VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

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VOLUME VIII

1950
ACADEMIC PRESS INC. PUBLISHERS
NEW YORK

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EDITORS' PREFACE

By this time *Vitamins and Hormones* needs no special introduction to the scientific public. The success of seven previous volumes can only mean that the series serves a useful purpose in surveying, reporting and assessing the progress of the rather broad field which it covers.

The present volume continues the general trend already set. The field shows no signs of contracting, and indeed the number of workers in it appears to be increasing steadily. Recent developments in medicine will probably lead to still further increases.

The critical and thorough nature of the reviews in the present volume testifies to the very large investment of time and trouble on the part of the authors, as well as to their familiarity with an extensive and scattered literature. The Editors have been fortunate in having such distinguished contributors, and take this opportunity of formally expressing their thanks.

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* Part 1 was published in *Vitamins and Hormones*, Vol. VII, 1949, pp. 389–435.

"Animal Protein Factor" and Vitamin B_{12} in the Nutrition of Animals

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I. Background and Outline of Recent Progress

It is difficult to say when or where the thought first arose that there may be a nutritional quality associated with animal protein which is not shared by vegetable food sources and is not dependent on amino acid make up. The idea was put into words by Mapson (1932, 1933) and by Byerly *et al.* (1933). The work of the former dealt with rats fed diets containing special purified caseins; it was almost overlooked at the time and even later did not receive the attention it deserved because of certain difficulties of interpretation. The more recent studies of Cary and Hartman re-establish Mapson as a pioneer in this field. Unfortunately Mapson did not continue these studies. Byerly's group (Office of Poultry Investigations, Beltsville) carried on and laid the groundwork both for more rational studies with vegetable proteins and for highly significant advances towards the isolation of the unknown factor.

As a period of scarcity of animal protein sources came on, with general wartime shortages, soybean meal was introduced into poultry feeding on a much larger scale. The unavailability of animal protein supplements made necessary an intensified study of both the slight amino acid deficiencies of soybeans (principally methionine) and the postulated unknown factor. From 1946 on, the poultry literature bristled with papers on various aspects of this problem. These will not be cited here but most of them will be referred to later on. A perusal of the table of contents of *Poultry Science* for 1946 and 1947 will give the reader a picture of both the intensity of effort and the types of problems which had to

be solved. For scientific purposes it was necessary step by step to differentiate the effects of various vitamin and amino acid deficiencies and for practical purposes to find suitable sources of the unknown factor. The main achievements will be summarized here. It was firmly established that an unknown factor not present in any vegetable feeds was a determinant in the quality of various animal protein supplements. Meat scrap was rated lower and fish meal higher than heretofore and a new product "fish solubles"[1] was introduced and shown to be very potent and apparently more dependable than many commercially available animal by-products in use. Liver and liver extracts were proved to be high potency sources for experimental work. The factor in question came to be known as "animal protein factor" and later the term "animal protein factor complex" reflected the conviction that more than one substance was involved.

The results of Bird and associates at Beltsville should be specifically mentioned here since they gave a new turn to the investigation. Hammond (1944) had shown earlier that cow manure contained very appreciable amounts of B factors and could find application as a substitute for alfalfa. Bird and coworkers (Rubin and Bird, 1946a, b) demonstrated that cow manure also carried the factor to which fish meal owed its distinctive value for hatchability and growth of chicks and gave methods for concentrating the factor a thousand fold (Bird, 1947b). Bird's additional finding that the factor occurred in hen droppings (Rubin *et al.*, 1946) was further developed by McGinnis *et al.* (1947a) who showed that the fresh droppings were practically inactive but on incubation high potency developed. These findings gave assurance that the factor was not necessarily dependent on feeding animal tissues but had an independent microbiological origin.

In the meantime, Cary and Hartman, without reference to the poultry investigations, picked up again the thread of Mapson's work. In a series of preliminary communications and short government reports (Bureau of Dairy Industry, U.S. Dept. Agriculture) from 1941 on, they showed that by extraction with hot alcohol a factor that is a nutritional essential for rats can be removed from crude casein. Liver and other material including dairy products were successful supplements.

Zucker and Zucker worked with rats on an all-plant diet progressively supplemented with more and more known factors. The resulting deficiency was relieved by liver or crude casein and not by yeast, a distribution of activity which caused them, in 1946, to suggest identity of their factor with Cary's. Activity was then established for the two

[1] Description of, and analytical data on, this product are given by Lassen and Bacon (1946) and by Mishler *et al.* (1948).

recently announced successful poultry supplements—Bird's cow manure concentrate and fish solubles—leading to the suggestion in 1947 that a similar deficiency could occur in both birds and mammals induced either by the removal of a factor from the diet materials, as in Cary's work, or by feeding a naturally deficient diet. Active supplements were the same for rats and chicks.

