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J. H. LONGWELL, *Director*

Vitamins Required by the Chick

A HISTORICAL REVIEW

BY A. G. HOGAN

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A. G. HOGAN

Author's Note

Several reviews of poultry nutrition have been published and considerable duplication of subject matter is unavoidable. In the preparation of this one, an effort was made to include some historical material that others were forced to disregard because of space limitations. In turn it was necessary to omit some important publications from this review that had been emphasized elsewhere recently. It is hoped this statement will explain some unevenness in the choice of subject matter.

There may be a difference of opinion as to who first recognized the existence of vitamins, but no student in this field would ignore Eijkman's contribution. While stationed as a physician in the Dutch East Indies by the home government he observed experimental beri-beri in hens that consumed white or milled rice (Eijkman 1897). Within three of four weeks after the chickens were brought to the laboratory they would lose weight and develop the characteristic symptoms which he described in detail. If the food was raw rice chicks did not develop the disease, or if diseased ones were fed the raw rice, they recovered. It seemed probable to Eijkman that rice starch contained a deleterious component, and that in some of the layers of the rice bran there was a substance which protected the chickens from the toxin. Some of Eijkman's associates recognized that beri-beri was a deficiency disease. In time, Eijkman adopted that point of view. He (Eijkman 1906) mentioned that Grijns had shown that when raw rice or a legume or flesh was heated to high temperatures (120° C) it lost its protective action against beri-beri. Eijkman extracted rice bran with water and obtained a concentrate that healed or prevented chicken polyneuritis. Beri-beri, then, was due to the absence of an essential nutrient, a vitamin in modern terminology.

Partly because of the timing of Eijkman's publications, some years passed before their importance was recognized. Pasteur and Koch had shown not so long before this that many diseases were due to specific pathogenic bacteria, and medical thinking of that time was dominated by the idea that disease was due to the effect of a pathogenic microorganism

or of some other harmful agent. It was some years before Eijkman's interpretation received serious consideration. Funk (1911) obtained the antipolyneuritis vitamin in a high degree of concentration and the active component was later called the beri-beri vitamin. Funk's publications were important and in all probability they hastened materially the acceptance of the vitamin hypothesis.

The more important constituents of a simplified diet supplied to rats by McCollum and Davis (1913) were casein, salts, dextrine or starch, lard and lactose. One would expect rats to fail promptly on such a diet. However, they gained fairly well for some weeks, and then the gains came to a standstill. If egg-yolk fat was added to the diet the animals again gained in weight, which was usually maintained. The fat of milk had similar activity. It was concluded that these two food products contained an organic nutrient of unique importance. With the benefit of more recent knowledge we can explain the results reported by McCollum and Davis. The fats of egg-yolk and of butter contained vitamin A. The lactose in the diet was impure and was contaminated with thiamine and with some of the other water soluble vitamins in milk.

A paper was published a month later by Osborne and Mendel (1913a) which stated that rats failed to grow on diets of isolated protein, fat, carbohydrate and inorganic salts. When the salts were removed from the diet and protein-free milk included, a rat would grow well on this diet for 80 days, hold its weight for a time, then decline and die. If milk fat replaced the lard before serious damage was done, the rats started to grow immediately. Later on in this same year Osborne and Mendel (1913b) mentioned an infectious eye disease which healed when butterfat was included in the diet. Egg fat and cod liver oil were equally effective (Osborne and Mendel 1914. McCollum and Davis (1915) were probably the first to state specifically that the diet of the rat must contain two previously unrecognized nutrients. One, they observed, was in milk fat, the other in rice polishings and in skim milk. McCollum and Kennedy (1916) proposed the terminology fat-soluble A and water-soluble B.

We can say the vitamin hypothesis was established in 1913, but it was after 1920 before this hypothesis had much effect in the feeding of poultry. W. A. Lippincott published the third edition of his book, Poultry Production, in 1921. In chapter VII, "The Nutrients and Nutrition," there are barely three pages on the topic, "The Vitamins," including two fairly large photographs, which illustrate vitamin deficiencies. The effects of a deficiency of water-soluble B are shown in a photograph of a rooster who was "down on his legs." However, the toes on both feet are curled, and it seems probable to the reviewer that the photograph illustrates the

effect of a deficiency of riboflavin. Another photograph shows a swollen and closed eye in a hen, due to a deficiency of fat-soluble A. Lippincott mentioned the antiscorbutic vitamin, and said its relation to the nutrition of birds was not known.

There was some delay in applying the new knowledge of vitamins to poultry. The chick is much more exacting in its vitamin requirements than mammals, at least under practical conditions. When a diet is deficient in several vitamins at the same time, it is exceedingly difficult to determine the number of deficiencies, or to acquire any definite information about any one deficiency. We will need to turn to investigations with the rat later in this review in order to become familiar with the early history of nutrition of the chick. If useful experimental work was to be done with chicks it was necessary to impose some minimum of experimental conditions as to food intake and especially as to quarters, or environment. It turned out to be extraordinarily difficult to rear chicks in restricted quarters. Drummond (1916) brought chicks to the laboratory in the early summer of 1915, but in spite of all his efforts to provide a satisfactory diet, the chicks developed abnormalities, failed, and practically all died. Drummond decided it was impossible to raise chicks in restricted quarters. We do not know what the first limiting factor was in Drummond's diet. Presumably it was vitamin D, though there were complications.

Other investigators in this field were somewhat more successful. Buckner, Nollau and Kastle (1916) were familiar with the studies of Osborne and Mendel on lysine and they attempted in 1916 to determine whether or not there was any probability that the rations of chicks could be deficient in this amino acid. The mash in the ration that gave the best results was moistened with skim milk and presumably this was a very important precaution. The chicks were in a basement room but since the experiment began May 13, the windows were undoubtedly open and the chicks were exposed to the sun from the east and south. There were also ration supplements such as a little sod, sprouted oats and cabbage. At eight weeks the average weight of the chicks was 511 grams, but the mortality was 50 percent, with no explanation.

One cannot be certain why Buckner *et al.* were more successful than Drummond. It is possible that the corn in the grain mixture was yellow, and that this with the sprouted oats or cabbage may have supplied enough carotene to prevent disaster. We do not know the length of exposure to the sun. It may have been enough to give at least partial protection. The results show that the other requirements were met to some degree at least by the ration itself.

Osborne and Mendel (1916) had used rats in their demonstration that

lysine was indispensable and sometimes deficient. They were much interested in the report of Buckner, Nollau and Kastle and decided to restudy the indispensability of lysine, but to use chicks instead of rats. The chicks were brought to a well lighted basement room when they were about three weeks old. During the next 3 weeks the chicks were placed out of doors for a few hours in good weather, and a small amount of green food was supplied. After the chicks were 7 weeks old they were kept in the laboratory continuously and green food was discontinued. During this time the chicks ate large quantities of blotting paper and the authors thought this was of some importance. The rations were of the simplified type and contained corn gluten, lactalbumin, protein-free milk, starch, milk fat and lard. The conclusion of this trial was described in a subsequent publication. The experimental conditions were not rigorous; still the chicks grew slowly. Looking back it seems that the chief weakness in the ration was poor quality of protein and a deficiency of vitamin D. The supply of manganese may have been inadequate. Also (Osborne and Mendel 1918) two of the chicks described in the preceding paper finally attained weights of over 6 pounds. They were in the laboratory continuously nearly a year, in cages about 2 feet square. They had good feathers, they were normal in appearance, and they were fertile but leg weakness was common. These and other data indicate that Osborne and Mendel attained some degree of success.

Hart and collaborators at the University of Wisconsin had used rats, swine, and cattle in studies of the comparative nutritional value of cereals and concluded that wheat in livestock rations was mildly toxic and inferior to corn. Hart, Halpin and McCollum (1917) continued these studies with poultry. One set of rations contained corn and corn gluten, the other contained wheat and wheat gluten. When chickens were started on these rations at 3 or 4 pounds, on either corn or wheat rations, they grew slowly, developed no pathological symptoms and produced fertile eggs. They were of the opinion that chickens respond differently from rats or swine, when supplied with an all-wheat ration. Hart, Halpin and Steenbock (1917) were evidently disturbed by this observation that chickens were more resistant to wheat toxicity than mammals. They repeated the trial, with a different result. For example, on rations of wheat meal, wheat gluten, and minerals, the mortality rate was 100 percent. On rations of corn meal, gluten feed, and minerals the mortality rate was 33 percent. They concluded that wheat was toxic for chickens and that fowls did resemble mammals in this respect. In view of knowledge acquired more recently we would offer a different explanation today. The authors did not describe the corn, but we would say it was yellow, and the cryptoxanthine

in the corn kept the chicks alive.

Plimmer and collaborators (Plimmer, Rosedale, Crichton and Topping (1922a, b) were quite successful in rearing chickens in confinement. However, their diet contained cod liver oil, a yeast extract with some quantity of skim milk, and it must have been a fairly good source of vitamins. The chicks grew slowly by modern standards and there were some cases of leg weakness. However, the hens laid fertile eggs which hatched. Bethke and Kennard (1924-25) gave baby chicks a practical diet which contained cod liver oil and they grew as rapidly and developed as normally in confinement as outdoors. They concluded chicks had high fat-soluble vitamin requirements, but did not distinguish between A and D. As was well recognized at the time, the key to success was McCollum's discovery of the antirachitic agent, vitamin D. The modern poultry business rests in large degree on McCollum's discovery. Before that time the poultry flock brought pin money to the housewife. After the discovery the poultry business expanded into a large scale industry.

Important discoveries in the nutrition of the chick have been made with experimental diets of the practical type, but over the years the simplified type of diet has been much more useful. One of the early studies of this nature was carried out at the Missouri Station. Hogan, Guerrant and Kempster (1925) attempted to raise chicks on synthetic diets in laboratory quarters. Casein was the only protein in the basal diet and yeast was the source of the water soluble vitamins. Looking back it is clear that there were two major deficiencies in that diet. The chicks did not receive enough arginine, and they did not receive enough vitamin E when lard was the source of fat in the diet. Many of the chicks also developed the condition later designated perosis. This may have been due to an inadequate supply of manganese but more probably was due to an inadquate intake of choline. Whatever the cause, the diets became adequate, or at least were vastly improved, when fortified with 10 percent of dried egg yolk, 15 percent of dried liver, or when corn starch was replaced with polished rice. One would suppose that the chief contribution of polished rice was arginine. Egg yolk and liver, in varying degrees, contributed arginine, vitamin E, choline and probably manganese. During the next 25 years our research was largely confined to an effort to separate the unrecognized vitamins and other nutrients that were present in these protective foodstuffs. Liver seemed to be the most promising material for this purpose and it was used almost exclusively.

Hogan and Boucher (1933) prepared a diet that contained various liver fractions and in which it was supposed all essential vitamins were in soluble form. This diet was fairly adequate for the chick. Hens laid fer-

tile eggs which hatched and the offspring in turn attained maturity and came into egg production. Hogan and Richardson (1940) expanded somewhat on the report by Hogan and Boucher, and showed that the insoluble protein fraction of liver made a vast improvement in the diet of the chick as evidenced both by rate of gain and freedom from deformities.

After the discovery of folic acid, the unrecognized vitamin or vitamins required by the chicks could be obtained in a much more concentrated form (Richardson, Hogan and Karrasch, 1942). It became apparent later that vitamin B_{12} was the only important vitamin in the concentrate. Savage, O'Dell, Kempster and Hogan (1950) considered the possibility that the superiority of liver meal over casein was entirely due to unrecognized vitamins, but later work showed that it was explained by the higher percentage of arginine in liver meal.

It is easy to see now why the diets of Hogan, Guerrant and Kempster were improved so strikingly by the inclusion of liver. Its protein supplied arginine, and possibly enough manganese to be of some consequence. An ether extract contained vitamin E. The water or alcohol extract contained choline. As a matter of fact, liver, as a source material, played an exceedingly important part in the discovery of practically all of the water-soluble vitamins, except thiamine and ascorbic acid. In the investigations at Missouri the fractions of liver have been used almost continuously from 1924 up until the present time.

VITAMIN A

Osborne and Mendel had assumed that the chick required the fat-soluble factor, but it was several years before the requirement was actually demonstrated. Beach (1923) gave fowls a ration of grain and meat scrap which contained no yellow corn. All of them developed a disease previously known as avian diphtheria or roup. There were no cases when the basal ration was supplemented with cod liver oil or lawn clippings and the disease was ascribed to a deficiency of vitamin A.

A more intensive study of the requirement of the chick for vitamin A was published by Emmett and Peacock (1923) who began with day-old White Leghorn chicks and supplied them with simplified diets. Chicks that consumed the synthetic diet grew at the normal rate, laid eggs on the 187th day, and leg weakness was not common. The authors stated that when chicks were started before they were three weeks old there was a high incidence of rickets, or weak legs. If started after 3 weeks of age, the number of cases of rickets was relatively small.

When the vitamin A extract was omitted from the diet, ophthalmia appeared in about 83 percent of all the chicks. However, if the chicks were only 10 to 14 days old when they were given the deficient diet they often died within a week or 10 days before ophthalmia developed. Emmett and Peacock also noted a deposit of white material, probably urates, on the liver, heart and other organs. The kidneys had a network of fine white lines, probably urate-filled tubules. The two investigators treated 126 cases of ophthalmia, orally, with cod liver oil and various extracts. Sixty-three recovered (50 percent).

