VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

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EDITORS' PREFACE

The fifteenth volume of *Vitamins and Hormones*, which the Editors are happy to present, is as international in its authorship as have been most of its fourteen predecessors. Of the eight contributions to this volume three come from the United States, three from Great Britain, two from Switzerland, and one from South Africa.

The equal distribution of articles between the two fields of interest which the Editors always endeavor to achieve has in fact been achieved in the present volume. Three of the articles are on vitamins and related compounds; three are on hormones; one is concerned with both vitamins and hormones; and the remaining one, on the biosynthesis of cholesterol, is relevant to both fields in so far as cholesterol is a precursor of the steroid hormones and of vitamin D_3.

In presenting this volume the Editors wish to take the opportunity of thanking all the contributors for their cooperation, and of reminding all readers that suggestions about topics which could be profitably reviewed in future volumes are always gratefully received.

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October, 1957

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By J. T. Irving, *Department of Experimental Odontology, and the Joint Dental Research Unit of the Council for Scientific and Industrial Research and the University of the Witwatersrand, Johannesburg, South Africa*

Chemistry of Vitamin B_{12}

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I. Introduction

Since the chemistry of the antipernicious anemia factor, vitamin B_{12}, was last reviewed in "Vitamins and Hormones" by Folkers and Wolf (1954), outstanding progress has been made, resulting in the complete structural elucidation of the vitamin. It is our purpose in the present article to bring that review up to date by reviewing results published between the date of its appearance and September, 1956. In the intervening period several review articles have appeared, each dealing with various aspects of the B_{12} problem (Smith, 1954, 1955, 1957; Heinrich and Lahmann, 1954; Kon, 1955; Hodgkin *et al.* 1955a; Briggs and Daft, 1955; Ford and Hutner, 1955; Pfiffner and Bird, 1956; Johnson, 1956).

II. Isolation

The demand for vitamin B_{12} has increased, and many recent papers on the preparation of the vitamin are concerned with commercial production which depends on fermentation methods; in fact vitamin B_{12} is synthesized almost entirely by microorganisms in nature (see Ford and Hutner, 1955). It is now more usual to employ special organisms for B_{12} production than to isolate it as a by-product from antibiotic fermentations. Various species of *Streptomyces* have been recommended for the preparation of vitamin B_{12}, e.g. *S. olivaceus* (Hester and Ward, 1954; Pfeifer *et al.*, 1954), as well as *Bacillus megatherium* (Garibaldi *et al.*, 1953; Hester and Ward, 1954), although no outstanding improvements have been reported in its isolation (e.g. Janicki *et al.*, 1953).

Vitamin B_{12} appears to exist as a conjugate (i.e. in combination with a protein) in animal tissues, and some progress has been made in the purification of this complex (Haùsmann, 1953; Wijmenga *et al.*, 1954; Hedbom, 1955; review, Ford and Hutner, 1955).

III. Physical Properties

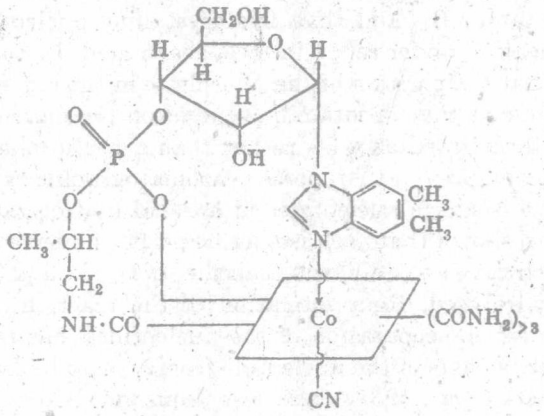
The isoelectric point of vitamin B_{12} is at pH 1.5 (Ericson and Nihlén, 1953a; Nihlén and Ericson, 1955), thus confirming the presence of weak basic groups in the molecule (cf. Alicino, 1951). The trivalent nature of the cobalt in the vitamin has been confirmed by an accurate wavelength measurement of the cobalt-K absorption line (Boehm *et al.*, 1953, 1954a,b).

