THE CHEMISTRY OF THE VITAMIN B_{12} GROUP

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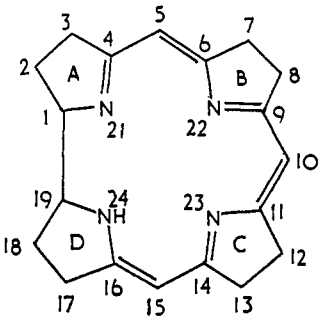
CONTENTS

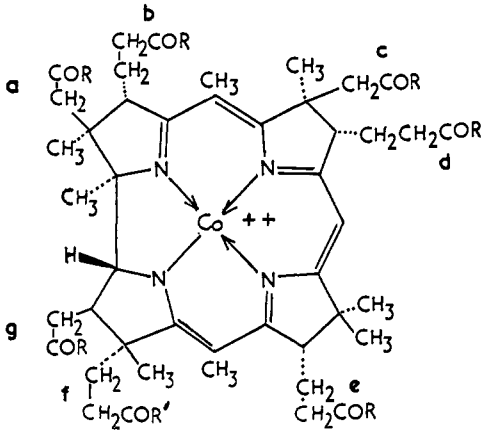
I. INTRODUCTION

The aim of this review is to summarize and discuss the chemistry of the vitamin B_{12} group. Such a discussion is made apposite by the recent developments in this series, by the challenge of synthetic work, and by the potential for investigation which this remarkable group of compounds offers in various branches of chemistry. Previous reviews (126, 170, 268, 271) have naturally tended to emphasize the question of structural elucidation; the proceedings of two conferences (162, 163) covering a wide field of interest in the vitamin have been published; and a monograph (222) is available which gives an excellent coverage of the broader aspects of the subject. In particular, and together with recent reviews (34, 267), it deals with biochemical aspects, and these will not, therefore, be treated in detail here.

It is fortunate that nomenclature in this series was tackled at an early stage. Figure 1 illustrates the basic systems employed (88); a derivative is named on the basis of the largest of the systems I–VII which is contained in that derivative. The names of the vitamin and its analogs are thus derived from cobamide (*e.g.*, α-(5,6-dimethylbenziminazolyl)cobamide cyanide is vitamin B_{12} itself). The cations are sometimes conveniently referred to (*e.g.*, as in aquocobalamin) without a full specification of axial ligands or anions: the naming of these ligands follows the methods of inorganic

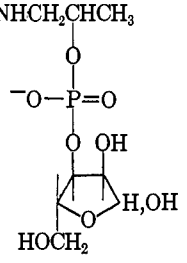


I. Corrin
The basic ring system, indicating position numbering. Large letters denote individual pyrroline rings. C-20 is omitted; it becomes the δ-carbon in the porphyrin nomenclature.



Small letters denote acyl residues starting at position 2 and moving clockwise

II, R = R′ = OH. Cobyrinic acid
III, R = NH_2; R′ = OH. Cobyric acid
IV, R = OH; R′ = $NHCH_2CH(OH)CH_3$. Cobinic acid
V, R = NH_2; R′ = $NHCH_2CH(OH)CH_3$. Cocinamide
VI, R=OH; R′=$NHCH_2CHCH_3$



Cobamic acid

VII, R = NH_2; R′ as in VI. Cobamide

Fig. 1.—Nomenclature in the vitamin B_{12} series.

chemistry and avoids prefixes which would denote substitution in the organic part of the molecule.

The term cobalamin is now officially restricted to vitamin B_{12} (cyanocobalamin), vitamin B_{12a} (aquocobalamin), and vitamin B_{12c} (nitritocobalamin). The suggested naming of III (Factor V_{1a}) as cobyric acid (223) is not official but is very convenient and will be used here. The terms nucleoside and nucleotide, respectively, will be used to refer to 5,6-dimethyl-1-α-D-ribofuranosylbenziminazole and its 3′-(2′-)phosphate and analogous compounds, although it must be borne in mind that these are not strictly parallel to the hydrolysis products of the nucleic acids. The corrinoid system occasionally will be represented by a parallelogram.

II. Structural Elucidation

The isolation from liver of the antipernicious anemia factor in 1948 by Folkers (253) and by Lester Smith (221) was followed by a period of intense chemical investigation, initially with very small amounts of material. Somewhat larger quantities became available with the discovery (254) that *Streptomyces* fermentations produced the vitamin. The investigations of Folkers (Merck) and of Lester Smith (Glaxo) and their co-workers continued, and contributions also came from the group led by Petrow (British Drug Houses). The Cambridge group under Todd made detailed studies of both the nucleotide and corrinoid fragments, while Dorothy Hodgkin (Oxford) and her associates, working with crystals of vitamin B_{12} and certain other derivatives (which included a degradation product isolated in Todd's laboratory) carried out X-ray analyses which, together with chemical considerations, allowed a complete structure to be drawn. A decade or so later the pattern was repeated: Barker (California) discovered and isolated members of the vitamin B_{12} coenzyme series. Some chemical work was done by his group, and by those of Johnson (Nottingham) and Bernhauer (Stuttgart), but again the pieces were assembled as a result of X-ray studies at Oxford.

By early 1949 vitamin B_{12} was known to have a molecular weight of about 1400 [ebullioscopic (methanol), 1490 ± 150, (65); early X-ray data, 1360–1575 (179)]. Elemental analyses (2, 65) suggested a molecular formula in the range $C_{61-64}H_{84-92}N_{14}O_{13-14}PCo$. The presence of the cyanide ligand was not established until 1950, when approximately 1 mole of hydrogen cyanide was detected when the vitamin was heated with aqueous oxalic acid or hydrochloric acid (64). The C≡N stretching frequency was identified in the infrared spectrum while certain analogs, *e.g.*, vitamin B_{12a} which did not show this absorption (187, 249), were converted into the vitamin by cyanide ions (118, 202, 285). At this stage the cobalamin nomenclature was introduced (202), vitamin B_{12} being termed cyanocobalamin.