Relation Between Yellow Pigments and Nutrition

Before anyone suggested that the fat-soluble factor might be carotene, L. S. Palmer was investigating the pigments of some animal tissues, milk, and hen's eggs. Palmer and Kempster (1919) commented on the paper of Osborne and Mendel (1918) in which they said they had raised a few chicks on diets that contained no plant carotinoids. Palmer and Kempster pointed out that the diet of Osborne and Mendel did contain butterfat, which contained both carotene and xanthophyll. They undertook the task of making up a ration that was adequate for growth and was at the same time free from carotinoids. In their third investigation, the chicks were in a pen on a board floor covered with dirt and had access to a dirt yard, with no vegetation. The feed was white corn as scratch, a mash of white corn-meal, and white corn bran, with skim milk and bone meal and paper pulp *ad lib.* The chicks did fairly well for the first 6 weeks, though there

were a few deaths. They were then supplied with raw pork liver, but in no definite amount, and the chicks began to improve immediately. They made a complete recovery and seemed to be entirely normal except they were practically free of carotinoids. The females laid fertile eggs; the eggs were incubated and normal chicks hatched and survived. It was concluded correctly, that xanthophyll, the natural yellow pigment of the fowl, had nothing to do with nutritional status. Kline, Schultze and Hart (1932) came to the same conclusion.

Palmer, Kennedy and Kempster (1921) continued their investigation of the relation of yellow pigments to vitamin A and used growth and reproduction of rats as an index of vitamin A content. Carotinoid-free egg yolk, they found, was an excellent source of vitamin A. They also found that milk fat of the ewe was almost entirely free of carotinoids, but could be an excellent source of the vitamin. The amount of corn that was the equivalent of one gram of ewe's milk in vitamin A content contained several hundred times as much carotinoids as the milk. From this they concluded again that carotene was not vitamin A. Palmer and Kempster reported their facts correctly but in time their conclusions were modified.

Other investigators besides Palmer and Kempster had considered the possibility that there was some relation between yellow plant pigments and fat-soluble A, but apparently Drummond (1919) was one of the first to study a specific compound. He depleted three rats of vitamin A and then gave them a diet which contained about 0.003 percent of carotene. The average gain in 21 days was 27 grams, which one could regard as a definite, though not a maximum response. However, the author commented that the crude preparation contained slight traces of vitamin A, and he evidently thought carotene had no fat-soluble factor activity. Drummond was of outstanding ability and it is unfortunate that he was not more persistent in this study.

The next investigator to be mentioned is Steenbock who may have had his curiosity stimulated by the observation that wheat was inferior to corn as a source of nutrients. He (Steenbock 1919) reported that yellow corn was at least a fair source of the fat-soluble vitamin, and white corn was not, and suggested that the fat-soluble factor was identical with one of the yellow plant pigments or closely related to it. There were numerous publications on this subject for a few years, then there seemed to be a lull. as apparently everyone decided that any relation between the fat-soluble factor and carotene was accidental. There was a great revival of interest, though, when Euler, Euler and Hellstrom (1928) reported that carotene did have fat-soluble vitamin activity.

Looking back, there seem to be two reasons for previous failures to

identify carotene with the fat-soluble factor: (1) carotene is highly labile and the early investigators probably destroyed the vitamin A activity by oxidation. (2) Vitamin D had not been discovered at that time and in all probabiliy vitamin A deficiencies were complicated with vitamin D deficiencies. Rachitic animals would not respond to carotene and the early investigators were misled. Moore (1930) reported that when rats were depleted of vitamin A, the oil extracted from their livers did not give the $SbCl_3$ color reaction. If the depleted rats were then supplied with carotene, the liver oil did give the vitamin A color reaction. Thus, the conclusion was reached that carotene is the precursor of vitamin A.

VITAMIN D

Practically all of the early students of poultry nutrition made some brief mention of leg weakness in chicks on experimental diets. Hart, Halpin and Steenbock (1920) made an attempt to discover the cause. They gave chicks a simplified diet made up of casein 18, dextrin 69.3, butterfat 5,, salts 3.7, agar 2, and yeast 2. A few chicks attained an apparently normal maturity, but most of them would soon start to lose weight, and would die in 3 to 5 weeks. Presumably the chicks were reared indoors, though nothing was said on that point. The symptoms of malnutrition included unsteady gait and difficult locomotion with a tendency to assume a squatting position. The leg joints would swell and after a time the swelling became permanent. Since vitamin D was not added to the diet, rickets probably developed. The diet was probably deficient also in manganese, choline, arginine, and possibly in glycine. In addition, the 2 percent of yeast probably supplied several water-soluble vitamins in inadequate amount. The authors gave heavy emphasis to the lack of roughage, or to the wrong kind of roughage, for they seemed to think a lack of indigestible ballast was a major cause of leg weakness. This idea was soon discarded.

Hart, Halpin and Steenbock (1922) gave chicks a diet of white corn 97, calcium carbonate 2, and sodium chloride 1, with skimmed milk *ad. lib.* with no water. The chicks soon had a tendency to squat; their feathers became ruffled and they developed leg weakness. The mortality rate was high and at six weeks there was only one survivor. A few chicks were sacrificed for blood analysis and at four weeks the inorganic blood phosphorus was low, ranging from 1.07 to 2.80 per 100 cc. The inorganic blood phosphorus in the group that received cod liver oil ranged from 2.50 to 5.15 mg. per 100 cc. The authors mentioned that when 5 percent of cod liver oil was included in the diet the chicks grew at a satisfactory rate and no leg weakness developed. But the heaviest chick weighed 175 gm. at 4 weeks—about one half the average by modern standards. The authors concluded that when baby chicks were reared in confinement, an ample supply of the fat-soluble vitamin was important. They were uncertain as to whether the results with cod liver oil were due to the fat-soluble vitamin alone, or to this vitamin plus the hypothetical rachitic vitamin. They also concluded that the chick required more of the fat-soluble vitamin than the rat. We know now they were led to this conclusion because chicks are exceedingly susceptible to a deficiency of vitamin D. On a normal diet the rat does not require this vitamin at all.

It is not possible to be entirely fair in apportioning credit for the discovery of vitamin D, but a major share belongs to E. V. McCollum. He

and his associates (McCollum, Simmonds, Parsons, Shipley and Park (1921) described a number of rations, made up chiefly of cereal or legume seeds, which would produce disturbances in the growth and formation of the skeleton of young rats. The rats developed normally when 2 percent of cod liver oil was included in the diet and the authors were convinced that rickets was a consequence of malnutrition. In a later report (Shipley, Park, McCollum, Simmonds and Parsons (1921) they told of giving young rats diets that were deficient in fat-soluble A until they developed xerophthalmia, then including cod liver oil in the diet of some of them for periods of two, four, seven and eight days. At the end of the experimental period some of the long bones were split longitudinally, and stained with silver nitrate. It was seen that healing began when cod liver oil was supplied, and that calcium salts were deposited between the cells of the proliferative zone of cartilage. The deposit began as a thin line, at a right angle to the diameter, and the width of the line depended on the length of time cod liver oil had been consumed. This was developed into the "line test," an exceedingly important research tool in this field.

It was later shown (Shipley, Park, McCollum and Simmonds (1921) by the Johns Hopkins group that an important feature of their experimental diet was a low level of phosphorus. This feature is of no consequence when the diet is supplied to chicks.

The first critical demonstration that the rickets-preventing and the ophthalmia-preventing factors were different substances was published by McCollum, Simmonds, Becker and Shipley (1922). The diet used most for the line test was No. 3143:

	Diet 3143		
Whole wheat Kernel	33.0	NaCl	1.0
Whole maize Kernel	33.0	Wheat glutin	15.0
Gelatin	15.0	$CaC0_3$	3.0

This diet is low in phosphorus and it has a wide Ca:P ratio. It was shown that oxidized cod liver oil was ineffective in preventing xerophthalmia but it was still effective in preventing rickets. Coconut oil had some effectiveness in preventing rickets, but none in preventing xerophthalmia. After this paper appeared there remained no doubt that there was an antirachitic vitamin, separate from the xerophthalmia-preventing factor.

Ultraviolet Irradiation.

Huldschinsky (1919) demonstrated the healing of rickets in children by exposure to ultraviolet irradiation and Hess and Unger (1921) confirmed the report.

The acquisition of antirachitic activity by certain materials on ex-

posure to ultraviolet irradiation is an important part of the vitamin D story. It actually began many years before McCollum's discovery but Huldschinsky's publication started a search for the mechanism of activation upon irradiation.

One of the first attempts to find an explanation of the effectiveness of ultraviolet irradiation was stimulating but proved to be erroneous. Hume and Smith (1923) gave rats a diet that was deficient in vitamin D and kept them in glass jars with sawdust as bedding. On alternate days they removed the rats from the jars, which were taken to another room and exposed from above to ultraviolet irradiation for 10 minutes. The investigators then covered the jars with glass plates to prevent loss of irradiated air, took them back to the animal room, and returned the rats to the jars. They left the glass plates on the jars for another 10 minutes and then removed them. The experimental rats survived for a longer period than the controls which did not breathe irradiated air and the authors concluded that the air itself had acquired antirachitic properties.

Steenbock and Black (1924) soon demonstrated that Hume and Smith had misinterpreted their observations. They carried out about the same type of study and demonstrated that the rate of growth and the degree of calcification were about the same when the food was irradiated as when the rats themselved were irradiated. Lard and olive oil acquired antirachitic properties during irradiation. Hume and Smith (1924) recognized the error in their observations almost at once. They reported that when sawdust in jars was exposed to ultraviolet irradiation some of it becomes activated. Some of this was consumed by the rats in their earlier experiment and this explained the protection from rickets, and the cures, previously reported. When sawdust was not placed in the jars, irradiation of the jars was ineffective.

A very important contribution was made by Hess, Weinstock, and Helman (1925). They had shown previously that the constituent of oils which became rachitic- preventive when activated was in the non-saponifiable fraction. They prepared recrystallized cholesterol from brain and found that it had no antirachitic activity. It became active, however, on irradiation. The authors suggested that cholesterol in the skin was activated by ultra-violet irradiation which was transported later by way of the circulation. Later, investigators demonstrated that it was not cholesterol itself, but an impurity in the cholesterol that was a close relative, which was activated.

It developed in time that there was more than one vitamin D and it was several years before the more important facts were uncovered. Rosenheim and Webster (1927) reported that when cholesterol was purified by chemical procedures it could not be activated, and that ergosterol

was probably the impurity in cholesterol that was provitamin D. They stated that it was the most potent of the known antirachitic substances. When rats were supplied with irradiated ergosterol, in a daily dose of 0.0001 mg., rats on a rachitogenic diet would recover from rickets, or be protected from rickets.

It was not long until students of nutrition were able to supply irradiated ergosterol to chicks—then unexpected discrepancies were noted. Massengale and Nussmeier (1930a) gave chicks a rachitogenic diet. When irradiated ergosterol was supplied it seemed to raise the calcium of the blood serum, but it did not raise the inorganic phosphorus of the blood. A later report by these investigators (Massengale and Nussmeier 1930b) stated that cod liver oil, on a basis of rat tests, was much more active than ergosterol in preventing leg weakness in chickens. These reports were confirmed at once, and Waddell (1934) made an observation that soon led to an explanation of these discrepancies. He irradiated crude cholesterol and determined the number of rat units per unit weight it contained. This material was much more potent than irradiated ergosterol, when the same number of rat units from both sources was supplied to chicks, and these two sources of vitamin D are not identical.

It was apparent that the provitamin D which was active for the chick was more closely related to cholesterol than to ergosterol. Windaus, Lettre and Schenck (1935) prepared 7-Dehydrocholesterol from cholesterol and found it to be activated on exposure to ultraviolet irradiation. Windaus and Bock (1937) identified 7-dehydrocholesterol as the provitamin D in pigskin. Schenck (1937) prepared free crystalline vitamin D_3 and found that its activity for rats was 40,000 I.U. per mg. The same material was obtained from liver oil of the tunny fish, by Brockman and Busse (1938). They reported that is was identical with the vitamin D_3 obtained by Schenck.

Milas and Heggie (1938) were able to transform substantial quantities of cholesterol into 7-dehydrocholesterol, by suitable manipulations. When exposed to ultraviolet irradiation it acquired marked antirachitic properties. To complete the story of Vitamin D for the chick it will be necessary to retrace our steps somewhat. Hart, Steenbock, Lepkovsky and Halpin (1923) gave chicks their standard diet of white corn 97, calcium carbonate 2, NaCl 1, skimmed milk *ad lib,* and demonstrated that rickets could be prevented by exposing the chicks to sunlight.

