).

[387] R. M. Bethke, P. R. Record, O. H. M. Wilder and C. H. Kirk, *Ibid.*, **15**, 336 (1936).

[388] J. van Niekerk and M. S. C. Bliek, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **9**, 25 (1939).

[389] A. F. Hess and M. Weinstock, *Am. J. Diseases Children*, **27**, 1 (1924); *J. Am. Med. Assoc.*, **83**, 1558 (1924). C. E. Bills, *J. Biol. Chem.*, **72**, 751 (1927). C. E. Bills and A. M. Wirick, *Ibid.*, **86**, 117 (1930).

[390] K. W. Toverud and G. Toverud, *Acta Paediat.* (Suppl. 2), **12**, 1 (1936).

[391] A. F. Hess, R. F. Light, C. N. Frey and J. Gross, *J. Biol. Chem.*, **97**, 369 (1932). A. F. Hess, M. Weinstock and J. Gross, *Proc. Soc. Exptl. Biol. Med.*, **30**, 1357 (1933).

In the broadest aspect, vitamin D stimulates growth.[392] While this property is apparently common to all vitamins, at least as a secondary reaction, it seems of primary significance in the case of vitamin D, since growth of all animals stops or is retarded in the absence of this vitamin. On the basis of this property a method for the determination of vitamin D has been proposed.[393] This retardation of growth is probably quite a fundamental metabolic process. Thus, eggs which contain an insufficient amount of vitamin D or which have been laid by hens that obtained suboptimal doses of this vitamin do not hatch.[394]

The growth of bones can easily be demonstrated to be related to the action of vitamin D, although the possibility should not be overlooked that the growth of cells other than bone cells may also be influenced.

The physiological study of the action of vitamin D must necessarily commence with a study of the organism deprived of this vitamin and proceed to a determination of the effects brought about by the action of the vitamin and finally end with an explanation of the vitamin action.

Whereas the clinical symptoms of a vitamin D deficiency will be discussed in the section on avitaminosis, the broad picture of the disease and the metabolic changes must be mentioned here. The clinical symptoms of the vitamin D deficiency are commonly known under the term "rickets" and the gross effect is obviously a disturbance of the mineral metabolism. Actually, the deposition of the inorganic calcium-phosphorus salts in the bones is retarded or stopped entirely, thus retarding or stopping the growth of bones. Furthermore, the inorganic material previously deposited may be withdrawn from the bones, causing a considerable softening. An early symptom of a vitamin D deficiency is a lowered content of phosphorus in the blood serum and later also a lowering of the calcium level is observed.[395, 396] Furthermore, a general decreased retention of phosphorus and at later stages of the deficiency also a decreased retention of calcium are found. Thus, the phosphorus metabolism is much more hampered than the calcium metabolism. All these changes are not necessarily the primary ones, but represent the ultimate effect of the vitamin deficiency which can be observed.

To study the physiology and the mode of action of vitamin D, animal experiments must be performed. Experimental rickets was first obtained

[392] G. Stearns, P. C. Jeans and V. Vandecar, *J. Pediatrics*, **9**, 1 (1936). F. Slyker, B. M. Hamil, M. W. Poole, T. B. Cooley and I. G. Macy, *Proc. Soc. Exptl. Biol. Med.*, **37**, 499 (1937).

[393] K. H. Coward, K. M. Key and G. E. Morgan, *Biochem. J.*, **26**, 1585 (1932).

[394] J. S. Carver, E. I. Robertson, D. Brazie, R. H. Johnson and J. L. St. John, *Washington Agr. Exptl. Station Bull.*, **299** (1934).

[395] J. Howland and B. Kramer, *Am. J. Diseases Children*, **22**, 105 (1921).

[396] P. Iversen and E. Lenstrup, *Forhandl. Forste nord. Kongr. Paediatr.* (*Copenhagen*), **1920**, 89.

in dogs,[397] but soon rats were used[398, 399] and most of the work has been done with these animals. Rats, however, have usually a very low requirement for vitamin D. That is, on a vitamin D-deficient, but otherwise normal diet, they do not develop classical rickets. A condition, however, which resembles human rickets, can be brought about by special rations which have a disproportion between calcium and phosphorus. Thus, a diet of high calcium and low phosphorus content or of low calcium and high phosphorus content produces experimental rickets in rats. In addition to the ratio of calcium to phosphorus, the absolute amount of each also determines the rachitogenic properties of the diet.[400] Very satisfactory results were obtained with the diets "Steenbock and Black No. 2965"[401] and with "McCollum No. 3143,"[402] which are similar in composition and which contain about 1.2% of calcium and about 0.25% of phosphorus, the ratio of calcium to phosphorus thus being about 5 : 1. Rats reared on such rations develop dietary rickets that can be cured by the administration of vitamin D.

Physiological studies on rats have given rise to a number of theories on the action of vitamin D. None of these theories, however, should be generalized until confirmatory evidence from experiments with other, more suitable species has been obtained. In contradistinction, the mode of action of vitamin D should be investigated in animals which develop rickets only by a deficiency of vitamin D and in which rickets cannot be prevented by a regulation of the calcium and phosphorus content of the diet. Thus, dogs,[403] pigs[404] and many birds,[405] especially the hen and the turkey, are useful experimental animals.

The histological changes that occur during bone formation must be discussed briefly in order to understand the action of vitamin D. There are three different types of tissue concerned with the growth of bones—the cartilage tissue, the osteoblasts and the bone tissue. During the course of normal bone growth, a certain number of cartilage cells de-

[397] E. Mellanby, *J. Physiol.*, **52**, LIII (1919); *Lancet*, **I**, 407 (1919).

[398] H. C. Sherman and A. M. Pappenheimer, *Proc. Soc. Exptl. Biol. Med.*, **18**, 193 (1920/1921); *J. Exptl. Med.*, **34**, 189 (1921).

[399] E. V. McCollum and N. Simmonds, *J. Biol. Chem.*, **47**, 111, 139, 175, 207, 235, 507 (1921). See also V. Korenchevsky, *Brit. Med. J.*, No. 3171, 547 (1921); *Special Rept. Sci. Med. Research Council*, No. 71 (1922).

[400] H. B. Brown, A. T. Shohl, E. E. Chapman, C. S. Rose and E. M. Sauerwein, *J. Biol. Chem.*, **98**, 207 (1932). A. Querido, *Arch. neerland. physiol.*, **20**, 487 (1935). A. T. Shohl and S. B. Wolbach, *J. Nutrition*, **11**, 275 (1936).

[401] H. Steenbock and A. Black, *J. Biol. Chem.*, **64**, 263 (1925).

[402] E. V. McCollum, N. Simmonds, P. G. Shipley and E. A. Parks, *Ibid.*, **51**, 41 (1922); **47**, 507 (1921); *Proc. Soc. Exptl. Biol. Med.*, **18**, 275 (1921); *Am. J. Hyg.*, **1**, 492 (1921)

[403] E. Mellanby, *J. Physiol.*, **52**, LIII (1919); *Lancet*, **I**, 407 (1919).

[404] Schoch, *Mitt. Lebensm., Hyg.*, No. 1/2, 176 (1939).

[405] O. Wanscher, *Avitaminosis D hos Kyllinger* (Thesis), Copenhagen, 1939.

generate and disappear completely and in their place bone-forming cells or osteoblasts arise. Soon capillaries invade this tissue. The osteoblasts are responsible for the deposition of the bony matrix and are transformed into bone cells. In turn new osteoblasts are formed from the cartilage cells. In normal growth there is always a continuous thin layer of osteoblasts.

The first sign of vitamin D deficiency in the bone is the cessation of the formation of osteoblasts. On the other hand, the growth of the cartilage tissue continues. The osteoid material which accumulates remains uncalcified. When the vitamin D deficiency becomes more severe, the ossified part of the bone slowly softens by conversion into osteoid material and loss of calcium phosphate.

The effect of vitamin D given to rachitic organisms becomes apparent within twenty-four hours. From the mass of apparently unorganized cartilage cells, orderly arranged osteoblasts are formed and calcification begins. This is preceded by a penetration of the tissue with blood vessels which carry the bone-forming salts.

There is no fundamental pathological condition in the bone that prevents calcification during rickets. The change is only brought about by the absence of vitamin D. This has been demonstrated in various ways. Rachitic cartilage when inserted into serum of a rachitic animal does not change its structure. In the presence of serum of a normal animal, however, calcification occurs.[406] The same effect can also be observed by transplantation of rachitic cartilage tissue into the abdomen.[407] Furthermore, when osteogenic tissue is cultured on a medium derived from normal animals (fowl were used in these experiments), calcified bone is formed, but unossified osteoid tissue and cartilage are formed when a vitamin D-deficient medium is used.[408] Addition of calcium and of phosphorus to the vitamin D-deficient medium did not bring about complete calcification.

These experiments demonstrate that the formation of bones is dependent upon the presence of vitamin D as such. It has often been discussed whether or not the action of vitamin D may not be due to a facilitation of the absorption of phosphorus and of calcium from the intestines as is the case in rats.[409] This is certainly not the case in man and in birds. The phosphorus content of blood can be raised by parenteral injection

[406] A. F. Hess, *J. Am. Med. Assoc.*, **91**, 783 (1928); *Proc. Soc. Expil. Biol. Med.*, **26**, 199 (1928). P. G. Shipley, *Bull. Johns Hopkins Hosp.*, **35**, 304 (1924).

[407] S. v. Pfaundler, *Jahrb. Kinderheilk.*, **60**, 123 (1904).

[408] C. F. Fischmann, *Arch. Zellforsch.*, **19**, 211 (1937).

[409] R. Nicolaysen, *Biochem. J.*, **31**, 107, 122 (1937). R. Nicolaysen and J. Jansen, *Acta Paediat* **23**, 405 (1939).

of glycerophosphate and the calcium content can be increased by the injection of calcium salts or of A.T.10, but rickets cannot be cured by these methods.[410] Increased permeability of the membranes of the intestines for calcium and phosphorus may occur to a limited extent upon administration of vitamin D but cannot be the main or sole reason for the cure of rickets.

The presence of phosphorus and calcium in the blood serum needs further consideration. Calcium occurs in the serum in four different forms,[411] two of which are diffusible. Of the diffusible forms, one is ionized the other consists of a calcium-phosphorus complex which can be adsorbed on barium sulfate. It is this complex which is believed to be the precursor of the bone salts. Its content in blood is believed to be independent of the total calcium or phosphorus content of the serum. The total calcium content (9–11 mg. % in the normal person) is not necessarily influenced by a rachitic state, although in severe cases a decrease of calcium in the blood serum occurs (to about 7–8 mg. %) and the partition of the several forms of calcium is profoundly changed.

The metabolism of phosphorus is somewhat different, as has previously been stated. Phosphorus occurs in blood serum bound to organic and to inorganic compounds. It occurs as lipoid-phosphorus, phospho-proteid, nucleids, ester-phosphates and as primary and secondary alkali-phosphate. The inorganic phosphorus and the ester-phosphate are constantly converted into each other. During vitamin D deficiency the serum phosphorus is reduced from 4–6 mg. % normal to 1–2 mg. %. The phosphorus is excreted[412] in considerable amounts even if abundant quantities are offered in the diet or if glycerophosphate is injected parenterally.[413] By means of radioactive phosphorus it was furthermore ascertained that the phosphorus metabolism in rachitic animals (chicks) is more intense than in normal animals.[414] In acute rickets phosphorus is removed from the bones. Simultaneously, the amount of the enzyme phosphatase, which splits organic compounds of phosphorus into a diffusible, inorganic form, is almost invariably greatly increased above normal[415] in the blood serum

[410] E. Rominger, *Ergeb. Vitamin Hormonforsch.*, **2**, 104 (1939). Grosser, *Z. Kinderheilk.*, **25**, 141 (1920).

[411] H. R. Benjamin and A. F. Hess, *J. Biol. Chem.*, **100**, 27 (1933); **103**, 629 (1933). H. R. Benjamin, *Ibid.*, **100**, 57 (1933).

[412] J. A. Schabad, *Arch. Kinderheilk.*, **54**, 83 (1910).

[413] Grosser and W. Heymann, *Z. Kinderheilk.*, **45**, 232 (1928); **46**, 575, 584 (1928).

[414] M. J. L. Dols, B. C. P. Jansen, G. J. Sizoo and G. J. van der Maas, *Proc. Acad. Sci. Amsterdam*, **42**, 499 (1939).

[415] R. Robison, *Biochem. J.*, **17**, 286 (1923). R. Robison and K. M. Soames, *Ibid.*, **18**, 740 (1924). H. D. Kay, *Physiol. Rev.*, **12**, 384 (1932). S. J. Folley and H. D. Kay, *Ergeb. Enzymforsch.*, **5**, 159 (1936).

and it has even been stated that its increase is the first definite evidence of the development of a rachitic condition before an actual change in the blood phosphorus can be detected and long before a change in the bone structure becomes evident from roentgenograms. The degree of increase is, in general, correlated to the severity of rickets. On the other hand, the phosphatase value does not decrease immediately after the administration of vitamin D and may not reach a normal level for several months after healing from the vitamin D deficiency becomes evident.[416]

The effect of an administration of vitamin D on the phosphorus metabolism of rachitic organisms is striking. No further loss through excretion occurs and an immediate increase of all forms of phosphorus in the serum is noted.[417] This evidence proves that vitamin D is intimately concerned with the phosphorus retention of the normal organism. This retention of phosphorus is brought about by the formation of the previously mentioned calcium-phosphorus complex and the theory has been advanced that vitamin D may act in this process as an activator, perhaps in combination with an enzyme system.[418] Further evidence leading to the same conclusion comes from studies with radioactive phosphorus which suggests that vitamin D acts in aiding the conversion of organic into inorganic phosphorus.[419]

Actually, during vitamin D deficiency the amount of this calcium-phosphorus complex is significantly reduced as has been shown[420] both on children and on rats, and is increased again upon administration of vitamin D.[421] Furthermore, it has been demonstrated that this complex must be present in serum in order to bring about calcification.[422] The mechanism of the actual deposition of this complex on the bones is still obscure. It has already been mentioned that the precipitation of the bone salts occurs only in specially prepared cells, the osteoblasts, and it is believed that this process can be explained on the basis of selective adsorption. An attempt has also been made to correlate the deposition of bone salts with their concentration in the body fluids, applying the physicochemical principle of mass action.[423] The deposited salt has the compo-

[416] A. Bodansky and H. L. Jaffé, *Arch. Intern. Med.*, 54, 88 (1934). N. Morris, M. M. Stevenson, O. D. Peden and J. M. D. Small, *Arch. Diseases Childhood*, 12, 45 (1937).

[417] A. F. Hess and M. G. Gutman, *J. Am. Med. Assoc.*, 78, 29 (1922).

[418] E. Rominger, *Ergeb. Vitamin Hormonforsch.*, 2, 104 (1939). Grosser, *Z. Kinderheilk.*, 25, 141 (1920).

[419] W. E. Cohn and D. M. Greenberg, *J. Biol. Chem.*, 130, 633 (1939).

[420] A. F. Hess and H. R. Benjamin, *Ibid.*, 100, 27 (1933).

[421] H. R. Benjamin and A. F. Hess, *Ibid.*, 100, 27 (1933); 103, 629 (1933). H. R. Benjamin, *Ibid.*, 100, 57 (1933).

[422] K. Klinke, *Klin. Wochschr.*, 7, 385 (1928).

[423] A. B. Hastings, *New England J. Med.*, 216, 377 (1937).

sition of two mols of tricalcium phosphate and one mol of calcium carbonate. Traces of other elements, such as magnesium and fluorine, may be present.

In the discussion of the phosphorus metabolism, the primary effect of vitamin D upon an organism affected with rickets has been emphasized, that is, the phosphorus content of the serum increases rapidly to normal and sometimes even reaches somewhat higher values. When cases of severe rickets are thus treated, a state occurs when the calcium content of the serum is still low (7–8 mg. % or less) but the phosphorus content is high. This is the typical disproportion which is found in hypoparathyroid tetany and which causes tetany. Experimentally tetany can be produced in rats by inducing such a disproportion of the phosphorus and the calcium content in the blood.[424] Tetany is therefore closely related to vitamin D deficiency. In man, this relation has repeatedly been observed when rachitic babies obtained insufficient amounts of vitamin D, for example, by a single ultraviolet light irradiation or by small doses of vitamin D.[425] Etiologically, vitamin D cures the tetany of children but not symptomatically, or only after a considerable length of time. It is interesting to note that a compound chemically closely related to vitamin D, namely, the previously discussed dihydro-tachysterol (A.T.10) acts symptomatically on tetany by raising immediately the calcium level in the serum and thus relieving the symptoms.[426]

Decreased phosphorus retention in the organism as the result of vitamin D deficiency is furthermore accompanied by acidosis which in turn, according to some investigators,[427] causes a disturbance in the calcification of the bone. Therefore, the acid-base content of the diet has repeatedly been investigated for its influence upon the occurrence of rickets. In rats such a relation can indeed be demonstrated, but not in man. In rats, rickets can also be cured through what has been called a "specific organic acid effect,"[428] that is, specific organic acids, such as citric acid and tartaric acid, when added to the diet, cause the disappearance of the symptoms of rickets. This effect is specific for rats and cannot be observed in chicks.[429]

[424] J. H. Jones and B. N. E. Cohn, *J. Nutrition*, **11**, 293 (1936). A. T. Shohl and H. B. Brown, *J. Biol. Chem.*, **84**, 501 (1929).

[425] H. J. Gerstenberger, J. I. Hartman, G. R. Russel and T. S. Wilder, *J. Am. Med. Assoc.*, **94**, 523 (1930). K. Huldschinsky, *Z. Kinderheilk.*, **26**, 207 (1920). Lust, *Monatsschr. Kinderheilk.*, **44**, 72 (1929).

[426] F. Holtz, *Klin. Wochschr.*, **13**, 104 (1934); *Deut. med. Wochschr.*, **I**, 560 (1934); **II**, 1830 (1934).

[427] E. Freudenberg and P. György, *Jahrb. Kinderheilk.*, **96**, 5 (1921); *Münch. med. Wochschr.*, **I**, 422 (1922); *Monatsschr. Kinderheilk.*, **28**, 503 (1924); *Handb. Kinderheilk. Pflaundler-Schlossman*, **I**, 4th ed., 1931.

[428] A. T. Shohl, *J. Nutrition*, **14**, 69 (1937). B. Hamilton and C. Schwartz, *Am. J. Diseases Children*, **46**, 669 (1933); **54**, 548 (1937).

[429] J. T. Correll, *J. Nutrition*, **21**, 515 (1941).

While only the relation of vitamin D to the calcium and phosphorus metabolism has been discussed, there is evidence that vitamin D is also concerned with a number of other reactions, either directly or indirectly. For example, vitamin D has an influence upon the metabolism of other minerals, especially of magnesium and iron. Vitamin D, furthermore, affects the carbohydrate metabolism, since during vitamin D avitaminosis the phosphorylation of carbohydrates is retarded.[430] Vitamin D, or ultraviolet light, leads to changes in carbohydrate metabolism (experiments with rats) which are very similar to those observed by administration of insulin. An increase in the glycogen in the liver, and to a lesser degree in the muscle, has been observed. Furthermore, the quotient carbohydrate/lactic acid goes up in blood, liver and muscle.[431] A relation of vitamin D to the fat metabolism has also been postulated.

26. Relation to Other Vitamins and Hormones

As has been postulated in the general chapter on the interrelationship of vitamins and on the relationship of vitamins to hormones (pages 29 to 31), no single vitamin is able to exert its full action in the absence of other necessary substances. The effects which occur when only vitamin D is taken out of the vitamin balance have been discussed. A multiple deficiency of vitamin D together with another or several other vitamins has not been observed other than in the form of added symptoms from each deficiency. It has been claimed that the effect of toxic amounts of vitamins D can be relieved by simultaneous administration of the vitamins of the B-group such as are present in yeast.[432]

At various times relations of vitamin D to different glands or hormones have been postulated. The thyroid gland and the parathyroid gland have been the center of discussion. It has been claimed that the administration of thyroid extracts cures rickets,[433] but it has also been claimed that thyroid intensifies the symptoms of rickets. The parathyroid gland influences the calcium metabolism, but in an entirely different manner from the influence that vitamin D exerts. Parathyroid raises primarily the calcium level in blood serum; vitamin D, however, raises the phos-

[430] E. Freudenberg and A. Welker, *Z. Kinderheilk.*, **41**, 466 (1926). H. Heutschel and E. Zöller *Ibid.*, **44**, 146 (1927).

[431] L. Pincussen, *Proc. Am. Soc. Biol. Chem.*, **1941**, CI.

[432] L. J. Harris and T. Moore, *Biochem. J.*, **22**, 1461 (1928); *Lancet*, **I**, 892 (1928). R. F. Light, G. Miller and C. N. Frey, *J. Biol. Chem.*, **84**, 287 (1929). E. R. Norris, and A. E. Church, *Ibid.*, **89**, 437 (1929). H. J. Jusatz, *Z. ges. exptl. Med.*, **87**, 529 (1933); *Z. Vitaminforsch.*, **3**, 268 (1934); *Klin. Wochschr.*, **II**, 1501 (1932). Herrmann, *Klin. Wochschr.*, **II**, 1752 (1929).

[433] A. Nitschke, *Z. ges. exptl. Med.*, **82**, 236 (1932); *Z. Kinderheilk.*, **54**, 233 (1933); *Klin. Wochschr.*, **12**, 1793 (1933).

phorus level. Parathyroid causes an increase of ionized calcium in blood, vitamin D of bound calcium. Parathyroid stimulates the withdrawal of calcium from the body, vitamin D stimulates its retention. All these facts caused various investigators to find either a synergistic or an antagonistic effect of parathyroid on rickets according to the type of experiments carried out. It now seems that the action of both compounds is independent.

The secretions of various other glands, for example, the anterior pituitary, have been claimed to be interrelated with the action of vitamin D. Thus the secretions of the lymph glands are said to influence the phosphorus and the calcium content of serum.[434] It has been suggested that the ovaries influence rickets, since mothers after the parturition sometimes become rachitic.[435] Finally, the thymus has been reported to be involved in the functioning of vitamin D.[436] All these findings and theories need further study, since there is no agreement, as yet, about the relation between these effects and vitamin D.

27. Hypovitaminosis and Avitaminosis

The clinical symptoms of vitamin D deficiency in infants and in young animals are called "rickets." This disease occurs most frequently in man, beginning around the fourth month of age, but also occurs in children of school age. Early signs of the disease are noticeable, continuous discomfort and perspiration on the head. Soon the typical skeletal changes especially in the ribs, forearm and wrist become apparent and can be recognized by roentgenograms. The retardation in ossification of the fontanelles is especially characteristic in babies.[437] The change in the bones is a lack of calcification which becomes especially noticeable at the epiphysis. As a result, the ends of the long bones become greatly enlarged by excessive cartilage formation. Also, enlargements of the junctions between the bones, especially between the ribs and the cartilages which are normally present, ("rachitic rosary") occur. In more serious cases, the skull is malformed. The jaw may be ill-shaped, the teeth appear late, grow too close to each other and possess ill-formed enamel.[438] Deficiency of vita-

[434] A. Nitschke, *Z. ges. exptl. Med.*, 65, 637, 651 (1929); *Deut. Med. Wochschr.*, 62, 629 (1936).
[435] Hanau, *Korrespbl. Schweiz. Ärzte*, 22, 497 (1892).
[436] G. Lucandri, *Boll. soc. ital. biol. sper.*, 13, 5 (1938). O. Hirota, *Folia Endocrinol. Japon.*, 13, 46 (1937).
[437] Farfl, E. J. Dalyell and Mackay, *Med. Research Council Rept. on Rickets in Vienna, Spec. Rept.* No. 77 (1923).
[438] M. M. Eliot, S. P. Southern, B. A. Anderson and S. Arnim, *Am. J. Diseases Children*, 46, 458 (1933).

min D is to a certain extent, but not altogether, responsible for the occurrence of dental caries.[439] The elastic properties of the bones are generally disturbed as evidenced, for example, in rat bones by breaking load and deflection stress.[440] A curvature of all bones occurs in later stages of the disease, and is especially significant in the limbs, the spine, etc.

A vitamin D deficiency, however, does not affect the bones exclusively but the entire body. Besides the changes noted on the bones, the effects on muscles are most obvious. During severe cases of rickets the muscles become weak and flabby.

During vitamin D deficiency the organism is particularly susceptible to a number of infectious diseases which sometimes cause death, such as bronchopneumonia, tuberculosis, infectious fevers, etc. It has also been reported[441] that milk fever can be prevented in cows by a vitamin D supplement fed prior to parturition.

A special symptom is spasmophilia (infantile tetany). As previously discussed, this disease when caused by a deficiency of vitamin D is considered to indicate the beginning of healing. In typical rickets, tetanic spasms often occur of either a general nature or localized in the hands and feet. Sometimes cramps occur even in the heart muscles and the bronchial muscles. Rickets of the adult is also called "osteomalacia" and occurs especially in women during and after pregnancy but has also been noted sporadically among men and women of all ages (osteoporosis). The general symptoms are exactly the same as those of the baby rickets, namely, decalcification of the bones leading to brittleness.

(a) *Clinical Test Methods*

1. **X-Ray Determination.** This method is the oldest and the one that is most commonly used, but reveals only cases of avitaminosis, whereas the state of hypovitaminosis cannot be detected. The roentgenographic examination of the bones of the forearm and the wrist is especially recommended both for diagnosis of rickets and for following the healing process.

2. **Determination of Blood Ca and P.** The normal level is 4–6 mg. % of P and 9–11 mg. % of calcium. Any value below these is considered to indicate vitamin D deficiency. The determination of Ca and of P is

[439] M. Mellanby and J. D. King, *Ergeb. Vitamin Hormonforsch.*, **2**, 1 (1939). G. F. Taylor and C. D. M. Day, *Brit. Med. J.*, **1**, 919 (1939).

[440] A. A. Schiller, H. C. Struck and C. I. Reed, *Proc. Am. Physiol. Soc.*, **1941**, 250.

[441] J. R. Grieg, *Scottish J. Agr.*, **13**, 369 (1930). B. Sjollema, *Nutrition Abstracts & Revs.*, **1**, 621 (1932). F H. Conover, *Vet. Med.*, **35**, 657 (1940). T. M. Olson, *South Dakota Expti. Station, Bull.* **319**, 1938.

carried out according to standardized procedures.[442] The product of the values found for Ca and P is also used as criterion and should be above 30 in normal individuals. This evaluation procedure is considered to give trustworthy results only in cases of severe avitaminosis and cannot be used to follow the progress during treatment.

3. Blood Phosphatase Test.[443] This test is based on the fact that the enzyme phosphatase occurs in increasing amounts in the blood during bone diseases such as rickets. When rickets is healed, the amount of phosphatase in blood is again slowly but not immediately reduced.

4. Mineral Metabolism Test.[444] This test in which the phosphorus[445] and calcium[446] balance is determined is considered, in the hands of experts, to give the best data as to the state of rickets. In early disease much more phosphorus is excreted than calcium. At later stages calcium and phosphorus are both excreted in increased amounts. The beginning of healing is characterized by a marked phosphorus retention and when healing is well under way a considerable retention of calcium and phosphorus is observed.

28. Hypervitaminosis

Vitamins D given in large excess to any experimental animal or man are toxic. It is therefore important to know the symptoms of such a D-hypervitaminosis and the minimum amount of vitamin D which may cause an intoxication.

The first sign of a D-hypervitaminosis is digestive disorder. There is a loss of appetite, vomiting and diarrhea. A considerable loss of weight, an inflammation of the kidneys and finally death occur. Excessive doses of vitamin D cause an increase of the calcium content of the serum, which may reach a value of 17 mg. %. As a result metastatic calcification occurs in various organs and tissues, especially in the kidneys, stomach, lungs, heart, blood vessels and bronchi. At first, a retention of calcium in the organism is observed, but at later stages a decalcification of the skeletal

[442] For example, E. Müller, *Z. Kinderheilk.*, **57**, 243 (1935); *Z. physiol. Chem.*, **237**, 35 (1935). See also L. Pincussen, *Mikromethodik.*, Leipzig, 1930.

[443] H. D. Kay, *J. Biol. Chem.*, **89**, 325 (1930); *Physiol. Rev.*, **12**, 384 (1932). N. Morris and O. D. Peden, *Quart. J. Med.*, **6**, 211 (1937); *Arch. Disease Childhood*, **12**, 45 (1937). D. J. Barnes and A. D. Carpenter, *J. Pediatrics*, **10**, 596 (1937).

[444] E. Rominger, *et al.*, *Arch. Kinderheilk.*, **80**, 195 (1927); **81**, 176 (1927). E. Rominger, H. Meyer and C. Bomskov, *Klin. Wochschr.*, **II**, 1391 (1930); **II**, 1293, 1342 (1931); *Z. ges. exptl. Med.*, **73**, 344 (1930); **78**, 259, 272 (1931).

[445] Phosphorus determination, for example, according to E. Müller, *Z. physiol. Chem.*, **237**, 35 (1935); *Z. Kinderheilk.*, **57**, 243 (1935).

[446] Calcium determination, for example, according to B. Kramer and F. F. Tisdall, see L. Pincussen, *Micromethodik.*, Leipzig, 1930.

bones sets in. Finally, phosphorus is excreted and calcium deposited in the tissues. Shortly before death an excretion of calcium through the kidneys also occurs.[447]

Unfortunately, no exact data for the toxic dose can be given. This is due mainly to the fact that the toxic threshold varies considerably among individuals. As an average figure a continued daily dose of about 20,000 International Units of vitamin D per kilogram of body weight may be considered to cause intoxication in man and dogs.

Clinically, in the so-called shock-therapy, single doses up to 1,000,000 International Units of pure crystallized vitamins D dissolved in a suitable medium have been used and no injuries have been observed from such treatments.

The over-all toxic effect of vitamin D_2 is believed to be somewhat greater than that of vitamin D_3 (studied on dogs). After feeding dogs excessive amounts of these two forms of vitamin D, the animals were allowed to recover. Functional recovery was rapid in the dog relieved from vitamin D_3 but the damage to tissues was more severe and less repaired than in the animal relieved from vitamin D_2.[448]

It should, furthermore, be noted that toxic symptoms have been observed only when vitamin D was given *per os* or parenterally, but never when vitamin D was supplied by ultraviolet irradiation. The deleterious effect observed on the normal organism upon over-irradiation is independent of vitaminization. This suggests that a special protective mechanism exists in the body, probably in the skin, which takes care of the potential effects of an over-irradiation of the provitamin D.

29. Requirements

The human requirements of vitamin D[449] are difficult to estimate correctly due to an individual variation in the utilization of dietary calcium and phosphorus without added vitamin D.[450] The optimum amount of vitamin D for babies, children and adolescents is believed to be about 400 to 800 International Units per day provided a sufficient amount of calcium and phosphorus is offered. The best combination of the necessary minerals with vitamin D is found in milk, which may be fortified with vitamin D. The need of adults for vitamin D appears to be somewhat smaller but exact data are not available. Pregnant and lactating women[451]

[447] C. A. Ashford, *Biochem. J.*, 24, 661 (1930). L. I. Harris and J. R. M. Innes, *Ibid.*, 25, 367 (1931).
[448] A. F. Morgan, J. B. Hendricks and R. M. Freytag, *Proc. Am. Soc. Biol. Chem.*, 1941, XCII.
[449] P. C. Jeans and G. Stearns, *J. Am. Med. Assoc.*, 111, 703 (1938).
[450] H. A. Hunscher, F. C. Hummel and I. G. Macy, *Proc. Soc. Exptl. Biol. Med.*, 35, 189 (1936).
[451] K. W. Tovernd and G. Tovernd, *Acta Paediat.* (Suppl. 2), 12, 1 (1936).

are advised to take at least 800 I. U. per day. Babies born prematurely and twins need increased amounts. (See also page 613 for the recommended daily allowances as established by the National Research Council.)

These requirements, as stated, pertain only to the ingested forms of vitamin D and to the optimum daily intake of normal organisms for protection against rickets. To cure rickets in infants a daily dose of from 500 to 1500 International Units is usually given. The method of supplying the body only once every three to six months instead of every day is applied in the so-called shock-therapy. Massive doses of 200,000 to 1,000,000 International Units have been recommended and used for this purpose.[452, 453, 454]

The vitamin D requirement of poultry has been studied extensively because of its practical importance and is usually expressed in vitamin D content per pound of feed. It has been recommended to incorporate about 180 A.O.A.C. chick units of vitamin D per pound of total feed for growing chicks, while for the laying stock 360 A.O.A.C. chick units and for the breeding stock 540 A.O.A.C. units are required.

The vitamin D requirement of species other than man and poultry has not been investigated quantitatively. Some data have been presented which indicate that dogs, especially dogs of large breeds such as Great Dane, Setter, Airdale and German Shepherd, need considerably more vitamin D than small breeds such as Terrier and Spaniel.[455] In terms of vitamin D_2, small breeds need about 28 U. S. Pharmacopoeia Units per kg. of body weight, while large breeds need ten times this amount or even more. It seems conceivable that this species difference is due to the compound specificity of the vitamin D_2 used and that the vitamin D requirement of dogs is more uniform and considerably lower when other forms of vitamin D, such as vitamin D_3, are fed. In dogs, a greater tendency toward rickets has been observed in males than in females.[456]

Swine and especially young pigs need vitamin D, and when reared on a low vitamin D intake became definitely rachitic especially in the winter months.[457] Vitamin D given to pigs resulted also in a considerable decrease in the amount of feed necessary for maximum growth.[458] A daily ad-

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[452] S. Gunnarson, *Acta Paediat.*, **25**, 69 (1939).

[453] J. Ström, *Ibid.*, **25**, 251 (1939).

[454] G. O. Harnapp, *Klin. Wochschr.*, **15**, 1043 (1936); *Monatsschr. Kinderheilk.*, **71**, 193 (1937); *Klin. Wochschr.*, **17**, 390 (1938).

[455] A. F. Morgan, *North Am. Vet.*, **21**, 462 (1940).

[456] C. R. Stockard, *Am. J. Diseases Children*, **36**, 310 (1928).

[457] G. Bohstedt, R. M. Bethke, B. H. Edgington and W. L. Robinson, *Ohio Expt. Station, Bull.* **395** (1926). W. C. Skelley, *N. J. Expt. Station, Bull.* **661** (1939).

[458] R. D. Sinclair, *Sci. Agr.*, **9**, 629 (1929).

ministration of approximately 110 U. S. Pharmacopoeia Units vitamin D_2 has been suggested for pigs per kg. of body weight. Sheep also benefit from an intake of vitamin D[459] and many reports have been published showing the need of cattle and especially of calves for vitamin D.[460] A daily intake of about 200 U. S. Pharmacopoeia Units vitamin D per kg. of body weight has been suggested for these animals. For horses 200 to 2000 U. S. Pharmacopoeia Units have been given per kg. of body weight for the cure of rickets.[461]

[459] D. W. Auchinachie and A. H. H. Fraser, *J. Agr. Sci.*, 22, 560 (1932).

[460] I. W. Rupel, G. Bohstedt and E. B. Hart, *Wisconsin Expt. Station, Research Bull.* 115 (1933). N. W. Hilston (Thesis), The Pennsylvania State College, 1937. T. W. Gulliken, L. S. Palmer and W. L. Boyd, *Minnesota Agr. Stat. Techn. Bull.* 105 (1935).

[461] J. H. Kinter and R. L. Holt, *Philippine J. Sci.*, 49, 1 (1932).

THE GROUP OF VITAMINS E

THE GROUP OF VITAMINS E

The physiological effect of vitamin E is brought about by a series of naturally occurring compounds, which are chemically very closely related, being homologs and isomers of each other. Three different compounds have been isolated, which are designated as α-, β-, and γ-tocopherol. The possibility that other vitamin E factors may occur in the animal or plant organism cannot be excluded, but no definite proof for the existence of other compounds can be offered.

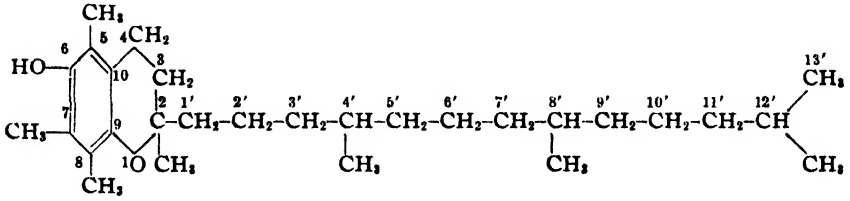
1. Nomenclature and Survey

Names:

Vitamin E.[1, 2]
Tocopherols[3] (tokos (Greek) meaning childbirth, phero (Greek) meaning to bear).
Anti-encephalomalacia vitamin.
Factor X.[1]
Antisterility factor.
Reproductive vitamin.
Sterilamine.[4]
Fertility vitamin.

The members of the group of vitamins E:

1. *α-Tocopherol*, $C_{29}H_{50}O_2$.
   Synonym: 5,7,8-Trimethyl-tocol.[5]



[1] H. M. Evans and K. S. Bishop, *Science*, **56**, 650 (1922); *Am. J. Physiol.*, **63**, 396 (1922); *J. Am. Med. Assoc.*, **81**, 889 (1922); *J. Metabolic Research*, **1**, 319, 335 (1922).

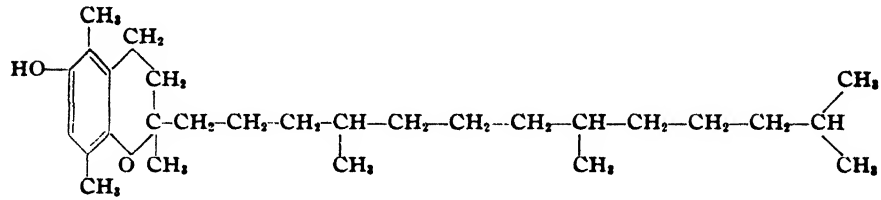
[2] B. Sure, *J. Biol. Chem.*, **59**, 19 (1924).

[3] H. M. Evans, O. H. Emerson and G. A. Emerson, *Ibid.*, **113**, 319 (1936).

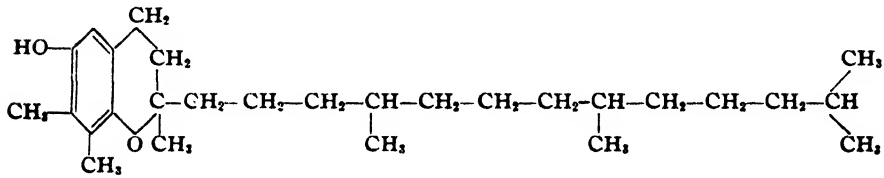
[4] R. L. Jones, *Science*, **68**, 480 (1928).

[5] P. Karrer and H. Fritzsche, *Helv. Chim. Acta*, **21**, 1234 (1938), proposed the term "tocol" for the general class of tocopherols without substituents in the benzene nucleus. The single members are indicated by following the Geneva-nomenclature principle for chromans. Accordingly, α-tocopherol is 5,7,8-trimethyl-tocol, β-tocopherol is 5,8-dimethyl-tocol, etc.

2. *β-Tocopherol*, $C_{28}H_{48}O_2$.
Synonyms: Cumo-tocopherol,[6] neo-tocopherol, *p*-xylo-tocopherol,[7] 5,8-dimethyl-tocol.[5]



3. *γ-Tocopherol*, $C_{28}H_{48}O_2$.
Synonyms: *o*-Xylo-tocopherol,[7] 7,8-dimethyl-tocol.[5]



Efficacy:

1 g. α-tocopherol = 400 Rat Units.
1 g. β-tocopherol = 200 Rat Units.
1 g. γ-tocopherol = 200 Rat Units.
1 g. synthetic racemic tocopherol acetate (*dl*-α-tocopherol acetate) = 1000 International units.

2. Chronology

1920 MATILL and CONKLIN[8] observed disturbances in the reproduction of rats on special milk diets.

1922 EVANS and BISHOP[9] reported failure of rats to reproduce when on a purified diet and recognized the missing factor as a vitamin.

1927 EVANS and BURR[10] recognized that vitamin E is non-saponifiable.

1928–1930 EVANS and BURR[11] and GOETTSCH and PAPPENHEIMER[12] described the

[6] W. John, *Z. physiol. Chem.*, **250**, 11 (1937).

[7] According to a suggestion by O. H. Emerson and L. I. Smith, *J. Am. Chem. Soc.*, **62**, 1869 (1940), the substitution in the aromatic ring of the tocopherol structure is indicated in the name of the tocopherol by a prefix which describes the substitution in terms of simple benzene derivatives, such as *o*-, *m*- and *p*-xylo-tocopherol, tolu-tocopherols, etc.

[8] H. A. Matill and R. E. Conklin, *J. Biol. Chem.*, **44**, 137 (1920).

[9] H. M. Evans and K. S. Bishop, *Science*, **56**, 650 (1922); *Am. J. Physiol.*, **63**, 396 (1922); *J. Am. Med. Assoc.*, **81**, 889 (1922); *J. Metabolic Research*, **1**, 319, 335 (1922).

[10] H. M. Evans and G. O. Burr, *Mem. Univ. Calif.*, No. 8 (1927).

[11] H. M. Evans and G. O. Burr, *J. Biol. Chem.*, **76**, 273 (1928).

[12] M. Goettsch, *Proc. Soc. Exptl. Biol. Med.*, **27**, 564 (1930). A. M. Pappenheimer, *Ibid.*, **27**, 567, 568 (1930). M. Goettsch and A. M. Pappenheimer, *J. Exptl. Med.*, **54**, 145 (1931).

occurrence of a specific muscular dystrophy in rats, rabbits and guinea pigs on a vitamin E-deficient diet.

1936 EVANS, EMERSON and EMERSON[13] isolated two different vitamins E (α- and β-tocopherol) in the form of crystallized esters.

1937–1938 FERNHOLZ[14, 15] elucidated the chemical structure of α-tocopherol.

1938 KARRER, followed by L. I. SMITH and by TODD synthesized tocopherol.[16]

3. Occurrence

The group of vitamins E occurs predominantly in plant materials. The animal organism contains only small amounts.

The best natural source of vitamins E is vegetable oils, such as wheat germ oil which contains especially high amounts. Varying amounts of this group of vitamins are found in cottonseed oil,[17] rice germ oil and other seed germ oils.[18] Olive oil does not contain any vitamins E, arachis oil contains traces.[19] Lettuce, alfalfa, etc., contain considerable amounts, oranges and bananas small amounts. Animal materials contain little vitamin E. The highest amount has been found[20] in livers (of horse and cattle, but not of rats[21, 22]) and small amounts are present in the muscles, heart, kidneys, placenta, milk and eggs. Fish liver oils, which are especially rich in the vitamins A and D, are poor in vitamin E.

Different vitamins E or different mixtures of vitamins E occur in the various natural sources. Thus, α- and β-tocopherols have been found in wheat germ oils but not always in the same relative proportions. In European oil the β-compound is the main principle, while in American sources the α-form is the predominant factor with smaller quantities of γ-tocopherol.[23] Cottonseed oil, palm oil and corn oil[24] contain predominantly γ-tocopherol besides small amounts of α-tocopherol.

The tocopherols occur in the free form,[25] at least to a considerable extent, in the seed oils. It is believed that some occur esterified, but no definite data concerning this are available.

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[13] H. M. Evans, O. H. Emerson and G. A. Emerson, *J. Biol. Chem.*, **113**, 319 (1936).

[14] E. Fernholz, *J. Am. Chem. Soc.*, **59**, 1154 (1937).

[15] E. Fernholz, *Ibid.*, **60**, 700 (1938).

[16] See the literature references on page 445.

[17] H. S. Olcott, *J. Biol. Chem.*, **107**, 471 (1934).

[18] H. M. Evans and G. O. Burr, *Proc. Natl. Acad. Sci. U. S.*, **11**, 334 (1925). H. S. Olcott and H. A. Matill, *J. Biol. Chem.*, **104**, 423 (1934).

[19] A. L. Bacharach, E. Allchorne and H. E. Glynn, *Biochem. J.*, **31**, 2287 (1937).

[20] P. Karrer, W. Jaeger and H. Keller, *Helv. Chim. Acta*, **23**, 464 (1940).

[21] W. F. J. Cuthbertson, R. R. Ridgeway and J. C. Drummond, *Biochem. J.*, **34**, 34 (1940).

[22] T. Moore, A. J. P. Martin and K. R. Rajagopal, *Soc. Chem. Ind. Food Group*, **1939**, 41.

[23] A. R. Todd, F. Bergel and T. S. Work, *Biochem. J.*, **31**, 2257 (1937).

[24] O. H. Emerson, G. A. Emerson and H. M. Evans, *Science*, **89**, 193 (1939).

[25] A. R. Moss and J. C. Drummond, *Biochem. J.*, **32**, 1953 (1938).

The tocopherols occur naturally together with other compounds of unknown constitution which are even stronger antioxidants than the vitamins E and which protect the vitamins against oxidation.

4. Isolation

The isolation of the vitamins E is usually carried out by first isolating the unsaponifiable part of the vitamin E-containing material. Wheat germs, for example, are dried and extracted with an organic solvent, such as chloroform, ether, etc. Another method is to press the germs, to collect the oil and to extract the residues. The total fats are then saponified at room temperature with, for example, 20% alcoholic potassium hydroxide in the absence of oxygen and the non-saponifiable part (approximately 5% of the oil) is extracted with an organic solvent. Sterols constitute up to 90% of the non-saponifiable material and are separated by crystallization from suitable solvents such as alcohols, pentane, etc. The last traces are then removed by precipitation with digitonin. The remaining oil can be purified by distillation, the vitamin E-containing fraction being carried over at 200–250° C. at 0.1 mm. pressure.[26, 27, 28] Some of the vitamin is, however, lost in this procedure. Some purification of the unsaponifiable mass can be achieved by partition between different solvents, such as methanol and petroleum ether,[29, 30] whereby the vitamin goes into the latter. By utilization of the principle of the chromatographic adsorption on aluminum oxide, a certain further purification can be achieved.[31, 32]

Instead of first isolating the non-saponifiable part, the vitamins E can also be obtained in improved yields directly from, for example, wheat germ oils, by application of the principle of the chromatographic adsorption method.[33]

Final isolation of the vitamins E is achieved by precipitation in the form of a crystallized ester. The allophanates are especially useful for this purpose.[34] By fractional crystallization of these esters, the members of the group of vitamins E are separated. The most insoluble fraction represents the α-tocopherol, and from its mother liquors the allophanates of the β-

[26] H. S. Olcott, *J. Biol. Chem.*, **107**, 471 (1934).
[27] H. S. Olcott and H. A. Matill, *Ibid.*, **104**, 423 (1934). H. S. Olcott, *Ibid.*, **110**, 695 (1935).
[28] F. W. Quackenbush, H. L. Gottlieb and H. Steenbock, *Ind. Eng. Chem.*, **33**, 1276 (1941).
[29] H. M. Evans, O. H. Emerson and G. A. Emerson, *Ibid.*, **113**, 319 (1936).
[30] A. R. Todd, F. Bergel and T. S. Work, *Biochem. J.*, **31**, 2257 (1937).
[31] J. C. Drummond, E. Singer and R. J. MacWalter, *Ibid.*, **29**, 456, 2510 (1935).
[32] J. C. Drummond and A. A. Hoover, *Ibid.*, **31**, 1852 (1937).
[33] A. R. Moss and J. C. Drummond, *Ibid.*, **32**, 1953 (1938).
[34] H. M. Evans, O. H. Emerson and G. A. Emerson, *J. Biol. Chem.*, **113**, 319 (1936).

isomer are recovered. The γ-isomer has been isolated from a different source by the same technic.[35]

An efficient separation of the α- and β-tocopherols is also achieved by the adsorption method.

5. Properties

The α-, β- and γ-isomers of tocopherol are oils which have not been obtained in the crystalline state. Certain esters, however, such as the

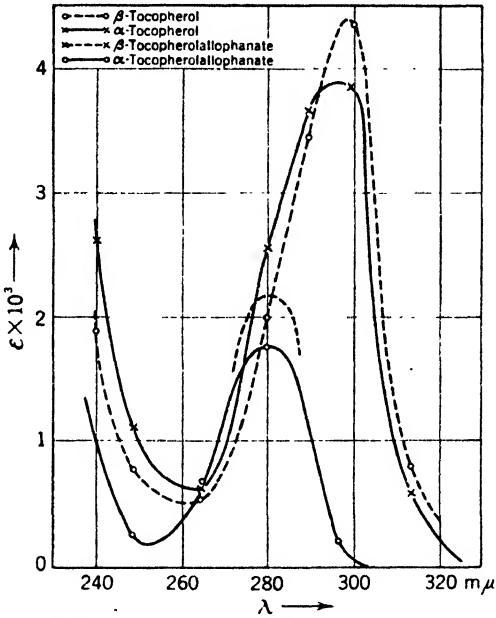


Fig. 23.—Absorption spectra of α- and β-tocopherol and of their allophanates. (H. Rudy in W. Stepp, *Ernährungslehre.*)

allophanates, *p*-nitrophenyl-urethanes and 3,5-dinitro-benzoates are crystalline. The tocopherols have a characteristic absorption spectrum in the ultraviolet with a maximum at 295 mμ which is displaced to 285 mμ in the esters, such as the acetates. The extinction coefficient for α-tocopherol, for example, is $E_{1\text{ cm.}}^{1\%}$ = approximately 77, while the coefficient of the esters is reduced to approximately 42 (Fig. 23).

In the absence of oxygen, the vitamins E are stable to heat treatment up to 200° C. and are not affected by sulfuric or hydrochloric acid up to 100° C.

[35] O. H. Emerson, G. A. Emerson and H. M. Evans, *Science*, **83**, 421 (1936).

Alkali destroys the vitamins of this group only very slowly, so that they can be obtained by alkaline saponification. The tocopherols are, however, quite sensitive to oxidation, which process destroys the biological activity.

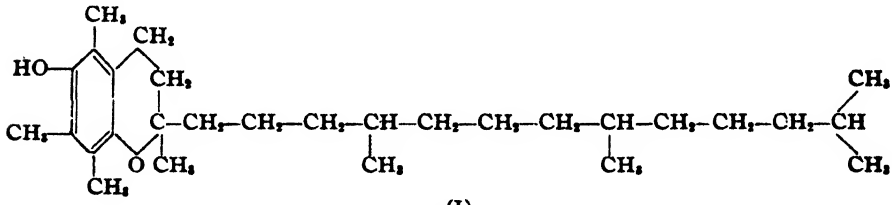
The vitamins E are soluble in all lipoid solvents, but are insoluble in water. They are fairly stable to visible light, but are destroyed readily by ultraviolet light.[36]

The tocopherols are effective antioxidants, the γ-isomer being more effective than the β-isomer, which in turn is more effective than the α-isomer.[37] Thus the antioxidant power is the reverse of the vitamin activity and is dependent upon the presence of a free phenolic hydroxyl group, which is not necessarily important for the vitamin action (see page 449).

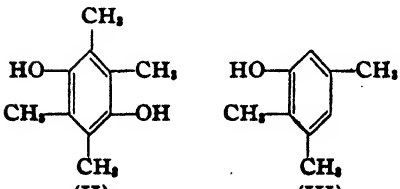
6. Chemical Constitution

(*a*) *α-Tocopherol*

α-Tocopherol has the empirical formula $C_{29}H_{50}O_2$. One of the oxygens is present in the form of a free hydroxyl group since the vitamin readily forms esters[38, 39, 40] and ethers. The phenolic character of this hydroxyl group



(I)
α-Tocopherol



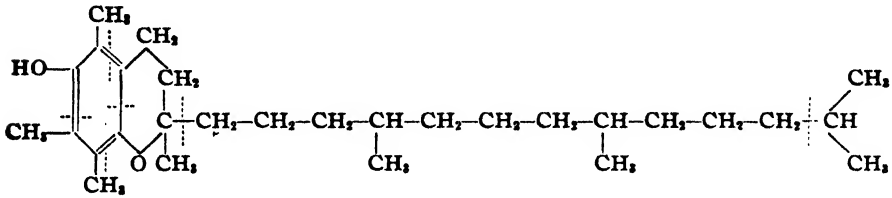
(II)
Duroquinone

(III)
Isopseudo-cumenol

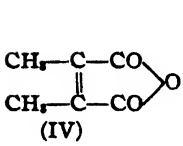
[36] J. C. Drummond, E. Singer and R. J. MacWalter, *Biochem. J.*, **29**, 456, 2510 (1935).
[37] H. S. Olcott and O. H. Emerson, *J. Am. Chem. Soc.*, **59**, 1008 (1937).
[38] H. S. Olcott, *J. Biol. Chem.*, **107**, 471 (1934).
[39] H. S. Olcott, *Ibid.*, **110**, 695 (1935).
[40] H. S. Olcott and H. A. Matill, *Ibid.*, **104**, 423 (1934).

was suspected on the basis of the change of the absorption spectrum upon esterification.[41] Upon pyrolysis, α-tocopherol yields[42] duroquinone (II), while isopseudo-cumenol (pseudo-cumenol-6) (III) is obtained by heating with hydriodic acid.[44] Upon hydrogenation four mols of hydrogen are absorbed.[43]

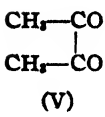
Further insight into the structure of α-tocopherol was obtained by oxidative degradation with chromic acid, whereby the following reaction products were isolated: dimethyl-maleic anhydride (IV), diacetyl (V), acetone (VI), a C_{21}-lactone (VII), a C_{18}-ketone (VIII) and a C_{16}-acid (IX).[15]



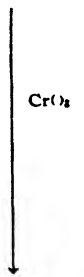
(I)

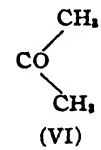


(IV)

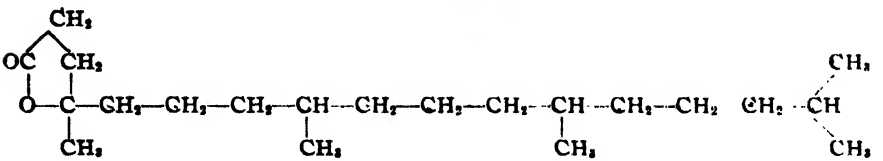


(V)





(VI)



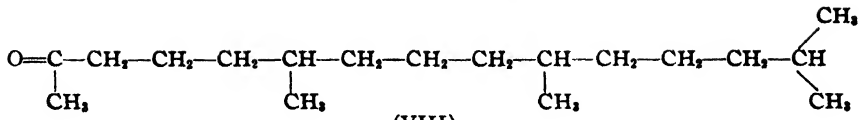
(VII)

[41] W. John, *Z. physiol. Chem.*, **250**, ii (1937). W. John, E. Dietzel and P. Günther, *Ibid.*, **252**, 208 (1938); *Naturwissenschaften*, **26**, 366 (1938). W. John, *Z. physiol. Chem.*, **252**, 222 (1938).

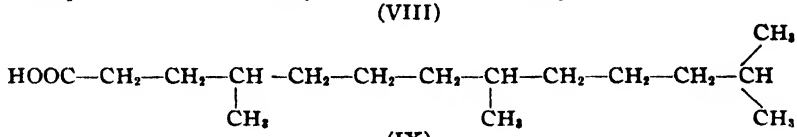
[42] E. Fernholz, *J. Am. Chem. Soc.*, **59**, 1154 (1937).

[43] F. Bergel, A. R. Todd and T. S. Work, *J. Chem. Soc.*, **1938**, 253.

[44] W. John, *Z. physiol. Chem.*, **250**, ii (1937). W. John, E. Dietzel and P. Günther, *Ibid.*, **252**, 208 (1938); *Naturwissenschaften*, **26**, 366 (1938). W. John, *Z. physiol. Chem.*, **252**, 222 (1938).



(VIII)



(IX)

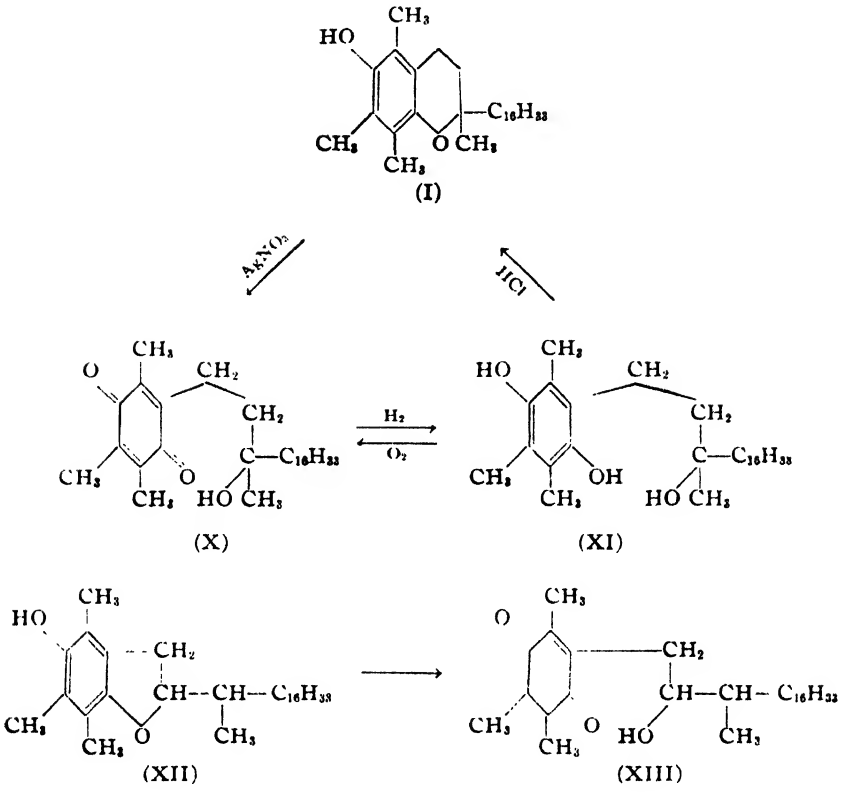
Of these, the C_{21}-lactone is of greatest importance. The hydroxy-acid, from which the lactone is derived, lactonizes readily thus indicating that the hydroxyl group is in either γ- or δ-position to the carboxyl group. The methyl-ester of the hydroxy-acid could not be oxidized to a keto-acid which proves that the hydroxyl group is tertiary. Additional evidence for this fact is furnished by the difficulty encountered upon attempted esterification of this hydroxyl group. The C_{16}-acid (IX) served for a determination of the side chain methyl groups, which indicated the presence of three. In analogy to many naturally occurring compounds of the terpene family which follow the isoprene rule, formula (IX) was postulated for the C_{16}-acid and formula (I) for α-tocopherol. The correctness of this formula, at least as far as the structure of the aliphatic side chain is concerned, is evident from the successful synthesis of this vitamin from phytol.

The formula of α-tocopherol (I) shows the presence of a chroman ring system. That such a ring system and not a coumaran structure occurs was proved[44] by careful oxidation with ferric chloride or with silver nitrate, which yielded a yellow quinone (X).[45] This undergoes reduction to a hydroquinone, which is a hydroxy-quinol ("Tocopheryl-quinol") (XI). Upon esterification of the phenolic hydroxyl groups the remaining aliphatic hydroxyl group was investigated and showed all the properties of a tertiary hydroxyl group in oxidation and esterification experiments. A coumaran compound (XII), on the other hand, should have given a secondary hydroxyl group (XIII). The hydroxy-quinol (XI) can be reconverted into α-tocopherol by treatment with strong mineral acids.

The tocopherols have three asymmetric centers, namely, at carbon atoms 2, 4′ and 8′. It is still an open question whether or not each of the naturally occurring tocopherols represents one of the eight possible isomers. Available evidence indicates that the natural products may be racemic about all three asymmetric centers.[46]

[45] P. Karrer, R. Escher, H. Fritzsche, H. Keller, B. H. Ringier and H. Salomon, *Helv. Chim. Acta*, **21**, 939 (1938).

[46] P. Karrer, H. Koenig, B. H. Ringier and H. Salomon, *Ibid.*, **22**, 1139 (1939).



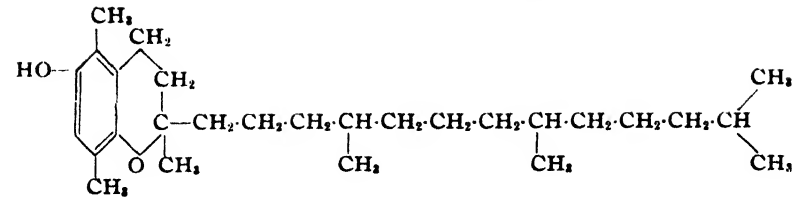
(b) β-Tocopherol

β-Tocopherol has the empirical formula $C_{28}H_{48}O_2$ and thus differs from the empirical formula of α-tocopherol only by one CH_2-group. Upon pyrolysis of the β-compound, trimethyl-hydroquinone[47, 48] (ψ-cumoquinol) (II) resulted and upon cleavage with hydriodic acid, *p*-xylenol (III) was obtained. Oxidation of β-tocopherol with chromic acid yielded the same C_{21}-lactone (IV)[49] which was obtained from the α-isomer. β-Tocopherol is thus the lower homolog of α-tocopherol with only two methyl groups in the aromatic ring of the molecule. These two methyl groups are in *p*-position to each other. β-Tocopherol has therefore the structure (I) which was proved by synthesis.

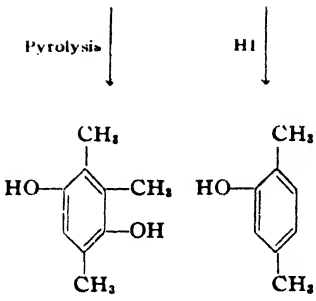
[47] W. John, *Z. physiol. Chem.*, **250**, ii (1937). W. John, E. Dietzel and P. Günther, *Ibid.*, **252**, 208 (1938); *Naturwissenschaften*, **26**, 366 (1938). W. John, *Z. physiol. Chem.*, **252**, 222 (1938).

[48] F. Bergel, A. R. Todd and T. S. Work, *J. Soc. Chem Ind.*, **56**, 1054 (1937).

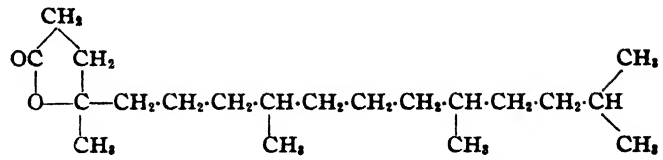
[49] O. H. Emerson, *J. Am. Chem. Soc.*, **60**, 1741 (1938).



(I)



(II) (III)



(IV)

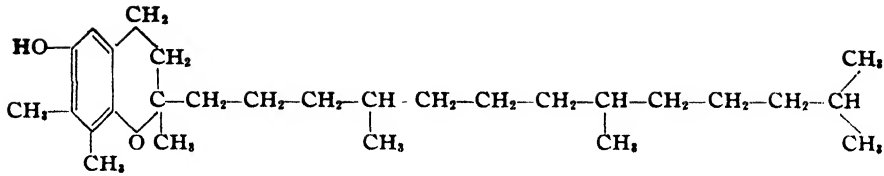
One position in the benzene nucleus of β-tocopherol is not substituted. This fact could be proved by the introduction of an allyl group by means of allyl bromide and zinc chloride.[50]

(*c*) *γ-Tocopherol*

γ-Tocopherol has the empirical formula $C_{28}H_{48}O_2$ and is therefore an isomer of β-tocopherol. Pyrolysis yields the same compound as does the pyrolysis of β-tocopherol, namely, trimethyl-hydroquinone. Oxidation with chromic acid yields dimethyl-maleic anhydride.[51] γ-Tocopherol is thus *o*-xylo-tocopherol (I):

[50] P. Karrer, R. Escher, H. Fritzsche, H. Keller, B. H. Ringier and H. Salomon, *Helv. Chim. Acta*, 21, 939 (1938).

