VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

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EDITORS' PREFACE

In introducing the present volume of *Vitamins and Hormones* the Editors venture to hope that it may enjoy as favorable a reception as have each of its eleven predecessors. The proportion of articles concerned with hormones is somewhat smaller in this volume than in many preceding ones. This does not, of course, reflect any slackening in the tempo of research in the hormone field in recent years; rather it is because many of the newer and more significant developments in this field had not, at the time when this volume was being planned, reached a stage at which they could be profitably reviewed in a comprehensive and critical manner. It is hoped that some of these newer developments will be reviewed in forthcoming volumes.

The Editors wish to take this opportunity of expressing their warm appreciation for the cooperation they have received from the contributors to this volume. They also wish to express their gratitude to those of their colleagues in many parts of the world who have lightened their editorial duties by coming forward with valuable suggestions and constructive criticisms.

Robert S. Harris
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CONTENTS

Chemistry of Vitamin B_{12}

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I. Introduction

Soon after the epoch-making recognition of the effectiveness of whole liver therapy for the control of pernicious anemia, various groups of investigators carried out programs on the fractionation of liver extracts for the purification of the active principle. Many investigators published chemical fractionation studies which can be found in an extensive review paper on "The Chemistry of Anti-Pernicious Anemia Substances of Liver" (Subba-Row *et al.*, 1945).

The combined chemical, clinical, and microbiological research led to the isolation of a red crystalline compound which had extremely high biological activity. It was designated vitamin B_{12} and announced in April of 1948 by Rickes, Brink, Koniuszy, Wood, and Folkers (1948a). Once this active principle of liver was established as a red compound, the color itself provided guidance in subsequent chromatographic and related manipulations and simplified the selection of fractions for biological tests. It is the purpose of this paper to discuss the development of the chemical investigation of vitamin B_{12} up to the present time.

II. Isolation

The isolation of vitamin B_{12} from liver was achieved by a lengthy process of fractionation using almost entirely physical methods (Rickes *et al.*, 1948a; Smith, 1948; Smith and Parker, 1948). With an original concentration of about 1 part per million in liver, the concentration of vitamin B_{12} involved numerous procedures (Smith and Parker, 1948; Fantes *et al.*, 1949; Smith, 1950a). When proteolyzed liver extract was used as the source of the vitamin, the initial steps in the treatment of minced liver were avoided. The active principle may be adsorbed on activated carbon or fuller's earth. The activity can be eluted with any of several solvents such as 65% ethyl alcohol, aqueous phenol, or an aqueous pyridine mixture. The activity is recovered by evaporation of the solvent or by extraction from phenol into water after dilution with an inert organic solvent such as ether.

Partition chromatography on damp silica using butanol containing 11% to 12% water was found valuable in the purification. Variations of this method were investigated; starch and kieselguhr served as solvent supports, and other solvents were used such as a mixture of butanol and phenol or mixtures of *n*- or *iso*propyl alcohol with 10% to 25% water. In the fractionation by this method the pink color of vitamin B_{12} was of great advantage in following activity.

Adsorption chromatography on silica or alumina was also employed. Aqueous solutions of the active principle when passed through an alumina

column lost part of the impurities while the activity ran through the column. Columns of bentonite or aluminum silicate were used for adsorption chromatography by Ellis *et al.* (1949a).

Several extraction steps were investigated and found to be of advantage at various stages in the fractionation scheme. Extraction with butanol of aqueous solutions containing fairly high concentrations of ammonium sulfate removes the active principle, which can then be extracted from the butanol by water (Ellis *et al.*, 1949a). Phenol or cresol in combination with a less efficient solvent such as butanol or toluene has also been useful in the extraction procedures.

The great difficulty encountered by early investigators in the purification of the anti-pernicious anemia factor was the absence of a convenient assay. The discovery of a microbiological method for the measurement of vitamin B_{12} by Shorb (1948) was of great value. By the use of *Lactobacillus lactis* Dorner a 23-hour growth period was sufficient for the assay procedure. With an assay method available other sources of vitamin B_{12} were soon discovered. *L. lactis* activity was found in milk powder, beef extract, and culture broths of strains of *Mycobacterium smegmatis*, of *Lactobacillus arabinosus*, of *Bacillus subtilis*, and of several *Streptomyces* species including *S. roseochromogenus*, *S. griseus*, and *S. antibioticus* (Rickes *et al.*, 1948c). Vitamin B_{12} was isolated in crystalline form from a strain of *S. griseus* and proved to be identical with the material isolated from liver. It was also isolated as a by-product from the production of streptomycin (Schindler and Reichstein, 1952).