In the same year Steenbock, Hart, Jones and Black (1923) aerated cod liver oil to destroy vitamin A and found that it was still effective in supporting growth, preventing leg-weakness or rickets, and maintaining normal levels of calcium and inorganic phosphorus in the blood. This paper

showed that the chick required vitamin D and confirmed the existence of the vitamin itself. In a subsequent study, this same group (Hart, Steenbock, Lepkovsky and Halpin 1924) found there was some improvement when yellow corn was substituted for white corn, though the results were still variable. Some of the chicks died quickly, but others grew fairly well for about six weeks, then declined. These chicks received some vitamin A but no vitamin D. If chicks on the white corn diet were exposed to sunlight they did well for about 14 weeks and then declined. These chicks received the equivalent of vitamin D but no vitamin A. If yellow corn replaced the white and there was also exposure to sunlight the chicks grew well continuously, without later failure, Evidently, the chick required both vitamin A and the antirachitic vitamin.

Numerous publications appeared which showed that window glass filtered out that portion of the spectrum which afforded protection against rickets. Only one of these will be mentioned: Wood (1926-27) gave chicks a rickets producing ration of yellow corn meal 97, $CaCO_3$ 2, NaCl, 1, and skim milk *ad lib.* (the Hart-Steenbock diet) and when chicks were exposed to sunlight filtered through window glass they developed rickets. If the light was filtered through a glass substitute rickets did not develop.

It was taken for granted during a considerable period that green feed was an excellent source of the antirachitic vitamin but it turned out that this was not the case. Hart, Steenbock, Lepkovsky and Halpin (1923) gave chicks a simplified basal ration and supplemented it with fresh green clover in such amounts as to add 5 percent of the material (on a dry basis). After a period of 23 days it was apparent that the chicks could not make significant gains in weight, or even survive, on this ration. Two of them were exposed to direct sunlight for 30 minutes daily. They improved and gained at an excellent rate. Thus the conclusion was reached that green feed was a poor source of the antirachitic vitamin.

Bethke, Kennard and Kik (1925) had the same experience; green clover, they found, was of no practical value as a source of vitamin D.

Reliable quantitative methods for the assay of vitamin D are important both for technical and for practical reasons. Bethke, Steenbock and Nelson (1923) developed the procedure now used in the determination of bone ash of both rats and chicks. The bones most commonly used are the femur or humerus of the rat, and the tibia of the chick. The bone selected is freed from adhering tissue, dried, crushed with pliers and then thoroughly extracted with ether and alcohol. The bone residue is then dried, weighed, and incinerated. The percentage of ash is calculated on the dry, fat-free basis. If the instructions are followed the data are an excellent index of vitamin activity.

VITAMIN E

Evans and Bishop (1922, 1923) were the discoverers of vitamin E. Female rats were the experimental animals and a typical diet was made up of casein 18, cornstarch 54, lard 15, butterfat 9, and salts 4, with 0.4 gm. of dried yeast daily for each animal. Animals which consumed this diet became sterile. The ovulation rate was usually normal, the ova were fertilized and the placental sign appeared on the fourteenth day. The placentae, however, were abnormal and the fetuses were usually completely resorbed. Evans and Bishop found that a factor, X, which prevented this type of female sterility in rats, was present in green lettuce leaves, beef muscle and wheat germ. Sterile females became fertile again if supplied with factor X.

Sure (1924) presented additional evidence for the existence of factor X and his suggestion that it be designated vitamin E was adopted almost at once. The isolation of vitamin E was accomplished by Evans, Emerson and Emerson (1936).

For some years after the discovery of vitamin E it was supposed that sterility was the only effect of a deficiency. However, Evans and Burr (1928) showed that when the vitamin E intake of female rats was restricted, many of their offspring were paralyzed. Somewhat later it was shown in other laboratories that when guinea pigs and rabbits were deprived of vitamin E they developed muscular dystrophy (Goettsch and Pappenheimer 1931; Mackenzie and McCollum 1940).

When the discovery of vitamin E, or factor X, was first announced, some investigators had difficulty in confirming the report. It was some time before the reason was apparent. We know now that 9 percent of butterfat in the diet used by Evans and Bishop would ordinarily be enough to maintain fertility, but factor X is readily destroyed by oxidation, especially in the presence of lard that has become rancid. It seems probable that the Evans-Bishop ration, when freshly prepared, was fairly well supplied with their factor X, but it was destroyed on standing by rancidity in the 15 percent of lard. Later on Evans, Emerson and Emerson (1939) made it a practice to let their experimental diets stand for two weeks at room temperature for the express purpose of destroying any vitamin E that might be present in their basal diet. At about this same time it was shown by Lease, Lease, Weber and Steenbock (1938) that when rancid fats and vitamin A were consumed together the vitamin could be destroyed before it left the stomach. It soon developed, according to Weber, Irwin and Steenbock (1939) that when the diet contains a rancid fat, any vitamin E it contains will soon be destroyed.

This destruction was an important source of difficulty in the earlier

studies on nutrition and in the years following it was subjected to intensive investigation. Mackenzie, Mackenzie and McCollum (1941) gave rabbits a diet supplemented with alpha-tocopherol and there were no symptoms of a deficiency. However, if cod liver oil was supplied along with the alpha-tocopherol the deficiency symptoms did develop. Presumably, the alpha-tocopherol was destroyed in the alimentary tract by cod liver oil, when they were consumed together.

Some of the effects of a deficiency of vitamin E had been observed in the chick long before they were observed in guinea pigs. For example, the deficiency of vitamin E in the basal rations of Hogan, Guerrant and Kempster (1925) was the most important single cause of the mortalities and growth failures they observed. However, the symptoms of a deficiency of vitamin E in the chick often resemble the symptoms caused by a deficiency of the antineuritic vitamin, and the investigators erroneously ascribed the abnormalities to this condition.

It is probably correct to say the story of vitamin E in chick nutrition begins with Pappenheimer and Goettsch (1931). They used a simplified diet, which contained 3 percent of lard and 2 percent of cod liver oil. After the chicks had consumed the experimental diet for three or four weeks, there would suddenly appear characteristic symptoms of a deficiency such as prostration, head retraction, flexed claws and clonic spasms of the legs. Examination at autopsy regularly disclosed degeneration in the cerebellum, rarely in the cerebrum. The variability on the experimental diet was enormous, as out of 19 chicks, 4 did not develop any deficiency symptoms. The authors made no suggestion as to the cause of the abnormality, though they had prepared the experimental diet for the purpose of studying the effect when chicks were deprived of vitamin E. The condition was later designated as nutritional encephalomalacia in chicks (Pappenheimer, Goettsch and Alexieff 1933). When this condition develops in either the cerebrum or cerebellum there is an increase in the weight and moisture content of the part affected as was pointed out by Goettsch and Pappenheimer (1936a, b). The authors concluded that the activity was not due to vitamin E. The most important bit of evidence they had for this conclusion was failure to prevent encephalomalacia by including feedstuffs in the diet that were good sources of vitamin E. Thus, when wheat or wheat by-products were added to the diet there were still a number of the abnormalities. Our explanation now would be that vitamin E in the diet was destroyed by the combination of cod liver oil and lard.

Apparently the first investigators to actually try vitamin E itself on chicks were Dam, Glavind, Bernth and Hagens (1938). They gave chicks

one of the diets of Pappenheimer and Goettsch and supplemented it with synthetic alpha-tocopherol. A daily dose of 7.5 mcg. per gm. body weight gave complete protection. Pappenheimer, Goettsch and Jungherr (1939) obtained complete protection with 1 mcg. per gm. body weight, but apparently the minimum protective dose had not been established.

During their investigations on vitamin K, Dam and Glavind (1938) observed a syndrome described as exudative diathesis. Large amounts of a clear fluid accumulated in the subcutaneous connective tissue, especially on the breast and abdomen. This fluid was rarely observed in the peritoneal cavity. In a later report these same workers (Dam and Glavind 1939) demonstrated that the exudative diathesis was prevented by alpha-tocopherol. According to Pappenheimer, Goettsch and Jungherr (1939) nutritional encephalomalacia occurs in farm flocks, but the incidence is highly variable. It was estimated that the mortality in affected flocks varied from 0.13 to 42.8 percent with an average of 7.9 percent. Various natural feedstuffs have been studied as sources of the anti-encephalomalacia factor (Pappenheimer, Goettsch and Jungherr 1939). The cereals were not highly effective, and green feed in small amounts gave incomplete protection. Meat scraps and a small amount of beef liver did not prevent the disease. The protective effect of natural foodstuffs was not reduced by autoclaving or by treatment with ethereal ferric chloride. Vegetable oils, including corn, and especially cottonseed, peanut, soybean and wheat germ were highly effective, especially when lard was not included in the diet. The activity of the oils was recovered in the non-saponifiable fraction.

VITAMIN K

Dam (1929) attempted to use chicks in a study of cholesterol metabolism, and supplied them with a simplified diet of casein, soluble starch, marmite, salts and filter paper, fortified with vitamins A and D. Since the diet was deficient in several factors the chicks did not thrive, but after about 3 weeks they developed characteristic hemorrhages recognized later as due to a specific nutritional deficiency. The chicks died when about four weeks old. The hemorrhages were both subcutaneous and intramuscular and appeared on the head, neck, back, breast, legs, wings and in the alimentary tract. Anemia was mentioned also but we know now it was a consequence of hemorrhage. The chicks also developed gizzard erosion, edema, and perosis, but these symptoms are not due to a deficiency of the antihemorrhagic vitamin.

McFarland, Graham, and Hall (1931) attempted to prepare a basal synthetic diet for chicks that would provide all the vitamins these animals required. The object was to compare proteins from different sources, but all of the rations were failures. Numerous symptoms were described and one of the most prominent was anemia with an average of about 6.3 percent hemoglobin. In some respects one of the most important symptoms was extensive hemorrhage, for example when the wing bands were inserted.

Holst and Halbrook (1933) were among the first, if not actually the first, to recognize that the hemorrhagic syndrome was the result of a specific vitamin deficiency. They were studying the nutrition of chicks and gave them a ration made up of ground yellow corn, fish meal, yeast, ground oyster shell, and either sardine oil or cod liver oil. The authors stated that the chicks developed a disease which resembled scurvy, and that the external symptoms were first observed when the chicks were about 3 weeks old. Gizzard erosion was also a very common symptom. The amount of hemoglobin in the blood was extremely low but, though the authors did not say so, this could have been due to loss of blood by hemorrhage. The chicks would recover, after symptoms had developed, if they were given a small amount of cabbage. The authors were unable to identify the antihemorrhagic substance correctly but, presumably, Dam, McFarland *et al.* and Holst and Halbrook had all observed the same condition.

After some apparent delay, Dam (1934) realized that the hemorrhagic disease was of nutritional origin and that it was caused by a deficiency of an antihemorrhagic factor which was not vitamin C, and which was present in cereals and seeds. When a powdered grain mixture was substituted for the sucrose in the basal diet there were no abnormal symptoms, (Dam and Schonheyder 1934). The symptoms of a deficiency of vitamin K are

most severe when the chicks consume a diet that permits rapid growth (Almquist, and Stokstad 1935). Dam (1935a) concluded that the hemorrhagic disease was the result of a deficiency of a fat-soluble vitamin and suggested that it be called vitamin K. This designation was suggested by the spelling in German, Koagulations-Vitamin. This same author (Dam 1935b) elaborating on these observations in another publication, observed that hog liver fat, hemp seed, tomatoes, kale and in lesser degree the cereals, were excellent sources of the new factor.

Schonheyder (1935) reported that there was no increase in the amount of antithrombin in the plasma, and no decrease in the amount of thrombokinase in the tissues of affected animals. However, normal plasma does contain an agent that shortens the clotting time of blood from affected animals. A deficiency of prothrombin was not mentioned. The experimental details were described in another publication by Schonheyder (1936).

The correct explanation of the biochemical defect in the hemorrhagic disease was supplied almost at once from the same laboratory group (Dam, Schonheyder, and Tage-Hansen, 1936.) When the deficiency of vitamin K is extreme, the amount of prothrombin in the plasma may be less than 2 percent of the normal amount (Schonheyder, 1938). Tidrick, Joyce, and Smith (1939) reported that when chicks consumed a diet that was deficient in vitamin K the amount of prothrombin declined from 58 units per cc. of plasma on the first day, to about 20 units per cc. on the seventh day, and to about 10 units on the 10th day. They reported that the clotting time of the blood was not prolonged until the prothrombin content fell to about 30 percent of the normal amount. Chicks did not become hemorrhagic until the prothrombin content fell to about 10 percent of the normal amount. According to Tidrick, Stamler, Joyce, and Warner (1941) the blood prothrombin fell to that level in about 13 days when chicks consumed a vitamin K-free diet from the time of hatching. The amount of prothrombin in the blood of newly hatched chicks is about 40 percent of the quantity present in the blood at maturity. Chicks with hemorrhagic disease do not have abnormal livers (Emmel and Dam, 1944).

The diets most commonly used in studies of vitamin K are usually similar to one that was described by Almquist and Klose (1939d), made of ether extracted fish meal 17.5, ether extracted brewer's yeast 7.5, ground polished rice 72.5, salt with added iron and copper 1.0, calcium carbonate 0.5, and cod liver oil 1.0. Inspection indicates that this diet is at least reasonably adequate, except for the absence of vitamin K.