[51] O. H. Emerson and L. I. Smith, *J. Am. Chem. Soc.*, 62, 1869 (1940).



(I)

7. Synthesis

The three known vitamins E, α-, β- and γ-tocopherol have been synthesized according to the same principle: Alkylated hydroquinone is reacted with phytyl halide in the presence of a catalyst to form, in one reaction, the desired chroman derivative. Thus trimethyl-hydroquinone (I) condenses with phytyl bromide (II) in benzene solution in the presence of zinc chloride to give in almost quantitative yield *dl*-α-tocopherol (III).[52] Instead of the phytyl halide, phytol[53] or phytadiene[54] can be used successfully. The condensation with any of these three phytyl compounds can be accomplished without catalysts. The synthetic *dl*-form (racemic about position 2 and perhaps about carbon atoms 4′ and 8′) can be resolved by esterification with bromo-camphor-sulfonic acid to yield an ester which appears to be identical with the naturally occurring α-tocopherol.[55, 56]

The syntheses of β- and of γ-tocopherol have been carried out by the same methods.[57] Isomeric xylo-hydroquinones have been reacted with phytyl compounds to yield the racemic β-tocopherol (V) from *p*-xylo-hydroquinone (IV) and the racemic γ-tocopherol (IX) from *o*-xylo-hydroquinone (VIII). A number of by-products are obtained in these reactions by condensation of the hydroquinone with two molecules of the phytyl compound. The yield of these by-products is greater when zinc chloride is used as a catalyst than when formic acid is employed.[58] The two types of by-products which are obtained from, for example, *p*-xylo-hydroquinone, are shown in formulas (VI) and (VII). Better yields of the tocols are also obtained when mono-esters of the quinols, for example, the mono-ben-

[52] P. Karrer, H. Fritzsche, B. H. Ringier and H. Salomon, *Helv. Chim. Acta*, 21, 520, 820 (1938); *Nature*, 141, 1057 (1937).

[53] F. Bergel A. M. Copping, A. Jacob, A. R. Todd and T. S. Work, *Nature*, 142, 36 (1938); *J. Chem. Soc.*, 1938, 1382.

[54] L. I. Smith, H. E. Ungnade and W. W. Prichard, *Science*, 88, 37 (1938).

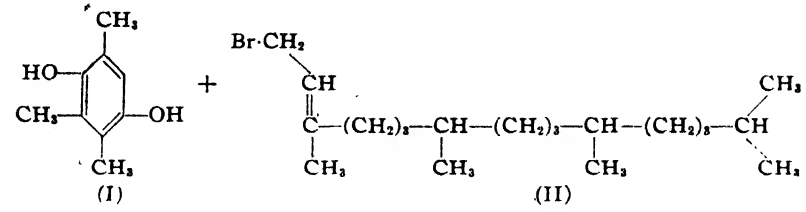
[55] P. Karrer, H. Fritzsche, B. H. Ringier and H. Salomon, *Helv. Chim. Acta*, 21, 520, 820 (1938); *Nature*, 141, 1057 (1937).

[56] P. Karrer, H. Koenig, B. H. Ringier and H. Salomon, *Helv. Chim. Acta*, 22, 1139 (1939).

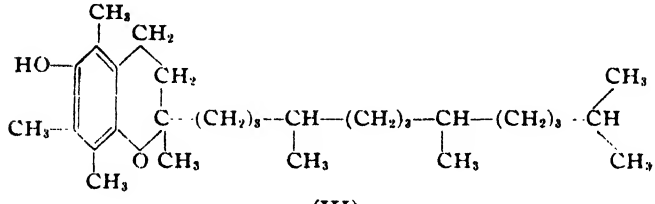
[57] P. Karrer and H. Fritzsche, *Ibid.*, 21, 1234 (1938); 22, 260 (1939).

[58] P. Karrer, H. Koenig, B. H. Ringier and H. Salomon, *Ibid.*, 22, 1139 (1939).

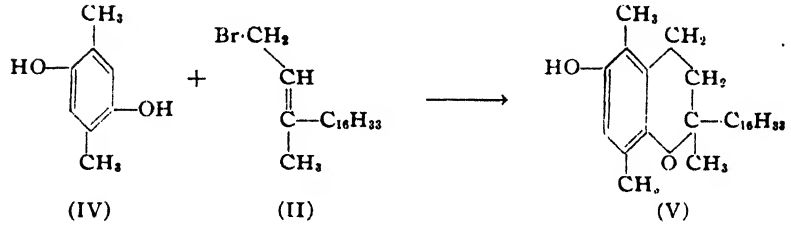
zoates, instead of the free quinols are used for the condensation.[59] Subsequently the ester group is removed by hydrolysis.



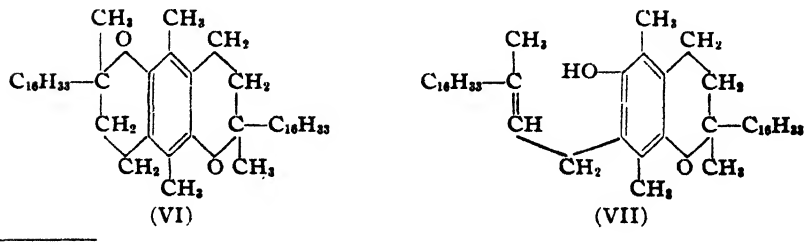
(I) (II)



(III)

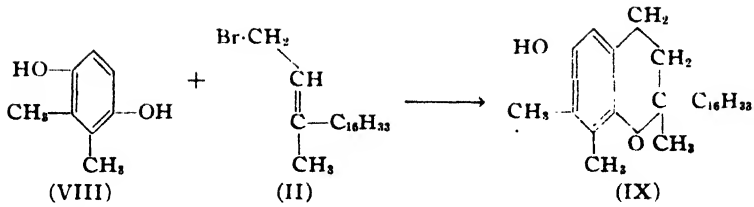


(IV) (II) (V)



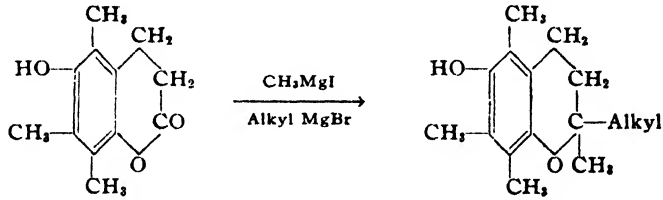
(VI) (VII)

[59] A. Jacob, M. Steiger and A. R. Todd, *J. Soc. Chem. Ind.*, **57**, 1188 (1938).



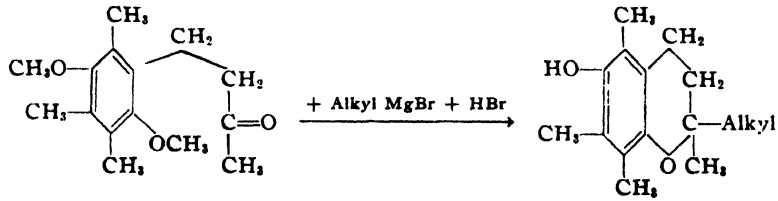
(VIII) (II) (IX)

An entirely different type of synthesis should be mentioned which has no utility for the preparation of the tocopherols proper, but which has been used successfully for the preparation of similar compounds with different aliphatic side chains. 5,7,8-Trimethyl-6-hydroxy-3,4-dihydro-coumarin is condensed with a mixture of methyl-magnesium-halide and alkyl-magnesium-halide to yield the desired tocopherol-homolog.[60]



(I) (II)

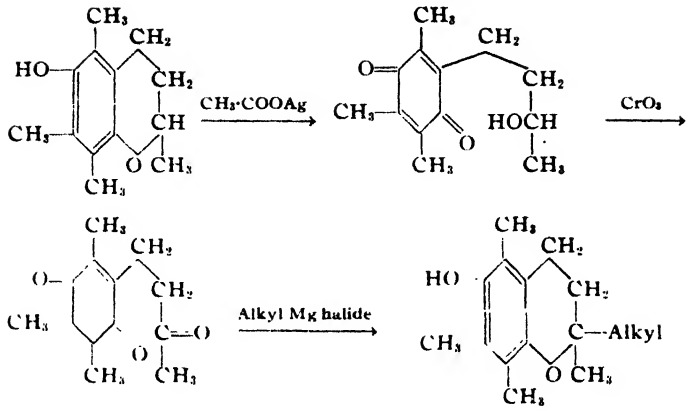
Again a different type of synthesis, not well suited for the tocopherols themselves but attractive for certain of their homologs (IV), is the Grignard reaction of the ketone (III)[61, 62] with an alkyl-magnesium-halide, followed by treatment with hydrobromic acid:



(III) (IV)

A last method should be mentioned which has been used for the preparation of tocol homologs. This consists of the introduction of a second alkyl group in the 2-position of a 2-methyl-6-hydroxy-chroman according to the reaction scheme:[63]

[60] W. John, P. Günther and M. Schmeil, *Ber.*, **71**, 2637 (1938).
[61] W. John and P. Günther, *Ibid.*, **72**, 1649 (1939).
[62] L. I. Smith, H. E. Ungnade, J. W. Opie, W. W. Prichard, R. B. Carlin and E. W. Kaiser, *J. Org. Chem.*, **4**, 323 (1939).
[63] W. John and M. Schmeil, *Ber.*, **72**, 1653 (1939).



8. Industrial Methods of Preparation

Vitamin E is marketed in the form of concentrates from natural sources and in the form of the racemic *dl*-α-tocopherol obtained by synthesis. The latter is preferred by the medical profession. The methods used for these processes are those which have already been described in the sections on the isolation and synthesis. The synthetic tocopherol is sold in the form of the acetate which is at least as active as the free phenol and has the advantage of greater stability toward oxidation.[64] The most important industrial method for the concentration of vitamins E from natural oils is the short path, high vacuum molecular distillation. The natural vitamin E can be stabilized by the addition of antioxidants, such as hydroquinone or ascorbic acid.[64]

9. Biogenesis

Nothing definite is known about the biogenesis of the vitamins E. They are not synthesized in the animal organism, but in the plant. It can be assumed that the synthesis in the plant cells is similar in principle to the laboratory methods for the synthesis of the tocopherols. Phytol occurs in relatively large quantities in the plant, mainly in esterified form in chlorophyll but also in the free state as, for example, in germ oils.[65] There is some evidence for the assumption that vitamins E are synthesized preferentially in the green parts. If this is true, the vitamin is transported into the seeds, which usually contain higher amounts than, for example, the leaves. Of special interest is the observation that plant embryos, for example, wheat

[64] O. Isler, *Helv. Chim. Acta.*, **21**, 1756 (1938).
[65] A. R. Todd, F. Bergel and T. S. Work, *Biochem. J.*, **31**, 2257 (1937).

germs, contain more vitamin E than the seed contained and more per unit weight than the plant will contain at any other stage of its life.

The animal organism (for example, rat) cannot synthesize vitamins E as has been stated before. Such a synthesis *in vivo* does not occur, even when the starting materials of the laboratory synthesis, namely, phytol and trimethyl-hydroquinone, are fed to the animals.[66]

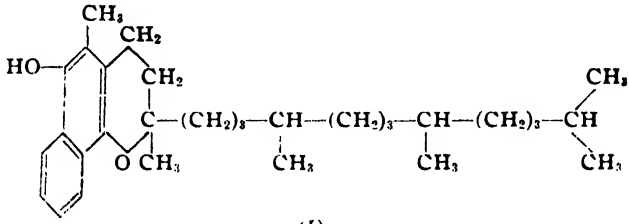
10. Specificity

Synthetic *dl*-α-tocopherol has the same biological efficacy as the naturally occurring α-tocopherol (2–3 mg. correspond to one Rat Unit). The β- and the γ-isomers are only half as active as the α-isomer (activity 5 mg.), whereas the *m*-xylo-tocopherol, which has not been found in nature, appears to be almost as active as the α-tocopherol (activity 3 mg.). Most esters of the tocopherols show excellent vitamin E efficacy,[67, 68] with the exception of the allophanates which are completely inactive. The acetate, propionate and butyrate are said to be even more active than the free vitamin.[68] The phosphoric acid ester of *dl*-α-tocopherol upon parenteral administration is more active than the vitamin itself.[69] Etherification brings about a complete loss of activity.

The tocopherol-quinones are not biologically active.[70, 71, 72, 73] The difference in activity (in rats) of the naturally occurring vitamins E points toward a relatively great specificity of this vitamin. This has been substantiated by the preparation and biological evaluation of a great number of compounds of more or less similar structure to the tocopherols. Varying the substituents of the benzene ring, it was found that 5,7-dimethyl-8-ethyl-tocol[74, 75] and a diethyl-methyl-tocol[76] are active in a minimum dose of 10 mg. while mono-methyl-tocol and 5,7-diethyl-tocol are not active even in doses of 40–50 mg.[77, 78] Tocol itself[78] and 6-desoxy-tocol,[79] at least up to 100 mg.-doses, are also inactive.

[66] H. M. Evans, O. H. Emerson, G. A. Emerson, L. I. Smith, H. E. Ungnade, W. W. Prichard, F. L. Austin, H. H. Hoehn, J. W. Opie and S. Wawzonek, *J. Org. Chem.*, **4**, 376 (1939).
[67] O. Isler, *Helv. Chim. Acta*, **21**, 1756 (1938).
[68] V. Demole, O. Isler, B. H. Ringier, H. Salomon and P. Karrer, *Ibid.*, **22**, 65 (1939).
[69] P. Karrer and G. Bussmann, *Ibid.*, **23**, 1137 (1940).
[70] P. Karrer and A. Geiger, *Ibid.*, **23**, 455 (1940).
[71] W. John, E. Dietzel and W. Emte, *Z. physiol. Chem.*, **257**, 180 (1939).
[72] P. Karrer, H. Salomon and H. Fritzsche, *Helv. Chim. Acta*, **21**, 309 (1938).
[73] M. D. Wright and J. C. Drummond, *Biochem. J.*, **34**, 32 (1940).
[74] P. Karrer, H. Koenig, B. H. Ringier and H. Salomon, *Helv. Chim. Acta*, **22** 1139 (1939).
[75] P. Karrer and O. Hoffmann, *Ibid.*, **22**, 654 (1939).
[76] P. Karrer and O. Hoffmann, *Ibid.*, **23**, 1126 (1940).
[77] P. Karrer and H. Fritzsche, *Ibid.*, **21**, 1234 (1938); **22**, 260 (1939).
[78] A. Jacob, F. K. Sutcliffe and A. R. Todd, *J. Chem. Soc.*, **1940**, 327.
[79] F. v. Werder, T. Moll and F. Jung, *Z. physiol. Chem.*, **257**, 129 (1939).

Of special interest is the naphtho-tocopherol (I) which shows vitamin E activity in 25-mg. doses, but also shows vitamin K activity in doses between 300 and 600 γ.[80]



(I)

The introduction of a double bond into the chroman ring with the formation of a *dl*-3,4-dehydro-α-tocopherol has no significant influence on the activity, which is about 6 mg.[81]

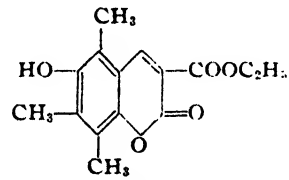
The structure of the long aliphatic side chain on the 2-position is of great importance for the biological efficacy. Shortening this chain by one isoprene unit,[82] that is by five carbon atoms, or by two such units[83] causes loss of the activity (tested up to 40 mg.). The same is true for similar substances which contain double bonds in the side chain, for example, for the compound prepared from trimethyl-hydroquinone and farnesyl-bromide.[84] Compounds which have instead of the long aliphatic side chain only a methyl group or no side chain at all are also inactive.[85] Activity in a 50–60-mg. dose has, however, been claimed for a 2-dodecyl-2,5,7,8-tetramethyl-6-oxy-chroman.[86]

A number of other compounds which are less closely related chemically to the tocopherols have been investigated for vitamin E activity. Surprisingly enough some have been found active, at least in the rat test which is commonly employed for the determination of vitamins E. All these other compounds, however, are active only in doses of an entirely different order of magnitude. Thus several hydroquinones, their esters and ethers have been found active. *o*- and *p*-Xylo-hydroquinone but not the *m*-derivative, are active in 100-mg. doses.[87] Trimethyl-hydroquinone and durohydroquinone, but not trimethyl-ethyl-hydroquinone, are active at the same level. A naphthoquinone, namely, the 2,3-dimethyl-5,6,7,8-

[80] M. Tishler, L. F. Fieser and N. L. Wendler, *J. Am. Chem. Soc.*, **62**, 1982 (1940).
[81] P. Karrer, R. G. Legler and G. Schwab, *Helv. Chim. Acta*, **23**, 1132 (1940).
[82] P. Karrer and K. A. Jensen, *Ibid.*, **21**, 1622 (1938).
[83] P. Karrer and K. S. Yap, *Ibid.*, **23**, 581 (1940).
[84] P. Karrer and K. A. Jensen, *Ibid.*, **21**, 1622 (1938).
[85] P. Karrer, R. Escher, H. Fritzsche, H. Keller, B. H. Ringier and H. Salomon, *Ibid.*, **21**, 939 (1938).
[86] W. John, P. Günther and M. Schmeil, *Ber.*, **71**, 2637 (1938).
[87] F. v. Werder, T. Moll and F. Jung, *Z. physiol. Chem.*, **257**, 129 (1939).

tetrahydro-1,4-naphthoquinone is reported to be active at high levels.[88] A considerable number of esters and of ethers of trimethyl- and of tetramethyl-hydroquinone have been tested and practically all of them show some activity at high levels.[87, 88, 89, 90] The simple phenols and phenol-ethers are inactive, but *o*-allyl-phenol, di-*o*-hexenyl-phenol and *p*-amino-*o*-allyl-phenol are active.[91]

Chroman itself is active, as are the 2,2-diethyl- and 2,2-di-*n*-butyl-derivatives but not the corresponding derivatives with odd numbers of carbon atoms. 2,5,7,8-Tetramethyl-chroman and 2,2,5,7,8-pentamethyl-6-hydroxy-chroman are also active. Quite a number of coumarins have been tested and were found to be inactive with the exception of compound (I) which is said to be active in doses as low as 20 mg. 2-Methyl-coumaran, 2,2,7-trimethyl-coumaran and 2,3,4,6,7-pentamethyl-5-hydroxy-coumaran show activity at about 100-mg. doses.



(I)

11. Determination

The only reliable method for evaluating vitamins E is the biological assay based on a comparison with the synthetic *dl*-α-tocopherol. The physical and chemical methods have a number of shortcomings, the most important of which is that it is impossible to differentiate between the α-, β- and γ-isomers, which have different biological efficacies. Furthermore, the necessity of applying isolation procedures in order to separate interfering substances causes considerable loss.

(*a*) *Physical Methods*

Spectroscopical Determination.[92, 93]—The determination of vitamin E in alcohol or cyclohexane solution by its characteristic absorption spectrum

[88] F. v. Werder and T. Moll, *Ibid.*, **254**, 39 (1938).
[89] H. M. Evans, O. H. Emerson and G. A. Emerson, *Science*, **88**, 193 (1938).
[90] E. Fernholz and J. Finkelstein, *J. Am. Chem. Soc.*, **60**, 2402 (1938).
[91] H. M. Evans, O. H. Emerson, G. A. Emerson, L. I. Smith, H. E. Ungnade, W. W. Prichard, F. L. Austin, H. H. Hoehn, J. W. Opie and S. Wawzonek, *J. Org. Chem.*, **4**, 376 (1939).
[92] T. Moore and K. R. Rajagopal, *Biochem. J.*, **34**, 335 (1940).
[93] W. F. J. Cuthbertson, R. R. Ridgeway and J. C. Drummond, *Ibid.*, **34**, 31 (1940).

with a maximum of 294 mμ can only be recommended for solutions of the pure or almost pure compounds. The naturally occurring substances with vitamin E activity comprise, besides α-tocopherol, its esters, which have different absorption maxima and less pronounced extinction coefficients, for example, α-tocopherol, $E_{1\,cm.}^{1\%}$, 284 mμ = about 77; α-tocopherol acetate, $E_{1\,cm.}^{1\%}$, 285 mμ = about 42. Furthermore, even traces of vitamin A interfere due to the strong absorption characteristics [$E_{1\,cm.}^{1\%}$, 328 mμ = 1725].

Natural materials, such as plant oils and the fat extract of animal materials, contain many other substances which have no vitamin E activity but exhibit absorption in the region of the vitamin E absorption spectrum and interfere therefore with the spectroscopical determination of the vitamin. Furthermore, any extraction of the vitamin from animal tissues causes some loss. In order to obtain any data, it is necessary to saponify the material, for example, the fat extract, germ oil, etc. The saponification destroys further amounts of the vitamin,[94] unless it is carried out under most carefully controlled conditions.[95]

A modification of the spectroscopical determination of the tocopherols consists in the spectroscopical determination of the oxidation product of the vitamin.[96] The vitamin is best oxidized[97] with a 5% solution of silver nitrate in 90% methyl alcohol. The quinone formed shows an absorption maximum at 265 mμ with an extinction coefficient about four times as high as that of the vitamin itself. Spectroscopical assays are run before and after the oxidation of the vitamin to exclude the values obtained from any previously oxidized material.

(*b*) *Chemical Methods*

Ferric Chloride-Dipyridyl Method.[98]—This method is based on the oxidation of vitamin E in alcoholic solution by ferric chloride.[99] An addition of α,α'-dipyridyl develops a characteristic red color with the resulting ferrous chloride. A blank is run with each determination. Working in subdued light is recommended, since sunlight tends to cause a development of color in the blank solution.

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[94] A. Emmerie and C. Engel, *Rec. trav. chim.*, **58**, 895 (1939).
[95] A. Emmerie, *Ibid.*, **59**, 246 (1940).
[96] W. John, *Soc. Chem. Ind. Food Group*, **1939**, 23.
[97] W. F. J. Cuthbertson, R. R. Ridgeway and J. C. Drummond, *Biochem. J.*, **34**, 34 (1940)
[98] A. Emmerie and C. Engel, *Nature*, **142**, 873 (1938); *Rec. trav. chim.*, **57**, 1351 (1938).
[99] J. Waddell and H. Steenbock, *J. Biol. Chem.*, **80**, 431 (1928).

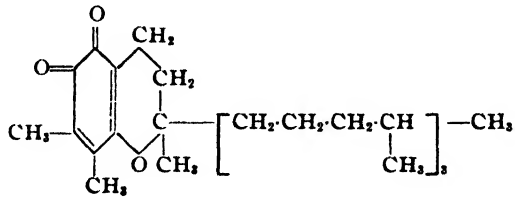
This method is not very specific since the color is given by all reducing compounds. Other substances, such as the carotenoids, interfere by obscuring the color of the ferrous dipyridyl. Although it is possible to estimate the vitamin E content of unsaponified oils, these oils often contain other reducing agents which can be removed by saponification. The best results are obtained by hydrolysis with 2 *N* KOH-methanol solution for 10 minutes.[100] It has, furthermore, been suggested that all interfering substances be adsorbed on, for example, floridin, which has been activated by treatment with hydrochloric acid.[101]

A modification of this procedure consists[102] in treating the material to be assayed in a hexane or petroleum ether solution with 85% sulfuric acid, followed by centrifuging and washing with dilute alkali. The interfering substances are largely removed and the vitamin E is determined in the remaining solution by ferric chloride and dipyridyl.

Gold Chloride Method.[103, 104]—This method consists in the oxidation of vitamin E with gold chloride at about 50° C. The progress of the reaction is followed electrometrically. The equilibrium is but slowly established.

The accuracy of this method, like that of the ferric chloride-dipyridyl method, is affected by other reducing substances, including carotenoids. For accurate determinations, this method requires larger quantities of the vitamin than needed in the ferric chloride-dipyridyl method.

Nitric Acid Method.[105]—Vitamin E, upon heating with alcoholic nitric acid, yields a characteristic red color. The coloration is caused by the production of the following compound:[106]



Since other naturally occurring substances give similar, or at least yellow colors, the use of a filter limited to the wave band 450–520 mμ has been

[100] A. Emmerie, *Rec. trav. chim.* **59**, 246 (1940).
[101] A. Emmerie and C. Engel, *Ibid.*, **58**, 283 (1939).
[102] W. E. Parker and W. D. McFarlane, *Can. J. Research*, **18**, 405 (1940).
[103] P. Karrer, R. Escher, H. Fritzsche, H. Keller, B. H. Ringier and H. Salomon, *Helv. Chem. Acta*, **21**, 939 (1938).
[104] P. Karrer and H. Keller, *Ibid.*, **21**, 1161 (1938).
[105] M. Furter and R. E. Meyer, *Ibid.*, **22**, 240 (1939).
[106] L. I. Smith, W. B. Irvin and H. E. Ungnade, *J. Am. Chem. Soc.*, **61**, 2424 (1939); *Science*, **90**, 334 (1939).

advocated. The color is then measured in one of the common types of colorimeters.

A distinct disadvantage of this method is the fact that the simple biologically inactive oxidation product of the tocopherols gives the same coloration with nitric acid. Thus, vitamin E destroyed, for example, by air oxidation or by ferric chloride, shows up in this test as being present as vitamin E. On the other hand, this method has the advantage that carotenoids do not interfere. Appropriately used, this assay procedure is very convenient and rapid.[107]

(*c*) *Biological Methods*

Fertility Test on Rodents.[108, 109] For biological determinations of the vitamin E, female animals (rats) are usually preferred, since they can be essentially healed several times from the avitaminosis, whereas in males (rats) the damage done by the absence of the vitamin is irreparable. Rats are used usually in these tests, although occasionally other animals, for example, the rabbit,[110] have been recommended. Female virgin rats preferably are used[111, 112] (at about 30 to 40 days after weaning), since the initial storage of the vitamin in the young can be controlled through placental and mammary transfer.

The tests can be carried out by prophylactic or by curative methods, but the latter is considered much more reliable. The criterion is the proof of the occurrence of gestation and resorption of the fetuses. This can be accomplished by following the weight increase and decrease, respectively, after mating. Conception is proved by the plug, that is, the bouchon vaginale. Successful implantation of the ova can be verified by the placental sign, that is, detection of blood in the vagina between the ninth and thirteenth days after mating. The percentage fertility is measured in this test. An improved method which is based on a graded response of the animals consists in the autopsy of all animals at the tenth day of pregnancy. The evaluation comprises a determination of the weight of the uterus, both before and after removal of living fetuses, dead fetuses and resorption sites. The results of this latter method are expressed in the so-called "uterine index."

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[107] H. E. Ungnade and L. I. Smith, *J. Org. Chem.*, **4**, 397 (1939).

[108] H. M. Evans and G. O. Burr, *Proc. Natl. Acad. Sci. U. S.*, **11**, 334 (1925). H. S. Olcott and H. A. Matill, *J. Biol. Chem.*, **104**, 423 (1934).

[109] H. M. Evans and G. O. Burr, *Mem. Univ. Calif.*, No. 8 (1927).

[110] C. G. Mackenzie and E. V. McCollum, *J. Nutrition*, **19**, 345 (1940).

[111] A. L. Bacharach, E. Allchorne and H. E. Glynn, *Biochem. J.*, **31**, 2287 (1937).

[112] A. L. Bacharach and E. Allchorne, *Ibid.*, **32**, 1298 (1938).

Fertility Test on Daphnia. The use of *Daphnia magna*, the transparent crustacea, as a test animal has been suggested for the quantitative estimation of vitamin E.[113] The criterion in this assay procedure is the growth of the ovaries and of the parthenogenic embryos in the brood sac.

12. Standards

Synthetic racemic alpha-tocopherol-acetate has been adopted as the international standard for vitamin E and it has been recommended that the International Unit for vitamin E be defined as the specific activity of 1 mg. of the standard preparation. This quantity is the average amount which when administered orally, prevents resorption-gestation in rats deprived of vitamin E.[114] The international standard is issued in the form of a solution in olive oil of which one International Unit is contained in 0.1 g.

One Rat Unit, or the "fertility dose," is the smallest amount of vitamin E which when given *per os* daily to resorption-sterile female rats for the entire period of gestation (21 days) brings about in 50% of the animals the birth of at least one living young.

One Rat Unit, or the fertility dose, is equal to 2–3 mg. of α-tocopherol.

The Pacini-Linn Unit is equal to $^1/_{10}$ of the "fertility dose."

The Bomskow Rat Unit[115] is the amount of vitamin E which when administered once during the first eight days of pregnancy prevents resorption.

13. Physiology of Plants and Microorganisms

The present-day knowledge of the significance of the occurrence of vitamins E in plants is very meager. It must be assumed that the tocopherols play a very definite role in the plant cells, and that this role is not primarily concerned with the antioxidative properties of the compounds. It is probably quite significant that the seeds usually contain higher amounts than any other part of the living plant. On the other hand, the germs, for example, wheat germs, contain even higher amounts. The possibility of an influence upon cell growth must therefore be considered.

Not all organisms contain tocopherols. The microorganism *Phycomyces*, for example, does not seem to contain any at all as evident from electrometric titration with gold chloride.[116]

[113] A. Vichoever and I. Cohen, *Am J. Pharm*., 110, 297 (1938). A Vichoever, *J. Assoc. Official Agr. Chem.*, 22, 715 (1939).

[114] E. M. Hume, *Nature*, 148, 472 (1941).

[115] C. Bomskow, *Arch. expil. Path. Pharmakol.*, 190, 627, 635 (1938)

[116] W. H. Schopfer and S. Blumer, *Z. Vitaminforsch.*, 9, 344 (1939)

Vitamin E, when injected into higher plants in olive oil solution, exerts an inhibitory effect upon the growth (experiments with *Mellandrium album* (Miller) Garke).[116]

14. Animal Physiology

(*a*) *Metabolism of Vitamins E*

Vitamins E and their esters are easily absorbed from the intestinal tract, especially in the presence of bile acids.[117] The vitamin is, however, not well utilized when given parenterally.[118] The vitamin is taken up by the blood in which its presence can be demonstrated. The blood of female rats contains approximately 5.6 γ and that of male rats 6.4 γ of tocopherols per 10 cc. of serum.[119] The blood level of rats can be increased up to 100 γ by oral administration of the vitamin. Upon an intake of esters, for example, the acetate, the free vitamin appears in the blood.

No significant excretion of vitamin E is observed[120] as long as normal doses of the vitamin are fed. The intake of excess doses causes the excretion of a certain amount in the feces,[119, 121] but only traces are found in the urine.[119] This means that the vitamin is inactivated in the organism, probably by an oxidation mechanism. Since, furthermore, no simple oxidation products of the tocopherols are excreted, the vitamin molecule must be broken down in the organism (rats).[119]

Vitamins E are stored to a small, but significant extent in animal body fats,[119, 122] in the muscles,[119, 122] and especially high amounts are found in the placenta and in the anterior lobe of the pituitary gland. Vitamin E is secreted in the milk,[123] and in the eggs of birds.

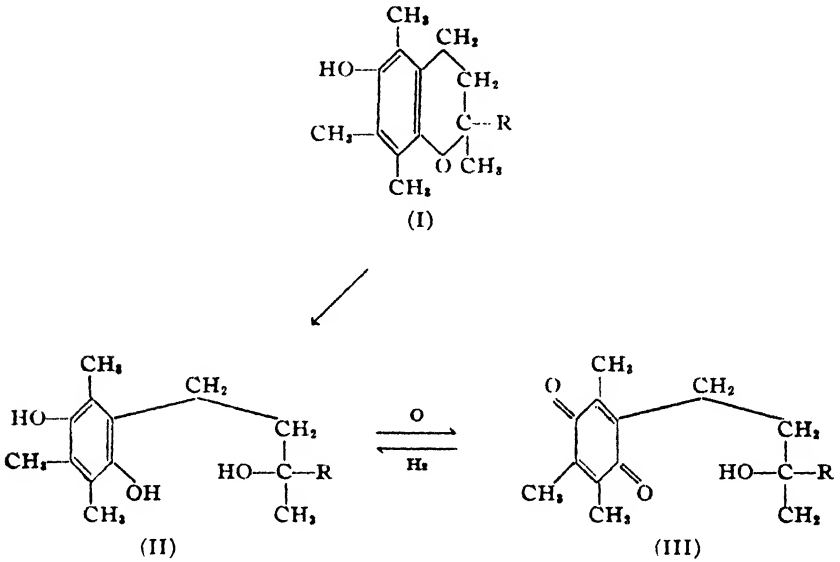
The liver of horse and cattle contains unusually high amounts,[124] but no vitamin E is stored in the liver of rats even on excessive dosage of the vitamin.[119, 125]

(*b*) *Physiological Action of Vitamins E*

Much work and much speculation concerning the physiological action of the vitamins E arose when the constitution of the vitamins became known.

[117] J. D. Greaves and C. L. A. Schmidt, *Proc. Soc. Exptl. Biol. Med.*, **37**, 40 (1937).
[118] M. Goettsch and A. M. Pappenheimer, *J. Nutrition*, **22**, 463 (1941).
[119] A. Emmerie and C. Engel, *Rec. trav. chim.*, **58**, 895 (1939); W. F. J. Cuthbertson, R. R. Ridgeway and J. C. Drummond, *Biochem. J.*, **34**, 34 (1940).
[120] C. S. McArthur and E. M. Watson, *Can. Chem. Proc. Ind.*, **23**, 350 (1939).
[121] A. Juhasz-Schäffer, *Arch. path. Anat. (Virchow's)*, **281**, 53 (1931).
[122] H. M. Evans and G. O. Burr, *Mem. Univ. Calif.*, No. 8 (1927).
[123] M. M. O. Barrie, *Biochem. J.*, **32**, 1474 (1938).
[124] P. Karrer, W. Jaeger and H. Keller, *Helv. Chim. Acta*, **23**, 464 (1940).
[125] T. Moore, A. J. P. Martin and K. R. Rajagopal, *Soc. Chem. Ind. Food Group*, **1939**, 11.

The fact that they are phenols and that they are antioxidants led to the supposition that vitamins E may take part in some oxidation-reduction mechanism. From a chemical standpoint it would be conceivable to assume that the vitamin acts in the following manner:



No support for this theory can, however, be offered. The quinone (III) which can be obtained by oxidation of the vitamin is completely inactive biologically.[126, 127, 128, 129] Thus a reduction to the quinol (II) in the cells seems quite improbable.

The many symptoms which can be observed during a deficiency of vitamin E points to the assumption that vitamin E is of importance to the tissues as a whole. Unconfirmed experimental findings to the effect that the organism of vitamin E-depleted animals contains unsaponifiable material which upon injection into rats causes the symptoms of a vitamin E deficiency, have tentatively been interpreted as indicating a disturbance of some metabolic processes, perhaps of the fat metabolism.[130]

A possible relation of vitamin E to the amino-acid metabolism is seen in the fact that during avitaminosis the creatine content of striated muscles is

[126] P. Karrer and A. Geiger, *Helv. Chim. Acta*, 23, 455 (1940).
[127] W. John, E. Dietzel and W. Emte, *Z. physiol. Chem.*, 257, 180 (1939).
[128] P. Karrer, H. Salomon and H. Fritzsche, *Helv. Chim. Acta*, 21, 309 (1938).
[129] M. D. Wright and J. C. Drummond, *Biochem. J.*, 34, 32 (1940).
[130] B. A. Kudrjashov, *Bull. biol. et med. expér. URSS.*, 3, 279 (1937).

greatly reduced[131] in rabbits[132] and in rats[133] and that the urinary excretion of creatine is considerably increased[132, 134] while the excretion of creatinine remains unchanged. A marked reduction of the creatine content in the urine is observed following the administration of vitamin E. No such changes have, however, been found in the urine of man suffering from progressive muscular dystrophy, amyotonia congenita and similar neuromuscular disturbances.[135]

The primary physiological action of vitamin E is apparently to direct certain activities of the cell nucleus.[136, 137] This conception of the mechanism of the vitamin E action, although much disputed, has proved to be of extreme value in an attempt to correlate the various experimental facts and observations of the many different symptoms of vitamin E deficiency.

The role which vitamin E plays in the metabolic activities of the cell nucleus cannot as yet be defined exactly. It seems that the vitamin function is more intimately concerned with the processes of cell maturation and differentiation than with mitosis. Thus the rate of growth of the Walker 256 mammary carcinoma is not essentially affected by continuous maintenance in vitamin E-deficient rats.[138] On the other hand, an increase in growth has been demonstrated in *in vitro* experiments with liver, spleen, heart and periosteal tissue cultures upon addition of vitamin E.[139] Retarded growth has as in the case of deficiencies of the other vitamins also been observed during vitamin E deficiency.[140] The considerable decrease in the weight of the testis even when calculated in relation to the reduced body weight is, however, specific for this vitamin.[141] In accordance with the conception that vitamin E is of special influence to all those tissues in which cellular proliferation and differentiation proceed at a high speed, it has been found that the application of this vitamin is especially useful, for example, to effect growth of prematurely born babies or to heal skin wounds in rats.[142, 143] Beneficial effects in the latter case have been observed upon oral or local application.

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[131] M. Goettsch and E. F. Brown, *J. Biol. Chem.*, **97**, 549 (1932).
[132] C. G. Mackenzie and E. V. McCollum, *J. Nutrition*, **19**, 345 (1940).
[133] F. Verzár, *Schweiz. med. Wochschr.*, **69**, 738 (1939).
[134] S. Morgulis and H. C. Spencer, *J. Nutrition*, **11**, 573 (1936).
[135] W. Fleischmann, *Proc. Soc. Exptl. Biol. Med.*, **46**, 94 (1941).
[136] A. Juhasz-Schäffer, *Arch. path. Anat. (Virchow's)*, **281**, 53 (1931).
[137] K. E. Mason, *Am. J. Anat.*, **52**, 153 (1933).
[138] K. E. Mason, *Soc. Chem. Ind. Food Group*, **1939**, 31.
[139] A. Juhasz-Schäffer, *Arch. path. Anat. (Virchow's)*, **281**, 35 (1931).
[140] H. Blumberg, *J. Biol. Chem.*, **108**, 227 (1935).
[141] A. M. Copping and V. Korenschevsky, *Soc. Chem. Ind. Food Group*, **1939**, **44**.
[142] G. Léranth and L. Frank, *Orvosi Hetilap*, **80**, 778 (1936); *Chem. Abstracts*, **30**, 7634 (1936).
[143] F. R. Adamstone, *J. Morphol.*, **52**, 47 (1931).

The histopathological changes which have been observed to occur in the testes of vitamin E-depleted rats are largely responsible for the conception of the function of vitamin E. The chromatin of the spermatozoa undergoes a typical change, a lysis, and the nuclei become crescentric. Upon further development of the deficiency disease, the mature and immature cells, the permatogonia, spermatocytes and spermatozoa, fuse into giant cells with many nuclei. Besides chromatolysis there appears to be an interference in the formation of chromatin.

Similar effects are observed in the fetus. The earliest signs of a vitamin E deficiency in the embryo is observed in the hematopoietic tissue, the growth of which is disturbed. The mesodermal tissues where rapid cellular activity prevails are similarly affected. The symptoms of vitamin E deficiency, to be discussed in the next section, namely, nervous lesions and degenerative lesions in the muscles, seem to be related to the cerebral cortex (observations on rats), a tissue of high cellular activity.

All experimental evidences lead to the conclusion that, secondary to the lesions in the gonads and in the neuro-muscular system and probably as the result of these rather than as their cause, abnormalities occur in the pituitary and thyroid glands. In young vitamin E-depleted rats a change in the fur has been observed which is considered characteristic for hypophysectomized animals.[144, 145] In the anterior lobe, the acidophil and basophil cells degenerate and show no distinct granulation.[146] The histological appearance of the anterior lobe is quite similar to the degeneration observed in the case of castrated animals. Actually many of the symptoms observed as the result of a vitamin E deficiency resemble very closely the syndromes of a hypophysectomy.[147, 148] The changes observed in the testes are, however, dissimilar.[149] Vitamin E deficiency causes a degeneration of the germinative tissue, whereas upon dysfunction of the pituitary the interstitial tissue is degenerated. In male rats deprived of vitamin E, the pituitaries were found to contain a greater gonadotropic action than found in the glands of rats fed with vitamin E.[150, 151] In female rats deprived of vitamin E the pituitary contains a decreased amount of luteinizing hormone.[152] Nevertheless, the important symptoms of vitamin E deficiency, namely, muscular dystrophy, gestation resorption and testicular degenera-

[144] F. Verzár, *Abderhaldens Handb. biol. Arbeitsmethoden*, Abt. 5, Pt. 36, No. 8, 1269 (1937).
[145] F. Verzár and E. Kokas, *Arch. ges. Physiol. (Pflügers)*, **227**, 511 (1931).
[146] M. M. O. Barrie, *Lancet*, **II**, 251 (1937).
[147] M. M. O. Barrie, *J. Soc. Chem. Ind.*, **55**, 1053 (1936).
[148] F. Verzár, *Arch. ges. Physiol.*, **227**, 499 (1931).
[149] K. E. Mason, *Am. J. Anat.*, **52**, 153 (1933).
[150] W. O. Nelson, *Anat. Record*, **56**, 241 (1933).
[151] J. C. Drummond, *Soc. Chem. Ind. Food Group*, **1939**, 27.
[152] I. W. Rowlands and E. Singer, *J. Physiol.*, **86**, 323 (1936).

tion, cannot be prevented or cured by administration of the hormones of the anterior lobe. The syndromes are, furthermore, not influenced by the injection of the sex hormones. On the other hand, vitamin E, when administered to immature or hypophysectomized adult rats, does not exert any effect on the ovaries, uterus or vagina.[153] In frogs an interesting relation of vitamin E to the secretion of the hormones of the anterior lobe of the pituitary has been observed. The glycogenotropic hormone could be detected in the livers only after an intake of vitamin E.[154]

In the thyroids of vitamin E-depleted animals hypoplasia occurs. The vesicles are enlarged and filled with colloid.[155, 156] In the kidneys a degeneration of the epithelium of the convoluted tubules is observed.[157] In the young of vitamin E-deficient rats cretinism is observed.[158]

(c) *Relation of Vitamins E to Other Vitamins, Hormones, Etc.*

Vitamin E has a remarkable influence upon the storage of vitamin A. In rats kept for prolonged periods on a diet deficient in vitamin E, the vitamin A reserves were consistently found lower than those of control animals receiving supplements of vitamin E.[159] Another relation of vitamin E to vitamin A is seen in the fact that the pathological changes caused by a vitamin E deficiency are retarded when a vitamin A deficiency exists simultaneously.[160]

The various relations of a vitamin E deficiency to the anterior lobe of the pituitary gland have already been discussed in the previous section.

15. Avitaminosis and Hypovitaminosis

Vitamin E deficiency has been studied in greatest detail on rats. Both male and female rats suffer from a decreased vitamin E intake. In male rats the symptoms appear earlier than in the female rat. Within a few weeks or months a considerable decrease in the weight of the testes compared with those of animals on a sufficient diet can be observed. Typical testicular changes set in. In cases of slight hypovitaminosis the spermatozoa become non-motile. The nucleus of the sperms begins to deform.

[153] J. C. Drummond, R. L. Noble and M. D. Wright, *J. Endocrinology*, **1**, 275 (1939).
[154] L. Képinov, *Compt. rend.*, **209**, 358 (1939); **210**, 188 (1940).
[155] M. M. O. Barrie, *Lancet*, II, 251 (1937).
[156] E. Singer, *J. Physiol.*, **87**, 287 (1936).
[157] C. M. Blumenfeld, *Endocrinology*, **18**, 367 (1934).
[158] M. M. O. Barrie, *Nature*, **139**, 286 (1937).
[159] T. Moore, *Biochem. J.*, **34**, 1321 (1940). A. L. Bacharach, *Quart. J. Pharm. Pharmacol.*, **13**, 138 (1940).
[160] K. E. Mason, *Am. J. Anat.*, **52**, 153 (1933).

On further vitamin E depletion, the spermatozoa degenerate and lose the power of fertilization. The nucleus becomes crescentric. Later, upon copulation, the bouchon vaginale does not contain any sperm at all. At this stage the seminal vesicles develop a brown discoloration. Upon further development the ability to form a bouchon is lost and finally even the sex instinct disappears. The end effect is a functional sterility through degeneration of the germinal epithelium. Once these patho-physiological changes begin to become histologically noticeable, they cannot be repaired, arrested or retarded by the administration of vitamin E.[161, 162, 163]

Similar pathological changes in the epithelium of the seminiferous tubules have been observed in mice, bulls[164] and in fowls.[165, 166]

The changes in the female organism are somewhat different. The female rat at times of an E-hypovitaminosis shows a normal oestrus cycle and normal ovulation. Conception is not disturbed and gestation sets in as normal. In cases of only slight hypovitaminosis births occur normally but considerable prolongation of the length of gestation occurs and the litters are not reared.[167] In more severely depleted animals, the young are born dead. In the case of typical E-avitaminosis gestation sets in as normal but the development of the fetuses is hampered in about the middle of the gestation period. Placental tissue, fetal tissue and the implantation site begin to degenerate. The fetuses die and are resorbed. This state is called resorption sterility. Upon completion of the resorption of the fetuses and of the placenta, normal oestrus cycle sets in and the animal is again able to conceive. Administration of vitamin E at this stage repairs the power to cast normal litters. Further depletion again causes resorption of the fetuses. These resorptions bring about certain changes in the uterus, especially brown pigmentation,[168] caused by the deposition of a pigment in the muscle layers.[169] Eventually, further uterine degenerations become apparent, especially fibrosis of the uterine muscle and sometimes fibromyomata of the uterus,[169] and when avitaminosis is advanced enough, absolute sterility results.

One of the early signs of vitamin E deficiency, observed on young as well as on old animals of both sexes, is a dystrophy of the cross-striated muscula-

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[161] H. M. Evans and G. O. Burr, *Mem. Univ. Calif.*, No. 8 (1927).
[162] K. E. Mason, *J. Expt. Zoöl.*, **45**, 159 (1926).
[163] H. M. Evans, *Proc. Natl. Acad Sci. U. S.*, **11**, 373 (1925).
[164] N. Lagerlöf, *Acta Path. Microbiol. Scand.*, **19**, Supplement (1934).
[165] L. E. Card, *Poultry Sci.*, **8**, 328 (1929).
[166] F. R. Adamstone, *J. Morphol.*, **52**, 47 (1931).
[167] M. M. O. Barrie, *Biochem. J.*, **32**, 1467 (1938).
[168] A. J. P. Martin and T. Moore, *J. Soc. Chem. Ind.*, **55**, 236 (1936).
[169] M. M. O. Barrie, *Biochem. J.*, **32**, 2134 (1938).

ture which has been found in rats,[170, 171, 172, 173, 174, 175] rabbits,[176, 177, 178] dogs,[179] guinea pigs[180, 181, 182, 183] and chicks.[184] Before the overt symptoms of such a dystrophy appear, the following syndromes can be detected:[185] increase in the concentration of water and of chlorides in the muscles, decrease in the maximum strength and focal hyaline necrosis of muscle fibers. These lesions are accompanied by degenerative changes in the central nervous system,[186] histologically evident by degeneration of nerve cells in the spinal cord.[187] In cases of severe avitaminosis, paralysis first of the hind and then also of the fore limbs is found in young[188] and in old rats. Carpopedal spasms and convulsions have been observed in the final stages when the mortality is 100%.[189]

Another pathological change observed in rats deprived of vitamin E is a considerable enlargement of the thymus both in male and in female animals.[190] The changes which occur in the pituitary, the thyroids and the kidney have already been discussed. In the young vitamin E-deficient rat also an uncalcified skull has been observed.

In beef cattle and in milk cows, sterility and early abortion have successfully been treated with vitamin E and the existing evidence leads to the conclusions that some of these animals suffered from a true E-avitamino-

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[170] See however M. Goettsch and J. Ritzmann, *J. Nutrition*, **17**, 371 (1939).

[171] H. M. Evans and G. O. Burr, *J. Biol. Chem.*, **76**, 273 (1938). H. Blumberg, *Ibid.*, **108**, 227 (1935). G. O. Burr, W. R. Brown and R. L. Mosely, *Proc. Exptl. Biol. Med.*, **36**, 780 (1937). A. Ringstead, *Biochem. J.*, **29**, 788 (1935).

[172] H. S. Olcott, *J. Nutrition*, **15**, 221 (1938).

[173] H. M. Evans and G. O. Burr, *J. Biol. Chem.*, **76**, 273 (1928).

[174] L. Einarson and A. Ringstead, *Effect of Chronic Vitamin E Deficiency on the Nervous System and the Skeletal Musculature in Adult Rats*, Copenhagen, 1938.

[175] M. D. Lipshutz, *Rev. neurol.*, **65**, 221 (1936). J. Morelle, *Compt. rend. soc. biol.*, **108**, 804 (1931). H. S. Olcott and H. A. Matill, *J. Biol. Chem.*, **104**, 423 (1934).

[176] C. G. Mackenzie and E. V. McCollum, *J. Nutrition*, **19**, 345 (1940).

[177] S. Morgulis, *Monographic Actualitées Scientifiques et Industrielles*, Paris, 1938, p. 74.

[178] S. G. Morris, *Science*, **90**, 424 (1939).

[179] H. D. Anderson, C. A Elvehjem and J. E. Gonce, *Proc. Soc. Exptl. Biol. Med.*, **42**, 750 (1939). K. M. Brinkhous and E. D. Warner, *Am. J. Path.*, **17**, 81 (1941).

[180] M. Goettsch and A. M. Pappenheimer, *J. Exptl. Med.*, **54**, 145 (1931).

[181] H. S. Olcott, *J. Nutrition*, **15**, 221 (1938).

[182] N. Shimotori, G. A. Emerson and H. M. Evans, *Science*, **90**, 89 (1939); *J. Nutrition*, **19**, 547 (1940).

[183] E. L. Wood and H. M. Hines, *Proc. Soc. Exptl. Biol. Med.*, **36**, 786 (1937).

[184] H. Dam and J. Glavind, *Naturwissenschaften*, **28**, 207 (1940); *Nature*, **143**, 810 (1939).

[185] G. C. Knowlton and H. M. Hines, *Proc. Soc. Exptl. Biol. Med.*, **38**, 655 (1938); **42**, 133, 804 (1939).

[186] D. Lipshutz, *Rev. neurol.*, **65**, 221 (1936). L. Einarson and A. Ringstead, *Effect of Chronic Vitamin E Deficiency on the Nervous System and the Skeletal Musculature in Adult Rats*, Copenhagen, 1938.

[187] M. Ekblad and G. Wohlfart, *Z. ges. Neurol. Psychiat.*, **168**, 144 (1940).

[188] H. M. Evans and G. O. Burr, *J. Biol. Chem.*, **76**, 273 (1928).

[189] M. M. O. Barrie, *Lancet*, **II**, 251 (1937).

[190] A. M. Copping and V. Korenschevsky, *Soc. Chem. Ind. Food Group*, **1939**, 44.

sis.[191, 192] There is also considerable evidence[193] that administration of vitamin E is of value in the treatment of the infectious abortion in cows, caused by the *Bacillus abortus* (Bang), probably due to the fact that vitamin E increases the resistance of the animals against infection.[194]

In sows barrenness has also successfully been overcome by vitamin E therapy.[195]

The pathological changes observed on rats are essentially similar to the changes observed in birds. Eggs of hens reared on a vitamin E-deficient diet cannot be hatched successfully. In the embryos a lethal ring develops in the blastoderm from cell proliferation in the mesoderm. The organism dies from starvation and hemorrhages after the blood vessels of the blastoderm are choked off.[196] Hatchability is greatly improved upon adding vitamin E to the chicken feed.[197, 198, 199] In the male fowl, degeneration of the testis has been observed.[196, 197] In chicks a vitamin E deficiency causes alimentary exudative diathesis followed by increased capillary permeability and muscular dystrophy.[200] Nutritional encephalomalacia of the chick is caused by vitamin E deficiency.[201]

The question as to whether or not an E-avitaminosis occurs in man has not definitely been decided. Considerable evidence exists for the fact that vitamin E may exert a beneficial influence in certain cases of habitual abortion[202, 203] and possibly also of threatened abortion and premature separation of the placenta. Certain forms of toxemia in pregnancy[204] have also been reported to respond favorably to a treatment with vitamin E. It has furthermore been stated that administration of vitamin E is beneficial

[191] F. Vogt-Möller and F. Bay, *Münch. tierärztl. Wochschr.*, **82**, 637 (1931); *Vet. J.*, **87**, 165 (1931); **90**, 288 (1934).

[192] A. Andersen, *Medlemsblad dansk. Dyrlaegeforen*, **17**, 113 (1934). H. Lehmke, *Berlin. tierärztl. Wochschr.*, **1938**, 367. J. P. Tutt, *Vet. J.*, **89**, 416 (1933).

[193] R. Moussu, *Compt. rend.*, **201**, 1228 (1935).

[194] A. E. Lange, *Tierärztl. Runds.*, **1938**, 239.

[195] R. W. Lentz, *Berlin. tierärztl. Wochschr.*, **1938**, 201.

[196] F. R. Adamstone, *J. Morphol.*, **52**, 47 (1931).

[197] L. E. Card, *Poultry Sci.*, **8**, 328 (1929).

[198] G. I. Barnum, *J. Nutrition*, **9**, 621 (1935).

[199] F. Ender, *Z. Vitaminforsch.*, **4**, 106 (1935); see however C. E. Holmes and W. W. Cravens, *Poultry Sci.*, **19**, 303 (1940).

[200] H. Dam and J. Glavind, *Naturwissenschaften*, **28**, 207 (1940); *Nature*, **143**, 810 (1939).

[201] A. M. Pappenheimer, M. Goettsch and E. Jungherr, *Connecticut Agric. Exp. Stat.*, **Bull. 229** (1939).

[202] P. Vogt-Möller, *Lancet*, **221**, 182 (1931); *Acta Obstet. Gyn. Scand.*, **13**, 219 (1933); *Klin. Wochschr.*, **15**, 1883 (1936).

[203] D. W. Currie, *Brit. J. Med.*, **II**, 1218 (1937). J. Gierhake, *Arch. Gynäkol.*, **156**, 348 (1933). A. Juhasz-Schäffer, *Ergeb. inn. Med.*, **45**, 129 (1933). H. Martius, *Med. Welt*, **1937**, 407. E. V. Shute, *J. Obstet. Gynaecol. Brit. Empire*, **42**, 1071, 1085 (1935); **43**, 74 (1936); *Am. J. Obstet. Gynecol.*, **33**, 429 (1937). E. M. Watson, *Canad. Med. Assoc. J.*, **34**, 134 (1936). E. M. Watson and W. P. Tew, *Am. J. Obstet. Gynecol.*, **31**, 252 (1935). J. Young, *Brit. Med. J.*, **I**, 953 (1937).

[204] E. V. Shute, *Am. J. Obstet. Gynecol.*, **33**, 429 (1937). J. Young, *Brit. Med. J.*, **I**, 953 (1937).

in cases of muscular dystrophy,[205, 206] amyotrophic lateral sclerosis,[205, 207, 208] myelopathy from pernicious anemia, neuromuscular syndromes, roaring sensations in the ears, anorexia[209] and primary fibrositis[210]. The information gained from these reports must, however, be regarded as provisional and all clinical use of vitamin E is still considered purely experimental.

(*a*) *Clinical Test Methods*

Determination of Vitamin E in Urine. *Urinary Creatine Determination:*[211]—Vitamin E-hypovitaminosis can be detected by urine analysis since the creatine output increases rapidly. In rabbits, for example, the daily creatine excretion rises from a normal level of less than 10 mg. to over 20 mg. By the time symptoms of muscular dystrophy occur, 50 to 150 mg. of creatine are excreted.[212] This method is only of restricted value since many other factors may influence the creatine output.

Determination of Vitamin E in Blood. *The Ferric Chloride-Dipyridyl Method:* For a successful determination of vitamins E in blood it is necessary to extract the active material. This can, for example, be accomplished by treating the serum with dilute alkali in the cold and in the presence of formaldehyde and ethyl alcohol. The vitamin is then extracted with ether and the ether solution is washed neutral with alkali and acid solutions. By selective adsorption, for example, on floridin,[213] the carotenoids are removed. The determination of the vitamin E in the remaining solution is then carried out according to the ferric chloride-dipyridyl method. As little as 5 cc. of blood can be used for this method. This method fails to be accurate for amounts of vitamin E less than 5 γ.

16. Hypervitaminosis

Vitamins E are non-toxic substances. E-hypervitaminosis is unknown. Synthetic *dl*-α-tocopherol causes no toxic symptoms in rats even when given orally in doses as high as 50 g. per kilogram of body weight. The administration of several grams of vitamin E daily over a period of one to

[205] F. Bicknell, *Lancet*, I, 10 (1940).
[206] S. Stone, *J. Am. Med. Assoc.*, **114**, 2187 (1940).
[207] I. S. Wechsler, *Ibid.*, **114**, 948 (1940). T. D. Spies and R. W. Vilter, *Southern Med. J.*, **33**, 663 (1940).
[208] L. Einarson and A. Ringstead, *Effect of Chronic Vitamin E Deficiency on the Nervous System and the Skeletal Musculature in Adult Rats*, Copenhagen, 1938.
[209] T. D. Spies, D. P. Hightower and L. H. Hubbard, *J. Am. Med. Assoc.*, **115**, 292 (1940).
[210] C. L. Steinberg, *Am. J. Med. Sci.*, **3**, 347 (1941).
[211] O. Folin, *J. Biol. Chem.*, **17**, 469 (1914).
[212] C. G. Mackenzie and E. V. McCollum, *J. Nutrition*, **19**, 345 (1940).
[213] A. Emmerie and C. Engel, *Rec. trav. chim.*, **58**, 283 (1939).

two months does not result in any adverse symptoms in rats, dogs and cats. The functions of the kidneys, the intestines, the nervous system, the muscles and the sex organs are not affected by such doses.[214]

17. Requirements

It has been proved experimentally that vitamin E is necessary for mice, rats, rabbits, guinea pigs, dogs, chicks and ducks. The crustacea Daphnia also needs vitamin E.[215] Goats can apparently get along without an external supply of this vitamin.[216] The requirements for man are largely unknown as stressed before.

The vitamin E requirement of the rat is different for the male and the female animal. It seems that the male animal needs only about one-seventh to one-tenth of the amount required by the female. A similar but perhaps more marked difference is observed in mice.

The average daily need of a female rat is from 2–3 mg. of α-tocopherol. The minimum fertility requirements of female rats are 15 mg. per kilogram body weight daily. For rabbits, the daily antimuscular dystrophy dose is about 1 mg. of α-tocopherol per kilogram body weight.[217] In clinical therapy, a dose of 6 mg. of α-tocopherol per day has been recommended for women.[218]

[214] V. Demole, *Z. Vitaminforsch.*, **8**, 338 (1939).
[215] A. Vichoever and I. Cohen, *Am. J. Pharm.*, **110**, 297 (1938).
[216] G. K. L. Underbjerg, *Iowa State College, J. Sci.*, **15**, 107 (1940).
[217] C. G. Mackenzie and E. V. McCollum, *J. Nutrition*, **19**, 345 (1940).
[218] D. W. Currie, *Soc. Chem. Ind. Food Group*, **1939**, 77.

VITAMIN H—
BIOTIN

VITAMIN H—BIOTIN

1. Nomenclature

Names:

Bios II (Lucas, 1924).
Factor X (Boas, 1927[1]).
Vitamin H (György, 1931[2]).
Coenzyme R (Allison, Hoover and Burk, 1933[3]).
Bios II *b* (Miller, 1934).
Anti-egg-white-injury factor (Lease and Parsons, 1934[4]).
Biotin (Kögl, 1935[5]).
Factor W (Elvehjem, 1936[6]).
Vitamin B_w (Lunde and Kringstad, 1940[7]).
S or Skin-Factor (Marshall, 1939[8, 9]).

Empirical formula:

$C_{10}H_{16}O_3N_2S$.

Efficacy:

One gram of biotin-methyl ester = 27,000,000 Rat Units.
= 25,000,000,000 Saccharomycetes Units.

2. Chronology

1901 WILDIERS recognized that yeast needs a special material, "bios," in addition to the known nutrients for optimal growth. This had already been postulated by LIEBIG in 1869.

1916 BATEMAN[10] made the casual observation that egg white exhibits a definite toxicity.

1923 FULMER discovered the multiple nature of "bios."

1924 LUCAS separated bios into two components, bios I and II.

1927 BOAS[11] found that certain foods contain an organic substance which protects against egg-white toxicity.

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[1] M. A. Boas, *Biochem. J.*, **21**, 712 (1927).
[2] P. György, *Z. ärztl. Fortbildung*, **28**, 377, 417 (1931).
[3] F. E. Allison, S. R. Hoover and D. Burk, *Science*, **78**, 217 (1933).
[4] J. G. Lease and H. T. Parsons, *Biochem. J.*, **28**, 2109 (1934).
[5] F. Kögl, *Ber.*, **68**, 16 (1935).
[6] C. A. Elvehjem, C. J. Koehn and J. F. Oleson, *J. Biol. Chem.*, **115**, 707 (1936).
[7] G. Lunde and H. Kringstad, *J. Nutrition*, **19**, 321 (1940).
[8] W. Marshall, *J. Invest. Dermatol.*, **2**, 205 (1939).
[9] W. Marshall, *Med. World*, **57**, 101 (1939).
[10] W. G. Bateman, *J. Biol. Chem.*, **26**, 263 (1916).
[11] M. A. Boas, *Biochem. J.*, **21**, 712 (1927).

1928 EASTCOTT[12] isolated bios I in the pure form and identified it with *i*-inositol.
1931 GYÖRGY[2] recognized the necessity of an anti-egg-white-injury factor (vitamin H) for man.
1933 MILLER showed that bios II contained at least two different factors.
1935 KÖGL[13] isolated "biotin," one of the compounds of bios II, in the pure form.
1940 GYÖRGY, MELVILLE, BURK and DU VIGNEAUD[14] identified vitamin H with biotin.
1942 DU VIGNEAUD, HOFMANN and MELVILLE suggested structural formulas for biotin on the basis of degradation reactions.[14a]

3. Occurrence

Vitamin H occurs in small amounts in all higher animals (mammals and birds). The only tissue found so far which is apparently devoid of this vitamin is the lens of the eye.[15] The highest concentrations have been observed in liver,[15, 16] kidney[15] and eggs[15] while milk[16] contains this vitamin in somewhat smaller concentrations.

Vitamin H is found widely distributed in the plant kingdom. Vegetables, grains, nuts, etc., contain considerable quantities.[16] Most strains of yeast contain some vitamin H.[16] In plants, the tips of the roots and of the coleoptiles usually have a somewhat higher concentration than the rest of the plant.[17] Relatively high concentrations have been observed in seeds and in pollen.[17]

Vitamin H occurs predominantly in the free state in fruits and in grasses. In grains, nuts and vegetables this vitamin is present partly in a bound form and partly free.[18] In yeast and in animal tissues, for example, in liver, vitamin H occurs mainly as a chemically bound compound.

