VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

Edited by

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EDITORS' PREFACE

It gives us considerable pleasure to announce that Professor G. F. Marrian is joining us as an Editor of *Vitamins and Hormones*, beginning with the next volume. Dr. Marrian is Professor of Biochemistry at the University of Edinburgh, Scotland. Because he is a chemical endocrinologist, and is located in Europe, he will strengthen and broaden the work of the Editorial Board.

We have pointed out in previous volumes that our contributors strive to maintain a very high standard of critical judgment in their reviews. Because of their continued success in this, as well as in the ambitious task of covering a rapidly expanding literature, it is difficult to single out any one article for special emphasis. However, since cortisone and ACTH represent one of the most striking developments in endocrinology in recent years we direct attention to the essentially complete summary of the present status of this important subject published here. At the same time the Editors desire to express their thanks to the authors of all the articles for their outstanding work.

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I. Introduction

During the past twenty years many investigations have been reported which dealt with the effect of liver extracts on the megaloblastic anemias in human subjects. Many reports appeared in the same period which dealt with nutritional deficiencies in rats and chicks resulting from the use of diets lacking in a factor associated with animal proteins. The isolation of vitamin B_{12} (Rickes *et al.*, 1948a; Smith, 1948) made it possible to reexamine the earlier data and to repeat and extend many of the findings. The role of vitamin B_{12} in microbiology has been studied intensively during 1949 and 1950 with the aid of the crystalline vitamin.

II. Chemistry

Several fragments resulting from the hydrolysis of vitamin B_{12} have been isolated and identified as follows:

1. *5,6-Dimethylbenzimidazole-1α-*D*-ribofuranosido-3-phosphate* "Benziminazole nucleotide" (Buchanan *et al.*, 1950; Brink *et al.*, 1950; Cooley *et al.*, 1950) (Fig. 1).

2. D*-1-Amino-2-propanol* (Wolf *et al.*, 1950). Two residues per vitamin B_{12} molecule (Cooley *et al.*, 1950; Chargaff *et al.*, 1950).

3. *Ammonia* (Cooley *et al.*, 1950) and *cyanide* (Brink *et al.*, 1950) have also been found present in hydrolyzates of vitamin B_{12} (cyanocobalamin); cyanide is absent from vitamin B_{12b} (hydroxocobalamin).

The remainder of the substance is an unidentified cobalt-complex which represents the major part of the molecule and is presumably esterified on the phosphate group shown in Fig. 1.

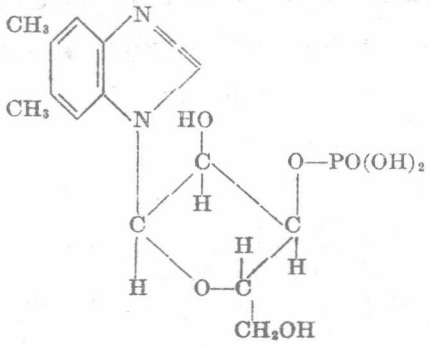

FIG. 1

III. BIOLOGICAL ACTIVITY

Vitamin B_{12} is effective at very low dosage levels. The requirement of various species for vitamin B_{12} is shown in Table I.

IV. BIOLOGICAL FORMATION OF VITAMIN B_{12}

There is no evidence for synthesis of vitamin B_{12} by vertebrates although their tissues are known to contain it. The green plants do not appear to supply significant amounts of vitamin B_{12} in the diet, but certain intestinal microorganisms produce the vitamin and there is evidence for its formation in the rumen. Yeast is a poor source of vitamin B_{12} as indicated on page 6. The addition of yeast to the diet of rats has been found to accentuate the signs of a deficiency of vitamin B_{12} (Cary and Hartman, 1947).