Almquist and Stokstad (1936) demonstrated that the feces of chicks contained vitamin K and that in experimental studies it was essential to

prevent coprophagy. Vitamin K will be synthesized by bacteria and it is necessary to adopt procedures that retard or prevent bacterial contamination in food, or water, or in the cages. The amount of vitamin K in the tissues of chicks is low, regardless of the amount consumed (Dam, Galvind, Lewis, and Tage-Hansen 1938). The kidneys, white muscle, and abdominal fat were the poorest sources. The red muscle, gizzard, and spleen contained 15 units per gm. of dry matter.

The liver of young chicks is practically devoid of vitamin K (Almquist and Stokstad 1936). Hog liver contains considerably more; green leaves are an excellent source (Dam and Glavind 1938). A diet which contains 2 percent of soybean oil meal is adequate in vitamin K, but 1 percent is not enough (Almquist and Stokstad 1937).

The first announcement of antihemorrhagic activity in a compound of known structure was published by Almquist and Klose (1939a) who stated that phthiocol at a level of 10 mg. per kg. of a K-deficient ration, was sufficient to maintain the blood clotting time at a normal value. However, its activity was of a low order. The simpler naphthoquinones were investigated immediately and Almquist and Klose (1939b) and Ansbacher and Fernholz (1939) reported that 2-methyl-1, 4-naphthoquinone had antihemorrhagic activity. For convenience this compound has been designated as menadione and as vitamin K_3.

Several groups of investigators isolated at least one form of vitamin K, but only one will be mentioned, McKee, Binkley, MacCorquodale, Thayer and Doisy (1939). They isolated vitamin K_1 from alfalfa and vitamin K_2 from putrefied sardine meal. E. Fernholz and Ansbacher (1939) observed the 2 mcg of vitamin K_1 gave about the same effect as would 2 mg. of phthiocol. According to the estimates of Almquist and Klose (1939c), the physiological effect of 1 mcg. of 2-methyl-1, 4-naphthoquinone was about the same as that of 4 mcg. of vitamin K_1. All estimates of this type are variable.

VITAMIN C

Scurvy in man has been known for many centuries but it is permissible to say its modern history began with Holst and Frolich (1907). They observed that when guinea pigs consumed a cereal diet they developed symptoms that were characteristic of scurvy in man. After the vitamin hypothesis was accepted some investigators began using the term "antiscorbutic vitamin", but others objected and it was some time before its existence was commonly acknowledged. This vitamin soon was shown to be an essential nutrient for guinea pigs, monkeys and men. Drummond (1919) stated that the antiscorbutic factor is a nutrient for the rat, which was a mistake. However, he suggested the terminology, water-soluble C, or antiscorbutic factor.

As a rule the evidence that dietary ascorbic acid is required by poultry, is either negative or doubtful. The nutritional status of chicks was not improved by including in the diet either orange juice (Hart, Halpin and Steenbock 1920) or tomato juice (Mitchell, Kendall and Card 1922-23). Plimmer, Rosedale and Raymond (1923) reported that a diet of white rice and dried skim milk, supplemented with a yeast extract and cod liver oil but devoid of ascorbic acid, was adequate for the chick from hatching until they were 14 months old. The hens laid eggs, and though the hatchability was low three chicks survived to maturity. Hauge and Carrick (1926a) had a similar experience. When the chicks were about 5 months old some of them were sacrificed and the livers and kidneys were administered to scorbutic guinea pigs. They recovered, showing that chicks synthesize vitamin C, and that they thrive on diets that do not contain the vitamin. The livers of pullets contain as much ascorbic acid when the diet is deficient in vitamin A as when it contains an abundance, therefore a deficiency of vitamin A does not interfere with ascorbic acid synthesis (Rubin and Bird 1943) It has been reported that the ascorbic acid content of chick blood is about 2 mg. percent, with a range of from 1.5 to 2.4 percent (Holmes, Tripp and Satterfield (1938)

Dietrich, Nichol, Monson and Elvehjem (1949) omitted folic acid from their basal diet and chicks which consumed it grew slowly. If either ascorbic acid or vitamin B_{12} was included in the diet the rate of gain was accelerated, and when the two supplements were included simultaneously the acceleration was still more rapid. The synthesis of folic acid *in vivo* is stimulated by both ascorbic acid and vitamin B_{12}. This same group (Dietrich, Monson and Elvehjem. 1950) repeated an earlier statement that the rate of gain of chicks was accelerated by including vitamin C in the ration. However if ascorbic acid and sulfasuxidine were added simultane-

ously there was no acceleration. The inclusion of vitamin C in the ration increased the amount of folic acid stored in the liver, but this increase did not occur if sulfasuxidine was included simultaneously with the ascorbic acid.

March and Biely (1953) gave chicks diets of practical feedstuffs, which supported a moderate rate of growth. Again, under some circumstances there was an acceleration in the rate of gain when ascorbic acid was included in the diet. It seemed that there were interrelations between certain dietary components, ascorbic acid, folic acid, fat and antibiotics such as aureomycin and penicillin. However, these interrelations seemed to be complicated and probably of little practical importance.

As was mentioned elsewhere (Hogan 1949-50) there are reports that vitamin C may raise the level of hemoglobin in the blood, accelerate the rate of growth of chicks and prevent some of the ill effects of heavy egg production by hens.

Thiamine

As was recounted briefly in the introduction, many would say the vitamin concept began its development with a deficiency of thiamine. One could also say that thiamine was the first vitamin to be discovered and unless carotene is classified as a vitamin it was the first to be isolated in a pure state. If all areas of the world are included, this vitamin is the one that is most frequently deficient in the diet of human beings. From all points of view, then, historical, sentimental, theoretical and practical, thiamine is of the highest importance. There is a tremendous volume of literature concerned with this vitamin. However, there are fewer publications in the field of chick nutrition on thiamine than on any of the major members of the vitamin B-complex. This is partly because other laboratory animals were used in working out the more significant observations on thiamine, and partly because any practical chick diet supplies an abundance of the vitamin, hence it is of no practical importance in chick feeding.

The crystalline vitamin was first isolated by Jansen and Donath in 1926. Williams (1936) determined its structure and Williams and Cline (1936) described its synthesis, a heroic achievement.

Much of the pertinent literature on this vitamin was reviewed a few years ago (Hogan 1949-50) so bare mention here of some of the more important publications will suffice. In the early days deficiencies of thiamine were probably complicated with other nutritional deficiencies, and in some few cases the symptoms ascribed to a deficiency of thiamine may have been the symptoms of a multiple deficiency. The gross symptoms

are well known and have been described by Jukes and Heitman (1940). There is no degeneration of nerve tissue (Engel and Phillips 1938). There is a biochemical defect (Lipschitz, Potter and Elvehjem, 1938) which interferes with the utilization of pyruvic acid.

There are large differences between strains or breeds in the amount of thiamine required (Lamoreux and Hutt, 1939). All of the thiamine of eggs is in the yolk (Westenbrink and Van Leer 1940-1941). Some investigators assert all of the vitamin disappears during incubation, others assert there is no destruction.

RIBOFLAVIN

Almost immediately after McCollum's designation water-soluble B had been accepted, evidence appeared which seemed to show that it was a mixture, and not a single entity. Mitchell (1919) reviewed the pertinent literature on the antineuritic vitamin and on water-soluble B. He concluded from their properties that they could not be identical. Emmett and McKim (1917) gave pigeons a diet of polished rice until they came down with polyneuritis. When given the Seidell autolyzed yeast vitamin concentrate in addition to the polished rice diet, they recovered from polyneuritis and regained part, but not all, of the loss in weight. If pigeons with polyneuritis were given brown rice alone, and none of the concentrate, the pigeons recovered all of the lost weight. The authors explained this by assuming that the brown rice contained a new factor in addition to the antineuritic vitamin.

In view of what we know now this vitamin could have been riboflavin, pyridoxine, or pantothenic acid, or some combination of these. In this study Emmett and McKim tested two different preparations on one species of animal. Emmett was a notably cautious investigator and he next used two species of animals on the same test material (A. D. Emmett and G. O. Luros, 1920). Trials with pigeons showed that the antineuritic vitamin was completely destroyed when autoclaved for 2 hours at 120° C and 15 pounds pressure. However, trials with rats on a diet that contained unmilled rice as a source of water-soluble B seemed to show that this vitamin was not destroyed by autoclaving for 6 hours at a temperature of 120° C. This was interpreted as evidence that water-soluble B was a mixture of two factors. At this distance the results with rats seem surprising, but whatever the explanation the authors did reach a correct conclusion.

Hauge and Carrick (1926b) used only the chick as an experimental animal. They relied on two dietary constituents to show that the antineuritic and the water-soluble growth-promoting factors were separate and distinct entities. Ration 1 contained 30 percent of corn as a source of the water-soluble vitamin. The chicks that received it made only slight gains in weight, but none of them developed polyneuritis. This showed that corn contained the antineuritic vitamin but not the growth-promoting vitamin. We also know now that corn contains vitamin E, and this was a highly important constituent of Ration 1. Ration 2 contained 30 percent of a yeast fraction that had been prepared about six years previously, which offered an opportunity for deterioration, but it contained no corn at all. The average weight of the chicks on this diet, at 6 weeks, was 256 grams; therefore, yeast was a good source of the growth-promoting substance.

However, 60 percent of the chicks developed a condition designated polyneuritis by the authors. They concluded that yeast was an excellent source of the growth-promoting factor, but was seriously defiicent in the antineuritic vitamin. This, they felt, seemed to be conclusive evidence that the water-soluble factor consisted of at least two components. Hauge and Carrick were certainly correct, though the evidence probably is not as conclusive now as it seemed to be at that time. For example, both rations contained 2 percent of cod liver oil, and only the corn ration contained vitamin E.

It is reasonably certain, then, that the polyneuritic described by Hauge and Carrick on Ration 2 was really a symptom of a deficiency of vitamin E, and not of water-soluble B or thiamine. Vitamin E had been identified before this paper appeared, but no one at that time was aware that the chick required vitamin E, or that the symptoms of a deficiency had a superficial resemblance to polyneuritis. Under those circumstances it was natural to confuse a deficiency of vitamin E for a deficiency of the antineuritic vitamin, thiamin. Hogan, Guerrant and Kempster (1925), under similar circumstances, had been misled in the same way.

Smith and Hendrick (1926) used rats in their studies, which was a fortunate selection for that period. The rat is less exacting than the chick in nutritional requirements, and this choice avoided serious nutritional complications other than those under investigation. This is evident from inspection of the ration these authors used:

Casein	18	Olive Oil	8	Cod Liver Oil	2
Starch	67	Salts	4	Vit. B. Conc.	1

As would be expected, rats on the diet described above made practically no growth. If the vitamin B concentrate was omitted, and if autoclaved yeast was added, there was a slow loss in weight, and death in 3 to 4 weeks. If both the concentrate and autoclaved yeast were included in the diet, the rats made reasonable gains in weight. One essential factor in brewer's yeast was destroyed by autoclaving. This factor was identical with the one in the vitamin B concentrate. The other factor was not destroyed by autoclaving and when both were added to the diet, growth proceeded at a normal rate. We know now that autoclaved yeast contains more than one essential nutrient, but riboflavin was probably the one required first. Goldberger, Wheeler, Lillie and Rogers (1926) and Chick and Roscoe (1927) followed essentially the same procedure and obtained about the same result. Aykroyd and Roscoe (1929) gave rats a synthetic diet supplemented with cod liver oil and a concentrate of the antineuritic vitamin. Their rats developed a symmetrical dermatitis, which was healed by adding egg white to the diet.

By this time it was almost universally accepted that water-soluble B was a mixture. The American workers retained the term vitamin B for the antineuritic factor but the British called it vitamin B_1. The American workers called the growth-promoting or anti-dermatitis factor vitamin G and the British called it vitamin B_2. At present it is more commonly known as riboflavin. By the year 1935 the vitamin had been isolated from milk, liver, and egg white and had been prepared synthetically.

Requirement of the Chick for Riboflavin.

A review of early studies on chick nutrition shows, as one would expect, that the symptoms of a riboflavin deficiency were observed for some time before the cause was established. Hogan, Shrewsbury and Kempster (1928) observed two types of leg weakness. One was perosis, also mentioned by Hogan, Guerrant and Kempster (1925). The other was characterized by curled toes. It was cured or prevented by supplying the chicks with yeast. We know now that this condition was the result of a riboflavin deficiency. However, in a group of chicks on the same diet some would develop perosis, some curled toes, some both, and the Missouri group was unable to determine whether the ration was deficient in one, or more than one, vitamin. The investigators demonstrated again that liver meal was remarkably effective in preventing abnormalities and in sustaining a superior rate of growth in the chick.

Norris, Heuser, and Wilgus, (1930) encountered accidentally a deficiency of riboflavin while studying the metabolism of calcium and phosphorus. The practical protein supplements such as milk, meat scrap and fish meal contained too much calcium and phosphorus; therefore, the authors tried casein as a protein supplement. Their basal ration was made up chiefly of corn meal, wheat middlings and casein. A peculiar type of leg weakness appeared at the third week in the lot on the basal diet. The authors stated, "The birds could use their legs only with great difficulty and frequently walked upon their hooks. Their toes curled up and in certain cases the birds walked upon the distal extremity of the tarsometatarsus." There were three photographs and each showed curled toes. There were also other, less well defined types of leg weakness. The curled toes did not appear in groups that received a supplement of a milk vitamin concentrate along with the basal diet. The authors concluded that milk contained an unrecognized vitamin, required to prevent the type of paralysis described.