Some further work has been carried out on radioactive modifications of vitamin B_{12}. In view of contradictory reports concerning the retention of radioactivity (Co^{60}) in the vitamin after irradiation in the nuclear pile ranging from 80% retention (Anderson and Delabarre, 1951), through 5% retention (Smith, 1952), to no retention at all (Numerof and Kowald, 1953; Woodbury and Rosenblum, 1953), Maddock and Pinto Coehlo (1954) repeated the experiment and confirmed the finding of Smith that there is only slight retention of radioactivity in the cobalt and phosphorus atoms. Bradley *et al.* (1954) have prepared vitamin B_{12} labeled with Co^{58} rather than Co^{60}; the lighter isotope has a much smaller half-life period and is therefore preferable for therapeutic work. Co^{56} and Co^{57} as well as P^{32} have also been incorporated into the vitamin (Smith, 1955). C^{14} can be conveniently introduced into the benzimidazole nucleus at C-2 (Weygand *et al.*, 1954); in an early proof that *S. griseus* could incorporate added 5,6-dimethylbenzimidazole into vitamin B_{12}, these authors added labeled dimethylbenzimidazole to the fermentation when vitamin B_{12} containing

C^{14} was produced. However the question has now been settled beyond any doubt by the preparation of known and new B_{12} analogs by biosynthetic methods as described later. In studies of the biogenesis of vitamin B_{12}, Smith (1956) and Shemin *et al.* (1956) have produced the vitamin, again containing C^{14}, by using C^{14}-labeled δ-aminolevulinic acid as a starting material (see Section VI below).

IV. Structural Investigation

The earlier review (Folkers and Wolf, 1954) summarized the experimental evidence on which the partial structure (I) was advanced for the vitamin in 1953 (Armitage *et al.*, 1953; Cooley *et al.*, 1953; Kaczka and Folkers, 1953). In view of the complexity of the subject it is desirable to deal with the newer structural work under various headings. Studies on D$_g$-1-amino-2-propanol and the benzimidazole nucleotide fragment will be discussed first and will be followed by those studies bearing on the nature of the central, planar, portion of the molecule.



I

1. D$_g$-1-Amino-2-propanol

D$_g$-1-Amino-2-propanol, a hydrolysis product of vitamin B_{12}, has been obtained (Clark *et al.*, 1954) by an improved method. The commercially available DL-compound was converted into DL-2-(1-benzylaminopropyl)-*p*-nitrobenzoate and then resolved as the L(+)-tartrate; the *N*-benzyl group was finally removed by hydrogenolysis. The ratio of one mole of D$_g$-1-amino-2-propanol per mole of vitamin B_{12} (Armitage *et al.*, 1953; Cooley *et al.*, 1953) has been confirmed by all later work.

2. 5,6-Dimethyl-1-α-D-ribofuranosyl-benzimidazole-3′-phosphate (α-Ribazole-3′-phosphate)

The isolation of a 5,6-dimethyl-1-α-D-ribofuranosyl-benzimidazole phosphate was first reported by Buchanan *et al.* (1950a,b), who obtained it as an amorphous barium salt following acid hydrolysis of vitamin B_{12}. The crystalline acid was later obtained by Kaczka *et al.* (1952) and again by Armitage *et al.* (1953). In considering the structure of this nucleotide it seemed a priori that the known course of hydrolysis of the ribonucleic acids (Brown and Todd, 1952) would operate in the case of vitamin B_{12} also, i.e. that the hydrolysis product would be an equilibrium mixture of the 2′- and 3′-phosphates of the parent nucleoside, the cyclic 2′,3′-phosphate being an intermediate in their production. This being so, the position of the phosphate group in the crystalline degradation product actually isolated (assuming it to be homogeneous) was not necessarily the same as in the parent vitamin.

However in a later paper (Kaczka and Folkers, 1953) it was stated that only one isomer of the nucleotide could be detected in an acid hydrolyzate of vitamin B_{12} and that this crystalline nucleotide gave two isomers after heating under reflux in 80% acetic acid. It was assumed by these authors that the position of the phosphate in the crystalline nucleotide was the same as that in vitamin B_{12}, and on the basis of relative R_f values, it was formulated as a 3′- rather than a 2′-phosphate. Todd and Johnson (1952) had previously reported chromatographic evidence for the existence of two isomeric nucleotides in an acid hydrolyzate of vitamin B_{12} and had also shown that each isomer could be converted into a cyclic phosphate which gave an equilibrium mixture of the 2′- and 3′-phosphates when again hydrolyzed. Reinvestigation of the reaction, using Dowex 1 × 2 columns for the separation of the nucleotides, has resulted in the isolation of both isomers of the nucleotide from an acid hydrolyzate of the vitamin (Bonnett *et al.*, 1957a) and also from an alkaline hydrolyzate. Each of the nucleotide isomers was unaffected by aqueous barium hydroxide at room temperature overnight, but each was isomerized to the equilibrium mixture by treatment with concentrated hydrochloric acid at room temperature overnight. The 2′,3′-cyclic phosphate was best obtained by treatment of either isomer with di*cyclo*hexylcarbodiimide, and hydrolysis of the cyclic phosphate either with acid or alkali gave an equilibrium mixture of the 2′- and 3′-nucleotides. The properties of these compounds are therefore similar to those of the pyrimidine and purine 2′- and 3′-ribonucleotides, and the hydrolysis of vitamin B_{12} is strictly analogous to that of the esters of the 2′- and 3′-ribonucleotides; contrary to the opinion of Kaczka and Folkers (1953), the failure to detect the phosphate of amino-