The isolation of a nutritional factor in pure form is always a significant turning point and opens new paths. This is what happened when from the laboratories of Merck and Co. in April, 1948 (Rickes *et al.*, 1948a) the announcement came that a red crystalline product had been isolated from liver which, in clinical tests carried out by West (1948), proved to be the antipernicious anemia substance. Almost simultaneously the laboratories of Glaxo House in England announced a red cobalt-containing product from liver with antipernicious anemia activity in microgram quantities (Smith, 1948a, b). Closely upon this followed communications from the Merck Therapeutic Institute showing that the new compound called B_{12} had both in rats and in chicks the properties of an "animal protein factor" (Emerson, 1948; Ott *et al.*, 1948). A microbiological origin for B_{12} was demonstrated when the Merck group announced the large scale preparation of the identical material from cultures of *Streptomyces griseus* (Rickes *et al.*, 1948c). This was somewhat preceded by production of clinically active concentrates from bacterial cultures from hen feces at the Lederle Laboratories (Stokstad *et al.*, 1948). About this same time a project was undertaken at the Western Regional Research Laboratories in collaboration with McGinnis (Stephenson *et al.*, 1948; McGinnis *et al.*, 1949) involving the microbiological production of active material from a number of organisms including as common a one as *Bacillus subtilis*.

The isolation work of the Merck group was made possible by a microbiological technique devised by Shorb (1947, 1948) using *Lactobacillus lactis* Dorner as test organism. Later *L. leichmannii* was also used for assay purposes (Skeggs *et al.*, 1948; Hoffman *et al.*, 1948).

Before we proceed to a detailed consideration it should be realized that progress was not as simple as it may seem from an outline given after the event. The microbiological assays rendered service efficient enough in isolation work but have otherwise presented knotty problems. Standardization of the material for pharmaceutical purposes is still uncertain. In the poultry field, which was really the proving ground for the nutritional phase, it was well known that poultry has a high and exigent requirement for amino acids and much attention had to be given to this phase of the protein problem. During practically the whole of the time of development of "animal protein factor" work, the literature

abounded also in statements such as "animal protein supplements are not essential in chick rations for rapid growth." How rapidly a complete change in attitude set in is illustrated by the fact that the man whose chicks in 1948 furnished the first proof that crystalline B_{12} has "animal protein factor" activity published in 1944 under the title: "Satisfactory early chick growth on a diet containing no animal protein."

The answer to the riddle of why chicks do and do not need B_{12} lies largely in two circumstances characteristic of B_{12}: an unusually effective storage in the maternal organism for transmissal to the offspring and the considerable production of B_{12} activity in the feces, making it available to the young unless access is prevented.

II. Chemistry of Vitamin B_{12}

1. The Crystalline Substance

Crystalline material has been obtained by investigators at Merck, Glaxo Laboratories, British Drug Houses, the Lederle Laboratories, and E. R. Squibb and Sons. Both British groups have had preparations of a substance believed to be identical with the Merck B_{12} (Brink *et al.*, 1949a; Smith, 1949). Accepting this as so, data will be reported from all three groups on the properties of B_{12}.

The red crystals, said to be hygroscopic (Merck and Co., 1949) darken at 190–215°, and do not melt below 300°C. (Rickes *et al.*, 1948c; Brink *et al.*, 1949a). Refractive indices of the crystals are (α) 1.616; (β) 1.652, (γ) 1.664 (Rickes *et al.*, 1948c). Data for solubility are fragmentary; it is soluble in H_2O at least to the extent of 26.3 mg. in 15 ml. (Kaczka *et al.*, 1949). It is crystallized by the addition of four or more parts of acetone to an aqueous solution. The absorption spectrum in water is characterized by maxima at 2780 A.($E^{1\%}_{1cm.} = 115$); 3610 A.(204); 5500 A.(63) and is not much affected by change in pH (Brink *et al.*, 1949a). An absorption curve has been published by Ellis *et al.* (1949a), and is said to differ only in fine detail from that of the Merck group (Brink *et al.*, 1949a). This spectrum is stated to be incompatible with the presence of any pterin group in the molecule. B_{12} is optically active, with $[\alpha]^{23°}_{6563A.} = -59 \pm 9°$, and is a polyacid base, whose basic groups are too weak to show up in potentiometric titration in aqueous solution, but can be demonstrated in glacial acetic acid (*ibid.*).