It has been apparent for some years that a fraction with nutritional activity for chicks now known to correspond to that of vitamin B_{12} is produced in the digestive tract or feces of cattle and chickens (Hammond, 1944; Rubin *et al.*, 1946). The first report of the bacterial synthesis of a factor with anti-pernicious anemia activity was made by Stokstad and coworkers (1948), who found that it was possible to prepare active concentrates from fermentation materials produced by a nonmotile rod-shaped organism which was originally isolated from hen feces. These concentrates did not furnish significant amounts of folic acid. The concentration was followed by assay with chicks on a corn-soybean

TABLE I

Requirements for Vitamin B_{12} under Various Conditions

Species	Conditions in which measured	Dose in μg.	Reference
Human beings	Pernicious anemia	1 to 3 injected daily	West, 1949 West and Reisner, 1949
Pigs	Nutritional deficiency	20 per kilo of diet	Nesheim *et al.*, 1950
Chicks	Nutritional deficiency	Between 15 and 30 per kilo of diet	Stokstad *et al.*, 1949
Chicks	Nutritional deficiency	About 0.5 injected weekly	Stokstad *et al.*, 1949
Rats	Nutritional deficiency	0.1 injected weekly	Frost *et al.*, 1949
Lactobacillus lactis Dorner	Purified culture media	0.013 per liter*	Rickes *et al.*, 1948a
Lactobacillus leichmannii 313	Purified culture media	0.15 per liter*	Hoffmann *et al.*, 1948
Lactobacillus leichmannii 4797	Purified culture media	0.011 per liter*	Broquist, 1950b
Euglena gracilis var. *bacillaris*	Purified culture media	0.01 per liter*	Hutner *et al.*, 1949

* for "half-maximum growth."

meal diet, using liver extract as a standard, and the final solution produced a good response when injected into patients with pernicious anemia. A few months later, Rickes and coworkers (1948b) reported the isolation of crystalline vitamin B_{12} from culture broths of *Streptomyces griseus* and the presence of activity for *L. lactis* Dorner in culture broths of several microorganisms including *Mycobacterium smegmatis*, *L. arabinosus*, *B. subtilis* and several *Streptomyces* species, although some of the activity of fermentation liquors was later found not to be due to vitamin B_{12} (Caswell *et al.*, 1949). The production of desoxyriboside-like-material, which will replace vitamin B_{12} in the nutrition of *L. leichmannii* and *L. lactis*, is a source of complication in such "screening" pro-

cedures unless suitable precautions are taken in the assay (Hoffmann *et al.*, 1949).

The stools of a patient with pernicious anemia were found to contain relatively substantial amounts of material with *L. lactis* activity (Bethell *et al.*, 1948) which was presumably vitamin B_{12} synthesized by intestinal bacteria. Similar results were found by Callender *et al.* (1949). Girdwood (1950) reported that the contents and secretions of the small intestine in patients with pernicious anemia contained negligible amounts of vitamin B_{12} activity, but about 5 μg. were found daily in the feces, perhaps indicating synthesis in the large bowel.

The isolation in crystalline form of vitamin B_{12} and a new substance vitamin B_{12b}, from cultures of *Streptomyces aureofaciens*, was described by Pierce and coworkers (1949). Both substances were equally biologically active.

A fermentation process for the production of vitamin B_{12} by *B. megatherium* was described by Lewis *et al.* (1949). Yields of 0.8 parts of vitamin B_{12} activity per million of whole culture medium were produced in a 12-hour batch fermentation of a medium containing sucrose, yeast extract, citric acid, ammonium and inorganic salts. Ansbacher *et al.* (1949a, b) mentioned the use of undescribed organisms in the production of "animal protein factor" activity by fermentation and indicated that choline was destroyed or utilized during the production of activity.

Halbrook *et al.* (1950) studied 142 isolated microbial colonies which were obtained from culturing poultry-house litter and chicken feces. Only four of the isolated organisms produced no detectable quantity of vitamin B_{12} activity for *L. leichmannii*. Choline was found to depress the production of vitamin B_{12} activity in many cases. One actively-producing organism was tentatively identified as a strain of *Aerobacter aerogenes*.