propanol in the hydrolysis products of vitamin B_{12} is therefore not surprising. The position of the phosphate group in vitamin B_{12} itself was finally established as being 3′ by X-ray crystallographic analysis (Hodgkin *et al.*, 1955a).

3. Polyamide Character

The polyamide character of vitamin B_{12} was determined by hydrolytic studies (Armitage *et al.*, 1953) which suggested the presence of six primary amide groups and a seventh amido function involving the D-1-amino-2-propanol grouping as shown in formula I. Electrophoretic evidence was presented for the presence of a heptacarboxylic acid in the mixture of acidic products obtainable from vitamin B_{12} by vigorous acid hydrolysis. Bernhauer and Friedrich (1954) produced similar evidence to suggest the presence of at least four primary amide groups in factor B (vitamin B_{12} lacking the 5,6-dimethylbenzimidazole nucleotide). By application of a modified Van Slyke determination (Diehl and Ellingboe, 1953; Brierly *et al.*, 1954), it was concluded that vitamin B_{12} contained five primary amide groups, but it is now known that the sixth amide group is fairly resistant to hydrolysis. Studies of the partial hydrolysis of the vitamin with acid indicate that three of the amide groups are hydrolyzed more readily than the others (Armitage *et al.*, 1953; Brierly *et al.*, 1954), but the conclusion that some of the amide groups are located sufficiently close to permit the formation of cyclic anhydrides or imides (Murie and Diehl, 1954) has not been confirmed. When the mono-, di-, and tricarboxylic acids derived from vitamin B_{12} without fission of the nucleotide were reconverted to the corresponding amides, the products were identical with vitamin B_{12} (Armitage *et al.*, 1953). However, when amines were substituted for ammonia in the above reaction, a variety of products related to vitamin B_{12} (i.e. with —NHR or —NRR′ instead of —NH_2 in the amide groups) were obtained, some of which had antivitamin activity (Smith *et al.*, 1956).

4. Crystalline Hexacarboxylic Acid Hydrolysis Product

For the determination of the chemical structure of the vitamin B_{12} chromophore, it was clearly desirable to obtain a crystalline degradation product lacking the nucleotide. An extensive study of the hydrolysis of the vitamin (Armitage *et al.*, 1953) had failed to reveal any conditions of hydrolysis which yielded a single product, but in the course of this work a series of nucleotide-free, cobalt-containing acids, containing from one to seven carboxyl groups, was recognized among the products of acid hydrolysis. Moreover, the acidic products derived from acid hydrolyses of

the vitamin were not identical with those derived from alkaline hydrolyses. Reaction of vitamin B_{12} with 30% aqueous sodium hydroxide at 150°C. for one hour was finally adopted for the preparation of nucleotide-free degradation products for further study; these particular conditions gave mainly a penta- and a hexacarboxylic acid together with a small amount of a tetracarboxylic acid fraction. In the initial separation, the mixed acids were subjected to a preliminary electrophoresis on thick paper in order to remove the free nucleotide, and then the individual acids were separated by ion-exchange chromatography on Dowex 1 × 2 resin (approximately 2% cross-linking) in the acetate form. Crystallization of the gummy hexacarboxylic acid from aqueous acetone eventually yielded a quantity of red prisms (Cannon *et al.*, 1954). Analysis of a sample of the acid dried at 50°C. gave values which, assuming the same degree of hydration as observed in the X-ray examination (see below), corresponded to a formula $C_{46}H_{58-60}O_{13}N_6CoCl\cdot 2H_2O$. The infrared spectrum of this hexacarboxylic acid suggested that there were no aromatic rings in the molecule (absence of bands between 690 and 870 cm.$^{-1}$) and that a cyanide group was present (band at 2141 cm.$^{-1}$). Chloride ion was detected by qualitative tests, and the strong resemblance of the ultraviolet absorption spectrum of the acid to that of vitamin B_{12}, both in aqueous and *N*/10 potassium cyanide solution, suggested that the vitamin chromophore had undergone little change during the hydrolysis. A sample of this acid was submitted to Dr. D. C. Hodgkin at Oxford for X-ray crystallographic examination; the results obtained (Brink *et al.*, 1954; Hodgkin *et al.*, 1955b, 1956), which led to the determination of the complete structure of the hexacarboxylic acid, are summarized below.