Hart, Kletzien, Scott and Halpin (1930) disputed this conclusion of Norris, Heuser and Wilgus concerning a new vitamin in milk. Although their chicks consumed large quantities of skimmed milk, they

thought the deformities they observed were similar to those described by Norris, Heuser and Wilgus and in all probability they were partly correct. It looks now as if both the Cornell and Wisconsin groups had a deficiency of manganese, or choline, or both. In addition, the Cornell group also had a deficiency of riboflavin. In reply, Norris, Heuser, Wilgus and Ringrose (1930-31) published four photographs which illustrate unmistakable symptoms of a riboflavin deficiency. Norris, Heuser and Wilgus were certainly correct in saying milk contained a new vitamin that was important for poultry. We know now, however, it was the same vitamin as was discovered by Smith and Hendrick, by Goldberger and by Miss Chick.

Lepkosvky and Jukes (1935a, 1936a) were probably the first to actually demonstrate the importance of riboflavin in nutrition of the chick. One experimental diet was made up chiefly of corn, wheat middlings, and casein; the other was of the simplified type. In each case the rate of gain was much accelerated when it was suppliemented with crystalline riboflavin.

The curled toe condition described by Norris and co-workers was observed also by Bethke, Record and Kennard (1931). Their typical diet was made up chiefly of corn, wheat and fish meal. Of the chicks on this diet, 20 to 30 percent developed the nutritional paralysis. There were none of these abnormalities when 5 percent of fish meal was replaced by 5 percent of dried buttermilk, and alfalfa-leaf meal and autoclaved yeast were also effective. Typical photographs of affected chicks indicate a deficiency of riboflavin. This same group (Bethke, Record and Wilder 1937) was probably the first to use crystalline riboflavine to demonstrate that curled toe paralysis can be cured or prevented by meeting the requirement of the chick for this vitamin. Additional details were supplied by Norris, Wilgus, Ringrose, Heiman and Heuser (1936); Bethke and Record (1942); Stokstad and Manning (1938); and Culton and Bird (1940). A number of other publications in this field were reviewed elsewhere (A. G. Hogan, 1949-1950).

PYRIDOXINE

Practically as soon as the existence of vitamin G, or riboflavin, had been established, evidence began to accumulate which showed that vitamin G itself was a mixture. Hogan and Hunter (1928) used both rats and chicks to demonstrate that the original vitamin B was a mixture of two components. The antineuritic vitamin was fairly stable to ultraviolet irradiation, the other was in large measure destroyed. It turned out (Richardson and Hogan 1936) that more than one vitamin was destroyed by irradiation but the first limiting factor was what is now known as pyridoxine. Rats that received the irradiated vitamin supplement developed a characteristic dermatitis on the feet, nose and ears. The essentials of these reports were confirmed by Gyorgy (1935b). The antidermatitis agent was designated vitamin H, but for various reasons this terminology was not widely accepted.

Chick and Roscoe (1929) developed a method of assaying for vitamin B_1 in which egg white was used as a source of protein, also of vitaman B_2. When young rats consumed this diet they grew for two or three weeks; then gains in weight came to a standstill for lack of vitamin B_1. If the rats were given the Peter's antineuritic concentrate they began to grow again and continued gaining at normal rate for several weeks, and then slowed down again. Chick and Roscoe concluded that the vitamin B complex contained at least one more component. This factor was present in autoclaved yeast, hence it was heat stable. In a later publication (Chick and Copping, 1930) the new vitamin was called factor Y.

Gyorgy (1934) gave rats a diet in which vitamin B_1 was provided in a yeast concentrate and vitamin B_2 in 10 mcg. daily of lactoflavine. The rats on this diet developed typical "rat pellagra," characterized by a symmetrical dermatitis. Gyorgy concluded that "rat-pellagra" was the result of a deficiency of a new member of the vitamin B-complex, which he called vitamin B_6. In a more detailed report (Gyorgy 1935a) one of the diets he described was made up of casein 18, rice starch 68, butter fat 9, cod liver oil 1, and salts 4. When crystalline vitamin B_1, crystalline riboflavin and crude vitamin B_1 concentrate were all added to the diet the rats grew fairly well. When rats were given vitamin B_1 and the crude concentrate they developed symptoms of a riboflavin deficiency. The most important symptom was a gradual thinning of the hair, especially on the back, chest and parts of the head. If the rats received vitamin B_1 and flavine, then skin lesions were the characteristic symptoms. Vitamin B_6 thus turned out to be identical with factor Y of Chick and Copping. Subsequently (Birch, Gyorgy and Harris 1935) the new vitamin was called

the "rat acrodymia" factor, but vitamin B_6 and pyridoxine (Gyorgy and Eckardt, 1939) are most commonly used now.

Pyridoxine Requirement of the Chick.

Investigations on the requirement of the chick for pyridoxine were comparatively late in getting started. Carter and O'Brien (1939) gave chicks a synthetic diet and each chick received a supplement of 20 mcg. of thiamine and 40 mcg. of riboflavin. Presumably. this was the daily dose, though the authors were not specific on that point. If this basal diet was supplemented with 20 mcg. of vitamin B_6, the chicks died in 28 days and most of them had developed dermatitis. If the diet was supplemented with a liver filtrate there was no dermatitis but the rate of gain was inferior. If the diet was supplemented with both pyridoxine and liver filtrate there was no dermatitis, the survival period was lengthened and the gains in weight were larger.

Jukes (1939b) prepared a basal diet which presumably was more complete than the one available to Carter and O'Brien. His diet 190 contained vitamins A, D, E, K, thiamine, riboflavin, nicotinic acid, and a pantothenic acid concentrate.

Three-fourths of the chicks on the basal diet died between the ages of 11 and 21 days. Their average weight did not reach 60 grams within that time. About 50 percent of the chicks developed deficiency symptoms, such as a jerky, abnormal gait and spasmodic convulsions. When 0.3 mg. of synthetic vitamin B_6 was added to the diet there were no deficiency symptoms and no deaths. Average weight at the end of three weeks was approximately 100 gm.

Hegsted, Oleson, Elvehjem and Hart (1939) followed a similar procedure and obtained a similar result.

There was little doubt that pyridoxine was required by the chick but it was impossible at that time to prepare a ration which was deficient in pyridoxine, and still contained all other important nutrients in adequate amounts. However, during the course of studies on another problem, Hogan, Richardson, Patrick, O'Dell and Kempster (1941) developed a simplified ration that permitted a satisfactory demonstration that pyridoxine was essential for the chick. Its essential features were liver residue, which supplied biotin and arginine, and a liver extract fraction which was comparatively free from pyridoxine. The growth rate on the basal diet was slow and as the symptoms became more acute the legs and wings would tremble. Death followed in a short time. The addition of 500 mcg. of pyridoxine to the diet reduced the mortality rate to zero and the rate of growth was vastly improved.

Lepkovsky and Kratzer (1942) also used a simplified diet, in which 15 percent of casein and 5 percent of gelatin were the chief sources of protein. Of seven chicks on the basal diet, two were dead in 3 weeks and the average weight of the survivors was 83.3 grams. There were no deaths in the group that received 0.2 mg. of pyridoxine in addition to the basal diet and their average weight in 3 weeks was 179.7 grams. The authors mentioned that the diet was not entirely adequate even when pyridoxine was included. We can not be sure of the complete explanation now, but it seems certain that the amount of protein was too low for the maximum growth rate. Furthermore, the amount of arginine was probably a limiting factor.

The nearest approach to a synthetic diet in studies of pyridoxine was described by Briggs, Mills, Hegsted, Elvehjem and Hart (1942). They prepared a diet which contained six different isolated vitamins, and a somewhat smaller than usual amount of the crude carriers of important unrecognized vitamins. In all probability, though, the diet was deficient in total protein and in arginine. When the diet contained 100 mcg. of pyridoxine per 100 gm. of diet, the chicks weighted 75 gm. at 3 weeks of age. When the diet contained 300 mcg., the average weight was 300 gm.

A similar diet (Cravens, Sebesta, Halpin, and Hart 1943) was used in studies of the requirement of laying hens for vitamin B_6. If no pyridoxine was added to the diet the eggs soon failed to hatch, and in a few days the rate of production dropped to zero. The effects of a deficiency of pyridoxine may be modified by other factors (Luckey, Briggs, Elvehjem and Hart, 1945) but there are reports of a decreased clotting time, hyperprothrombinemia, small spleens and anemia in chicks that received insufficient pyridoxine. The activity of pyridoxal and of pyridoxamine was somewhat lower than that of pyridoxine.

PANTOTHENIC ACID

Pyridoxine was the third member of the vitamin B-complex to be isolated, but some would say the story of pantothenic acid was the first of these two to begin. Wildiers (1901) observed over 50 years ago that yeast cells grew slowly in a medium which contained ammonium salts as the only source of nitrogen, but the cells multiplied rapidly if a small amount of a yeast extract was added to the culture. He supposed that the extract contained an unknown substance which was indispensable for the growth of yeast and gave it the name "bios." Williams (1919) observed that the yeast growth factor was adsorbed on fuller's earth, and then could be eluted again. He thought it was identical with the beri-beri-preventing vitamin and he was partly correct. In a continuation of his work (Willams and Roehm 1930) however, he became aware that the bios of Wildier was a mixture and the different varieties of yeast varied as to the ones that were required. Some required an unidentified substance which was not adsorbed on fuller's earth, and thus remained in the filtrate. This filtrate factor became quite important, for it turned out to be pantothenic acid.

Apparently the symptoms of a pantothenic acid deficiency in the chick were first described by Ringrose, Norris and Heuser (1931). They made up a series of rations in which yellow corn meal and wheat middlings were, quantitatively, the most important constituents and casein was the source of protein. These rations contained autoclaved yeast as a source of vitamins, with one exception which did not contain a vitamin supplement. When the diet contained 10 percent of yeast there were no cases of nutritional paralysis. When the diets contained 5 percent or less of autoclaved yeast the number of cases of paralysis was inversely proportional to the amount of autoclaved yeast. Presumably these cases of paralysis were due to a deficiency of riboflavine. When the diet contained 2.5 percent of autoclaved yeast, or none at all, pellagra-like lesions developed around the eyes, the corners of the mouth, and on the feet. It seems probable now that these cases of dermatitis were due to a deficiency of pantothenic acid. Ringrose, Norris and Heuser were of the opinion that the growth-promoting vitamin G and the antipellagric vitamin G were separate, and different, entities.

Kline, Keenan, Elvehjem and Hart (1932) used a basal ration much like the one described by Ringrose *et al.* but apparently their chicks did not develop dermatitis. The chicks gained at a fairly uniform rate, but the heaviest did not weigh over 200 gm. at four weeks and 300 gm. at six weeks. Ration 240 of Kline and co-workers was heated dry at 100° for

144 hours and was called Ration 240-H. Presumably this was the first report of the effect of dry heat on vitamins. The chicks on this ration failed to grow and they developed pellagra-like symptoms when about three weeks old, similar to those described by Ringrose, Norris and Heuser. Feathering was retarded and the feathers were ruffled. The lesions appeared around the eyes, the corners of the mouth, and on the legs and feet. "The crusty scabs at the corners of the mouth gradually enlarge and often involve the margins of the skin around the nostrils and underneath the lower mandible. The condition develops gradually and the animals often survive two weeks or more after the first appearance of the disease." When 6 percent of autoclaved yeast was added to Ration 240-H the chicks grew normally and there was no pellagra. It developed later that the lesions were due to a deficiency of pantothenic acid, and presumably this same deficiency explains the symptoms described by Ringrose *et al.* There is no ready explanation, though, for the fact that one group observed the symptoms only when the ration supplied to the chicks was heated. The antidermatitis agent was found in various liver fractions (Elvehjem and Koehn (1935). The active agent was not adsorbed on fuller's earth, but remained instead in the filtrate, as was the case with the "bios" fraction of Williams and Roehm (1930). Elvehjem and Koehn pointed out that their so-called antipellagric factor was not a flavin, and in all probability this was the pantothenic acid of today. They designated it as vitamin B_2 but this terminology was not accepted, and other laboratories used this designation for riboflavin.