4. Isolation

As pointed out previously, vitamin H occurs in many sources chemically bound and cannot be dissolved by any solvent. In order to liberate the vitamin, for example, from yeast,[19] the material is autolyzed from four to six days. Liver, on the other hand, does not possess the necessary enzyme system for the autolytic liberation of this vitamin.[20] Vitamin H can, how-

[12] E. V. Eastcott, *J. Phys. Chem.*, **32**, 1094 (1928).
[13] F. Kögl, *Ber.*, **68**, 16 (1935).
[14] P. György, D. B. Melville, D. Burk and V. du Vigneaud, *Science*, **91**, 243 (1940).
[14a] V. du Vigneaud, K. Hofmann and D. B. Melville, *J. Am. Chem. Soc.*, **64**, 188 (1942).
[15] F. Kögl and W. van Hasselt, *Z. physiol. Chem.*, **243**, 189 (1936).
[16] G. Lunde and H. Kringstad, *J. Nutrition*, **19**, 321 (1940).
[17] F. Kögl and A. J. Haagen-Smith, *Z. physiol. Chem.*, **243**, 209 (1936).
[18] J. O. Lampen, A. A. Kline and W. H. Peterson, *Proc. Am. Soc. Biol. Chem.*, **1941**, LXXIV. J. O. Lampen, G. P. Bahler and W. H. Peterson, *J. Nutrition*, **23**, 11 (1942).
[19] P. György, *J. Biol. Chem.*, **131**, 733 (1939).
[20] P. György, R. Kuhn and E. Lederer, *Ibid.*, **131**, 745 (1939).

ever, be liberated by proteolytic digestion of liver[20, 21, 22, 23] or by hydrolysis with acids,[21] for example, with 2*N* mineral acids with or without pressure.[20] In contrast to fresh liver, the vitamin can be liberated from liver powder by hydrolysis at high pressure without addition of acids. After the hydrolysis has been achieved according to any of these procedures, the solution is filtered and impurities may be removed from the filtrate by precipitation with alcohols or lead-acetate. The vitamin can be dissolved in acetone from a concentrated filtrate, whereby considerable amounts of impurities separate. Further concentration has been achieved by precipitation with phosphotungstic acid which precipitates only the impure compound but not the pure vitamin.[24, 25] Mercuric chloride,[26] gold chloride and H_2PtCl_4 also precipitate the vitamin from impure solutions. The vitamin is, however, not precipitated by flavianic acid, rufianic acid, Reinecke salts, picric acid, picrolonic acid, barium salts,[25] quinine or alkaloids.[27] Alcohol-insoluble salts are obtained from barium or calcium hydroxide.

Vitamin H is adsorbed on charcoal (Norit and Carboraffin), but not on fuller's earth, aluminum oxide, acid clay or benzoic acid. Vitamin H can be eluted with a mixture of pyridine, methanol and water. The methyl-ester, on the other hand, can be purified by adsorption on aluminum oxide.[27a]

Further purification of the vitamin can be accomplished by electrodialysis[28] and by high vacuum distillation.[29]

By a combination of these procedures 1.1 mg. crystallized biotin have been obtained from 250 kg. of dried Chinese egg yolk.[29]

5. Properties

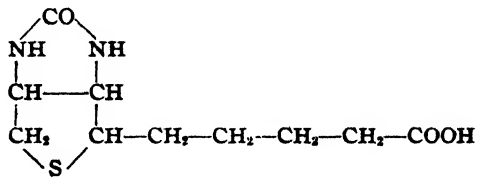
The free vitamin H is water- and alcohol-soluble but is relatively insoluble in chloroform, ether and petroleum ether. The vitamin is essentially heat-stable, readily dialyzable and resistant to treatment with acid or alkali. It is easily adsorbed on charcoal. The isoelectric point appears to be between pH 3 and 3.5. The pure vitamin melts at 230–232° C. and the methyl-ester at 166–167° C. Biotin is optically active, $[\alpha]_D^{22} = +92°$

[21] J. G. Lease, *Z. Vitaminforsch.*, **5**, 110 (1936).
[22] J. G. Lease and H. T. Parsons, *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **105**, I. (1934).
[23] P. György, *Ibid.*, **119**, XLIII (1937).
[24] J. G. Lease, *Z. Vitaminforsch.*, **5**, 110 (1936).
[25] P. György, R. Kuhn and E. Lederer, *J. Biol. Chem.*, **131**, 745 (1939).
[26] G. Drumel and L. Hubert, *Arch. intern. physiol.*, **46**, 141 (1938).
[27] T. W. Birch and P. György, *J. Biol. Chem.*, **131**, 761 (1939).
[27a] V. du Vigneaud, K. Hofmann, D. B. Melville and P. György, *J. Biol. Chem.*, **140**, 643 (1941).
[28] P. György, D. B. Melville, D. Burk and V. du Vigneaud, *Science*, **91**, 243 (1940).
[29] F. Kögl and B. Tönnis, *Z. physiol. Chem.*, **242**, 43 (1936).

in 0.1 *N* NaOH. It shows no characteristic absorption in the ultraviolet region.[30]

6. Chemistry

The empirical formula of biotin is $C_{10}H_{16}O_3N_2S$. By means of electrodialysis it was shown that vitamin H is an ampholyte with acidic properties.[31] The presence of a carboxyl-group is indicated by the formation of esters such as the methyl-ester. Biotin contains a basic group since at pH = 1 the vitamin cannot be extracted with organic solvents.[32] The nitrogens are present in urea configuration. By treatment of biotin with barium hydroxide at 140° C. a diamino-carboxylic acid is obtained with the loss of one carbon atom and one oxygen atom. By treatment of the diamino-carboxylic acid with phosgene the urea group and thereby biotin, is resynthesized. The sulfur is present as a thio-ether, since a sulfone results from peroxide oxidation. Since biotin does not contain any double bonds, it must contain a bicyclic ring system.[32a] Upon oxidation of the diamino-carboxylic acid with nitric acid or alkaline permanganate, adipic acid is isolated.[32b] One of the carboxyl groups of the adipic acid is the carboxyl group originally present in biotin, since upon Curtius degradation of the biotin methyl ester followed by oxidation of the obtained amine no adipic acid could be isolated.[32c] On the basis of these and additional evidences, [32d] biotin has been assigned the structure of 2-keto-3, 4-imidazolido-2-tetrahydro-thiophene-*n*-valeric acid (I). This conclusion has been confirmed by a total synthesis of biotin[32e].



[30] V. du Vigneaud, K. Hofmann, D. B. Melville and J. R. Rachele, *J. Biol. Chem.*, **140**, 763 (1941).

[31] T. W. Brich and P. György, *J. Biol. Chem.*, **131**, 761 (1939).

[32] H. Kringstad and G. Lunde, *Ibid.*, **261**, 110 (1939).

[32a] F. Kögl and L. Pons, *Z. physiol. Chem.*, **269**, 61 (1941). F. Kögl and T. J. de Man, *Ibid.*, **269**, 81 (1941). K. Hofmann, D. B. Melville and V. du Vigneaud, *J. Biol. Chem.*, **141**, 207 (1941). D. B. Melville, K. Hofmann and V. du Vigneaud, *Science*, **94**, 308 (1941).

[32b] K. Hofmann, D. B. Melville and V. du Vigneaud, *J. Am. Chem. Soc.*, **63**, 3237 (1941).

[32c] V. du Vigneaud, K. Hofmann and D. B. Melville, *J. Am. Chem. Soc.*, **64**, 188 (1942).

[32d] Refer to review papers by V. du Vigneaud, *Science*, **96**, 455 (1942) and K. Hofmann, *Advances in Enzymology*, Vol. III, Interscience, New York, 1943, p. 289.

[32e] S. A. Harris, D. E. Wolf, R. Mozingo and K. Folkers, *Science*, **97**, 447 (1943).

7. Industrial Methods of Preparation

Pure vitamin H is marketed in small quantities prepared by tedious isolation procedures. Concentrates are also manufactured from liver, yeast and certain bacteria. The preparation of the concentrates from liver is coordinated with the manufacture of the factor which prevents the development of pernicious anemia, since the residues from this manufacture contain relatively high concentrations of vitamin H.[33, 34, 35]

8. Specificity

Very little is known about the specificity of vitamin H. The methyl-ester is fully active. Benzoylation and acetylation do not effect the activity, probably because of the ability of the organism to hydrolyze these derivatives. Vitamin H in its naturally occurring chemically bound form is also essentially active. Inactive, however, is the biotin symplex with avidin (see page 476).

9. Determination

(*a*) *Biological Methods*

There are only biological methods available for the determination of biotin. The most valuable and accurate methods are those in which micro-organisms are used and in which the biotin concentration is measured by the growth of yeast or of bacteria, or by the amount of acid produced by specific organisms. The historically important bio-assay with rats is based upon a secondary deficiency disease brought about by egg-white injury and is quite time consuming. The chick can also be used for quantitative assays in a procedure which uses a primary biotin deficiency and which is more expedient than rat assays, but less so than the micro-biological methods.

Rat Tests. Male albino rats are fed rations which are well balanced except for a large proportion of egg white as the sole source of protein.[36, 37] The average time necessary for the appearance of the first skin symptoms is five to seven weeks. A rat growth method has also been used.[38]

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[33] J. G. Lease and H. T. Parsons, *Biochem. J.*, **28**, 2109 (1934).
[34] P. György, *J. Biol. Chem.*, **131**, 733 (1939).
[35] J. G. Lease, *Z. Vitaminforsch.*, **5**, 110 (1936).
[36] M. A. Boas, *Biochem. J.*, **21**, 712 (1927).
[37] P. György, *J. Biol. Chem.*, **131**, 733 (1939).
[38] G. Lunde and H. Kringstad, *J. Nutrition*, **19**, 321 (1940).

Chick Test. The chick has been suggested as test animal for the determination of vitamin H since chicks can be depleted readily from this vitamin on a diet low in this constituent.[39]

Coenzyme R Assay.[40] In this test, the increase in respiration of rhizobium is measured upon addition of vitamin H to the culture medium. This method is far more sensitive than the rat assay method for vitamin H.

Yeast Growth Method. The growth of special yeast strains such as *Saccharomycetes cerevisiae* is observed, for example, by measuring the resulting turbidity in a photoelectric colorimeter[41] or nephelometer.[42]

Staphylococcus Growth Test.[43] The response of the addition of vitamin H preparations to a culture medium of *Staphylococcus aureus* has been suggested as a quantitative method for the assay of vitamin H.

Colostridum Butylicum Growth Test.[44] The organism *Colostridum butylicum* has been suggested for vitamin H assays. The growth is measured by turbidity studies. In this test only the free vitamin is measured and not the bound vitamin.

Acidimetric Lactobacillus Test.[45] The amount of lactic acid produced by *Lactobacillus arabinosus* cultivated in a medium containing all the other necessary growth factors can be used as a measure of the vitamin H content.

10. Standards

One "Rat Unit" is the daily dose of a given preparation or foodstuff which in four weeks brings about complete cure of the egg-white injury in rats (on a special diet).[46] This corresponds to an activity of 27 million Rat Units of vitamin H per gram of the methyl-ester of biotin.[47]

One "Saccharomycetes Unit"[48] (S. U.) is the amount of biotin which produces an increase of 100% in cell growth of a special strain of yeast under defined conditions. One gram of biotin contains 25,000,000,000 Saccharomycetes Units.

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[39] D. M. Hegsted, J. J. Oleson, R. R. Mills, C. A. Elvehjem and E. B. Hart, *J. Nutrition*, **20**, 599 (1940). S. Ansbacher and M. Landy, *Proc. Soc. Expll. Biol. Med.*, **48**, 3 (1941).

[40] F. E. Allison, S. R. Hoover and D. Burk, *Science*, **78**, 217 (1933). F. E. Allison and F. W. Minor, *Soil Sci.*, **46**, 473 (1938).

[41] E. E. Snell, R. E. Eakin and R. J. Williams, *J. Am. Chem. Soc.*, **62**, 175 (1940).

[42] F. Kögl and B. Tönnis, *Z. physiol. Chem.*, **242**, 43 (1936).

[43] J. R. Porter and M. J. Pelczar, *Science*, **91**, 576 (1940).

[44] J. O. Lampen, A. A. Kline and W. H. Peterson, *Proc. Am. Soc. Biol Chem.*, **1941**, LXXIV

[45] E. E. Snell and L. D. Wright, *Ibid.*, **1941**, CXIX.

[46] P. György, *J. Biol. Chem.*, **131**, 733 (1939).

[47] P. György, C. S. Rose, K. Hofmann, D. B. Melville and V. du Vigneaud, *Science*, **92**, 609 (1940).

[48] F. Kögl and B. Tönnis, *Z. physiol. Chem.*, **242**, 43 (1936).

11. Physiology of Plants and Microorganisms

Vitamin H is necessary for the growth of certain bacteria, for example, various strains of *clostridium*,[49, 50] *rhizobium*,[51, 52] *staphylococcus*,[53, 54, 55] etc., and certain yeast strains.[56] Other bacterial species have been shown to synthesize biotin in varying amounts.[57] Vitamin H is also a true growth hormone for higher plants. This fact is indicated by the occurrence of increased amounts in plant seeds and in the tips of roots and coleoptiles, and has been proved experimentally with isolated pea roots.[58] The roots of higher plants excrete biotin into the soil.[59]

The role which biotin plays in plants and in microorganisms may be that of a true growth stimulant but an influence upon other factors is by no means excluded. Thus, it has been observed that in yeast biotin increases fermentation more directly than respiration, and respiration again more directly than growth.[60] It has also been shown for *rhizobium* that the respiration can be markedly increased without concomitant growth by the addition of biotin to the culture medium.[61]

12. Animal Physiology

(*a*) *Metabolism of Vitamin H*

Vitamin H is easily absorbed from the intestinal tract. The naturally occurring bound form seems to be equally well utilized, perhaps due to hydrolysis in the intestine. Some destruction appears to occur during the adsorption of biotin since the requirement by subcutaneous administration is only about one-fifth of that required for oral administration.

The animal and human organism is apparently able to store a certain amount of vitamin H especially in the liver and the kidneys. On the other hand, newborn infants have practically no such reserves. Similarly the secretion into milk, although not negligible, is markedly low. Excretion in the feces has been observed, but occurs predominantly through the urine.

[49] W. H. Peterson, L. E. McDaniel and E. McCoy, *J. Biol. Chem.*, **133**, LXXV (1940).
[50] E. E. Snell and R. J. Williams, *J. Am. Chem. Soc.*, **61**, 3594 (1939).
[51] P. M. West and P. W. Wilson, *Enzymologia*, **8**, 152 (1940).
[52] R. Nilsson, G. Bjälfve and D. Burström, *Ann. Landw. Hochschule Schwedens*, **7**, 301 (1939); *Naturwissenschaften*, **26**, 661 (1938); **27**, 389 (1939).
[53] J. R. Porter and M. J. Pelczar, *Science*, **91**, 576 (1940).
[54] F. Kögl and W. J. van Wagtendonk, *Rec. trav. chim.*, **57**, 747 (1938).
[55] G. A. Hottle, J. O. Lampen and A. M. Pappenheimer, *J. Biol. Chem.*, **137**, 459 (1941).
[56] E. E. Snell, R. E. Eakin and R. J. Williams, *J. Am. Chem. Soc.*, **62**, 175 (1940).
[57] M. Landy and D. M. Dicken, *Proc. Soc. Exptl. Biol. Med.*, **46**, 449 (1941).
[58] F. Kögl and A. J. Haagen-Smith, *Z. physiol. Chem.*, **243**, 209 (1936).
[59] P. M. West, *Nature*, **144**, 1050 (1939).
[60] D. Burk, R. J. Winzler and V. du Vigneaud, *Proc. Am. Soc. Biol. Chem.*, **1941**, XXI.
[61] F. E. Allison, S. R. Hoover and D. Burk, *Science*, **78**, 217 (1933).

During vitamin H deficiency the tissues, for example, of chicks, are practically devoid of this vitamin.[62]

(*b*) *Physiological Action of Vitamin H*

The physiological action of vitamin H is not clearly understood. A close relationship between this vitamin and fat metabolism has been postulated. A certain increase in the fat and cholesterol synthesis in the liver of rats upon feeding vitamin H has been observed. This abnormal activity can be prevented by the addition of lipocaic (an internal secretion of the pancreas) to the diet.[63]

Biotin is made unavailable to the living organism (experiments with rats, chicks and yeast) by the formation of a complex with a protein constituent of raw egg white, and the typical syndromes of a biotin deficiency occur when, instead of the free biotin, the protein-symplex is administered. (This is the historically important egg-white toxicity.) The combination of biotin with the protein is stoichiometric and biotin cannot be recovered by dialysis. The protein which has the peculiar capacity of binding biotin is called "avidin" and has been obtained in crystalline form.[64]

13. Avitaminosis

Rats develop peculiar and impressive skin changes on a diet deficient in vitamin H. These are accompanied by progressive emaciation and finally led to death. The skin lesions are differentiated from the pellagra or acrodynia syndrome since they are not predominantly a peripheral dermatosis.[65] The dermatitis can best be regarded as a seborrheid desquamative type. At the same time, biotin is involved to a certain extent in the maintenance of the normal pigment metabolism of the fur in rats and mice.[66] In human infants[67] and in rats[68] a deep brown pigmentation has been observed on the back. Male rats exhibit a greater sensitivity to vitamin H deficiency than female rats.[69, 65] A high incidence of a special type of pneumonia which could be improved and even completely cured by the

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[62] R. E. Eakin, W. A. McKinley and R. J. Williams, *Science*, **92**, 224 (1940).
[63] E. W. McHenry and G. Gavin, *Proc. Am. Soc. Biol. Chem.*, **1941**, LXXXVII.
[64] R. E. Eakin, E. E. Snell and R. J. Williams, *J. Biol. Chem.*, **136**, 801 (1940); **140**, 535 (1941).
[65] D. Pennington, E. E. Snell and R. E. Eakin, *J. Am. Chem. Soc.*, **64**, 469 (1942).
[66] P. György, *J. Biol. Chem.*, **131**, 733 (1939).
[67] P. Gyorgy and C. E. Poling. *Proc. Soc. Exptl. Biol. Med.*, **45**, 773 (1940).
[68] E. Moro, *Ekzema Infantum und Dermatitis Seboerhoides*, Berlin, 1932, p. 2.
[69] H. T. Parsons, *J. Biol. Chem.*, **90**, 351 (1931).
[70] M. A. Boas, *Biochem. J.*, **21**, 712 (1927).

administration of vitamin H[65] has been noted.[70] Certain disturbances of the nervous system in rats on a vitamin H-deficient diet have been observed.[71] Furthermore, biotin deficiency in rats causes a typical denudation around the eyes. This syndrome is known as the "spectacle eye" condition[71a] and is prevented and cured by the administration of biotin.[71b]

Vitamin H deficiency in chicks results in a specific dermatitis.[71c] The bottoms of the feet become rough and calloused and in more severe cases encrusted and hemorrhagic cracks appear. In addition, mandibular lesions occur in the corners of the mouth and around the beak, and the eyelids become swollen and stick together.

Experimental biotin deficiency in man on a diet in which about 30% of the total calories was supplied by desiccated egg white, resulted in the occurrence of a number of clinical symptoms similar to those of spontaneous avitaminosis. A striking ashy pallor of the skin and mucous membranes appeared, followed by an increasing dryness of the skin with marked reticulation and a fine branny desquamation. Extreme lassitude and somnolence, muscle pains, precordial distress and anorexia were observed. Parenteral administration of biotin cured these symptoms rapidly.[71d]

In man, vitamin H therapy has given curative results in a few isolated cases of acne vulgaris[72] and rosacea and of furunculosis. Certain cases of baldness in men are caused by seborrhoic conditions and can be improved by vitamin H administration. More severe cases of seborrhea (overaction of the sebaceous glands) seem to be related to the skin disease called psoriasis, which consists of an eruption of circumscribed rounded patches, occurring chiefly on the elbows and knees, scalp and back. Encouraging results have been attained in these cases by administration of vitamin H.

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[70] M. Gundel, P. György and W. Pagel, *Z. Hyg. Infektionskrankh.*, **113**, 629 (1932)

[71] C. M. Findlay and R. O. Stern, *Arch. Disease Childhood*, **4**, 1 (1929). J. G. Lease, H. T. Parsons and E. Kelly, *Biochem. J.*, **31**, 433 (1937).

[71a] J. Goldberger and R. D. Lillie, *Pub. Health Rep., U. S. P. H. S.* **41**, 1025 (1926). A. Bourquin and H. C. Sherman, *J. Am. Chem. Soc.*, **53**, 3501 (1931). H. E. Robinson and R. C. Newton, *Abstracts Div. of Biol. Chem., A. C. S.*, Kansas City, April 13–17, 1936. S. Lepkovsky, T. H. Jukes and M. E. Krause, *J. Biol. Chem.*, **115**, 557 (1936). B. Sjollema, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.*, **102**, 148 (1937). *Tijdschr. Diergeneesk.*, **64**, 986 (1937). P. Karrer, L. Laszt and F. Verzár, *Arch. ges. Physiol.*, **239**, 644 (1937). E. M. Mackay and R. H. Barnes, *Proc. Soc. Exptl. Biol. Med.*, **46**, 353 (1941). J. J. Oleson, H. R. Bird, C. A. Elvehjem and E. B. Hart, *J. Biol. Chem.*, **127**, 23 (1939). J. J. Oleson, C. A. Elvehjem and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.*, **43**, 161 (1940). P. L. Pavcek and H. M. Baum, *Science*, **93**, 502 (1941).

[71b] E. Nielsen and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.*, **48**, 349 (1941).

[71c] D. M. Hegsted, J. J. Oleson, R. C. Mills, C. A. Elvehjem and E. B. Hart, *J. Nutrition*, **20**, 599 (1940). D. M. Hegsted, R. C. Mills, G. M. Briggs, C. A. Elvehjem and E. B. Hart, *Ibid.*, **23**, 175 (1942).

[71d] V. P. Sydenstricker, S. A. Singal, A. P. Briggs, N. M. DeVaugh and H. Isbell, *Science*, **95**, 176 (1942).

[72] W. Marshall, *J. Invest. Dermatol.*, **2**, 205 (1939).

14. Requirements

Vitamin H is required by all animals investigated, such as rats, chicks,[73] guinea pigs, rabbits, monkeys,[74] dogs[75] and man. Man needs 50 Rat Units of vitamin H when administered subcutaneously or about three to five times this amount when given *per os*.[76] Cattle do not necessarily need an external supply of vitamin H since the microorganisms in the rumen synthesize the vitamin.[77] The requirements of microorganisms (bacteria) have been discussed previously (see page 475).

Men have responded favorably to parenteral administration of 150–300 gamma of biotin per day. Chicks need about 2.5–10 gamma and rats about 1–3 gamma per day.

[73] A. T. Ringrose, L. C. Norris and G. F. Heuser, *Poultry Sci.*, **10**, 166 (1931).

[74] J. G. Lease, H. T. Parsons and E. Kelly, *Biochem. J.*, **31**, 433 (1937).

[75] P. György, *J. Biol. Chem.*, **131**, 733 (1939).

[76] P. György, *Ibid.*, **119**, XLIII (1937). F. Schultz, *Med. u. Chem. Abhandl. med.-chem. Forschungstätten I. G. Farbenind.*, **3**, 143 (1936). P. György and C. S. Rose, *Proc. Soc. Expll. Biol. Med.*, **43**, 73 (1940).

[77] M. I. Wegner, A. N. Booth, C. A. Elvehjem and E. B. Hart, *Proc. Soc. Expll. Biol. Med.*, **45**, 769 (1940).

THE GROUP OF VITAMINS K

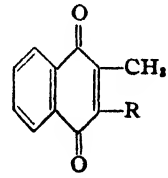
THE GROUP OF VITAMINS K[1]

1. Nomenclature and Survey

Names:

Vitamins K.[2]
Phylloquinones,[3] α-, β-, etc.
Koagulations Vitamin. (German and Scandinavian term, from which the present term "vitamin K" was derived.)
Coagulation vitamin.
Prothrombin factor.
Antihemorrhagic vitamin.

Structure:



Vitamin K_1: $R = -CH_2 \cdot CH = C(CH_3) \cdot CH_2 \cdot [CH_2 \cdot CH_2 \cdot CH(CH_3) \cdot CH_2]_2 \cdot CH_2 \cdot CH_2 \cdot CH(CH_3) \cdot CH_3$.

Vitamin K_2: $R = -CH_2 \cdot [CH = C(CH_3) \cdot CH_2 \cdot CH_2]_5 \cdot CH = C(CH_3) \cdot CH_3$.

2-Methyl-1,4-naphthoquinone: R=H.

2. Chronology

1929 DAM[4] observed subcutaneous and intramuscular hemorrhages in chicks raised on an artificial diet.

1934 DAM and SCHØNHEYDER[5] concluded that the hemorrhagic conditions of the chicks were caused by an avitaminosis and called the new factor vitamin K.[6]

[1] H. R. Butt and A. M. Snell, *Vitamin K*, Philadelphia, 1941.
[2] H. Dam, *Nature*, **135**, 652 (1935).
[3] P. Karrer and A. Geiger, *Helv. Chim. Acta*, **22**, 945 (1939).
[4] H. Dam, *Biochem. Z.*, **215**, 475 (1929); **220**, 158 (1930).
[5] H. Dam and F. Schønheyder, *Biochem. J.*, **28**, 1355 (1934).
[6] H. Dam, *Nature*, **135**, 652 (1935).

1935 DAM, SCHØNHEYDER and co-worker[7] found that vitamin K takes part in restoring and maintaining the normal prothrombin level of blood.

1938 WARNER, BRINKHOUS and SMITH,[8] BUTT, SNELL and OSTERBERG,[9] and DAM and GLAVIND[10] demonstrated clinically the usefulness of vitamin K for man.

1939 DAM, KARRER and co-workers,[11] isolated pure vitamin K_1 and DOISY and co-workers,[12] isolated pure vitamin K_2. The synthesis of vitamin K_1 was achieved independently in three different laboratories, namely, in those of ALMQUIST,[13] DOISY[14] and FIESER.[15] ANSBACHER and FERNHOLZ[16] discovered the antihemorrhagic activity of 2-methyl-1,4-naphthoquinone.

1940 DOISY and co-workers[17] elucidated the chemical structure of vitamin K_2.

3. The Group of Vitamins K

The existence of only two naturally occurring vitamins K of high activity, namely, vitamins K_1 and K_2, has been proved with certainty. Phthiocol, a naturally occurring compound, is also somewhat active but in considerably higher amounts. The inclusion of this compound into the class of naturally occurring vitamins K depends upon the proof that this compound is not an artefact from the alkaline hydrolysis procedure used for its isolation.[18, 19] It is, however, conceivable that besides vitamins K_1 and K_2 other members of similar structure occur naturally, especially of the type with β-unsaturated isoprenoid side chains in the 3-position of 2-methyl-1,4-naphthoquinone, such as 3-farnesyl- or 3-geranyl-derivatives.[20]

Indication for the occurrence of at least one other factor has been obtained through the isolation of colorless fractions.[21, 22] This factor is highly active and resembles in its behavior the oxides of vitamins K. It is, therefore, possible that this compound or compounds are oxides obtained during the procedure of isolation.

[7] H. Dam, *Nature*, **135**, 652 (1935). F. Schønheyder, *Ibid.*, **135**, 652 (1935). H. Dam, F. Schønheyder and E. Tage-Hansen, *Biochem. J.*, **30**, 1075 (1936).

[8] E. D. Warner, K. M. Brinkhous and H. P. Smith, *Proc. Soc. Exptl. Biol. Med.*, **37**, 628 (1938).

[9] H. R. Butt, A. M. Snell and A. E. Osterberg, *Proc. Staff Meetings Mayo Clinic*, **13**, 74 (1938).

[10] H. Dam and J. Glavind, *Lancet*, **234**, 720 (1938); *Acta Med. Scand.*, **96**, 108 (1938).

[11] H. Dam, A. Geiger, J. Glavind, P. Karrer, W. Karrer, E. Rothschild and H. Salomon, *Helv. Chim. Acta*, **22**, 310 (1939).

[12] R. W. McKee, S. B. Binkley, D. W. MacCorquodale, S. A. Thayer and E. A. Doisy, *J. Am. Chem. Soc.*, **61**, 1295 (1939).

[13] H. J. Almquist and A. A. Klose, *Ibid.*, **61**, 2557 (1939).

[14] S. B. Binkley, L. C. Cheney, W. F. Holcomb, R. W. McKee, S. A. Thayer, D. W. MacCorquodale and E. A. Doisy, *Ibid.*, **61**, 2558 (1939).

[15] L. F. Fieser, *Ibid.*, **61**, 2559 (1939).

[16] S. Ansbacher and E. Fernholz, *Ibid.*, **61**, 1924 (1939).

[17] S. B. Binkley, R. W. McKee, S. A. Thayer and E. A. Doisy, *J. Biol. Chem.*, **133**, 721 (1940).

[18] L. F. Fieser, *J. Am. Chem. Soc.*, **61**, 2559 (1939).

[19] L. F. Fieser, *Ibid.*, **61**, 3467 (1939).

[20] L. F. Fieser, M. Tishler and W. L. Sampson, *J. Biol. Chem.*, **137**, 659 (1941).

[21] S. Ansbacher, E. Fernholz and H. B. MacPhillamy, *Proc. Soc. Exptl. Biol. Med.*, **42**, 655 (1939).

[22] E. Fernholz, S. Ansbacher and M. L. Moore, *J. Am. Chem. Soc.*, **61**, 1613 (1939).

4. Occurrence

Generally speaking, vitamins K occur only in plants and in microorganisms. Which one of the group of vitamins K is present in each source is largely unknown, but it is assumed that the green leaves of plants contain predominantly or solely vitamin K_1, whereas the microorganisms contain vitamin K_2.

The best sources of vitamin K_1 are the green leafy tissues of alfalfa, spinach, cabbage,[23, 24] kale, cauliflower, nettle, chestnut,[25] etc. Vitamin K is also present in tomatoes, hempseed, seaweed[25] and soybean oil.[26] Fruits and cereals are very poor sources of the vitamin.

Vitamin K_2 occurs in many microorganisms, especially in most bacteria,[27] while molds, yeasts and fungi contain no, or practically no, vitamin K. From a practical standpoint it is important that the microorganisms in the intestinal tract contain abundant quantities. Most putrefied animal and plant materials contain high amounts due to bacterial growth.

Animal materials contain very little vitamin K, although milk and eggs contain small amounts. Hog liver[28] is the richest animal source found so far. The livers of chicks[28, 29, 30] and of rats[28] contain very little vitamin K.

5. Isolation

The isolation of vitamin K_1 from, for example, alfalfa leaf meal, is carried out by first extracting the dried plant material with petroleum ether, hexane[31] or acetone,[32] followed by an adsorption of the chlorophyll present on zinc carbonate,[33] magnesium oxide[34] or phosphotungstic acid.[34] Upon cooling the remaining solution, some material precipitates and is discarded. The solution is then subjected to fractional distillation.[35, 36] Vitamin K_1 distills between 120° and 140° C. at 10^{-4} mm. Hg. Several compounds, especially sterols, are separated from the distillate by crystallization from

[23] H. Dam, *Nature*, **135**, 652 (1935); *Biochem. J.*, **29**, 1273 (1935).
[24] H. J. Almquist and E. L. R. Stokstad, *Nature*, **136**, 31 (1935); *J. Biol. Chem.*, **111**, 105 (1935).
[25] H. Dam and J. Glavind, *Biochem. J.*, **32**, 485 (1938).
[26] H. J. Almquist and E. L. R. Stokstad, *J. Nutrition*, **14**, 235 (1937).
[27] H. J. Almquist, C. F. Pentler and E. Mecchi, *Proc. Soc. Exptl. Biol. Med.*, **38**, 336 (1938).
[28] H. Dam and F. Schønheyder, *Biochem. J.*, **30**, 897 (1936).
[29] H. Dam and J. Glavind, *Lancet*, **234**, 720 (1938).
[30] H. J. Almquist and E. L. R. Stokstad, *J. Nutrition*, **12**, 329 (1936).
[31] H. J. Almquist and E. L. R. Stokstad, *Ibid.*, **12**, 329 (1936).
[32] H. Dam and F. Schønheyder, *Biochem. J.*, **30**, 897 (1936).
[33] P. Karrer, A. Geiger, R. Legler, A. Rüegger and H. Salomon, *Helv. Chim. Acta*, **22**, 1464 (1939).
[34] A. A. Klose and H. J. Almquist, *J. Am. Chem. Soc.*, **61**, 532 (1939).
[35] B. Riegel, C. E. Schweitzer and P. G. Smith, *J. Biol. Chem.*, **129**, 495 (1939).
[36] H. J. Almquist, *Ibid.*, **120**, 635 (1937). H. Dam and L. Lewis, *Biochem. J.*, **31**, 17 (1937).

acetone. By application of the chromatographic adsorption technic using first dehydrated magnesium sulfate and then zinc carbonate, the pure vitamin can be obtained.[33] Other adsorbing agents such as calcium sulfate,[37] weakly acidic fuller's earth (Florex),[38] Permutit,[39, 40] Darco,[39] etc., have been used for the isolation of both vitamins K_1 and K_2.

The isolation of vitamin K according to these procedures is relatively difficult. A very simple method has, however, been devised for the isolation of vitamin K in the reduced form.[41] This consists in reducing a crude vitamin K concentrate in alcohol solution with sodium hydrosulfite and extracting the reduced vitamin with petroleum ether. The solvent solution is then extracted with 5% sodium hydroxide containing hydrosulfite, which removes considerable amounts of foreign matter. The petroleum ether solution is then extracted with Claisen's alkali solution,[42] prepared from potassium hydroxide, water and methanol, to which hydrosulfite has been added. The alkaline solution is diluted with water and the reduced vitamin is extracted with ether. The ether solution is washed with hydrosulfite solution and the reduced vitamin is obtained by evaporation of the solvent. The vitamin itself is obtained from the reduced compound by shaking in ether solution with silver oxide and magnesium sulfate.

Vitamin K can also be isolated in the form of the diacetate of the reduced form which is prepared by dissolving the vitamin in acetic anhydride, and adding zinc dust and pyridine.[43, 44]

6. Properties

The vitamins K are soluble in most of the common organic solvents, especially in ether, petroleum ether, hexane, acetone, etc., but are insoluble in water and only sparingly soluble in methanol. The vitamins are essentially thermostable.[45] They are very sensitive to alkali[46] and to light from various sources, such as sunlight,[47, 48] the illumination from ordinary

[37] H. Dam, *Z. Vitaminforsch.*, **8**, 248 (1938 39).

[38] B. Riegel, C. E. Schweitzer and P. G. Smith, *J. Biol. Chem.*, **129**, 495 (1939).

[39] S. B. Binkley, D. W. MacCorquodale, S. A. Thayer and E. A. Doisy, *Ibid.*, **130**, 219 (1939).

[40] R. W. McKee, S. B. Binkley, S. A. Thayer, D. W. MacCorquodale and E. A. Doisy, *Ibid.*, **131**, 327 (1939).

[41] L. F. Fieser, *J. Am. Chem. Soc.*, **61**, 3467 (1939).

[42] L. Claisen, *Ann.*, **418**, 96 (1919).

[43] L. F. Fieser, *J. Am. Chem. Soc.*, **61**, 3467 (1939).

[44] S. B. Binkley, D. W. MacCorquodale, L. C. Cheney, S. A. Thayer, R. W. McKee and E. A. Doisy, *Ibid.*, **61**, 1612 (1939).

[45] H. J. Almquist, *J. Biol. Chem.*, **120**, 635 (1937).

[46] H. J. Almquist and E. L. R. Stokstad, *J. Nutrition*, **14**, 235 (1937).

[47] D. W. MacCorquodale, S. B. Binkley, R. W. McKee, S. A. Thayer and E. A. Doisy, *Proc. Soc. Exptl. Biol. Med.*, **40**, 482 (1939).

[48] P. Karrer and A. Geiger, *Helv. Chim. Acta*, **22**, 945 (1939).

Mazda light bulbs[47] and ultraviolet light.[49] The vitamins K exhibit typical absorption characteristics in the ultraviolet region with maxima at 243, 248, 261, 270 and 328 mμ (Fig. 24).

Vitamin K_1 is a yellow oil, which melts at about −20° C. The redox potential is $E_m = +0.005$ volt. Vitamin K_1 exhibits a white fluorescence in the light of an argon lamp.[50] This phenomenon has not been observed in simple naphthoquinones without side chains in the 3-position.

Vitamin K_2 is a yellow crystalline solid, m. p. 53.5–54.5° C.[51]

2-Methyl-1,4-naphthoquinone is a lemon-yellow crystalline powder with faint but characteristic odor, melting at 106° C. It is soluble in

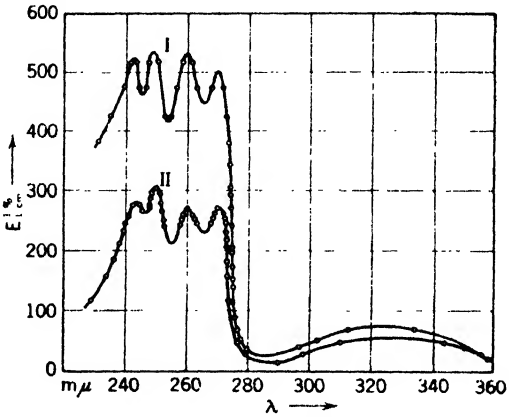


Fig. 24.—Absorption spectra of vitamin K_1 (I) and of vitamin K_2 (II). (D. T. Ewing, J. M. Vandenbelt and O. Kamm.)

water to the extent of 0.13 mg. per ml. The absorption spectrum of 1,4-naphthoquinone and the diacetate of the hydroquinone are shown in Fig. 25.

7. Chemical Constitution

(a) *The Constitution of Vitamin K_1*

Vitamin K_1 has the empirical formula $C_{31}H_{46}O_2$ and, according to potentiometric titrations with sodium hydrosulfite, a molecular weight of 450.[52] The presence of the oxygen atoms as quinone oxygens was deduced from the redox potential which proved to be very similar to that

[49] H. J. Almquist, *J. Biol. Chem.*, **117**, 517 (1937).
[50] H. J. Almquist and A. A. Klose, *J. Am. Chem. Soc.*, **61**, 2557 (1939).
[51] S. B. Binkley, R. W. McKee, S. A. Thayer and E. A. Doisy, *Ibid.*, **133**, 721 (1940).
[52] P. Karrer and A. Geiger, *Helv. Chim. Acta*, **22**, 945 (1939).

of many 1,4-quinones such as, for example, the anthraquinone-hydroanthraquinone system.[52] The presence of a quinoid structure was also suspected from the absorption spectrum of the vitamin[53] which proved to be very similar to 2,3-disubstituted 1,4-naphthoquinones. Further indications were the instability of the vitamin toward alkali and light and finally the absorption of eight atoms of hydrogen upon catalytic hydrogenation[53] (I → II). The reduced compound is colorless, but upon exposure to light it is oxidized to a yellow compound (III) the color of which is similar to that of the vitamin itself. This suggests that the original

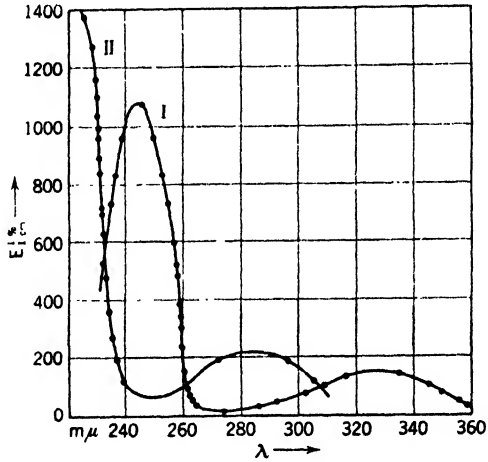
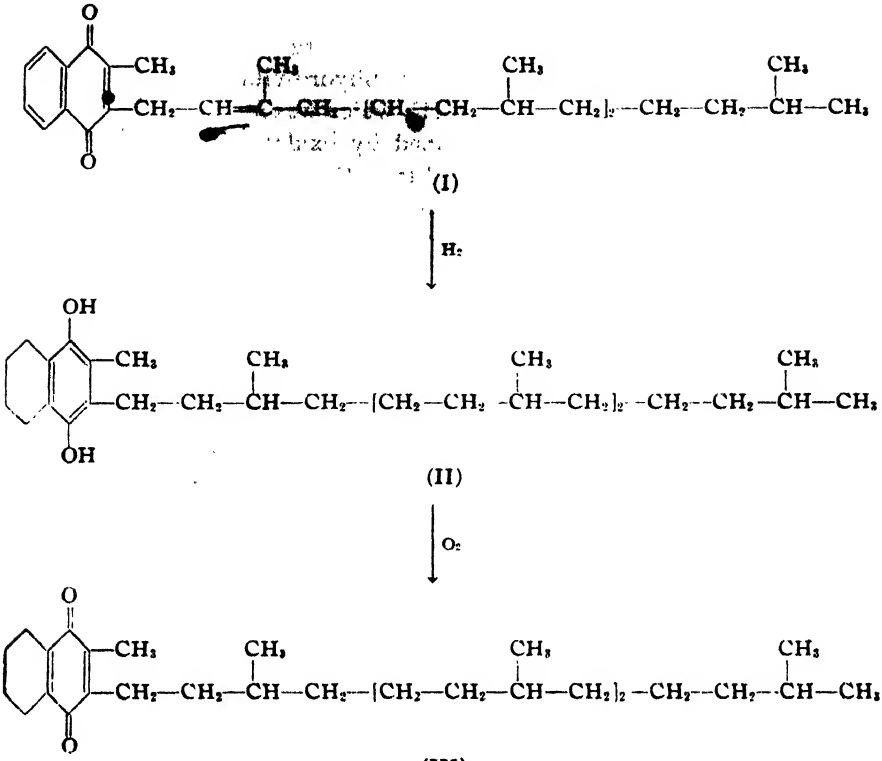


Fig. 25.—Absorption spectra of 1,4-naphthoquinone (I) and the diacetate of 1,4-naphthohydroquinone (II). (D. T. Ewing, J. M. Vandenbelt and O. Kamm.)

vitamin is a quinone which is reduced catalytically and partially reoxidized. Further proof was furnished by reductive acetylation which yielded a crystalline diacetate (IV) (formula on page 488) from which the vitamin could be recovered by hydrolysis by means of a Grignard reaction, followed by air oxidation.[54] The color of the quinone is yellow, which suggests a 1,4-position of the oxygen atoms, since the only other possible quinones, namely, the 1,2-quinones, are red.

[52] R. W. McKee, S. B. Binkley, D. W. MacCorquodale, S. A. Thayer and E. A. Doisy, *J. Am. Chem. Soc.*, **61**, 1295 (1939).

[53] S. B. Binkley, D. W. MacCorquodale, L. C. Cheney, S. A. Thayer, R. W. McKee and E. A. Doisy, *Ibid.*, **61**, 1621 (1939).



(I)

(II)

(III)

Vitamin K_1 upon oxidation with an excess of chromic acid yielded phthalic acid (V).[55] This shows that the aromatic non-quinoid ring has no side chains attached and suggests that a 1,4-naphthoquinone is present which then must be substituted in the 2- and 3-positions. Mild chromic acid oxidation yielded 2-methyl-1,4-naphthoquinone-3-acetic acid (VI)[55] which was identified by comparison with a synthetic specimen.[56] Similarly, chromic acid oxidation of the diacetate of dihydro-vitamin K_1 yielded 1,4-diacetoxy-2-methyl-naphthalene-3-acetic acid,[56] the methyl-ester of which proved to be identical with the synthetic compound of the indicated structure.

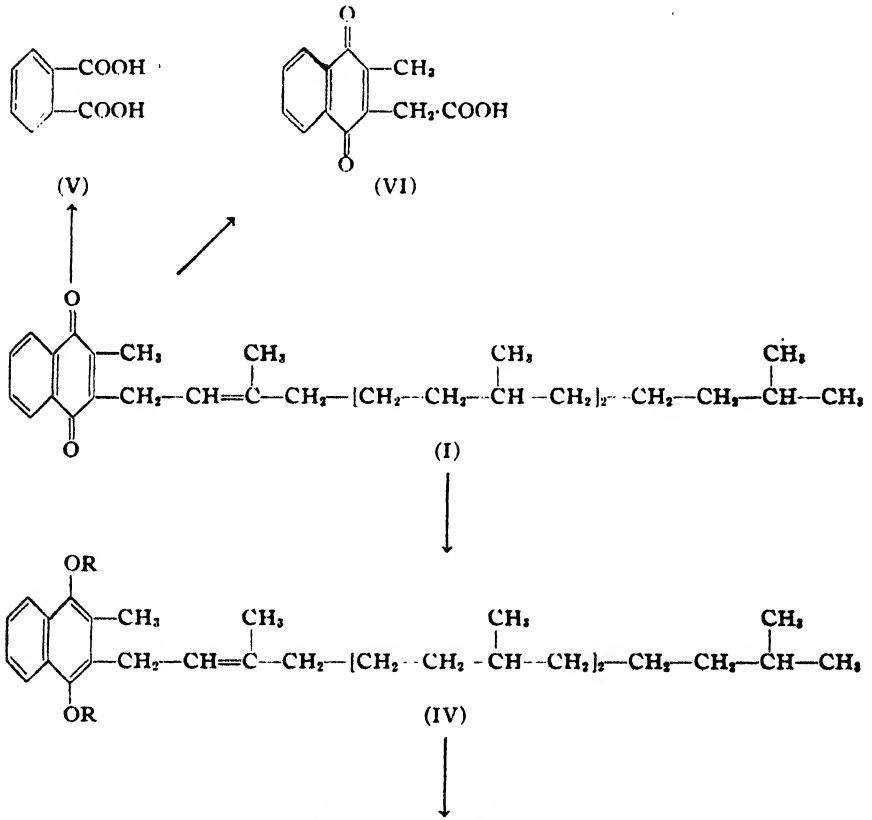
As indicated above, catalytic hydrogenation of vitamin K_1 yielded a hydroquinone by absorption of four mols of hydrogen. Since hydrogena-

[55] D. W. MacCorquodale, S. B. Binkley, S. A. Thayer and E. A. Doisy, *J. Am. Chem. Soc.*, **61**, 1928 (1939).

[56] S. B. Binkley, L. C. Cheney, W. F. Holcomb, R. W. McKee, S. A. Thayer, D. W. MacCorquodale and E. A. Doisy, *Ibid.*, **61**, 2558 (1939).

tion of a naphthoquinone nucleus would require only three mols, the presence of a double bond in a side chain was indicated. This was proved by oxidation of the diacetate of dihydro-vitamin K_1 with ozone which yielded a ketone, $C_{18}H_{36}O_{6}$ (VIII), which was found to be identical[56] with the corresponding ketone obtained by oxidation of phytol.[57] The constitution of this ketone is 2,6,10-trimethyl-pentadecanone-14. The other reaction product from the ozonolysis is 1,4-diacetoxy-2-methyl-naphthalene-3-acetaldehyde (VII) which was characterized as the semicarbazone.[58, 59]

These degradation reactions prove conclusively the structure of vitamin K_1 as 2-methyl-3-phytyl-1,4-naphthoquinone.

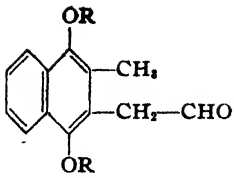


(Formula continued on following page.)

[57] F. G. Fischer and K. Löwenberg, *Ann.*, **464**, 69 (1929).

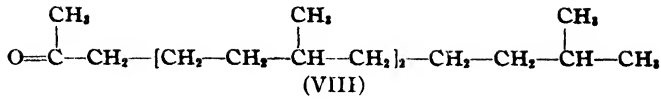
[58] S. B. Binkley, R. W. McKee, S. A. Thayer and E. A. Doisy, *Proc. Am. Soc. Biol. Chem.*, **1940**, XII.

[59] S. B. Binkley, R. W. McKee, S. A. Thayer and E. A. Doisy, *J. Biol. Chem.*, **133**, 721 (1940).



(VII)

+



(VIII)

(*b*) *The Constitution of Vitamin K_2*

The methods used for the elucidation of the structure of vitamin K_1 and the knowledge gained from the proof of the structure of this vitamin were successfully applied for the determination of vitamin K_2.

Vitamin K_2 has the empirical formula $C_{41}H_{56}O_2$. Upon reductive acetylation, a diacetate of dihydro-vitamin K_2 is formed[60] (I → II) which proves the presence of the two oxygens in a quinone structure. The quinone structure is also revealed by the absorption spectrum, which is very similar to that of vitamin K_1. (See Fig. 24 on page 485.)

Upon catalytic hydrogenation, vitamin K_2 absorbs 9 mols of hydrogen.[61] Since three mols are required for reduction of the quinone structure, the presence of six double bonds in the side chains is indicated. This assumption is confirmed by the fact that dihydro-vitamin K_2-diacetate adds 12 atoms of bromine. These six double bonds are not in conjugation to each other since no addition product is formed with maleic anhydride and since the ultraviolet absorption spectrum does not indicate the presence of any conjugated double bonds in addition to the quinone structure.

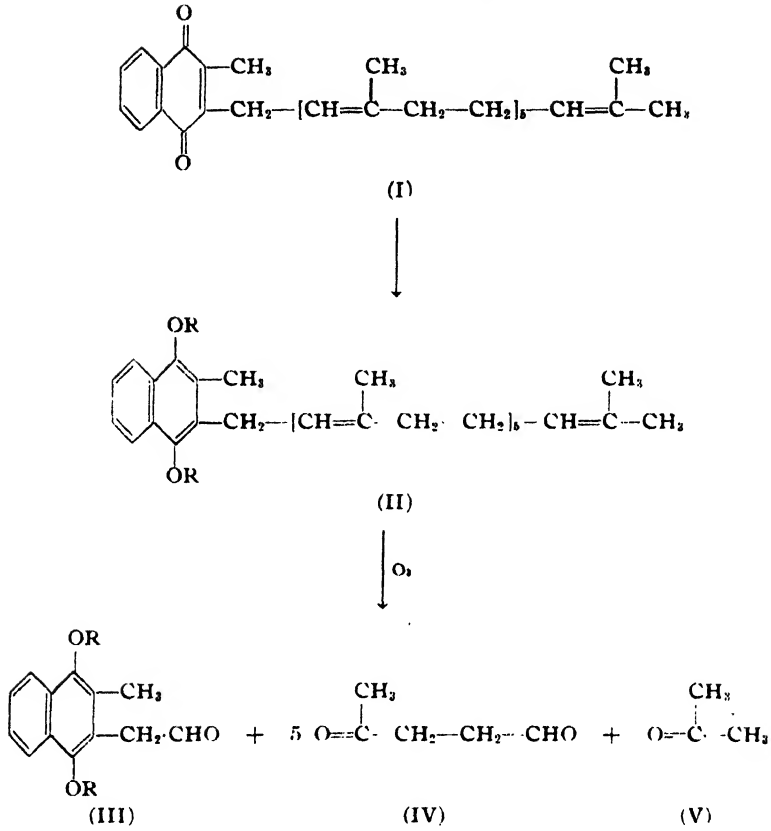
Oxidation of vitamin K_2 with ozone[62, 63] yields 1,4-diacetoxy-2-methyl-acetaldehyde (III) which was characterized as the semicarbazone. Levulin-aldehyde (IV) was further isolated from the oxidation product and identified as the bis-2,4-dinitro-phenyl-hydrazone. Assuming that 1 mol of vitamin K_2 would yield 5 mols of levulin-aldehyde, this compound was obtained in an 81% yield. Acetone (V) was obtained also from the ozonolysis in the form of its 2,4-dinitro-phenyl-hydrazone in a yield of 56%, assuming that one mol of acetone originates from one mol of the vitamin.

[60] S. B. Binkley, D. W. MacCorquodale, L. C. Cheney, S. A. Thayer, R. W. McKee and E. A. Doisy, *J. Am. Chem. Soc.*, **61**, 1612 (1939).

[61] R. W. McKee, S. B. Binkley, D. W. MacCorquodale, S. A. Thayer and E. A. Doisy, *Ibid.*, **61**, 1295 (1939).

[62] S. B. Binkley, R. W. McKee, S. A. Thayer and E. A. Doisy, *Proc. Am. Soc. Biol. Chem.*, 1940, XII.

[63] S. B. Binkley, R. W. McKee, S. A. Thayer and E. A. Doisy, *J. Biol. Chem.*, **133**, 721 (1940).



On the basis of these degradation products vitamin K_2 has the constitution (I) of a 2-methyl-3-difarnesyl-naphthoquinone-1,4.[64]

8. Synthesis

(a) *Synthesis of 2-Methyl-1,4-naphthoquinone*

The simplest method for the synthesis of 2-methyl-1,4-naphthoquinone is the oxidation of 2-methyl-naphthalene. The latter compound is a by-product obtained from the coal tar industry.[65] The oxidation is best accomplished by means of chromic acid in acetic acid[66, 67] at temperatures

[64] S. B. Binkley, R. W. McKee, S. A. Thayer and E. A. Doisy, *J. Biol. Chem.*, **133**, 721 (1940). P. Karrer and A. Epprecht, *Helv. Chim. Acta*, **23**, 272 (1940).

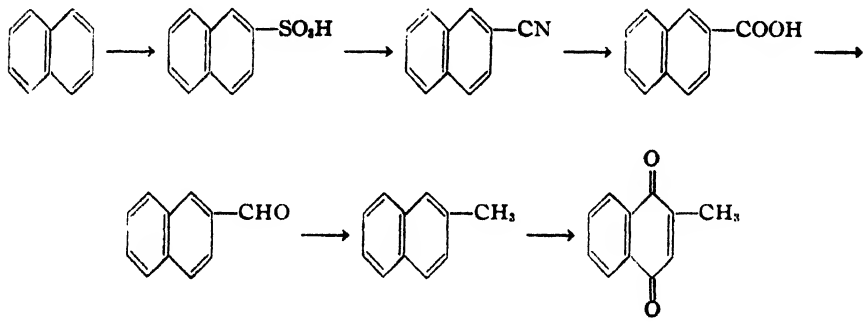
[65] E. A. Coulson, *J. Soc. Chem. Ind.*, **60**, 123 (1941).

[66] K. Fries and W. Lohmann, *Ber.*, **54**, 2918 (1921).

[67] R. J. Anderson and M. S. Newman, *J. Biol. Chem.*, **103**, 406 (1933).

below 50° C.[68, 69] This oxidation can, however, also be carried out by other oxidizing agents, such as molecular oxygen (air), hydrogen peroxide,[70] etc. Yields between 30% and 60% of theory have been reported for this oxidation.

2-Methyl-1,4-naphthoquinone has also been synthesized by a simple and practical reaction sequence from naphthalene.[71] Naphthalene is sulfonated in the β-position and the sodium salt yields β-naphthoic acid nitrile upon treatment with $K_4Fe(CN)_6$. The nitrile is hydrolyzed to the free β-naphthoic acid, the Ba-salt of which gives upon distillation with barium formate the corresponding aldehyde in 60% yield. Clemmensen reduction of the oxo-compound yields 2-methyl-naphthalene which is converted by oxidation into 2-methyl-naphthoquinone.



A total synthesis of 2-methyl-1,4-naphthoquinone has been accomplished[72] by condensing benzene by means of aluminum chloride with the anhydride of methyl-succinic acid, which is obtained from either citric acid or *d*-tartaric acid. The reaction product, α-methyl-β-benzoyl-propionic acid, is reduced by the Clemmensen method to yield α-methyl-γ-phenyl-butyric acid. Ring-closure of its chloride with $AlCl_3$ yields 2-methyl-α-tetralone, which is converted into 2-methyl-1,2,3,4-tetrahydro-naphthalene by reduction. Dehydrogenation with sulfur or with selenium yields 2-methyl-naphthalene, which is oxidized to the quinone.

[68] L. I. Smith and I. M. Webster, *J. Am. Chem. Soc.*, **59**, 662 (1937).

[69] L. F. Fieser, W. P. Campbell, E. M. Frey and M. D. Gates, *Ibid.*, **61**, 2559, 3216 (1939).

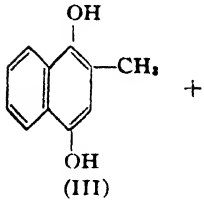
[70] R. T. Arnold and R. Larson, *J. Org. Chem.*, **5**, 250 (1940).

[71] P. P. T. Sah, W. Brüll and H. Holzen, *Ber.*, **73**, 762 (1940). P. P. T. Sah, *Rec. trav. chim.*, **59**, 161 (1940).

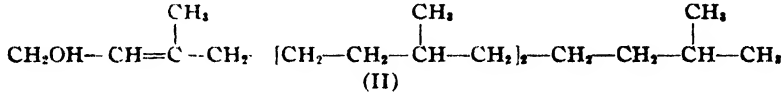
[72] P. P. T. Sah and W. Brüll, *Ber.*, **73**, 1430 (1940).

(*b*) *Synthesis of Vitamin* K_1

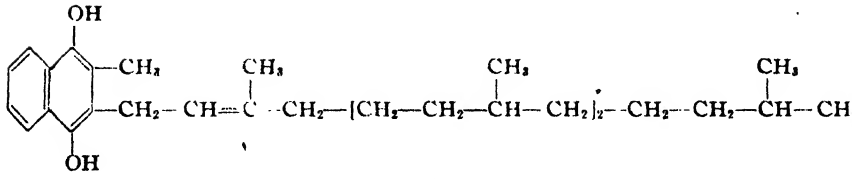
The synthesis of vitamin K_1 (I) has been carried out from 2-methyl-1,4-naphthoquinone and phytol (II) by a number of different methods. Thus the synthesis has been effected from these compounds alone,[73] or, better, from 2-methyl-1,4-naphthohydroquinone (III) and phytol in the



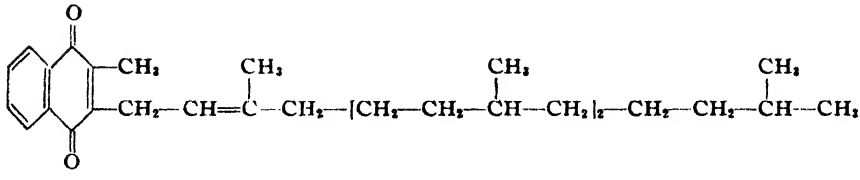
(III)



(II)



(IV)



(I)

[73] H. J. Almquist and A. A. Klose, *J. Am. Chem. Soc.*, 61, 2557 (1939).

presence of catalysts, such as oxalic acid or trichloro-acetic acid[74] using dioxane as a solvent. Another variation is to use the mono-sodium salt of 2-methyl-1,4-hydroquinone and phytyl bromide.[75] The hydroquinone has also been condensed with phytol in benzene solution using zinc chloride as the condensing agent. Vitamin K_1-hydroquinone (IV) is obtained from the methods involving the use of the hydroquinone and is then oxidized to the vitamin (I) by air or silver oxide.

The best yields can be obtained by working under mildly acidic conditions. In the presence of strong acids, such as mineral acids, the condensation is carried further, giving rise to compounds of the vitamin E type. Condensation in alkaline medium, on the other hand, gives low yield and undesirable dark brown reaction mixtures from which it is difficult to isolate the pure vitamin K_1.

Vitamin K_1 has also been synthesized in low yields from phthiocol, in its reduced form, and phytol.[76]

(c) *Synthesis of Vitamin K_2*

The synthesis of vitamin K_2 has not been accomplished as yet.

9. Industrial Methods of Preparation

The concentrates of natural vitamins K, such as alfalfa concentrates, are still of considerable industrial interest, since they contain the natural vitamin K_1 which is clinically used. Synthetic vitamin K_1 is available, prepared according to the methods previously described, but is not widely used due to the high cost. The synthetic 2-methyl-naphthoquinone, which is at least as active although at high doses somewhat more toxic than the natural vitamins K, is offered on the market and is used clinically.

Water-soluble forms of vitamin K have been introduced on the market. While the natural vitamins and 2-methyl-naphthoquinone are fat-soluble and require the presence of bile acids for proper absorption from the intestinal tract, the water-soluble forms are absorbed as such and are also important for intravenous administration. The following water-soluble compounds of vitamin K action are used: 2-methyl-1,4-naphtho-

[74] L. F. Fieser, *J. Am. Chem. Soc.*, **61**, 2559 (1939).

[75] S. B. Binkley, L. C. Cheney, W. F. Holcomb, R. W. McKee, S. A. Thayer, D. W. MacCorquodale and E. A. Doisy, *Ibid.*, **61**, 2558 (1939). D. W. MacCorquodale, L. C. Cheney, S. B. Binkley, W. F. Holcomb, R. W. McKee, S. A. Thayer and E. A. Doisy, *J. Biol. Chem.*, **131**, 357 (1939).

[76] M. Tishler, L. F. Fieser and N. L. Wendler, *J. Am. Chem. Soc.*, **62**, 1982 (1940).

hydroquinone-3-sodium-sulfonate,[77] 4-amino-2-methyl-1-naphthol hydrochloride[78, 79, 80] and sodium 2-methyl-1,4-naphthohydroquinone diphosphate.[81, 82, 83]

10. Biogenesis

The vitamins K are synthesized by plants and by certain microorganisms. In plants, the site of synthesis is in the green leaves. Thus the tops of carrots contain a considerable amount of vitamin K while the roots contain practically none.[84] The synthesis is greatly influenced by sunlight. Peas grown in the dark contain only small amounts, while control plants raised in light contain considerably more.[85] The outer leaves of cabbage contain about four times more vitamin K than the inner leaves.[85]

The synthesis of vitamin K_1 is presumably accomplished by methods similar to those used in the laboratory, that is, using phytol and methylnaphthoquinone as building units.[86] Vitamin K_2 is synthesized in certain microorganisms which live on vitamin K-free diets.[87] Coli bacteria synthesize vitamin K on a synthetic medium which contains as organic constituents only glucose, citrate and asparagin.[88]

11. Specificity

The physiological activity of the vitamins K_1 and K_2 is by no means restricted to these compounds. A number of other similar compounds have the same property. From the vast amount of work done to elucidate the specificity of the vitamin K action the following conclusions can be drawn: The quinones may be converted into the corresponding hydroquinones, their esters and ethers without essential loss of efficacy as long as the derivatives can be reconverted into the quinones under more or less simple conditions which also may occur in the organism. The unsub-

[77] M. B. Moore and F. J. Kirchmeyer, quoted in H. R. Butt and A. M. Snell, *Vitamin K*, Philadelphia, 1941. M. B. Moore, *J. Am. Chem. Soc.*, **63**, 2049 (1941).

[78] E. A. Doisy, D. W. MacCorquodale, S. A. Thayer, S. B. Binkley and R. W. McKee, *Science*, **90**, 407 (1939).

[79] H. J. Almquist and A. A. Klose, *Proc. Soc. Exptl. Biol. Med.*, **45**, 55 (1940).