In the examination of small amounts of vitamin B_{12}-containing materials, paper chromatography has been of value. Because of its red color vitamin B_{12} can be observed visually. Of particular interest has been the use of bioautographic procedures which are applicable on a micro-scale where direct visualization is impossible. In these the filter paper chromatogram of Vitamin B_{12}-containing material is developed with one of several solvents, the most commonly used being wet *n*- or *sec*-butyl alcohol or one of these containing a little acetic acid. The positions of the microbiologically active fractions are visualized by "zones of exhibition" produced on sheets of nutrient agar seeded with *L. lactis* Dorner after contact with the paper strips and incubation (Cuthbertson and Smith, 1949; Smith *et al.*, 1950; Winsten and Eigen, 1949; Kocher *et al.*, 1950).

III. Properties

Vitamin B_{12} is a red compound, crystallizing from water or aqueous acetone in needlelike crystals which possess the refractive indices of α, 1.616; β, 1.652; and γ, 1.664 after drying. The crystals darken to black at about 210–220° C. but fail to liquefy below 300° C. (Rickes *et al.*,

1948a). Vitamin B_{12} normally contains about 12% moisture, is odorless and tasteless, and soluble in water to the extent of 1.25% at 25° C. to give a neutral solution (Macek and Feller, 1952). The ultraviolet absorption spectrum of aqueous solutions of vitamin B_{12} is characterized by three maxima at 278, 361, and 550 mμ with extinction coefficients ($E^{1\%}_{1\,cm.}$) of 115, 204, and 63, respectively (Fantes *et al.*, 1949; Wijmenga *et al.*, 1949; Brink *et al.*, 1949). The absorption spectrum is practically unchanged by shifts in the pH of the solution.

The optical activity of vitamin B_{12} in aqueous solution has been measured at two wave lengths, $[\alpha]^{23}_{6563} = -59° \pm 9°$, (Brink *et al.*, 1949) and $[\alpha]^{20}_{6438} = -110° \pm 10°$ (1.98 mg. in 0.4 ml. of water) (Fantes *et al.*, 1949). The infrared absorption spectrum has been published (Fantes *et al.*, 1949). Electrometric titration in aqueous solution failed to reveal the presence of either acidic or basic groups, but in glacial acetic acid vitamin B_{12} behaves as a polyacidic base (Brink *et al.*, 1949). In aqueous solution the specific conductivity at 25° C. was found to be 11.8 gemmhos at a molar concentration of 2.2×10^{-4}. If it is assumed that a single ionizing group is present, the molar conductivity is 53 (Fantes *et al.*, 1949). Vitamin B_{12} has been subjected to electrical mobility experiments designed to separate it from related compounds of natural origin. In 0.5 *N* acetic acid containing 0.01% potassium cyanide, its mobility is zero (Holdsworth *et al.*, 1953).

The exact molecular formula of vitamin B_{12} is still in question. The molecular weight determined ebullioscopically in methanol solution gave a value of 1490 ± 150 (Brink *et al.*, 1949). From the X-ray crystallographic data, Dr. Hodgkin has calculated a value of 1360 to 1575 (Hodgkin *et al.*, 1949). Analyses of samples dried in a weighing pig at 100° C. support a formula of $C_{61-64}H_{89-92}N_{14}O_{13}PCo$ (Brink *et al.*, 1949). Analysis of a carefully purified perchloric acid salt of vitamin B_{12} supported the formula $C_{63}H_{84}N_{14}O_{14}PCo.6HClO_4$ (Alicino, 1951).

Vitamin B_{12} is a cobalt co-ordination complex containing one replaceable cyano group bound co-ordinatively to the cobalt atom (Brink *et al.*, 1950a). Magnetic susceptibility measurements indicate its diamagnetic character and show that the cobalt is trivalent (Grün and Menasse, 1950; Wallmann *et al.*, 1951; Diehl *et al.*, 1950). The polarographic behavior of cyanocobalamin in 0.1 *M* sodium enta, pH 9.5, is characterized by a single reductive wave, $E_{\frac{1}{2}} = 1.021$ volts vs. saturated calomel electrode. These results lead to the conclusion that the valence states of cobalt in vitamin B_{12} are the normal 2 and 3 (Boos *et al.*, 1953).

Stability. Vitamin B_{12} has an optimum stability in aqueous solution at pH 4.5 to 5.0. No significant loss in activity at room temperature has been noted for periods of two years or longer (Macek and Feller, 1952). At pH

2 or pH 9 to 12 a decomposition of vitamin B_{12} has been noted which is accelerated at higher temperatures (Hartley *et al.*, 1950).