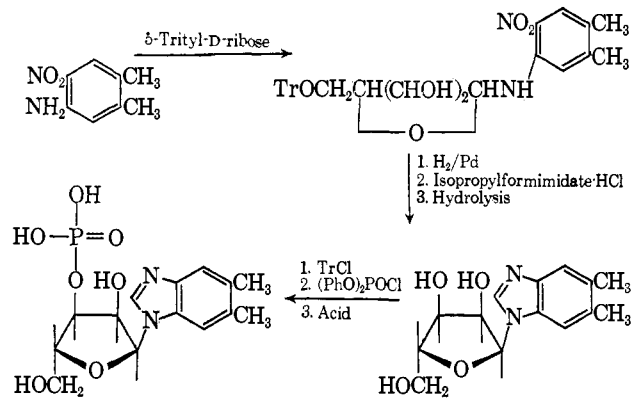
Hydrolytic studies gave complex results, the close study of which was to prove most valuable. Considerable fragmentation of the molecule occurred under acidic or basic conditions, and furnished ammonia (5–6 moles) (5, 60, 83, 90, 239), a volatile organic base which gave a ninhydrin reaction, phosphate associated with fluorescent substances, and an intractable mixture of red acidic compounds. The volatile organic base was eventually (92, 119) identified as D-1-aminopropan-2-ol by making use of the crystalline dibenzoate (287), and it was synthesized by the reduction of D*g*-lactamide

with lithium aluminum hydride. It was originally claimed (83) that 2 moles of propanolamine were evolved in the hydrolysis, but later workers concluded that only one was present (5, 90).

The fluorescence in ultraviolet light of the fragments associated with phosphate facilitated their examination and they received early attention. It was shown that vigorous acid hydrolysis produced three colorless fluorescent substances (22, 91) which represented successive stages of degradation. Chromatographic and spectroscopic evidence (22) suggested that 5,6-dimethylbenziminazole was the ultimate product, and this previously unknown substance was indeed isolated and synthesized (62). The two other components were shown to be the "nucleoside" and "nucleotide" derived from this base. The sugar derivative was isolated as the picrate (63), which had a molecular formula indicating a pentose, and which, since it consumed approximately 1 mole of periodate, was evidently a furanose. Because the product from this oxidation and that from the oxidation (2 moles of periodate) of 5,6-dimethyl-1-β-D-glucopyranosylbenziminazole picrate were not the same, the α-configuration was postulated for the ribosylamine linkage. This conclusion was remarkable in view of the uniform β-configuration of the natural purine and pyrimidine ribonucleosides, but the structure was confirmed as 5,6-dimethyl-1-α-D-ribofuranosylbenziminazole (VIII) by syntheses, one of which involved the preformed benziminazole (193), while others involved cyclization at a later stage (185) (Fig. 2). It may be noted that the benziminazole–ribose linkage is cleaved with great difficulty (section V-F-1), and under conditions which liberate the free base the ribose is destroyed. Hence the sugar itself was not detected among the degradative products.

The nucleotide was first isolated as the amorphous barium salt (74), and later as the free acid (200). The nucleotide was unaffected by periodate, and hence was the 2′- (or 3′-)phosphate. The crystalline free acid isolated from the vitamin was identical with the synthetic substance (Fig. 2) and this compound was formulated tentatively but reasonably as the 3′-phosphate on the basis of chromatographic behavior. In spite of suggestions to the contrary (199), the 3′-phosphate is not the only nucleotide formed on hydrolysis of the vitamin, which gives (44) a mixture of the 2′- and 3′-phosphates (section V-F-2) as would be expected from the reactions of the more common ribonucleotides (68). It was not, in fact, possible to establish the position of the phosphate–ribose linkage in the vitamin on the chemical evidence available.

The major problems now remaining were the nature of the chromophore and the manner of its linkage to the fragments found thus far. A spectroscopic study (23, 93) of the vitamin in relation to the nucleotide and some benziminazole complexes of platinum indicated that a



IX, 5,6-Dimethyl-1-α-D-ribofuranosylbenziminazole 3′-phosphate

VIII, 5,6-Dimethyl-1-α-D-ribofuranosylbenziminazole

Fig. 2.—Synthesis of the nucleoside and nucleotide fragments derived from vitamin B_{12}.

minor peak at about 288 mμ was associated with compounds containing a free N-3 position. Vitamin B_{12} contained no such peak, and on this basis it was suggested that the 5,6-dimethylbenziminazole moiety was coordinated to the cobalt atom, and was extruded in the presence of cyanide at pH 10, when the 288 mμ peak developed. Careful and detailed hydrolytic studies (5) gave further information. Acid hydrolysis gave a range of acids from monobasic to heptabasic. Mild hydrolysis produced a series of crimson carboxylic acids (three mono-, three di-, and one tribasic) in which the nucleotide had been retained. Since the tribasic acid could be converted [*via* the mixed anhydride with ethyl hydrogen carbonate (5)] into vitamin B_{12} by treatment with ammonia, the vitamin contained three readily hydrolyzable amide groups. The possibility of three more amide groups had been indicated by the early ammonia estimations, and was supported by the observation that nitrous acid, which is known to catalyze the hydrolysis of hindered amides (265) markedly accelerated the formation of the higher carboxylic acids, and under mild conditions gave a mixture of nucleotide-containing pigments (up to six carboxyl groups) with nucleotide-free pigments (up to seven carboxyl groups). This, together with the observation that the aminopropanol was retained until the nucleotide had been liberated, was in accord with the suggestion that the aminopropanol was esterified by the phosphoric acid and linked as an amide to the rest of the molecule (5, 73, 90).

The problem of the cobalt-containing fragment remained. The cobalt atom was shown to be tervalent (section III-A) and was firmly bound; vigorous hydrolysis did not appear to remove it, and exchange with radioactive cobaltous salts was not observed (6, 52). This behavior was reminiscent of certain metalloporphyrins, and, indeed, distillation of the nucleotide-free pigment with sodium hydroxide gave crude products

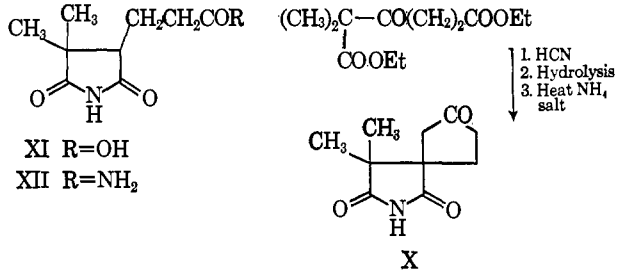
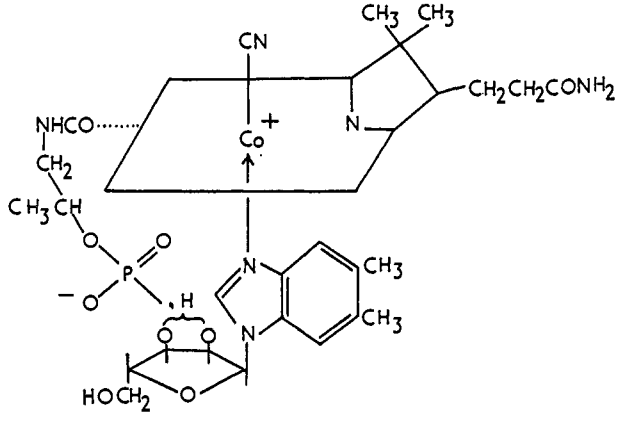