[80] D. Richtert, S. A. Thayer, R. W. McKee, S. B. Binkley and E. A. Doisy, *Ibid.*, **44**, 601 (1940).

[81] L. F. Fieser and E. M. Frey, *J. Am. Chem. Soc.*, **62**, 228 (1940).

[82] R. H. K. Foster, J. Lee and U. V. Solmssen, *Ibid.*, **62**, 453 (1940).

[83] S. Ansbacher, E. Fernholz and M. A. Dolliver, *Proc. Soc. Exptl. Biol. Med.*, **43**, 652 (1940).

[84] H. J. Almquist, *J. Biol. Chem.*, **117**, 517 (1937).

[85] H. Dam and J. Glavind, *Biochem. J.*, **32**, 485 (1938).

[86] L. F. Fieser, W. P. Campbell and E. M. Frey, *J. Am. Chem. Soc.*, **61**, 2206 (1939).

[87] H. J. Almquist, C. F. Pentler and E. Mecchi, *Proc. Soc. Exptl. Biol. Med.*, **38**, 336 (1938). A. E. Osterberg, *Proc. Staff Meetings Mayo Clinic*, **13**, 72 (1938).

[88] H. Dam, J. Glavind, S. Orla-Jensen and A. D. Orla-Jensen, *Naturwissenschaften*, **29**, 287 (1941).

stituted benzene ring of the vitamin molecule cannot be substituted without loss of activity. The 2-methyl group cannot be replaced by hydrogen or higher alkyl groups without considerable decrease in activity. The substituents on the 3-position, however, can vary considerably and the change in efficacy is only slight. The most remarkable fact is that when the substituent in 3-position is hydrogen, that is, when 2-methyl-1,4-naphthoquinone is tested, an activity of from two to four times that of vitamin K_1 per weight unit is observed. This unique activity of 2-methyl-naphthoquinone has caused widespread interest which led to the introduction of this compound into clinical therapy. Its unusual efficacy has been explained by the hypothesis that this compound itself does not act as a vitamin in the organism but that it is converted in the organism into a quinone of the true vitamin K type. On the other hand, it has been postulated that the vitamins K_1 and K_2 owe their activity to their degradation in the organism to 2-methyl-1,4-naphthoquinone.[89] While the latter hypothesis appears less attractive, no experimental proof for the validity of the former can be offered other than the fact that such a synthesis is easily accomplished in the laboratory and that the building units for a side chain of the vitamin K_1 or K_2 type are readily available.

The effect of variations of the side chain in 3-position are noteworthy. A double bond in the β-γ-position contributes to the potency while unsaturation at points more remote from the quinoid nucleus is without influence. A branched side chain, built from isoprene units, is more active than a strictly straight chain and maximum activity is reached when some 20 or 30 carbon atoms are present.

Finally, the fact that the activity of the 2,3-oxides of the vitamins K is almost equivalent to that of the vitamins is noteworthy. Compounds which may be identical with these have been isolated from natural materials, but their identity has not been established as yet and the possibility of an artefact during the process of isolation cannot be ruled out.

These conclusions have been drawn from a large number of experiments with many compounds. In the following tables, comparative data are given for the minimum effective dose of various series of compounds which are chemically closely related. The effective dose in these tables is defined as the minimum amount of material which, when administered in 0.1 cc. of peanut oil, will reduce the blood clotting times of 60 to 80% of vitamin K-deficient chicks to less than 10 minutes during an 18-hour period.[90]

[89] H. J. Almquist, *Physiol. Rev.*, **21**, 194 (1941)

[90] L. F. Fieser, M. Tishler and W. L. Sampson, *J. Biol. Chem.*, **137**, 659 (1941).

TABLE I[90]

2-METHYL-3-ALKYL- AND 3-β-ALKENYL-1,4-NAPHTHOQUINONES

Compound	Effective dose in γ
2-Methyl-3-phytyl-1,4-naphthoquinone[91, 92] (Vitamin K_1)	1
2-Methyl-3-difarnesyl-1,4-naphthoquinone[93, 94] (Vitamin K_2)	1.6
2-Methyl-3-farnesyl-1,4-naphthoquinone[95, 96]	5
2-Methyl-3-(β,γ-dihydrophytyl)-1,4-naphthoquinone[95, 97, 98]	8
2-Methyl-3-geranyl-1,4-naphthoquinone[92]	25
2-Methyl-3-cinnamyl-1,4-naphthoquinone[99]	25
2-Methyl-3-(β,γ,γ-trimethyl-allyl)-1,4-naphthoquinone[99]	50
2,3-Dimethyl-1,4-naphthoquinone[100]	50
2-Methyl-3-benzyl-1,4-naphthoquinone[99]	200
2-Methyl-3-hydrocinnamyl-1,4-naphthoquinone[97]	300
2-Methyl-3-octadecyl-1,4-naphthoquinone[98, 100]	Inactive at 1000

TABLE II[101]

2-ALKYL- AND 2-β-ALKENYL-1,4-NAPHTHOQUINONES

Compound	Effective dose in γ
2-Methyl-1,4-naphthoquinone[102]	0.3
2-Phytyl-1,4-naphthoquinone[96, 103, 104]	50
2-Farnesyl-1,4-naphthoquinone[96, 103]	500
2-(β,γ-Dihydro-phytyl)-1,4-naphthoquinone	600
2-*n*-Hexadecyl-1,4-naphthoquinone[105]	More than 600
2-*n*-Octadecyl-1,4-naphthoquinone[105]	More than 600
2-Allyl-1,4-naphthoquinone[106, 107]	800
2-Geranyl-1,4-naphthoquinone[96, 103]	1000
2-Ethyl-1,4-naphthoquinone[105, 108, 109]	Inactive at 1000
2-*n*-Propyl-1,4-naphthoquinone[105, 108, 109]	Inactive at 1000

[91] L. F. Fieser, *J. Am. Chem. Soc.*, **61**, 2559 (1939)

[92] L. F. Fieser, *Ibid.*, **61**, 3467 (1939).

[93] R. W. McKee, S. B. Binkley, D. W. MacCorquodale, S. A. Thayer and E. A Doisy, *Ibid.*, **61**, 1295 (1939).

[94] H. Dam, J. Glavind and P. Karrer, *Helv. Chim. Acta*, **23**, 224 (1930).

[95] L. F. Fieser, M. Tishler and W. L. Sampson, *J. Am. Chem. Soc.*, **62**, 996, 1628 (1940)

[96] L. F. Fieser, M. Tishler and N. L. Wendler, *Ibid.*, **62**, 2861 (1940).

[97] M. Tishler, L. F. Fieser and N. L. Wendler, *Ibid.*, **62**, 2866 (1940).

[98] P. Karrer and A. Epprecht, *Helv. Chim. Acta*, **23**, 272 (1940).

[99] L. F. Fieser, W. P. Campbell, E. M. Frey and M. D. Gates, *J. Am. Chem. Soc.*, **61**, 2559, 3216 (1939).

[100] E. Fernholz, S. Ansbacher and H. B. MacPhillamy, *Ibid.*, **62**, 430 (1940).

[101] L. F. Fieser, M. Tishler and W. L. Sampson, *J. Biol. Chem.*, **137**, 659 (1941).

[102] S. Ansbacher and E. Fernholz, *J. Am. Chem. Soc.*, **61**, 1924 (1939).

[103] L. F. Fieser, M. Tishler and W. L. Sampson, *Ibid.*, **62**, 996, 1628 (1940).

[104] H. Dam, J. Glavind and P. Karrer, *Helv. Chim. Acta*, **23**, 224 (1940).

[105] E. Fernholz, S Ansbacher and H. B. MacPhillamy, *J. Am. Chem. Soc.*, **62**, 430 (1940).

[106] L. F. Fieser, W. P. Campbell and E. M. Frey, *Ibid.*, **61**, 2206 (1939).

[107] L. F. Fieser, D. M. Bowen, W. P. Campbell, M. Fieser, E. M. Frey, R. N. Jones, B. Riegel, C. E. Schweitzer and P. G. Smith, *Ibid.*, **61**, 1925 (1939).

[108] M. Tishler and W. L. Sampson, *Ibid.*, **61**, 2563 (1939).

[109] B. Sjögren, *Z. physiol. Chem.*, **264**, 1 (1939).

TABLE III[110]

HIGHLY ALKYLATED NAPHTHOQUINONES

Compound	Effective dose in γ
2-Ethyl-3-phytyl-1,4-naphthoquinone[111, 112]	1000
2,3-Diallyl-1,4-naphthoquinone[113, 114]	1000
2,5-Dimethyl-1,4-naphthoquinone[115]	500
2,6-Dimethyl-1,4-naphthoquinone	Inactive at 1000
2,7-Dimethyl-1,4-naphthoquinone	1000
2,8-Dimethyl-1,4-naphthoquinone[116]	500
6,7-Dimethyl-1,4-naphthoquinone[113, 114]	Inactive at 1000
2,6-Dimethyl-3-phytyl-1,4-naphthoquinone[116]	Inactive at 1000
1,1-Dimethyl-3-*tert*-butyl-1,4-dihydro-anthraquinone[117]	Inactive at 1000
2-(δ-Methyl-γ-pentenyl)-1,4-dihydro-anthraquinone[117]	Inactive at 1000
1,2,4-Trihydroxy-anthraquinone[118]	100

TABLE IV[119]

CARBETHOXY- AND HYDROXY-NAPHTHOQUINONES

Compound	Effective dose in γ
2-Methyl-3-carbethoxy-1,4-naphthohydroquinone[120]	25
2-Methyl-5-hydroxy-1,4-naphthoquinone[121]	400
2-Methyl-3-hydroxy-1,4-naphthoquinone (Phthiocol)[122]	500
5-Hydroxy-1,4-naphthoquinone (Juglone)[123]	Inactive at 1000, feebly active at 10 mg.
2-Hydroxy-1,4-naphthoquinone (Lawsone)[123, 124]	Inactive at 1000, active at 10 mg.
2-Hydroxy-3-dimethyl-allyl-1,4-naphthoquinone (Lapachol)[123]	Inactive at 1000, active at 5 mg.
2-β-Heptenyl-3-hydroxy-1,4-naphthoquinone	Inactive at 1000
2-Farnesyl-3-hydroxy-1,4-naphthoquinone[125]	Inactive at 1000
2-Methyl-3-(γ-hydroxy-dihydrophytyl)-1,4-naphthoquinone[126]	Inactive at 1000
Hydroquinone-diacetate[126]	Inactive at 1000

[110] L. F. Fieser, M. Tishler and W. L. Sampson, *J. Biol. Chem.*, **137**, 659 (1941).
[111] L. F. Fieser, *J. Am. Chem. Soc.*, **61**, 2559 (1939).
[112] L. F. Fieser, *Ibid.*, **61**, 3467 (1939).
[113] L. F. Fieser, W. P. Campbell and E. M. Frey, *Ibid.*, **61**, 2206 (1939).
[114] L. F. Fieser, D. M. Bowen, W P. Campbell, E. M. Frey and M. D. Gates, *Ibid* , **61**, 1926 (1939).
[115] M. Tishler, L. F. Fieser and N. L. Wendler, *Ibid.*, **62**, 2866 (1940).
[116] L. F. Fieser, *Ibid.*, **61**, 3467 (1939).
[117] L. F. Fieser and C. W. Wieghard, *Ibid.*, **62**, 153 (1940).
[118] G. J. Martin and C. F. Lischer, *J. Biol. Chem.*, **137**, 169 (1941).
[119] L. F. Fieser, M. Tishler and W. L. Sampson, *Ibid.*, **137**, 659 (1941).
[120] C. F. Koelsch and D. J. Byers, *J. Am. Chem. Soc.*, **62**, 560 (1940).
[121] L. F. Fieser and J. T. Dunn, *Ibid.*, **58**, 572 (1936).
[122] H. J. Almquist and A. A. Klose, *Ibid.*, **61**, 1611 (1939).
[123] R. Kuhn, K. Wallenfels, F. Weygand, T. Moll and L. Hepding, *Naturwissenschaften*, **27**, 518 (1939).
[124] H. Dam, J. Glavind and P. Karrer, *Helv. Chim. Acta*, **23**, 224 (1940).
[125] M. Tishler, L. F. Fieser and N. L. Wendler, *J. Am. Chem. Soc.*, **62**, 2866 (1940).
[126] M. Tishler, L. F. Fieser and N. L. Wendler, *Ibid.*, **62**, 1982 (1940).

TABLE V[127]

NAPHTHOQUINONE OXIDES

Compound	Effective dose in γ
Vitamin K_1 oxide[128, 129]	1.2
2-Methyl-1,4-naphthoquinone oxide[130, 131]	5
2,3-Dimethyl-1,4-naphthoquinone oxide[128, 129]	25
2-Methyl-3-cinnamyl-1,4-naphthoquinone oxide[128, 129]	150
2-Phytyl-1,4-naphthoquinone oxide[128, 129]	200
2-Farnesyl-1,4-naphthoquinone oxide[128, 129]	1000
2,7-Dimethyl-1,4-naphthoquinone oxide[130]	Inactive at 1000

TABLE VI[132]

MISCELLANEOUS QUINONES

Compound	Effective dose in γ
2,3,5-Trimethyl-1,4-benzoquinone	Inactive at 1000
Duroquinone[133]	10000
2,3,5-Trimethyl-6-phytyl-1,4-benzoquinone[134, 135]	Inactive at 1000
α-Tocopherylquinone[135]	Inactive at 1000
9-Methyl-perinaphthenone-7[136]	Inactive at 1000
2-Methyl-2-phytyl-2,3-dihydro-1,4-naphthoquinone[135, 137]	50
Naphthotocopherol[134, 137]	500
2,5-Dimethyl-benzoquinone (Phlorone)[138, 139]	3000

TABLE VII[140]

ESTERS AND ETHERS OF HYDROQUINONES

Compound	Effective dose in γ
Sodium 2-methyl-1,4-naphthohydroquinone diphosphate[141, 142, 143]	0.5
Sodium 2-methyl-1,4-naphthohydroquinone disulfate[141, 143]	2
Vitamin K_1 hydroquinone diphosphoric acid[141]	50
Sodium 2,3-dimethyl-1,4-naphthohydroquinone disulfate[141]	500
Potassium vitamin K_1 hydroquinone disulfate[141]	Inactive at 500
Diacetate of 2-methyl-1,4-naphthohydroquinone[144, 145]	1
Dibenzoate of 2-methyl-1,4-naphthohydroquinone[144, 145]	1
Dimesitoate of 2-methyl-1,4-naphthohydroquinone[146, 147]	300
Monoethyl ether of 2-methyl-1,4-naphthohydroquinone[148]	1
Dimethyl ether of 2-methyl-1,4-naphthohydroquinone[145]	5
Dibenzyl ether of 2-methyl-1,4-naphthohydroquinone[144]	7
Vitamin K_1 hydroquinone diacetate[149]	2
Vitamin K_2 hydroquinone diacetate[149]	3.2

(See footnotes on opposite page.)

TABLE VIII

AMINO-COMPOUNDS

Compound	Effective dose in γ
4-Amino-2-methyl-1-naphthol hydrochloride[150, 151, 152]	About 1
4-Amino-3-methyl-1-naphthol hydrochloride[151]	About 1
2-Methyl-1-naphthylamine[153, 154]	5

TABLE IX[155]

HYDRIDES OF VITAMIN K AND OF METHYL-NAPHTHOQUINONE

Compound	Effective dose in γ
5,8-Dihydro-vitamin K_1[156, 157]	4
β,γ,5,6,7,8-Hexahydro-vitamin K_1[156, 158]	1000
2-Methyl-5,8-dihydro-1,4-naphthohydroquinone[157]	6
2-Methyl-5,8,9,10-tetrahydro-1,4-naphthoquinone[157]	8
2-Methyl-5,6,7,8-tetrahydro-1,4-naphthoquinone[158]	500

[127] L. F. Fieser, M. Tishler and W. L. Sampson, *J. Biol. Chem.*, **137**, 659 (1941).
[128] L. F. Fieser, M. Tishler and W. L. Sampson, *J. Am. Chem. Soc.*, **62**, 996, 1628 (1940).
[129] M. Tishler, L. F. Fieser and N. L. Wendler, *Ibid.*, **62**, 2866 (1940).
[130] L. F. Fieser, W. P. Campbell, E. M. Frey and M. D. Gates, *Ibid.*, **61**, 2559, 3216 (1939).
[131] J. Madinaveita, *Anales soc. españ. fis. quim.*, **31**, 750 (1933).
[132] L. F. Fieser, M. Tishler and W. L. Sampson, *J. Biol. Chem.*, **137**, 659 (1941).
[133] G. J. Martin and C. F. Lischer, *Ibid.*, **137**, 169 (1941).
[134] L. F. Fieser, M. Tishler and W. L. Sampson, *J. Am. Chem. Soc.*, **62**, 996, 1628 (1940).
[135] L. F. Fieser, M. Tishler and N. L. Wendler, *Ibid.*, **62**, 2861 (1940).
[136] M. Tishler, L. F. Fieser and N. L. Wendler, *Ibid.*, **62**, 2866 (1940).
[137] M. Tishler, L. F. Fieser and N. L. Wendler, *Ibid.*, **62**, 1982 (1940).
[138] S. Ansbacher and E. Fernholz, *J. Biol. Chem.*, **131**, 399 (1939).
[139] H. J. Almquist, *Physiol. Rev.*, **21**, 194 (1941).
[140] L. F. Fieser, M. Tishler and W. L. Sampson, *J. Biol. Chem.*, **137**, 659 (1941).
[141] L. F. Fieser and E. M. Frey, *J. Am. Chem. Soc.*, **62**, 228 (1940).
[142] R. H. K. Foster, J. Lee and U. V. Solmssen, *Ibid.*, **62**, 453 (1940).
[143] S. Ansbacher, E. Fernholz and M. A. Dolliver, *Proc. Soc. Exptl. Biol. Med.*, **43**, 652 (1940).
[144] L. F. Fieser, W. P. Campbell, E. M. Frey and M. D. Gates, *J. Am. Chem. Soc.*, **61**, 2559, 3216 (1939).
[145] S. Ansbacher, E. Fernholz and M. A. Dolliver, *Ibid.*, **62**, 155 (1940).
[146] M. Tishler, L. F. Fieser and N. L. Wendler, *Ibid.*, **62**, 2866 (1940).
[147] M. Tishler, L. F. Fieser and W. L. Sampson, *Ibid.*, **62**, 1881 (1940).
[148] M. Tishler, L. F. Fieser and N. L. Wendler, *Ibid.*, **62**, 1982 (1940).
[149] S. B. Binkley, D. W. MacCorquodale, L. C. Cheney, S. A. Thayer, R. W. McKee and E. A. Doisy, *Ibid.*, **61**, 1612 (1939).
[150] E. A. Doisy, D. W. MacCorquodale, S. A. Thayer, S. B. Binkley and R. W. McKee, *Science*, **90**, 407 (1939).
[151] H. J. Almquist and A. A. Klose, *Proc. Soc. Exptl. Biol. Med.*, **45**, 55 (1940).
[152] D. Richtert, S. A. Thayer, R. W. McKee, S. B. Binkley and E. A. Doisy, *Ibid.*, **44**, 601 (1940).
[153] M. Tishler, L. F. Fieser and N. L. Wendler, *J. Am. Chem. Soc.*, **62**, 2866 (1940).
[154] M. Tishler, L. F. Fieser and W. L. Sampson, *Ibid.*, **62**, 1881 (1940).
[155] L. F. Fieser, M. Tishler and W. L. Sampson, *J. Biol. Chem.*, **137**, 659 (1941).
[156] L. F. Fieser, M. Tishler and W. L. Sampson, *J. Am. Chem. Soc.*, **62**, 996, 1628 (1940).
[157] L. F. Fieser, M. Tishler and N. L. Wendler, *Ibid.*, **62**, 2861 (1940).
[158] M. Tishler, L. F. Fieser and N. L. Wendler, *Ibid.*, **62**, 2866 (1940).

TABLE X[159]

METHYL-NAPHTHOLS, METHYL-TETRALONES AND RELATED COMPOUNDS

Compound	Effective dose in γ
2-Methyl-1,4-naphthohydroquinone	0.5
2-Methyl-1-naphthol[160, 161]	1
3-Methyl-1-naphthol[160, 161]	0.6
4-Methyl-1-naphthol[160, 161]	Inactive at 1000
1-Methyl-2-naphthol[160, 161]	Inactive at 1000
3-Methyl-2-naphthol[160, 161]	Inactive at 1000
1-Naphthol	1000
2-Methyl-1-tetralone[160, 161]	0.6
3-Methyl-1-tetralone[160, 161]	1
β-Methyl-naphthalene	1000

12. Determination

(a) *Physical Methods*

Spectroscopic Examination. The vitamins K_1 and K_2 and 2-methyl-1,4-naphthoquinone can be estimated by means of their absorption spectra,[162] provided the compounds are essentially free from other absorbing materials and are not present in the reduced hydroquinone form. For a description of the spectra see page 485.

(b) *Chemical Methods*

Colorimetric Redox Method.[163, 164] This method involves a catalytic hydrogenation of the vitamin K-quinone to the hydroquinone stage using butanol as the solvent and phenosafranin as the indicator. An excess of 2,6-dichlorophenol-indophenol is added to the reduced material in the absence of air. The decrease in color is determined and this determination is a measure of the quinone originally present.

This method is, of course, not very specific since all quinones present will give the same reaction. Highly colored solutions cannot be used but, after reduction, the hydroquinone can be extracted with Claisen's alkali yielding colorless solutions which can then be used in this test.

[159] L. F. Fieser, M. Tishler and W. L. Sampson, *J. Biol. Chem.*, **137**, 659 (1941).
[160] M. Tishler, L. F. Fieser and N. L. Wendler, *J. Am. Chem. Soc.*, **62**, 2866 (1940).
[161] M. Tishler, L. F. Fieser and W. L. Sampson, *Ibid.*, **62**, 1881 (1940).
[162] J. I. Pinder and J. H. Singer, *Analyst*, **65**, 7 (1940).
[163] J. V. Scudi, *Proc. Am. Physiol. Soc.*, **1941**, 252.
[164] N. R. Trenner and F. A. Bacher, *J. Biol. Chem.*, **137**, 745 (1941).

Reaction with Sodium Alcoholates.[165, 166] The natural vitamins K give a purple-blue color reaction with sodium ethylate and sodium methylate. The color is unstable and turns into red and finally into brown. During this reaction, the vitamins are degradated to 2-methyl-3-hydroxy-1,4-naphthoquinone (phthiocol).[167] This color reaction is specific for those 1,4-naphthoquinone derivatives which have β-unsaturated side chains in the 3-position. Carotenoids, which may hinder the quantitative determination of the vitamin, may be removed when the color has reached the red-brown stage by extraction with a hydrocarbon solvent. The color developed from the vitamin K remains in the alcohol phase.

The sensitivity of this color reaction can be increased by testing the 2,4-dinitro-phenyl-hydrazones of the vitamins instead of the free vitamin.[168] For this purpose an alcoholic solution of the vitamin (quinone) is mixed with a solution of 2,4-dinitro-phenyl-hydrazine in diluted hydrochloric acid and is gently heated. A bluish green color is then developed with sodium methylate or a green color can be produced with ammonia and amyl-alcohol. The colors produced are stable.

Titanous Chloride Titration.[169] Vitamins K as quinones can be reduced quantitatively to the corresponding hydroquinones by means of titanous chloride, the end-point being shown by the use of an internal oxidation-reduction indicator, for example, potassium indigo-disulfonate or phenosafranin. The determination is carried out under carbon dioxide in alcohol-acetic acid solution in the presence of sodium carbonate and sodium potassium tartrate (Rochelle salt) in order to prevent the solution from becoming too acid during the titration.

Ethyl-cyano-acetate Method.[169, 170] Vitamin K in alcoholic solution containing ammonia is mixed with ethyl-cyano-acetate followed by the addition of alkali. A yellow color develops, which is measured in a photometer. The same reagent without the addition of alkali gives a violet color, which, however, is too unstable to be useful.

(*c*) *Biological Methods*

There are two important biological methods for the determination of vitamins K. Both of these methods measure in principle the clotting power of blood.

[165] P. Karrer, *Helv. Chim. Acta*, **22**, 1146 (1939).
[166] H. J. Almquist and A. A. Klose, *J. Am. Chem. Soc.*, **61**, 1611, 1923 (1939).
[167] L. F. Fieser, W. P. Campbell and E. M. Frey, *Ibid.*, **61**, 2206 (1939).
[168] A. Novelli, *Science*, **93**, 358 (1941).
[169] J. L. Pinder and J. H. Singer, *Analyst*, **65**, 7 (1940).
[170] R. Carven, *J. Chem. Soc.*, **1931**, 1605.

1. **Spontaneous Blood Clotting Time Determination.** In the perfected form[171] of this assay procedure, day-old chicks are kept on a vitamin K-deficient diet, care being taken to prevent coprophagy. Within about 15 days severe avitaminosis develops. The determination of the blood clotting time is then carried out by puncturing a wing vein and placing a tube containing the blood in a thermostat which is subjected to continuous shaking. The clotting time is then defined as the time necessary to form a solid clot. While the coagulation time of the blood of normal chicks is about 4–6 minutes, the blood of avitaminotic birds may not coagulate for several hours. The determination of the vitamin K efficacy can be carried out according to this method, using either a prophylactic or a curative procedure. The latter is usually more reliable. After oral ingestion of the vitamin preparation or after injection of the material to be tested, the blood clotting time is determined after six hours[171] or in a modified procedure after 18 hours.[172] Avitaminotic birds which have previously been bled may show a decrease in the clotting time upon repeated bleeding several hours later without treatment with antihemorrhagic substances[173] and therefore should not be used in actual vitamin K assays.

2. **Prothrombin Clotting Time Determination.** In this test, the actual amount of prothrombin present in blood is determined[174, 175] and therefore the test is independent of various other factors which may affect the clotting time.[176] In the most convenient form of this test[177] the ability of chicken blood to form clots in a minimum of time is determined. The blood is obtained by decapitation or from the carotid artery and is run into a tube containing sodium oxalate solution. Thromboplastin is prepared from chicken breast muscle[178] or from rabbit brain, and then a calcium chloride solution is added. The time from the addition of the calcium chloride until a firm clot is formed is measured. Blood of normal birds clots within 25–30 seconds.

Many variations of this procedure have been suggested and in practice each laboratory uses its own modified technic. A practical important modification[178] is to dilute the blood plasma with an equal amount of Ringer solution and to mix this solution with thromboplastin extracts of

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[171] S. Ansbacher, *J. Nutrition*, **17**, 303 (1937).

[172] S. A. Thayer, R. W. McKee, S. B. Binkley, D. W. MacCorquodale and E. A. Doisy, *Proc. Soc Exptl. Biol. Med.*, **40**, 478 (1939); **41**, 194 (1939).

[173] G. Cheney, *J. Lab. Clin. Med.*, **24**, 919 (1939).

[174] A. J. Quick, *Am. J. Physiol.*, **118**, 260 (1937).

[175] A. J. Quick, *J. Biol. Chem.*, **34**, LXXVIII (1940).

[176] R. T. Tidrick, F. T. Joyce and H. P. Smith, *Proc. Soc. Exptl. Biol. Med.*, **42**, 853 (1939).

[177] H. J. Almquist and A. A. Klose, *Biochem. J.*, **33**, 1055 (1939)

[178] H. Dam and J. Glavind, *Ibid*, **32**, 1018 (1938).

different concentrations in a series of parallel experiments. The concentration which produces blood clotting in a defined length of time, for example, within three minutes, is a measure of the amount of prothrombin present.

In the curative procedure the effect of vitamin K may be determined after one[179] or four days. While, as pointed out above, severe bleeding may reduce the whole blood clotting time, no such effect has been observed on the prothrombin time.[180]

Besides chicks, rabbits have also been recommended for use in this assay procedure.[181] A photoelectric apparatus has also been devised for registering the clotting time.[182]

The symptoms of a vitamin K deficiency can be obtained, besides by dietary means, by intoxication with chloroform[183] or with *p*-toluenediamine.[184]

13. Standards

No nationally or internationally accepted standard of vitamin K has been adopted so far. Both 2-methyl-1,4-naphthoquinone[184, 185] and 2-methyl-1,4-naphthohydroquinone-diacetate[186, 187] have been proposed as a reference standard, since they can be obtained easily in pure form, can be characterized by physical constants and are relatively stable.

The efficacy of various vitamin K preparations has been expressed in different units. In the table[188] given below, these various units are correlated. The relation of these units to vitamin K_1 and to 2-methyl-1,4-naphthoquinone is given by the Thayer-Doisy Unit which defines[189] the activity of 1 mg. of vitamin K_1 as 1000 Thayer-Doisy Units. The efficacy of 2-methyl-1,4-naphthoquinone is about 3.3 times as great as that of vitamin K_1.

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[179] A. J. Quick, *J. Biol. Chem.*, **133**, 78 (1940).

[180] M. C. Elliott, B. Isaacs and A. C. Ivy, *Proc. Soc. Exptl. Biol. Med.*, **43**, 240 (1940).

[181] P. Meunier, H. Hinglais, D. Bovet and A. Dreyfuss, *Compt. rend.*, **210**, 454 (1940).

[182] K. K. Nygaard, *J. Lab. Clin. Med.*, **24**, 517 (1939).

[183] H. P. Smith, E. D. Warner and K. M. Brinkhous, *J. Exptl. Med.*, **66**, 803 (1937).

[184] S. A. Thayer, S. B. Binkley, D. W. MacCorquodale, E. A. Doisy, A. D. Emmett, R. A. Brown and O. D. Bird, *J. Am. Chem. Soc.*, **61**, 2563 (1939).

[185] E. Fernholz, S. Ansbacher and H. B. MacPhillamy, *Ibid.*, **62**, 430 (1940).

[186] H. Dam, J. Glavind and P. Karrer, *Helv. Chim. Acta*, **23**, 224 (1940).

[187] D. T. Ewing, J. M. Vandenbelt and O. Kamm, *J. Biol. Chem.*, **131**, 345 (1939).

[188] S. Ansbacher, *Proc. Am. Soc. Biol. Chem.*, **1940**, 111.

[189] S. A. Thayer, S. B. Binkley, D. W. MacCorquodale, E. A. Doisy, A. D. Emmett, R. A. Brown and O. D. Bird, *J. Am. Chem. Soc.*, **61**, 2563 (1939)

1 Thayer-Doisy Unit[189] = 0.5 Ansbacher Unit.[190]
= 0.5 Thayer Unit (1938).[191]
= 10 Dam Units.[192]
= 0.25 Dann (1938) Unit.[193]
= 0.625 Dann (1939) Unit.[194]
= 0.08 ml. Almquist Reference Standard.

14. Physiology of Plants and Microorganisms

Plants and many microorganisms produce relatively large amounts of vitamins K. This suggests that this vitamin is needed for the maintenance of life, and has actually been found to be a growth factor for Johne's bacillus.[195] No information is available which would indicate the action of this compound in plants.

15. Animal Physiology

(a) *Metabolism of Vitamins K*

Vitamin K occurs in the intestinal tract from the daily food intake and from synthesis by microorganisms in the bowels. Since the natural vitamins K are fat-soluble compounds, they are absorbed only in the presence of bile salts,[196, 197] especially desoxy-cholic acid.[198] Thus, in rats with bile fistula, vitamin K deficiency occurs even when large doses are given orally unless bile salts are administered simultaneously. Besides the presence of sufficient amounts of bile, normal digestion of fats and apparently also a proper functioning of the liver[199] are necessary for vitamin K absorption. Intestinal lesions interfere with vitamin K absorption. The feeding of high doses of mineral oil together with vitamin K prohibits the normal absorption.[200] This effect is not noted when the vitamin is injected. Vitamins K are generally active upon parenteral injection. Water-soluble forms of vitamin K are absorbed from the intestinal tract

[190] S. Ansbacher, *Proc. Soc. Exptl. Biol. Med.*, **44**, 248 (1940).
[191] S. A. Thayer, D. W. MacCorquodale, R. W. McKee and E. A. Doisy, *J. Biol. Chem.*, **123**, CXX (1938).
[192] H. Dam and J. Glavind, *Z. Vitaminforsch.*, **9**, 71 (1939).
[193] F. P. Dann, *Am. J. Physiol.*, **123**, 48 (1938).
[194] F. P. Dann, *Proc. Soc. Exptl. Biol. Med.*, **42**, 663 (1939).
[195] D. W. Woolley and J. R. McCarter, *Ibid.*, **45**, 357 (1940).
[196] J. D. Greaves and C. L. A. Schmidt, *Ibid.*, **37**, 43 (1937).
[197] H. R. Butt, *Am. J. Digestive Diseases Nutrition*, **6**, 127 (1939). H. R. Butt, A. M. Snell and A. E. Osterberg, *Proc. Staff Meetings Mayo Clinic*, **13**, 74 (1938).
[198] C. L. A. Schmidt, *Pacific Coast Med.*, **5**, 7 (1938).
[199] R. L. Clark, C. F. Dixon, H. R. Butt and A. M. Snell, *Proc. Staff Meetings Mayo Clinic*, **14**, 407 (1939).
[200] M. C Elliott, B. Isaacs and A. C. Ivy, *Proc. Soc. Exptl. Biol. Med.*, **43**, 240 (1940).

even in the absence of bile salts[201, 202] and are especially useful for intravenous injections.

Vitamins K are found only in very small amounts in blood (apparently only during times of transport) but in somewhat larger amounts in livers. The animal organism has apparently no storage organs for this vitamin and metabolizes this compound fairly rapidly. The mechanism of this metabolism is unknown. No vitamin K is excreted through the kidneys,[203] but considerable amounts are found in feces, mainly due to the bacterial synthesis in the intestinal tract.

Vitamin K is secreted in small but definite amounts in milk and eggs.[204] There is apparently a special mechanism which regulates the passage of this vitamin through the placenta. It has generally been observed that pregnant women just prior to parturition have an increased need for vitamin K. The newborn infant usually has a slight K-hypovitaminosis, which can be overcome by feeding vitamin K to the infant or by giving it to the mother before delivery. Better results are usually obtained by maternal administration than by giving vitamin K after birth.[205]

(*b*) *Physiological Action of Vitamins K*

Vitamins K are necessary for the maintenance of normal blood coagulation. According to the classical theory[206] two different phases are involved in this process of coagulation. The end-effect, which constitutes the second phase of the total process, is the transformation of fibrinogen, a protein dissolved in the blood plasma, into fibrin, a solid and essentially insoluble protein derivative. This precipitation reaction is carried out by a postulated enzyme, the thrombin. All experimental evidences point to the actual existence of this compound. Thrombin does not occur in the blood, since otherwise the blood would not be liquid. But the precursor of thrombin, namely, prothrombin, is present in plasma. The first phase of the blood coagulation, therefore, is the conversion of prothrombin into thrombin. This process involves the action of another enzyme, thromboplastin (or thrombokinase) in the presence of calcium ions. While the latter are present in blood plasma, thromboplastin is a normal cell constituent, but not a blood constituent.

[201] E. D. Warner, K. M. Brinkhous and H. P. Smith, *Proc. Soc. Exptl. Biol. Med.*, **44**, 607 (1940).
[202] H. P. Smith and C. A. Owen, *J. Biol. Chem.*, **134**, 783 (1940).
[203] H. Dam, A. Geiger, J. Glavind, P. Karrer, W. Karrer, E. Rothschild and H. Salomon, *Helv. Chim. Acta*, **22**, 310 (1939).
[204] H. J. Almquist and E. L. R Stokstad, *J. Nutrition*, **12**, 329 (1936).
[205] L. B. Shettles, E. Delfs and L. M. Hellman, *Bull. Johns Hopkins Hosp.*, **65**, 419 (1939).
[206] O. Hammarsten, *Z. physiol. Chem.*, **28**, 98 (1899). P. Morawitz, *Biochem. Z.*, **18**, 30 (1909).

$$\text{Prothrombin} \xrightarrow{\text{Ca + Thromboplastin}} \text{Thrombin} \qquad (1)$$

$$\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin} \qquad (2)$$

During vitamin K deficiency only the prothrombin concentration is altered, that is, reduced.[207] Vitamin K, however, is not identical with prothrombin since this vitamin does not cause blood coagulation *in vitro*.[208] Vitamin K,[207] apparently, is also not a constituent of prothrombin, since prothrombin fractions exert practically no vitamin K action[209]. Since, however, vitamin K influences the prothrombin concentration it is assumed that vitamin K is involved in the prothrombin formation. This synthesis is apparently accomplished in the liver,[209, 210] since partial extirpation of the liver markedly reduces the prothrombin level in blood[211, 212] and since symptoms of a vitamin K deficiency even in the presence of normally adequate amounts of this vitamin have been observed frequently in cases of liver damages such as cirrhosis, hepatitis, atrophy and carcinoma.[213] In all these cases the prothrombin concentration could not be influenced by vitamin K when given orally or injected intravenously or intramuscularly. That a special mechanism for the formation of prothrombin actually exists is furthermore suggested by the fact that excessive feeding of vitamins K will not cause the prothrombin to rise above a certain level.[214]

The mechanism of the vitamin K action is unknown. It has been suggested[215] that the quinone structure of the vitamin takes part in an oxidation-reduction system involving the oxidation of SH-groups to —S—S- linkages. The hypothesis has been advanced[216] that the formation of the blood clot also involves an oxidation of SH-groups of fibrinogen to —S—S- groups in fibrin.

[207] H. Dam, *Nature*, **135**, 652 (1935). F. Schønheyder, *Ibid.*, **135**, 652 (1935). H. Dam, F. Schønheyder and E. Tage-Hansen, *Biochem. J.*, **30**, 1075 (1936). F. Schønheyder, *Ibid.*, **30**, 890 (1936).

[208] H. Dam, J. Glavind, L. Lewis and E. Tage-Hansen, *Skand. Arch. Physiol.*, **79**, 121 (1938).

[209] C. L. A. Schmidt, *Pacific Coast Med.*, **5**, 7 (1938).

[210] A. M. Snell, *J. Am. Med. Assoc.*, **112**, 1457 (1939).

[211] W. D. Amdrus, J. W. Lord and R. A. Moore, *Surgery*, **6**, 899 (1939).

[212] E. D. Warner, *J. Exptl. Med.*, **68**, 831 (1938).

[213] P. M. Aggeler, S. P. Lucia and L. Goldman, *Proc. Soc. Exptl. Biol. Med.*, **43**, 689 (1940). W. D. Andrus and J. W. Lord, *J. Am. Med. Assoc.*, **114**, 1336 (1940). H. R. Butt, A. M. Snell, A. E. Osterberg and J. L. Bollman, *Proc. Staff Meetings Mayo Clinic*, **15**, 69 (1940). F. J. Pohle and J. K. Stewart, *J. Clin. Investigation*, **19**, 365 (1940). G. H. Scanlon, K. M. Brinkhous, E. D. Warner, H. P. Smith and J. E. Flynn, *J. Am. Med. Assoc.*, **112**, 1898 (1939).

[214] A. J. Quick, *Proc. Am. Soc. Biol. Chem.*, **34**, LXXVIII (1940).

[215] F. Bernheim and M. L. C. Bernheim, *J. Biol. Chem.*, **134**, 457 (1940).

[216] J. P. Baumberger, *Proc. Am. Physiol. Soc.*, **1941**, 18.

16. Hypovitaminosis and Avitaminosis

In vitamin K deficiency the prothrombin content of blood is markedly decreased and the blood clotting time is considerably prolonged. The principle symptom during times of vitamin K deficiency is, besides a prolonged bleeding time, the occurrence of hemorrhages. In chicks subcutaneous and intramuscular hemorrhages occur on the head, neck, breast, abdomen, back, wings and legs.[217] In man, hemmorrhagic syndromes caused by a vitamin K deficiency are frequently observed in an infant during the first few days of life, when an extremely low prothrombin concentration generally is observed which lasts until a normal intestinal flora has been developed by the ingestion of food,[218, 219, 220, 221] but they may also occur in adults.[222] Low prothrombin levels are furthermore generally observed in humans when the absorption of the vitamin is impaired, such as in cases of obstructive jaundice,[223, 224, 225] sprue,[226] biliary fistula,[227, 228] and ulcerative colitis. Hemophilia and thrombopenia, however, are not due to vitamin K deficiency and cannot be influenced by the administration of this vitamin.[229, 230, 231]

(*a*) *Clinical Test Methods*

1. **Bleeding Time Determination.** A stab is made in the tip of a finger and the amount of time which it takes to stop the bleeding is observed. In order to obtain measurable figures it has been suggested that the blood be absorbed every 15 seconds on a piece of filter paper. The

[217] S. Ansbacher, *J. Nutrition*, **17**, 303 (1937).

[218] G. D. Johnson, *J. South Carolina Med. Assoc.*, **36**, 336 (1940).

[219] H. Dam, *et al.*, *Lancet*, **2**, 1157 (1939).

[220] W. W. Waddell and D. Guerry, *J. Pediatrics*, **15**, 802 (1939).

[221] A. J. Quick and A. M. Grossman, *Am. J. Med. Sci.*, **199**, 1 (1940); *Proc. Soc. Exptl. Biol. Med.*, **40**, 647 (1939); **41**, 227 (1939).

[222] R. Kark and E. L. Lozner, *Lancet*, **2**, 1162 (1939).

[223] S. T. Townsend and E. S. Mills, *Can. Med. Assoc. J.*, **41**, 111 (1939); **42**, 541 (1940).

[224] K. M. Brinkhous, H. P. Smith and E. D. Warner, *Am. J. Med. Sci.*, **196**, 50 (1938). E. D. Warner, K. M. Brinkhous and H. P. Smith, *Proc. Soc. Exptl. Biol. Med.*, **37**, 628 (1938). A. M. Snell, T. B. Magath, E. W. Boland, A. E. Osterberg, H. R. Butt, J. L. Bollman and W. Walters, *Proc. Staff Meetings Mayo Clinic*, **13**, 65 (1938).

[225] A. J. Quick, M. Stanley-Brown and F. W. Bancroft, *Am. J. Med. Sci.*, **190**, 501 (1935).

[226] P. M. Aggeler, S. P. Lucia and L. Goldman, *Proc. Soc. Exptl. Biol. Med.*, **43**, 689 (1940). R. L. Clark, C. F. Dixon, H. R. Butt and A. M. Snell, *Proc. Staff Meetings Mayo Clinic*, **14**, 407 (1939). R Engel, *Med. Welt*, **13**, 120 (1939). H. Hult, *Nord. Med.*, **3**, 2428 (1939).

[227] I. C. Zuckerman, B. Kogut, M. Jacobi and J. Y. Cohen, *Am. J. Digest. Dis.*, **6**, 332 (1939).

[228] L. K. Ferguson and D. G. Calder, *Ibid.*, **6**, 722 (1939). H. P. Smith, S. R. Ziffern, C. A. Owen, G. R. Hoffman and J. E. Flynn, *J. Iowa State Med. Soc.*, **29**, 377 (1939).

[229] H. Dam and J. Glavind, *Ugeskrift Laeger*, **100**, 248 (1938).

[230] H. Dam, F. Schønheyder and E. Tage-Jansen, *Biochem. J.*, **30**, 1075 (1936).

[231] G. H. Scanlon, K. M. Brinkhous, E. D. Warner, H. P. Smith and J. E. Flynn, *J. Am. Med. Assoc.*, **112**, 1898 (1939).

blood drops should become continually smaller. The bleeding normally stops after 1–3 minutes. Continued bleeding may be due to many causes such as deficiency in vitamins C or K, hemophilia, etc.

2. **Coagulation Time Determination.** About 1 cc. of fresh blood is drawn in a tube containing thromboplastin. The tube is shaken and the clotting time is observed. The determination is carried out with the blood of a normal person as a control. This test[232] has been recommended[233] as a "bedside" method since it is easy to carry out and is very reliable.

3. **Prothrombin Time Determination.** This method has already been described among the methods used for the determination of vitamin K and has been most successfully used clinically in various modifications.[233, 234, 235, 236] Various micromethods have been worked out[237] which require only one drop of blood.

4. **Two-Stage Determination of Prothrombin.** In this test[238] the blood is first mixed with a sodium oxalate solution and is centrifuged. The plasma is then defibrinated by adding thrombin. The remaining fluid contains the prothrombin which is completely converted into thrombin by mixing various dilutions with exactly defined solutions of saline, calcium ions and thromboplastin. A specified amount of fibrinogen is then added and the amount of thrombin previously formed is measured by determination of the clotting time.

5. **Serum Volume Test.**[239] The volume of the blood serum, adjusted to the red cell count, decreases during early stages of vitamin K hypovitaminosis and its determination in conjunction with an examination of the blood clot for friability has been claimed to serve as an indication of danger of bleeding.

17. Hypervitaminosis

The naturally occurring vitamins K_1 and K_2 are non-toxic, even when given in excessive doses.[240] Thus, in mice no lethal effect could be pro-

[232] H. P. Smith, S. E. Ziffern, C. A. Owen and G. R Hoffman, *J. Am. Med. Assoc.*, **113**, 389 (1939); S. E. Ziffern, C. A. Owen, G R Hoffman and H. P. Smith, *Proc. Soc. Exptl. Biol. Med.*, **40**, 595 (1939).

[233] H. R. Butt and A M. Snell, *Vitamin K*, Philadelphia, 1941.

[234] A. J. Quick, M. Stanley-Brown and F. W. Bancroft, *Am. J. Med. Sci.*, **190**, 501 (1935)

[235] A. J. Quick, *Am. J. Clin Path.*, **10**, 222 (1940).

[236] T. B. Magath, *Am. J. Clin. Path.* (Tech. Suppl.), **3**, 187 (1939)

[237] A. J. Quick, *Proc. Soc. Exptl. Biol. Med*, **42**, 788 (1939). W. E. Bray and O. R. Kelly, *Am. J. Clin. Path.*, **10**, 154 (1940). O. R. Kelly and W. E. Bray, *J. Lab. Clin. Med.*, **25**, 527 (1940). K. Kato, *Am. J. Clin. Path.*, **10**, 147 (1940)

[238] E. D. Warner, K. M. Brinkhous and H. P. Smith, *Am J. Physiol.*, **114**, 667 (1936).

[239] E. F. Boyce and E. M. McFetridge, *New Orleans Med. Surg. J*, **91**, 357 (1939).

[240] S. Ansbacher, *Proc. Am. Soc. Biol. Chem.*, **1940**, 111.

duced with doses as high as 25 g. of vitamin K_1 per kilogram of body weight[241] (studies on man[242]). The easily available substitutes 2-methyl-1,4-naphthoquinone and the 2-methyl-1,4-naphthohydroquinone, however, show definite toxic symptoms[243, 244] when fed to dogs, rabbits or humans in excessive amounts. Vomiting and porphyrinuria and occasionally albuminuria have been observed when 180 mg. of these compounds was given orally to humans or when 30–60 mg. per kilogram body weight was fed to dogs. In rabbits, excess feeding of 2-methyl-1,4-naphthoquinone has been noted to cause a prolongation of the blood-clotting time, which amounts to an effect which is contrary to that obtained by feeding normal doses of vitamin K.[245] In mice, small quantities of 2-methyl-1,4-naphthoquinone and, similarly, also phthiocol, cause a drop in the erythrocyte count and hemoglobin, while quantities from 0.2 to 0.5 g. per kilogram body weight cause death.[241]

18. Requirements

Vitamins K are required by all animals experimentally investigated, such as the chick, duck, goose, canary, pigeon,[246] turkey,[247] rat,[248, 249] rabbit,[250] mouse,[251] dog[252] and man.[253] The actual amount of this vitamin required by different species is unknown. In human therapy amounts varying from 1 to 10 mg. of 2-methyl-1,4-naphthoquinone have been recommended.[254] In general, pregnant and lactating women need increased amounts of this vitamin in order to protect the newborn from hypovitaminosis. (See also page 614.)

Vitamins K are not required by most microorganisms, such as yeast, fungi and most bacteria, but seem necessary for growth of Johne's bacillus.[255]

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[241] H. Molitor and H. J. Robinson, *Proc. Soc. Exptl. Biol. Med.*, **43**, 125 (1940).
[242] H. R. Butt and A. M. Snell, *Vitamin K*, Philadelphia, 1941.
[243] F. Koller, *Schweiz. med. Wochschr.*, **45**, 1159 (1939).
[244] H. Molitor and H. J. Robinson, *Proc. Soc. Exptl. Biol. Med.*, **43**, 125 (1940).
[245] P. Meunier, H. Hinglais, D. Bovet and A. Dreyfuss, *Compt. rend.*, **210**, 454 (1940).
[246] H. Dam, F. Schønheyder and L. Lewis, *Biochem. J.*, **31**, 22 (1937).
[247] H. J. Almquist, *Proc. Seventh World's Poultry Congress, Cleveland*, **1939**, 138.
[248] H. Dam and J. Glavind, *Z. Vitaminforsch.*, **9**, 71 (1939).
[249] J. D. Greaves, *Am. J. Physiol.*, **125**, 429 (1939).
[250] H. Dam and J. Glavind, *Acta Med. Scand.*, **96**, 108 (1938).
[251] R. Murphy, *Science*, **89**, 203 (1939).
[252] W. B. Hawkins and K. M. Brinkhous, *J. Exptl. Med.*, **63**, 795 (1936).
[253] R. Kark and E. L. Lozner, *Lancet*, **237**, 1162 (1939).
[254] H. Dam, *Ann. Rev. Biochem.*, **9**, 373 (1940).
[255] D. W. Woolley and J. R. McCarter, *Proc. Soc. Exptl. Biol. Med.*, **45**, 357 (1940).

VITAMIN P

VITAMIN P

1. Nomenclature and Survey

Names:

Vitamin P.[1, 2]
Citrin.[1, 2]
Permeability vitamin.

Composition:

Vitamin P is the name given to a crude extract which contains besides other physiologically active compounds the glucosides eriodictin and hesperidin which yield upon hydrolysis eriodictyol and hesperitin.

2. Chronology

1936 ARMENTANÓ, BENTSÁTH, BÉRES, RUSZNYÁK and SZENT-GYÖRGYI[1, 2] reported the occurrence of a substance, other than vitamin C, which controls hemorrhages in man. The active material was identified as a flavanone.

1939 SCARBOROUGH[3] presented evidence from experiments on human subjects which established the existence of a factor decreasing capillary fragility.

3. Occurrence

The distribution of vitamin P in nature has not been investigated for a large number of foods. It has been shown[4, 5] that this vitamin is present in citrus fruits, such as lemon, orange and grapefruit. The skin of these fruits is generally richer than the juice from the pulp. Lemon juice contains more vitamin P than does orange juice and this in turn contains more than grapefruit juice. Vitamin P is also found in the juice of other plants, such as paprika, and is believed to be widely distributed over the entire plant kingdom.

It has not been possible to demonstrate the presence of vitamin P (eriodictyol or hesperitin) in animal materials, such as milk, liver or kidney.[6, 7]

[1] L. Armentanó, A. Bentsáth, T. Béres and I. Ruszynák, *Deut. med. Woschr.*, **62**, 1326 (1936).
[2] A. Bentsáth, I. Rusznyák and A. Szent-Györgyi, *Nature*, **138**, 798 (1936).
[3] H. Scarborough, *Biochem. J.*, **33**, 1400 (1939).
[4] I. Rusznyák and A. Szent-Györgyi, *Nature*, **138**, 27 (1936).
[5] A. Szent-Györgyi, *Z. physiol. Chem.*, **255**, 126 (1938).
[6] I. Robeznieks, *Z. Vitaminforsch.*, **8**, 27 (1938–39).
[7] W. Neuweiler, *Ibid.*, **9**, 338 (1939).

Vitamin P appears to occur in plants predominantly in the form of glucosides.[8]

4. Isolation

The isolation of vitamin P is based on its property of being precipitated by lead salts in neutral solution and by alkali in anhydrous alcoholic solution. The actual isolation procedure, for example, from lemons, is carried out as follows:[9] The ripe fruit is pressed and the peels are ground and extracted with 96% alcohol. By the addition of Ba- and Pb-acetate solutions to the combined juice and extracts considerable amounts of impurities are precipitated. The vitamin is then precipitated by neutralizing the solution with ammonium hydroxide. The precipitate can be purified by reprecipitation and is then suspended in alcohol to which dilute sulfuric acid is added. Lead sulfate precipitates, and the vitamin goes into solution. The vitamin is obtained from the alcoholic solution by fractional precipitation with alkali. The free vitamin is obtained from the alkali salt by acidification. The procedure is essentially the same for the isolation of vitamin P from dried material, but the operations are reversed. After an initial alcohol extraction the vitamin is precipitated with alkali, the precipitate is dissolved in acetic acid and the vitamin is precipitated with lead acetate and ammonium hydroxide.

Two final products are obtained: a crystalline material consisting essentially of hesperidin with some eriodictin,[10] and the mother liquor which contains mainly eriodictin plus some hesperidin. The crystalline material and the solution apparently contain other physiologically active components in addition to eriodictin and hesperidin. An efficient method for the preparation of absolutely pure eriodictin or hesperidin has not been worked out and a separation has not been accomplished satisfactorily. The crystalline material can be recrystallized from pyridine and water.[11]

The glucosides eriodictin and hesperidin yield upon hydrolysis with dilute acids eriodictyol and hesperitin.

5. Properties

The chemical and physical properties of vitamin P cannot be stated, since the chemistry is not definitely established. Concentrates contain eriodictin and hesperidin besides other unknown components.

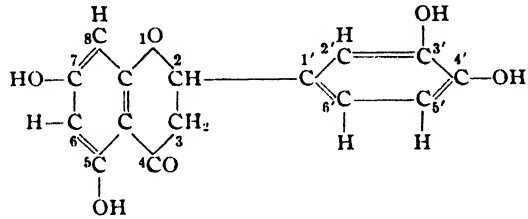
[8] V. Bruckner and A. Szent-Györgyi, *Nature*, **138**, 1057 (1936).
[9] A. Szent-Györgyi, *Z. physiol. Chem.*, **255**, 126 (1938).
[10] V. Bruckner and A. Szent-Györgyi, *Nature*, **138**, 1057 (1936).
[11] H. Scarborough, *Biochem. J.*, **33**, 1400 (1939).

Eriodictyol forms light yellow crystals which melt at 267° C. These crystals are sparingly soluble in water, but very soluble in organic solvents and in alkali.

Hesperitin melts at 224° C. and is readily soluble in water and in organic solvents.

6. Constitution

There are probably two glucosides which are vitamins P,[11a] namely *hesperidin*, which is hesperitin-*l*-rhamnosido-*d*-glucose, and *eriodictin*, which is eriodictyol-*l*-rhamnoside. Indications are that these compounds may be active only when the flavanone rings are opened to the corresponding chalcones. In the glucosides the sugar residues are attached to the 7-position. Hesperitin is the 4′-methyl-ether of eriodictyol. The latter has the following constitution:



Eriodictyol
5,7,3′,4′-Tetrahydroxy-flavanone

7. Biogenesis

The method which is used in the plant organism for the synthesis of vitamin P is not known. Various theories concerning the formation of flavanones in plants have been advanced.[12] The biogenetic scheme implies a synthesis from carbohydrates. Specifically, the flavanones appear to be built up from two hexoses and one triose by means of aldol condensations. Coniferyl alcohol, a compound which is believed to be a building unit of lignin, may be an intermediate.

8. Specificity

Little is known about the specificity of vitamin P. Besides the hydrolyzed vitamin concentrate containing eriodictyol and hesperitin, the naturally occurring glucosides have been shown to be active.

[11a] A. Mager, *Z. Phys. Chem.*, **274**, 109 (1942). G. Zemplen and R. Bognar, *Ber.*, **76**, 773 (1943). C. Z. Wawra and J. L. Webb, *Science*, **96**, 302 (1942). R. H. Higby, *J. Am. Pharm. Assoc.*, **32**, 74 (1942).

[12] R. Robinson, *Nature*, **137**, 172 (1936).

9. Determination

(a) *Chemical Methods*

There is no chemical method by which vitamin P can be detected. This is due mainly to the fact that the chemical composition of vitamin P is not known. Certain color reactions for the flavanones, such as the ones isolated from vitamin P preparations, cannot be accepted as tests for the vitamin as long as their vitamin nature is not better defined.

Vitamin P is said to give a yellow color with boric acid.[13] This color reaction may be used for the determination of flavones in natural products. The flavanones, on the other hand, give no color reaction with boric acid. Since vitamin P preparations contain predominantly flavanones (eriodictin and hesperidin), the positive color reaction must be due to some other unknown constituent.

(b) *Biological Methods*

The biological methods recommended for the demonstration of the presence of vitamin P are based on the fact that during vitamin P deficiency the capillary resistance of experimental animals is diminished. The occurrence of hemorrhages (type and number) has also been used as a criterion for vitamin P deficiency, but with less success.[14, 15, 16] Guinea pigs have been used predominantly[17, 18, 19, 20, 21] as experimental animals but the rat responds equally well to a vitamin P-deficient diet and can be healed by the administration of this vitamin.[22]

10. Standards

No standard of vitamin P has been designated.

11. Physiology of Plants

The physiological reason for the presence of vitamin P in the plant organism is not known. The flavanones may constitute structural building units.

[13] C. W. Wilson, *J. Am. Chem. Soc.*, **61**, 2303 (1939).
[14] A. Szent-Györgyi, *Z. physiol. Chem.*, **255**, 126 (1938).
[15] S. S. Zilva, *Biochem. J.*, **31**, 915, 1488 (1937).
[16] T. Moll, *Klin. Wochschr.*, **16**, 1653 (1937).
[17] A. Bentsáth, I. Rusznyák and A. Szent-Györgyi, *Nature*, **138**, 798 (1936).
[18] A. Bentsáth, I. Rusznyák and A. Szent-Györgyi, *Ibid.*, **139**, 326 (1937).
[19] E. N. Todhunter, R. C. Robbins, G. Ivey and W. Brewer, *J. Nutrition*, **19**, 113 (1940).
[20] A. Bentsáth and N. B. Das, *Z. physiol. Chem.*, **247**, 258 (1937).
[21] C. E. Zacho, *Acta Path. Microbiol. Scand.*, **16**, 1411 (1939).
[22] I. Rusznyák and A. Benko, *Science*, **94**, 25 (1941).

It has been suggested[23] that vitamin P acts in plants as a detoxifying agent and that considerable amounts of this agent are held available for times of need in the form of a glucoside. This is feasible since certain other biologically important compounds are stored in the plant organism in the form of inactive glucosides and, when needed, the glucosides are hydrolyzed and the biologically active compounds are set free.

12. Animal Physiology

Vitamin P (and probably the glucosides after hydrolysis in the intestines) is absorbed from the intestinal tract. Excess amounts are excreted through the urine.[24, 25] Vitamin P is also active when given by intramuscular or intravenous injection or through the rectum. The physiological action of vitamin P is concerned with the maintenance of normal conditions in the walls of the small blood vessels and the absence of this vitamin causes increased capillary fragility and permeability. It has been suggested that the action of vitamin P in protecting capillary resistance may be due to a detoxifying power.[26] The action of vitamin P, however, is not due to a change in concentration of any of the blood-clotting factors such as prothrombin, fibrinogen or platelets. It has been demonstrated, on the other hand, that vitamin P is able to increase the calcium level in the blood.[27] The view has also been expressed that vitamin P may be required for the absorption and retention of ascorbic acid.[28]

Vitamin P, but apparently not the isolated constituents hesperidin and eriodictin, causes upon intravenous injection a definite drop in blood pressure[29, 30] as demonstrated on the cat, rabbit, frog and turtle.[31]

Vitamin P also promotes growth and cell division in the fertilized ovum of sea urchins.[32]

13. Avitaminosis

A state of vitamin P deficiency is known for the guinea pig, rat and for the human organism. In patients suffering from vitamin P deficiency,

[23] I. N. Kugelmass, *J. Am. Med. Assoc.*, **115**, 519 (1940).
[24] L. Armentanó, E. B. Hatz and I. Rusznyák, *Klin. Wochschr.*, **17**, 739 (1938).
[25] I. Huszak, *Z. physiol. Chem.*, **249**, 214 (1937).
[26] I. N. Kugelmass, *J. Am. Med. Assoc.*, **115**, 519 (1940).
[27] M. Raunert, *Z. Urol.*, **32**, 630 (1938).
[28] A. Elmby and E. Warburg, *Lancet*, **1937, II**, 1363.
[29] A. Szent-Györgyi, *Z. physiol. Chem.*, **255**, 126 (1938).
[30] L. Armentanó, *Z. ges. exptl. Med.*, **102**, 219 (1937).
[31] A. J. Leser, C. F. Lombard, C. H. Thienes, C. Wawra and J. L. Webb, *Proc. Am. Soc. Pharmacol. Exptl. Therap.*, **1941**, 26.
[32] F. Ludwig, *Arch. exptl. Path. Pharmakol.*, **189**, 243 (1938).

capillary resistance is decreased and vascular permeability is increased. The clinical symptoms comprise hemorrhagic conditions of the skin (nutritional purpura) which cannot be cured by vitamin C.[33, 34, 35, 36, 37, 38] Vitamin P therapy has also been found of value in hemorrhagic conditions of otherwise non-specific character, such as in cases of bleeding in the kidney (nephritis) and in the stomach.[39]

14. Requirements

The exact vitamin P requirement of the human organism is not known. In clinical cases 50–150 mg. of the natural mixture of compounds have been administered. Rats with diminished capillary resistance have been given 3–4 mg. per day subcutaneously.

[33] L. Armentanó, A. Bentsáth, T. Béres and I. Ruszuyák, *Deut. med. Wochschr.*, **62**, 1326 (1936).
[34] H. Scarborough, *Biochem. J.*, **33**, 1400 (1939).
[35] I. N. Kugelmass, *J. Am. Med. Assoc.*, **115**, 519 (1940).
[36] S. Lajos, *Klin. Wochschr.*, **16**, 1615 (1937).
[37] C. T. Decker, *Münch. med. Wochschr.*, **86**, 292 (1939).
[38] T. Jersild, *Lancet*, **1938, I**, 1445.
[39] M. Raunert, *Z. Urol.*, **32**, 630 (1938).

NON-IDENTIFIED VITAMINS

THE NON-IDENTIFIED VITAMINS

In addition to the vitamins which are identified and which have been discussed in the preceding chapters of this monograph, there is an unknown number of non-identified vitamins. Their existence has, as a general rule, been postulated since it was found that an animal species kept on a more or less purified diet ceased to grow or developed specific diseases which could be alleviated by the feeding of special foods. As a result, a great number of unknown vitamins have been claimed. It remains to be seen how many of these dietary essentials will prove to be separate entities. On the other hand, there is the definite possibility that future research will uncover the existence of other vitamins, the existence of which is unknown today.

1. Vitamin B_3

In the early work on vitamin B_1 from 1911 to 1920, it was noted[1] that an additional factor (called vitamin B_3[2]) was necessary in order to cause pigeons to gain weight. Vitamin B_3 was described by Williams and Waterman in 1928.[3] A clearer definition of this vitamin was offered in 1936 by Carter and O'Brien.[4] Vitamin B_3 occurs in liver, yeast, whole grains and malt. It is soluble in water and dilute alcohol and sensitive to heat and alkali. Vitamin B_3 is present in the filtrate fraction after adsorption of an extract on fuller's earth.[5] It is possible that vitamin B_3 is identical with pantothenic acid.