The report of Elvehjem and Koehn was confirmed by Lepkovsky and Jukes (1935b) in all respects, by a similar procedure, and a little later these same investigators (Lepkovsky and Jukes 1936b) were able to make some improvements in the experimental diet. Lepkovsky and Jukes called the antidermatitis vitamin the "filtrate factor" from the mode of preparation described by Elvehjem and Koehn (1935). Later on they (Lepkovsky, Jukes and Krause 1936) used the order in which members of the vitamin B complex were adsorbed on fuller's earth to show that the third, or filtrate, factor was itself a mixture. The antineuritic vitamin (B_1) and vitamin G (or riboflavin) had been removed by adsorption on relatively small quantities of fuller's earth. When the adsorption procedure with fuller's earth was repeated, the authors' factor 1 was adsorbed. It was then eluted with barium hydroxide and concentrated. In the preparation of Factor 2 the liver filtrate was treated exhaustively with fuller's earth to complete the adsorption of Factor 1, and this last filtrate was concentrated to the original volume. This concentrate contained Factor 2, or the new filtrate factor. Rats were given the basal diet supplemented with vitamin B_1 and

flavin. When Factor 2 was added the rats grew slowly and developed dermatitis. If Factor 1 was now added, the animals grew and the dermatitis disappeared. Factor 1, then, was pyridoxine. When Factor 1 alone was included in the diet the rats did not grow and the eyelids became swollen, with a tendency to stick together. The eyes were watery and the nose was inflamed. If Factor 2 was now added, the animals begin to gain and the eye condition was prevented or cured. Factor 2, the filtrate factor, was pantothenic acid. The dermatitis in chicks which developed on heated diets was not prevented or cured by Factor 1. When Factor 2 was added to the heated diet the dermatitis was cured or prevented and the chicks again began to grow.

Another observation of Lepkovsky, Jukes and Krause may have had some bearing on terminology. They included in their basal diet a vitamin B extract of white corn prepared by the method of Goldberger and Lillie (1926). When the basal diet was given to rats there were practically no gains in weight, and they developed a dermatitis characteristic of a riboflavin deficiency. When the basal diet was supplement with a concentrate which contained Factors 1 and 2 there was only slight improvement in the rate of gain. When crystalline riboflavin was added in addition to factors 1 and 2 the rate of gain was excellent and the skin symptoms disappeared. It seems quite certain that Goldberger's original diet was deficient in more than one vitamin but riboflavin was the first limiting factor. His pellagra-preventing or P-P factor, then, was riboflavin, and this was the first vitamin G.

It is easy to see now why there was so much confusion about "pellagra-like" diseases and methods of prevention. There were five different syndromes in all that were characterized by skin abnormalities but at one time or another they were all ascribed to a single deficiency. Pellagra in man, due to a niacin deficiency, was the first one observed and, originally, each of the other four types of dermatitis in experimental animals was supposed to be a symptom of pellagra. Actually, none was due to a deficiency of niacin and this caused enormous confusion. Two of the others, one due to a deficiency of riboflavin and the other to a deficiency of pyridoxine, were observed only in the rat. Most of the experimental work leading to their identification was done with that animal. The other two, pantothenic acid and biotin, were accompanied by dermatitis in both the rat and chick and in each case the chick was useful, and probably most used, in their identification.

As an example, Koehn and Elvehjem (1937) continued their attempts to obtain the chick antipellagra factor in higher concentration, and when the final concentrate was supplied to chicks at a level of 0.7 mg.

daily, the pellagra-like symptoms were prevented. This same preparation cured dogs of black tongue, which made it seem probable that the symptoms were due to the same deficiency. However, Elvehjem, Madden, Strong and Wolley (1937) had shown that nicotinic acid was effective as a cure of black tongue in dogs and Mickelsen, Waisman and Elvehjem (1938) demonstrated that the symptoms were the result of different deficiencies. A few additional observations on the properties of the filtrate factor were described by Woolley, Waisman, Mickelsen, and Elvehjem (1938), and by Jukes (1939a).

Williams, Lyman, Goodyear, Truesdail and Holaday (1933) continued their attempts to identify various members of the Bios mixture. They prepared a crude fraction from a rice bran extract which was remarkably effective in promoting the growth of yeast. It was given the name pantothenic acid, taken from a Greek word meaning from everywhere. The authors suggested that it was one of the unidentified water-soluble vitamins and this suggestion soon became a certainty. Later on they prepared a salt of the vitamin, in a high degree of purity. Jukes (1939c) obtained a sample and showed that pantothenic acid is the filtrate factor, or chick antidermatitis agent. Essentially the same findings were published simultaneously by Woolley, Waisman and Elvehjem (1939). Williams and Major (1940) determined the structure of pantothenic acid and accomplished its synthesis.

NICOTINIC ACID

Nicotinic acid was actually discovered in 1867. It was recently shown to be a constituent of important enzyme systems, but from our point of view the most important discovery was made by Elvehjem and collaborators (Elvehjem, Madden, Strong and Woolley (1937). They discovered that canine black tongue was healed or prevented by nicotinic acid, or nicotinic acid amide, and it was shown almost at once that it would also heal or prevent pellagra in man.

The lesions of the skin of pellagrins are striking and of all symptoms developed during pellagra, these are probably most emphasized. It was commonly assumed at first that dermatitis in the chick or rat must have the same explanation as pellagra in man. However, it was soon realized that this assumption was erroneous. Dann and Subbarow (1938) carried out a similar study, with a similar result, but there was some indication that nicotinic acid had improved the nutritional state of the chicks that received it.

Dann and Handler (1941) reported that a fresh egg yolk contains 19 mcg. of nicotinic acid and the white contains 61 mcg., a total of 80 mcg. The tissues of a newly hatched chick contain about 820 mcg., a 10-fold increase. They concluded that the chick synthesized nicotinic acid and did not need a dietary supply. Nicotinic acid then would not be a vitamin for the chick. Snell and Quarles (1941) came to the same conclusion. It was supposed for some time that nicotinic acid was not a dietary requirement for the chick, but it was soon shown that on some types of diets the response of the chicks was vastly improved by supplying them with supplementary nicotinic acid. Briggs, Mills, Elvehjem and Hart (1942) gave chicks a basal diet which contained 18 percent of casein and 10 percent of gelatin. This combination as we now know contained an excess of some amio acids, probably glycine, and not enough tryptophan. The chicks developed symptoms of black tongue, which disappeared when the total amount of dietary nicotinic acid was raised to 0.8 mg. per 100 gm. of diet. One would conclude from the report of Briggs, Luckey, Tepley, Elvehjem and Hart (1943) that dermatitis will develop on the legs and feet of chicks if the deficiency of nicotinic acid is sufficiently severe. Chicks on the basal diet also developed perosis. It was shown later by Sarma and Elvehjem (1946) and Scott, Singsen and Matterson (1946) that under some circumstances the requirement for nicotinic acid is increased by including corn in the diet.

A partial explanation for some of the interrelations just described was provided by Briggs, Groschke and Lillie (1946). The weights were low

and there was a high incidence of black tongue and perosis on a diet they used that contained 18 percent of casein and 10 percent of gelatin. If this diet was supplemented with 5 mg.% of nicotinic acid, or with 0.2 percent of dl-tryptophan there was a large increase in the rate of gain, black tongue was eliminated, and perosis was reduced to a much lower level. Under some circumstances, then, trypotophan was a precursor of nicotinic acid. When the inhibitory effects of gelatin and zein were first observed it was supposed the damage caused was due to an imbalance of only one or two amino acids. It was shown, however, by Anderson, Combs, Groschke and Briggs (1951) that imbalances of practically all amino acids did have larger or lesser effects on the interrelation between nicotinic acid and tryptophan.

It seemed certain that under some circumstances the chick required dietary nicotinic acid, but there was some doubt as to whether there would be a deficiency under practical conditions. In an attempt to answer that question, Richardson, Hogan and Kempster (1945) diluted a ration of natural feedstuffs with a synthetic diet, in order to reduce the content of water-soluble vitamins to approximately 50 percent of the normal level. The chicks grew normally when the ration was fortified with the missing vitamins, but if nicotinic acid was omitted from the mixture the weights were reduced and the incidence of perosis was increased seven fold. Dermatitis and the inflammation observed by Briggs *et al.* were not observed. These data indicated that a deficiency of nicotinic acid in poultry production was possible, but not probable. However, Childs, Carrick and Hauge (1952) gave chicks a typical corn-soybean oil meal type diet and observed that chicks were unable to gain at the maximum rate unless the diet contained added niacin. The differences were not large or entirely consistent.

Usually newly hatched chicks are used in estimates of nutritional requirements, but Sunde (1955) estimated the requirement for niacin between the 6th and 11th weeks of age. The experimental diet was of the synthetic type commonly used and contained 18 percent of casein, 5 percent of gelatin and 0.3 percent of methionine. The data indicated that the amount of added niacin required for the maximum rate of growth was slightly over 7 mg. per kilo, but less than 12 mg. per kilo. By way of comment, the amino acid composition of the diet was not well adapted to the chick, and this imbalance may have increased the requirement for dietary niacin. It would be desirable to know whether dietary niacin is required when the protein mixture is of desirable biological value.

CHOLINE

Choline was isolated from hog bile nearly 100 years ago and not long after that its structure was determined and the compound was synthesized. It is present in most animal tissues, especially in the brain and central nervous system. Chiefly for that reason, it was studied intensively by biochemists over a long period of time. However, it was approximately 85 years after this compound was first isolated before it was discovered that it might be of nutritional importance. Best, Hershey and Huntsman (1932) gave rats a diet that provided each animal with approximately 2.5 gm. of fairly saturated fat for a period of 3 weeks and large amounts of fatty acids accumulated in the liver. This deposition did not occur in animals that consumed the same diet with added lecithin. When the hydrolytic products of lecithin were examined, only choline inhibited this accumulation of fat. Betaine seemed to have the same property. Likewise, when the diet of the rat contains large amounts of cholesterol there is an accumulation of fat in the livers, and this is prevented by including either choline or betaine in the diet (Best and Ridout 1933). This group published numerous papers on the physiology of choline and Best, Huntsman, and Ridout (1935) coined the term lipotropic to describe the effect of choline on the amount of fat in the liver. This could be a decrease in the rate at which fat is deposited, or an increase in the rate at which it is removed.

Another noteworthy series of papers was published by Griffith and collaborators which provided additional evidence that dietary choline is important (Griffith and Wade 1939, 1940, and Mulford and Griffith 1942). Typical synthetic diets contained casein 10 percent, and fibrin 5 percent, and young male rats which consumed these diets developed hemorrhagic kidneys with massive tubular degeneration, and in the more severe cases there were hemorrhages into the eyeballs. The abnormal symptoms were intensified by cholesterol and by cystine, but were alleviated or prevented by methionine, choline and betaine.

So far as we are aware now the lipotropic and antihemorrhagic effects are not of great practical importance for the chick, but some of the vitamin's other biochemical properties are of very considerable practical importance.

Probably the first serious study of choline in the diet of poultry was published by Abbott and DeMasters (1940). They began with week-old chicks and gradually transferred them to a basal ration made up of 70 parts of polished rice and 30 parts of skim milk powder. This diet was supplemented with vitamins A and D, and in addition with what the authors regarded as suitable amounts of vitamin B_1 and B_2. The pullets on the basal diet had a high mortality rate and fatty livers were common.

There were some aborted egg yolks and half the pullets did not lay at all. Some of them were supplied each day with 75 mg. of choline for each individual; they developed few abnormalities. These observations were not exploited and at the present time they seem to be largely forgotten. However, the suggestion that choline may be nutritionally important was not overlooked in some quarters and, as will be shown later, it probably hastened the solution of one of the important problems in poultry nutrition.

A Deficiency of Choline and Perosis.

Hogan, Guerrant and Kempster (1925) observed the most characteristic symptom of a choline deficiency, the condition now known as slipped tendon, hock disease or perosis, but a deficiency of manganese may have been an important contributing factor. The most common abnormality was shortening and thickening of the long bones, but curvature of the tarsometatarsal bone occurred frequently. In more extreme conditions, the tendon of Achilles was out of position and the tarsometatarsal bone was rotated in such a way that the toes pointed to one side. All of the early workers in chick nutrition must have observed some type of leg weakness, and most of them made some mention of the fact. However, it seems probable that most of these observers also assumed at first that all cases of leg weakness were the result of the same anatomical derangement or weakness and had the same cause. Gradually it became apparent that there were different types and causes of leg weakness. Hunter and Funk (1930) were probably the first to publish a specific description of the condition they described as hock disease or slipped tendon. A restudy of their diet indicates the perosis was a result of a lack of manganese but it may have been the result of a multiple deficiency. Hunter and Funk were well aware that the mineral composition of the diet was an important, if not the determining factor in the condition they described. It was called perosis by Titus (1932).

In all probability the Missouri investigators in this field did at some time or other encounter perosis which was the result of a manganese deficiency. However, the same year Hunter and Funk reported their findings, Hogan and Schrewsbury (1930)) found that their diet was not improved by the addition of the ash of wheat. This would seem to show that the leg weakness they observed was not due to a deficiency of any mineral element, though there is little doubt that it was identical with the perosis of Titus.

It seemed surprising, then, to the Missouri investigators when Wilgus, Norris and Heuser (1936) reported that perosis in the chick was a conse-

quence of a deficiency of manganese. This was an important discovery and easily verified on our rations. It was noted, however, that on simplified diets of certain types chicks would develop perosis regardless of the amount of manganese added. The Missouri diets contained liver fractions (Hogan, Richardson, Patrick and Kempster 1941) of the type shown below.

Alcohol Extract: Dry liver extracted with 95% alochol (contains choline)
Water Extract: Residue from alcohol extraction is extracted with hot water.
Liver Residue: The fraction remaining, insoluble in hot water.