The fundamental source of vitamin B_{12} in animal nutrition thus appears to be in non-photosynthetic organisms. A microbiological synthesis of vitamin B_{12} may take place in the rumen and elsewhere in the digestive tract of cattle, providing a supply of the vitamin which is accumulated in the liver, thus accounting for the effectiveness of extracts of this organ in the treatment of pernicious anemia.

V. Vitamin B_{12} and the "Intrinsic Factor"

An extensive series of investigations by Castle and coworkers (1934) and by other clinical groups has shown that patients with pernicious anemia are deficient in their ability to secrete a thermolabile substance, the "intrinsic factor," in the gastric juice. This substance is needed for the utilization of a heat-stable dietary essential, the "extrinsic factor"

in the prevention of pernicious anemia. The "extrinsic factor" has been found present in various foods including lean beef, milk, crude casein, eggs, yeast, rice polishings, wheat germ, and liver. The "intrinsic factor," on the other hand, is found only in the gastric and intestinal mucosa of animals (Sturgis and Isaacs, 1929) or in normal gastric juice.

The effect of the two factors was studied by oral administration to patients with pernicious anemia in relapse, in whom a remission may be shown by a transient reticulocytosis, by hemopoiesis and by other characteristic therapeutic changes. When given singly at intervals separated by 12 hours or more neither factor is effective in producing a remission. However, when the factors are mixed together and ingested, or when they are given separately by mouth within 6 hours of each other, a remission is produced.

It was shown that the intrinsic-factor activity of gastric juice was destroyed by heating to 70 to 80° for 30 minutes. The mixture of the extrinsic and intrinsic factors was ineffective if it was similarly heated before administration (Castle *et al.*, 1937; Formijne, 1940). This point was of importance because the extrinsic factor was repeatedly shown to be stable to boiling, and the ineffectiveness of the heat-treated mixture thus showed that the intrinsic factor had not, in spite of the suggestions by some investigators (Klein and Wilkinson, 1934), reacted with the extrinsic factor to form a new thermostable product which could be utilized orally in the treatment of pernicious anemia in the absence of unheated intrinsic factor.

The oral ineffectiveness of extrinsic factor in the absence of intrinsic factor was not absolute, for certain patients were found to respond to massive doses of the extrinsic factor when administered alone. This could possibly be explained by the existence of an incomplete deficiency of the intrinsic factor in the digestive juices of such patients (Goldhamer, 1936). Injectable liver extracts, effective in the parenteral treatment of pernicious anemia, were found also to contain the extrinsic factor (Fouts *et al.*, 1935; Napier, 1938).

Studies with vitamin B_{12} showed that daily doses of 5 or 10 μg. were ineffective when given by mouth unless a source of the intrinsic factor was given simultaneously (Berk *et al.*, 1948). This may be compared with the effective level of vitamin B_{12} when injected, which is in the neighborhood of one microgram daily. Similar findings were reported by Hall *et al.* (1949).

From these observations it could be concluded that the defect in pernicious anemia was an absence of intrinsic factor in the digestive tract, occasioned by a loss of ability to produce this factor in the gastric secretions and possibly also a simultaneous loss in the secretions of the

small intestine. This absence led to the inability of the patient to absorb vitamin B_{12} or related food substances from the digestive tract. Pernicious anemia thus emerges as a tissue deficiency of vitamin B_{12} which is occasioned by a degenerative loss of the mechanism for uptake of this vitamin.

The question remains: Are vitamin B_{12} and its closely related analogues such as vitamin B_{12b} the only substances which have "extrinsic factor" activity? The potency of many of the materials such as meat, milk, and liver which were shown in early investigations to have such activity, can be explained in terms of their vitamin B_{12} content. The "extrinsic factor" activity of yeast extract ("Vegex") (Strauss and Castle, 1932) needs further explanation because yeast has been reported to be effective in the absence of gastric juice (Wintrobe, 1939). The oral administration of folic acid will, of course, produce a hemopoietic response in pernicious anemia in the absence of intrinsic factor, and yeast is a good source of folic acid, but yeast does not appear to have vitamin B_{12} activity in experiments with animals (Hartman *et al.*, 1949).