2. Vitamin B_4

The existence of a special factor (or factors) designated as vitamin B_4 which prevents the occurrence of a typical paralysis in rats[6] and in chicks[7]

[1] H. Schaumann, *Trans. Soc. Tropical Med. Hyg.*, **5**, 59 (1911). E. A. Cooper, *J. Hyg.*, **12**, 436 (1912). A. D. Emmett and L. H. McKim, *J. Biol. Chem.*, **32**, 409 (1917).

[2] R. R. Williams and W. H. Eddy, *Carnegie Inst. Wash. Yearbook*, **27**, 378 (1928).

[3] R. R. Williams and R. E. Waterman, *J. Biol. Chem.*, **78**, 311 (1928).

[4] C. W. Carter and J. R. O'Brien, *Biochem. J.*, **30**, 43 (1936).

[5] C. W. Carter and J. R. O'Brien, *Ibid.*, **33**, 1810 (1939).

[6] V. Reader, *Biochem. J.*, **23**, 689 (1929). O. L. Kline, C. A. Elvehjem and E. B. Hart, *Ibid.*, **30**, 780 (1936).

[7] J. A. Keenan, O. L. Kline, C. A. Elvehjem and E. B. Hart, *J. Biol. Chem.*, **103**, 671 (1933).

has been postulated. This factor is present in yeast and in liver. Dried grass, wheat germ, pork brain and pork kidney are good sources while grains are relatively poor sources.[8] Rats on a vitamin B_4-deficient diet show general muscular weakness, spastic gait, swollen paws and a tendency to sit in a hunched position.[9] Chicks which are deficient in vitamin B_4 show a disturbed gait, a lack of coordination and a tendency to fall on their side with their legs in tension, pulled against the abdomen. It has been suggested[10] that vitamin B_4 may be identical with a mixture of arginine and glycine, since a deficiency of these amino-acids causes the occurrence of symptoms in the chick which resemble closely those observed in vitamin B_4 deficiency.

3. Vitamin B_5

The term vitamin B_5 was given in 1930[11] to a fuller's earth eluate fraction from yeast which was shown to be a factor essential for maintaining weight in pigeons. Vitamin B_5 was found to be stable toward heat and alkali. Later investigations have shown[12] that the vitamin B_5 concentrate contains vitamin B_6, which latter compound has weight-maintaining properties for the pigeon, but the effects of which are less pronounced than those of the vitamin B_5 concentrate. The remaining factor is now called vitamin B_5. It seems probable that vitamin B_5 is identical with nicotinic acid[12] since nicotinic acid exhibits the weight-maintaining properties of vitamin B_5 and has been isolated from eluate fractions.[13] It has been reported independently that nicotinic acid is required by the pigeon.[14]

4. Vitamin B_7 —Vitamin I

Vitamin B_7 or Vitamin I is a name given to a substance present in rice polishings, which is soluble in methanol and in ethyl alcohol. In the absence of this factor, pigeons develop digestive disturbances.[15]

5. Vitamin B_8 —Adenylic Acid

It has repeatedly been suggested[16] that adenylic acid (or its degradation product, adenine) might exert vitamin activity and the term vitamin B_8

[8] O. L. Kline, H. R. Bird, C. A. Elvehjem and E. B. Hart, *J. Nutrition*, 11, 515 (1936); 12, 455 (1936).
[9] V. Reader, *Biochem. J.*, 24, 1827 (1930).
[10] D. M. Hegsted, G. M. Briggs, C. A. Elvehjem and E. B. Hart, *J. Biol. Chem.*, 140, 201 (1941).
[11] C. W. Carter, H. W. Kinnersly and R. A. Peters, *Biochem. J.*, 24, 1832, 1844 (1930).
[12] C. W. Carter and J. R. O'Brien, *Ibid.*, 33, 1810 (1939).
[13] T. F. Macrae and C. E. Edgar, *Ibid.*, 31, 2225 (1937).
[14] L. J. Harris, *J. Soc. Chem. Ind.*, 58, 471 (1939).
[15] E. Centanni, *Biochim. terap. sper.*, 22, 137 (1935).
[16] H. v. Euler, F. Schlenk, L. Melzer and B. Högberg, *Z. physiol. Chem.*, 258, 212 (1939).

has been assigned to this compound. Adenine has been shown to be necessary for certain strains of lactic acid bacteria[17] and it has been claimed that adenylic acid is the coenzyme of the fatty acid dehydrogenase.[18] In clinical studies[19] on pellagrins, adenylic acid seemed to increase the effect of nicotinic acid. When 3 to 20 mg. of yeast-adenylic acid were injected intravenously into patients, deep, involuntary, gasping inspiration, a fluttering sensation in the upper part of the abdomen and a feeling of fullness in the head were observed.

6. Vitamin B_c

The occurrence of anemia has been observed in chicks as the result of a deficiency of an unknown member of the vitamin B complex.[20] The postulated vitamin is present in liver but has not been identified. The deficiency syndrome comprises a decreased red blood cell count, decreased per cent of hemoglobin in the blood and decreased red cell volume. In addition to showing the symptoms of anemia, young animals deficient in this vitamin grow only slowly.

7. Vitamin B_p (Anti-Perosis Vitamin)

Perosis, a deficiency disease characterized by the occurrence of deformed legs and known also as hock disease or slipped tendon, has frequently been observed in chicks on synthetic diets.[21] The lower bones in perotic chicks are abnormally short and twisted. Perosis can be treated to a certain extent prophylactically by manganese[22] and by choline.[23] In addition there seems to exist another perosis-preventing compound of organic chemical nature.[24]

8. Vitamin J[25]

Vitamin J, which has at times also been called vitamin C_2, has been postulated as an anti-pneumonia factor. The existence of such a factor has been

[17] E. F. Möller, *Z. angew. Chem.*, **52**, 466 (1939).
[18] K. Lang and H. Mayer, *Z. physiol. Chem.*, **262**, 120 (1939).
[19] T. D. Spies, D. P. Hightower and L. H. Hubbard, *J. Am. Med. Assoc.*, **115**, 292 (1940).
[20] A. G. Hogan and E. M. Parrott, *J. Biol. Chem.*, **132**, 507 (1940).
[21] A. G. Hogan, N. B. Guerrant and H. L. Kempster, *Ibid.*, **64**, 113 (1925). A. G. Hogan and C. L. Shrewsbury, *J. Nutrition*, **3**, 39 (1930). A. G. Hogan, L. R. Richardson and H. Patrik, *Ibid.*, **19**, Proc. 14 (1940).
[22] H. S. Wilgus, L. C. Norris and G. F. Heuser, *J. Nutrition*, **14**, 155 (1937).
[23] T. H. Jukes, *J. Biol. Chem.*, **134**, 789 (1940).
[24] A. G. Hogan, L. R. Richardson, H. Patrik and H. L. Kempster, *J. Nutrition*, **21**, 327 (1941).
[25] H. v. Euler, H. Soder and M. Malmberg, *Z. Hyg. Infektionskrankh.*, **116**, 672 (1935).

postulated because guinea pigs infected with pneumococci show remarkable resistance when lemon juice is administered. Since it is known that vitamin C is present in lemon juice and since vitamin C is known to detoxify bacteria, the power of ascorbic acid for protecting guinea pigs against pneumonia was studied experimentally. The effect of ascorbic acid was found to be considerably less than that of lemon juice. It was therefore concluded that a special anti-pneumonia factor exists in lemon juice. As a further differentiation from vitamin C, vitamin J does not occur in paprika and in the eye lens. In addition to lemons, it is also found in black currants, rowanberries and elderberries. The chemical nature of vitamin J is not known. No studies have been published on the use of this vitamin in man.

9. Vitamins L_1 and L_2—Lactation Vitamins

The existence of two different water-soluble vitamins, necessary for the onset of normal lactation, has been postulated.[26] Vitamin L_1 is present in liver, while vitamin L_2 is present in baker's yeast. Neither of these factors can replace the other. Much higher amounts of these vitamins are apparently necessary to induce the first lactation than are necessary for a second litter. The vitamins seem to be of functional importance in the maturation of lactation tissue.

Evidence for the existence of a factor essential for lactation has also been presented by another laboratory.[27] The factor was found in rice polishings, defatted wheat embryo and brewer's yeast, but most abundantly in liver and in rice bran extracts.

The factor or factors necessary for lactation appear to be different from all other known vitamins.[26, 27, 28]

10. Vitamin M

The existence of a vitamin M[29] has been postulated, because monkeys kept on a diet supposedly containing all recognized food factors are known

[26] W. Nakahara, F. Inukai and S. Ugami, *Science*, **87**, 372 (1938); *Sci. Papers Inst. Phys. Chem. Research Tokyo*, **31**, 42 (1937); **34**, 250 (1938); *Proc. Imp. Acad. Tokyo*, **14**, 9 (1938).

[27] B. Sure, *J. Biol. Chem.*, **140**, CXXX (1941).

[28] W. Nakahara, F. Inukai and S. Ugami, *Science*, **91**, 431 (1940).

[29] P. L. Day, W. C. Langston and W. J. Darby, *Proc. Soc. Expll. Biol. Med.*, **38**, 860 (1938). W. C. Langston, W. J. Darby, C. F. Shukers and P. L. Day, *J. Expll. Med.*, **68**, 923 (1938). P. L. Day, W. C. Langston, W. J. Darby, J. G. Wahlin and V. Nims, *Ibid.*, **72**, 463 (1940). M. Janota and G. M. Dack, *J. Infectious Diseases*, **65**, 217 (1939).

to suffer from anemia, cytopenia and loss of weight, often accompanied by ulceration of the gums and by diarrhea. Monkeys on a diet devoid of the postulated vitamin M die in 26 to 100 days. The vitamin is present in yeast and in liver and is therefore probably a member of the vitamin B complex. Vitamin M deficiency causes a lowered microbic resistance in the gastrointestinal mucosa, and gingivitis and ulcerative colitis result from the action of pathogenic saprophytes (for example, *Bacterium dysenteriae* (Flesner), *Shiga bacillus*, etc.) normally present in the intestines. There is a possibility[30] that a nutritional deficiency is also an essential etiological factor in human dysentery.

11. Factor T

The existence of a fat-soluble factor T has been postulated.[31] The absence of this factor causes a decrease in the number of blood platelets (thrombocytosis) in rats and man. Factor T is reported to occur in sesame oil and in egg yolks, but not in cod liver oil or olive oil. The chemical nature of this compound is not known, but it has been observed that its activity is destroyed by ultraviolet irradiation.

12. Factor U

Factor U is a growth-promoting substance required by chicks.[32, 33] It is present in yeast, wheat bran, middlings, alfalfa meal and, to a lesser extent, in corn. The factor is insoluble in ether, acetone and isopropyl alcohol, but soluble in 50 per cent methanol and can be adsorbed on fuller's earth and charcoal. The factor is stable in yeast when autoclaved for half an hour at pH 1.7 or 11.0, but is destroyed in alfalfa when autoclaved for five hours at 120° C.

13. Folic Acid

Folic acid[34] (derived from the latin *folium*—leaf) occurs in green leaves of many kinds, including grass. Mushrooms and yeast are good sources. It is also present in a number of animal tissues of which liver and kidney are the best sources.

[30] G. F. McGinnes, A. L. McLean, F. Spindle and K. F. Maxcy, *Am. J. Hyg.*, **24**, 552 (1936). Editorial, *J. Am. Med. Assoc.*, **116**, 2169 (1941).

[31] E. Schiff and C. Hirschberger, *Am. J. Diseases Children*, **53**, 32 (1937). *Jahrb. Kinderheilk.*, **146** [N. F. 96], 181, 191, 293 (1936); **147** [N. F. 97], 81 (1936).

[32] E. L. R. Stokstad and P. D. V. Manning, *J. Biol. Chem.*, **125**, 687 (1938); *Science*, **88**, 35 (1938).

[33] E. L. R. Stokstad, P. D. V. Manning and R. E. Rogers, *J. Biol. Chem.*, **132**, 463 (1940).

[34] H. K. Mitchell, E. E. Snell and R. J. Williams, *J. Am. Chem. Soc.*, **63**, 2284 (1941).

Folic acid has been isolated from spinach by adsorption on charcoal, precipitation with lead and silver salts and chromatographic adsorption on fuller's earth. Folic acid has a molecular weight of about 500, contains nitrogen but no phosphorus or sulfur, and is acidic in character.

Folic acid stimulates growth of rats and of various bacteria including *Streptococcus lactis* R., *Lactobacillus delbrückii* and *Lactobacillus casei*.[35]

14. Growth Factor for *Lactobacillus casei*

The existence of a growth factor for *Lactobacillus casei* was demonstrated by the same method which was used for the isolation of folic acid (see page 525). The chemical characteristics of the growth factor are, however, different from those of folic acid. This factor was isolated[36] from liver by adsorption on norite, followed by elution with ammonium hydroxide in methanol solution, followed by fractional precipitation with manganese salts. The product obtained has the properties of a dinucleotide containing a purine and a pyrimidine nucleus. Guanine was isolated from the hydrolysis products.

15. Grass Juice Factor

The existence of a special factor necessary for optimal growth of rats[37] and of guinea pigs[38, 39, 40] has been postulated. This factor is present in fresh grass[37] and in its juice[38] and has therefore been called "grass juice factor." Cereal grasses, rye grass, young white clover, peas, pea shells, cabbage, turnip tops and spinach are excellent sources. Young berries, cauliflower, canned peas and beans contain less. Relatively little of this factor is present in apples, celery, molasses, peanuts, turnips, lettuce and oats.[41] Animal materials, such as liver, contain only small quantities. The amount present in grass varies with the age of the plant, the maximum concentration being present in the growing plant while the mature and old plants contain considerably less.[38, 41] The active material can be preserved in forages by careful ensiling.[42] Acid methods of ensiling are superior

[35] E. E. Snell and W. H. Peterson, *J. Bact.*, **39**, 273 (1940).
[36] E. L. R. Stokstad, *J. Biol. Chem.*, **139**, 475 (1941).
[37] G. O. Kohler, C. A. Elvehjem and E. B. Hart, *J. Nutrition*, **14**, 131 (1937).
[38] G. O. Kohler, C. A. Elvehjem and E. B. Hart, *Ibid.*, **15**, 445 (1938).
[39] M. D. Cannon and G. A. Emerson, *Ibid.*, **18**, 155 (1939).
[40] G. O. Kohler, S. B. Randle, C. A. Elvehjem and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.*, **40**, 154 (1939).
[41] S. B. Randle, H. A. Sober and G. O. Kohler, *J. Nutrition*, **20**, 459 (1940).
[42] B. C. Johnson, C. A. Elvehjem, W. H. Peterson and H. J. Fagen, *Ibid.*, **18**, 527 (1939).

to other methods, especially to the molasses method and the dry method. The factor is destroyed by heat and by oxidation.[41]

The "grass juice factor" appears to be different from all other known vitamins. It can be precipitated from grass juice with acetone and then removed from this precipitate by means of acidified acetone. When grass juice is deproteinized with chloroform and alcohol, the factor remains in the water phase and can be adsorbed on norite.[43]

The grass juice factor is apparently readily absorbed by experimental animals and is secreted into milk.[37, 42] Therefore, the summer milk of cows contains considerable amounts of this factor while winter milk is practically devoid of it.

In the absence of the grass juice factor, rats and guinea pigs show a decline in weight and finally die. The rat is less sensitive to a deficiency than the herbivorous guinea pig. In addition to the loss in weight, some respiratory trouble was noted in most cases. The lungs show inflammation and congestion and in isolated cases necrotic areas were observed.[38]

16. Mouse Factor

Mice on a synthetic diet, but not on stock diets, have been shown[44] to develop sore eyes, uni- or bilateral, characterized by swelling and inflammation of the eyelids leading to closure of the eyes and blindness in many cases. Simultaneously a dermatitis on the ventral aspect of the neck between the forelegs and extending almost up to the lower lip, and some loss of hair, especially on the face and neck, occur. Finally the animals die. Rats, on the other hand, do not develop this disease when kept on the same diet. The symptoms can be cured by normal diet. Aqueous liver extracts and fresh flaked wheat germs are especially potent cures. In many cases the symptoms could not be cured completely, suggesting that the damage, if too extensive, is irreparable.

17. Anti-Pernicious Anemia Factor

There is the possibility that the postulated "extrinsic factor," in the absence of which pernicious anemia occurs in man, will eventually be found to be a vitamin. Little is known about this factor. In the presence of an "intrinsic factor" the extrinsic factor is supposedly converted into the compound which prevents the onset of pernicious anemia. The latter com-

[41] G. O. Kohler, S. B. Randle and J. R. Wagner, *J. Biol. Chem.*, 128, LV (1939).
[44] C. Carruthers, *Science*, 93, 44 (1941).

pound is found predominantly in the liver, but the natural distribution of the extrinsic factor and its chemical characteristics are unknown. This is due to the fact that it has been impossible to reproduce the human macrocytic hyperchromic anemia in animals.

APPENDIX:
THE VITAGENS

THE VITAGENS

ESSENTIAL FATTY ACIDS

1. Nomenclature and Survey

Names:

Essential fatty acids.
Vitamin F.[1, 2]
Vitamin F_1.

List of known essential fatty acids:

Linoleic acid: $C_{18}H_{32}O_2$.
Linolenic acid: $C_{18}H_{30}O_2$.
Arachidonic acid: $C_{20}H_{32}O_2$.

Formulas:

Linoleic acid:

$$CH_3\cdot(CH_2)_4\cdot CH{=}CH\cdot CH_2\cdot CH{=}CH\cdot(CH_2)_7\cdot COOH$$

Linolenic acid:

$$CH_3\cdot CH_2\cdot CH{=}CH\cdot CH_2\cdot CH{=}CH\cdot CH_2\cdot CH{=}CH\cdot(CH_2)_7\cdot COOH$$

Arachidonic acid:

$$CH_3\cdot CH{=}CH\cdot CH_2\cdot CH_2\cdot CH{=}CH\cdot CH_2\cdot CH_2\cdot CH{=}CH\cdot CH_2\cdot CH_2\cdot CH{=}CH\cdot(CH_2)_4\cdot COH$$

2. Chronology

1929 G. O. BURR and M. M. BURR[3] showed that the presence of highly unsaturated acids, such as linoleic and linolenic acid, in the diet is essential for normal growth of rats.

1938 TURPEINEN[4] found arachidonic acid more potent than any other of the investigated unsaturated acids and introduced the tentative hypothesis that the body needs this acid primarily. NUNN and SMEDLEY-MACLEAN[5] established that arachidonic acid was deposited in the liver fat when linoleic acid was fed to rats.

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[1] H. M. Evans, S. Lepkovsky and E. A. Murphy, *J. Biol. Chem.*, **106**, 431 (1934).

[2] The Council on Pharmacy and Chemistry of the American Medical Association, the American Society of Biological Chemists and the American Institute of Nutrition recommended "that the term vitamin F should not be used in referring to linoleic or linolenic acids, or any fatty acid or mixture of fatty acids." *J. Am. Med. Assoc.*, **113**, 589 (1939).

[3] G. O. Burr and M. M. Burr, *J. Biol. Chem.*, **82**, 345 (1929); **86**, 618 (1930).

[4] O. Turpeinen, *J. Nutrition*, **15**, 351 (1938).

[5] L. C. A. Nunn and I. Smedley-MacLean, *Biochem. J.*, **32**, 2178 (1938).

3. The Essential Fatty Acids as Vitagens

Much controversy has arisen over the question of whether or not the essential fatty acids should be classified as vitamins. In view of the definition of vitagens, as given on pages 5–6, the essential fatty acids are vitagens, since they are needed only in small quantities (0.1% of the food) and since they contribute to the mechanism of the transformation of energy, but are also building units of the phospholipides.

Which of the fatty acids are really essential is not known. The early work was done with linoleic and linolenic acids, both of which are able to counteract the deficiency symptoms, but apparently neither of these is stable in the organism. It has, however, been demonstrated[5] that after an intake of linoleic or linolenic acid, arachidonic acid was deposited in the liver fats. This may suggest that linoleic and linolenic acids are active precursors, whereas arachidonic acid is physiologically the most important compound. There are quite a number of other highly unsaturated fatty acids which occur naturally, but their vitagen nature has not been investigated as yet. In the following pages only the acids with known vitagen character will be discussed.

4. Occurrence

The essential fatty acids occur naturally both in plants and in animals. For a consideration of their occurrence, each fatty acid must be studied separately.

1. *Linoleic acid* occurs as a glyceride in most drying oils such as cottonseed, poppyseed, corn[6] and other oils. In animals, linoleic acid is present chiefly in the fatty acids of the phospholipides and to a lesser extent in the neutral fat.

2. *Linolenic acid*, like linoleic acid, occurs as a glyceride in most drying oils, such as linseed and perilla oils. Linolenic acid is usually absent from animal fats.

3. *Arachidonic acid* occurs predominantly in animal tissues. It is found mainly in the phospholipide fraction as a constituent of lecithin and cephalin,[7] but occurs also as a constituent of neutral fat. This acid has been isolated from heart[8], spleen,[8] liver,[9] adrenal[8] and brain[10, 11] fatty acids.

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[6] T. C. Taylor and J. M. Nelson, *J. Am. Chem. Soc.*, **42**, 1726 (1920).
[7] P. A. Levene and I. P. Rolf, *J. Biol. Chem.*, **51**, 507 (1922); **54**, 91, 99 (1922).
[8] E. Klenk and O. von Schoenebeck, *Z. physiol. Chem.*, **209**, 112 (1932).
[9] P. Hartley, *J. Physiol.*, **38**, 353 (1909).
[10] E. Klenk and J. Dittmar, *Z. physiol. Chem.*, **244**, 203 (1936).
[11] D. Wesson, *J. Biol. Chem.*, **60**, 183 (1924).

5. Isolation

The isolation of the essential fatty acids comprises the isolation of the total fatty acids from plant or animal materials followed by the separation of the individual fatty acids.

Plant material is either saponified as such or the oil present in plants, such as in seeds, is pressed out first, and then saponified. Alkali or alkaline earth hydroxide is usually used for these saponifications. The acids are usually extracted from the saponification mixture with an organic solvent. Linoleic and linolenic acids are separated from the other fatty acids by the addition of bromine whereby the tetra- and the hexabromide, respectively, precipitate. The brominated acids are separated and debrominated by means of zinc and alcohol. Thus, a mixture of linoleic and linolenic acids is obtained which can be separated by means of the different solubilities of their zinc salts in alcohol.

Animal materials are either saponified as such or the total fat is extracted or separated into the phospholipides and true fats. The latter procedure is carried out by dehydrating the material either by a drying process or, better, by means of acetone. The total fatty material is then extracted with alcohol, preferably at elevated temperature and in the presence of inert gases, such as nitrogen. After removal of the solvent, the phospholipide fraction is precipitated with acetone while the true fats go into solution. This division into neutral fats and phospholipides is not a quantitative procedure, but serves for a gross separation. The fractions are then saponified and worked up as described for plant material. Upon bromination, the octabromide of arachidonic acid is obtained which yields arachidonic acid upon debromination.

The yields of the unsaturated acids obtained by the bromination-debromination procedure are rather low, but the purity is considered to be high, at least in the case of linoleic and linolenic acids. To a certain extent, however, these acids and especially arachidonic acid undergo a rearrangement of the position of the double bonds.

A principally different method can be used for the isolation of the unsaturated fatty acids, namely, the direct isolation by fractional crystallization at low temperature. The yields by this method are high but the purity of the product is not as satisfactory. Practically, the phosphatide fraction is used for the isolation and the phosphatides are converted into methylesters by direct alcoholysis.[12] Fractional crystallization is then carried out at various low temperatures, for example, consecutively at $-20°$,

[12] G. Y. Shinowara and J. B. Brown, *Oil & Soap*, **15**, 151 (1938).

$-40°$ and $-80°$ C. By this method linoleic acid has been obtained from cottonseed and from corn oils in 90–94% purity,[13] linolenic acid from linseed and from perilla oils in 85–88% purity[14] and arachidonic acid from adrenal phosphatides in 70–75% purity.[15] Further purification, for example, of the methyl-arachidonate, can be effected by fractional distillation, whereby a product of 95% purity is obtained.[15]

6. Properties

All essential fatty acids have similar solubility characteristics. They are insoluble in water, but soluble in alkali and in organic solvents.

(1). **Linoleic acid:** Colorless liquid at room temperature. M. p. 11° C.
(2). **Linolenic acid:** Colorless liquid. B. p. 230–232° C./17 mm. Hg.
(3). **Arachidonic acid:** Colorless crystals. M. p. 77° C.

7. Chemical Constitution

All essential fatty acids are carboxylic acids as evident from the formation of various salts, analytical data and the formation of various esters.

1. Linoleic acid has the empirical formula $C_{18}H_{32}O_2$ and contains two double bonds as evident from the formation of a tetrabromide and of tetrahydroxy-stearic acid (sativinic acid) upon mild oxidation with permanganate.[16] Other oxidation products are azelaic acid, oxalic acid and *n*-caproic acid.[17] Upon reduction with hydriodic acid and phosphorus, stearic acid is obtained.[18] Linoleic acid is, therefore, Δ-9, 12-octadecadienoic acid:

$$CH_3\cdot(CH_2)_4\cdot CH{=}CH\cdot CH_2\cdot CH{=}CH\cdot(CH_2)_7\cdot COOH$$

2. Linolenic acid has the empirical formula $C_{18}H_{30}O_2$ and contains three double bonds since upon addition of bromine a hexabromo-derivative is formed. Upon reduction stearic acid is obtained. Linolenic acid is therefore a straight chain fatty acid.[19] Oxidation with ozone yields propionaldehyde, malonic-aldehyde-carboxylic acid and azelaic-aldehyde-

[13] J. B. Brown and G. C. Stoner, *J. Am. Chem. Soc.*, **59**, 3 (1937). J. B. Brown and J. Frankel, *Ibid.*, **60**, 54 (1938).
[14] G. Y. Shinowara and J. B. Brown, *Ibid.*, **60**, 2734 (1938).
[15] G. Y. Shinowara and J. B. Brown, *J. Biol. Chem.*, **134**, 331 (1940).
[16] A. Rollett, *Z. physiol. Chem.*, **62**, 410 (1909).
[17] G. L. Goldsobet, *Chem.-Ztg.*, **30**, 825 (1906).
[18] K. Peters, *Monats?.*, **7**, 552 (1886).
[19] E. Erdmann and F. Bedford, *Ber.*, **42**, 1324 (1909).

carboxylic acid. The three double bonds of linolenic acid are, therefore, in Δ-9,12,15-positions according to the formula:

$$\overset{16}{\overset{\cdot}{}}\quad\overset{15}{}\qquad\overset{13}{}\quad\overset{12}{}\qquad\overset{10}{}\quad\overset{9}{}$$
$$CH_3\cdot CH_2\cdot CH{=}CH\cdot CH_2\cdot CH{=}CH\cdot CH_2\cdot CH{=}CH\cdot [CH_2]_7\cdot COOH$$

3. Arachidonic acid has the empirical formula $C_{20}H_{32}O_2$ and contains four double bonds. An octabromo-arachidonic acid is formed upon bromination. The straight chain structure is established by total hydrogenation and comparison with a synthetic *n*-eicosanic acid.[20] The position of the four double bonds has been established by ozonolysis which yielded caproic, acetic, succinic, malonic and glutaric acids, acetaldehyde and carbon dioxide. Arachidonic acid, therefore, has been assigned the structure of Δ-5, 8, 11, 14-eicosa-tetrenoic acid:[20a]

$$CH_3\cdot[CH_2]_3\cdot[CH=CH\cdot CH_2]_4\cdot CH_2\cdot CH_2\cdot COOH$$

An eicosa-tetrenoic acid which is apparently different from arachidonic acid, has been isolated from sardine oils. This acid has the double bonds in 4-, 8-, 12- and 16-positions since upon oxidation succinic and butyric acids exclusively are obtained in almost quantitative yields.[21] The biological efficacy of this acid has not been investigated as yet.

8. Synthesis

None of the essential fatty acids has been synthesized.

9. Biogenesis

The origin and the biogenesis of linoleic acid and of linolenic acid in plant material are largely unknown. It has been speculated that certain plants may contain a dehydrogenation mechanism whereby saturated fatty acids are converted into unsaturated fatty acids. However, it is just as probable that the unsaturated fatty acids are obtained by total synthesis as is the case of the saturated fatty acids.

A better insight exists concerning the biogenesis of arachidonic acid. Upon feeding of linoleic or linolenic acid to rats, arachidonic acid is deposited in the tissues.[22] This proves a partial synthesis of this acid

[20] G. Y. Shinowara and J. B. Brown, *J. Biol. Chem.*, **134**, 331 (1940).
[20a] D. E. Dolby, L. C. A. Nunn and I. Smedley-MacLean, *Biochem. J.*, **34**, 1422 (1940). D. T. Mowry, W. B. Brode and J. B. Brown, *J. Biol. Chem.*, **142**, 679 (1942). C. L. Arcus and I. Smedley-MacLean, *Biochem. J.*, **37**, 1 (1943).
[21] Y. Toyama and T. Tsuchiya, *Bull. Chem. Soc. Japan*, **10**, 296 (1935).
[22] L. C. A. Nunn and I. Smedley-MacLean, *Biochem. J.*, **32**, 2178 (1938).

in the organism of the rat and indicates that rats are incapable of synthesizing a precursor of arachidonic acid.

10. Specificity

It has already been mentioned that of all the fatty acids investigated, only linoleic, linolenic and arachidonic acids show activity. Besides the free acids, the esters are active. The esters of the lower aliphatic alcohols have been demonstrated to be active and it is expected that all esters which can be hydrolyzed in the organism will prove effective.

The relative efficacy of the active acids, tested as their methanol esters, is recorded in the following table showing the symptom efficacy according to the skin test and growth test on a comparative basis arbitrarily designating the activity of linoleic acid in both tests as 1.

Acid	Efficacy in skin test	Efficacy in growth test
Arachidonic acid[23]	1	6
Linoleic acid	1	1
Linolenic acid	1/6	1/3
Docosa-hexaenoic acid ($C_{22}H_{32}O_2$)[24]	0	Little
Linusic and isolinusic acid	0	0

Tetrahydroxy-stearic acids, dioxido-stearic acid, 9,10,12-trihydroxy-stearic acid and chaulmoogric acid are inactive.

11. Determination

(a) *Chemical Methods*

The only known chemical method for the determination of the essential fatty acids is the precipitation of their bromine addition compounds which has been described on page 533.

A number of color reactions have been proposed[25] for the determination of the essential fatty acids, which are based upon reactions of the double bonds. Thus, the transfer of hydrogen from aromatic amines such as *p*-phenylene-diamine either alone or in the presence of phenols, for example, *o*-cresol, to the essential fatty acids has been studied. The colors developed depend upon the concentration used and the time. Initially a violet, then

[23] E. M. Hume, L. C. A. Nunn, I. Smedley-MacLean and H. H. Smith, *Biochem. J.*, **34**, 879 (1940).
[24] E. H. Farmer and F. A. Van de Heuvel, *J. Chem. Soc.*, **1938**, 427.
[25] G. Woker and P. Bernhard, *Helv. Chim. Acta*, **24**, 98 (1941).

an olive-green and finally a gray color are observed. This reaction can be made considerably more sensitive by the addition of hydrogen peroxide. Another reaction, which has been studied, is the addition of iodine to the double bonds. All these reactions are, however, not specific for the essential fatty acids since they are given by other unsaturated but non-essential fatty acids, by carotenoids, etc.

(*b*) *Biological Methods*

(a) **The Rat Growth Method.**[26] This method led to the discovery of the essential fatty acids and can be used either prophylactically or as a curative method and is based on increase in weight following the feeding of the essential fatty acids to young rats which have been depleted of these acids. The depletion of this factor is a very slow process and may take many months.

(b) **The Rat Skin Test.**[27] In this curative method, the criterion is the healing of the characteristic dryness and scurfiness on the dorsal surface of the hind feet and front of the ankles. Instead of feeding the essential fatty acid preparation, the material may also be applied topically to the skin.[28]

(c) **The Oestrus Cycle Test.**[29] Irregular ovulation, observed during essential fatty acid deficiency, has been used for the determination of the deficiency of these factors, and the restoration of the oestrus cycle toward normal has been used for the determination of the presence of the essential fatty acids.

12. Standards

There is no recognized standard fatty acid for comparison of its biological effect with that of unknown material. Since it is desirable to set up such a standard reference essential fatty acid, it is recommended that arachidonic acid be adopted, since all available evidence indicates that this is the acid which is produced in the organism from the other known essential acids.

13. Physiology

Little is known about the physiology and the mode of action of the essential fatty acids. Practically all experimental work has been carried out with rats.

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[26] G. O. Burr and M. M. Burr, *J. Biol. Chem.*, **82**, 345 (1929); **86**, 618 (1930).
[27] E. M. Hume, L. C. A. Nunn, I. Smedley-MacLean and H. H. Smith, *Biochem. J.*, **32**, 2162 (1938).
[28] M. I. Shepherd and D. R. Linn, *Drug Cosmetic Ind.*, **38**, 629 (1936).
[29] O. Turpeinen, *J. Nutrition*, **15**, 351 (1938).

Linoleic and linolenic acids and their simple esters are absorbed from the intestinal tract. Linolenic acid is metabolized directly after entering the organism and is not stored as such unless it is administered in excessive doses.[30] Linoleic acid, on the other hand, has been found invariably in all investigated animals and in man in the fatty acids of the phospholipides and to a lesser extent in the neutral fats. The main unsaturated acid in the phospholipides is arachidonic acid. The essential fatty acids are held in the phospholipides with extreme tenacity[31] and it takes, therefore, several months before a rat is completely depleted of these acids.

It is possible that linoleic and linolenic acids are really precursors of the active form. Linolenic acid cannot be demonstrated to be present in the organism although its biological efficacy is established. Linoleic and linolenic acids are converted in the organism into arachidonic acid[32, 33] which is the most active acid known. Besides arachidonic acid, other highly unsaturated fatty acids such as an acid $C_{20}H_{34}O_2$, dihydro-arachidonic acid, docosa-penta-enoic acid, etc., are synthesized in the body from linoleic and linolenic acids. Whether or not some of these act as "essential fatty acids" is not known.

The mechanism of the action of the essential fatty acids is not quite clear. Apparently these acids act in phosphatides and take part in the mechanism of utilizing fats. It has been suggested[34] that the first action is to load up the connective tissue cells of the fat depots with fats. If only small amounts of the essential fatty acids are administered, this effect can be demonstrated separately. After the depots are filled up with fat and additional amounts of the essential fatty acids are offered, growth takes place. Simultaneously the excessive fat deposits disappear.

The essential fatty acids are, however, not only connected with the movement of fats but also with the utilization of fats. It has been shown in *in vitro* experiments that unsaturated fatty acids catalyze the oxidation of saturated fatty acids without being affected themselves.

From experiments with fat-starved rats, the conclusion was drawn[35] that the essential fatty acids are necessary for the formation of new tissue but not for the maintenance of the normal metabolism of the cell.

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[30] N. R. Ellis and H. S. Isbell, *J. Biol. Chem.*, 69, 219 (1926). E. Klenk, *Z. physiol. Chem.*, 206, 25 (1932). R. H. Snider and W. R. Bloor, *J. Biol. Chem.*, 99, 555 (1933).

[31] R. G. Sinclair, *Proc. Soc. Exptl. Biol. Med.*, 27, 1059 (1930).

[32] N. R. Ellis and H. S. Isbell, *J. Biol. Chem.*, 69, 219 (1926). H. C. Eckstein, *Ibid.*, 81, 613 (1929). J. M. Spadola and N. R. Ellis, *Ibid.*, 113, 205 (1936).

[33] L. C. A. Nunn and I. Smedley-MacLean, *Biochem. J.*, 32, 2178 (1938).

[34] I. Smedley-MacLean and L. C. A. Nunn, *Ibid.*, 34, 884 (1940).

[35] I. Smedley-MacLean and E. M. Hume, *Ibid.*, 35, 990 (1941).

14. Deficiency Syndrome

A deficiency of the essential fatty acids causes a typical dermatosis in rats characterized by general dryness of the skin and thinness with much scurf especially on both the fore and hind paws and on the ears and tail. The latter may appear to be annulated. It should be noted that there is no edema present as is in the otherwise quite similar symptoms caused by a vitamin B_6 deficiency. In fat deficiency, the lacrimal glands are also affected and irregular ovulation and lesions of the kidney and of the urinary tract occur. Abnormal reproduction and deficient lactation have been observed. In young rats, cessation of increase in length and weight occurs. Sexual development is also delayed.

Clinical symptoms of a deficiency of the essential fatty acids in man are not known. There are, however, some observations which indicate that in certain eczema, and especially in infantile eczema, favorable clinical results can be obtained when oils containing unsaturated fatty acids are administered.[36] In such pathological cases it was found that the fatty acids from the blood serum contained a significantly lower concentration of unsaturated compounds than those of healthy individuals. By the time a clinical improvement was noticed, the concentration of unsaturated fatty acids had risen to essentially normal levels.

(*a*) *Clinical Test Methods*

There are no accurate methods for determining a deficiency of the essential fatty acids. It has been proposed to assay the actual concentration of unsaturated fatty acids in the serum since it was found that in rats,[37] goats[38] and dogs[39] the degree of unsaturation diminished with a lowered intake of the unsaturated fatty acids. These methods, however, have not been perfected and do not permit a differentiation of the essential fatty acids from non-essential, unsaturated fatty acids.

15. Requirements

The requirements of the essential fatty acids are largely unknown. In rats, a daily intake of about 14 mg. methyl-arachidonate produces optimum effects,[40] but decreased amounts show less favorable responses.

[36] A. E. Hansen, *Proc. Soc. Exptl. Biol. Med.*, **30**, 1198 (1933); **31**, 160, 161 (1933). T. Cornbleet, *Arch. Dermatol. Syphilol.*, **31**, 224 (1934).

[37] A. E. Hansen and G. O. Burr, *Proc. Soc. Exptl. Biol. Med.*, **30**, 1200, 1201 (1933).

[38] H. H. Williams and L. A. Maynard, *J. Dairy Sci.*, **17**, 223 (1934).

[39] A. E. Hansen, W. R. Wilson and H. H. Williams, *J. Biol. Chem.*, **114**, 209 (1936).

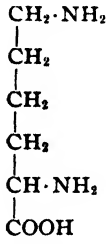
[40] E. M. Hume, L. C. A. Nunn, I. Smedley-MacLean and H. H. Smith, *Biochem. J.*, **34**, 879 (1940).

ESSENTIAL AMINO-ACIDS

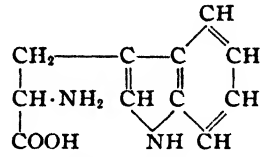
Among the amino-acids which are normal constituents of dietary proteins, ten are essential. Those amino-acids are defined[1, 2] as "essential" which must be present in the diet of young rats, in addition to all other energy and building unit requisites and the vitamins, to insure optimal growth. That is, these amino-acids cannot be synthesized in the animal organism or at least not at a rate necessary for normal growth. These ten amino-acids are:[1, 2] lysine, tryptophane, histidine, phenyl-alanine, leucine, isoleucine, threonine, methionine, valine, arginine.

A deficiency of any one or of all of these ten amino-acids causes an inhibitory effect upon growth. The same amino-acids which are essential for rats are also essential for dogs.[3] It is reasonable to assume that they are also indispensable for the human body, but no definite information regarding the special amino-acid requirements of man is available.

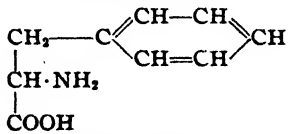
Arginine holds a somewhat unique position. Rats on an arginine-free diet are able to synthesize some of their requirements since the animals show a



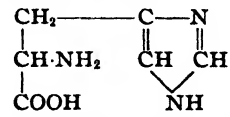
Lysine
(α,ϵ-Diamino-caproic acid)



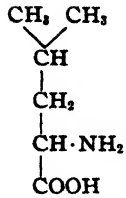
Tryptophane
(β-Indolyl-alanine)



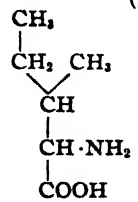
Phenyl-alanine



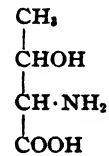
Histidine
(β-Iminazolyl-alanine)



Leucine (α-Amino-iso-caproic acid)

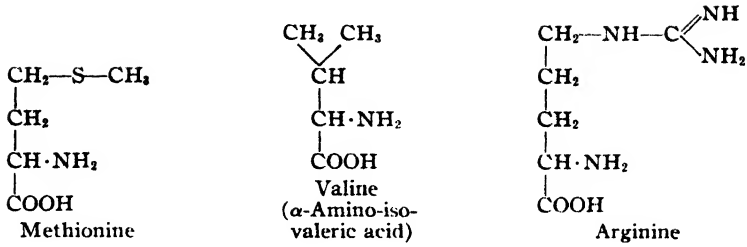


Isoleucine (α-Amino-β-methyl-valeric acid)



Threonine (α-Amino-β-hydroxy-butyric acid)

[1] W. C. Rose, *Physiol. Rev.*, **18**, 109 (1938). [2] W. C. Rose, *Science*, **86**, 298 (1937).
[3] W. C. Rose and E. E. Rice, *Ibid.*, **90**, 186 (1939).



Methionine Valine (α-Amino-iso-valeric acid) Arginine

growth of 70–80% in comparison with animals which received arginine.[4] In mice the synthesis of arginine from ornithine has been demonstrated by means of labeling the compounds with deuterium.[5] Adult dogs can get along without an external supply of this amino-acid. Chicks, on the other hand, must rely upon dietary arginine[6] since this amino-acid is needed as a structural building unit of creatine for feather formation.

While retarded growth is a somewhat non-specific symptom for a vitamin or vitagen deficiency, specific deficiency symptoms are known for a few of the indispensable amino-acids. The fright disease of dogs has, for example, been correlated with inadequate amino-acid supply.[7] A deficiency of lysine causes anemia in rats.[8] Cataract and other ocular changes have been observed in young rats on a tryptophane-deficient diet.[9] Rats deprived of valine show a disorder of the nervous system characterized by sensitivity to touch and severe lack of coordination in movement.[10] Young chicks fed a diet devoid of arginine and glycine develop a typical paralysis.[6] For the effects caused by a deficiency in methionine see pages 543 and 549. Other specific deficiency syndromes will undoubtedly be observed upon further studies.

The exact requirements of the essential amino-acids for man and various animal species are not known. The minimum amount necessary to support normal growth has been determined only for rats.[10] The total amount of essential amino-acids corresponds to 5.8% of the food consumed with the following figures for the individual amino-acids: lysine 1.0%, leucine 0.9%, phenyl-alanine 0.7%, valine 0.7%, threonine 0.6%, methionine 0.6%, isoleucine 0.5%, histidine 0.4%, tryptophane 0.2% and arginine 0.2%.

[4] C. W. Scull and W. C. Rose, *J. Biol. Chem.*, **89**, 109 (1930).
[5] R. F. Clutton, R. Schoenheimer and D. Rittenberg, *Ibid.*, **132**, 227 (1940).
[6] A. Arnold, C. L. Kline, C. A. Elvehjem and E. B. Hart, *Ibid.*, **116**, 699 (1936). D. M. Hegsted, G. M. Briggs, C. A. Elvehjem and E. B. Hart, *Ibid.*, **140**, 191 (1941).
[7] A. Arnold and C. A. Elvehjem, *J. Am. Vet. Med. Assoc.*, **95**, 303 (1939).
[8] A. G. Hogan, E. L. Powell and R. E. Guerrant, *J. Biol. Chem.*, **137**, 41 (1941).
[9] J. R. Totter and P. L. Day, *Ibid.*, **140**, CXXXIV (1941).
[10] W. C. Rose, *Science*, **86**, 298 (1937).

ESSENTIAL CARBOHYDRATES

In analogy to the discovery that besides the energy-bearing and building unit supplying compounds of the class of fats and proteins there are some fats and proteins which are also protective food constituents and which are therefore classified as vitagens, there may be specific carbohydrates which are vitagens. Ascorbic acid and inositol are examples of essential carbohydrates which are vitamins. Whether or not other carbohydrates exist which are essential food constituents is not known. If carbohydrates as food constituents would be utilized only to supply energy, it should be possible to replace all carbohydrates with the exception of the carbohydrates of vitamin character by some other energy-bearing foods such as fats or proteins. This is, however, not the case. The animal organism maintains, for example, in the blood, a certain normal glucose concentration and it has been shown that the organism is able to synthesize a certain amount of glucose for this purpose.[1, 2, 3] Animals on a completely carbohydrate-free diet, however, do not survive, apparently because the amount of glucose synthesized is not sufficient to maintain the necessary carbohydrate concentration in the organism. It is thus possible that either carbohydrates in general or some specific carbohydrates may, eventually, be found to be essential protective food constituents.

There is some evidence for the existence of an additional growth factor of the carbohydrate class, at least for the growing chick. This growth factor was first encountered in rice and has therefore been called "rice factor." This factor is apparently not present in yeast.[4] It has also been found in cartilage and the effect has been shown to be caused by the glucuronic acid component of chondroitin.[5] The effect does not appear to be very specific but is also brought about by a number of other carbohydrates, such as gum arabic, sodium alginate, gluconic acid, galactonic lactone, arabinose and xylose, while sugars like *d*-ribose and rhamnose are apparently inactive.[6]

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[1] R. D. Cramer and G. B. Kistiakowsky, *J. Biol. Chem.*, **137**, 549 (1941).

[2] J. B. Conant, R. D. Cramer, A. B. Hastings, F. W. Klemperer, A. K. Solomon and B. Vennesland, *Ibid.*, **137**, 557 (1941).

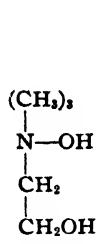
[3] A. K. Solomon, B. Vennesland, F. W. Klemperer, J. M. Buchanan and A. B. Hastings, *Ibid.*, **140**, 171 (1941).

[4] D. W. Hegsted, J. J. Oleson, C. A. Elvehjem and E. B. Hart, *Poultry Sci.*, **19**, 167 (1940). E. L. R. Stokstad, P. D. V. Manning and R. E. Rogers, *Ibid.*, **19**, 167 (1940).

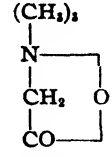
[5] H. J. Almquist, E. L. R. Stokstad, E. Mecchi and P. D. V. Manning, *J. Biol. Chem.*, **134**, 213 (1940). H. J. Almquist, E. Mecchi, E. L. R. Stokstad and P. D. V. Manning, *Ibid.*, **134**, 465 (1940).

[6] E. L. R. Stokstad, H. J. Almquist, E. Mecchi, P. D. V. Manning and R. E. Rogers, *Ibid.*, **137**, 373 (1941).

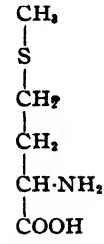
CHOLINE AND RELATED COMPOUNDS AND THE ESSENTIAL, TRANSFERABLE METHYL GROUP



Choline



Betaine



Methionine

1. Chronology

1894 HOFMEISTER[1] postulated the transfer of methyl groups in metabolism as the result of *in vivo* formation of methyl selenides and tellurides.

1917 THOMPSON[2] concluded that the synthesis of creatine in the animal organism is the result of methyl-transfer.

1932 BEST and co-workers[3] showed that in rats the addition of choline or betaine to a diet high in fat prevents the deposition of excess amounts of fats in the liver.

1935 BEST and co-workers[4] presented evidence that choline is an accessory food factor for rats.

1937 TUCKER and ECKSTEIN[5] found that methionine exerted an effect on liver fat similar to that of choline.

1939 DU VIGNEAUD and co-workers[6] observed that the "essential amino-acid" methionine can be replaced in the diet of rats by homocysteine when choline or betaine is given simultaneously, and suggested that this effect is due to the anabolic formation of methionine by methyl-transfer.

1940 BORSOOK and DUBNOFF[7] and DU VIGNEAUD and co-workers[8] proved that methyl-transfer occurred in the synthesis of creatine in the animal organism. DU VIGNEAUD[8] expressed the view that the transferable methyl group may be an essential dietary constituent.

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[1] F. Hofmeister, *Arch. exptl. Path. Pharmakol.*, **33**, 198 (1894).

[2] Thompson, *J. Physiol.*, **51**, 347 (1917).

[3] C. H. Best, J. M. Hershey and M. E. Huntsman, *Am. J. Physiol.*, **101**, 7 (1932); *J. Physiol.*, **75**, 56 (1932). C. H. Best and M. E. Huntsman, *Ibid.*, **75**, 405 (1932).

[4] C. H. Best, M. E. Huntsman, E. W. McHenry and J. H. Ridout, *J. Physiol.*, **84**, 38P (1935).

[5] H. F. Tucker and H. C. Eckstein, *J. Biol. Chem.*, **121**, 479 (1932); **126**, 117 (1938).

[6] V. du Vigneaud, J. P. Chandler, A. W. Moyer and D. M. Keppel, *Ibid.*, **131**, 57 (1939).

[7] H. Borsook and J. W. Dubnoff, *Ibid.*, **132**, 559 (1940).

[8] V. du Vigneaud, J. P. Chandler, M. Cohn and G. B. Brown, *Ibid.*, **134**, 787 (1940).

2. The Active Compounds and Their Properties

The number of naturally occurring compounds which may serve, among other purposes, as suppliers of the essential transferable methyl group is not known. However, besides choline, methionine and betaine are known to act in this capacity, especially in the absence of the former.

Choline. Choline is a colorless, viscid, strongly alkaline liquid and is very hygroscopic. It absorbs carbon dioxide from the air and easily forms salts—for example, the chloride, borate, picrate, etc. Choline is very soluble in water and alcohol, but is insoluble in ether. The salts are white, hygroscopic crystals which are soluble in water and in alcohol. Their aqueous solutions are practically neutral.

Methionine. M. p. 283° C. under decomposition. (Darkening at 278° C.) $[\alpha]_D^{25} = -6.87°$ C. in water.

Betaine. Betaine forms very hygroscopic crystals which lose their water at 100° C. Betaine melts at 293° C. under rearrangement into the methyl-ester of dimethyl-amino-acetic acid.

3. Pathological States Caused by a Deficiency of the Active Compounds

A deficiency of choline, betaine or methionine in the diet of young rats or dogs causes the deposition in the liver of fat[9] and to a lesser extent also of cholesterol esters.[10] These are removed or their deposition prevented when choline or one of the other mentioned compounds is fed. These substances are called lipotropic factors. At the same time a diffuse nodular cirrhosis of the liver occurs and has been observed in rats,[11, 12] dogs,[13] rabbits [14, 15] and guinea pigs.[15] The deficiency of these compounds furthermore causes hemorrhagic degeneration of the kidneys, characterized by symmetrical hemorrhagic necrosis of the cortex.[16, 17, 18, 19] An involution of the thymus gland, an enlargement of the spleen, a transformation of the

[9] C. H. Best, J. M. Hershey and M. E. Huntsman, *Am. J. Physiol.*, **101**, 7 (1932); *J. Physiol.*, **75**, 56 (1932). C. H. Best and M. E. Huntsman, *Ibid.*, **75**, 405 (1932).

[10] C. H. Best and J. H. Ridout, *J. Physiol.*, **78**, 415 (1933); **84**, 7P (1935). A. V. Stresser, I. McQuarrie and J. A. Anderson, *Proc. Soc. Exptl. Biol. Med.*, **33**, 595 (1936). H. P. Himsworth, *Acta Med. Scand.*, **Supplement 90**, 158 (1938).

[11] H. Blumberg, *U. S. Pub. Health Service Pub. Health Repts.*, **55**, 534 (1940). H. Blumberg and H. G. Grady, *Proc. Am. Soc. Biol. Chem.*, April, 1941. H. Blumberg and E. V. McCollum, *Science*, **93**, 598 (1941). R. D. Lillie, F. S. Daft and W. H. Sebrell, *Pub. Health Rep.*, **56**, 1255 (1941). F. S. Daft, W. H. Sebrell and R. D. Lillie, *Proc. Soc. Exptl. Biol. Med.*, **48**, 228 (1941).

[12] P. György and H. Goldblatt, *Proc. Soc. Exptl. Biol. Med.*, **46**, 492 (1941). P. György, E. C. Poling and H. Goldblatt, *Ibid.* **47**, 41 (1941).

[13] I. L. Chaikoff and C. L. Connor, *Ibid.*, **43**, 638 (1940).

[14] A. R. Rich and J. D. Hamilton, *Bull. Johns Hopkins Hosp.*, **66**, 185 (1940).

[15] M. A. Spellberg and R. W. Keeton, *Am. J. Med. Sci.*, **200**, 688 (1940).

[16] W. H. Griffith and N. J. Wade, *J. Biol. Chem.*, **131**, 567 (1939); **132**, 627 (1940).

[17] W. H. Griffith, *J. Nutrition*, **21**, 291 (1941). W. H. Griffith and D. J. Mulford, *J. Am. Chem. Soc.*, **63**, 929 (1941).

[18] P. György and R. E. Eckhardt, *Biochem. J.*, **34**, 1143 (1940).

[19] L. Rane and Y. Subbarow, *J. Biol. Chem.*, **134**, 455 (1940).

lymph nodes to hemolymph nodes, and in more severe cases hemorrhages in the eye have been observed.[20, 21] These compounds are also necessary for normal growth and lactation of the rat.[22] In the nursing young rat a flaccid paralysis of the hind leg occurs.[18, 22] In albino rats a rustiness of the fur has been observed as the result of a diet low in choline.[23]

In chicks, choline deficiency causes decreased or discontinued egg production, increased mortality and abortion of egg yolks.[24] In turkeys and in chicks choline deficiency is characterized by perosis and by slow growth.[25, 26, 27]

Choline deficiency is also manifested by high non-protein nitrogen in the blood.[28] Prolonged deficiency causes a loss of the ability of the liver to store glycogen and to excrete dye.[29]

4. Specificity Studies

The naturally occurring suppliers of the transferable methyl group show a relatively high specificity. Many proteins are active due to their content of methionine or due to the fact that betaine may be formed from some of the amino-acids in the protein.[30] However, the methyl groups of all compounds, which have methyl groups attached to quaternary nitrogen or sulfur, are not transferable. In order to be available for transmethylation, the methyl group must be bound on specific molecules. Thus in choline the hydroxyl group must be free or in a readily available form, since the ethers are inactive.[31] Choline salts, such as choline-chloride, and betaine-aldehyde are active.[31] On the other hand, the nitrogen of choline may be replaced by phosphorus[31] without loss of activity. The methyl groups of creatine,[32] S-methyl-cysteine[33, 34] or of the betaines from threonine, serine or allothreonine[35] are not transferable.

[20] K. Christensen, *Proc. Am. Soc. Biol. Chem.*, 1940, XX.
[21] R. W. Engel and W. D. Salmon, *J. Nutrition*, 22, 109 (1941).
[22] B. Sure, *Ibid.*, 19, 71 (1940).
[23] H. S. Owens, M. Trautman and E. Woods, *Science*, 93, 406 (1941).
[24] O. D. Abbott and C. U. DeMasters, *J. Nutrition*, 19, 47 (1940).
[25] T. H. Jukes, *J. Biol. Chem.*, 134, 789 (1940); *J. Nutrition*, 20, 445 (1940).
[26] D. M. Hegsted, R. C. Mills, C. A. Elvehjem and E. B. Hart, *J. Biol. Chem.*, 138, 459 (1941).
[27] T. H. Jukes, *Proc. Soc. Exptl. Biol. Med.*, 46, 155 (1941).
[28] W. H. Griffith and D. J. Mulford, *J. Nutrition*, 21, 633 (1941). R. W. Engel and W. D. Salmon, *Ibid.*, 22, 109 (1941).
[29] D. L. MacLean, J. H. Ridout and C. H. Best, *Brit. J. Exptl. Path.*, 18, 345 (1937).
[30] A. W. Beeston, H. J. Channon, J. V. Loach and H. Wilkinson, *Biochem. J.*, 30, 1040 (1936). C. H. Best, R. Grant and J. H. Ridout, *J. Physiol.*, 86, 337 (1936).
[31] A. D. Welch and M. S. Welch, *Proc. Soc. Exptl. Biol. Med.*, 39, 7 (1938).
[32] V. du Vigneaud, J. P. Chandler, M. Cohn and G. B. Brown, *J. Biol. Chem.*, 134, 787 (1940).
[33] H. J. Channon, M. C. Manifold and A. P. Platt, *Biochem. J.*, 34, 866 (1940).
[34] A. D. Welch, *J. Biol. Chem.*, 137, 173 (1940).
[35] H. E. Carter and D. B. Melville, *Ibid.*, 133, 109 (1940).

Choline derivatives which contain alkyl groups other than the methyl group and even the methyl-diethyl homolog[34] are unable to support growth of rats on a diet containing homocysteine,[36] but are potent lipotropic agents[37] and prevent the occurrence of hemorrhagic kidneys.[34] Arsenocholine, which is active as a lipotropic factor[31, 38] and prevents the hemorrhagic kidney condition in rats and perosis in turkeys,[34] does not transfer the methyl group to homocysteine.[34]

5. The Physiological Action of the Active Compounds

According to the tentative classification of choline as a vitagen, this substance is utilized in the animal organism as a building unit and is at the same time concerned with the regulation of metabolic processes. Choline serves as a building unit in many phosphatides, for example, in lecithins and in sphingomyelins. Choline is present in the phospholipides of practically all cells. In addition, choline regulates the turnover of fat[39] and of phospholipides,[40] for example, in the liver, and has for that reason often been classified as a vitamin.[41] In the absence of choline, fat is deposited in the liver, but this deposition is removed when choline becomes available. A dietary deficiency of choline becomes apparent from disturbances of the fat metabolism in spite of the presence in the body of very large amounts of choline-containing phospholipides.[42] In addition to these functions, choline is utilized in the animal organism after acetylation as a blood pressure-lowering substance. The process of producing acetylcholine is intimately connected with nerve impulses and special enzyme systems are readily available in the organism to acetylate choline when needed and to hydrolyze the active acetyl-compound to the free choline which is relatively incapable of influencing the blood pressure.

Choline can be synthesized to a limited extent in the animal organism. This is apparent, for example, from the fact that deuterium-containing choline can be isolated after feeding methionine, the sulfur-bound methyl group of which contained deuterium.[43] Choline synthesis is furthermore indicated by the fact that the choline content of rats on choline-free diets

[36] V. du Vigneaud, J. P. Chandler, A. W. Moyer and D. M. Keppel, *J. Biol. Chem.*, **131**, 57 (1939).

[37] H. J. Channon and J. A. B. Smith, *Biochem. J.*, **30**, 115 (1936). H. J. Channon, A. P. Platt and J. A. B. Smith, *Ibid.*, **31**, 1736 (1936).

[38] A. D. Welch, *Proc. Soc. Exptl. Biol. Med.*, **35**, 107 (1936). C. H. Best and J. H. Ridout, *Can. Med. Assoc. J.*, **39**, 188 (1938).

[39] See the review by C. H. Best and J. H. Ridout, *Ann. Rev. Biochem.*, **8**, 349 (1939).

[40] I. Perlman and I. L. Chaikoff, *J. Biol. Chem.*, **127**, 211 (1938); **128**, 735 (1939); **130**, 593 (1939).

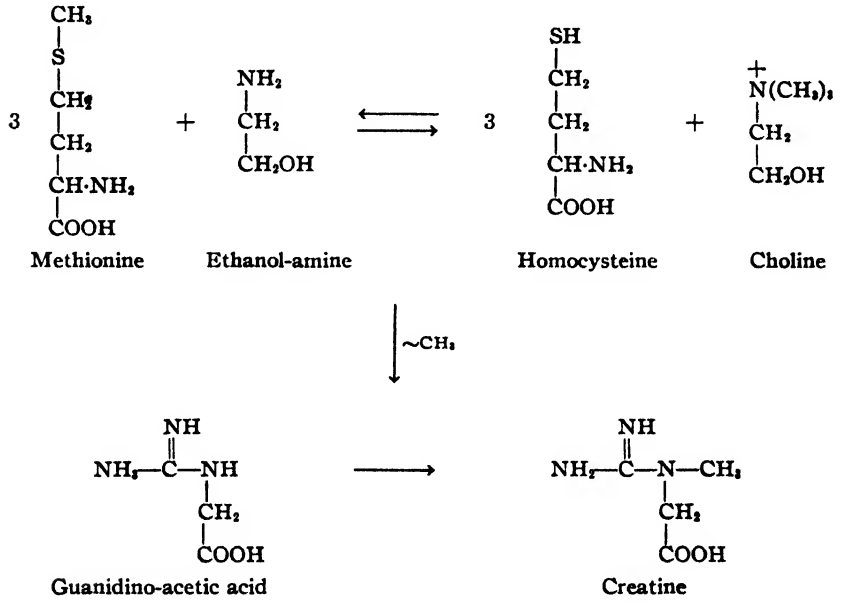
[41] P. György and H. Goldblatt, *J. Exptl. Med.*, **72**, 1 (1940). C. G. King, *Ann. Rev. Biochem.*, **8**, 371 (1939). H. C. Sherman, *Chemistry of Food and Nutrition*, New York, 1941, p. 395.

[42] F. X. Aylward, H. J. Channon and H. Wilkinson, *Biochem. J.*, **29**, 169 (1935).

[43] V. du Vigneaud, J. P. Chandler, M. Cohn and G. B. Brown, *J. Biol. Chem.*, **134**, 787 (1940).

increased with the weight of the animals.[44] Preformed choline must, however, be supplied to the animal for optimal physiological performance.

The methyl groups attached to the quaternary nitrogen are partly responsible for the physiological action of choline. They serve as methylating agents in anabolic processes, for example, in the synthesis of creatine from guanidino-acetic acid. Choline can be replaced in this reaction by other compounds with transferable methyl groups ($\sim CH_3$) such as methionine and betaine. Another physiologically important methylation is the synthesis of methionine from homocysteine and choline. On the other hand, choline can be built up from methionine and ethanol-amine. This series of reactions is shown in the following scheme:



The action of choline, however, cannot be due solely to the available methyl groups. For example, the ethyl-derivatives of choline cannot transfer methyl groups since the compounds are devoid of them and either do not transfer the ethyl group or the ethyl group is inactive. In any event, these compounds are unable to support growth of rats on a choline- and methionine-free diet in the presence of homocysteine.[45] Arsenocholine

[44] H. P. Jacobi, C. A. Baumann and W. J. Meek, *J. Biol. Chem.*, 138, 571 (1941).
[45] V. du Vigneaud, J. P. Chandler, A. W. Moyer and D. M. Keppel, *Ibid.*, 131, 57 (1939).

also is unable to transfer methyl groups.[46] However, these compounds are active as lipotropic factors, and they prevent the hemorrhagic kidney condition and the perosis encountered in animals kept on a diet devoid of choline.

6. Requirements

Choline (methionine or betaine) is required by all investigated animals, such as the rat, dog, rabbit, guinea pig, chick, turkey, etc. Choline is also an essential growth factor for bacteria, for example, for the *Pneumococcus bacillus*.[47]

Generally, the young, growing organism needs more choline than the adult organism. During lactation the requirements are increased. Rats need 10–20 mg. choline per day to prevent the deposition of excess amounts of fats in the liver and to maintain the fat deposition at a normal level.[48] The choline requirement of dogs is about 35 mg. per kilogram of body weight.[49] Chicks need about 75 mg. daily.[50]

In the absence of choline, the animal organism is able to utilize effectively methionine or betaine. The efficacy of these compounds is, however, somewhat lower than that of choline (estimated efficacy: 30%).