It was observed (Hogan, Richardson and Patrick 1940) that chicks regularly developed perosis when only the water extract and the liver residue were included in the diet. When the alcohol extract was included with the other two, perosis did not develop. It was concluded that an organic factor as well as manganese was concerned in the perosis syndrome. We know now the organic factor was choline.

Jukes (1940a) used turkey poults to show that perosis may be prevented by choline and in a later publication (Jukes 1940b) he demonstrated that the chick also requires choline in order to prevent this abnormality. His basal synthetic diet contained 25 percent of casein and 5 percent of gelatin, which should be less deficient in arginine than were most of the diets previously used. The water-soluble vitamins were supplied by thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, and by 5% of dried yeast. The incidence of perosis at 32 days on the basal diet was 75%. When the ration was supplemented with 100 mg. of choline per 100 gm. of diet the incidence of perosis fell to 9%.

Hegsted, Mills, Elvehjem and Hart (1941) gave chicks a diet that contained casein 18%, cartilage 15% and yeast 10%, with added thiamine, riboflavin and pyridoxine; about 60% of the chicks developed perosis. This abnormality was almost completely prevented by including 0.1% of choline in the diet. Hogan, Richardson, Patrick and Kempster (1941) concluded that choline was not the only organic substance that was required for the prevention of perosis, a view that is now well established.

The voluminous literature on the functions of choline, and on its interrelations with other nutrients, is of interest to the biochemist, but limitations of space make it necessary to omit the publications in this field. Those who wish to pursue the subject further should review, among others, the publications of Almquist, Jukes, Bethke, Norris, and their various collaborators.

BIOTIN

It seems to have been the rule that before any one vitamin was discovered, a number of apparently unrelated observations were made which depended in some way on the same previously unrecognized nutrient. This is true of biotin, for several biochemical studies were described which seemed at first to have no connection with each other, but later work showed that all of them were concerned with the same substance.

Isolation of biotin. As mentioned before, Wilders (1901) attempted to discover what nutrients had to be present in a medium for yeast cells to survive and grow. He concluded that the medium had to contain a nutrient previously unknown and called it Bios, which means "life." When attempts were made to concentrate the active agent it turned out to be a mixture. One component was finally isolated in pure form by Kogl and Tonnis (1936). The enormous difficulty of their accomplishment is illustrated by the fact that from 250 kilograms of dried egg yolk they obtained a little over 1 milligram of the crystalline material.

A little before the isolation of biotin was achieved, Allison, Hoover and Burk (1933) discovered that some unknown substance was required for normal respiration of the root-nodule bacterium, Rhizobium. They concluded that the unknown factor was a coenzyme in the respiratory enzyme system, hence the name it eventually received, Co-enzyme "R".

The egg-white disease. A third observation developed more slowly. Batemen (1916) reported that raw egg-white was poorly utilized by rats, rabbits, dogs and man. The most prominent symptom mentioned was diarrhea. After the egg white was coagulated by heat it was well utilized and there were no symptoms of toxicity. Boas (1924a) included an imported dried egg-white preparation in a simplified diet for young rats. They grew well for about 5 weeks and then growth came to a standstill. This was accompanied by a rapid loss of hair and inflammation of the eyelids. If casein was substituted for the egg white the rats recovered and grew well. Boas concluded that egg white, even when it made up 20% of the diet, was inadequate for rats. Not long thereafter, she (Boas 1924b) published a brief note to the effect that her earlier observations on commercial Chinese dried egg white were not repeated when it was replaced with the coagulated whites of fresh hen's eggs. This fresh product was adequate in maintaining health and supporting growth. In a later publication (Boas 1927) she considered two possibilities: (1) An essential, unrecognized nutrient is destroyed by dehydration. This hypothetical substance was factor X. (2) A toxic substance is formed when egg white is desiccated and this toxin is responsible for the symptoms. The toxin then would be neutralized by factor X. Factor X is present in numerous foods

such as potato starch, yeast, milk, spinach and cabbage. As the amount of egg white in the diet increases the amount of factor X needed for protection increases also. Additional observations by this same author (Boas-Fixsen 1931) led her to the conclusion that her second hypothesis was correct, and that the process of desiccation resulted in the formation of a toxic substance in egg white. Her factor X neutralized this substance and liver was an excellent source of the protective factor.

Parsons and co-workers made many important contributions to our knowledge of the egg-white disease, beginning in 1931 and ending about 15 years later (Parsons 1931 and McGregor, Parsons and Peterson 1947). These investigators, and several others not cited specifically, reported that if egg white was heated at 80° C for 5 minutes it caused no damage. They also found that egg white that had been dried was more resistant to detoxification than the fresh material. If egg-white was subjected to peptic digestion, or to mild heating with hydrochloric acid it lost its toxicity. Purified ovalbumin was not toxic and there was no evidence that the trypsin inhibitor of egg white was responsible for the toxicity observed. Pork kidney, egg yolk and liver are excellent sources of the protective factor. The factor that prevents chick dermatitis (pantothenic acid) is not the same as the one that prevents the egg-white disease in chicks. The egg-white disease has also been described by Ringrose, Norris and Heuser (1931) and by Tully and Franke (1934).

Gyorgy (1931) stated that he had prepared a diet on which rats developed pellagra-like symptoms that were not prevented by any vitamin then recognized. Characteristic features were scaly dermatitis, erythema of the skin and seborrhea. Yeast and liver were good sources and Gyorgy prepared a concentrate which induced prompt remission of the symptoms. It was given the designation Haut faktor, or vitamin H. The diet was not described and there were practically no experimental details. However, a photograph of one of the animals indicates that Gyorgy had produced a biotin deficiency, and one would come to that conclusion by a process of elimination. Presumably Gyorgy had used a raw egg white diet. He (Gyorgy, Kuhn and Lederer 1939) attained a high degree of success in concentrating vitamin H from liver and at this point the problem of the anti-egg white factor was started on the way to a rapid solution. Gyorgy, Melville, Burk and du. Vigneaud (1940) recognized that the properties of vitamin H, of biotin, and of coenzyme R, were all similar. The distribution of these three factors was also similar, and liver and yeast were especially good sources of all three. They had two concentrates of vitamin H, one of which was almost 30 times as potent as the other. The ratio of biotin and of coenzyme R in these samples was almost exactly the same. It

seemed highly probable, therefore, that a single compound had been given three different names.

du Vigneaud, Melville, Gyorgy and Rose (1940) obtained a solution of 150 mcg. of crystalline biotin methly ester from Professor Kogl and administered it subcutaneously to rats that had developed symptoms of a vitamin H deficiency. The rats recovered on a daily dose of 0.1 mcg. daily for 30 days, a total of 3 mcg. Gyorgy, Rose, Hofmann, Melville and du Vigneaud (1940) isolated a crystalline compound from liver which had the same crystalline form as was ascribed to biotin by Kogl and this material was highly active as a source of vitamin H, and of coenzyme R.

The relation of egg white to biotin deficiencies was still obscure and the solution to this problem was provided by Eakin, McKinley and Williams (1940). They prepared two chick diets which were similar except for the protein concentrates. One diet contained 20% casein, the other 20% of dried egg white. The casein diet contained 0.39 mcg. of biotin per gm., the egg white diet contained 0.67 mcg. The tissues of the chicks on the egg white diet contained much less biotin than did the tissue of chicks on the casein diet, and this showed that the injury caused by egg white was due to the fact that the biotin was unavailable. Eakin, Snell and Williams (1940) demonstrated that biotin is inactivated in vitro by egg white, and then isolated the protein fraction that combines with biotin. Presumably this was the fraction responsible for egg-white injury. Woolley and Longsworth (1942) prepared an antibiotin factor from egg white, a protein fraction, which was 15,000 times as effective in inactivating biotin as was the fresh egg white from which it was prepared. According to the usual criteria this was a pure substance. Gyorgy and Rose (1942) concluded the amount of biotin in the egg yolk was insufficient to neutralize all of the avidin in the white. This group (Gyorgy, Rose, Eakin, Snell and Williams 1941) gave the name avidalbumin to the toxic fraction of raw egg white and prepared a concentrate which in one case amounted to 231 mg. from 100 gm. of dried fresh egg white. The biotin in the diet is fixed by the avidalbumin and is unavailable. Presumably, it is excreted in the feces. The name was later changed from avidalbumin to avidin (Eakin, Snell and Williams 1941). Additional details are found in another review Hogan 1949-1950).

Perosis in chicks is one of the consequences of a deficiency of biotin (Richardson, Hogan and Miller 1942). If laying hens receive an insufficient supply the hatchability of fertile eggs declines and there is a large increase in the number of deformed embryos. Hegsted, Oleson, Mills, Elvehjem and Hart (1940) observed incipient lesions in chicks after about 3 weeks on a simplified diet that contained casein 18%, cartilage 15% and a liver extract fraction 3 to 8%. Observations of Parsons have already been mentioned.

INOSITOL

Inositol is one of the bios factors but as a vitamin it is not highly important. Woolley (1940) reported loss of hair in mice that consumed a simplified diet and reported that the anti-alopecia factor was inositol. McIntire, Schweigert and Elvehjem (1944) gave cotton rats a synthetic diet and found that they grew much more rapidly when inositol was included. Hamilton and Hogan (1944) found no evidence that the rate of gain by hamsters was accelerated by adding inositol to the diet but in the absence of inositol, few females bore living young. Gavin and McHenry (1941) reported that the biotin type of fatty liver in rats was prevented by inositol, and Pavcek and Baum (1941) produced a spectacled eye condition in rats and obtained rapid and spectacular cures by the administration of inositol. There was also an acceleration of the rate of growth.

Publications were mentioned in another review (Hogan, 1949-1950) which stated that under prescribed circumstances perosis in chicks was prevented by the injection of inositol, and on some diets there was an acceleration in the rate of gain when inositol was included in the diet. One group of investigators stated that inositol was synthesized by the chick embryo, another could not confirm the statement. It has also been reported that the hatchability of hens' eggs declines if they are deprived of inositol. Chicks develop an exudative diathesis and encephalomalacia when deprived of vitamin E and there are claims that these abnormalities may be prevented or reduced in severity by including inositol in the diet.

When all the evidence is considered there can be little doubt that inositol is of some importance in animal physiology, and practically all investigators in poultry nutrition include inositol in their experimental diets. However, a limited study at Missouri yielded no evidence that inositol was important in poultry nutrition, and it is included in experimental diets as a form of insurance.

FOLIC ACID

There is some reason to suppose that the first studies on folic acid were conducted by Lucy Wills and coworkers (Wills and Stewart 1935. Wills, Clutterbuck and Evans 1937). She gave monkeys a diet that contained polished rice 40%, white bread 45%, margarine 15%, salt, cod liver oil, and tomato or carrot. There were various symptoms of ill-health, but the hemoglobin declined to about one-half the normal level and the number of both red and white blood cells decreased to approximately one-third the normal number. The condition was cured by both a yeast fraction and a liver extract but it was not cured by the liver fraction used to treat pernicious anemia.

Similar studies on men and women with a tropical macrocytic anemia were described by Wills and Evans (1938). Various extracts were administered with about the same response as had been previously reported for monkeys. Again, the liver extracts manufactured for the treatment of pernicious anemia were ineffective, but crude liver or autolyzed yeast extracts were effective. It was concluded that the active agent was probably identical with the one that prevented nutritional macrocytic anemia in monkeys and it was not identical with any of the vitamins identified at that time.

Vitamin M.

Day, Langston and Shukers (1935) gave a diet made up of casein, wheat, polished rice, cod liver oil, salts, sodium choloride and orange to monkeys and they developed the same symptoms described by Wills and collaborators. These abnormalities included anemia, diarrhea, gingivitis and leukopenia. In some cases the red cell count was one-fifth and the white cell count one-tenth of the normal number. The animals recovered, or remained healthy when supplied with brewer's yeast and presumably the unrecognized nutrient in yeast was a member of "the vitamin B complex."

The same symptoms appeared in monkeys that were supplied with the Goldberger diet (Day, Langston, Darby, Wahlin and Mims 1940) and they disappeared when a liver extract was added to the diet. The factor that prevented the nutritional cytopenia in the monkey was designated vitamin M (Day, Langston and Darby 1938).

Factor U.

Stokstad and Manning (1938) attempted to devise a diet that would be satisfactory for a riboflavin assay with chicks and noticed that yeast and

wheat middlings had a growth promoting effect not accounted for by their content of the filtrate factor or of riboflavin. It was concluded that a new growth factor was concerned and studies were begun on its distribution and chemical properties. A typical experimental diet contained washed fish meal, polished rice, a rice bran filtrate, a whey adsorbate, and thiamine, the more important constituents in a consideration of adequacy. Chicks which received Diet 1 grew slowly and in 5 weeks reached a growth plateau of about 150 grams. When the diet was reinforced with yeast the chicks grew at a normal rate. Other sources of the factor were wheat bran, sun-dried alfalfa leaf meal and rice bran. The growth factor could not be identified with any of the known vitamins and it was designated factor U. It developed a little later (Stokstad, Manning and Rogers 1940) that the experimental diet previously used was also deficient in pyridoxine, and the original factor U was a mixture. Subsquent studies leave little room to doubt that the activity of Factor U was chiefly due to a vitamin B_c conjugate.

Vitamin B_c.