In a later study of the separation of the acids obtained from the vigorous alkaline hydrolysis of vitamin B_{12} (Bonnett *et al.*, 1957c), an attempt was made to minimize anionic ligand exchange on the cobalt atom by using Dowex 1 × 2 resin in the chloride form and by carrying out the separation in the presence of excess cyanide. By this means both the hexacarboxylic acid and the pentacarboxylic acid were obtained as crystalline dicyano derivatives. The mixture of cobalt-containing acids resulting from vigorous acid hydrolysis of the vitamin was also subjected to fractionation on Dowex 1 × 2 (chloride form) by the above method and the individual tetra-, penta-, hexa-, and heptacarboxylic acids were separated. None of these have yet been obtained in crystalline form, but it is clear that these acids are not identical with those obtained from the alkaline hydrolysis, and they probably represent a separate series.

The first X-ray studies were carried out on vitamin B_{12} itself as well as on the corresponding selenocyanate (selenocyanato-cobalamin).

The investigators were faced with almost insuperable difficulties as all of the electron-density calculations had to be made in three dimensions; moreover, the cobalt atom was not heavy enough to dominate the phases completely and at the outset of this work none of the chemical structure was known, although the partial formula (I) had been deduced by the end of 1953. However, by using the cobalt contributions it was possible to obtain approximate electron-density patterns revealing atomic positions

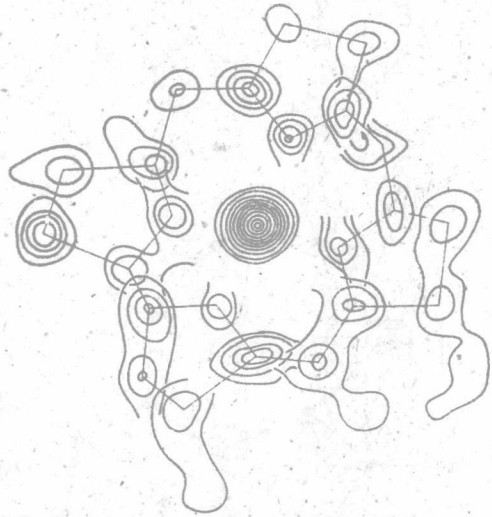
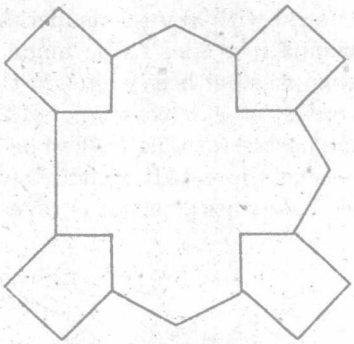
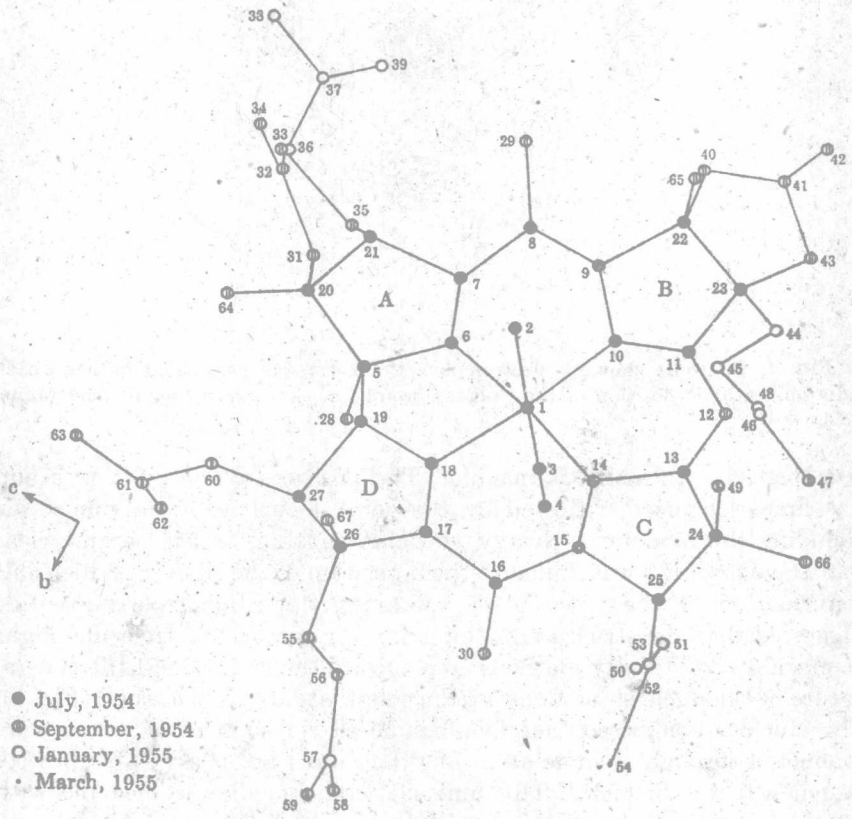