The mechanism of action of the intrinsic factor is the subject of current investigations. It was observed by Ternberg and Eakin (1949) that a B_{12}-binding effect was exerted by normal gastric juice and by extracts of the mucosa of the stomach and small intestine of the hog. This effect was measured by assay with *E. coli* in a medium containing sulfanilamide. The addition of vitamin B_{12} promotes growth of the organism in such a medium but the growth-promoting effect is abolished by simultaneous addition of unheated protein fractions from gastric juice. It was concluded that the protein fraction contained an active non-dialyzable heat-labile substance, termed "apoerythein," which was probably identical with the intrinsic factor or was an important component thereof. The B_{12}-binding activity of such extracts has been confirmed with respect to *L. leichmannii* (Broquist, 1950; Meyer *et al.*, 1950; Prusoff *et al.*, 1950). However, Prusoff and coworkers have reported that fractionation of preparations of hog stomach led to separation of intrinsic factor from most of the B_{12}-binding activity. These investigators used saline extracts of desiccated hog stomach ("Ventriculin") and prepared fractions by adding increasing amounts of ammonium sulfate. Three principal fractions were prepared: (A) 0 to 35% saturation, (B) 35 to 55%, (C) 55 to 100%. Fraction C, which exerted the greatest B_{12}-binding activity, contained less intrinsic factor activity than fraction B. The relation of the intrinsic factor to "apoerythein" as measured by *E. coli* assays is thus undefined. No clinical tests of "apoerythein" have been described by the Texas group.

It was occasionally, although not consistently observed, that aureo-

mycin had a "sparing effect" on the vitamin B_{12} requirement of chicks for growth (Stokstad and Jukes, 1950) while Davis and Mingioli (1950) reported that wild type *E. coli* rapidly removes vitamin B_{12} from culture media. The possibility thus occurred that aureomycin, by decreasing or changing the properties of the *E. coli* population of the gut, might facilitate the uptake of vitamin B_{12} by the host. Furthermore, it was shown by Ternberg and Eakin (1949) that gastric juice obtained from normal subjects had a marked effect in rendering vitamin B_{12} unavailable to *E. coli*, while gastric juice from subjects with pernicious anemia showed little of such effect. This led to the speculation that aureomycin might be used instead of gastric juice in conjunction with vitamin B_{12} in the oral treatment of pernicious anemia. It was reported by Lichtman and coworkers (1950) that four patients with Addisonian pernicious anemia in relapse showed definite although submaximal hematological improvement to aureomycin when given orally. No response was obtained in a patient who received aureomycin intravenously.

The interchangeability of vitamin B_{12} and folic acid in producing a hemopoietic remission in pernicious anemia remains unexplained. However, an interplay between folic acid and vitamin B_{12} has been observed in several metabolic processes, such as in the formation of thymidine and in biological "methylations," which are discussed elsewhere in this review. These close relationships in intermediary metabolism may well prove to be a sufficient explanation for the overlapping therapeutic effects of vitamin B_{12} and folic acid.

VI. Vitamin B_{12} and Combined System Disease

Sensory and motor disturbances associated with involvements of the posterior columns and pyramidal tracts may be encountered in untreated or relapsed cases of pernicious anemia. The condition progresses if untreated and destructive changes may take place in the spinal cord. It has long been recognized that the symptomatology may be ameliorated by prompt treatment with concentrated liver extracts; simultaneously a hemopoietic response takes place. Such liver extracts were shown to contain little or no "folic acid activity" (O'Dell and Hogan, 1943) so that observations in 1945 that folic acid would produce remission in pernicious anemia (Jukes and Stokstad, 1948) were unexpected. Within a year, and before folic acid was made freely available, it became evident that folic acid would not prevent or relieve the neurological disturbances that occur in a certain percentage of cases of pernicious anemia, although a few exceptions were noted. Recommendations that liver extract should always be used to treat pernicious anemia therefore accompanied the commercial introduction of folic acid.