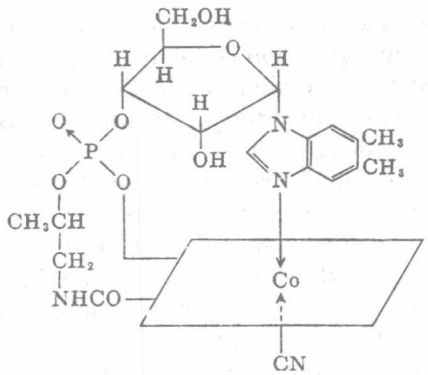
In preparations containing other B vitamins, vitamin B_{12} demonstrates a high order of stability (Macek and Feller, 1952). Aqueous solutions of vitamin B_{12} are incompatible with ascorbic acid (Trenner *et al.*, 1950). Solutions of vitamin B_{12} are unstable in the presence of either oxidizing or reducing substances and exposure to daylight (Hartley *et al.*, 1950; Fantes *et al.*, 1949; Lang and Chow, 1950).

IV. Structural Investigation

Early in the investigation of the chemical nature of vitamin B_{12} it was found to be a cobalt co-ordination complex (Rickes *et al.*, 1948b). As the study of the structure of this molecule progressed, the stability of this complex became of significant interest. A number of products of hydrolysis and other decomposition reactions have been identified; in all but the most severe reactions a red acidic cobalt complex moiety has always been present. The nature of this central complex system is still not fully known. Because of the known physiological relationship to anemia it was early suspected that this portion of the molecule contained a cobalt complex of a polypyrrole, related to either the porphyrins or bile pigments. Indeed early evidence in support of this concept was obtained in the isolation of a pyrrole-containing distillate from alkaline fusion of vitamin B_{12} (Brink *et al.*, 1949). Other evidence has been derived from the study of cobalt co-ordination complexes of known porphyrin compounds (McConnel *et al.*, 1953). It has also been suggested on the basis of the behavior of vitamin B_{12} under specific polyene oxidation conditions that a terpene-type structure is present. The manner in which such a moiety is combined in the molecule remains unanswered (Schmid *et al.*, 1953).

In reviewing the classical chemistry involved in the degradative study of vitamin B_{12} it is of interest to list the component degradation products so far established as portions of the molecule. Early hydrolytic experiments revealed the presence of ammonia (Ellis *et al.*, 1949d). Later and more careful investigation showed that five molecules of ammonia in addition to that originating from cyanide are liberated by acid hydrolysis (Schmid *et al.*, 1953). The isolation of 5,6-dimethylbenzimidazole was followed by the identification of a phosphate of α-ribazole (1-α-D-ribofuranosyl-5,6-dimethylbenzimidazole) as a portion of the vitamin B_{12} molecule (Kaczka *et al.*, 1952). The ninhydrin reacting substance observed in early experiments was found to be D$_g$-1-amino-2-propanol (Wolf *et al.*, 1950). The question of whether there are one or two molecules of this moiety present in vitamin B_{12} must still be answered, but the most recent

evidence indicates that only one is present (Armitage *et al.*, 1953). Although it is not possible to make an exact arithmetical accounting, it can be said that the known parts of the molecule account for only about 18 carbon atoms and 9 (or 10) nitrogens and leaves more than half the molecular weight without structural representation. As a working basis for consideration a graphic formula has been postulated (Armitage *et al.*, 1953; Cooley *et al.*, 1953; Kaczka and Folkers, 1953). This unknown structural unit appears to contain the acidic cobalt co-ordination complex which is formed in many hydrolytic reactions.



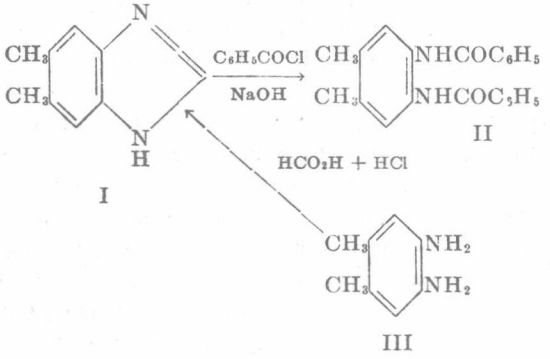
In considering the chemistry of these known portions of the molecule the identification of each one is discussed under the headings below.