Fig. 3.—Succinimide fragments and synthesis of 3,3-dimethyl-2,5-dioxo-4-hydroxypyrrolidine-4-propionic acid lactone.

with a positive Ehrlich reaction (234). However, the absorption spectrum, although it possessed a strong band at 361 mμ, did not have a true Soret band in the 400 mμ region, and, moreover, early oxidation experiments did not yield maleimides (5) but only small fragments (see section V-D-I). Chromic acid oxidation did yield useful heterocyclic products however (210). The oxidation of the acid-hydrolyzed vitamin gave two products. The less polar compound X, $C_9H_{11}NO_4$, had infrared bands at 3.02, 5.65, and 5.80 μ and was hydrolyzed to a substance that behaved as a lactone dicarboxylic acid. The more polar oxidation product XI, $C_9H_{13}NO_4$, was hydrolyzed to the known 2-methylpentan-2,3,5-tricarboxylic acid, the ammonium salt of which, when pyrolyzed, gave back XI, which was formulated as indicated. The structure of X, which was evidently an imide γ-lactone, was shown by synthesis (Fig. 3). The oxidation of the vitamin itself gave none of the acid XI; instead the corresponding amide XII was obtained together with some of the lactone–imide (211). Of these three oxidation products only the amide had detectable optical activity.

The partial structure XIII for the vitamin could thus be advanced, the indications for the internal salt formulation being (i) the instability of 3′-ribonucleotide diesters which had just been demonstrated (67) and (ii) the observation that cobinamide (the vitamin

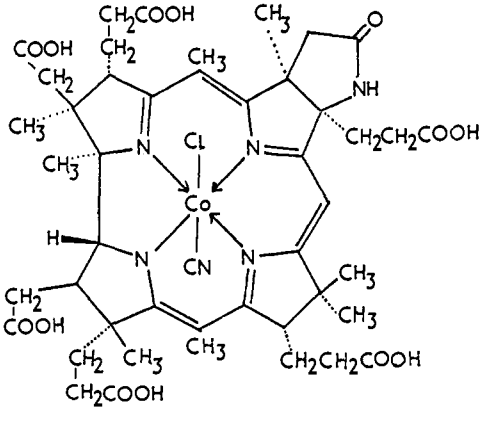


XIII

without the nucleotide) was a monoacidic base (XXXIII, Table IV) (5).

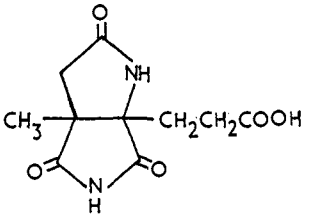
At this stage detailed structure began to emerge from the formidable X-ray analyses of vitamin B_{12} and its derivatives, in particular from that of the hexacarboxylic acid obtained by vigorous alkaline hydrolysis of the vitamin (61, 174, 177, 178). The positions of atoms, other than hydrogen, so revealed are shown in Fig. 4. From this representation, and a similar but somewhat less complete one of the vitamin, it was possible, with the help of chemical considerations, to formulate both compounds (47, 177, 178).

The hexacarboxylic acid, the analysis of which accorded with such formulas as $C_{46}H_{58}N_6O_{13}CoCl \cdot 2H_2O$ gave a positive chloride test, while the infrared spectrum indicated the presence of a coordinated cyanide (48). This identified the axial ligands. The nucleus (Fig. 4) was evidently of the porphyrin type, but heavily reduced and with one of the meso bridges replaced by a direct linkage. Since six carboxyl functions were to be accommodated, the six branched β-substituents were evidently acetic and propionic residues, and the stability of the substance, together with the elementary analysis, suggested that the eight single atom substituents almost certainly represented methyl groups; thus the oxidation product XI stemmed from ring C. The five-membered ring fused in *cis* fashion to ring B was shown to be a lactam on the basis of the infrared spectrum (a band at ~1720 cm.$^{-1}$; no band at 1780 cm.$^{-1}$), the resistance of the hexacarboxylic acid to alkali, and the nitrogen analysis. This led to structure XV (shown with six double bonds) for the hexacarboxylic acid (8-aminocobyrinic acid-*c*-lactam chloride cyanide).



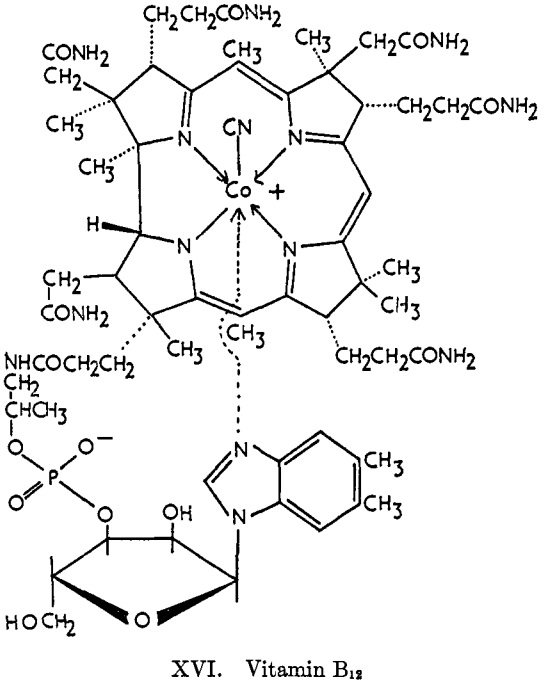
XV The hexabasic carboxylic acid

Further work has confirmed the lactam formulation since (i) the oxidation of the hexacarboxylic acid gives the lactam–imide XIV, which is not obtained on oxidizing the vitamin (section V-D-1), and (ii) the closure of the lactam ring has been observed in isolation (section V-D-2).