The isolation of vitamin B_{12}, the active hemopoietic substance in concentrated liver extract, enabled further studies of the neurological syndrome to be made and, as might have been anticipated, vitamin B_{12} had an alleviatory effect on the nervous symptoms (Ungley, 1948; Berk *et al.*, 1948b; Hall and Campbell, 1948). The use of larger doses of vitamin B_{12} than usually employed for hemopoietic treatment has been suggested by several groups of investigators for treatment of combined system disease.

Folic acid, by maintaining hematological remission, may allow the eventual development of nervous changes in patients whose pernicious anemia, if untreated, would presumably have progressed to a fatal termination prior to such neurological involvements. The appearance of these changes in patients who were incompletely treated by the administration of folic acid without liver extract has led to speculations that folic acid may provoke the symptomatology (Anonymous, 1947). These speculations are not in accordance with the observation that the symptoms do not occur when folic acid and liver extract are given simultaneously (Dameshek, 1948) and are alleviated by vitamin B_{12} regardless of the continued administration of folic acid (Heinle, 1950).

In chicks, the neurological disturbances and paralytic signs caused by a lack of folic acid are aggravated by the administration of vitamin B_{12} (Nichol *et al.*, 1949).

VII. Vitamin B_{12} and Cobalt in the Nutrition of Ruminants

A wasting disease in ruminants, recognized and described as occurring in various areas in many parts of the world, was identified in 1935 as being due to a deficiency of inorganic cobalt (Marston and Lines, 1935; Underwood and Filmer, 1935). The subject was reviewed by Marston (1939), who described the disease in sheep as follows: "The demeanor of the sheep changes from the vigorous alertness of normal health to one of listlessness; their eyes become rheumy and their mucosae bloodless; their appetite fails and the lethargy and weakness progresses to a fatal termination. Autopsy reveals little other than the general findings associated with hunger edema although hemosiderosis of the liver, spleen, and pancreas are frequently apparent. During the course of the disease the hemoglobin content of the blood has been observed to fall to less than half the normal of about 14 vol. % of oxygen, and in extreme cases an oxygen-carrying capacity of less than 3 vol. % of oxygen is often encountered." Nonruminants, such as horses and rabbits, can subsist on forage crops grown in the deficient areas and can reproduce without signs of dietary disturbance, indeed, no untoward effects have been reported in nonruminants as a result of a lack of cobalt (Thompson and

Ellis, 1947). It was found by Filmer (1933) that whole liver would cure the disease of cattle and sheep caused by cobalt deficiency although Filmer and Underwood (1937) noted that liver contained insufficient cobalt to account for its therapeutic effect. Martin (1944) stated that cobalt was effective by mouth but not by injection in treating the deficiency in sheep, leading to the suggestion by McCance and Widdowson (1944) that cobalt exerted its beneficial effect by acting upon some of the microorganisms in the rumen. In young calves, cobalt deficiency was stated not to be observed until the rumen had started to function (Comar and Davis, 1947).

It was reported by Marston and Lee (1949) and by Becker and coworkers (1949) that cobalt-deficient lambs did not respond to the feeding or injection of vitamin B_{12}. No significant response was obtained by injecting amounts of vitamin B_{12} up to 125 μg. in two weeks or by feeding 120 μg. as a concentrate weekly for 6 weeks. Hale and coworkers (1949) found that the injection of vitamin B_{12} did not alleviate cobalt deficiency in sheep, and they noted that the rumen contents of cobalt-supplemented sheep produced a greater response in the chick assay for vitamin B_{12} than did the rumen contents of cobalt-deficient sheep. It must be assumed that due precautions were taken to prevent the formation of vitamin B_{12} by fermentation subsequent to the removal of the rumen contents, for Lewis *et al.* (1950) found in studies with cobalt-supplemented rats that the addition of toluene to liver samples was necessary to prevent marked increases in vitamin B_{12} content *in vitro*.