FIG. 1. Contours showing electron density in sections passing at or near each individual atomic position of the "planar group" of the hexacarboxylic acid (June, 1954).

interspersed with spurious maxima. The position of the cyanide group was first recognized, followed by the general outline of the nucleotide including the moderately heavy phosphorus atom, and it became clear that the phosphate was linked to the 3-position of the ribose residue. This confirmation of the presence of 5,6-dimethylbenzimidazole nucleotide suggested that the structure of the unknown part of the molecule might be elucidated by X-ray methods, and toward the end of 1954 the general nature of the nucleus surrounding the cobalt atom was discerned (Fig. 1). This nucleus comprised four five-membered rings, three of which were connected through a single atom but the fourth appeared to be directly attached to the first (II). This unusual arrangement was accepted with reserve until it was detected again in the hexacarboxylic acid degradation product (Brink *et al.*, 1954).



II



III

The calculations of structure factors and three-dimensional Fourier syntheses were accelerated with the aid of the National Bureau of Standards computer (SWAC) in Los Angeles, and by gradually inserting known atoms into the phasing calculations it was possible to construct an atomic projection of all of the atomic positions (excluding hydrogen) in the molecule (III), (Hodgkin *et al.*, 1955a,b).

Much of the chemical structure of the hexacarboxylic acid could be derived from the projection (III). Thus the central cobalt atom was attached to both cyanide and chloride and the remaining four valences of

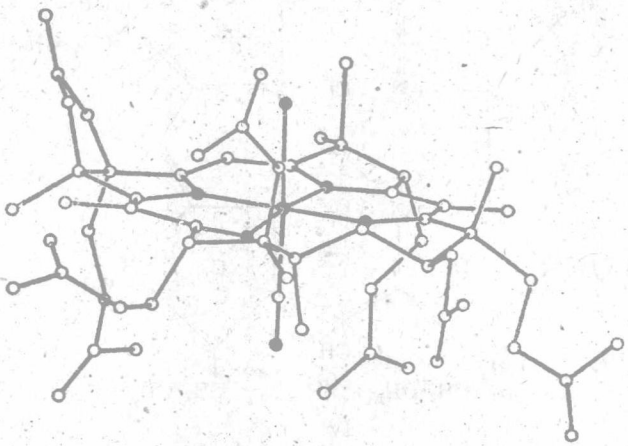


FIG. 2. Atomic positions found in the hexacarboxylic acid projected on the *C*-plane. The chemical bonding deduced is outlined to emphasize the stereochemical form of the molecule.

the cobalt atom were involved in bonds to nitrogen atoms. Each of the nitrogen atoms formed part of a five-membered ring. The six carboxyl groups were also evident, four as propionic acid residues and two as acetic acid residues, and all six were placed as β-substituents in the five-membered rings. Each of the propionic acid groups projected on the same side of the ring as the cyanide and each of the acetic acid groups, on the same side as the chlorine atom (Fig. 2). Fused to one of the five-membered rings (ring B in IV) there was an additional five-membered ring which, from its geometry, was either a lactone or a lactam. The lactam formulation was adopted because of the stability of the hexacarboxylic acid in alkaline solution and because of a band at 1720 $cm.^{-1}$ (γ-lactam carbonyl group) in the infrared spectrum of the acid. In addition, there were eight single-atom substituents (excluding hydrogen) and these were all formulated as methyl groups because of the molecular formula (all the oxygen and nitrogen atoms were already accounted for) and because of the lack