XIV

The remaining parts of the vitamin molecule were already known. The conclusions (172, 175, 176, 284) from the X-ray studies on the vitamin itself (air-dried; and with the mother liquor) and on the selenocyanate confirmed the results of earlier chemical work summarized in partial structure XIII, and in addition indicated a 3′-phosphate linkage. The lactam observed in the hexacarboxylic acid was not present in the vitamin. The stereochemistry of the macrocycle emerged in absolute terms, since not only was the absolute configuration of D-ribose already known, (245) but a direct check was possible by making use of the Bivjoet effect for the X-ray reflections from the hexacarboxylic acid (276). The number of double bonds in the conjugated system remained unsettled, although five were preferred, largely on the basis of earlier halogenation experiments, which suggested that the chromophore could be extended. A reinvestigation showed that this was not the case (section V-E), and attempted dehydrogenation of the chromophore using high-potential quinones which readily dehydrogenate the hydroporphyrins (115) was unsuccessful, suggesting that the unsaturation was already extended to six double bonds



XVI. Vitamin B_{12}

(46). These conclusions were in agreement with those from further refinements of the X-ray analyses (173),

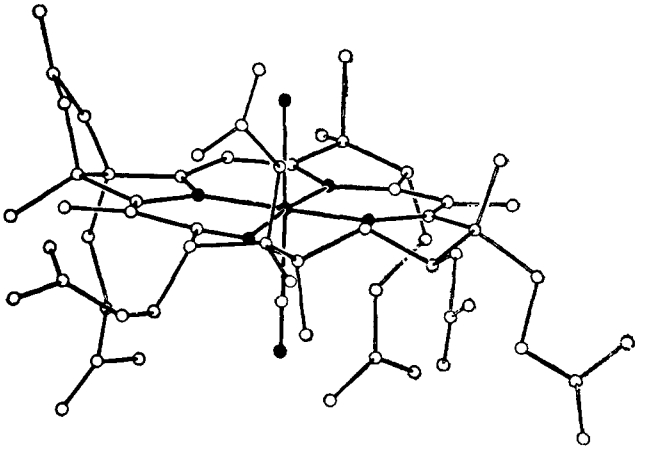


Fig. 4.—Atomic positions found in the hexacarboxylic acid chloride cyanide projected on the *c* plane [reproduced by permission of Professor D. C. Hodgkin, F. R. S., and the Editors of *Nature* from ref. 178].

and the vitamin was formulated as XVI ($C_{63}H_{88}N_{14}O_{14}PCo$, mol. wt. 1355).

The structure thus deduced was unique for a natural product in many respects, most notably in that it contained cobalt, the α-anomer of a new riboside, and the directly-linked pyrroline rings in a novel macrocycle. Although it may be considered to join the chlorophylls, bacteriochlorophyll, and stercobilin as a reduced tetrapyrrolic compound, it differs from these in that the reduction level is fixed by methyl groups. As a consequence its discovery has introduced into the chemistry of porphyrins and related compounds a stereochemical interest which has hitherto not been particularly prominent. An intriguing feature is the degree of symmetry in the disposition of the "extra" methyl groups. The resemblance to the natural type III porphyrins, and especially uroporphyrin III, is striking, as is the fact that the propionamide groups are all directed to that side of the molecule on which the nucleotide lies, while the acetamide groups are uniformly directed in the opposite sense.

In 1958 the question of structure in this series took on a new interest with the isolation of the light-sensitive cobamide coenzymes (13, 283). These were obtained from strains of *Clostridium tetanomorphum* and *Propionibacterium shermanii*, and have been shown (11, 222) to catalyze a series of remarkable enzymatic reactions including (i) succinate → methyl malonate; (ii) L-glutamate → L-*threo*-β-methyl aspartate, and (iii) propane-1,2-diol → propanal. The mechanisms of these reactions, and the form in which the coenzyme is involved, remain of much interest.

The coenzyme appears to be the state in which a major part of the vitamin occurs in liver and other sources (272). Cyanocobalamin is, in many cases at least, an artifact, the cyanide being introduced deliber-

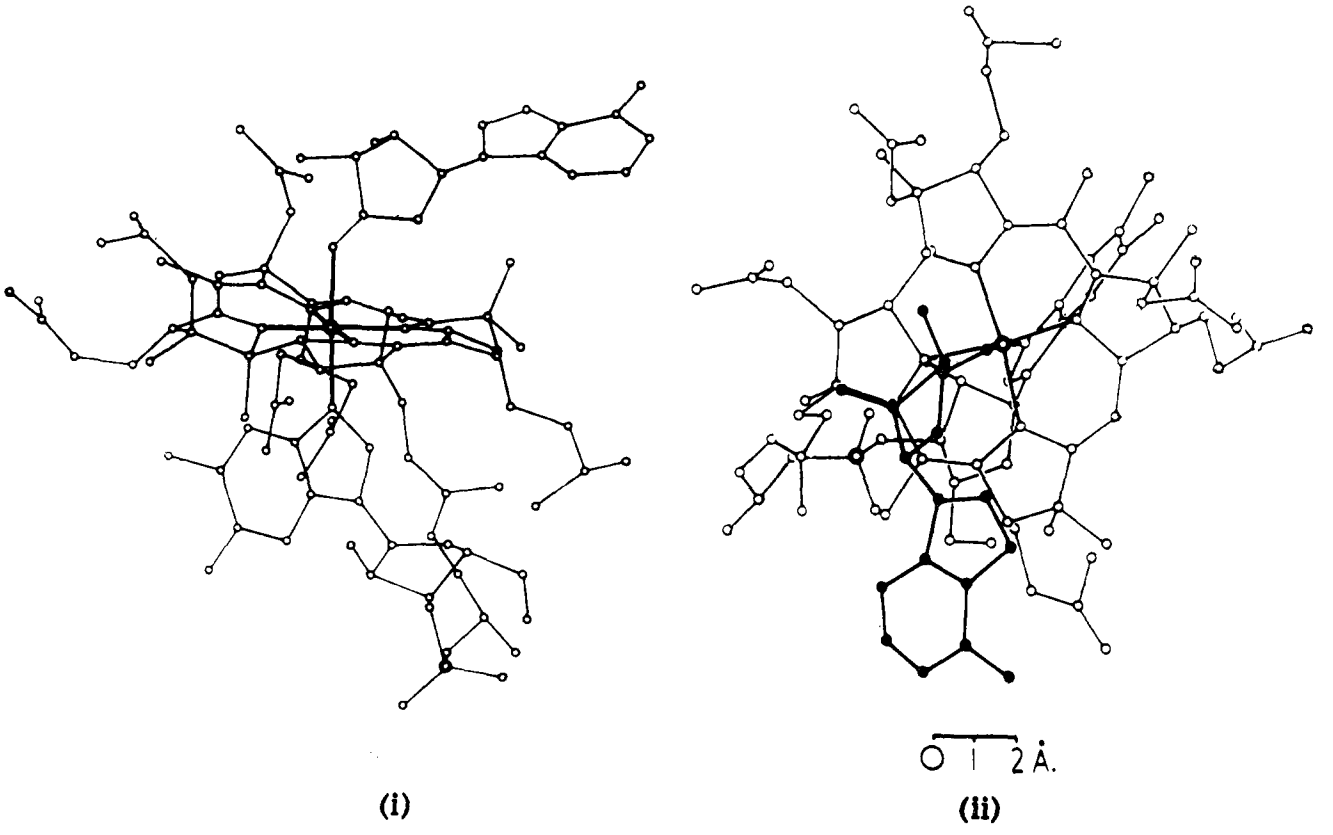
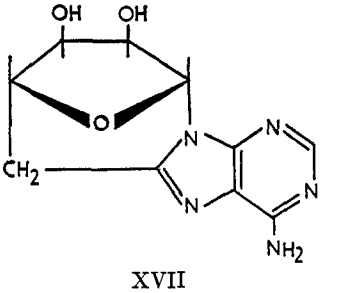