As mentioned previously, the Missouri investigators had discovered that their simplified diets seemed adequate when supplemented with dried liver. Hogan and Parrott (1940) included in one diet that portion of a liver extract fraction that was soluble in 95% alcohol. This preparation improved the basal ration materially, as shown by lengthening of the survival period, but the chicks made negligible gains in weight. They also became extremely anemic, and the anemia was ascribed to a deficiency of an unidentified vitamin. This vitamin belonged to the vitamin B complex. It was essential for the chick, therefore it was designated as vitamin B_c. Improvements in the experimental technique were developed by O'Dell and Hogan (1943). The isolation of the vitamin in crystalline form was accomplished by Pfiffner, Binkley, Bloom, Brower, Bird, Emmett, Hogan and O'Dell (1943), also by Stokstad (1943). The synthesis of the vitamin was announced by the Lederle Laboratories in 1946 and it was given the name pteroylglutamic acid.

It soon became evident that, according to microbiological assay, yeast was a poor source of vitamin B_c, although it was an excellent source when supplied to the chick. It soon developed (Binkley, Bird, Bloom, Brown, Calkins, Campbell, Emmett and Pfiffner 1944) that this vitamin was present in yeast as a conjugate, and this was the probable explanation of later difficulties in its identification.

Norite Eluate Factor.

Snell and Peterson (1940) had described a norite eluate from a yeast extract which was essential for L. casei, and which was often called the L. casei factor. Mills, Briggs, Elvehjem and Hart (1942) tested the norite eluate of a liver fraction as a nutrient and concluded that it was essential for the chick. In the absence of this factor growth is retarded, the feathers are faulty, and the number of red blood cells is reduced. Later studies (Briggs, Luckey, Elvehjem and Hart 1943) led the Wisconsin group to believe the norite eluate, prepared from liver extract, contained two unrecognized nutrients. One of them was designated vitamin B_{10} and was regarded as essential for the normal development of feathers. The other, vitamin B_{11}, was essential for normal gains in weight.

Alcohol precipitate factor.

Bauernfeind and Norris (1939) likewise concluded that the chick required a vitamin not yet identified. Both liver and yeast were good sources and it was precipitated from solution by alcohol. In subsequent studies (Hill, Norris and Heuser 1944) it was stated that the alcohol precipitate factor was a mixture of at least two components, factors R and S. One would conclude now that conjugates of vitamin B_c (Folic acid) were at least an important part of vitamins B_{10} and B_{11}, and of factors R and S. The possibility that another important nutrient is yet to be identified out of this group of four will be decided in the future.

Folic Acid.

Mitchell, Snell and Williams (1941) reported the isolation of a product from spinach which stimulated the growth of L. casei and obtained some evidence that this product was a vitamin for the rat. It was given the name, "folic acid." These same investigators (Mitchell, Snell and Williams 1944) achieved an exceedingly high degree of concentration but apparently they were unable to prepare folic acid, or a derivative, in crystalline form. The concentration of this proruct was a noteworthy accomplishment but by an unhappy chance spinach was not a suitable source material. The name, folic acid, was an excellent choice and gained immediate acceptance, but its concentration from spinach came too late to make an important contribution to animal nutrition.

VITAMIN B_{12}

It is impossible to give an exact date when our knowledge of vitamin B_{12} began, for the reputed superiority of animal over vegetable proteins has been emphasized for many years. As an example, Kempster (1924) reported that when the protein supplement supplied to laying hens was linseed meal, gluten meal, or cottonseed meal, the rate of egg production was much lower than when the protein supplement was meat scrap or buttermilk. Kinder and Kempster (1953) returned to this problem some years later, however, and demonstrated that animal proteins were not highly important in broiler feeding, if the protein supplement was soybean oil meal, and if the allowance of riboflavin was adequate. It was becoming apparent, then, that the inferiority of vegetable protein could be due in some cases to vitamin deficiencies but this explanation developed slowly.

Byerly, Titus and Ellis (1933) compared soybean meal with a mixture of meat meal, fish meal and dried buttermilk as protein supplements for laying hens. The rate of production and the percentage of hatchability were lower on the soybean oil meal ration than when animal protein was included, and the percentage of chondrodystrophic embryos was higher. The authors apparently ascribed these differences to the quality of the protein consumed. In this same year, Halpin, Holmes and Hart (1933) reported that the hatchability of hen eggs was 11% when the laying ration was made up of corn, oats, wheat bran, wheat middlings, meat scraps, cod liver oil and salt and rose to 70% when supplements such as skim milk or alfalfa or 5% of autoclaved yeast were included in the diet. The improvement was ascribed to vitamin G. However Nestler, Byerly, Ellis, and Titus (1936) gave hens a basal diet that included 5.8% of alfalfa leaf meal and concluded that the diet was deficient in a factor not yet recognized.

With the advantage of hindsight, students of nutrition can see that time might have been saved by adopting more quickly a suggestion in a publication by Johnson, Carrick, Roberts and Hauge (1942). They made up a practical diet, except for casein, which was used as a protein supplement. If the casein was thoroughly extracted with alcohol, the rate of gain was slow. However, if the alcohol extract was added back to this diet, or if the casein was not extracted, the rate of gain was greatly accelerated. Presumably Johnson and co-workers were extracting the vitamin later designated by Cary as Factor X and later given the name of vitamin B_{12}.

Hammond (1944) was apparently searching for a source of riboflavin when he began his investigations on cow manure as a constituent of poul-

try feeds. As had become customary by this time, soybean oil meal was the protein supplement in all-vegetable type diets, but chicks which consumed them grew slowly. When 8% of dried cow manure was included in the diet the rate of gain was markedly increased. Hammond did not suggest that the increase was due to an unrecognized nutrient.

Bird, Rubin, Whitson and Haynes (1946), in confirmation of earlier reports, observed that the hatchability of eggs was 66% when the diet of the hens contained 30% of soybean oil meal as the only source of protein. When 5% of cow manure, or 10% of fish meal or 10% of dried skim milk was included in the diet the hatchability was approximately 80%. Another striking observation was the low viability of chicks on the basal diet. The viability of the chicks was not improved by adding supplements to the diet of the chicks, which would seem to show they had suffered irreparable damage during the embryonic stage. Presumably this was an effect of a vitamin B_{12} deficiency. Rubin and Bird (1946) had a considerable degree of success in concentrating the cow manure factor. Optimum growth was obtained when 3.75 to 7.5 mg. of the material per 100 gm. of diet was fed. When this concentrate was included in a laying ration that contained no animal protein (Rubin, Groschke and Bird 1947) the hatchability of the eggs rose to a normal percentage. This degree of concentration was a noteworthy accomplishment, but from some points of view it was not properly rewarded. The actual isolation of the factor was accomplished in another laboratory, by a different procedure.

We can see now that the accomplishments previously described were preparing the way for the discovery of the last vitamin of major importance that has been announced. Thus, some years previously, Richardson, Hogan and Karrasch (19425) had been able to reduce the number of supplements previously included in their basal diet. Gelatin could replace the protein fraction of liver. We know now this replacement was effective largely because gelatin is a fairly good source of arginine. The alcohol extract was dispensable because pyridoxine, pantothenic acid and choline had become available. After folic acid became available the water extract was still indispensable because it was the source of vitamin B_{12}.

The story of the isolation of vitamin B_{12} probably began when Cary, Hartman, Dryden and Likely (1946) announced that an unidentified nutrient was essential for growth of the rat and designated it Factor X. Unless rigid precautions were taken, casein was contaminated with this new vitamin. The report of Cary *et al.* was confirmed by Derse, Nath, Elvehjem and Hart (1948). A number of investigators were trying to isolate the contaminant described by Cary *et al.*, but the end was near

when Mary S. Shorb (1947) reported that one of the bacterial media she prepared was deficient in two unidentified nutrients required by Lactobacillus lactis Dorner. One of them, LLD, is in liver extract fractions used in treating pernicious anernia and the amounts of the bacterial growth factor and of the anti-pernicious anenia factor were parallel. The fact that the potency of liver fractions could be assayed quickly by microbiological methods was undoubtedly a major factor in the early isolation of the active agent. Rickes, Brink, Koniuszy, Wood, and Folkers (1948) isolated the LLD factor in crystalline form. This was a remarkable achievement and the substance was designated vitamin B_{12}. Shorb (1948) showed that vitamin B_{12} was the LLD factor and R. West (1948) showed that it was the anti-pernicious anemia factor.

Vitamin B_{12} is not found in significant amounts in the higher plants (Robbins and Stebbins 1950) but it is synthesized by many bacteria and antimomycetes in the soil. Apparently microorganisms are the original source of practically all of the vitamin B_{12} in the world.

Ott, Rickes and Wood (1948) and Ott (1951) estimated that the required amount of vitamin B_{12} for the chick is probably less than 3 mcg. per 100 gm. of food. When the chicks come from undepleted hens the amount of vitamin B_{12} required is reduced. Davis and Briggs (1951) used chicks from hens that had been supplied with liberal amounts of vitamin B_{12}. The chicks consumed a practical corn-soybean oil meal ration, and their requirement for the vitamin was estimated at between 0.15 and 0.20 mcg. per 100 gms. of diet. Additional data on this point were supplied by Miller, Norris and Heuser (1956). They showed that when hens received liberal amounts of vitamin B_{12} the amount transferred to the egg would supply all that the chick required until it was 6 weeks old. When the diet of the hens contained enough vitamin B_{12} to maintain an 83% hatchability rate the chicks required approximately 0.125 mcg. of vitamin B_{12} per 100 gm. of feed and when the hatchability rate was 63% the requirement was not over 0.25 mcg. Chicks on a corn-soy bean oil meal type ration do not become anemic if vitamin B_{12} is withheld (Nichol, Harper and Elvehjem 1949. Stern, Hsu and McGinnis 1952).

Savage, Turner, Kempster and Hogan (1952) depleted hens of vitamin B_{12} by giving them an all plant laying ration but did not obtain an increase in rate of egg production when vitamin B_{12} was added to the diet. There was, however, a considerable increase in the hatchability of fertile eggs. There is, however, a considerable body of evidence which shows that the rate of egg production declines when the intake of vitamin B_{12} by hens is severely restricted. The minimum requirement for vitamin

B_{12} by hens in laying cages was probably not over 2.0 mcg. of vitamin B_{12} per pound of feed. When hens were on built-up litter the requirement was not over 0.75 mcg. per lb. of feed (Petersen, Wiese, Milne and Lampman 1953).

The story of built-up litter deserves some comment. Not long after Hammond (1944) announced that all-vegetable chick diets were improved by the addition of cow manure it was shown that chicken manure was also effective, under suitable conditions. This preceded the isolation of vitamin B_{12}. Later on Halbrook, Cords, Winter and Sutton (1950) found that nearly all the microorganisms isolated from poultry house droppings synthesized a considerable amount of vitamin B_{12}. Kennard and Chamberlin (1948) made a practical application of this discovery when they demonstrated that built-up floor litter is a source of the animal protein factor. If chicks have access to built-up floor litter they make excellent gains even on all-vegetable type rations. This built-up litter may also improve the hatchability of eggs when hens consume a diet devoid of animal protein supplements (Kennard, Bethke, and Chamberlin 1948). In one trial the hatchability of the eggs on fresh litter was 32%, on built-up litter it was 78%.

Olcese, Couch, Quisenberry and Pearson (1950) gave hens a synthetic diet, which contained no added vitamin B_{12}. There was a high rate of embryonic mortality, and there were numerous embryonic abnormalities. These included perosis, hemorrhage, malposition and myoatrophy.

Are there any more vitamins?

After vitamin B_{12} became available the Missouri investigators still obtained a marked growth response when liver protein was included in the diets of baby chicks and at the time this response was ascribed to an unidentified nutrient. However, a rather lengthy investigation showed that the activity of liver protein was due to its arginine content, and not to the presence of a new, unidentified vitamin (Hogan, Craghead, Savage, Cole and O'Dell 1957).

Other studies at times seemed to show that various natural products, egg yolk for example, contained at least one important, unrecognized vitamin, but as time went on every such indication seemed to become fainter, and finally it would disappear. At the present time there are two aspects of the search for new vitamins that may deserve comment. In the first place, well known compounds which are supposed to be of no special consequence as components of the diet, do frequently seem to accelerate the rate of growth. Examples are found in ascorbic acid, arabinose and glucuronic acid. In the second place, results with chicks are often variable

in a surprising manner. Thus in one, two, or three trials a preparation may give a marked and consistent response. The numbers of experimental animals and controls may be large and the differences are mathematically significant. If, however, the trials are repeated inconsistent data appear, and mathematical significance is lost. One could assume that the seemingly positive results secured at first, merely show that extremely large numbers are necessary to insure a representative sample. However, some investigators are convinced that an obscure nutritional circumstance may exist. On one occasion this circumstance may make the presence of a nutrient mandatory, on another it may make its presence dispensable. Whether or not this hypothetical circumstance is related to an unrecognized nutrient is another matter.

It seems possible that important vitamins or nutrients still remain to be discovered. To the Missouri investigators, however, their existence is not regarded as a certainty. One can be certain, however, that many important facts, based on the nutrients now known, have not yet been disclosed.

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