1. 5,6-Dimethylbenzimidazole

Acid hydrolysis of vitamin B_{12} yielded a new basic compound which was identified by its reactions and by synthesis as 5,6-dimethylbenzimidazole (I) (Brink and Folkers, 1949, 1950). A solution of vitamin B_{12} in 6*N* hydrochloric acid was heated at 150° C. for 20 hours; then the hydrolyzate was extracted continuously with chloroform. The chloroform extractives yielded an ether-soluble fraction which was further purified by sublimation and crystallization. The following properties of the compound were noted: basic to litmus paper, m.p. 205–206° C., optically inactive, molecular composition $C_9H_{10}N_2$, 1.1 moles of acetic acid per mole of compound in Kuhn-Roth determination, an acid solution of the compound showed absorption maxima at 2745 A. (E_M 7500) and at 2840 A. (E_M 8100). In alkaline solution maxima were observed at 2470 A. (E_M 3900), 2775 A. (E_M 4900), 2810 A. (E_M 5250), and 2880 A. (E_M 5700). The change in absorption with pH is reversible.

The molecular formula of the degradation product and its properties ndicated that it might be a benzimidazole, and a close resemblance of the

ultraviolet absorption spectra with that of 2,5-dimethylbenzimidazole was observed. When the degradation product was treated with benzoyl chloride in aqueous alkali, a new product having the composition $C_{22}H_{20}N_2O_2$ was formed; this product of benzoylation was identified as 4,5-dibenzamido-1,2-dimethylbenzene (II) by a comparison with a synthetic sample. Although the dibenzamido derivative was not experimentally hydrolyzed to 4,5-diamino-1,2-dimethylbenzene (III), this diamine may be considered as a degradation product of vitamin B_{12}.



I II III

Synthetic 5,6-dimethylbenzimidazole was obtained from the reaction of 4,5-diamino-1,2-dimethylbenzene and formic acid. The synthetic compound melted at 204–205° C. and caused no depression of the melting point when it was mixed with the product of degradation. The absorption spectrum of the synthetic benzimidazole was identical with that of the degradation product.

The yield of 5,6-dimethylbenzimidazole in this hydrolysis was consistently about 70% of one molar equivalent, and on the basis of the stability of the compound, it seemed that there was no more than 1 mole of 5,6-dimethylbenzimidazole per mole of vitamin B_{12}. It is noteworthy that the 1,2-diamino-4,5-dimethylbenzene moiety (IV) appears in the 5,6-dimethylbenzimidazole from vitamin B_{12} and also in the riboflavin molecule.

IV

5,6-Dimethylbenzimidazole as a degradation product of vitamin B_{12} was also identified on the basis of spectrophotometric evidence involving the use of highly refined micro-operations (Holiday and Petrow, 1949).

Paper chromatograms of an acid hydrolyzate of vitamin B_{12} which were developed with an *n*-butanol-acetic acid solvent revealed three blue fluorescent spots in the light of a low-pressure mercury vapor lamp with a Corning 9863 glass filter. The three compounds responsible for the fluorescent spots were designated components alpha, beta, and gamma. The R_F values were 0.62, 0.77, and 0.85, respectively. The identification of the γ component as 5,6-dimethylbenzimidazole was accomplished by comparison of its properties with a series of 22 methylated benzimidazoles synthesized for the purpose. Components β and γ were concluded to represent different stages of degradation of the α component, α-ribazole phosphate (Cooley *et al.*, 1950a, b, c; Buchanan *et al.*, 1950a, b; Kaczka *et al.*, 1952).

Interesting details of the spectrophotometric evidence for the identification of 5,6-dimethylbenzimidazole have been described by Beaven *et al.* (1949). The ultraviolet absorption of compound γ pointed to a dicyclic chromaphore of unsaturated or aromatic character. Since the spectra of aromatic compounds of this type are different, a heterocyclic chromaphore was indicated. The marked fine structure indicated that the heterocyclic compound probably contained nitrogen. Compounds containing one or more hydrogen atoms and two fused six-membered rings showed absorption at longer wave lengths and could be excluded. Of the compounds containing a six-membered ring fused to a five-membered ring, only benzimidazole and indazole showed spectra resembling that of component γ.

The spectra of components α, β, and γ resembled that of benzimidazole, but the characteristic long wave length fine structure band of the latter compound lay at a much shorter wave length. The shifted bands of the three components led these investigators to conclude that components α, β, and γ were all substituted benzimidazoles. Components α and β were evidently 1-substituted benzimidazoles and corresponded to α-ribazole phosphate and α-ribazole, respectively.