Fig. 5.—Atomic positions in α-(5,6-dimethylbenziminazolyl)-cobamide coenzyme as found in the wet crystals and viewed along (i) the *b* axis, (ii) the *a* axis [reproduced by permission of Professor D. C. Hodgkin, F. R. S., and the Editors of *Nature* from ref. 217].

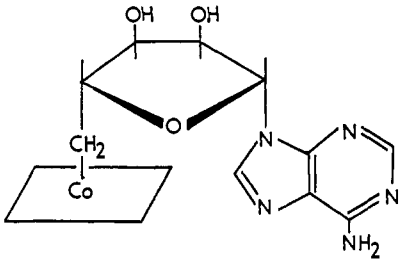
ately in the isolation process or accidentally as a trace contaminant of the charcoal absorbent.

Analysis of the crystalline α-(5,6-dimethylbenziminazoyl)cobamide coenzyme suggested (12) a molecular formula in the range $C_{72-75}H_{112-115}N_{18}O_{19-20}PCo$. The coenzyme did not contain cyanide. Mild acid hydrolysis gave aquocobalamin, adenine, and an unsaturated sugar which was identified as D-*erythro*-2,3-dihydroxypent-4-enal (section V-C). The stability of the benziminazole–riboside linkage was a valuable feature here, since hydrolysis of the nucleotide did not interfere as it did, for example, in the similar hydrolysis of the adenylcobamide coenzyme (section IV-C). With potassium cyanide solution in the dark (195) the coenzyme gave adenine, dicyanocobalamin, and the cyanohydrin of D-*erythro*-2,3-dihydroxypent-4-enal. Photolysis in the presence of oxygen produced aquocobalamin together with two adenine-containing nucleoside derivatives, one neutral and one acidic, and a trace of adenine. The acidic nucleoside was a 9-substituted adenine (ultraviolet spectrum) and hydrolyzed to adenine and D-riburonic acid. It was identified as adenosine-5′-carboxylic acid by comparison with an authentic sample prepared by the catalytic oxidation of adenosine (235). Thus, this nucleoside had the common 9-β-glycosylamine linkage. In another laboratory the 5′-aldehyde, rather than the acid, was isolated from this reaction (183); this difference presumably arises from variations in experimental method. The second, neutral, nucleoside, $C_{10}H_{11}N_5O_3$, was present in larger amount and under anaerobic conditions (31, 195) was the only nucleoside obtained (together with vitamin B_{12r}—section V-B-1). It has been provisionally formulated as 8,5′-cycloadenosine XVII (see section V-C).



XVII

Many of the foregoing results predated the appearance of the full structure and were the basis of various suggested modifications of the ligand pattern (194, 282). However, the structure which emerged from the X-ray crystallographic analysis was amazing (217); it demonstrated for the first time in a natural product the presence of a metal–alkyl bond, and for the first time ever the existence of a stable alkylcobalt. Figure 5 shows the atomic positions derived for the



XVIII

vitamin B_{12} coenzyme, and XVIII represents the chemical structure of the novel ligand (5′-deoxyadenosyl) which takes the same position as the cyanide ligand of the vitamin B_{12} structure. All the other detectable atoms of vitamin B_{12} appear to be present, but there are still some uncertainties about the extent of the conjugated system and the valence of the cobalt atom. The similarity of the spectrum of the coenzyme to that of the reduced system (*e.g.*, vitamin B_{12r}, which has been considered to contain divalent cobalt—section V-B-1) led to the suggestion that it was a Co(II) complex (29, 150) and some measurements indicating paramagnetism supported this (28, 194, 208, 243). However, other measurements (168) of magnetic susceptibility suggest that in solution the coenzyme is diamagnetic, indicating trivalent cobalt. X-Ray absorption edge studies reach the same conclusion (121), while e.s.r. measurements also accord with the essential absence of unpaired electrons in the coenzyme (182). The chemical reactions of the coenzyme are probably most readily interpreted in terms of a trivalent cobalt complex (section V-C) in which the cyanide group of the vitamin has been replaced by a carbanion, but the metal atom can be regarded as Co(III), Co(II), or Co(I) depending on whether the alkyl ligand is visualized as contributing two, one, or zero electrons to the coordination sphere.

Although the shape of the corrinoid ring in the coenzyme appears to be very similar to that in the vitamin (171), some doubt also remains about the extent of the double bond system (section V-C). It is hoped that neutron diffraction experiments will resolve this question (171). The partial synthesis of the coenzyme from cobyric acid has been achieved using reduced precursors (section VIII-A).

The structural work on vitamin B_{12} and the coenzyme has emphasized a changing situation which has become increasingly apparent since Robertson's work on phthalocyanine (255). X-Ray crystallographic analysis is much more than just another physical tool; it can, in certain cases, solve very complex structures which would yield slowly or scarcely at all to other approaches, and it provides at the same time much valuable stereochemical detail, complete with bond lengths and bond angles. For many cases where it is applicable, it will inevitably replace older methods, and is to be welcomed unreservedly, for while it is true that in the past much fundamental organic chemistry has derived from structural study by chemical methods, it is clear that in some ways, and especially perhaps with natural products, this can be a rather wasteful (albeit fascinating) process. It is important, however, that when structures are determined solely or largely by X-ray analysis that the structure is not regarded as the end of the matter, but that advantage is taken of it to plan a logical study of both reactivity and synthesis in a manner that has not hitherto been possible. This is especially important when new types of structure are revealed.