[46] A. D. Welch, *Ibid.*, **137**, 173 (1940).
[47] L. Rane and Y. Subbarow, *Ibid.*, **134**, 455 (1940).
[48] H. J. Channon, J. V. Loach and G. R. Tristram, *Biochem. J.*, **32**, 1322 (1938).
[49] C. Entenman and I. L. Chaikoff, *J. Biol. Chem.*, **138**, 477 (1941).
[50] O. D. Abbott and C. U DeMasters, *J Nutrition*, **19**, 47 (1940).

ESSENTIAL ORGANIC SULFUR-CONTAINING COMPOUNDS

There is the possibility that organic chemical compounds containing sulfur belong to the class of protective foods[1] and should be classified as vitagens. Sulfur-containing organic compounds act not only as structural building units, for example, in hair and nails, but are also of specific and functional importance for growth and maintenance of life. A deficiency of sulfhydryl-compounds brings about death. Sulfhydryl-compounds apparently act as activators (and inhibitors) of a number of enzyme systems,[2] probably by virtue of their ability to undergo reversible oxidation to disulfide compounds and reduction to the original sulfhydryl-compounds. They act, for example, as activators in the aerobic and anaerobic fermentation and in the oxidation of glucose in propionic acid bacteria.[3] In experiments with the *Amoeba proteus* it has been found[4] that sulfhydryl-compounds regulate nuclear growth and fission, and in experiments with non-nucleated blue-green algae, a stimulating influence on cell multiplication has been observed.[5]

The demonstration of the physiological significance of sulfhydryl-compounds is, however, in itself, no proof for their vitagen nature. This rests with the demonstration that the organism is unable to synthesize a particular sulfur-containing compound which may be essential, such as cysteine or the cysteine-containing glutathione, or any other normal body constituent of this class. There is actually ample experimental evidence that cystine is synthesized in the organism[6,7] from methionine.[8] On the other hand, the essential nature of methionine has been established in the search for the indispensable amino-acids (see page 540) and its efficacy is at least partly due to the available methyl group which acts in transmethylations (see page 543). Whether or not the vitagen action of methionine involves a participation of the sulfur in the molecule is not known. Future research must decide whether or not sulfur- or sulfhydryl-compounds belong to the class of vitagens and if the activity is due to the presence of the sulfur group, sulfhydryl-group or due to the entire molecule.

Among the known vitamins is a sulfur-containing compound, namely,

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[1] W. C. Rose, *Science*, **86**, 298 (1937). W. H. Griffith, *J. Nutrition*, **21**, 291 (1941).
[2] See the review by T. Bersin, *Ergeb. Enzymforsch.*, **4**, 68 (1935).
[3] P. Chaix and C. Fromageot, *Enzymologia*, **6**, 33 (1939).
[4] C. Voegtlin and H. W. Chalkley, *U. S. Pub. Health Service Pub. Health Repts.*, **45**, 3041 (1930). *J. Natl. Cancer Inst.*, **1**, 63 (1940).
[5] F. S. Hammett and L. Walp, *Growth*, **3**, 427 (1940).
[6] W. C. Rose, *Physiol. Rev.*, **18**, 109 (1938).
[7] E. F. Beach and A. White, *J. Biol. Chem.*, **127**, 87 (1939).
[8] H. Tarver and C. L. A. Schmidt, *Ibid.*, **130**, 67 (1939).

vitamin B_1. While vitamin B_1 is not a sulfhydryl-compound but a sulfide, the mechanism of the vitamin action is believed to be due to the ability of the vitamin to undergo reversible oxidation-reactions with the intermediary formation of a disulfide (see page 143). It is also known that in this case the vitamin action is due to the specific structure of the entire molecule.

PATENT INDEX

PATENT INDEX

In the following pages United States, British, German and French patents dealing with vitamins are listed. They are divided according to subject matter and arranged numerically under each subject. The essential data of each patent are given, including the number of the patent and the country in which it issued (U. S. for United States, B. for Great Britain, G. for Germany and F. for France). This is followed by the date on which the patent was issued. The inventor and the assignment recorded at the date of issue are indicated. A short abstract of the claim of the patent follows.

In addition to patents from the United States, Great Britain, Germany and France, a few patents from other countries such as Austria, Australia (Austral.), Belgium (Belg.), Canada (Can.). Denmark (Den.), Japan (Jap.), Norway (Norw.), Russia (Russ.), Sweden (Swed.) and Switzerland (Swiss) are mentioned because of their outstanding interest.

Abbreviations have been used for a number of industrial organizations.

Ciba stands for Chemical Industries of Basel
DuPont stands for E. I. du Pont de Nemours & Co.
Glaxo stands for Glaxo Laboratories, Ltd.
Hilger stands for Adam Hilger, Ltd.
I.C.I. stands for Imperial Chemical Industries
I. G. stands for Interessen Gemeinschaft der Farbenindustrie
Pfizer stands for Charles Pfizer & Co., Inc.
Squibb stands for E. R. Squibb & Son

VITAMINS: GENERAL

Patent No.	Date	Patentee	Abstract
U. S. 883,174	Mar. 31, 1908	F. S. Davidson and W. P. Burra	Extracts from hops and yeasts by steaming under pressure followed by pressing to separate the condensed liquid extract.
U. S. 1,461,703	July 10, 1923	D. Chidlow	Preserving the vitamin content of cereals by heating to 132° C.
U. S. 1,474,746	Nov. 20, 1923	G. S. Ward	Bread enriched with all vitamins of natural origin.
U. S. 1,479,418	Jan. 1, 1924	N. Mininberg	Separation of vitamins of whole grain, cooking of the starchy extracted material, addition of the vitamin extract to the cooked extracted material and drying.
U. S. 1,479,502	Jan. 1, 1924	G. Heffele	Yeast mixture as vitamin food.
U. S. 1,480,520	Jan. 8, 1924	J. R. Eoff	Vitamin elixir in wine from yeast or yeast concentrate.
U. S. 1,481,671	Jan. 22, 1924	T. J. Allen	Vitamin-containing food by fermentation of yeast.

Patent No.	Date	Patentee	Abstract
U. S. 1,488,815	April 1, 1924	I. F. Harris	Vitamin extract from yeast.
U. S. 1,502,563	July 22, 1924	W. P. Heath	Preserving vitamins in bread by working in an atmosphere of CO_2.
U. S. 1,526,032	Feb. 10, 1925	J. A. Wessener	Vitamin-containing food by mixing steep water with yeast followed by evaporation.
U. S. 1,538,366	May 19, 1925	R. Willstätter and H. Sobotka	Yeast rich in vitamins.
U. S. 1,540,883	June 9, 1925	I. F. Harris	Vitamin preparations, especially water-soluble vitamins, are preserved from deterioration by admixture with non-hygroscopic sugars.
U. S. 1,541,263	June 9, 1925	C. Hoffman, H. D. Grigsby and N. M. Cregor	Vitamin-containing food ingredients from rice polishings, wheat bran or cereal germ by cooking or by proteolytic enzymatic action followed by saccharification by malt diastase.
U. S. 1,552,176 B. 242,645	Sept. 1, 1925 Nov. 8, 1925	H. Liebers	Production of nutrient materials rich in vitamins by mixing yeast with concentrated extracts of germinated cereals.
U. S. 1,552,549 B. 258,704 F. 611,760	Sept. 8, 1925 Oct. 6, 1926 Oct. 11, 1926	B. L. Eicher	Charcoal tablets containing medicinals and fish oils.
U. S. 1,568,196	Jan. 5, 1926	O. Stiner, A. Hauswirth and A. Gams	Vitamin malt preparation.
U. S. 1,574,776	Mar. 2, 1926	R. Willstätter and H. Sobotka	Yeast preparation rich in vitamins.
U. S. 1,589,192	June 15, 1926	T. C. Manchester	Preservation of the vitamin content of milk by replacing the air in receptacles containing the milk with an inert gas such as CO_2.
U. S. 1,590,837	June 29, 1926	H. Liebers	Addition of yeast to cheese to increase its vitamin content.
U. S. 1,624,154	April 12, 1927	M. Winckel	Vitamin preparation from fermented skim milk and yeast.
U. S. 1,633,711	June 28, 1927	R. K. Prince Ass. to Vitamin Food Co.	Process for sealing vitamins A and D from cod liver oil into a mixture of yeast or other vegetable products with a gum solution.
U. S. 1,690,091 B. 289,798 G. 545,268 F. 655,799	Oct. 30, 1928 April 30, 1927 May 1, 1928 April 27, 1928	J. K. Marcus	Separation of vitamins A, D and E, along with unsaponifiable matter from oils by saponification and extraction with ethylene dichloride.
U. S. 1,708,914	April 9, 1929	B. Dass Ass. to Ellis-Foster Co.	Incorporation of dried yeast into peanut butter.
U. S. 1,722,175	July 23, 1929	W. S. Bowen	Spray desiccation for material containing vitamins.
U. S. 1,746,657	Feb. 11, 1930	W. J. Kemp	Preparation of vitamin-containing tomato juice.
U. S. 1,753,531	April 8, 1930	R. K. Prince Ass. to Vitamin Food Co.	Endocrine gland substances are mixed with vitamin preparations.
U. S. 1,756,574	April 29, 1930	J. Takamine, J. Takamine and N. Fujita Ass. to Takamine Ferment Co.	Vitamin product from propagated fungi such as aspergillus.

Patent No.	Date	Patentee	Abstract
U. S. 1,764,085	June 17, 1930	H. Placek Ass. to G. E. Conkey Co.	Vitamin-containing granular products from cod liver oil, yeast or soy bean meal and paraffin.
U. S. 1,775,966 U. S. Reissue 18,542 U. S. 1,775,967 U. S. Reissue 18,523 B. 256,765 B. 274,051	Sept. 16, 1930 July 26, 1932 Sept. 16, 1930 July 12, 1932 Aug. 19, 1926 July 8, 1926	E. H. Miles and G. Reilly	Preparation of vegetable extracts rich in vitamins by mixing the juices of vegetables containing vitamins and preservatives, adding acid material and heating the mixture to accomplish hydrolysis.
U. S. 1,796,027	Mar. 10, 1931	H. Iscovesco Ass. to Health Products	Process of isolating lipoids by acetone extraction.
U. S. 1,845,813 U. S. 1,918,983	Feb. 16, 1932 July 18, 1933	R. K. Prince Ass. to Vi-Foods Co.	Vitamin concentrate from a blend of different vegetables.
U. S. 1,861,677 B. 268,655 G. 486,228	June 7, 1932 Sept. 24, 1926 Sept. 25, 1926	L. A. Agopian	Vitamin concentrate from fruits or vegetables by precipitation with a Pb- or Cu-salt followed by extraction of the precipitate.
U. S. 1,879,762	Sept. 27, 1932	F. W. Nitardy Ass. to Squibb	Vitamin-containing tablets are provided with a coating containing an antioxidant such as hydroquinone.
U. S. 1,886,931	Nov. 8, 1932	E. R. Alexander Ass. to Vitamin Co. of America	Mixing of vitamins with citrus fruit masses.
U. S. 1,896,490 U. S. 1,896,520 U. S. 1,896,521	Feb. 7, 1933 Feb. 7, 1933 Feb. 7, 1933	E. Komm	Vitamin-containing food product from wheat germ.
U. S. 1,913,515 B. 317,554 F. 655,181	June 1, 1933 May 25, 1928 June 2, 1928	C. Schmitt	Vitamin extract from cotton seeds.
U. S. 1,919,297 G. 508,503	July 25, 1933 Oct. 23, 1928	W. Kropp, F. Lange and A. Bohne Ass. to Winthrop	Stable aqueous solutions of fat soluble vitamins by using water-soluble amides of lower fatty acids as solvents for the vitamins.
U. S. 1,929,786	Oct. 10, 1933	A. E. Meyer Ass. to Chappel Brothers	Isolation of chondroitin compounds from cartilage.
U. S. 1,942,943	Jan. 9, 1934	C. F. Schnabel	Poultry-feed rich in vitamins from grass.
U. S. 1,964,867 Continuation of U. S. 1,633,711	July 3, 1934	L. B. Allyn Ass. to Vitamin Food Co.	Granules of dried yeast impregnated with a vitamin A-containing oil and paraffin.
U. S. 1,965,355	July 3, 1934	C. L. Patterson	Vitamin-containing foodstuff from milk and yeast.
U. S. 1,975,169	Oct. 2, 1934	A. B. O. Norrbin	Extraction of water-soluble vitamins by dilute acids and addition of a substance soluble in acid solution which precipitates upon neutralization and carries the vitamins in the precipitate.
U. S. 1,984,858 B. 441,513 F. 782,314	Dec. 18, 1934 Jan. 21, 1936 June 3, 1935	C. L. Barthen Ass. to Health Products Co.	Countercurrent extraction of vitamins from an aqueous alkaline soap.
U. S. 1,988,677 U. S. 1,988,678	Jan. 22, 1935 Jan. 22, 1935	G. D. Arnold	Portable apparatus for drying vegetable matter while preserving the vitamin content.

PATENT NO.	DATE	PATENTEE	ABSTRACT
U. S. 1,999,789	April 30, 1935	E. Schmierer Ass. to Baker Perkins Co.	Apparatus for extracting vitamin-containing juices from fruits and vegetables in a vacuum.
U. S. 2,006,700	July 2, 1935	G. C. Supplee, G. E. Flanigan and R. C. Bender Ass. to The Borden Co.	Method for separating vitamins from casein.
U. S. 2,017,942	Oct. 22, 1935	S. Botcharsky Ass. to S. Teitelbaum	Fat-soluble vitamins are obtained from animal or vegetable material by plasmolysis, followed by extraction.
U. S. 2,041,056	May 19, 1936	W. L. Fleisher	Vitamin-containing compounds are injected into freshly baked products, such as bread.
U. S. 2,041,129	May 19, 1936	C. Hoffman Ass. to Ward Baking Co.	Cereal food is enriched in vitamins by adding natural vitamin-containing ingredients to dough.
U. S. 2,052,218	Aug. 25, 1936	C. Dickens	Vitamin product by concentrating milk.
U. S. 2,052,219	Aug. 25, 1936	C. Dickens	Vitamin concentrate from asparagus.
U. S. 2,060,389	Nov. 10, 1936	A. F. Wigelsworth	Apparatus for drying organic materials while preserving their vitamin content.
U. S. 2,065,332	Dec. 22, 1936	G. W. Kirby and C. N. Frey Ass. to Standard Brands	Vitamin product from yeast.
U. S. 2,072,402	Mar. 2, 1937	E. Kretschmer Ass. to Krause Medico	Vitamin-containing food product from unripe apples.
U. S. 2,128,845	Aug. 30, 1938	R. P. Myers and S. M. Weisberg Ass. to Sealtest System Laboratories	Whey is fermented with a lactose-fermenting microorganism to produce a product rich in vitamins
U. S. 2,128,946	Sept. 6, 1938	M. B. Katzman	Tetraphosphates of aliphatic hydroxy-compounds are used to retard rancidification of vitamin-containing preparations.
U. S. 2,132,656	Oct. 11, 1938	A. R. Smith Ass. to Combustion Engineering Co.	Flash drying of materials containing vitamins
U. S. 2,133,362	Oct. 18, 1938	C. F. Schnabel Ass. to American Dairies	Vitamin concentrate from grass juices.
U. S. 2,141,155	Dec. 27, 1938	C. Waizmann	Vitamin products from yeast and vegetable material.
U. S. 2,145,344	Jan. 31, 1939	F. Draisbach Ass. to Hall Laboratories	Vitamin in oil-water emulsions, using an alkali metaphosphate as the emulsifying agent.
U. S. 2,151,644	Mar. 21, 1939	H. C. Stephens Ass. to Natural Food Products	Deaeration of fruit juices to preserve the vitamin content.
U. S. 2,157,755	May 9, 1939	C. G. Harrel and A. W. Lindert Ass. to Pillsbury Flour Mills Co.	Addition of an oil soluble dye to vitamin preparations to facilitate the degree of mixing with other feed.
U. S. 2,167,144	July 25, 1938	R. W. Barton and W. M. Cox Ass. to Mead Johnson	Vitamin emulsions containing glycerol, a mono-oleic acid ester of diglycerol and sucrose.

Patent No.	Date	Patentee	Abstract
U. S. 2,170,520	Aug. 22, 1939	J. A. Raynolds Ass. to Atlantic Coast Fisheries Co.	Gelatine capsules for vitamin-bearing liquids.
U. S. 2,183,053 B. 489,970 B. 490,001	Dec. 12, 1939 Aug. 2, 1938 Aug. 2, 1938	H. F. Taylor Ass. to Atlantic Coast Fisheries Co.	Incorporation of vitamins in gelatine droplets.
U. S. 2,186,282	Jan. 9, 1940	W. W. Cowgill	Apparatus for drying vitamin containing foods in film form.
U. S. 2,188,319	Jan. 30, 1940	G. F. Siemers Ass. to McKesson & Robbins and to Vital	Composition of vitamin A with an adsorbate of vitamins B_1 and B_2.
U. S. 2,189,438	Feb. 6, 1940	L. H. Smith and C. F. Schnabel Ass. to American Dairies	Vitamin-containing fodder by mixing dried greens with a milk product.
U. S. 2,194,672	Mar. 26, 1940	C. M. Porter, L. V. Porter and E. Hurlock	Food product rich in vitamins from lactobacillus cultures.
U. S. 2,195,595	April 2, 1940	F. W. Nitardy Ass. to Squibb	Stabilization of fatty materials containing vitamins by the addition of an anti-acid substance such as MgO
U. S. 2,195,596 U. S. 2,206,113	April 2, 1940 July 2, 1940	F. W. Nitardy Ass. to Squibb	Vitamin tablets containing Ca-gluconate or Ca-phosphate.
U. S. 2,197,095 B. 458,399 B. 473,506 F. 797,654	April 16, 1940 Dec 18, 1936 Oct. 14, 1937 May 1, 1936	B. Cuenod Ass. to Soc. d'études et applications industrielles	Addition of vitamins to milk preparations.
U S 2,206,319	July 2, 1940	F. Geitz	Addition of vitamins to ground coffee by a spray process.
U S. 2,236,517	April 1, 1941	F. J. Cahn and B. R. Harris Ass. to Emulsol Co.	Emulsification of vitamins with a reaction product of an anhydride of a carboxylic acid ester of a hydroxy-polycarboxylic acid and a partial ether or ester of an aliphatic poly-hydroxy-compound.
B. 195,343	April 5, 1923	P. M. Travis and C. Glaban	Vitamin-containing milk by addition of fish oils, malt extracts and yeast.
B. 199,043	June 11, 1923	P. Farup	Addition of alcohol to fish oils. Also addition of plant extracts.
B. 217,282 G. 434,158	Mar. 5, 1923 Sept. 20, 1926	Kellogg Co.	Vitamin extracts from bran, milk or other sources are added to dough or bread preparations.
B. 222,073	Sept. 17, 1923	J. Schmidt	Vitamin-containing materials, such as yeast, are added to products similar to malt extracts.
B. 225,252	May 28, 1923	Fleischmann Corp.	Addition of vitamins to yeast nutrient solutions.
B. 226,549	Dec. 21, 1923	Mellemeuro Paeisk	Vitamin preparations are added to cheese.
B. 237,242	July 16, 1924	O. Mustad and Sön	Margarine and other edible fatty materials are mixed with substances rich in vitamins in the presence of an inert gas.
B. 249,746 G. 456,387 F. 604,077	April 8, 1926 Feb. 21, 1928 April 8, 1926	P. M. W. Grelck	Vitamin-containing food from malted grain.

Patent No.	Date	Patentee	Abstract
B. 254,724	July 1, 1925	F. H. Peck	Addition of vitamins to beverages. Also ultraviolet light irradiation of beverages.
B. 271,626	July 20, 1927	H. Haddan	Vitamin-containing tablets from dried vegetables or fruits.
B. 280,212	May 17, 1928	S. Grönningsaeter and Fischer-Hollinshed Co.	Process of transferring vitamins by extracting the vitamins from vitamin-containing material and adding the substance to be vitaminized to the solution containing the extract.
B. 293,735	April 11, 1927	Vitamin Food Co.	Dried yeast and cod liver oil are mixed with a gum and dried to form an air-excluding coating on the granular particles.
B. 297,256	Dec. 14, 1927	J. E. Nyrop	Production of vitamin-containing powders, suitable for adding to margarine, by spray-drying emulsions containing cream or skimmed milk.
B. 315,340	Jan. 10, 1928	W. Kollath and H. Magistris Ass. to L. W. Gans A.G.	Vitamin extract by precipitation and adsorptions.
B. 320,369	July 2, 1928	J. Korselt	Vitamin solutions from plant juices are treated with an acid Ca salt to remove oxalic acid.
B. 326,742 G. 482,494	July 25, 1929 June 18, 1921	Van Den Bergh's Margarine-Ges.	Incorporation of emulsions of vitamins into margarine.
B. 328,942 B. 362,023 F. 655,343 and Add. 39,331	Feb. 4, 1929 Sept. 11, 1930 Sept. 8, 1930	Matro Ges.	Vitamins are extracted from rootlets of grain from malt houses.
B. 340,580 B. 360,282 F. 681,093	Nov. 24, 1928 Jan. 23, 1931 Sept. 2, 1929	VanHouten & Zoon	Addition of vitamins to chocolate and cocoa.
B. 343,086 G. 570,158	Nov. 13, 1929 Nov. 14, 1929	A. B. O. Norbin and A. Astra Apotekarnas Kemiska Fabrieker	Extraction of water-soluble vitamins by diluted acids, precipitations and extractions.
B. 346,574 G. 519,778 F. 696,910	June 15, 1929 June 16, 1929 April 15, 1930	U. Brinch, H. Spehr and C. E. Sander Ass. to Brinch and Spehr	Preservation of vitamins in dried fruits by the addition of acids and salts prior to the drying.
B. 350,684	May 14, 1930	E. Maybury	Food seasonings and sauces containing vitamins.
B. 357,732 G. 598,027 G. 609,744 G. 618,899 G. 625,075 G. 636,434 F. 698,375	Sept. 13, 1929 Jan. 3, 1935 Feb. 22, 1935 Oct. 31, 1935 Feb. 3, 1936 Oct. 9, 1936 July 4, 1930	H. Van de Sandt	Addition of vitamins to beer.
B. 362,023 G. 501,844 G. 647,542	Dec. 9, 1931 July 9, 1930 July 7, 1937	Matro Ges.	Production of vitamin extracts.
B. 365,256	Dec. 1, 1930	H. Van de Sandt	Dealbuminized vitamin concentrate from yeast, tomatoes, spinach, etc.

Patent No.	Date	Patentee	Abstract
B. 366,516	Oct. 19, 1929	L. Bernardini	Fat- and water-soluble vitamins from germs and cereals are added to food products.
B. 367,063 B. 367,909 B. 368,919 G. 579,369	Nov. 29, 1929 Dec. 3, 1929 Jan. 30, 1930 June 24, 1933	H. Krönig Ass. to F. Lux	Enrichment of vitamins in beer by the addition of opened yeast.
B. 369,633 F. 709,423	Dec. 23, 1930 Aug. 6, 1931	W. W. Triggs Ass. to Tropical Vitamin Co.	Vitamin products from citrus fruits, liver oils, wheat germ oils, etc.
B. 370,926	April 6, 1932	J. E. Nyrop	Vitamin-containing feed for animals.
B. 378,399	Mar. 24, 1931	L. W. Mapson, J. T. MacCurdy, H. O. Nolan and Cambio Products Ltd.	Edible vitamin-containing food products by autolysis or fermentation of animal or plant material.
B. 395,957	July 27, 1933	A. R. Jahn	Multiple-effect evaporator for the concentration of vitamins.
B. 418,214	Oct. 8, 1934	Salsterol Lab.	Aqueous vitamin concentrate from raw fresh plant tissue.
B. 425,998	Mar. 26, 1935	I. G.	Emulsions of fat-soluble vitamins in oil with water and an emulsifying agent, such as gelatin, gum arabic, yolk of egg, etc.
B. 454,528	Oct. 2, 1936	A. Nyrop	Vitamin extract from pulped raw material by treatment with steam under pressure.
B. 459,467	Jan. 8, 1937	Nyegaard & Co.	Vitamins are incorporated in CO_2-evolving compounds containing substances of acid reaction.
B. 506,092	May 23, 1939	I. G.	Manufacture of edible glycerides to which vitamins may be added.
G. 362,367	Oct. 27, 1922	T. Hamburger	Dry vitamin preparation from plant juices and calcium-lactate.
G. 392,442	Mar. 21, 1924	Bayer	Food product from plasmolized yeast and calcium-phosphate.
G. 470,035	Jan. 3, 1929	M. Winkel	Vitamin-containing yoghurt.
G. 483,394	Aug. 12, 1925	H. Aman	Extraction of vitamins and inositol-phosphoric acid.
G. 489,186	May 13, 1926	O. Reinke	Vitamin extract from asparagus.
G. 492,281	Feb. 20, 1930	J. Wolf	Extraction of vitamins from fish livers by acidic solvents.
G. 499,384	Aug. 1, 1925	Aktienfabrik z. Erzeugung von Chemikalien	In the preparation of vitamin extracts, the starting material is plasmolyzed followed by saccharification.
G. 504,816	June 27, 1922	Diamalt A. G.	Vitamin products from yeast.
G. 505,356	Aug. 18, 1930	P. Grube	Vitamin-containing food product from the berries of *Sorbus aucuparia*.
G. 511,993	Mar. 28, 1929	H. Jena and J. Jena	Addition of vitamin-containing fruit juices to cheese.
G. 516,820	July 29, 1927	R. Neugebauer	Food rich in vitamins from germinating cereals.
G. 521,126	Aug. 20, 1926	Knoll, A. G.	Purification of vitamins by fractional diffusion or dialysis.

Patent No.	Date	Patentee	Abstract
G. 528,250	Feb. 12, 1930	A. Keddi	A vitamin extract from barley to be added to dough.
G. 532,521	Mar. 20, 1927	H. Netzow & Co.	Vitamin preparations from irradiated yeast are added to margarine.
G. 537,057	May 19, 1928	Enossis, S. A.	An aqueous vitamin extract from cotton seeds is added to milk and milk products.
G. 549,304	Feb. 26, 1930	H. Van de Sandt	Stabilization of beverages to which vitamins have been added by adjusting the pH to about 4.
G. 561,686	April 12, 1927	Vitamin Food Co.	Bird food rich in vitamins from yeast, cod liver oil and gum.
G. 565,901	April 22, 1931	A. Hölscher	Vitamin product by fermenting vegetable material containing vitamins with yeast.
G. 575,546 G. 577,622 G. 631,182	April 13, 1933 May 11, 1933 June 15, 1936	H. Van de Sandt	Vitamin-containing beer.
G. 581,143 Add. to G. 561,725	July 21, 1933	H. Krönig	Beer is enriched in vitamin content by adding disintegrated yeast before the end of the fermentation.
G. 586,589	Oct. 25, 1933	F. Weinmann	*d*-Glucuronic acid from plant germs by hydrolysis.
G. 586,959	Oct. 27, 1933	W. Lesselberg	Vitamin preparations are mixed with albuminous material and dried.
G. 618,482	Sept. 9, 1935	H. Sander & Co	Food preparations containing extracts of water- and fat-soluble vitamins.
G. 623,610	Dec. 30, 1935	K. Bodendorf	Food preparation by extracting vegetable matter with cod liver oil.
G. 623,657	Jan. 2, 1936	W. Kropp, F. Lange and A. Bohne Ass. to I. G.	Preparation of water emulsions of fat-soluble vitamins by dissolving the vitamins in water-soluble ethers or esters of polyhydroxy-compounds followed by incorporation into water.
G. 626,776	Mar. 2, 1936	Hoffmann-LaRoche	Extraction of vitamins with solutions of bile acids followed by separation of the acids.
G. 642,307	Mar. 1, 1937	F. Laquer Ass. to I. G.	Dispersions of fat-soluble vitamins in water by means of albuminous materials.
G. 661,503	June 20, 1938	P. Lindner	Assimilation of alcohol and/or CO_2 by yeast or algae to form vitamins.
F. 694,443	July 24, 1929	L. M. Rayband	Preservation of vitamins in germinated seeds by a coating of sugar chocolate.
F. 715,445	Aug. 26, 1930	A. A. Gourrier	Food product rich in vitamins from cereals and milk.
F. 717,067	May 15, 1931	I. G.	Dispersion of vitamins in water by the aid of albumins.
F. 723,537	July 18, 1931	G. Dubois	Extraction and concentration of vitamins.
F. 735,596	April 20, 1932	Établissements Byla	Food products from yeast.

Patent No.	Date	Patentee	Abstract
F. 757,454	Dec. 27, 1933	H. C. Meyers	Food product rich in vitamins from the germs of grain.
F. 771,059	Sept. 29, 1934	M. C. Gulbransen	Concentrated vegetable juices are mixed with vitamin-containing liquids.
F. 796,101	Mar. 30, 1936	M. Ernotte	Vitamin composition containing lecithin, sugar and vitamins, especially vitamin D.
F. 797,186	April 22, 1936	E. A. Barbet	Vitamin-enriched drinks by the addition of concentrates from grapes.
F. 797,229	April 23, 1936	International Vitaerine Lab. Co.	Fresh foods are dried to preserve their vitamin content by a liquid which causes exosmosis of the juice.
F. 805,598	Nov 24, 1936	Soc. Française Des Sucres	Addition of vitamins to sugar.
F. 810,961	April 3, 1937	C. G. V. Bouillon	Apparatus for generating vitamins in grain.
F. 833,984	Nov. 8, 1938	"Ocean"	The vitamins in a purée of paprika are preserved by sealing under vacuum.
F. 845,046	Aug. 9, 1939	G. Sandulesco and A. Girard Ass. to Les Laboratories Français de Chimiothérapie	Separation of hydroxyl group-containing vitamins by transformation into water-soluble quaternary ammonium compounds.
Austral. 29,933/35	Jan. 16, 1936	Nestlé and Anglo-Swiss Condensed Milk Co.	Fat- and water-soluble vitamins are emulsified in condensed milk.
Austrian 121,078	Aug. 15, 1930	E. Reinisch	Yeast is enriched in vitamins by ultraviolet rays.
Austrian 131,289	July 15, 1932	H. Oleoth	Extraction of vitamins from vegetable materials or microorganisms in the presence of nascent hydrogen.
Austrian 137,427	May 11, 1934	M. Klein	Vitamin preparations from cellular vegetable tissue by dialysis followed by precipitation and extraction procedures.
Dan. 54,611	April 19, 1938	H. C. E. Tillisch	Vitamin-containing material for coloring margarine is extracted from plants with oils.
Norw. 43,892	Mar. 28, 1927	A. W. Owe	Vitamin extract from vegetable material by saponification and extraction.
Norw. 44,019	Dec. 29, 1930	A. W. Owe	Edible fat is mixed with a vitamin-bearing material.
Vitamins A and D			
U. S. 1,162,907	Dec. 7, 1915	C. Funk	Extraction of vitamins from cod liver oil with ligroin or another organic solvent, precipitation with alcohol or acetone.
U. S. 1,326,968	Jan. 6, 1920	G. D. Rogers	Oil is separated from fatty materials, *e. g.*, from fish livers, by adding sodium chloride followed by subjection to an electrical discharge.
U. S. 1,368,148 B. 137,514	Feb. 8, 1921 Dec. 6, 1919	P. M. Heyerdahl	Extraction and purification of oils, *e. g.*, fish oils, by water.
U. S. 1,519,779	Dec. 16, 1924	E. M. Johnson	Oil is pressed from frozen cod livers.

PATENT NO.	DATE	PATENTEE	ABSTRACT
Vitamins A and D (*Continued*)			
U. S. 1,629,074	May 17, 1927	C. Funk and H. E. Dubin	Vitamins A and D are obtained from fish oils by extraction with acetic acid, saponification and digitonin precipitation of non-vitamin products.
U. S. 1,629,618	May 24, 1927	S. Grönningsaeter	Vitamin-containing fats are saponified with alcoholic alkali and the vitamins extracted by a vegetable oil.
U. S. 1,638,700	Aug. 9, 1927	D. Molofsky Ass. to Silmo Chemical Co.	Powdered fish oil product in a non-metallic mineral carrier.
U. S. 1,649,520	Nov. 15, 1927	C. Funk and H. E. Dubin	Stabilizing vitamin-containing oils by mild hydrogenation.
U. S. 1,678,454 B. 208,145 B. 227,121 B. 227,122 G. 484,993 F. 579,734	July 24, 1928 April 3, 1925 April 3, 1925 April 3, 1925 Oct. 24, 1929 Oct. 22, 1924	T. F. Zucker Ass. to University Patents Corp.	Extraction of liver oils with alcohol, saponification of the extract, precipitation of the fatty acids as calcium soaps and extraction of the vitamins from the soaps.
U. S. 1,715,945 G. 472,814	June 4, 1929 Oct. 11, 1924	A. W. Owe	Vitamin-containing oils are saponified with an alkaline earth hydroxide and extracted with an edible fat.
U. S. 1,725,964	Aug. 27, 1929	F. W. Nitardy Ass. to Squibb	Vitamin-bearing oils from fish livers by heating under subatmospheric pressure.
U. S. 1,753,790	April 8, 1930	K. Kawai	Partial saponification of cod liver oil.
U. S. 1,786,095 B. 220,697 F. 566,695	Dec. 23, 1930 Aug. 14, 1924 Feb. 18, 1924	K. Takahashi Asst. to Z. H. R. Kenkyujo, Japan	Extraction of fat-soluble vitamins from fish oils by saponification and precipitation of the fatty acids with an alkaline earth metal followed by extraction of the vitamins with an organic solvent.
U. S. 1,805,593 B. 266,905 G. 501,834	May 19, 1931 Mar. 2, 1926 Nov. 4, 1924	A. W. Owe	Vitamin extracts from marine oils by saponification followed by extraction with a vegetable oil.
U. S. 1,845,370	Feb. 16, 1932	T. B. Wagner	Emulsions of cod liver oil with a calcium-phosphorus compound derived from steepwater of corn.
U. S. 1,879,734	Sept. 27, 1932	W. G. Christiansen and E. Moness Ass. to Squibb	Extraction of vitamins from a saponified material by means of acetone.
U. S. 1,896,185	Feb. 7, 1933	H. O. Nolan Ass. to Ellis-Foster Co.	Partially hydrogenated cod liver oil containing its original vitamin content.
U. S. 1,897,039	Feb. 14, 1933	W. G. Christiansen, W. S. Jones and E. Moness Ass. to Squibb	Precipitation of fatty acids in the saponification mass of cod liver oil as the Al-salt.
U. S. 1,919,369	July 25, 1933	H. A. Holaday and A. Black Ass. to Squibb	Saponification of vitamin-containing oils and extraction with ether, acetone or dichloro-ethyl-ether.
U. S. 1,925,489	Sept. 5, 1933	E. Langfeldt and R. Hellerud	Apparatus for the extraction of vitamins from solutions containing water-soluble soaps by means of a vaporized solvent.

PATENT NO.	DATE	PATENTEE	ABSTRACT
Vitamins A and D (*Continued*)			
U. S. 1,935,042	Nov. 14, 1933	A. Black Ass. to Squibb	Refining the vitamin-containing unsaponifiable part of fish oils by dissolving in alcohol or dioxane and washing with a vegetable oil.
U. S. 1,947,315	Feb. 13, 1934	W. O. Snelling	Vitamin concentrate from fish oils by saponification in pentane with aqueous alkalies.
U. S. 1,947,432	Feb. 13, 1934	R. C. Huston and H. D. Lightbody Ass. to State Board of Agriculture of Michigan	Preservation of vitamin-containing fish liver oils by the addition of hydroquinone, resorcinol or the like.
U. S. 1,983,654	Dec. 11, 1934	A. Black Ass. to Squibb	Refining the vitamin-containing unsaponifiable matter of oils by treatment with carbon in the presence of an antioxidant.
U. S. 1,988,969	Jan. 22, 1935	A. F. O. Germann Ass. to S.M.A. Corp.	Vitamin A and D concentrate from palm oil by separation of the fraction permanently liquid at low temperature and irradiation or addition of viosterol.
U. S. 2,007,108 B. 463,655	July 2, 1935 April 5, 1937	H. Bresnick	Vitamin preparation by mixing an edible dehydrated hard vegetable fat with a fatty material of high vitamin content.
U. S. 2,026,395	Dec. 31, 1935	H. P. Loomis Ass. to Silmo Chemical Co.	Vitamin concentrate from oils by partial saponification.
U. S. 2,051,257	Aug. 18, 1936	H. N. Holmes Ass. to Parke, Davis	Materials such as fish liver oil, carotene or irradiated ergosterol are stabilized by adding phospholipides such as lecithin, sphingomyelin, etc.
U. S. 2,067,279	Jan. 12, 1937	F. W. Nitardy and W. S. Jones Ass. to Squibb	Vitamin-containing oil is extracted from fish livers, the protein of which has been coagulated, by means of ethylene chloride or other chlorinated solvents.
U. S. 2,090,738	Aug. 24, 1937	A. O. Tischer Ass. to Eastman Kodak	Purification of vitamin A- and D-containing natural oils by extraction of the impurities with an aldehyde having a furane nucleus.
U. S. 2,136,453	Nov. 15, 1938	H. M. Merker Ass. to Parke, Davis	Purification of vitamin-containing ether extracts from fish oils by means of sodium aluminum silicate.
U. S. 2,136,481	Nov. 15, 1938	F. H. Young and H. D. Robinson Ass. to Abbott	Vitamins A and D concentrate by partial saponification of livers.
U. S. 2,150,315 B. 506,730	Mar. 14, 1939 June 5, 1939	A. E. Briod and B. R. East Ass. to National Oil Products	Process for emulsifying vitamins A and D concentrates with cream or evaporated milk.
U. S. 2,161,882	June 13, 1939	James A. Patch	Method of extracting vitamins from fish liver oils by saponifying, adding organic solvents to produce a homogeneous solution and subsequently adding excess water.
U. S. 2,173,629	Sept. 19, 1939	N. A. Milas Ass. to Research Corp.	Process for the isolation of substantially pure vitamins A and D from fish liver oils by a series of fractional crystallizations.

Patent No.	Date	Patentee	Abstract
Vitamins A and D (*Continued*)			
U. S. 2,179,917	Nov. 14, 1939	H. Brinton	Addition of iodine to vitamin A- and D-containing oils as a stimulating effect for poultry.
U. S. 2,201,061 2,201,062 2,201,063 2,201,064	May 14, 1940	B. H. Thurman Ass. to Refining Inc.	Stabilization of fats and oils by a vegetable phosphatide.
U. S. 2,207,712	July 16, 1940	J. G. Blaso Ass. to Natural Vitamins Co.	Concentration of vitamins in oils by hydrogenation of the oil, removal of the hardened material and recovery of the vitamin-containing mother liquors.
U. S. 2,255,875 B. 535,014	Sept. 16, 1941 Mar. 26, 1941	L. O. Buxton and E. J. Simons Ass to National Oil Products	Purification of fat-soluble vitamin-containing oils by treatment with activated carbon.
U. S. 2,258,671	Oct. 14, 1941	L. O. Buxton Ass. to National Oil Products	Refinement of vitamin-containing oils by treatment with sugars.
U. S. 2,258,672	Oct. 14, 1941	L. O. Buxton and H B. Colman Ass to National Oil Products	Process for refining vitamin-containing oils by treatment with aliphatic aldehydes.
U. S. 2,258,673	Oct 14, 1941	L. O. Buxton Ass. to National Oil Products	Process for refining vitamin-containing oils by treatment with edible gum.
U. S. 2,266,719	Dec. 16, 1941	L. O Buxton and E J. Simons Ass. to National Oil Products	Process for refining a fat-soluble vitamin-containing material with an adsorbent in a non-polar solvent containing about 10% of a polar solvent.
U. S. 2,266,830	Dec. 23,.1941	H. F. Taylor, A W. Wells and V. A. Nedzvedsky Ass. to Atlantic Coast Fisheries	Vitamin concentrate from oils by saponification followed by extraction.
B. 3,075	Feb. 25, 1915	Boehringer	Extraction of vitamins from cod liver oil with ligroin and precipitation with phosphotungstic acid and extraction with acetone.
B. 207,545	Dec. 5, 1923	P. M. Heyerdahl	Liver oils are added to food products, *e. g.*, to chocolate or plant juices.
B. 214,238	April 9, 1923	F. W. Nitardy Ass. to Squibb	Packing of cod liver oil and similar food substances in the presence of an inert gas.
B. 217,363	April 5, 1923	T. W. F. Clark and E. T. Pearson & Co.	Improvement in odor and flavor of fish oils or their unsaponifiable part by passing a stream of inert gas through the oil followed by heating.
B. 227,474	Jan. 11, 1924	D. A. Hausen	Oils are obtained from fish livers by passing inert gas through the material at 40–50° C.
B. 266,139	Mar. 2, 1926	A. W. Owe	Vitamin concentrates are added to oils and the mixture subjected to a mild refining treatment.
B. 267,410	Mar. 17, 1927	H. Iscovesco and A. B. Adams	Extraction of vitamins from cod livers by saponification followed by extraction of the vitamins with a solvent.

Patent No.	Date	Patentee	Abstract
Vitamins A and D (*Continued*)			
B. 289,187	Feb. 7, 1927	F. H. Carr Ass. to British Drug Houses	Extraction of livers with an oil.
B. 293,777	April 5, 1927	L. H. Lampitt and J. H. Bushill Ass. to J. Lyons & Co.	Fats of high vitamin content from frozen viscera by heating with alkali solution and separating the fat.
B. 305,929	Feb. 11, 1928	K. Helholt	Vitamin-containing oils are added to non-saponifiable oils, saponified, extracted with a solvent and added to another oil.
B. 316,656	Aug. 2, 1928	H. F. Taylor Ass. to Atlantic Coast Fisheries	Fish livers are preserved by an oil solution of NaF.
B. 334,950 B. 361,343	Nov. 5, 1930 Dec. 2, 1931	E. Langefeldt and E. Hellerud	Extraction of soap solutions from liver oils by means of benzene introduced in vapor form.
B. 381,342	Oct. 12, 1932	K. Kawai	Oil from fish livers by treatment with dilute alkali and centrifuging the separated oil.
B. 382,060	Oct. 21, 1930	I. G.	Vitamin-containing hydrogenated fish oils are made aromatic by adding peppermint oil, anise oil or the like.
B. 433,930	Aug. 22, 1935	Ferrosan A. G.	Isolation of the vitamin-containin unsaponifiable fraction of oils.
B. 433,938	Sept. 4, 1935	I. G.	Use of hydroxy-alkyl-ethers and alkoxy-ethyl-ethers of polyhydroxy-compounds as water-miscible solvents for fat-soluble vitamins.
B. 441,545	Jan. 16, 1936	B. A. Rewald	Extraction of vitamins from fish tissue.
B. 461,202	Feb. 12, 1937	Aarus Oliefabriek and C. E. Christensen	Isolation of vitamins from oils and fats by simultaneous extraction and saponification.
B. 494,262 G. 664,021	Oct. 24, 1938 Aug. 18, 1938	E. Auhagen Ass. to I. G.	Manufacture of stable vitamin A and vitamin D preparations by mixing oily vitamin preparations with fat-containing plant material, *e. g.*, germinated wheat.
B. 500,770	Feb. 15, 1939	G. H. Lubarsky Ass. to Vitamol, Inc.	Manufacture of vitamin compositions for feeding poultry and livestock by emulsifying an oil concentrate of vitamins A and D in molasses.
B. 506,730	June 5, 1939	A. E. Briod & B. R. East	Vitamin concentrates and use thereof in making food products, particularly milk products.
B. 533,323	Feb. 11, 1941	G. H. Lubarsky Ass. to Vitamol, Inc.	Vitamin composition by mixing a fish liver oil vitamin concentrate emulsified in molasses with an animal feed meal followed by drying.
B. 535,383	April 8, 1941	L. O. Buxton Ass. to National Oil Products	Vitamin concentrate from the unsaponifiable fraction of marine oils by adsorption followed by elution.
B. 537,403	June 20, 1941	L. O. Buxton and E. J. Simons Ass. to National Oil Products	Refining of fat-soluble vitamin-containing oils by activated carbon.

Patent No.	Date	Patentee	Abstract
Vitamins A and D (*Continued*)			
B. 541,003	Nov. 10, 1941	H. P. Kaufmann	Refinement of vitamin-containing oils by chromatographic adsorption.
G. 452,646	Nov. 15, 1927	I. G.	Liver oils are refined by treatment with alcohols in the absence of oxygen.
G. 468,301	Nov. 4, 1924	A. W. Owe	Vitamin extracts, *e. g.*, carotene, by saponification, neutralization and removal of solvent.
G. 492,281	June 3, 1927	J. Wolf	Extraction of livers or fish liver oil with an acidic agent, having a pH between 3 and 6.
G. 448,870 G. 496,597	Sept. 1, 1927 April 26, 1930	H. Sander & Co.	Vitamin-containing margarine from cod liver oil.
G. 560,146 G. 567,648	Feb. 26, 1926 Mar. 31, 1927	A. G. f. Medizinische Produkte	Extraction of vitamins from oils by means of hot alcohol and/or acetone.
G. 568,901	Oct. 12, 1930	J. D. Riedel-E. de Haen	Petroleum ether extraction of the saponifiable part of cod liver oil.
G. 593,395	Feb. 26, 1934	Pentosin-Werke	Emulsion from liver oil, lime and calcium-saccharate.
G. 692,711	Sept. 27, 1940	F. Unger Ass. to Heyl & Co. and to F. Unger	Extraction of vitamins A and D by means of esters of low M. W. acids and low M.W. alcohols.
Belg. 430,869	Nov. 30, 1938	M. Vermeulen	Natural products containing vitamins are saponified directly and extracted.
Can. 259,310	Mar. 30, 1926	P. M. Heyerdahl	Incorporation of vitamins in margarine by mixing cod liver oil with olive oil prior to the addition to margarine.
Can. 374,395	June 14, 1938	H. N. Brocklesby Ass. to the Fisheries Research Board of Canada	Process of preparing natural fish liver oils of high vitamin A and D potency by digestion of the liver proteins with pepsin, peptization of the digested material with a mild alkali and separation of the liberated oil.
Norw. 34,895	May 8, 1922	T. B. Lexow	Margarine is enriched in vitamins by the addition of animal fats high in vitamin content, such as cod liver oil.
Norw. 41,688	July 27, 1925	Smoerfabrikken Flora	Fish liver oil to be added to margarine is treated to cover the unpleasant taste, *e. g.*, by adding aromatic substances and emulsification.
Distillation Procedure			
U. S. 1,260,072 B. 120,820	Mar. 19, 1918 Dec. 31, 1917	W. P. Schuck Ass. to Superior Oil & Process Co.	Purification of oils by distilling off impurities.
U. S. 1,925,559 U. S. reissue 20,705	Sept. 5, 1933 April 26, 1938	K. C. D. Hickman Ass. to Eastman Kodak	Vacuum distillation of fish oils.

Patent No.	Date	Patentee	Abstract
Distillation Procedure (*Continued*)			
U. S. 2,113,302 U. S. 2,124,879 B. 479,802 F. 825,979 F. 834,540	April 5, 1938 July 26, 1938 Feb. 11, 1938 Mar. 18, 1938 Nov. 23, 1938	K. C. D. Hickman Ass. to Eastman Kodak	Process for the separation of vitamins A and D from natural oils by molecular distillation in the presence of dyes as indicators for the distillation points of the vitamins.
U. S. 2,117,802	May 17, 1938	K. C. D. Hickman Ass. to Eastman Kodak	Apparatus for molecular distillation of organic substances in degassed condition.
U. S. 2,117,803	May 17, 1938	K. C. D. Hickman Ass. to Eastman Kodak	Apparatus for molecular distillation by spraying the compound to be distilled under vacuum into the hot vapor of a distilling substance.
U. S. 2,126,466	Aug. 9, 1938	K. C. D. Hickman Ass. to Eastman Kodak	High-vacuum short-path distillation of oils containing vitamins.
U. S. 2,126,467 B. 487,697 F. 817,036	Aug. 9, 1938 June 24, 1938 Aug. 24, 1937	K. C. D. Hickman and J. C. Hecker Ass. to Eastman Kodak	Short-path high-vacuum distillation and process for removing the distillate by a flow of an inert liquid over the condensing surface.
U. S. 2,128,223	Aug. 30, 1938	R. G. J. Fraser Ass. to I.C.I.	Fractional short path distillation.
U. S. 2,136,774	Nov. 15, 1938	K. C. D. Hickman Ass. to Distillation Products	Replacing the air by an inert gas or vapor prior to molecular distillation.
U. S. 2,143,587 B. 452,442 G. 659,217 F. 802,177	Jan. 10, 1939 Aug. 22, 1936 April 28, 1938 Aug. 29, 1936	H. I. Waterman, C. van Vlodrop and A. VanDijk Ass. to I.C.I.	Preparation of vitamin A and D concentrates, which are free from taste and odors, by molecular distillation of fish oils, followed by mild hydrogenation.
U. S. 2,144,906	Jan. 24, 1939	H. I. Waterman and C. van Vlodrop Ass. to I.C.I.	Flavoring matters and vitamins from butter fat by distillation.
U. S. 2,146,894	Feb. 14, 1939	K. C. D. Hickman Ass. to Distillation Products	Separation of sterols and vitamin D from oils by high vacuum distillation.
U. S. 2,150,683 B. 480,885 F. 812,734	Mar. 14, 1939 Mar. 2, 1938 May 15, 1937	K. C. D. Hickman Ass. to Distillation Products	Distillation of vitamin-containing oils in the presence of antioxidants.
U. S. 2,150,684 F. 825,978	Mar. 14, 1939 Mar. 18, 1938	K. C. D. Hickman Ass. to Distillation Products	Solid materials containing vitamins are subjected to molecular distillation.
U. S. 2,150,685 B. 482,883	Mar. 14, 1939 April 6, 1938	K. C. D. Hickman Ass. to Distillation Products	Apparatus for molecular distillation.
U. S. 2,165,378 B. 476,134 B. 482,882 B. 490,433 F. 811,766 F. 825,974	July 11, 1939 Dec. 2, 1937 Apr. 6, 1938 Aug. 15, 1938 Apr. 22, 1937 Mar. 18, 1938	K. C. D. Hickman Ass. to Eastman Kodak and to Distillation Products	Distillation in the presence of a substance of low volatility such as tripelargonine.
U. S. 2,169,195 B. 481,189 G. 664,745	Aug. 8, 1939 Mar. 7, 1938 Sept. 5, 1938	K. C. D. Hickman and A. O. Tischer Ass. to Distillation Products	Esters of vitamin A and unsaturated fatty acids having at least 8 carbon atoms.
U. S. 2,180,051 U. S. 2,210,926	Nov. 14, 1940 Aug. 13, 1940	K. C. D. Hickman Ass. to Distillation Products	Method of distilling fish oils to yield products of high vitamin content by subjecting the oil to a degassing operation prior to distillation.

Patent No.	Date	Patentee	Abstract
Distillation Procedure (*Continued*)			
U. S. 2,180,356	Nov. 21, 1939	K. C. D. Hickman Ass. to Distillation Products	Distillation of vitamins A and D from fish liver oils.
U. S. 2,186,669	Jan. 9, 1940	E. W. Fawcett and G. Burrows Ass. to I.C.I.	Apparatus for short-path high-vacuum distillation of vitamin-containing fish oils.
U. S. 2,199,994 U. S. 2,199,995	May 7, 1940 May 7, 1940	K. C. D. Hickman Ass. to Distillation Products	Distillation of fish oils.
U. S. 2,205,925	June 25, 1940	K. C. D Hickman Ass. to Distillation Products	Concentrates of vitamin A and of vitamins A and D are obtained from naturally occurring animal oils and fats by molecular distillation.
U. S. 2,210,927 G. 700,764 F. 834,936	Aug. 13, 1940 Nov. 28, 1940 Dec. 6, 1938	K. C. D. Hickman Ass. to Distillation Products	Vacuum distillation process.
U. S. 2,229,173	Jan. 21, 1941	K. C. D. Hickman Ass. to Distillation Products	A concentrate of vitamin A and D in ester form is made by molecular distillation of the crude ester, saponification, concentration of the vitamin and re-esterification.
U. S. 2,249,524	July 15, 1941	K. C. D. Hickman and J. C. Hecker Ass. to Distillation Products	Process for removing substances of undesirable odor and taste from vitamin-containing oils by short-path distillation of the undesirable materials, leaving the purified vitamin oil as distillation residue.
U. S. 2,249,525 B. 528,994	July 15, 1941 Nov. 21, 1940	K. C. D. Hickman Ass. to Distillation Products	Purification of vitamin A esters by alcohol extraction of impurities.
B. 415,088 G. 670,016 F. 767,191	Aug. 17, 1934 Jan. 10, 1939 July 19, 1934	F. H. Carr and W. Jewell Ass. to British Drug Houses	High vacuum short path distillation of vitamins from unsaponified liver oils.
B. 464,395 F. 811,920	April 19, 1937 April 26, 1937	E. W. Fawcett and D. Whittaker Ass. to I.C.I.	Concentration of vitamins by partial saponification followed by high vacuum distillation.
B. 479,816	Feb. 11, 1938	K. C. D. Hickman Ass. to Eastman Kodak	The undistilled residue of a molecular distillation is recirculated over the vaporizing element of the distillation apparatus.
B. 482,881 B. 493,948	April 6, 1938 Oct. 18, 1938	Eastman Kodak	High vacuum short-path distillation in the presence of "constant yield oil."
B. 485,549	May 18, 1938	K. C. D. Hickman Ass. to Eastman Kodak	Molecular distillation of unsaturated oils to be used for blending vitamin concentrates.
B. 487,367 F. 834,375	June 20, 1938 Nov. 18, 1938	G. G. R. Smith Ass. to Eastman Kodak	Saponification of the vitamin fraction obtained by high vacuum distillation and separation of the unsaponifiable part followed by high vacuum short path distillation.
B. 488,878	July 15, 1938	Eastman Kodak	High vacuum distillation of sterols and vitamins.
B. 489,623	July 29, 1938	K. C. D. Hickman and A. O. Tischer Ass. to Eastman Kodak	High vacuum distillation of materials containing sterols.

Patent No.	Date	Patentee	Abstract
Distillation Procedure (*Continued*)			
B. 500,195 Add. to 482,883	July 29, 1937	Eastman Kodak	Apparatus for high vacuum distillation.
B. 501,841	Mar. 2, 1939	J. G. Baxter Ass. to Eastman Kodak	Distillation of hydrocarbons and vitamins from marine animal oils.
B. 508,469	June 27, 1939	K. C. D. Hickman Ass. to Kodak Ltd.	Isolation of vitamin A, vitamin A esters and vitamin D from fish oils by molecular distillation.
B. 532,770	Jan. 30, 1941	K. C. D. Hickman Ass. to Distillation Products	Short-path, high-vacuum distillation of vitamins.
B. 539,089	Aug. 27, 1941	K. C. D. Hickman Ass. to Distillation Products	Extraction of fish tissues with a solvent of a vapor pressure lower than vitamins, followed by high vacuum distillation.
F. 825,406	Mar. 3, 1938	Eastman Kodak	Process for treating vitamin A consisting in the esterification of the distillate obtained by the molecular distillation of an animal oil containing vitamin A.
F. 825,973	Mar. 18, 1938	Eastman Kodak	Cholane derivatives, particularly sterols, are concentrated by high vacuum short path distillation.
F. 834,935 F. 834,937	Dec. 6, 1938 Dec. 6, 1938	Eastman Kodak	Method and apparatus for short path high vacuum distillation of vitamins A and D.

VITAMINS A

Provitamins A			
U. S. 1,328,278	Jan. 20, 1920	N. A. Gavin	Extraction of palm oil by cooking, centrifuging, screening and pressing.
U. S. 1,953,607	April 3, 1934	H. N. Holmes and H. M. Leicester Ass. to S.M.A. Corp.	Carotene from green plant material by alkali hydrolysis followed by chloroform extraction.
U. S. 1,967,121	July 17, 1934	H. N. Holmes and H. M. Leicester Ass. to S.M.A. Corp.	Carotene from carrots by cooking and acetone extraction.
U. S. 1,978,981	Oct. 30, 1934	H. M. Barnett Ass. to S.M.A. Corp.	Carotene from palm oil by precipitation with iodine.
U. S. 1,988,031	Jan. 15, 1935	H. M. Barnett Ass. to S.M.A. Corp.	Carotene from carrot oil or carrot powder by extraction.
U. S. 2,029,722	Feb. 4, 1936	V. Jersey Ass. to S.M.A. Corp.	Refining carotene-containing oils by separating the free fatty acids present as alkali salts.
U. S. 2,031,991	Feb. 25, 1936	O. Ungnade and W. F. Richards Ass. to S.M.A. Corp.	Acetone extraction of carotene from dry soaps.
U. S. 2,032,006	Feb. 25, 1936	R. J. Cross Ass. to S.M.A. Corp.	Addition of water binding materials to carotene-containing soaps and extraction of carotene from the dry material.
U. S. 2,032,165	Feb. 25, 1936	H. M. Barnett, W. O. Frohring and A. F. Germann Ass. to S.M.A. Corp.	Carotene from carrots or spinach by benzene extraction, crystallization of the main amounts of carotene and incorporation of the mother liquor into oil.

Patent No.	Date	Patentee	Abstract
Provitamins A (*Continued*)			
U. S. 2,131,394	Sept. 27, 1938	W. H. Test	Concentrate of pigments from vegetable origin by extraction with an organic solvent from an acidified aqueous solution or suspension.
U. S. 2,170,872	Aug. 29, 1939	D. D. Peebles	Macerated fresh plant materials are digested with alkali under pressure, followed by solvent extraction.
B. 364,113	Sept. 24, 1930	P. S. Voigt	Provitamin A and Vitamin C are extracted from lemon peel with alcohol.
G. 567,683	Jan 7, 1933	R. Kuhn	Separation of alpha- and beta-carotene by two methods: 1. chromatographic adsorption and 2. precipitation with iodine.
G. 685,390	Dec. 16, 1939	T. Buhr and W. Schoenenberger Ass. to W. Schoenenberger	Provitamin A-containing plant juices by centrifuging the plant juice, heating the liquid to coagulate and filtering and adding the sediment from the centrifugation to the liquid.
Hung. 116,057	Mar. 16, 1937	Ocean Magyar Konizervyar	Carotene is mixed with pulp of paprika and added to food products.
Isolation			
U. S. 2,076,901 G. 634,760	April 13, 1937 Sept. 2, 1936	F. Laquer Ass. to Winthrop	Preparation of vitamin A by direct saponification of fish livers with aqueous alcoholic caustic alkali metal hydroxide followed by extraction.
U. S. 2,111,049	Mar. 15, 1938	H. N. Holmes Ass. to Parke, Davis	Purification of vitamin A by chromatographic adsorption.
U. S 2,125,215 B. 500,087	July 26, 1938 April 25, 1938	A. D. Barbour Ass. to Ontario Research Foundation	Process for the production of a vitamin-containing fish oil partially hydrogenated in the presence of a highly active nickel catalyst.
B. 243,907	Dec. 10, 1925	Aarhus Oliefabrik and K. H. Hausen	Incorporation of vitamin A into oil, by mixing a vitamin A-containing soap from saponified oil with oil and separating the aqueous soap layer.
B. 283,265 F. 622,912	Feb. 2, 1928 June 9, 1927	K. Kawai	50–75% Saponification of cod liver oils.
B. 306,881 F. 670,114	May 19, 1930 Feb. 21, 1929	T. Shimizu	Precipitation of vitamin A from aqueous solutions by means of bile acids.
B. 393,883 G. 612,369	June 15, 1933 April 23, 1935	Hoffmann-La Roche	Purification of crude vitamin A preparation by freezing out impurities and precipitating the vitamin A with water. The precipitates are filtered through a layer of solid carbon dioxide.
B. 401,095	Nov. 9, 1933	Abbott	Vitamin A and D concentrates are obtained from fish liver oils by steaming the livers and extracting the oil with an organic solvent.
B. 434,432 G. 636,227 F. 768,217	Sept. 2, 1935 Sept. 17, 1936 Aug. 2, 1934	K. Ritsert Ass. to Merck	Purification of vitamin A in tuna fish liver oil by saponification at room temperature, followed by ether extraction.

Patent No.	Date	Patentee	Abstract
Isolation (*Continued*)			
B. 465,547	May 10, 1937	K. Kawai	Vitamin concentrates from fish oils by saponification followed by extraction with a fatty oil.
B. 500,645	Not accepted	Aktiebolaget Separator Ass. to Bergedorfer Eisenwerk, A.G., Astra-Werke	Process and apparatus for the extraction of oil from fish liver by breaking up the cell structure with acids or alkali without dissolving the oil and separating the oil by centrifugal treatment.
G. 468,301	Nov. 10, 1928	A. W. Owe	Partial saponification of plant material while extracting provitamin A.
G. 540,701	Dec. 24, 1931	J. A. de Loureiro	Obtaining vitamin A and vitamin D separately from cod liver oil by extraction with acetic acid followed by petroleum ether extraction of the acetic acid extract. Vitamin A is claimed to be in the petroleum ether while vitamin D is in the remaining acetic acid.
G. 660,621	June 3, 1933	Hoffman-La Roche	Separation of components of vitamin A by chromatographic adsorption.
G. 681,730	Sept. 29, 1939	R. Rosenbusch and G. Reverey Ass. to Riedel-de Haën	Isolation of vitamin A from fish oils by saponification in the presence of minute amounts of water, extraction of the solid saponification mass with acetone, removal of the acetone by distillation and ether extraction of the residue.
G. 697,762	Oct. 22, 1940	E. J. Wolf Ass. to Nordmark-Werke	Separation of vitamin A from animal tissues by enzymes followed by extraction with organic solvents.
F. 847,816	Oct. 17, 1939	M. Vermeulen	Concentration of vitamin A (or provitamins A) by direct saponification in the absence of oxygen, extraction of the saponification mass with ether, freezing out sterols and distilling off the ether. The residual product is claimed to contain from 3,200,000 to 3,600,000 I.U. vitamin A/gr.
Jap. 128,808	Feb. 15, 1939	K. Kituta	Concentration of vitamin A of fish liver oil by incomplete saponification (50–95%) and simultaneous extraction.
Synthesis			
U. S. 1,999,110 B. 418,723 G. 601,070	April 23, 1935 Oct. 30, 1934 Aug. 16, 1934	L. Ruzicka Ass. to Ciba	Synthesis of tetrahydro-vitamin A.
U. S. 2,175,843	Oct. 10, 1939	R. Kuhn and C. Grundmann Ass. to Winthrop	Polyene carboxylic acids and esters and manufacture thereof.
U. S. 2,233,375 G. 696,084	Feb. 25, 1941 Aug. 15, 1940	R. Kuhn and C. J. O. R. Morris Ass. to Winthrop	Condensing beta-ionylidene-acetaldehyde in the presence of a secondary amine with beta-methyl-crotonaldehyde to form ε-(beta-ionylidene)-beta-methyl-sorbin-aldehyde and reducing the aldehyde group of the latter compound by an aluminate of a secondary alcohol.