Synthetic 5,6-dimethylbenzimidazole showed an ultra-violet absorption spectrum identical with that of component γ where the absorption in both acid and alkaline solutions were compared. These spectra are so characteristic that they offer more presumptive evidence than is usual. This interpretation was supported by experiments on the paper chromatographic behavior of component γ and 5,6-dimethylbenzimidazole which revealed no differentiation. The absorption of synthetic 1,5,6-trimethylbenzimidazole supported speculations of the identity of components α and β with 1-substituted 5,6-dimethylbenzimidazoles.

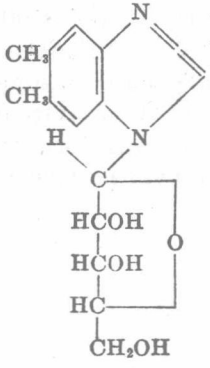
The following new benzimidazoles were prepared for these spectroscopic studies:

1,6-, 1,7-, 2,4-, 4,5-, 5,6-dimethylbenzimidazole;
1,2,7-, 1,4,5-, 1,5,6-, 2,4,5-, 2,5,6-trimethylbenzimidazole;
1,2,4,5-, 1,2,5,6-tetramethylbenzimidazole.

In general, the alkylated benzimidazoles were prepared by reaction of the respective *o*-phenylenediamine with formic acid or acetic acid in the presence of 4*N* hydrochloric acid. The *o*-diamines were prepared by catalytic reduction of the corresponding *o*-nitroanilines. *N*-Methyl-*o*-nitroanilines were prepared by methylation of *p*-toluenesulfonyl derivatives of the corresponding *o*-nitroanilines in order to obtain 1-methyl-substituted benzimidazoles.

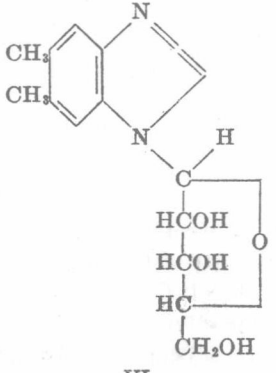
2. 1-α-D-Ribofuranosyl-5,6-dimethylbenzimidazole (α-Ribazole)

a. Degradation. The degradation of vitamin B_{12} to 1-α-D-ribofuranosyl-5,6-dimethylbenzimidazole (α-ribazole) (V) and the synthesis of the degradation product have been communicated by Brink *et al.* (1950b).



V

The anomeric riboside (VI) was also synthesized.

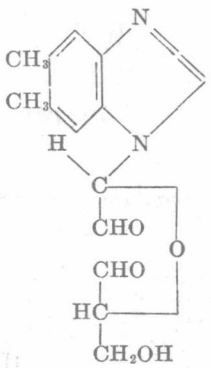


VI

The names α-ribazole (V) and β-ribazole (VI) were designated for the corresponding 1-D-ribofuranosyl-5,6-dimethylbenzimidazoles.

The degradation of vitamin B_{12} to 1-α-D-ribofuranosyl-5,6-dimethylbenzimidazole was accomplished by hydrolysis of vitamin B_{12} in 6*N* hydrochloric acid at 120° C. for 8 hours (Brink and Folkers, 1952). A basic fraction, isolated from the hydrolyzate, showed an absorption spectrum which was significantly different from that of 5,6-dimethylbenzimidazole; it was only sparingly soluble in ether, and the ether-insoluble material was shown to contain carbohydrate by the test involving dehydration to furfural or its derivatives. The crude product was a mixture of a new benzimidazole derivative and 5,6-dimethylbenzimidazole. When the hydrolysis was carried out at 100° C. overnight, the splitting of the glycoside was negligible, and the new benzimidazole derivative was readily isolated as a pure picrate. The picrate was dextrorotatory and showed an absorption spectrum in acidic ethanol solution with maxima at 2760 A. (E_M 10,950), 2850 A. (E_M 10,600), and 3590 A. (E_M 13,000). The picrate had the composition $C_{14}H_{18}N_2O_4.C_6H_3N_3O_7$.

The crystalline glycoside picrate consumed 0.92 mole of periodate per mole of base and a picrate of the oxidation product was obtained. The periodate oxidation product was formulated as α-(5,6-dimethylbenzimidazole-1)-α'-hydroxymethyldiglycolic aldehyde (VII).



VII

Similar oxidation of a model compound, 1-β-D-glucopyranosyl-5,6-dimethylbenzimidazole picrate with 2 moles of periodate led presumably to the anomeric compound (VIII). The reaction of the unknown glycoside of 5,6-dimethylbenzimidazole with 1 mole of periodate permitted the assignment of a furanoid ring structure to the pentose moiety; the pentose formulation was also shown by the molecular formula. Since the oxidation