III. Physical Properties

A. General

Vitamin B_{12} is a crimson solid which crystallizes as needles or prisms. The air-dried crystals are hydrated ($\approx$10% water); the water is removed on drying *in vacuo* at 100°, while at about 200° the vitamin begins to decompose. It does not melt below 300°. It is not soluble in hydrocarbons and other nonpolar solvents, ether, or dry acetone, but is fairly soluble in water, the lower alcohols, acetic acid (without apparent decomposition), dimethylformamide, acetone cyanohydrin, phenols, and dimethyl sulfoxide. After isolation employing columns of cellulose, cellulose ion-exchangers, or ion-exchange resins, a useful purification procedure for the vitamin and many of its analogs and derivatives involves extraction into a phenolic solvent, followed by transfer to an aqueous medium with ether (5, 141). Aqueous acetone is the solvent of choice for recrystallization, but crystallinity is not a proof of homogeneity in this series.

Suitable solvent systems for paper chromatography which, together with paper electrophoresis at various pH values, provide a fairly stringent test of homogeneity, include butan-2-ol saturated with water, and butanol–acetic acid–water (5). The presence of salts (*e.g.*, $KClO_3$, $NaBPh_4$) often gives a better separation in various forms of chromatography (142, 145), and normally this type of examination is carried out in subdued light (essential for the coenzyme) and (except for the coenzyme) in the presence of cyanide ions. Partition coefficients, notably for the benzyl alcohol–water system, have been reported (75, 157).

Electrophoresis (122) and titration (65, 122) show that vitamin B_{12} is essentially neutral in aqueous solution; in fact it is a very weak polyacidic base, and a hexaperchlorate has been prepared in glacial acetic acid (2). The isoelectric point has been reported (242) to be 1.5, but even so there is no discernible movement, apart from a small electroosmotic migration, on paper electrophoresis at pH 7.

The vitamin is optically active, although due to its intense light absorption accurate measurements are

TABLE I

VITAMIN B_{12} AND ITS ANALOGS

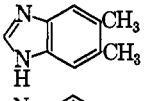
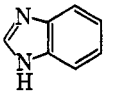
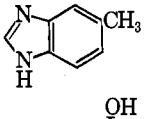
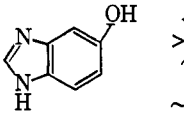
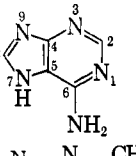
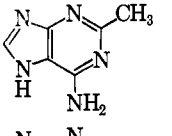
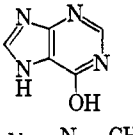
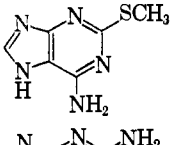
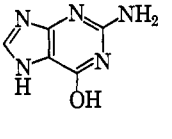
Systematic name (cyanide form at pH 7)	Name in common use	Other names	State and electrophoretical character	Base present in nucleotide	pH	Axial ligands	Light absorption[a] γ		β	α	Ref.
Cobyrinic acid	Cobyric acid	.....	Needles	None	2.5	CN, H_2O	275, 319	353 403	495	527	40
abcdeg-Hexaamide dicyanide (cobyric acid dicyanide)	Factor V_{1a}		Acidic (COOH)		>7.5	2CN	276, 308 (352)	367 415	538	578	40
Cobinamide dicyanide	Factor B	Etiocobalamin B_{12p}	Amorphous	None	7	OH, H_2O	(271)	348	492	517	35
					~7	CN, H_2O	(271) (320)	353	495	521	35
		Factor I	Basic		~7	CN, H_2O	276 315	355	503	530	129
					>7	2CN	277 308 (350)	367	540	580	35
Cobamide dicyanide	.....	Etiocobalamin-phosphoribose	Amorphous	None			As for cobinamide				105
		Factor E?									
		Factor Ib	Neutral								
Cobinamide phosphate dicyanide	Factor B phosphate	Factor C?	Amorphous acidic (PO_4H_2)	None	~7	CN, H_2O			500	530	8
					~10	2CN			540	580	8
α-(5,6-Dimethylbenziminazolyl)cobamide cyanide	Vitamin B_{12}	Factor II	Needles or prisms		2–10	CN, Nt	279 306 322	361	520	550	93
			Neutral		>8	2CN	279 289 308	368	540	582	93
α-Benziminazolylcobamide cyanide	.....	.....	Cryst.		6	CN, Nt	274	(As for vitamin B_{12})			139
			Neutral		10.5	2CN	275 280 306–313	(As for vitamin B_{12})			139
α-(5-Methylbenziminazolyl)cobamide cyanide	.....	.....	Cryst.		6	CN, Nt	278	(As for vitamin B_{12})			139
			Neutral		10.5	2CN	278 288 306–313	(As for vitamin B_{12})			139
α-(5-Hydroxybenziminazolyl)cobamide cyanide	Factor III	Factor I	Cryst.		4	H_2O, Nt		351	500	526	153
			Neutral		<9	CN, Nt	295	361	518	550	153
					>10.6	CN, Nt	309, 323	361			153
					~8.3	2CN	278 303	368			153
					~11	2CN	278 313	368	540	580	153
α-Aden-7-ylcobamide cyanide	ψ vitamin B_{12}	ψ B_{12b} Factor IV B_{12f}	Cryst. Basic (adenine)		~7	CN, Nt	278 308 320	361	518	548–550	111
α-(2-Methyladen-7-yl)-cobamide cyanide	Factor A	B_{12m} ψB_{12d}	Cryst. Basic		~7	CN, Nt	278 308 320	361	518	548–550	111
α-Hypoxanth-7-yl, cobamide cyanide	Factor G	.....	Cryst. Neutral		~7	CN, Nt		359	516	540	69
α-(2-Methylhypoxanth-7-yl)cobamide cyanide	Factor H	.....	Cryst. Neutral		~7	CN, Nt		358.5	517	540	69
α-(2-Methylmercaptoaden-7-yl)cobamide cyanide	.....	Factor F?	Cryst. Neutral		~7	CN, Nt[b]	303	360 410	520	550	137
α-Guan-7-ylcobamide cyanide	.....	.....	Cryst. Neutral		6	CN, Nt	279	361	520	550	144
					10.5	2CN	277 302	367	540	580	144