Patent No.	Date	Patentee	Abstract
Synthesis (*Continued*)			
U. S. 2,239,491	April 22, 1941	R. Kuhn and C. J. O. R. Morris Ass. to Winthrop	5 - (2′ - Methyl - 6′,6′ - dimethyl-cyclohexenyl - 1′) - 3 - methyl - 2,4-pentadienal, is claimed as an intermediate in the synthesis of vitamin A, and its preparation from 4-(2′ - methyl - 6′,6′ - dimethyl - cyclohexenyl - 1′) - 2 - methyl - 1,3-butadiene-1-carboxylic acid esters is also claimed.
B. 510,540	Aug. 2, 1939	I. M. Heilbron and J. W. Batty	Condensation of citral with beta-methyl-croton aldehyde.
G. 683,030	Oct. 27, 1939	R. Kuhn and K. Wallenfels Ass. to I. G.	Production of polyene compounds, *e. g.*, 1,30-diphenylpentadecaene, by converting polyene alcohols into thio- or seleno-aldehydes and removing from 2 molecules of the latter sulfur or selenium.
Analysis			
U. S. 2,065,953	Dec. 29, 1937	F. Twyman and D. H. Follet Ass. to Hilger	Photometric apparatus for estimating substances such as cod liver oil, vitamin A concentrates and fruit juices which have characteristic selective radiation absorption.
U. S 2,123,573	July 12, 1938	R. L. McFarlan and J. W. Reddie Ass. to United Drug Co.	Apparatus for measuring the concentration of vitamin A by light of 330 mμ.
F. 760,676	Feb. 28, 1934	Hilger	Determination of vitamin A by light of 328 mμ.
Derivatives and Utilization			
U. S. 2,183,084	Dec. 12, 1939	S. Reynolds Ass. to Atlantic Coast Fisheries	Composition of gelatine with vitamin A, etc.
U. S. 2,198,214	April 23, 1940	S. Musher Ass. to Musher Foundation	Stabilizing alfalfa against oxidation for the retention of its carotene content during drying and storage, by the use of a sugar and a phosphatide or H_3PO_4 in small proportions.
U. S. 2,218,591	Oct. 22, 1940	H. F. Taylor Ass. to The Atlantic Coast Fisheries	Dispersion of vitamin A in gelatine solution, addition of glycerine and subsequent drying.
U. S. 2,218,592	Oct. 22, 1940	H. F. Taylor Ass. to Atlantic Coast Fisheries	Substantially dry vitamin A preparations are made by dispersing the oil containing the vitamin in a matrix composed of a gelable colloid, *e. g.*, gelatine and an invert sugar, *e. g.*, molasses, honey, etc.
B. 491,212	Aug. 24, 1938	S. Reynolds Ass. to Glaxo	Process for the production of droplets of gelatine containing vitamin A.
B. 503,517	Not accepted	J. Verne and C. Mille	Process of protecting fatty substances from becoming rancid by the addition of carotenoids.
B. 507,471	June 12, 1939	Eastman Kodak	Retarding oxidation of animal and vegetable oils by the addition of a small quantity of a fraction possessing antioxidant properties taken from the first 20% distillate obtained by a high vacuum short path distillation of vegetable oils.

Patent No.	Date	Patentee	Abstract
Derivatives and Utilization (*Continued*)			
G. 658,957	April 20, 1938	I. G.	Preparation of an emulsion of fat-soluble vitamin concentrates from fish livers.
		VITAMIN B COMPLEX	
U. S. 1,058,927 G. 266,211 B. 25,322	April 15, 1913 Feb. 6, 1911 Nov. 12, 1912	J. Tsuzuki, Japan	Isolation of vitamin B concentrates from rice bran by alcohol extraction.
U. S. 1,162,908	Dec. 7, 1915	C. Funk	Extraction of vitamins from yeast, rice-bran, etc., by addition of phosphotungstic acid and extraction with acetone.
U. S. 1,173,317	Feb. 29, 1916	A. Seidell	Vitamins from yeast by autolysis and adsorption on fuller's earth followed by elution with dilute acid and alcohol.
U. S. 1,235,198	July 31, 1917	A. Gams and B. Schreiber	Vitamin concentrate from yeast, rice-bran, beans, etc., by precipitation of inactive materials with lead salts and precipitation of the active material with an alkaloid precipitant such as phosphotungstic acid.
U. S. 1,431,525 B. 186,633	Oct. 10, 1922 Oct. 2, 1922	C. Hoffman, H. D. Grigsby and N. M. Cregor Ass. to Ward Baking Co.	Addition of vitamin B-containing substances, such as defatted rice polishings, to dough.
U. S. 1,474,029	Nov. 13, 1923	I. F. Harris	Alcohol extraction of vitamins from yeast boiled in dilute acetic acid.
U. S. 1,617,702	Feb. 15, 1927	P. Caccia	Extraction of vitamin B with alcohol, precipitation with ethylene dichloride and crystallization in the presence of sulfuric acid.
U. S. 1,737,279	Nov. 26, 1930	L. Wallerstein Ass. to Wallerstein & Co.	Manufacture of extracts rich in vitamin B from malt and wheat germs.
U. S. 1,842,933	Jan. 26, 1932	B. W. Dedrick	Water extraction of the residues obtained by making flour from wheat, followed by filtration and recovery of the water-soluble materials.
U. S. 1,895,977 B. 373,028	Jan. 31, 1933 Sept. 1, 1931	J. W. Dressel	Liver concentrate by extraction with water.
U. S. 2,006,023	June 25, 1935	F. Lange and L. Taube Ass. to Winthrop	Preparations containing vitamin B from yeast extract.
U. S. 2,006,699	July 2, 1935	G. C. Supplee and G. E. Flanigan Ass. to The Borden Co.	Recovery of vitamins B_1 and B_2 from milk.
U. S. 2,095,638	Oct. 12, 1937	G. A. Jeffreys	Liquid binder for food products rich in vitamin B from fermented material.
U. S. 2,184,748 B. 428,044	Dec. 26, 1939 April 30, 1935	R. F. Light and C. N. Frey Ass. to Standard Brands	Preparation of vitamins B_1 and B_2 from yeast by plasmolysis, coagulation of the protein material and concentration of the water solution.

Patent No.	Date	Patentee	Abstract
U. S. 2,193,876	Mar. 19, 1940	B. Maizel Ass. to Vico Products Co.	Isolation of the vitamin B complex from yeast by means of 90% methyl or ethyl alcohol, distilling off part of the solvent to separate gummy constituents or precipitating these by alkali earth materials, and mixing the vitamin-containing material with extracted yeast or with coagulated egg white.
U. S. 2,202,161	May 28, 1940	C. S. Miner Ass. to Commercial Solvents	Recovery of vitamin B components from fermentation of molasses with butyl alcohol-producing bacteria.
U. S. 2,202,307	May 28, 1940	L. E. Booher	Extraction of the vitamin B complex.
B. 103,294 F. 485,649	Jan. 4, 1917 Jan. 24, 1918	Ciba	Extraction of vitamins from rice husks, yeast, etc., by alcohol and precipitation of inactive products by lead-salts.
B. 345,669 G. 566,174	Nov. 22, 1929 Oct. 10, 1929	I. G.	Aqueous solution of yeast rich in vitamin B for beverages.
B. 376,149 Jap. 91,250	Nov. 10, 1931 April 28, 1931	S. Ota and K. Umeda Ass. to K. Beer	Pressed yeast is fermented with cane sugar, etc. The product is extracted with alcohol to yield a nutrient material containing vitamin B.
B. 434,058	Aug. 26, 1935	W. W. Triggs	Concentrate of vitamins B from whey.
B. 477,528	Dec. 30, 1937	Standard Brands	Manufacture of a product containing vitamins of the B-complex by extraction of seeds or grain germs.
B. 485,079 F. 824,837	May 10, 1938 Feb. 17, 1938	I. G.	Plant growth stimulant containing vitamins of the B-complex.
B. 486,054	May 30, 1938	Eli Lilly	Solution of a vitamin of the B-complex in a polyhydric alcohol.
B. 536,510	May 16, 1941	Standard Brands	Yeast is grown on a culture medium containing the vitamins B_1, B_6, inositol, pantothenic acid and "biotic acid" (extracted from sheep liver).
B. 538,191	July 24, 1941	Emulsions Process Corp.	Extraction of the vitamins of the B-complex from yeast after breaking down the yeast cells by application of pressure followed by a sudden release of the pressure.
B. 539,825	Sept. 25, 1941	Standard Brands	Improvement in the propagation of yeast by the addition to the culture medium of vitamins B_6 and B_1 (or a pyrimidine and thiazol compound) and additional growth promoting factors.
G. 295,361	Mar. 25, 1914	Boehringer	Purification of phosphotungstate precipitates of vitamins B by acetone extraction.
G. 607,512 G. 634,909	Dec. 29, 1934 Sept. 10, 1936	P. György, R. Kuhn and T. Wagner-Jauregg Ass. to I. G.	Manufacture of vitamins of the B-complex by adsorption followed by elution with amines or ammonia.

Patent No.	Date	Patentee	Abstract
G. 630,772	June 5, 1936	I. G.	Isolation of vitamin B_4 by adsorption, elution and precipitation methods, making use of the strong bluish fluorescence characteristics of vitamin B_4.
G. 646,548 Continuation of G. 630,772	June 17, 1937	I. G.	Elution of vitamin B_4 by alkali and by amines. Vitamin B_4, according to this patent, has the formula $C_{12}H_{14}N_4OS$ and seems to resemble vitamin B_1 and thiochrome rather than the description of vitamin B_4.
G. 672,078 Continuation of 661,929 and 670,742	Feb. 18, 1939	I. G.	Method of concentrating growth-promoting compounds of the "bios" type.
G. 703,400	Mar. 7, 1941	Ass. to G. Henning	Adenosinphosphoric acid from adenosin and phosphoric acid by yeast.
Jap. 91,202	April 25, 1931	S. Izume, M. Sato, I. Seto Ass. to the South Manchurian Railway Co.	The alcoholic extract from soy bean is freed from impurities and contains large amounts of vitamin B.
Jap. 101,137	May 16, 1933	K. Taguchi Ass. to K. Katakura	Rice, bran or the like is extracted with methanol to yield a substance rich in vitamin B.

VITAMIN B_1—THIAMIN

Isolation			
U. S. 1,869,721 U. S. 1,889 427 B. 354,421 F. 714,416	Aug. 2, 1932 Nov. 29, 1932 Aug. 13, 1931	B. Sure	Purification of crude vitamin B_1 concentrates by dissolving in acetic acid, precipitating impurities with acetone and adsorbing the vitamin on charcoal.
U. S. 1,937,671	Dec. 5, 1933	A. Seidell	Elimination of non-vitaminic substances from vitamin B_1 concentrates by benzoylation.
U. S. 1,990,961	Feb. 12, 1935	E. H. Stuart Ass. to Eli Lilly	Removal of vitamin B_1 from an adsorbate by solutions of mineral acids.
U. S. 2,002,519	May 28, 1935	R. J. Block and G. R. Cowgill Ass. to S. J. Dannenberg	Purification of vitamin B_1 concentrates by oxidation of impurities.
U. S. 2,015,876	Oct. 1, 1935	B. Sure and E. H. Stuart Ass. to B. Sure	Concentration of vitamin B from acid alcoholic extracts from rice brans by adsorption on active carbon.
U. S. 2,049,988	Aug. 4, 1936	R. R. Williams and R. E. Waterman Ass. to Research Corp.	Elution of the fuller's earth adsorbate of vitamin B_1 with an acid polynitrogenous alkaloid salt, *e. g.*, with quinine sulfate solution.
U. S. 2,114,775 B. 497,081 F. 818,702	April 19, 1938 Not accepted Oct. 2, 1937	L. R. Cerecedo	Isolation of vitamin B_1 from natura sources by adsorption on zeolite followed by elution. Further purification by precipitation of the vitamin as silver compound or silicotungstate.
B. 390,378	April 6, 1933	S. J. Dannenberg	Vitamin B_1 concentrate by extracting the vitamin at pH 10–13 followed by an acid extraction.

Patent No.	Date	Patentee	Abstract
Isolation (*Continued*)			
G. 311,074	Jan. 12, 1916	Ciba	Extraction of animal or plant material with dilute alcohol and precipitation of impurities with lead salts.
G. 320,785	April 28, 1920	R. Bosshard and F. Hefti	Vitamin B_1 concentrates from plant material, *e. g.*, rice brans, yeast, etc., by total hydrolysis with dil. mineral acids at 80° C.
G. 359,878 Continuation of G. 311,074	Sept. 28, 1922	Ciba	Hydrolysis of the starting material with enzymes.
Jap. 109.268	Jan. 22, 1935	R. Otake	Isolation of crystallized vitamin B_1 from yeast by adsorption on acid clay, extraction with $Ba(OH)_2$ and precipitation with a silver salt.
Synthesis			
U. S. 2,127,446 B. 500,519 G. 676,980 F. 831,110	Aug. 16, 1938 Feb. 10, 1939 June 16, 1939 Aug. 23, 1938	M. Klingenfuss Ass. to Hoffmann-LaRoche	Synthesis of vitamin B_1 by condensation of 2-methyl-4-amino-5-thioformylamino - methyl - pyrimidine with 2-methyl-2-alkoxy-3-chlorotetra-hydro-furane.
U. S. 2,166,233	July 18, 1939	E. R. Buchman Ass. to Research Corp.	Process of preparing 2-methyl-6-amino-5-pyrimidyl-bromacetic acid by bromination of the corresponding pyrimidine acetic acid and method of synthesizing vitamin B_1 therefrom by condensation with 4-methyl - 5 - beta - hydroxy - ethyl-thiazole.
U S. 2,184,720	Dec. 26, 1939	T. Matukawa and M. Ohta Ass. to Kabusiki-Kaisha Takeda-Chobei Shoten	Synthesis of vitamin B_1 from 2-methyl - 4 - amino - 5 - formamino-methyl-pyrimidine and gamma, gamma-diaceto - gamma - mercapto-propyl alcohol.
U. S. 2,209,244 B. 456,735 B. 471,416 G. 685,032 F. 816,432	July 23, 1940 Nov. 11, 1936 Aug. 30, 1937 Dec. 11, 1939 Aug. 7, 1937	H. Andersag and K. Westphal Ass. to I. G.	(1) Synthesis of vitamin B_1 by condensation of 2-methyl-4-amino-5-thio - formylamidomethyl - pyrimidine with 2-keto-3-halogeno-pentanol-5 or its acetate. (2) Synthesis of vitamin B_1 by condensation of 4-methyl-5-beta-hydroxy-ethyl thiazole with 2-methyl-4 - amino - 5 - halogeno - methyl-pyrimidine. (3) Synthesis of vitamin B_1 by condensation of an ester of gamma-(4-methyl - 5 - acyloxy - ethyl - N-thiazolium - bromide) - alpha - cyano-butyric acid with acetamidine.
U. S. 2,235,862 B. 507,918 G. 669,187 Continuation of G. 681,638	Mar. 25, 1941 June 22, 1939 Dec. 19, 1938	O. Zima Ass. to Merck	Synthesis of vitamin B_1 by condensation of 2-methyl-4-amino-5-oxy-methyl-pyrimidine HCl with 4-methyl-5-beta-hydroxy-ethyl-thiazole-HCl.
U. S. 2,252,921	Aug. 19, 1941	Z. Földi and A. Gerecs	Synthesis of vitamin B_1 from 2-methyl - 4 - amino - 5 - thioform-amidomethyl-pyrimidine and 2-methyl- 2 - hydroxy - 3 - halogeno-tetrahydrofurane in the presence of salts of weak organic bases and strong acids.

Patent No.	Date	Patentee	Abstract
Synthesis (*Continued*)			
B. 496,726 F. 834,682	Dec. 2, 1938 Nov. 29, 1938	Research Corp.	Synthesis of vitamin B_1 by condensation of 2-methyl-4-amino-5-bromo-methyl-pyrimidine HBr with 4-methyl-5-(beta-hydroxyethyl)-thiazole.
B. 532,013	Jan. 15, 1941	Standard Brands and International Yeast	The vitamin B_1 content of yeast is increased by growing the yeast in a wort to which has been added a pyrimidine compound with or without a thiazole compound. 2-Methyl-5-ethoxymethyl-6-amino-pyrimidine and 4-methyl-5-beta-hydroxy-ethyl-thiazole are specifically mentioned as additive agents.
G. 681,638 Continuation of G. 669,187.	Sept. 27, 1939	O. Zima Ass. to Merck	Synthesis of vitamin B_1 by condensation of 2-methyl-4-amino-5-alkoxy-methyl-pyrimidine hydrochloride with the hydrochloride of 4-methyl-5-beta-hydroxy-ethyl-thiazole.
G. 703,775 Addition to G. 685,032	Mar. 15, 1941	H. Andersag and K. Westphal Ass. to I. G.	Synthesis of vitamin B_1 according to G.P. 685,032 but using 5-amino-alkyl-thiazole compounds for the condensation followed by conversion into vitamin B_1.
G. 705,432 Addition to G. 685,032	April 28, 1941	K. Westphal and H. Andersag Ass. to I. G.	Synthesis of vitamin B_1 by condensation of 4-methyl-5-beta-hydroxy-ethyl-thiazole with 2-methyl-4-amino-5-methyl-pyrimidine, the 5-methyl group of which is substituted by a reactive group (Example: benzol sulfonic acid ester of the 5-hydroxy-methyl derivative).
Synthesis of the Thiazole Part			
U. S. 2,123,653 B. 496,801 G. 684,587 F. 831,111	July 12, 1938 Dec. 6, 1938 Dec. 1, 1939 Aug. 23, 1938	Hoffmann-LaRoche	2-Methyl-2-alkoxy-3-chloro-tetra-hydro-furane from alpha-acetyl-alpha-chloro-butyrolactone and an aliphatic primary alcohol.
U. S. 2,133,969 B. 472,459 G. 673,174 G. 675,617 F. 803,495	Oct. 25, 1938 Sept. 17, 1937 Mar. 17, 1939 July 21, 1939 Oct. 1, 1936	E. R. Buchman Ass. to Research Corp.	4-Methyl-5-beta-hydroxy-ethyl-thiazole from gamma-aceto-gamma-halogeno-propanol and thio-formamide.
U. S. 2,134,015	Oct. 25, 1938	R. R. Williams Ass. to Research Corp.	Composition of matter claims for 4-methyl-5-beta-hydroxy-ethyl-thiazole and its salts.
U. S. 2,139,570 B. 456,751 G. 704,236 F. 811,224	Dec. 6, 1938 Nov. 13, 1936 Mar. 26, 1941 April 9, 1937	H. Andersag and K. Westphal Ass. to Winthrop	Condensation of gamma-bromo-gamma-aceto-propanol-esters with a rhodanide followed by acid treatment to form 2-hydroxy-4-methyl-thiazolyl-5-ethanol-esters.
U. S. 2,160,867 G. 670,131	June 6, 1939 Jan. 12, 1939	O. Hromatka Ass. to Merck	4-Methyl-5-beta-hydroxy-ethyl-thiazole from gamma-halogeno gamma-aceto-propyl alcohol, formamide and phosphorus pentasulfide.

Patent No.	Date	Patentee	Abstract
Synthesis of the Thiazole Part (*Continued*)			
U. S. 2,179,984 B. 492,637 G. 678,153	Nov. 14, 1939 Sept. 23, 1938 July 13, 1939	H. Spiegelberg Ass. to Hoffmann-LaRoche	4-Methyl-5-beta-hydroxy-ethyl-thiazole from the corresponding 2-mercapto-derivative by hydrogen peroxide oxidation.
U. S. 2,193,858	Mar. 19, 1940	E. R. Buchman Ass. to Research Corp.	Composition of matter claims for alpha-halogeno-alpha-aceto-γ-butyro-lactone and method of preparation by halogenation of alpha-aceto-γ-butyro-lactone.
U. S. 2,194,179 Continuation of U. S. 2,133,969	Mar. 19, 1940	E. R. Buchman Ass. to Research Corp.	4-Methyl-5-beta-acetoxy-ethyl-thiazole from gamma-halogeno-gamma-aceto-propylacetate and thio-formamide.
U. S. 2,209,092	July 23, 1940	D. Price and F. D. Pickel Ass. to National Oil Products	Preparation of 4-methyl-5-beta-amino-ethyl-thiazole, by brominating levulinic ester, reacting the halogenated compound with thio-formamide, treating the reaction product with ammonia, dehydrating the amide thus obtained to the corresponding cyano-derivative and catalytically hydrogenating the cyano-group to an amino-group.
U. S. 2,216,574 B. 472,396	Oct. 1, 1940 Sept. 17, 1937	T. D. Perrine Ass. to Research Corp.	Halogeno-aceto-propyl alcohol from aceto-propyl alcohol by direct halogenation.
U. S. 2,218,349	Oct. 15, 1940	E. R. Buchman Ass. to Research Corp.	Composition of matter claims for γ-halogeno-γ-aceto-propyl alcohol and methods of preparation by halogenation of γ-aceto-propyl-alcohol.
U. S. 2,218,350	Oct. 15, 1940	E. R. Buchman Ass. to Research Corp.	Method of producing γ-halogeno-γ-aceto-propyl alcohol by simultaneous hydrolysis and decarboxylation of an alpha-halogeno-alpha-aceto-γ-butyro-lactone.
U. S. 2,223,885	Dec. 3, 1940	E. R. Buchman Ass. to Research Corp.	gamma-Halogeno-gamma-aceto-propyl esters and their preparation by halogenation of a gamma-aceto-propyl-ester.
U. S. 2,263,014	Nov. 18, 1941	W. Scott Ass. to Wingfoot Corp.	Preparation of thiazoles from 2-mercapto-thiazoles by pyrolysis.
U. S. 2,267,313	Dec. 23, 1941	J. R. Stevens and G. A. Stein Ass. to Research Corp.	Production of gamma-aceto-propyl-ether.
B. 490,571 F. 826,067	Aug. 17, 1938 Mar. 22, 1938	Research Corp.	Process for the manufacture of γ-halogeno-γ-aceto-propyl-alcohol and of alpha-halogeno-alpha-aceto-γ-butyro-lactone from alpha-aceto-γ-butyro-lactone.
G. 664,789	Sept. 5, 1938	A. Wenz Ass. to Merck	4-Methyl-5-beta-hydroxy-ethyl-thiazole by halogenation of alpha-aceto-butyrolactone followed by condensation with thio-formamide.

Patent No.	Date	Patentee	Abstract
Synthesis of the Thiazole Part (*Continued*)			
G. 702,831	Feb. 17, 1941	H. Andersag and K. Westphal Ass. to I. G.	4 - Methyl - 5 - beta - hydroxy-ethyl-thiazole from gamma-aceto-gamma - halogeno - propyl compounds and thioformamide, followed by saponification.
G. 705,434	April 28, 1941	K. Westphal and H. Andersag Ass. to I. G.	2 - Methyl - 2 - alkoxy - 3 - halogeno-tetra-hydro-furane from 1-aceto - 1 - halogeno - 1 - alkoxy-ethyl-acetone and a mineral acid.
Synthesis of the Pyrimidine Part			
U. S. 2,153,016	April 4, 1939	R. R. Williams Ass. to Research Corp.	2 - Methyl - 5 - amino - methyl - 6-amino-pyrimidine and derivatives.
U. S. 2,184,964	Dec. 26, 1939	G. A. Stein Ass. to Research Corp.	2 - Methyl - 5 - chloromethyl - 6-amino-pyrimidine from the corresponding 5-alkoxy compound.
U. S. 2,194,190	Mar. 19, 1940	R. R. Williams Ass. to Research Corp.	2 - Methyl - 5 - methyl - 4 - halogeno-pyrimidines.
U. S. 2,220,243 B. 478,993 G. 675,881 F. 822,533	Nov. 5, 1940 Jan. 28, 1938 May 20, 1939 Dec. 31, 1937	M. Hoffer Ass. to Hoffmann-LaRoche	2 - Methyl - 4 - amino - 5 - thio-formyl - amino - methyl - pyrimidine and its manufacture from 2-methyl-4 - amino - 5 - amino - methyl-pyrimidine di-hydrochloride and potassium di-thioformate.
U. S. 2,235,638 G. 667,990	Mar. 18, 1941 Nov. 24, 1938	O. Hromatka Ass. to Merck	2 - Methyl - 4 - amino - 5 - cyano-pyrimidine from amino-methylene-malonitrile and acetimino-ethyl ether.
B. 473,193 G. 670,095 F. 819,596	Oct. 4, 1937 Jan. 11, 1939 Oct. 21, 1937	H. Andersag and K. Westphal Ass. to I. G.	2 - Methyl - 4 - amino - 5 - amino-methyl-pyrimidine from acetamidine and formyl-malonic ester.
B. 475,507	Nov. 19, 1937	I. G.	4 - Amino - 5 - hydroxy - alkyl-pyrimidines from the corresponding 5-amino-compounds by nitrous acid.
B. 475,559 G. 671,787	Nov. 19, 1937 Feb. 15, 1939	H. Andersag and K. Westphal Ass. to I. G.	Synthesis of 2-methyl-4-amino-methyl-pyrimidine by condensation of acetamidine with alpha-cyano-succinic ester to the corresponding 6-hydroxy-pyrimidine derivative and elimination of the 6-hydroxyl group *via* the chloride.
B. 486,414	June 2, 1938	Hoffmann-LaRoche	Manufacture of 2-methyl-4-amino-5-cyano-pyrimidine from acetimino-ethyl-ether and amino-methylene-malonitrile.
B. 496,738	Dec. 2, 1938	Research Corp.	2 - Methyl - 4 - amino - 5 - hydroxy-methyl-pyrimidine by condensation of acetamidine with formyl-beta-ethoxy-propionic ester, followed by chlorination and replacement of the chlorine by an amino group.
B. 522,531	June 20, 1940	Merck	2 - Methyl - 5 - chloro - methyl - 4-amino-pyrimidine from the corresponding 5-alkoxy-compound by hydrogen chloride.

Patent No.	Date	Patentee	Abstract
Synthesis of the Pyrimidine Part (*Continued*)			
B. 538,743	Aug. 14, 1941	Chinoin Gyogyszer es Vegyeszeti Termekek	2 - Alkyl - 4 - amino - 5 - carbalkoxy-pyrimidines from alpha-cyano-beta-amidino-acrylic acid esters.
G. 670,635	Jan. 23, 1939	O. Hromatka Ass. to Merck	2 - Methyl - 4 - amino - 5 - cyano-pyrimidine from alkoxy-methylene-malonic acid derivatives, ammonia and acetimido-ethyl ether.
Derivatives and Utilization			
U. S. 2,188,323 G. 704,172	Jan. 30, 1940 Mar. 25, 1941	H. Tauber Ass. to Merck	Synthesis of cocarboxylase from vitamin B_1, pyrophosphate and phosphoric acid.
U. S. 2,205,807	June 25, 1940	J. Bjorksten	Composition of matter claims for vitamin B_1 and biotin in a solution of a pH below 4.5, for the thiazole and pyrimidine parts of the vitamin B_1 molecule either separately or together in the presence of biotin at a pH between 2 and 4.5 as plant growth stimulants.
U. S. 2,224,174	Dec. 10, 1940	J. Weijlard Ass. to Merck	Purification of synthetic cocarboxylase from vitamin B_1 by fractional precipitation of the silver salts at various pH.
G. 663,588	Aug. 9, 1938	K. Lohmann and P. Schuster Ass. to I. G.	Isolation of cocarboxylase.
Swed. 94,746	Feb. 22, 1939	H. v. Euler and R. Vestin	Vitamin B_1-phosphate by enzymatic phosphorylation of the vitamin.
Swiss 197,717	Aug. 1, 1938	Hoffmann-LaRoche	HCl salts of vitamin B_1 by precipitating a picrate and converting the latter to the HCl salt.
		VITAMIN B_2—RIBOFLAVIN	
Isolation			
U. S. 2,139,857	Dec. 13, 1938	H. F. Seibert Ass. to S.M.A. Corp.	Precipitation of riboflavin in mixture with a precipitation of lead sulfide, followed by extraction of the filtered sulfide.
U. S. 2,175,014	Oct. 3, 1939	L. E. Booher and L. T. Work	Isolation of riboflavin from adsorbates by elution with water, alcohol or acetic acid, preferably at an elevated temperature.
U. S. 2,186,314	Jan. 9, 1940	S. Ansbacher, G. E. Flanigan and G. C. Supplee Ass. to The Borden Co.	Extraction of riboflavin adsorbates with aqueous acetone.
U. S. 2,188,008	Jan. 23, 1940	S. H. Lassen Ass. to P. R. Park, Inc.	Isolation of the vitamins of the B-complex and especially of vitamin B_2 from fish press water.
U. S. 2,222,306	Nov. 19, 1940	A. G. Atwood	Simultaneous production of alcohol for bourbon and rye whiskies and a riboflavin-containing material from grains.

Patent No.	Date	Patentee	Abstract
Isolation (*Continued*)			
U. S. 2,239,285	April 28, 1941	G. E. Flanigan and G. C. Supplee	Isolation of vitamin B_2 (riboflavin) from concentrates by precipitation of impurities in an acetone-water solution with ethyl alcohol, followed by ether extraction, precipitation of foreign matter with acetone and final crystallization from the concentrated acetone solution.
B. 524,445	Aug. 7, 1940	N. V. Organon	Separation of riboflavin from its phosphoric acid ester by elution of an adsorbate of these substances with a dilute aqueous solution of an amine or acid amide, or by selective adsorption of riboflavin from a dilute solution of an amine or acid amide.
B. 524,515	Aug. 8, 1940	N. V. Organon	Process for the separation of riboflavin from its phosphoric acid ester by distribution between water, a water-immiscible solvent, such as a phenyl substituted aliphatic alcohol or a mixture of a phenol with an aliphatic alcohol or a mixture of a phenol with a hydrocarbon.
G. 667,806	Nov. 21, 1938	K. Feudenberg Ass. to I. G.	Elution of physiologically active adsorbates from metal sulfides by oxidation of the sulfides, *e. g.*, with H_2O_2.
Jap. 110,826	May 17, 1935	W. Nakahara and B. Inukai Ass. to R. Kenkyujo Co.	Concentrate of vitamin B_2 by adsorption followed by enzymatic digestion of the adsorbate.
Synthesis			
U. S. 2,155,555	April 25, 1939	P. Karrer Ass. to Hoffmann-LaRoche	Product claims for 7-methyl- (or 7-alkyl-) 9-(*d*,l'-ribityl)-iso-alloxazine.
U. S. 2,238,874 B. 441,692 F. 792,070	April 15, 1941 Jan. 20, 1936 Dec. 21, 1935	R. Kuhn Ass. to I. G.	Synthesis of vitamin B_2 and of other iso-alloxazine derivatives by condensation of alloxan with N-monosubstituted aromatic o-diamines, especially in acid solution and in the presence of boric acid.
U. S. 2,261,608	Nov. 4, 1941	M. Tishler and J. W. Wellman Ass. to Merck	Synthesis of riboflavin by reductive condensation of tetra-acetyl-ribononitrile with 4,5-dimethyl-aniline, coupling with para-nitro-phenyl-diazonium chloride and reduction to N - tetra - acetyl - ribityl - amino-2-amino-4,5-dimethylbenzene followed by condensation with 5,5-dichlorobarbituric acid and final hydrolysis.
B. 457,984	Dec. 10, 1936	Hoffmann-LaRoche	Process for the manufacture of iso-alloxazine derivatives which consists in condensing derivatives of o-phenylenediamine, which possess a hydroxylated aliphatic side chain attached to an amino group, with alloxan.

Patent No.	Date	Patentee	Abstract
Synthesis (*Continued*)			
Swiss 187,938	Mar. 1, 1937	Hoffmann-LaRoche	6 - Methyl - 9 - (d - ribityl) - isoalloxazine by condensation of 4-methyl - 2 - amino - phenyl - ribamine with alloxan.
Intermediates for the Synthesis			
U. S. 2,152,662	April 4, 1939	F. P. Phelps Ass. to the Government of the United States	Manufacture of ribose by hydrolysis of nucleic acids.
U. S. 2,159,804	May 23, 1939	W. E. Lawson and C. P. Spaeth Ass. to DuPont	N - Ribityl - 6 - nitro - 3,4 - xylidine from ribitylamine and 6-nitro-3,4-dimethyl-chlorobenzene.
U. S. 2,193,433	Mar. 12, 1940	P. L. Salzberg Ass. to DuPont	Composition of matter claims for N-ribityl-3,4-xylidine and process of preparation from 3,4-xylidine and ribose followed by hydrogenation.
U. S. 2,237,074 B. 457,178 G. 677,515	April 1, 1941 Nov. 23, 1936 June 27, 1939	P. Karrer Ass. to Hoffmann-LaRoche	1 - Ribitylamino - 2 - amino - 4,5-dimethyl-benzene from N-ribityl-3,4-xylidine by coupling with a diazonium salt followed by reduction of the azo dye.
U. S. 2,250,999	July 29, 1941	R. Pasternack and E. V. Brown Ass. to Pfizer	Composition of matter claims for tetra - acetyl - (3,4 - dimethyl-phenyl)-ribityl-amine and tetra-acetyl - (3,4 - dimethyl - 6 - carbethoxy - amino - phenyl) - d-ribityl-amine and process of making these compounds by condensation of tetra-acetyl-d-ribose with an aromatic amine followed by hydrogenation.
B. 461,245 G. 642,148	Feb. 8, 1937 March 4, 1937	R. Kuhn and R. Ströbele Ass. to I. G.	Condensation of sugars with ortho-nitro-anilines, followed by reduction.
G. 664,048 Continuation of 642,148	Aug. 19, 1938	R. Kuhn and R. Ströbele Ass. to I. G.	Reduction of acyl-derivatives of condensation products of ortho-nitro-anilines and sugars followed by saponification.
G. 664,439 Continuation of 642,148	Sept. 1, 1938	R. Kuhn and R. Ströbele Ass. to I. G.	Condensation of ortho-nitro-anilines with sugars in the presence of ammonium halides or amine-hydrogen halides.
G. 679,001 Continuation of 642,148	July 27, 1939	R. Kuhn and R. Ströbele Ass. to I. G.	Reduction of the condensation products of ortho-nitro-anilines and sugars in the presence of borates in slightly alkaline solution to the corresponding phenylene diamines.
Derivatives and Utilization			
U. S. 2,068,623	Jan. 19, 1937	O. Warburg Ass. to Schering	Isolation of the yellow oxidation enzyme from yeast.
U. S. 2,111,491 B. 451,938 G. 666,791 G. 647,721 F. 809,884	Mar. 15, 1938 Aug. 10, 1936 Oct. 28, 1938 Dec. 7, 1937 Mar. 11, 1937	R. Kuhn, F. Weygand and H. Rudy Ass. to Winthrop	Preparation of 5'-phosphoric acid ester of vitamin B_2.

Patent No.	Date	Patentee	Abstract
Derivatives and Utilization (*Continued*)			
U. S. 2,256,604 G. 698,815 Continuation of G. 686,793	Sept. 23, 1941 Nov. 18, 1940	E. Auhagen Ass. to Winthrop	Water solutions of vitamin B_2 using nicotinic acid in the form of its salts or *N*-non-substituted amide to increase the solubility.
B. 430,571 G. 638,822 Continuation of G. 638,138	June 17, 1935 Nov. 23, 1936	O. Warburg Ass. to Schering	Cleavage of the yellow oxidation ferment with sodium hydroxide at 50–60° C. to produce lumi-lactoflavin.
B. 438,126 Continuation of 411,179	Nov. 7, 1935 June 7, 1934	G. B. Walden Ass. to Eli Lilly	Vitamin B_2 is added to preparations for the treatment of pernicious anemia.
B. 451,779 G. 637,503 F. 785,490	Aug. 11, 1936 Oct. 29, 1936 Aug. 10, 1935	Schering	Preparation of the yellow oxidation ferment by precipitation in aqueous solution at pH 4 in the presence of an acetate buffer.
B. 495,718	Nov. 18, 1938	Ass. to I. G.	Purification of the yellow oxidation ferment by adsorption, followed by elutriation with solutions of weakly alkaline reacting phosphates.
B. 504,721 G. 672,018	April 26, 1939 June 22, 1939	J. Eisenbrand and H. Picher Ass. to I. G.	Decomposition of vitamin B_2 during sterilization is restrained by adding urea and an acid, *e. g.*, HCl, to obtain a pH below 5, which pH after sterilization is between 5 and 7.
G. 632,131	July 3, 1936	R. Kuhn and H. Rudy Ass. to I. G.	Manufacture of a diacetone-vitamin B_2.
G. 632,366	July 7, 1936	Schering	Riboflavin phosphoric acid ester from the yellow oxidation ferment from aqueous yeast extract.
G. 633,392	July 25, 1936	H. T. Theorell Ass. to Schering	Riboflavin phosphoric acid ester from the yellow oxidation ferment by treatment with methanol.
G. 637,386 Continuation of 633,392	Oct. 27, 1936	Schering	Purification of the riboflavin-phosphoric acid ester by conversion into an alkali-earth salt.
G. 638,138 Continuation of G. 638,822	Nov. 10, 1936	Schering	Cleavage of the yellow oxidation ferment with sodium hydroxide.
G. 686,793	Jan. 16, 1940	E. Auhagen Ass. to I. G.	Solution of vitamin B_2 in mono-*N*-alkyl derivatives of amides of lower fatty acids.
G. 687,197	Jan. 24, 1940	Ciba	Preparation of vitamin B_2—phosphoric acid ester by phosphorylation of the vitamin by means of phosphatases from the mucous membranes of the intestines (in the presence of phosphates) and in the presence or absence of compounds of the adrenal cortex.
G. 688,047 Continuation of G. 686,793	Feb. 10, 1940	E. Auhagen Ass. to I. G	Solutions of vitamin B_2 in water using phenol- or polyphenol-sulfonic acid salts to increase the solubility.

VITAMIN B_6—PYRIDOXIN

PATENT NO.	DATE	PATENTEE	ABSTRACT
U. S. 2,248,078 B. 534,916	July 8, 1941 Mar. 21, 1941	S. A. Harris Ass. to Merck	Synthesis of vitamin B_6 by reacting ethoxyacetyl-acetone and cyano-acetamide to form 3-cyano-4-ethoxy-methyl-6-methyl-pyridone-2, hydrolyzing to form the lactone of 3-carboxy-4-hydroxymethyl-6-methyl-pyridone-2, treating with nitric acid, chlorinating and reducing to form the lactone of 3-carboxy-4-hydroxy-methyl-5-amino-6-methyl-pyridine, diazotizing to form the lactone of 3-carboxy-4-hydroxymethyl-5-hydroxy-6-methylpyridine and reducing the latter compound to form vitamin B_6.
U. S. 2,250,396 G. 699,555	July 22, 1941 Dec. 2, 1940	W. Salzer Ass. to Winthrop	Synthesis of vitamin B_6 by degradation of 3-alkoxy-quinaldine-4-carboxylic acid.
U. S. 2,261,188	Nov. 4, 1941	J. V. Scudi Ass. to Merck	Boric acid salts of vitamin B_6.
U. S. 2,266,754	Dec. 23, 1941	S. A. Harris Ass. to Merck	Catalytic reduction of 3-cyano-4-ethoxy-methyl-5-nitro-6-methyl-pyridone-2 and acetylation of 3-cyano-4-ethoxy-methyl-5-amino-6-methyl-pyridone.
U. S. 2,296,167	Sept. 15, 1942	Ass. to I. G.	Fat soluble derivatives of vitamin B_6 by acylation.
B. 534,917	Mar. 21, 1941	Ass. to Merck	The vitamin B_6 base from its hydrochloride.
B. 536,249	May 8, 1941	Merck	2-Methyl-3-methoxy-pyridine-4,5-dicarboxylic acid ester from the corresponding acid by esterification.
B. 538,000	July 16, 1941	Merck	Lactones of 2-methyl-3-alkoxy-4-hydroxymethyl-5-carboxy-pyridine by alkylating the lactone of 2-methyl-3-hydroxy-4-hydroxy-methyl-5-carboxy pyridine.
G. 684,975	Dec. 8, 1939	R. Kuhn and G. Wendt Ass. to I. G.	Isolation of a vitamin B_6-protein symplex from animal or plant material.
G. 702,829	Feb. 17, 1941	K. Westphal and H. Andersag Ass. to I. G.	Vitamin B_6 by degradation of 3-methyl-4-alkoxy-isoquinoline.
G. 702,830	Feb. 17, 1941	K. Westphal Ass. to I. G.	2-Methyl-3-methoxy-4,5-bis-amino-methyl-pyrimidine from the corresponding 4,5-dinitrile by catalytic reduction.
G. 704,761	April 7, 1941	K. Westphal Ass. to I. G.	Synthesis of vitamin B_6 by the action of nitrous acid on 4,5-bis-amino-methyl-methoxy-pyridine.

VITAMIN B_5

U. S. 1,976,175 B. 396,135	Oct. 9, 1934 Aug. 3, 1933	C. L. Lautenschläger and F. Lindner Ass. to Winthrop	Production of muscle adenosine phosphoric acid from yeast adenosine triphosphoric acid by hydrolysis.

Patent No.	Date	Patentee	Abstract
U. S. 2,098,976	Nov. 16, 1937	S. L. Ruskin Ass. to F. R. Ruskin	Solutions or tablets for pharmaceutical uses comprising isotonic solutions of compounds of an isolated nucleotide such as adenylic acid and Hg, Ca, Fe, Au, Ag, or Al.
B. 396,647	Aug. 10, 1933	I. G.	Alkali metal salt of adenyl-pyrophosphoric acid.
B. 413,430	July 19, 1934	I. G.	Muscle adenylic acid from muscle adenyl-pyrophosphoric acid by hydrolysis with an alkaline earth metal hydroxide.
B. 426,856 G. 638,418	April 10, 1935 Nov. 14, 1936	Ciba	Nucleotides, *e. g.*, adenylic acid, are acylated.
G. 591,926	Jan. 29, 1934	C. L. Lautenschläger and F. Lindner Ass. to I. G.	Isolation of adenosine-phosphoric acid by hydrolysis of yeast extracts containing adenosine-triphosphoric acid.

NICOTINIC ACID

U. S. 1,403,117 B. 184,625 G. 351,085	Jan. 10, 1922 Sept. 14, 1922 April 3, 1922	M. Hartmann and M. Seiberth Ass. to Ciba	Product claims for dialkyl amides as medicinals. Process claims reaction of nicotinic acid halide and a salt of a dialkylamine.
U. S. 1,611,978	Dec. 28, 1926	R. Wolffenstein	Terpene alcohol esters of nicotinic acid.
U. S. 1,617,332 G. 441,707	Feb. 15, 1927 Mar. 10, 1927	M. Hartmann and M. Seiberth Ass to Ciba	Manufacture of pyridine-3-carboxylic acid amides, substituted in the amide group, by reaction of a quinolinic acid anhydride with a secondary amine followed by decarboxylation.
U. S. 1,938,253	Dec. 5, 1933	M. Hartmann and M. Seiberth Ass. to Ciba	Addition product of nicotinic acid and its derivatives with water-soluble salts of alkaline earth metals.
U. S. 2,136,501	Nov. 15, 1938	M. Hartmann and L. Panizzon Ass. to Ciba	Mercuric - N - propyl - nicotinamides.
U. S. 2,136,503 Division of U. S. 2,136,501	Nov. 15, 1938	M. Hartmann and L. Panizzon Ass. to Ciba	N-substituted nicotinamide mercuric compounds.
U. S. 2,141,128	Dec. 20, 1938	H. v. Euler, H. Albers and F. Schlenk Ass. to E. Bilhuber	Purification of codehydrogenase I by precipitation with barium hydroxide and fractional adsorption on alumina. Also by precipitation with a monovalent copper halide followed by precipitation with barium hydroxide.
U. S. 2,146,899	Feb. 14, 1939	P. Karrer Ass. to Hoffmann-LaRoche	Manufacture of 3-carboxylic acid amides of acetylated N-glycosido pyridinium-bromide.

Patent No.	Date	Patentee	Abstract
U. S. 2,188,244 B. 503,780 F. 824,042 F. 824,042/40,165 (1st add.) F. 824,042/49,840 (2nd add.)	Jan. 23, 1940 April 12, 1939 Jan. 31, 1938 Nov. 28, 1938 Aug. 8, 1939	G. A. Langlois and A. M. Deloison Ass. to Fabriques de Produits de Chimie Organique de Loire	Morpholine amide of nicotinic acid.
U. S. 2,233,419	Mar. 4, 1941	E. E. Moore Ass. to Abbott	Composition of matter claims for straight chain aliphatic amine salt of nicotinic acid for parenteral injection.
B. 254,747	Dec. 20, 1914	Ciba	Dialkylamides of nicotinic acid.
B. 450,051 G. 653,873 F. 791,783	July 9, 1936 Nov. 18, 1937 Dec. 17, 1935	K. Fricker	Synthesis of N-substituted amides of pyridine-carboxylic acids by condensation of the acid with secondary aliphatic or cyclic amines in the presence of phosphorous pentoxide.
G. 529,319	July 11, 1931	Knoll, A. G.	Purification of cozymase by precipitation with picric acid followed by decomposition with silicotungstic acid.
G. 544,389	Mar. 12, 1931	Ciba	Double compound of nicotinic acid with salts such as $CaCl_2$, $MgCl_2$, etc.
G. 554,008 Continuation of G. 529,319	July 4, 1932	Knoll, A. G.	Purification of cozymase by precipitation with aluminum sulfate and decomposition by means of phosphoric acid. The cozymase obtained is further purified according to G. 529,319.
G. 603,733	Oct. 6, 1934	Ciba	Urethanes of nicotinic acid.
G. 654,960	Jan. 8, 1938	O. Warburg and W. Christian Ass. to Schering	Isolation of codehydrogenase II.
G. 667,696 G. 668,388	Mar. 14, 1939 Mar. 22, 1939	H. Albert and F. Schlenk Ass. to I. G.	Cozymase.
F. 849,519	Nov. 25, 1939	Ass. to G. Henning	Enzymatic synthesis of muscle adenylic acid and of cozymase from adenosine.
Hung. 110,083	June 1, 1934	Chinoin Gyógyszer es Vegyeszeti	Free nicotinic acid is heated with substituted carbamyl chlorides.
Russ. 35,836	April 30, 1934	I. L. Knunyantz and M. M. Katznel'son	Dialkyl amino ethyl esters of nicotinic acid.
Russ. 44,554	Oct. 31, 1935	Y. L. Goédfarb.	Preparation of 2-naphthyl-nicotinate.

PANTOTHENIC ACID

U. S. 2,234,680	Mar. 11, 1941	M. B. Moore Ass. to Abbott	Synthesis of pantothenic acid from beta-alanine and alpha, gamma-dihydroxy-beta, beta-dimethyl-n-butyric acid or alpha-hydroxy-beta, beta - dimethyl - gamma - butyrolactone.
U. S. 2,271,872	Feb. 3, 1942	H. K. Mitchell Ass. to Research Corp.	Production of pantothenic acid analogues having in the beta position of the butyric acid part one or two methylol groups.

Patent No.	Date	Patentee	Abstract
B. 535,988	April 29, 1941	Merck	Synthesis of pantothenic acid by condensation of beta-alanine or a salt or ester thereof with alpha-hydroxy - beta, beta - dimethyl-gamma-butyrolactone or the corresponding hydroxy- or keto-acid.
		INOSITOL	
U. S. 1,072,989 (Also Swiss 62,459	Sept. 9, 1913 June 5, 1912	E. Preiswerk Ass. to Ciba)	Iron salts of inositol-phosphoric acid absorbed on albumin.
U. S. 1,721,214 B. 308,403	July 16, 1929 Jan. 24, 1928	F. Goedecke	Dietary composition containing vitamins and inositol hexaphosphate and its Ca-salts from the same plant source.
U. S. 2,112,553	Mar. 29, 1938	E. Bartow and W. W. Walker	Inositol from phytin by decomposition with $Ca(OH)_2$.
B. 216,982	Mar. 28, 1923	G. Bruni	Methoxyinositol pentaphosphate.
G. 684,946	Dec. 8, 1939	F. Fischler	Isolation of alkaline earth salts of inositol tetraphosphate.
Swiss 91,727	Nov. 16, 1921	S. Pasternak	Inositolpolyphosphate by phosphorylation of inositol.
Swiss 141,522	Dec. 8, 1928	Cristallo, A. G.	Extraction of Ca-inositol-hexaphosphate and vitamins from vegetable matter.
		VITAMIN C	
Isolation			
U. S. 1,468,251 B. 193,831 G. 417,020 F. Add. 26,034 and 27,271 to F. 532,398	Sept. 18, 1923 May 6, 1924 Aug. 8, 1925	L. A. Agopian	Extraction of vitamins from fresh plant juices to which organic acids have been added.
U. S. 1,745,788	Feb. 4, 1930	C. Funk	Antiscorbutic vitamin from lemon juice by precipitation as lead salt.
U. S. 2,078,237	April 27, 1937	O. Dalmer and H. Wieters Ass. to Merck	Isolation of ascorbic acid from gladiola leaves.
U. S. 2,233,417	Mar. 4. 1941	C. G. King and W. A. Waugh	Isolation of vitamin C from lemon juice.
B. 133,183	Oct. 9, 1919	Ciba	Vitamin C preparations from vegetable materials by adding acids as stabilizers and concentrating the mixture.
B. 168,903 B. 268,655 G. 415,313 F. 532,398	Mar. 6, 1923 April 7, 1927 June 19, 1935 Feb. 2, 1922	L. A. Agopian	Preparation of vitamins from fresh plant juices by precipitation with salts of heavy metals and by evaporation in the absence of air. Extraction of the vitamins with suitable solvents.
B. 272,376 F. 595,537	June 16, 1927 Oct. 5, 1925	L. A. Agopian	Pure vitamin C from plant juices by precipitation with lead salts and recrystallization from alcohol and acetone.

Patent No.	Date	Patentee	Abstract
Isolation (*Continued*)			
G. 397,886	June 27, 1924	G. Eichelbaum	Antiscorbutic calcium preparation from fruit or plant juices by the addition of calcium hydroxide.
G. 637,258	Oct. 27, 1936	K. L. Lautenschläger and F. Lindner Ass. to I. G.	Isolation of ascorbic acid from leaves of plants of the Polygonaceae family.
G. 661,176	June 14, 1938	K. L. Lautenschläger and F. Lindner Ass. to I. G.	Improved method of isolating ascorbic acid by soaking plant material in solutions of strong acids and further processing according to known methods.
F. 850,539	Dec. 19, 1939	Établissements Byla	Vitamin C concentrates are purified by dialysis in acid solution.
F. 852,981	Mar. 7, 1940	Établissements Byla	Vitamin C extraction from agaves
Hung. 108,184	Feb. 1, 1934	Ass. to Chinoin Gyógyszer	Purification of vitamin C-containing aqueous plant extracts by precipitation with lead and barium salts.
Russ. 48,318	Aug. 31, 1936	A. A. Schmidt and K. S. Tutschinskaja	Concentrate of ascorbic acid from hips.
Synthesis			
U. S. 2,056,126 B. 425,198 G. 624,509 F. 770,816	Sept. 29, 1936 Mar. 8, 1935 Feb 11, 1936 Sept. 21, 1934	T Reichstein Ass. to Hoffmann-LaRoche	*l*-Ascorbic acid from *l*-xylosone by condensation with hydrocyanic acid followed by hydrolysis.
U. S. 2,068,453 B. 460,586 G. 637,448	Jan. 19, 1937 Feb. 1, 1937 Oct. 29, 1936	B. Helferich and O. Peters	Vitamin C by condensation of glyoxylic acid ester with *l*-threose.
U. S. 2,073,207 B. 443,901 F. 794,221	Mar 9, 1937 Mar. 2, 1936 Feb. 11, 1936	W N. Haworth, E. L. Hirst, J. K. N. Jones and F. Smith Ass. to British Drug Houses	Ascorbic acid is prepared by oxidizing *l*-sorbose with nitric acid to 2-keto-*l*-gulonic acid and enolizing.
U. S. 2,129,317	Sept. 6, 1938	F. Elger Ass. to Hoffman-LaRoche	Ascorbic acid by acid treatment of 2-keto-*l*-gulonic acid, its ester or diacetone-compound.
U. S. 2,159,191 B. 461,790 G. 641,639 F. 806,926	May 23, 1939 Feb. 24, 1937 Feb. 13, 1937 Dec. 29, 1936	W. Wenner Ass. to Hoffmann-LaRoche	Ascorbic acid from bis-methylene ethers of 2-keto-*l*-gulonic acid or diacetone-2-keto-*l*-gulonic acid by means of acids at elevated temperatures.
U. S. 2,160,621 B. 430,264 G. 648,311	May 30, 1939 June 17, 1935 Aug. 20, 1937	H. Ohle	Ascorbic acid from 2-keto-gulonic acid by basic agents.
U. S. 2,165,151 B 521,831	July 4, 1939 May 31, 1940	R. Pasternack and P. P. Regna Ass. to Pfizer	*l*-Ascorbic acid from esters of 2-keto-*l*-gulonic acid by treatment with a metal, *e. g.*, iron, nickel, cobalt, manganese, cadmium and zinc, in aqueous solution.
U. S. 2,165,184	July 4, 1939	R. Pasternack and P. P. Regna Ass. to Pfizer	*l*-Ascorbic acid from esters of 2-keto-*l*-gulonic acid by treatment with magnesium in water and alcohol or dioxane solution.
U. S. 2,179,977	Nov. 14, 1939	F. Elger Ass. to Hoffmann-LaRoche	*l*-Ascorbic acid from 2-keto-*l*-gulonic acid or derivatives thereof by acid treatment in a mixture of alcohol and chloroform.

Patent No.	Date	Patentee	Abstract
Synthesis (*Continued*)			
U. S. 2,179,978 G. 704,760 G. 673,485	Nov. 14, 1939 April 7, 1941 Mar. 2, 1939	F. Elger Ass. to Hoffmann-LaRoche	*l*-Ascorbic acid from 2-keto-*l*-gulonic acid or its alkyl esters by heating in the presence of an alkali salt of a weak acid in anhydrous alcohol.
U. S. 2,185,383 B. 516,115	Jan. 2, 1940 Dec. 22, 1939	R. Pasternack and G. O. Cragwall Ass. to Pfizer	*l*-Ascorbic acid from 2-keto-*l*-gulonic acid by the action of glacial acetic acid and a mineral acid.
U. S. 2,189,830 G. 676,011	Feb. 13, 1940 May 24, 1939	O. Zima Ass. to Merck	*l*-Ascorbic acid from diacetone-2-keto-gulonic acid by acid treatment
U. S. 2,189,778	Feb. 13, 1940	O. Dalmer and K. Heyns Ass. to Merck	*l*-Ascorbic acid from sorbose by catalytic oxidation followed by acid treatment.
U. S. 2,190,167 G. 684,725	Feb. 13, 1940 Dec. 4, 1939	O. Zima Ass. to Merck	*l*-Ascorbic acid from methylene-ether derivatives or dibenzal derivatives of 2-keto-*l*-gulonic acid by treatment with concentrated HCl.
U. S. 2,206,374	July 2, 1940	I. Stone Ass. to Wallerstein Co.	Ascorbic acid from osones plus hydrocyanic acid, the osone being prepared by oxidation of sugars with cupric ion in aqueous organic acid solution.
U. S. 2,207,680 G. 683,954	July 9, 1940 Nov. 18, 1939	B. Helferich Ass. to Winthrop	Ascorbic acid by condensation of mesoxalic acid ester with *l*-threose.
U. S. 2,265,121	Dec. 2, 1941	T. Reichstein Ass. to Hoffmann-LaRoche	Ascorbic acid from *l*-sorbose *via* 2-keto-*l*-gulonic acid or methylene ether derivative thereof.
B. 428,814 G. 673,485 F. 779,883	May 20, 1935 Mar. 28, 1939 April 13, 1935	T. Reichstein Ass. to Hoffmann-LaRoche	Ascorbic acid from 2-keto-*l*-gulonic acid esters by alkali saponification followed by lactonization in acid solution.
B. 428,815	May 20, 1935	T. Reichstein	Ascorbic acid from 2-keto-*l*-gulonic acid by acid treatment.
B. 459,207	Jan. 4, 1937	T. Reichstein	Ascorbic acid from 2-keto-*l*-gulonic acid or its derivatives which are easily split with acids, by acids in alcoholic solution.
B. 466,548	May 31, 1937	T. Reichstein	Ascorbic acid by heating 2-keto-gulonic acid or its derivatives which are easily split with acids.
B. 469,157	July 20, 1937	T. Reichstein	Ascorbic acid from 2-keto-gulonic acid by treating with alkali salts of weak acids and converting the so-formed alkali salts of ascorbic acid by means of strong acids into ascorbic acid.
G. 707,024 Continuation of G. 673,485	June 12, 1941	Hoffmann-LaRoche	Ascorbic acid from 2-keto-*l*-gulonic acid or derivatives which are hydrolyzed easily by acids, by treatment with acids in the presence of a solvent in which the formed ascorbic acid is insoluble.

Patent No.	Date	Patentee	Abstract
Intermediates for the Synthesis			
U. S. 2,140,480 U. S. 2,039,929 B. 428,815 B. 427,286 B. 435,971 G. 699,877 G. 703,227 F. 780,055 F. 45,774	Dec. 13, 1938 May 6, 1936 May 20, 1935 April 18, 1935 Oct. 2, 1935 Nov. 7, 1940 Mar. 4, 1941 April 18, 1935 Dec. 3, 1935	T. Reichstein Ass. to Hoffmann-LaRoche	2-Keto-*l*-gulonic acid from sorbose *via* a bis-methylene-ether derivative or acetals of cyclic ketones, by oxidation with permanganate.
U. S. 2,153,311	April 4, 1939	R. Pasternack and P. P. Regna Ass. to Pfizer	Oxidation of *l*-gulonic acid to 2-keto-*l*-gulonic acid by chromic acid.
U. S. 2,157,137	April 9, 1939	R. T. Major and E. W. Cook Ass. to Merck	Xyloseen-tribenzoates and process for producing the same.
U. S. 2,188,777 B. 534,746	Jan. 30, 1940 Mar. 17, 1941	R. Pasternack and P. P. Regna Ass. to Pfizer	Oxidation of soluble aldonates to 2-keto-aldonates by means of chlorates in the presence of a vanadium catalyst.
U. S. 2,190,377 B. 495,050 F. 829,236	Feb. 13, 1940 Nov. 7, 1938 June 16, 1938	O. Dalmer and K. Heyns Ass. to Merck	2-Keto-*l*-gulonic acid from sorbose by catalytic oxidation at pH of about 6 to 11.
U. S. 2,194,476 See U. S. 2,198,628	Mar. 26, 1940 April 30, 1940	R. T. Major and E. W. Cook Ass. to Merck	Nitriles of fully acetylated 2-keto-sugar acids and process for their production.
U. S. 2,198,628	April 30, 1940	R. T. Major and E. W. Cook Ass. to Merck	Fully acetylated sugar acid chlorides. To be used in U. S. 2,194,476
U. S. 2,207,991	July 16, 1940	R. Pasternack and P. P. Regna Ass. to Pfizer	2-Keto-aldonic acid esters from aldonic acids by chlorates in the presence of a vanadium catalyst and a mineral acid, using non-aqueous lower alcohols as solvents.
U. S. 2,222,155	Nov. 11, 1940	R. Pasternack and P. P. Regna Ass. to Pfizer	2-Keto-aldonic acids by subjecting an aldonate to anodic oxidation in a weakly acid aqueous solution containing a soluble chromium compound and a member selected from the group consisting of alkali metal and alkaline earth metal chlorides and bromides.
G. 544,666	Feb. 20, 1932	J. Muller and U. Hoffman Ass. to I. G.	Manufacture of polyvalent alcohols by catalytic reduction of sugars with hydrogen.
G. 627,249	Mar. 23, 1936	Hoffmann-LaRoche	*l*-Xylose from *d*-sorbitol acetate by oxidation.
G. 644,962	May 19, 1937	Hoffmann-LaRoche	2-Keto-*l*-gulonic acid from *l*-sorbose by direct oxidation with H_2O_2.
F. 851,347	Jan. 6, 1940	A. Corbellini	The acetone condensation product of a 2-keto-laevo-gulonic acid is heated with methanol and sulfuric acid to form methyl ethers with the hydroxyl groups of the acid. These methyl ethers are of value in the synthesis of laevo-ascorbic acid (vitamin C).

Patent No.	Date	Patentee	Abstract
Intermediates for the Synthesis (*Continued*)			
Swiss 175,347	May 1, 1935	T. Reichstein	Preparation of the methyl ester of keto-hexonic acid from the free acid with HCl or H_2SO_4 in methanol.
Derivatives and Utilization			
U. S. 1,886,931	Nov. 2, 1932	E. R. Alexander Ass. to the Vitamin Corp.	Process of preserving a concentrated vitamin extract by incorporation into a vitamin-preserving acidic fruit mass.
U. S. 2,035,153 B. 439,935 G. 660,703	Mar. 24, 1936 Dec. 17, 1935 June 1, 1938	F. Elger Ass. to Hoffmann-LaRoche	Ascorbic acid as stabilizer for neo-salvarsan.
U. S. 2,058,220 G. 629,723 F. 788,014	Oct. 20, 1936 May 9, 1936 Oct. 10, 1935	F. Elger Ass. to Hoffmann-LaRoche	Addition of 2-keto-*l*-gulonic acid esters to foods.
U. S. 2,117,777 G. 702,185	May 17, 1938 Feb. 4, 1941	K. Warnat Ass. to Hoffmann-LaRoche	Double salts of calcium-ascorbate and of calcium salts of poly-hydroxy-mono-carboxylic acids.
U. S. 2,132,662	Oct. 11, 1938	E. H. Volwiler and M. B. Moore Ass. to Abbott	Aliphatic amine salts of ascorbic acid and process for producing them.
U. S. 2,134,246 B. 480,503	Oct. 25, 1938 Feb. 23, 1938	F. Elger Ass. to Hoffmann-LaRoche	Ascorbic acid salts of histidine.
U. S. 2,140,989 B. 499,798 B. 499,840	Dec. 20, 1938 Jan. 30, 1939 Jan. 30, 1939	J. Eisenbrand and M. Lienz Ass. to Winthrop	Molecular compounds from ascorbic acid or iso-ascorbic acid and quinine or quinidine.
U. S. 2,150,140	Mar. 7, 1939.	K. Warnat Ass. to Hoffmann-LaRoche	3,5,6-Tribenzoyl-ascorbic acid and its preparation from ascorbic acid salts and benzoyl chloride.
U. S. 2,159,214	May 23, 1939	S. Klein	Double salt of calcium ascorbate and calcium acetylsalicylate.
U. S. 2,159,986	May 30, 1939	P. P. Gray and I. Stone Ass. to Wallerstein	Addition of ascorbic acid to aqueous-oil emulsions.
U. S. 2,187,467	Jan. 16, 1940	E. H. Stuart Ass. to Eli Lilly	Aqueous solution of a salt of ascorbic acid, *e. g.*, of alkali metals, alkaline earth metals, ammonium, the lower alkyl-substituted amines, and containing sulfur compounds, *e. g.*, sodium hydrosulfite.
U. S. 2,212,831	Aug. 27, 1940	F. Hoffman and P. Marquardt Ass. to Byk-Guldenwerke	Manufacture of stable derivatives of adrenaline by reacting adrenaline with ascorbic acid.
U. S. 2,213,977	Sept. 10, 1940	W. G. Christiansen Ass. to Squibb	Vitamin C-amine solution stabilized by means of hypophosphite.
U. S. 2,232,699	Feb. 25, 1941	U. H. Engels, J. Weijlard and R. T. Schenck Ass. to Merck	Stabilization of ascorbic acid solutions by water soluble edible colloids of the group consisting of albumen, acacia, casein, raw milk, gelatin and starch.
U. S. 2,232,712	Feb. 25, 1941	R. T. Major and E. W. Cook Ass. to Merck	Process for the production of fully acetylated sugar acids from corresponding aldehydo-sugar acetates and corresponding δ-lactones. Composition of matter claims for fully acetylated sugar acids which do not contain a keto group.