The empirical formula is given as $C_{61-64}H_{86-92}N_{14}O_{13}PCo$ (*ibid.*). The presence of Co and P was first reported by Smith (1948b). The minimum molecular weight based on a molecule containing 1 atom of Co (Co concentration being 4.5%) is 1300 (Brink *et al.*, 1949a). A rough ebullioscopic determination in methanol indicates a molecular weight of 1490 ± 150 but the value based on Co content is preferred (Brink *et al.*,

1949a; Wolf *et al.*, 1949). X-ray studies indicate an approximate molecular weight of 1550–1750 (Smith, 1948b). The compound is characterized as a cobalt coordination complex with six groups around the cobalt, and the red color is attributed to this structural feature (Rickes *et al.*, 1948b). No statement has appeared as to whether the Co complex in B_{12} carries a net charge; i.e., whether the groups attached to the Co carry enough negative charges to neutralize the charge on the Co. The stated molecular weight from the boiling point elevation is apparently based on the assumption that B_{12} does not dissociate in solution. The state of oxidation of the Co has not been reported. B_{12} is subject to reduction by hydrogen in the presence of a catalyst, with accompanying change in color from red to brown, and the reduced product spontaneously oxidizes in air (Kaczka *et al.*, 1949). These facts would fit a tervalent Co in B_{12}, although other interpretations might be made since it is not demonstrated that the Co was specifically involved in the reduction.

Michaelis (1948) has recently considered the properties of Co complexes which show reversible oxygenation, i.e., the addition of an O_2 molecule. The Co is in the divalent state and the oxygenation results in a binuclear cobaltous complex, with O_2 as a bridge between two cobalts. If B_{12} has, as stated, only 1 Co per molecule and if this should also be true for all pertinent derivatives, then of course these considerations do not apply. The redox properties of B_{12} and derivatives are of particular interest in view of the relation between degree of oxygenation of the medium and the requirement for B_{12} of the lactobacilli (see Section V, 1).

B_{12} is stable in neutral aqueous solution at room temperature for over a year (Merck and Co., 1949) and a solution of 74 μg./.5 ml. withstands autoclaving for 15 minutes at 121°C. (Rickes *et al.*, 1948b). In .01 *N* HCl at room temperature a solution of 10 μg./ml. has lost 18% of its potency after 3 hours, 75% after 23 hours (*ibid.*). In .015 *N* NaOH a solution of 0.2 μg/ml. deteriorates somewhat more rapidly, losing 20% activity after $\frac{2}{3}$ hour, 45% after 6 hours, 90% after 23 hours (*ibid.*). According to Smith (1948a), boiling in alkaline solution discharges the red color. Boiling with normal HCl for 1 hour does not apparently alter the color, but leads to the formation of two pigments, both insoluble in ether as is B_{12}, but one extractable with $CHCl_3$ the other with *n*-butanol (*ibid.*). The P can be hydrolyzed off in 20% HCl at 100° after about 6 hours, although no liberation of phosphate is detected at room temperature after 17 days (Ellis *et al.*, 1949b). A product of this reaction is a black Co complex with a visible spectrum (in dioxane) similar to that of B_{12}. It is insoluble in water but soluble in dilute alkali (Ellis *et al.*, 1949a).

Pyrolysis and alkaline fusion yield some evidence for the presence of

pyrroles or other N-containing 5-membered rings (Brink *et al.*, 1949a). "Degradation of vitamin B_{12} by acid hydrolysis" yields a product identified as 5,6-dimethylbenzimidazole, which is thought to be bound to the rest of the molecule according to Fig. 1 (Brink *et al.*, 1949b).

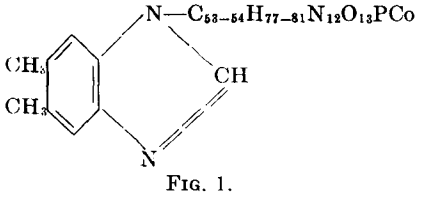

FIG. 1.

While crystalline preparations have yielded evidence of small amounts of alpha amino acids liberated by acid hydrolysis, in very well purified samples no amino acids can be detected by paper chromatography, and are presumably present to less than 0.2% (Brink *et al.*, 1949a).