TABLE I *Continued*

Systematic name (cyanide form at pH 7)	Name in common use	Other names	State and electrophoretical character	Base present in nucleotide	pH	Axial ligands	Light absorption[a] λ_{max}	γ	β	α	Ref.
P^1-Guanosine-5′	.....	Factor C	Amorphous acidic	(Guanine)	1-10	CN, H_2O?	273, 320	356	500	530	8
P^2-Cobinamide pyrophosphate		Factor y_1			10	2CN	276, 307.5	367.5	540	580	8
.....	α-(5,6-Dimethylbenzimidazolyl)-cobamide coenzyme	Vitamin B_{12} coenzyme; cobalamin conjugate	Cryst.	5,6-Dimethylbenzimidazole	2	...	263, 284, 303, 458				213
					7	...	260, (288), 315, 340, 375, 522				12
.....	α-Adenylcobamide coenzyme	.....	Amorphous	Adenine	1-8	...	263, 303, 375, 458				12
.....	Cobinamide coenzyme	SB_{12p}	Amorphous	None	7	...	264, 304, 375, 462				244

[a] $\lambda_{max.}$ for aqueous solution in mμ; values in parentheses indicate inflections. [b] Approximate values read from published curve.

somewhat difficult. Values of $[M]_{6563}^{23}$ −800 ± 120°, $[M]_{6438}^{20}$ −1490 ± 150°, $[M]_{5780}^{20}$ −1640°, $[M]_{4360}^{20}$ +4090°, and $[M]_{4050}^{20}$ −30,300° have been reported (40, 65, 122). Measurements for aquocobalamin, nitritocobalamin (225), and cobyric acid (40) have also been made. The optical rotatory dispersion curve (multiple negative Cotton effect) has been recorded for the visible region (114). Circular dichroism curves extending to 300 mμ have been presented (216).

Magnetic susceptibility measurements have shown that vitamin B_{12} is diamagnetic, a result which indicates a Co(III) complex (106, 154, 280). Evidence derived from work on polarographic reduction (51, 87, 107, 188) and on X-ray absorption edge studies (43) has been adduced to support this conclusion.

Neutron irradiation (229), concerning which there was some early controversy, appears to generate a small amount of Co^{60}–vitamin B_{12}, but evidently causes much decomposition (78), as does high-energy γ-radiation (165, 263, 264) which has been reported to yield over 40% free cobaltous ion after 8 hr. at 180 r./min. The vitamin has been labeled with Co^{56}, Co^{57}, Co^{58}, and Co^{60} by biosynthetic procedures: the Co^{60} compound is reported to decompose spontaneously on storage (14, 220), producing some mono and dicarboxylic acids with the same electronic absorption spectrum as the vitamin.

The vitamin B_{12} coenzyme is red and forms flattened diamonds or rosettes of needles from aqueous acetone. Its solubility characteristics are similar to those of the vitamin. On electrophoresis in acid solution (213) it behaves as a cation due to the protonation of the adenine moiety (see also section V-A-4).

B. SPECTRA

The infrared spectrum of the vitamin has been published (10, 187). A weak but sharp absorption at 2136 $cm.^{-1}$ is assignable to the coordinated cyanide, while NH (amide) and OH (ribose + water) groups are subject to strong hydrogen bonding and appear as a broad absorption with maxima at about 3200 and 3350 $cm.^{-1}$. The amide I band appears at 1670 $cm.^{-1}$, while the amide II is not resolved and occurs as a shoulder at 1625 $cm.^{-1}$. The bands at about 1574 and 1500 $cm.^{-1}$ probably represent contributions from both the corrinoid system and the benziminazole nucleus; the hexacarboxylic acid also shows bands at 1587 and 1504 $cm.^{-1}$ (48). Bands at 847 and 867 $cm.^{-1}$, not present in the hexacarboxylic acid, are probably associated with the C–H out-of-plane deformations of the benziminazole (53). The examination of vitamin B_{12} crystals with polarized infrared radiation (80) has given some information on the orientation of the cyanide ligand within the crystal.

As in the porphyrin series, electronic absorption spectra are valuable for characterization, although they are not wholly reliable for estimating purposes or as criteria of purity since, in contrast to the porphyrin series, changes at the β-positions have little or no effect on the chromophore. Vitamin B_{12} in water has maxima at 279, 306, 322, 361, 520, and 550 mμ (93), and the absorption is not much affected by changes in pH. The hexacarboxylic acid dicyanide (0.1 *N* KCN) has maxima at 276, 301, 311, 348, 363, 412, 537, 576–577 mμ (log ε 3.97, 3.92, 3.96, 4.16, 4.48, 3.32, 3.94, 3.97, respectively) (48). The spectra of other derivatives are indicated in Tables I, II, III, and V. Figure 6 shows the spectra of the vitamin and the coenzyme in water. The strong band at about 360 mμ and the weaker bands at ~520 mμ (sometimes an inflection) and ~ 550 mμ are characteristic (at least in the absence of an alkyl ligand) of the corrinoid unit, and it is convenient to refer to them as the γ, β, and α bands, respectively (168). Kuhn has considered the spectrum of the vitamin in terms of the electron gas model (113, 212), and has associated bands in the 280 and 550 mμ region with a transition moment parallel to the C-5–C-15 axis, while the γ band corresponds to a transition moment perpendicular to that axis. The latter band may thus correspond to the *cis* peak of the polyene series (293). Some of the absorp-

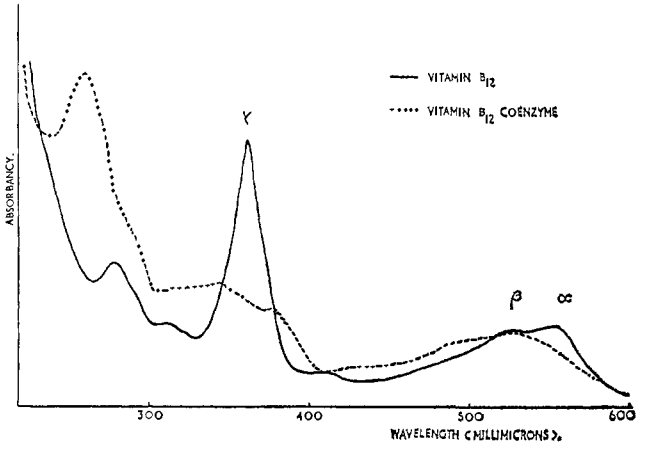


Fig. 6.—Absorption spectra of vitamin B_{12} and vitamin B_{12} coenzyme in water.

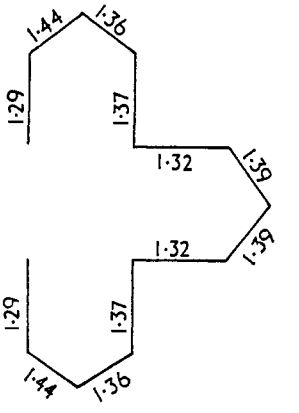
tion in the 280 mμ region must also be due to the 5,6-dimethylbenziminazole fragment.