Gall and coworkers (1949) noted that cobalt-deficient sheep had a simpler rumen flora and a lower bacterial count than did sheep on the same ration with added cobalt. It was found by Becker and Smith (1949) that the daily injection of vitamin B_{12}, 1 μg., and cobalt, 1 mg., was ineffective in the treatment of cobalt deficiency in sheep. The administration of 15 U.S.P. units of concentrated liver extract was ineffective by mouth but was beneficial when injected subcutaneously. As noted above, the young calf has been stated not to show cobalt deficiency before the rumen has started to function (Comar and Davis, 1947) and it is of interest that vitamin B_{12} deficiency has been described in young calves on purified diets which contain inorganic cobalt (Johnson, 1950). The calves responded to vitamin B_{12} when injected, providing a further contrast to the experiments reported with sheep.

In a later study (Hale and coworkers, 1950) it was pointed out that the dosages used in earlier investigations may well have been too small, especially in view of the high content of vitamin B_{12}, 50 to 60 μg. per gram of dry matter, found in the rumen contents of cobalt-supplemented sheep. The investigators observed increases in the blood hemoglobin

levels of two cobalt-deficient lambs which received respectively 100 μg. of vitamin B_{12} injected and 200 μg. by mouth daily for 4 weeks. The appetite remained poor and only 4 or 5 pounds of weight increase were obtained. In another approach, various mixtures of vitamins, not including B_{12}, were fed to cobalt-deficient lambs. Two lambs were used in each group and the group receiving a supplement including thiamine, riboflavin, niacin, calcium pantothenate, pyridoxine, folic acid, *p*-aminobenzoic acid, choline, biotin, and vitamin K showed a growth response and increases in hemoglobin levels. Hale and coworkers suggest that the beneficial effect of cobalt upon ruminants may be through enabling microorganisms to grow that produce B-vitamins in the rumen.

VIII. Vitamin B_{12} in Bacterial Metabolism

In a study of the effect of natural materials in reversing the toxic effect of "x-methyl folic acid" on *Leuconostoc mesenteroides*, a compound active in this reversal was isolated from liver and identified as thymidine (Shive *et al.*, 1948a). Thymine, however, was inactive in this system, showing that under certain conditions microorganisms are incapable of converting this pyrimidine base to the corresponding desoxyriboside. Following this initial observation, Shive, Ravel, and Eakin (1948b) deduced that thymidine was capable of replacing vitamin B_{12} in the nutrition of *Lactobacillus lactis* Dorner. Snell *et al.* (1948) and Wright *et al.* (1948) reported that thymidine was a growth factor for several lactic acid bacteria, in the absence of a folic acid antagonist. Thymine was ineffective in replacing thymidine. An interchangeability of crystalline vitamin B_{12} and thymidine was first established for a lactic acid organism, *L. leichmannii* 313, by Hoffmann *et al.* (1948).

The desoxyribosides of hypoxanthine, adenine and cytosine were found to be as effective as thymidine in promoting growth of certain organisms (Kitay *et al.*, 1949). A hydrolyzate of guanine desoxyriboside which presumably contained free desoxyribose was inactive. Some organisms were able to use intact desoxyribonucleic acid in place of the desoxyribosides while others were not.

The ability of a number of different desoxyribosides to replace vitamin B_{12} suggests that the carbohydrate moiety can be transferred from one purine or pyrimidine base to another. Friedkin *et al.* (1949) found that guaninedesoxyriboside underwent phosphorolysis in the presence of liver nucleoside phosphorylase to give desoxyribose phosphate which was capable of reacting with hypoxanthine in the presence of the liver enzyme to give the corresponding hypoxanthine desoxyriboside. Later work by McNutt (1950) casts doubt on whether this conversion by way of the