Patent No.	Date	Patentee	Abstract
Derivatives and Utilization (*Continued*)			
U. S. 2,249,903 G. 693,375	July 22, 1941 July 6, 1940	C. L. Lautenschläger and F. Lindner Ass. to I. G.	Preparation of stable water solutions of ascorbic acid by partial or complete neutralization with organic bases.
U. S. 2,251,526 B. 524,319	Aug. 5, 1941 Aug. 2, 1940	A. Salomon Ass. to N. V. Orgachemia	Stable concentrated quinine solutions comprising dissolving a monosalt of quinine with the acid of ascorbic acid.
U. S. 2,255,483	Sept. 9, 1941	G. F. D'Alelio Ass. to General Electric	Addition of ascorbic acid as an inhibitor to polymerizable materials containing a methylene group.
U. S. 2,260,870	Oct. 28, 1941	S. L. Ruskin	Manganese compound of ascorbic acid.
B. 455,221 F. 792,675	Oct. 28, 1936 July 1, 1936	Dansk Gaering Industri	Addition of ascorbic acid to flour and dough.
B. 469,335	July 23, 1937	I. G.	Stable solutions of partly or completely neutralized ascorbic acid for ampules.
B. 472,531 F. 817,578	Sept. 20, 1937 Sept. 6, 1937	Ciba	Process for the manufacture of ferrous salts of ascorbic acid.
B. 485,612 F. 829,982	May 23, 1938 July 18, 1938	H. Lotze	Addition of catalase to preparations containing vitamins A, B, C and/or D for protecting vitamin C against destruction.
B. 486,055	May 20, 1938	H. W. Rhodehamel and E. C. Kleiderer Ass. to Eli Lilly	Anhydrous solution of vitamin C in propandiol.
B. 486,546	June 7, 1938	P. K. Henriksen Ass. to Canned Cream & Milk Co.	Addition of vitamin C to milk or liquid milk products.
B. 486,757 G. 682,875	June 9, 1938 Oct. 24, 1939	F. B. Dehn Ass. to Promonta	Preparation of ferrous salts of ascorbic acid.
B. 488,784	July 13, 1938	S. L. Ruskin	Process for the production of the following salts of ascorbic acid: alkali, calcium, barium, strontium, iron, manganese, bismuth, arsenic, gold, silver, copper, mercury, zinc, aluminum and tin.
B. 495,675	Nov. 17, 1938	Hoffmann-LaRoche	Process for the manufacture of calcium double salts of ascorbic acid and poly - oxy - mono - carboxylic acids.
B. 503,476	Mar. 30, 1939	N. V. Industrielle Maatschapij vorheen Noutry & van der Lande	Mixture of ascorbic acid with calcium phosphates, gypsum, silica, talc, magnesium carbonate, potassium sulfate, wheat, rice, maize or potato starch.
B. 509,709	July 19, 1939	N. V. Orgachemia	Stabilization of ergometrine by ascorbic acid.
B. 511,585	Aug. 21, 1939	"Ocean"	Addition of vitamin C-containing paprika to foodstuffs.
B. 514,047	Oct. 30, 1939	E. E. Wells and Rountree Co.	Particles of ascorbic acid coated with gelatine, gum or hard sugar.

Patent No.	Date	Patentee	Abstract
Derivatives and Utilization (*Continued*)			
B. 517,348 F. 829,547	Jan. 26, 1940 June 29, 1938	P.R.A. Maltha	Addition of ascorbic acid to form in a mixture with metals or salts of copper, manganese, cobalt, iron salts, proteic acid salts or proteins containing such metals, or powder of squash, cucumbers, cabbage leaves or tomatoes.
B. 533,480	Feb. 13, 1941	Hoffmann-LaRoche	Ascorbic acid salts of cinchona alkaloids.
G. 470,012	Jan. 3, 1929	J. Korselt	Preparation of pressed plant juices rich in vitamins from chlorophyll-containing plants by the addition of calcium salts and aliphatic oxy-acids.
G. 639,776 (See continuation 701,561)	Dec. 12, 1936	Hoffmann-LaRoche	Preparation of esters of ascorbic acid and higher fatty acids.
G. 663,987	Aug. 18, 1938	Merck	Solution of mono alkali or alkaline earth salts of ascorbic acid for filling ampules followed by sterilization.
G. 681,859	Oct. 3, 1939	Madaus & Co.	Preparation of stable colloidal solutions of phosphorus in alcohol by the addition of ascorbic acid.
G. 696,794	Sept. 30, 1940	Madaus & Co.	Stabilization of colloidal bismuth-containing aqueous solutions for injections by adding ascorbic acid in the presence of protective colloids.
G. 699,327 Continuation of G. 693,375	Nov. 7, 1940	C. L. Lautenschläger and F. Lindner Ass. to I. G.	Preparation of stable water solutions of ascorbic acid by partial neutralization with inorganic bases.
G. 701,561 Continuation of G. 639,776	Jan. 18, 1941	Hoffmann-LaRoche	Esters of ascorbic acid by reaction of salts of ascorbic acid with acid halides of aromatic carboxylic acids or by reaction of ascorbic acid with acid halides in the presence of organic bases.
F. 749,742	July 28, 1933	C. Groll and E. Stirnimann	Addition of sterilized plants containing vitamin C to evaporated milk.
F. 800,592	July 8, 1936	Soc. Française Des Sucres	Addition of solutions of vitamins C and D to sugar or sugar syrup.
F. 805,335	Nov. 17, 1936	F. Arloing, A. Morel and A. Josserand	Preparation of complex salts of ascorbic acid.
F. 805,335/47,155	Feb. 6, 1937	F. Arloing, A. Morel and A. Josserand	Preparation of ammonium or amine-containing complex salts of ascorbic acid.
F. 807,877	Jan. 23, 1937	F. Arloing, A. Morel and A. Josserand	Preparation of complex salts of ascorbic acid.
F. 823,732	Jan. 25, 1938	F. Arloing, A. Morel and A. Josserand	Preparation of complex salts of iso-vitamin C.
F. 827,142	April 20, 1938	N. V. Industrielle Maatschappij vorheen Noutry & van der Lande	Preservation of food by the addition of ascorbic acid together with a non-hygroscopic substance.

Patent No.	Date	Patentee	Abstract
Derivatives and Utilization (*Continued*)			
F. 831,054	Aug. 22, 1938	L. Flodquist	Yeast, which naturally contains no or only traces of vitamin C, is enriched in this vitamin by fermentation in fruit juices.
Hung. 115,639	Dec. 15, 1936	E. Tornya	Ascorbic acid in mixture with glycerin and talc to preserve rubber.
Jap. 128,714	Feb. 9, 1939	Z. Nukida Ass. to T. Takeda Co.	Preparation of adrenaline ascorbate.
		VITAMINS D	
Provitamins D			
U. S. 1,724,706 U. S. 1,840,756 B. 322,465 G. 517,499 G. 520,853	Aug. 13, 1929 Jan. 12, 1932 Jan. 2, 1930 Feb. 4, 1931 Mar. 16, 1931	E. Walz, R. Griessbach, and O. Ambros Ass. to Winthrop	Extraction of ergosterol from autolized yeast.
U. S. 1,733,009 B. 292,133 G. 549,110	Oct. 22, 1929 June 14, 1927 June 17, 1927	A. Gams and F. Locher Ass. to Ciba	Isolation of ergosterol from yeast or other fungi by saponification with aqueous alkali and skimming off the crystallized ergosterol.
U. S. 1,775,548	Sept. 9, 1930	C. E. Bills Ass. to Mead Johnson	Ergosterol is purified by crystallization from a mixture of acetone and ether.
U. S. 1,842,929	Jan. 26, 1932	C. E. Bills Ass. to Mead Johnson	Preparation of ergosterol by dissolving yeast fat in acetone and saponifying the material with potassium hydroxide.
U. S. 1,893,317	Jan. 3, 1933	A. Zimmerli Ass. to Acetol Products	Process of isolating ergosterol from mycelium of aspergillus niger, by extracting the mycelium with an alcoholic alkali, and re-extracting the alcoholic residue with ether.
U. S. 1,912,440	June 6, 1933	C. N. Frey and R. F. Light Ass. to Standard Brands	Isolation of ergosterol from yeast by saponification followed by crystallization.
U. S. 1,941,097	Dec. 26, 1933	R. F. Light and C. N. Frey Ass. to Standard Brands	Process of obtaining sterols from crude substances by saponification at about 20 lbs. pressure with a water solution of an alkaline material until the sterols are liberated followed by extraction with a water immiscible solvent.
U. S. 2,028,364	Jan. 21, 1936	J. Waddell Ass. to DuPont	Process for producing antirachitic substances by heating cholesterol in the presence of water followed by ultraviolet irradiation.
U. S. 2,056,992 B. 485,452	Oct. 13, 1936 May 17, 1938	J. Waddell Ass. to DuPont	Production of antirachitic substances by subjecting a sterol-containing substance to a mild oxidation treatment, thus producing a provitamin, and subsequently or simultaneously activating the provitamin produced.
U. S. 2,059,980	Nov. 3, 1936	W. G. Bennett Ass. to Standard Brands	By cultivation of yeast in the presence of an oxidizing agent the ergosterol content is increased.

Patent No.	Date	Patentee	Abstract
Provitamins D (*Continued*)			
U. S. 2,098,984 B. 454,260 G. 701,601	Nov. 16, 1937 Sept. 28, 1936 Jan. 20, 1941	A. Windaus and F. Schenck Ass. to Winthrop	Synthesis of 7-dehydro-sterols, especially 7-dehydro-cholesterol, by reduction of 7-oxo-sterol compounds to 7-hydroxy-sterols followed by esterification and splitting out of an acid.
U. S. 2,098,985	Nov. 16, 1937	A. Windaus and F. Schenck Ass. to Winthrop	7-Hydroxy-cholesterol, an intermediate for the synthesis of provitamin D_3.
U. S. 2,112,200 B. 489,083	Mar. 22, 1938 July 19, 1938	M. Kharasch and S. Weinhouse Ass. to Eli Lilly	Process for increasing the provitamin D content of cholesterol by heating solid cholesterol and solid benzoyl peroxide together to a temperature of about 120° to 250° C.
U. S. 2,128,198	Aug. 23, 1938	A. Windaus Ass. to Winthrop	Preparation of 22, 23-dihydro-ergosterol from the ergosterol-maleic anhydride addition product by hydrogenation, followed by thermal decomposition.
U. S. 2,163,659 B. 471,994 F. 826,925	June 27, 1939 Sept. 15, 1937 April 13, 1938	A. G. Boer, J. van Niekerk, E. H. Reerink and A. van Wijk Ass. to N. V. Philips' Gloeilampenfabrieken	Isolation of a new provitamin D from invertebrata and conversion to a chicken active vitamin D by irradiation.
U. S. 2,167,272	July 25, 1939	W. A. Carlson Ass. to General Mills	Production of large crystals of ergosterol by recrystallization from a slowly cooled solution in an organic solvent, the solution containing water in a proportion between 2 and 30 parts by volume of water per 1000 parts of solvent.
U. S. 2,209,934 B. 537,036	July 30, 1940 June 6, 1941	H. R. Rosenberg Ass. to DuPont	Production of 7-dehydro-sterols from esterified 7-hydroxy sterols by treatment with organic nitrogen-containing bases.
U. S. 2,215,727 B. 537,030	Sept. 24, 1940 June 6, 1940	H. R. Rosenberg and J. M. Tinker Ass. to DuPont	Production of 7-oxo-cholesterol-benzoate, 7-hydroxy-cholesterol-3-mono-benzoate and 7-hydroxy-cholesterol-dibenzoate.
U. S. 2,237,762 B. 509,063	April 8, 1941 July 11, 1939	R. E. Marker Ass. to Parke, Davis	7-Keto and 7-hydroxy-cholesteryl-chloride.
U. S. 2,255,815 F. 867,189	Sept. 16, 1941 July 7, 1941	H. R. Rosenberg and T Parsons Ass. to DuPont	7-Dehydro-sterol-compounds from esterified 7-hydroxy-sterol-compounds by treatment with oxonium salt-forming compounds.
U. S. 2,266,674	Dec. 16, 1941	A. G. Boer, J. van Niekerk, E. H. Reerink and A. van Wijk Ass. to Hartford National Bank and Trust Co.	Purification of provitamin D preparations from invertebrata by esterification and fractional adsorption.
B. 396,206	Aug. 10, 1933	Intern. Yeast	Cultivation of yeast rich in ergosterol by aeration.
B. 477,283 G. 673,277 F. 825,971	Dec. 22, 1937 Mar. 20, 1939 Mar. 18, 1938	A. G. Boer, J. van Niekerk, E. H. Reerink and A. van Wijk Ass. to N. V. Philips' Gloeilampenfabrieken	Production of a purified provitamin D from a sterol mixture by esterification, fractional adsorption and recrystallization. By irradition of the provitamin, the vitamin can be obtained.

Patent No.	Date	Patentee	Abstract
Provitamins D (*Continued*)			
B. 504,051	April 19, 1939	The International Yeast Co. and W. G. Bennett	Extraction of ergosterol from yeast by plasmolysis or boiling with acid followed by extraction with an organic fat solvent and saponification.
G. 542,667	Jan. 28, 1932	Merck	Ergosterol from yeast residues
G. 553,915	July 2, 1932	Hoffmann-LaRoche	Isolation of sterols from yeast by heating yeast with aqueous alkali solutions in an open vessel.
Austrian 140,190	Jan. 10, 1935	W. Halden	Cultivation of yeast high in ergosterol under aerobic conditions.
Belg. 416,160	June 19, 1936	N. V. Philips' Gloeilampenfabrieken	Purification of ergosterol by esterification followed by absorption.
Swiss 129,879	Jan. 2, 1939	Ciba	Isolation of ergosterol from fungi by alkali saponification.
Swiss 201,169	Feb. 1, 1939	I. G.	Preparation of 7-dehydro-cholesterol from 7-oxo-cholesterol by reduction to 7-hydroxy-cholesterol, diacylation, heat decomposition to a 7 - dehydro - cholesterol - ester and final saponification.
Conversion of Provitamins D to Vitamins D			
U. S. 1,680,818 B. 236,197 F. 587,187	Aug. 14, 1928 June 30, 1924 April 14, 1935	H. Steenbock Ass. to Wisconsin Alumni Research Foundation	Antirachitic activation of foods by ultraviolet irradiation.
U. S. 1,681,120	Aug. 14, 1928	A. J. Pacini Ass. to M. Richter	Production of antirachitic material from growth-producing substances by irradiation with light of wave lengths longer than 3022 Å.
U. S. 1,682,318	Aug. 28, 1928	F. C. Beardslee Ass. to F. C. Beardslee, B. M. Huffine and J. I. Huffine	Apparatus for ultraviolet irradiation of food materials.
U. S. 1,704,173	Mar. 5, 1929	J. W. D. Chesney Ass. to Solar Research Corp.	Method of making the ultraviolet rays of the sun available for the production of the anti-rachitic principle in substances susceptible to such activation, by separating solar ultraviolet rays of wave lengths within the range of substantially 2900 to 3700 Angström Units from the remaining rays, and intensifying the separated ultraviolet rays by concentration.
U. S. 1,723,603	Aug. 6, 1929	J. W. D. Chesney Ass. to Chesney Process, Inc.	Process for activating pasteurized liquids by addition of an organic acid followed by ultraviolet irradiation.
U. S. 1,754,434	April 15, 1930	J. Perino	Vegetable, alimentary materials are subjected in the absence of oxygen, and in the presence of soluble phosphates to an irradiation by ultraviolet rays and are heated to not above 60° C. during the irradiation.
U. S. 1,762,105 B. 356,793	June 3, 1930 June 6, 1930	A. J. Pacini Ass. to C. M. Richter	Vitamins D by irradiation of sterols or fats with infra-red or ultraviolet light in inert gas.

Patent No.	Date	Patentee	Abstract
Conversion of Provitamins D to Vitamins D *(Continued)*			
U. S. 1,771,343	July 22, 1930	A. J. Pacini Ass. to C. M. Richter	Production of vitamin D by irradiation of sterols in the presence of a suitable photocatalyst such as halogen, or by rays longer than 3022 Å or by electromagnetic radiation in the presence of a catalyst.
U. S. 1,796,134 G. 564,401 G. 608,277 G. 647,522	Mar. 10, 1931 Nov. 18, 1932 Jan. 19, 1935 July 6, 1937	A. Wörner and F. Kielwein	Claims a baking oven containing a mercury vapor lamp and a reflector for indirect irradiation and also claims irradiation of yeast therein.
U. S. 1,808,760	June 9, 1931	C. E. Bills Ass. to Mead Johnson	Apparatus for irradiating liquids, such as oils, mixed with ergosterol.
U. S. 1,817,936	Aug. 11, 1931	G. C. Supplee Ass. to The Borden Co.	Process of irradiating milk with ultraviolet rays.
U. S. 1,842,313	Jan. 19, 1932	N. K. Chaney Ass. to National Carbon Co.	Apparatus for irradiating foods with ultraviolet rays.
U. S. 1,848,305	Mar. 8, 1932	C. E. Bills Ass. to Mead Johnson	Method of irradiating a stream of fluids.
U. S. 1,871,135	Aug. 9, 1932	H. Steenbock Ass. to Wisconsin Alumni Research Foundation	Preparation of antirachitic food substances by ultraviolet irradiation of cereals.
U. S. 1,871,136 B. 314,942 G. 605,960	Aug. 9, 1932 Aug. 28, 1929 Nov. 22, 1934	H. Steenbock Ass. to Wisconsin Alumni Research Foundation	Antirachitic product by ultraviolet irradiation of lipoids.
U. S. 1,873,942 B. 283,557	Aug. 23, 1932 Not accepted	A. Windaus (In Great Britain—E. Merck)	Antirachitic preparations by irradiation of ergosterol solutions until digitonin yields only a small precipitate.
U. S. 1,880,977	Oct. 4, 1932	A. J. Pacini Ass. to Sun-A-Sured, Inc.	Production of vitamin D by heating lipoids, such as cholesterol with a photocatalyst such as uranium-acetate.
U. S. 1,880,978	Oct. 4, 1932	A. J. Pacini Ass. to Sun-A-Sured, Inc.	Process of producing vitamin D by heat-extracting ergosterol from natural sources in the presence of a photocatalyst.
U. S. 1,894,158	Jan. 10, 1933	N. K. Chaney Ass. to National Carbon Co.	Antirachitic activation of foodstuffs by irradiation from an arc.
U. S. 1,896,191 B. 296,093 B. 316,803 G. 499,524 F. 659,448	Feb. 7, 1933 Aug. 26, 1927 Aug. 29, 1929 June 7, 1930 Aug. 24, 1928	W. Zimmermann and W. Frankenburger Ass. to Winthrop	Production of vitamin D from ergosterol by ultraviolet rays and interrupting the exposure before the maximum absorption in the range of the spectrum between $\lambda = 300m\mu$ and $\lambda = 230m\mu$ has been reached.
U. S. 1,904,751 B. 343,528 G. 634,146 F. 700,312 F. 40,142	April 18, 1933 Mar. 19, 1931 Aug. 18, 1936 Feb. 27, 1931 April 20, 1932	E. H. Reerink and A. van Wijk Ass. to N. V. Philips Gloeilampenfabrieken	Irradiation of ergosterol with wave lengths from 270 to 300 $m\mu$.
U. S. 1,920,587	Aug. 1, 1933	A. J. Pacini Ass. to Sun-A-Sured, Inc.	Method of producing vitamin D comprising suspending lipoids, such as cholesterol, and treating them with aspergillus oryzae.

Patent No.	Date	Patentee	Abstract
Conversion of Provitamins D to Vitamins D (*Continued*)			
U. S. 1,928,397	Sept. 26, 1933	E. D. Shumway Ass. to Quaker Oats	Production of an antirachitically activated cereal by removing the husk or skin from cereal and subjecting the whole kernel to the action of activating rays.
U. S. 1,954,065	April 10, 1934	J. H. Bragg	Apparatus for increasing the vitamin content of liquid food comprising a food chamber having openings for the passage of liquid food, a source of ultraviolet rays and means to effect a cooling of the contents of the chamber.
U. S. 1,955,554 B. 394,408	April 17, 1934 June 29, 1933	R. F. Light and C. N. Frey Ass. to Standard Brands	Process of activating antirachitically activatable unsaponifiable lipoids in dioxane by light containing frequencies below the visible spectrum.
U. S. 1,966,546 U. S. 1,966,547	July 17, 1934 July 17, 1934	G. P. Goode Ass. to General Development Lab.	Apparatus for irradiating solutions of ergosterol, etc., with filtered ultraviolet light.
U. S. 1,980,971	Nov. 13, 1934	H. G. Campsie	Production of vitamin D from provitamin D by means of radiant energy of 2536 to 2540 Angström Units.
U. S. 1,982,028	Nov. 27, 1934	G. Sperti Ass. to General Development Laboratories	Process of increasing the vitamin content of food substances by subjecting them to soft x-rays having wave lengths between approximately 2 and 13 Angström Units.
U. S. 1,982,029	Nov. 27, 1934	G. Sperti, R. J. Norris, R. B. Withrow and H. Schneider Ass. to General Development Laboratories	Process for treating food substances with ultraviolet light.
U. S. 1,983,944	Dec. 11, 1934	A. J. Pacini Ass. to American Research Products	Process of activating provitamin D by treatment with cathode rays in the presence of a suitable catalyst such as chlorine or bromine.
U. S. 2,007,765 B. 292,926	July 9, 1935 Aug. 22, 1929	A Knudson Ass. to Sun-A-Sured, Inc.	Process of increasing the vitamin D content of food, fats or ergosterol by treatment with high velocity electrons.
U. S. 2,015,264 U. S. 2,015,265	Sept. 24, 1935 Sept. 24, 1935	R. M. Fraps	Apparatus for irradiating materials, such as ergosterol, by sunlight.
U. S. 2,015,282	Sept. 24, 1935	A. J. Pacini Ass. to American Research Products, Inc.	Production of vitamin D from provitamin D by radioactive substances.
U. S. 2,057,399	Oct. 13, 1936	H. Steenbock Ass. to Wisconsin Alumni Research Foundation	Yeast is antirachitically activated by treatment with light rich in ultraviolet rays.
U. S. 2,104,681	Jan. 4, 1938	G. C. Supplee Ass. to The Borden Co. and to National Carbon Co.	Method of irradiating liquid milk products with ultraviolet energy to give increased antirachitic potency by impinging the ultraviolet energy in such a manner that all rays of energy impinge obliquely upon the surface.

Patent No.	Date	Patentee	Abstract
Conversion of Provitamins D to Vitamins D (*Continued*)			
U. S. 2,106,779	Feb. 1, 1938	C. C. Whittier	Production of vitamin D by passing a vaporized provitamin D through a zone of electrically-induced antirachitically-activating discharge in a vacuum containing indium emanations.
U. S. 2,106,780	Feb. 1, 1938	C. C. Whittier	Method of producing vitamin D which consists in vaporizing ergosterol, passing the same through a zone of electrical discharge in a vacuum tube, condensing the treated ergosterol vapor and subjecting the condensate to a transversely directed electrical discharge in the vacuum tube.
U. S. 2,106,781	Feb. 1, 1938	C. C. Whittier Ass. to Nutrition Research Laboratories	Apparatus for the activation of provitamin D by the electrical discharge method.
U. S. 2,106,782	Feb. 1, 1938	C. C. Whittier Ass. to Nutrition Research Laboratories	Apparatus for the activation of provitamin D by the electrical discharge method.
U. S. 2,112,242	Mar. 29, 1938	B. Kramer and A. E. Sobel	Process for the antirachitic activation of provitamins D by an electrical discharge method.
U. S. 2,117,100	May 10, 1938	N. A. Milas Ass. to DuPont	Preparation of antirachitic substances from provitamins D by the action of a high frequency electrical oscillating discharge.
U. S. 2,128,199 G. 642,759	Aug. 23, 1938 Mar. 16, 1937	A. Windaus Ass. to Winthrop	Preparation of an antirachitically active substance by ultraviolet irradiation of 22,23-dihydro-ergosterol.
U. S. 2,151,645	Mar. 21, 1939	H. C. Stephens and S. B. Hoar Ass. to Natural Food Products	Method for deaerating a liquid food product in vacuum and, while under vacuum, exposing the completely deaerated liquid to ultraviolet irradiation.
U. S. 2,183,933	Dec. 19, 1939	J. K. Elderkin and E. Hofman Ass. to Chemical Products Co.	Foods are antirachitically activated by the action of ozone.
U. S. 2,202,611	May 28, 1940	G. C. Supplee and J. Dorcas Ass. to The Borden Co. and National Carbon Co.	Method and apparatus for irradiation of milk in turbulent flow to increase the vitamin D content.
U. S. 2,231,870	Feb. 18, 1941	W. Baeckler Ass. to Union Carbide & Carbon	Apparatus for open-air irradiation of liquids such as milk by flowing the liquid in a substantially rectilinear direction along a smooth surface.
U. S. 2,231,871	Feb. 18, 1941	W. Baeckler Ass. to Union Carbide & Carbon	Apparatus for open-air irradiation of liquids, such as milk, by using a conical type of support for the liquid in order to maintain a constant rate of flow.
U. S. 2,234,554	Mar. 11, 1941	H. W. Elley and J. Waddell Ass. to DuPont	The process which comprises dissolving a provitamin D-containing material in an organic solvent, adding a sugar-amine, exposing the solution to ultraviolet light and recovering a vitamin D concentrate.

Patent No.	Date	Patentee	Abstract
Conversion of Provitamins D to Vitamins D (*Continued*)			
U. S. 2,243,632	May 27, 1941	M. L. Johnson Ass. to Vitamin Technologists	Production of vitamin D_2 from ergosterol by irradiation with ultraviolet light of 2536–2540 Angström Units.
U. S. 2,260,823	Oct. 28, 1941	E. S. Bettis Ass. to Pet Milk Co.	Irradiation of milk in a thin film.
B. 265,910	April 7, 1927	K. Hoefelmayr	Milk in concentrated form or in a curdled state is subjected to the action of artificially produced violet rays, by which it is converted into an invalid food effective for the cure of certain diseases.
B. 266,101	Feb. 24, 1927	O. A. Elias	Method of manufacturing biscuits, bread, cakes and similar food products characterized by carrying out the baking process in the presence of artificially produced ultraviolet rays.
B. 270,296	April 28, 1926	H. C. E. Tillisch	Antirachitic vitamin by ultraviolet irradiation of oils or fats.
B. 285,083	Jan. 24, 1929	Merck	Manufacture of antirachitic preparations, by esterifying the unsaponifiable constituents of yeast, fat or of the corresponding extracts from ergot or similar lower fungi and exposing the esters to the action of an activating radiation, or by first activating and afterwards esterifying the material.
B. 286,665	Sept. 6, 1928	Merck	Manufacture of antirachitic substances by irradiation of provitamins D in the presence of photochemical sensibilisers, such as eosin or iodine.
B. 290,195	Mar. 7, 1929	C. Jaeger	Ultraviolet irradiation of dried bananas to produce an antirachitic food.
B. 293,255	Nov. 28, 1927	T. D. Kelly	Oils, fats or emulsions are treated with beta-rays and with ultraviolet rays to produce vitamins.
B. 295,757	Aug. 23, 1928	I. S. MacLean	Yeast is incubated in a solution containing phosphates and carbohydrates and the sterols or sterols and fats obtained are subjected to activating radiation.
B. 296,053	Aug. 24, 1927	A. J. Pacini Ass. to M. Richter	Process of treating materials to form antirachitic substances by various rays other than ultraviolet rays, *e. g.*, by x-rays, canal rays, cathode rays, etc.
B. 298,585	Dec. 5, 1928	Dry Milk Co.	Ultraviolet irradiation of milk.
B. 302,980	Sept. 24, 1927	N. Bendixen	Rotary device for treating liquids with rays or emanations.
B. 309,601 F. 672,318	April 13, 1928 Mar. 29, 1929	E. Oppenheim	Ultraviolet irradiation of chocolate.

Patent No.	Date	Patentee	Abstract
Conversion of Provitamins D to Vitamins D (*Continued*)			
B. 313,558 G. 524,874	June 14, 1928 Sept. 13, 1928	G. Zecher Ass. to N. V. Philips' Gloeilampenfabrieken	Apparatus for irradiating substances with ultraviolet light.
B. 314,267	Aug. 20, 1928	I. G.	Apparatus for the irradiation of ergosterol in circulating solvent liquids.
B. 316,264	Sept. 18, 1929	O. Ried	Treatment of foods and fats with short waves, ultraviolet rays or x-rays.
B. 318,268	Sept. 2, 1929	I. M. Heilbron	Activation of provitamins D by metallic catalysts at elevated temperature
B. 318,269	Sept. 2, 1929	I. G.	Production of antirachitic products by treating ergosterol or substances containing ergosterol with corona discharges or with electric corpuscular rays or Roentgen rays, while excluding oxygen.
B. 321,992 F. 675,558	Nov. 25, 1929 May 10, 1929	I. G.	Production of vitamin D by irradiation of sterols in the presence of substances which exert a protective action on the vitamin formed, such as ether, olefinic hydrocarbons or caustic alkalies.
B. 324,503	Mar. 19, 1930	Patent-Treuhand Ges. f. elektr. Glühlampen.	Apparatus for ultraviolet irradiation of milk.
B. 325,470	July 12, 1929	J. O. Hickman and N. V. Hickman	In a process for the irradiation of flowing milk in thin layers, the intensity of irradiation and rate of flow of the milk are regulated.
B. 342,500 F. 697,367	Feb. 15, 1929 Jan. 30, 1930	T. Reiter	Vitamin D by irradiation of ergosterol with light of wave lengths about 280 mμ.
B. 346,682	Oct. 15, 1929	V. C. From, C. D. Rowley and A. W. Larsky	Simultaneous infra-red and ultraviolet light irradiation of milk.
B. 357,223	Jan. 24, 1930	F. F. Tisdall	Wheat germ, whole wheat and yeast, treated with ultraviolet light, are used in the manufacture of bread, cakes, etc.
B. 385,626 F. 714,827	Dec. 20, 1932 April 4, 1931	N. V. Philips' Gloeilampen fabrieken	Irradiation of ergosterol with ultraviolet light using a filter which absorbs light of wave lengths 312-313 mμ, *e. g.*, CS_2.
B. 403,650	Jan. 10, 1934	J. Waerham	Vitamin D-containing soaps by irradiation of soaps containing ergosterol or of oils containing ergosterol, followed by saponification.
B. 489,142	July 20, 1938	H. F. Rost	Ultraviolet irradiation of thin films of provitamins D in solution or suspension in the presence of air with rays of wave lengths between 2600 and 3000 Angström Units.
B. 497,165	Dec. 14, 1938	Nutrition Research Laboratories	Antirachitic activation of provitamins D by subjecting the vapor to a silent non-luminous flow of electricity.

Patent No.	Date	Patentee	Abstract
Conversion of Provitamins D to Vitamins D (*Continued*)			
B. 498,068	Jan. 3, 1939	H. F. Glunz	Process for the irradiation of liquids and the sterile filling of containers therewith in which a zone of ultraviolet rays is maintained within a vacuum chamber to irradiate the liquid as it is directed in a thin film past the zone, the containers being filled with the treated liquid by direct connection with the interior of the vacuum chamber.
G. 502,726	April 3, 1927	H. Geffcken and H. Richter	Foods, such as milk, are subjected to ultraviolet rays.
G. 523,257	Feb. 8, 1927	H. Geffcken and H. Richter Ass. to A. G. f. Elektricitäts-Ind.	Apparatus for irradiating foodstuffs.
G. 526,141	May 17, 1929	H. Geffcken and H. Richter	Apparatus for irradiating foods with ultraviolet rays.
G. 530,877	Nov. 4, 1926	F. Heinemann	Foodstuffs are vitaminized by ultraviolet radiation, using a calc spar filter.
G. 545,080	Feb. 25, 1932	H. Geffcken and H. Richter	Irradiation of skimmed milk.
G. 556,716	Mar. 7, 1928	Merck	Stable aqueous colloidal solutions of irradiated ergosterol are obtained by irradiating ergosterol in its dispersed form and separating the unchanged ergosterol after irradiation.
G. 564,401 G. 608,277	Nov. 1, 1927 Jan. 19, 1935	F. Kielwein	Apparatus for irradiating bread or yeast for incorporation into bread.
G. 564,736 G. 566,744	Nov. 22, 1932 Dec. 20, 1932	E. Latacz	Apparatus for irradiation of liquids.
G. 567,333	Dec. 31, 1932	Hoffmann-LaRoche	Production of water-soluble sterol compounds by irradiating sterols in the form of their ester salts, *e. g.*, a salt of phthalic acid monoergosterol ester, with ultraviolet light.
G. 568,900 G. 577,170	Jan. 5, 1933 Aug. 10, 1933	K. Hembd and Vitamin Fabrik	Irradiation of extracts of high viscosity.
G. 572,491	Mar. 17, 1933	A. Schindler	Ultraviolet irradiation of milk.
G. 577,531	June 1, 1933	G. Kersten and O. K. Schultz	Irradiation of skimmed milk followed by addition of the original cream.
G. 583,791	Sept. 9, 1933	I. G.	Production of an antirachitically active material by treating lumisterol or its esters with ultraviolet light and method of crystallizing the antirachitic product as the dinitro-benzoate.
G. 622,373	Nov. 27, 1935	Leo-Werke	Vitamin-containing cosmetics from wool fat by irradiation with ultraviolet light.
G. 624,325	Jan. 17, 1936	H. Heitan and Kuntze's Verwaltungs G.	Vitamins are produced in malt beer by light from a Mg-arc.
G. 632,783	July 13, 1936	Hanovia	Irradiation of milk with mercury-vapor lamps.
G. 648,326	July 29, 1937	E. Freitag	Irradiation of milk in bottles.

Patent No.	Date	Patentee	Abstract
Conversion of Provitamins D to Vitamins D (*Continued*)			
G. 673,852	Mar. 30, 1939	K. Wolff and R. Havemann	Method for obtaining transformation products of ergosterol and similar materials by irradiating with electrons from a glowing cathode in which the material to be activated is passed in thin layers over the anode.
F. 666,959	Oct. 8, 1929	Osa Part. Ind. Soc.	Apparatus for the irradiation of liquids.
F. 667,660	Oct. 19, 1929	A. Tribout	Pasteurization and irradiation of milk.
F. 677,010	Mar. 3, 1930	J. Seipi	Irradiation of beverages.
F. 677,111	June 17, 1929	I. G.	Preparation of antirachitic substances by irradiation of provitamins at a temperature above 70° C.
F. 697,367	Jan. 16, 1931	T. Reiter	Conversion of ergosterol to vitamin D by means of light of wave lengths longer than 280 mμ.
F. 700,036	Nov. 16, 1929	H. Labbé	Sterols extracted from cacao beans are activated by ultraviolet light.
F. 708,548	July 24, 1931	N. V. Philips' Gloeilampenfabrieken	Irradiation of provitamins in solution in special apparatus.
F. 752,261	Sept. 20, 1933	J. Major	Vitamin-containing flour by special treatment of grain and ultraviolet irradiation.
F. 779,847	April 13, 1935	C. Devret & Co.	Irradiation of cholesterol-containing foods.
F. 851,421	Jan. 9, 1940	Hermes Patentverwertungs-Gesellschaft	Method and apparatus for irradiating milk sprays in a carbon dioxide atmosphere.
Austrian 118,762	Mar. 15, 1930	O. Ried	The irradiation effect is enhanced by the addition of mineral substances, *e. g.*, ZnO.
Austrian 118,764	Mar. 15, 1930	O. Ried	Enhancement of the biological effect of irradiated substances by the addition of trypaflavin.
Austrian 137,455	May 11, 1934	Leo-Werke	Apparatus for irradiating wool fat or wool fat alcohols to enrich the vitamin D content.
Belg. 386,419	Mar. 31, 1932	Soc. Rémiz	Vitamin-containing foods by irradiation while stirring.
Hung. 100,696	Aug. 4, 1927	G. Feher	Yeast is saponified and extracted. The isolated ergosterol is irradiated to form vitamin D.
Hung. 104,227	April 14, 1931	A. Jendrassik	Production of vitamin D from solid ergosterol in contact with a solution which dissolves the irradiated ergosterol and which solution is continuously removed.
Vitamins D			
U. S. 1,563,134	Nov. 24, 1925	T. F. Zucker Ass. to University Patents	Process of assaying the antirachitic strength of a substance by determining the pH value of the feces of experimental animals.

Patent No.	Date	Patentee	Abstract
Vitamins D (*Continued*)			
U. S. 1,902,745	Mar. 21, 1933	A. Windaus Ass. to Winthrop	The antirachitic vitamin obtained by ultraviolet irradiation of ergosterol is purified by the formation of addition products of the non-vitamin material with compounds of the maleic anhydride type.
U. S. 1,902,785 B. 370,743 B. 405,321 G. 565,900 G. 576,021	Mar. 21, 1933 April 14, 1932 Feb. 5, 1934 Dec. 7, 1932 May 6, 1933	O. Linsert Ass. to Winthrop	Process which comprises freeing an antirachitically active product from unchanged ergosterol by subjecting the product to the action of at least one mole of maleic or citraconic acid anhydride.
U. S. 2,030,377 G. 603,088	Feb. 11, 1936 Sept. 22, 1934	O. Linsert Ass. to Winthrop	Crystalline vitamin D_3 from irradiated ergosterol by formation of the 3,5-dinitrobenzoate followed by saponification.
U. S. 2,099,550 B. 491,653 G. 661,686	Nov. 16, 1937 Sept. 6, 1938 June 24, 1938	A. Windaus and F. Schenck Ass. to Winthrop	Process for the purification of vitamin D_3, obtained by activation of 7-dehydro-cholesterol, by esterification with meta-dinitro-benzoyl-chloride, fractional crystallization of the esters formed and saponification of the meta-dinitro-benzoate of the vitamin D_3. Purification may also be accomplished by precipitation of 7-dehydro-cholesterol as the digitonide or by the formation of addition products with compounds of the maleic anhydride type.
U. S. 2,179,560	Nov. 14, 1939	S. E. Miller Ass. to General Mills	Process of concentrating vitamin D from either naturally occurring or synthetic vitamin D concentrates by dissolving vitamin D-containing material in an organic solvent, passing the solution through tricalcium phosphate whereby a major portion of the vitamin D is adsorbed by the phosphate, washing the phosphate with an organic solvent, and then separating the vitamin D from the solvent by distillation.
U. S. 2,216,719	Oct. 8, 1940	A. G. Boer, J. van Niekert, A. van Wijk and E. H. Reerink Ass. to Hartford National Bank & Trust Co.	Process for producing a new chicken active vitamin D by activation of a new provitamin D derived from periwinkles.
B. 335,277 F. 698,040	Sept. 25, 1930 June 11, 1930	Société des Usines Chimiques Rhone-Poulenc	Separation of ergosterol from its crude irradiation products by crystallization from an organic solvent particularly alcohol, acetone and ethyl-acetate.
B. 464,066 G. 678,533 F. 815,545	April 12, 1937 July 17, 1939 July 13, 1937	N. V. Philips' Gloeilampenfabrieken	Process of producing a preparation which has a high antirachitic activity for chickens, wherein the unsaponifiable fraction or the sterol fraction of duck eggs is irradiated with ultraviolet light. The provitamin D is identical with 7-dehydro-cholesterol.

Patent No.	Date	Patentee	Abstract
Vitamins D (*Continued*)			
B. 482,880	June 17, 1937	Eastman Kodak	Isolation of a new vitamin D, by molecular distillation of degassed vitamin D-containing oils.
B. 491,007	Aug. 23, 1938	Eastman Kodak	Esterification of vitamin D or of provitamin D with unsaturated higher fatty acids, especially with linolenic acid. The provitamin D ester is activated to vitamin D ester.
B. 517,214	Jan. 24, 1940	Eastman Kodak	High vacuum-short path distillation of natural vitamin D esters, resulting in the separation of 5 or 6 different antirachitic materials.
G. 550,496	April 27, 1930	L. Brauer and H. Seel	Vitamins by oxidation of cholesterol with benzoyl peroxide.
G. 659,882	May 17, 1938	H. Brockmann Ass. to I. G.	Isolation of the antirachitic vitamin from natural products.
G. 672,000	Feb. 18, 1939	H. Fincke Ass. to Gebrüder Stollwerck	Fats with high vitamin D content are obtained by extracting ground cocoa shells with cocoa butter.
Can. 379,424	Feb. 9, 1939	L. Yoder Ass. to Iowa State College Alumni Assoc.	Antirachitic substance by heating cholesterol with a mixture of concentrated sulfuric acid, acetic anhydride and acetic acid.
Derivatives and Utilization			
U. S. 1,824,653 B. 334,002	Sept. 22, 1931 July 1, 1929	E. Brauchli Ass. to Hoffmann-LaRoche	Irradiated ergosterol is stabilized by the addition of a small quantity of a dihydroxy-phenol such as hydroquinone.
U. S. 1,974,808	Sept. 25, 1934	C. F. Dietz Ass. to Commander Larabee Corp.	The oil from a germ-bearing cereal grain is irradiated to form vitamin D and is incorporated into flour.
U. S. 2,010,792	Aug. 6, 1935	J. Siegel Ass. to A. M. Siloan	Colloidal dispersion of sterols in water containing bonemeal and alfalfa extract.
U. S. 2,022,464	Nov. 26, 1935	L. A. Hall Ass. to C. L. Griffith	An emulsified vitamin D concentrate containing gum tragacanth and gum acacia, the emulsion having a pH of 5.5 to 6.0.
U. S. 2,030,792 B. 436,713 B. 469,150 G. 642,261	Feb. 11, 1936 Oct. 16, 1935 July 20, 1937 Feb. 27, 1937	C. W. Hooper Ass. to Winthrop	Propanediols and butanediols as solvents for fat soluble vitamins, especially for vitamins D.
U. S. 2,070,117 G. 624,231	Feb. 9, 1937 Jan. 15, 1936	O. Dalmer and F. v. Werder Ass. to Winthrop	Hydrogenation of tachysterol-dinitrobenzoate to form dihydro-tachysterol.
U. S. 2,150,316	Mar. 14, 1939	A. E. Briod and B. R. East Ass. to National Oil Products	Vitamin D milk is prepared by homogenizing a cod liver oil concentrate with cream or evaporated milk followed by canning and sterilization.
U. S. 2,175,340	Oct. 10, 1939	J. W. D. Chesney Ass. to New Discoveries, Inc.	A vitamin D concentrate is added to a product such as beer, containing at least 0.25% of alcohol and dissolved CO_2.

Patent No.	Date	Patentee	Abstract
Derivatives and Utilization (*Continued*)			
U. S. 2,185,969	Jan. 2, 1940	H. E. Schultze Ass. to Winthrop	Preparation of clear aqueous therapeutic solution of vitamin D with triolein, polyethylene glycol oleyl ether and physiological NaCl solution.
U. S. 2,194,188	Mar. 19, 1940	G. C. Supplee Ass. to The Borden Co.	Method for producing a vitamin D protein symplex of enhanced antirachitic activity.
U. S. 2,228,491	Jan. 14, 1941	F. v. Werder Ass. to Winthrop	Isolation of pure dihydro-tachysterol.
U. S. 2,245,418	June 10, 1941	R. C. Sherwood and C. G. Ferrari Ass. to General Mills	Production of a sterile emulsion of evaporated milk fortified with a concentrate of vitamin D_2 by dissolving activated ergosterol in butter fat, and then dispersing the butter-fat concentrate in evaporated milk and canning and sterilizing the resulting product.
U. S. 2,264,320	Dec. 2, 1941	O. Linsert Ass. to Alba	Vitamin D_2 double compounds with cholesterol, cholestanol or coprosterol.
U. S. 2,265,320	Dec. 9, 1941	R. C. Sherwood and C. G. Ferrari Ass. to General Mills	Emulsion of vitamin D_2 in evaporated milk.
B. 405,791	Feb. 15, 1934	Bell and Sons Ltd. and J. Sowler	Vitamin D-containing oils are absorbed in flour or other carriers of porous nature and mixed with mineral matter for animal feeds.
B. 406,629 F. 737,234	Not accepted May 17, 1932	N. V. Philips' Gloeilampenfabrieken	Vitamin D is preserved by adding oil or fat to a solution of ergosterol before, during or immediately after irradiation in the absence of oxygen.
B. 412,535	July 11, 1934	N. V. Philips' Gloeilampenfabrieken	Poultry feeds containing vitamin D_2.
B. 449,888	April 13, 1935	Standard Brands	Increase in vitamin D content of eggs by feeding hens vitamin D.
G. 495,450	June 30, 1927	Hoffmann-LaRoche	Sterols other than cholesterol, *e. g.*, ergosterol, are water solubilized by the formation of mono-esters of dicarboxylic acids.
G. 501,954 Add. to 495,450	Aug. 6, 1927	Hoffmann-LaRoche	Photo-activated ergosterol is water solubilized by the formation of mono-esters of dicarboxylic acids.

VITAMINS E

Isolation			
U. S. 2,188,878 G. 651,474	Jan. 30, 1940 Oct. 15, 1937	C. L. Lautenschläger and F. Lindner Ass. to Winthrop	Process for preparing crystallized vitamin E allophanates by esterification with cyanuric acid, followed by purification by adsorption on aluminum oxide followed by elution.

Patent No.	Date	Patentee	Abstract
Isolation (*Continued*)			
U. S. 2,203,400 B. 531,224	June 4, 1940 Dec. 31, 1940	J. S. Andrews Ass. to General Mills	Vitamin E concentrate from wheat germ oil by catalytically hydrogenating the oil, extracting the hydrogenated oil with alcohol and further concentrating by separating the sterols and glycerides at low temperatures, saponifying and extracting.
U. S. 2,263,550	Nov. 18, 1941	J. S. Andrews Ass. to General Mills	Preparation of vitamin E concentrate from wheat germ oils by esterification with a monohydric alcohol, followed by distillation of the vitamin E ester.
B. 531,226	Dec. 31, 1940	General Mills	Process for preparing a concentrate of vitamin E from vegetable oils by ester-interchange of the glycerol-esters with a lower aliphatic monohydric alcohol to yield glycerol and esters of monohydric alcohols, followed by separating the glycerol and the sterols, and saponifying the remaining mixture.
Synthesis			
U. S. 2,208,585	July 23, 1940	P. Karrer Ass. to Hoffmann-LaRoche	Synthesis of vitamin E by condensation of alkylated hydroquinones with halogen derivatives of phytol.
U. S. 2,215,398 B. 528,371	Sept. 17, 1941 Oct. 28, 1940	P. Karrer Ass. to Hoffmann-LaRoche	Separation of alpha-tocopherol from its racemic synthetic mixture, comprising the treatment with 3-bromo-camphor-sulphonic acid chloride, isolation of the condensation product and hydrolysis to yield the free, pure alpha-tocopherol.
U. S. 2,230,659	Feb. 4, 1941	F. v. Werder Ass. to Merck	Process for the production of alpha-tocopherol comprising condensing trimethyl-hydroquinone with phytol in the presence of phosphorus pentoxide.
U. S. 2,245,147	June 10, 1941	W. John and P. Günther Ass. to Merck	Synthesis of compounds of the general formula of vitamin E by reacting a dihydro-coumarin with a mixture of methyl-magnesium-halogenide and the Grignard compound from a higher halogenated hydrocarbon
U. S. 2,249,054 B. 529,082 (Add. to B. 529,081)	July 15, 1941 Nov. 13, 1940	L. I. Smith and H. E. Ungnade Ass. to University of Minnesota	Manufacture of tocopherol-like compounds by reacting hydroquinones or their monoethers with dienes in the presence of an acidic substance.
B. 526,884	Sept. 27, 1940	Hoffmann-LaRoche	Process for the manufacture of *dl*-tocopherols in which methyl-substituted hydroquinones are reacted with phytol, isophytol, phytyl-halides or 3-halogeno-dihydro-phytyl-halides in the presence of zinc chloride or formic acid.
B. 527,396	Oct. 8, 1940	Hoffmann-LaRoche	Condensation of trimethyl hydroquinones and alkylene-halides (with the exception of phytyl-halides).

Patent No.	Date	Patentee	Abstract
Synthesis (*Continued*)			
B. 528,372	Oct. 28, 1940	Hoffmann-LaRoche	Condensation of alkyl-hydroquinones with halides of alpha, beta-unsaturated carboxylic acids in the presence of an acid condensing agent.
B. 529,081 F. 855,414	Nov. 13, 1940 May 10, 1940	University of Minnesota	Manufacture of coumarins and chromanes by condensation of hydroquinones, alkylated or not alkylated, with substituted or unsubstituted alkyl-halides.
B. 532,364	Jan. 22, 1941	Hoffmann-LaRoche	Acetyl-*dl*-alpha-tocopherol by acetylation of *dl*-alpha-tocopherol.
B. 537,774	July 7, 1941	Hoffmann-LaRoche	Manufacture of tocols from phytyl halides and alkylated hydroquinones.
B. 539,697	Sept. 22, 1941	Hoffmann-LaRoche	Synthesis of vitamin E from trimethyl-hydroquinone *via* the mono-alkylene ether (*e. g.*, of phytol) by intramolecular condensation.
B. 540,907	Nov. 5, 1941	Hoffmann-LaRoche	Synthesis of ethers and esters of tocols by condensation of phytol-compounds with mono-ethers or mono-esters of dimethyl-hydroquinones.
B. 541,008	Nov. 10, 1941	Hoffmann-LaRoche	Manufacture of ring homologues of vitamin E by condensation of phytol compounds with alkylated hydroquinones.
B. 541,011	Nov. 10, 1941	Hoffmann-LaRoche and J. E. Pollak	Synthesis of vitamin E by rearrangement of trimethyl-hydroquinone-mono-phytyl-ether, followed by condensation under acidic conditions.
G. 374,142	April 20, 1923	L. Claisen	Synthesis of chromanes by condensation of phenols with butadiene-hydrocarbons in the presence of acidic condensing agents.
G. 703,957	Mar. 20, 1941	O. Hromatka Ass. to Merck	Synthesis of vitamin E by condensation of 3-amino-6-oxy-1,2,4-trimethyl-benzene with phytol compounds followed by conversion of the amino into a hydroxyl group.
Intermediates for the Synthesis			
U. S. 2,229,573 G. 683,908 Add. to G. 676,198	Jan. 21, 1941 Nov. 18, 1939	F. Jung Ass. to Merck	Trimethyl-hydroquinone from trimethyl-quinone by catalytic hydrogenation.
U. S. 2,229,574	Jan. 21, 1941	F. Jung Ass. to Merck	Xylohydroquipone by catalytic hydrogenation of xyloquinone.
U. S. 2,259,936 G. 676,198	Oct. 21, 1941 May 30, 1939	F. Jung Ass. to Merck	Duro-hydroquinone from duroquinone by catalytic hydrogenation.
B. 508,292	June 28, 1939	Hoffmann-LaRoche	Manufacture of phytyl-bromide.
B. 537,793	July 7, 1941	Hoffmann-LaRoche	Condensation of trimethyl-hydroquinone with acetyl-phytol to yield phytyl - trimethyl - o - mono - acetyl-hydroquinone.

Patent No.	Date	Patentee	Abstract
Derivatives and Utilization			
U. S. 2,167,002	July 25, 1939	A. J. Pacini Ass. to U. S. Vitamin Corp.	Composition of matter claims for vitamin E and magnesium distributed therein in an amount not less than 0.05%.
U. S. 2,212,531	Aug. 27, 1940	F. v. Werder Ass. to Merck	Process claims for the reaction of durohydroquinone with long chain alkyl halides, alkylene halides and hydro-aromatic halides to form compounds of the type $C_6(CH_3)_4(OH)OR$, R = 6–14 carbons. Product claims for the latter compounds with vitamin E activity.
U. S. 2,212,532	Aug. 27, 1940	F. v. Werder Ass. to Merck	Process claims for the reaction of trimethyl-hydroquinone with long chain alkyl halides, alkyene halides and hydro-aromatic halides to form compounds of the type $C_6H(CH_3)_3(OH)OR$, R = 6–14 carbon atoms. Product claims for the latter compounds with vitamin E activity.
U. S. 2,216,841 B. 506,569 B. 527,006	Oct. 8, 1940 May 31, 1939 Sept. 30, 1940	O. Isler Ass. to Hoffmann-LaRoche	Composition of matter claims to duro - hydroquinone - mono - phytyl-ether and process for preparation by reacting durohydroquinone with a phytyl halide in the presence of an alkali metal carbonate
U. S. 2,231,125 B. 536,602	Feb. 11, 1941 May 21, 1941	P. Karrer Ass. to Hoffmann-LaRoche	Composition of matter claims for tocopherol-oleate and stearate
U. S. 2,235,884	Mar. 25, 1941	W. John and O. Dalmer Ass. to Merck	Process for the manufacture of monoethers of trimethyl-hydroquinone by treatment with aliphatic or aromatic, saturated or unsaturated alcohols, esters or halides. Product claims for the octadecyl and nonadecyl mono-ethers.
U. S. 2,245,480 B. 529,023 G. 702,491	June 10, 1941 Nov. 12, 1940 Feb. 8, 1941	P. Karrer Ass. to Hoffmann-LaRoche	Process for the manufacture of condensation products from beta-tocopherol and alpha, beta-unsaturated alkyl-halides.
U. S. 2,247,364 G. 704,171	July 1, 1941 Mar. 25, 1941	E. Fernholz Ass. to Merck	Mono- and di-alkyl-ethers of duro-hydroquinone.
B. 517,932	Feb. 13, 1940	Ciba	Manufacture of mono-ethers from alkylated para-dihydroxy-benzenes and aliphatic alcohols containing 10 or 11 carbon atoms forming a branched carbon chain to which may also be linked an alicyclic residue. Also esterification of the free hydroxyl group of the reaction product.
		VITAMIN H	
U. S. 2,193,523	Mar. 12, 1940	F. Schultz Ass. to Winthrop	Vitamin H is liberated from tissue protein by hydrolysis at elevated temperatures and extraction with organic solvents

Patent No.	Date	Patentee	Abstract
U. S. 2,202,307	May 28, 1940	L. E. Booher	Concentration of vitamin H by adsorption of an alcoholic solution on activated carbon followed by elution with alcohols, benzene-alcohol or acetone.
B. 459,524 G. 645,414	Jan. 11, 1937 May 27, 1937	I. G.	Purification of vitamin H preparations by electrodialysis.
B. 463,698 G. 664,088 G. 651,435	April 5, 1937 Aug. 19, 1938 Oct. 15, 1937	F. Schultz Ass. to I. G.	Extraction of vitamin H from animal organs by energetic hydrolysis followed by acetone addition which precipitates impurities and leaves the vitamin in solution.
B. 484,981 F. 824,878	May 9, 1938 Feb. 17, 1938	I. G.	Treatment of seeds with biotin or derivatives in various carriers.
G. 661,929	June 30, 1938	I. G.	Biotin extracts from yeast, seeds, germs, egg yolk, etc., by precipitation of impurities in crude extracts by means of Reinecke-salt, picrolonic or oxalic acid or by acetylation. Precipitation of biotin with phosphotungstic acid or mercuric chloride.
G. 670,098	Jan. 11, 1939	I. G.	Precipitation of biotin with H_2PtCl_6.
G. 670,922 Continuation of 670,098	Jan. 27, 1939	I. G.	Isolation of biotin after previous esterification.

VITAMINS K

U. S. 2,233,279	Feb. 25, 1941	S. Ansbacher, E. Fernholz and M. L. Moore Ass. to Squibb	Process for concentrating vitamin K by adsorption on activated charcoal followed by elution.
B. 533,513	Feb. 14, 1941	St. Louis University	Isolation of vitamins K by adsorption on a base exchange silicate, *e. g.*, sodium aluminum silicate, followed by extraction with non-polar solvents, *e. g.*, benzene.
B. 539,471	Sept. 11, 1941	Parke, Davis	Manufacture of 2-methyl-4-aminonaphthol from 1,4-naphthoquinone or nitro-methyl-naphthalene.
B. 541,138	Nov. 13, 1941	Hoffmann-LaRoche	Manufacture of acid succinic acid esters of alkylated 1,4-naphthohydroquinones.

VITAMIN P

U. S. 2,224,807	Dec. 10, 1940	M. Brockmühl and E. Bartholomäus Ass. to Winthrop	Process for the production of glucosides of polyhydroxy-flavanones.
B. 486,898 Can. 398,341	June 13, 1938 July 29, 1941	A. Szent-Györgyi Ass. to Winthrop	Process for the extraction of flavones from vegetable material by means of an organic solvent miscible with water, precipitation with an alkali or earth-alkali-metal hydroxide followed by acid decomposition.

CHOLINE

Patent No.	Date	Patentee	Abstract
U. S. 1,904,696 B. 345,713	April 18, 1933 Jan. 13, 1930	G. Roy Ass. to Usines Rhône Poulenc	Solutions of acetyl-choline salts in ethylene glycol monoacetate or glycerol mono- or di-acetate.
U. S. 1,957,443	May 8, 1934	J. K. Cline Ass. to Merck	Salts of acetylated cholines.
U. S. 1,957,461	May 8, 1934	L. W. Jones and R. T. Major Ass. to Merck	Acetyl choline salts.
U. S. 2,049,463	Aug 4, 1936	R. T. Major and J. Kline Ass. to Merck	Salts of beta-alkylated choline alkyl ethers.
B. 8,031/14	Mar. 30, 1914	J. Y. Johnson	Salts of choline.
B. 379,260 F. 736,107	Aug. 25, 1932 April 29, 1932	F. Körner	Preparation of salts of choline.
G. 290,523	Aug. 12, 1913	Hoffmann-LaRoche	Non-hygroscopic salts of choline with dihalohydroxybenzoic acids.
G. 305,772	May 15, 1918	Chem. Werke Grenzach	Arsonium compounds of the choline type.
G. 590,311	Jan. 10, 1934	Merck	Quaternary salts of choline derivatives
G. 593,258	Feb. 23, 1934	E. Glucksmann	Choline salts of bile acids.
G. 638,641	Nov. 20, 1936	Hoffmann-LaRoche	Alcoholic solutions of hygroscopic salts of acetylcholine.

RECOMMENDED DIETARY ALLOWANCES[a]

Recommended by the Food and Nutrition Board, National Research Council

Group	Calories	Protein, g.	Calcium, g.	Iron, mg.	Vitamin A[c], I.U.	Thiamin (B_1), mg.[b]	Riboflavin, mg.	Niacin (nicotinic acid), mg.	Ascorbic acid, mg.[b]	Vitamin D, I.U.
Man (70 kg.)										
Sedentary	2500	...	...	...	...	1.5	2.2	15	...	...
Moderately active	3000	70	0.8	12	5000	1.8	2.7	18	75	
Very active	4500	...	...	...	...	2.3	3.3	23	...	...
Woman (56 kg.)										
Sedentary	2100	...	...	...	...	1.2	1.8	12	...	...
Moderately active	2500	60	0.8	12	5000	1.5	2.2	15	70	
Very active	3000	...	...	...	...	1.8	2.7	18	...	...
Pregnancy (latter half)	2500	85	1.5	15	6000	1.8	2.5	18	100	400–800
Lactation	3000	100	2.0	15	8000	2.3	3.0	23	150	400–800
Children up to 12 years:										
Under 1 yr.[d]	100/kg.	3-4/kg.	1.0	6	1500	0.4	0.6	4	30	400–800
1–3 yrs.[e]	1200	40	1.0	7	2000	0.6	0.9	6	35	[f]
4–6 yrs.	1600	50	1.0	8	2500	0.8	1.2	8	50	...
7–9 yrs.	2000	60	1.0	10	3500	1.0	1.5	10	60	...
10–12 yrs.	2500	70	1.2	12	4500	1.2	1.8	12	75	...
Children over 12 years:										
Girls, 13–15 yrs.	2800	80	1.3	15	5000	1.4	2.0	14	80	
16–20 yrs.	2400	75	1.0	15	5000	1.2	1.8	12	80	...
Boys, 13–15 yrs	3200	85	1.4	15	5000	1.6	2.4	16	90	[f]
16–20 yrs.	3800	100	1.4	15	6000	2.0	3.0	20	100	...

Footnotes to Table

[a] Tentative goal toward which to aim in planning practical dietaries; can be met by a good diet of natural foods. Such a diet will also provide other minerals and vitamins, the requirements for which are less well known.

[b] 1 mg. thiamin equals 333 I.U.; 1 mg. ascorbic acid equals 20 I.U.

[c] Requirements may be less if provided as vitamin A; greater if provided chiefly as the provitamin carotene.

[d] Needs of infants increase from month to month. The amounts given are for approximately 6–8 months. The amounts of protein and calcium needed are less if derived from human milk.

[e] Allowances are based on needs for the middle year in each group (as 2, 5, 8, etc.) and for moderate activity.

[f] Vitamin D is undoubtedly necessary for older children and adults. When not available from sunshine, it should be provided probably up to the minimum amounts recommended for infants.

Further Recommendations, Adopted 1942:

The requirement for *iodine* is small; probably about 0.002 to 0.004 milligram a day for each kilogram of body weight. This amounts to about 0.15 to 0.30 milligram daily for the adult. This need is easily met by the regular use of iodized salt; its use is especially important in adolescence and pregnancy.

The requirement for *copper* for adults is in the neighborhood of 1.0 to 2.0 milligrams a day. Infants and children require approximately 0.05 per kilogram of body weight. The requirement for copper is approximately one-tenth of that for iron.

The requirement for *vitamin K* is usually satisfied by any good diet. Special consideration needs to be given to newborn infants. Physicians commonly give vitamin K either to the mother before delivery or to the infant immediately after birth.

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SUBJECT INDEX

[*ABBREVIATIONS:* acty. (activity); defn. (definition); defcy. (deficiency); detn. (determination); dehydn. (dehydrogenation); hydn. (hydrogenation); oxidn. (oxidation); provit. (provitamin); vit. (vitamin).]

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