Two other biologically active compounds besides B_{12} have been characterized—B_{12a} and B_{12b}. B_{12a} (Kaczka *et al.*, 1949) is formed on exposure to air of a solution of B_{12} that has been catalytically reduced. It is a red crystalline compound differing only slightly in physical properties from B_{12}, but with only $\frac{1}{6}$ to $\frac{1}{2}$ of the biological activity (*ibid.;* Wolf *et al.*, 1949; assays clinical, rat, chick, microbiological). B_{12b} and B_{12} form two separate pink bands on silicic acid columns, using a concentrate from *Streptomyces aureofaciens* as starting material (Pierce *et al.*, 1949). B_{12b} has been crystallized in small red rods. It contains Co and P, is biologically active (chicks, *L. leichmannii*) and has a spectrum differing slightly from that of B_{12}. It has been recently reported to have the same potency as B_{12} in chick, microbiological and clinical assays (Stokstad *et al.*, 1949d).

Smith, who first reported on this chromatographic procedure, also obtained two separate pink bands using liver concentrates (Smith, 1948a; Smith and Parker, 1948); both fractions were clinically active, "one apparently resulting from the proteolysis of the other" since the faster moving one was prominent only in preparations of papain digested liver, not in preparations from undigested liver. Possibly one of these was B_{12b}. Winsten and Eigen (1949a) and Winsten *et al.* (1949) using paper strip chromatography have reported the presence of two zones, both active microbiologically, in commercially available crystalline B_{12} specimens. Their evidence indicates that these were not formed from one substance during the chromatography that lasted overnight, although Hendlin (Hendlin and Woodruff, 1949) stated that in similar experiments lasting 3 days, an originally homogeneous B_{12} is converted to an equilib-

rium mixture of B_{12} and B_{12a}, containing about 10% of the latter. (Statement not included in printed abstract.)

Smith first erroneously reported three P atoms per molecule of his red crystals (Smith, 1948b) indicating a different product from that of the Merck group and Ellis and coworkers, but this error was later corrected (Smith, 1949).

2. Concentrates

In the present state of knowledge the earlier observations on the behavior of B_{12} activity in crude concentrates are still of interest although the applicability of these properties to pure B_{12} must be regarded as questionable. First of all it is obvious that impurities present may affect the behavior of the activity. Secondly, the activity in crude sources may actually be present largely in various conjugated forms. The process of preparing pure B_{12} has not been described by anyone. Smith found it necessary to digest his concentrates with trypsin (Smith and Parker, 1948) or "mixed bacterial enzymes" (Smith, 1948a) to get final purification. It is not clear whether this procedure was effective simply by changing the adsorption and other properties of accompanying impurities, or whether the compound(s) carrying the activity were also altered by the removal of a peptide portion not necessary for activity. If the latter is the case the active material in natural sources may have quite different properties from pure B_{12}. Keeping in mind the reservation that the properties of the activity in crude materials or concentrates may be referable to larger molecules, conjugates of B_{12}, we will briefly summarize what properties have been consistently reported for what appears to be B_{12} activity in liver and fish solubles and the digest of Bird's acid precipitate from cow manure. These properties should continue to be of interest as long as there is a point in fractionating liver to concentrate other biologically active compounds.

The earlier work on concentrating anti-pernicious anemia activity has been reviewed by SubbaRow *et al.* (1945). The papers which are most informative on the properties of the primary liver factor (B_{12} active materials) are those of Cohn *et al.* (1928), Dakin *et al.* (1935, 1936), Karrer (1941), Kyer (1935), Sladek and Kyer (1938), Laland and Klem (1936). Of the recent papers, only Smith (1948a) describes any properties of liver concentrates. In the work on concentration of B_{12} active materials tested on laboratory animals, the papers of Bird *et al.* (1948), Jaffe and Elvehjem (1947), Pensack *et al.* (1949), Robblee *et al.* (1948a) and Zucker and Zucker (1948a) contain pertinent data. In addition, we have drawn upon a few unpublished results.

Solubilities—the active material is soluble in water, glycerine, glacial acetic acid, 90% phenol, and insoluble in ether, petroleum ether, chloro-

