VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

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EDITORS' PREFACE

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

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

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

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

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Intestinal Synthesis of Vitamins in the Nonruminant

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I. INTRODUCTION

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

The realization that viable bacteria are present in the gastrointestinal tract of normal individuals raised the question in Pasteur's mind whether they were necessary for the well-being of the host (Johansson and Sarles, 1949; Rettger and Cheplin, 1921). It was demonstrated as early as 1874 that the meconium of the newborn infant is sterile (Billroth, 1874). The characteristic flora that is established in the infant shortly after birth depends to a large degree upon the nature of its food (Escherich, 1885; Tissier, 1900). The work of Tissier (1900) showed that the flora in the breast-fed infant is predominantly *Lactobacillus bifidus*, whereas that in the bottle-fed baby is *Lactobacillus acidophilus*. The flora becomes more complex as supplementary foods are added to the child's diet. The varieties of bacteria in the gastrointestinal tract increase to a certain extent with age (Rettger and Cheplin, 1921).

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

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

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

2. Refection

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

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

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

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

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

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

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

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

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

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

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

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

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

d. In Animals Other than the Rat. Very little work has been done to determine whether refection can be established in animals other than the rat. In 1897 Eijkman was unable to produce polyneuritis in hens fed corn starch instead of polished rice (quoted by Kon, 1953). Whether this was due to a refected state in the hens was never determined. The type of dietary carbohydrate influences the synthesis of folic acid (Luckey *et al.*, 1946b) and biotin in the chick (Couch *et al.*, 1948). Although these situations may be related to refection, they may not be the same, since the original work of Fridericia *et al.* (1927) showed that if the diet of refected rats was supplemented with 5% brewer's yeast, they lost their refected state within 24 hours as shown by the change in the color, size, and consistency of their feces. Refection could be produced only on a vitamin B-deficient diet. These workers also reported that when mice fed a vitamin B-deficient diet were given feces from refected rats, one mouse out of 19 appeared to have become refected as shown both by the appearance of characteristic stools and a gain in body weight. A number of other mice in the above group showed the characteristic stools but died before any weight gains were evident. Three pigeons fed polished rice supplemented with feces from refected rats failed to become refected.

3. *Coprophagy*[4]

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

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

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

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

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

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

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

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

4. Survey of Work on Sulfonamides

The advent of the relatively insoluble sulfonamides permitted intensive investigations on the effect of the intestinal microflora on the vitamin requirements of animals. One of the first reports on the use of sulfonamides in nutrition work was that by Black *et al.* (1941). They used a purified diet on which they secured good growth in rats. The addition of 0.5% sulfaguanidine to that diet produced a marked inhibition in growth. The addition of liver extract or *p*-aminobenzoic acid (PABA) to the diet overcame the effect of the sulfonamide. The observed effects were explained on the assumption that sulfaguanidine had inhibited, in the gastrointestinal tract of the rats, the bacterial synthesis of certain nutrients not known at that time. This, and the work that followed, led to the recognition that vitamin K (Black *et al.*, 1942), folic acid (Nielsen and Elvehjem, 1942; Spicer *et al.*, 1942), and biotin (Daft *et al.*, 1942) were required by the rat. There was a suggestion from the work of Light *et al.* (1942) that the feces of rats fed sulfonamides had a reduced content of B vitamins. There was, however, no correlation between the reduction in vitamin content of the feces and the growth-depressing effect of the different sulfonamides studied.

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

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

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

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

5. Survey of Earlier Work on Nutritional Effects of Antibiotics

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

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

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

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

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

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

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