form, anhydrous acetone and anhydrous pyridine. It is soluble in aqueous ethanol and aqueous acetone. Somewhat more water is needed for a given degree of solubility with acetone than with ethanol. *Liquid-liquid distribution*—phenol-water: the activity is all in the phenol, but the addition of ether shifts the activity to the aqueous phase. *n*-BuOH-water: the activity is largely if not entirely in the aqueous phase, but the distribution ratio is shifted somewhat in favor of the butanol phase by the addition of phenol or ammonium sulfate. Aqueous acetone-saturated aqueous ammonium sulfate: activity mostly in acetone phase. *Salting out*—one-third to full saturation of aqueous solution with ammonium sulfate precipitates activity, but incompletely; precipitation at pH 3 gives better recovery. *Precipitating reagents*—the activity is not precipitated by Ca^{++}, La^{+++}, Ba^{++}, picric acid, trichloracetic acid, or basic lead acetate at a pH below 7.5. It is precipitated by phosphotungstic acid at pH 3 or below and also by Reinecke salt. These last reagents still give precipitates with very potent concentrates of B_{12}, but it was not stated whether the activity is precipitated (Smith, 1948a). *Adsorption*—the activity can be adsorbed on charcoal from aqueous solution. Neutral or slightly acid solutions have usually been used. Some failure to adsorb, or incomplete adsorption has been reported for animal protein factor activity (McGinnis *et al.*, 1947b; Bird *et al.*, 1948). Obviously unless a substance in a given solvent has a very favorable adsorption isotherm, it will be distributed between solution and adsorbate during either adsorption or elution procedure when one or at most two equilibrations are made. Then, depending on the concentration of the substance or substances to be adsorbed and the concentration of the charcoal suspension any result can be obtained from pretty complete adsorption to little adsorption. In our hands fairly complete adsorption of all activity from crude concentrates requires large amounts of charcoal; thus starting with 25 g. of a crude concentrate with activity corresponding to 4 mg. B_{12}, in 750 ml. volume, 25 g. Norit adsorbed 25% of the activity, and 120 g. was required to adsorb 90% of the activity. It cannot be said at this time whether this behavior is directly that of B_{12}, of a B_{12} conjugate, of a series of B_{12} active substances of differing adsorption properties, or to what extent the results are affected by impurities present which have displacing properties. Ninety percent phenol is an extremely efficient eluting agent; hot 50 or 65% ethanol is less efficient but more selective. *Chromatography*—Smith and Parker (1948) describe the use of alumina columns with water as solvent to remove dark impurities, and the use of columns of wet silicic acid or dry starch, with aqueous *n*-butanol, *n*-propanol or isopropanol with or without dissolved phenol to adsorb and purify the active material. Winsten and Eigen (1949a)

and Winsten *et al.* (1949) describe paper strip chromatography using *n*-butanol saturated with water, which separates B_{12}-like factors from desoxyribosides, and the same solvent containing glacial acetic acid, which separates 2 or 3 B_{12}-like factors (B_{12}, B_{12a}, B_{12b}?) from each other.

3. Terminology

The term *animal protein factor* (APF) is generally applied to the factor or factors necessary for the maintenance of normal hatchability and growth of young chicks subsisting on a practical ration which does not contain a crude animal protein supplement. Subsequent usage has branched out in several directions; the term has been applied to a factor needed by mice on a purified casein diet (Bosshardt *et al.*, 1949c) by rats on cottonseed or soybean meal diets (Zucker and Zucker, 1948b; Van Landingham and Lyon, 1947), by *Lactobacillus casei* (Daniel *et al.*, 1948). It has now been shown that a deficiency common to all these conditions (except possibly the last), is supplied by crystalline vitamin B_{12} which is certainly therefore the principal animal protein factor.

The term *vitamin* B_{12} is apparently being used by its originators for a single chemical entity of defined crystalline form, solubilities, spectral absorption, etc. Closely related compounds sharing the same kind of biological activity are designated as B_{12} with a subscript—B_{12a}, B_{12b}, etc. We have tried to use the term B_{12} *activity* for that biological activity which can be obtained in higher animals from B_{12}. Then B_{12a}, B_{12b} and possibly still other relatives and conjugates thereof show B_{12} activity, although not necessarily the same amount of activity per molecule or the same relative activity for different species.

There is currently an attempt to restrict the term animal protein factor to a chick growth factor demonstrated by use of rations based on soybean meal and corn which have been typically used in much of the chicken work. The term *animal protein factor complex* has been used by Stokstad *et al.* (1949c) for a group of factors needed on such a diet. Their experiments indicate that crystalline B_{12} does only part of the job. APF complex might then be understood to include the B_{12} family which provide B_{12} activity, plus sources of one or more other biological activities, all of them needed by poultry on a corn-soy ration.

The terms *cow manure factor* (Rubin and Bird, 1946a, b; Bird *et al.*, 1948), *X factor* (Hartman, 1946), *zoöpherin* (Zucker and Zucker, 1948a), *Lactobacillus lactis Dorner* or *LLD factor* (Shorb, 1948), *L. leichmannii factor* (Skeggs *et al.*, 1948) and *extrinsic factor* (Castle *et al.*, 1944) are used for activities as tested by particular procedures in all of which crystalline B_{12} is effective. The alternate technique with *L. leichmannii* (Stokstad *et al.*, 1949a) using thioglycolic acid in the medium may lead