C. MOLECULAR DIMENSIONS AND CONFORMATION

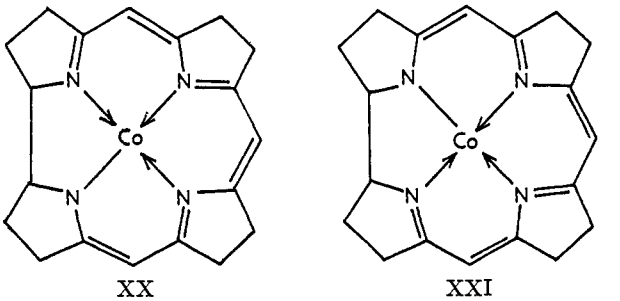
The X-ray analysis has, of course, furnished not only the gross structure but extensive details of molecular dimensions of much interest (176). Even certain hydrogen atoms (for example, those of the methyl groups at C-15, and those of the methyl groups of the benziminazole) have shown up in the later stages of refinement. Although the following results apply specifically to the crystal, it is likely that, insofar as the corrinoid system is concerned, they also apply in solution, although the conformation at the β-positions may vary to a certain degree.

In the vitamin the ribofuranose ring assumes the envelope (C_8) conformation, C-2′ being 0.74 Å. out of the plane of the other four atoms. The benziminazole system, which is essentially planar, is not quite orthogonal to the corrinoid portion (interplanar angle 80°), a deformation which is probably to be ascribed to the nonbonded interaction of the angular methyl group (at C-1) which is only 3.32 Å. from N-3 of the benziminazole. This interaction, and the absence of a δ-meso bridge, result in an irregular ligand environment about cobalt, the largest deformation in the plane being the angle N-21–Co–N-24 (80.5°). The conjugated system is approximately planar, but is, perhaps, even better described as two near-planar systems inclined at a small angle [19° for the "wet" B_{12} crystal (171)] about the Co–C-10 axis. One plane encompasses the atoms Co, C-10, C-11, N-23, C-14, C-15, C-16, and N-24 and includes the carbon of the methyl substituent at C-15; the other plane includes Co, N-21, C-4, C-5, C-6, N-22, C-9, and C-10, but on this side the carbon of the methyl group at C-5 (in one of the most crowded parts of the structure) deviates from planarity by 0.5 Å.

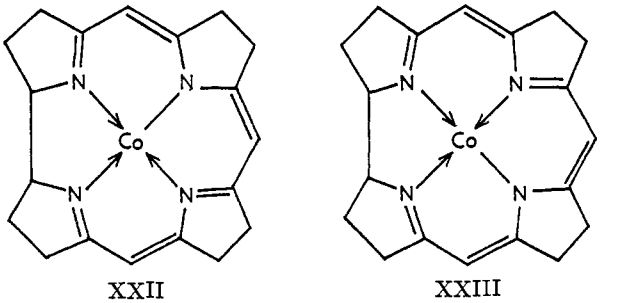
The macrocycles of the hexacarboxylic acid and of cobyric acid show similar features, although the warping of the conjugated system about the Co–C-10 axis is less pronounced than in the vitamin or the coenzyme. The structure of the cobyric acid (section IV-A) has been determined most accurately (99, 100, 171) and the dimensions found for the corrin ring are given in Fig. 7. It is remarkable how closely the bond lengths observed in the conjugated system agree with those (indicated in XIX) calculated on the basis of equal contributions of the four resonance structures XX–XXIII (172).



XIX



XX XXI



XXII XXIII

For comparison the C–N bonds in nickel phthalocyanine (255) are about 1.38 Å. long; in nickel etioporphyrin II (98) they are given as 1.35 ± 0.05 Å., while the C–C bonds involving meso bridges are 1.43 ± 0.05 Å. The distance between opposite nitrogen atoms observed in cobyric acid (~3.8 Å.) compares with distances of 3.84 Å. (phthalocyanine), 3.66 Å. (nickel phthalocyanine), and 3.68 Å. (nickel etioporphyrin II).

The pyrroline rings are nonplanar and generally irregular in shape. Figure 8 illustrates the atoms of the corrin system of cobyric acid viewed from the cobalt atom. The conjugated system is readily discerned and

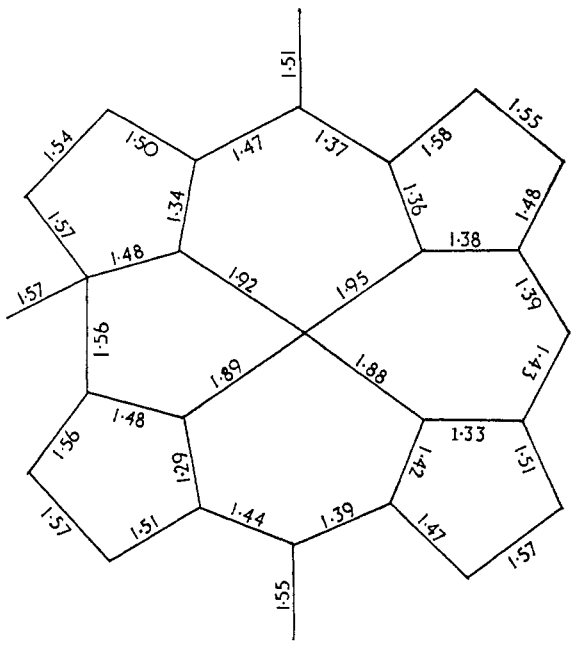


Fig. 7.—Bond lengths in corrinoid system of cobyric acid aquocyanide. (These bond lengths, and the dimensions upon which Fig. 8 is based, were kindly provided by Professor D. C. Hodgkin, F. R. S., prior to full publication.)

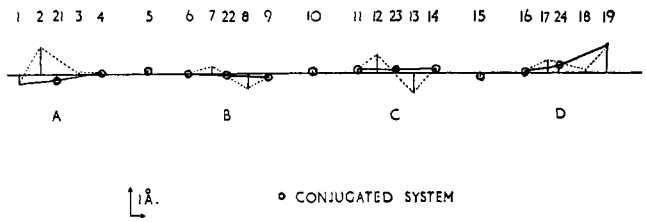


Fig. 8.—Corrin ring of cobyric acid aquocyanide viewed from cobalt. Vertical distances represent projections onto the least-squares plane passing through Co, N-21, C-4, C-5, C-6, N-22, C-9, C-10, C-11, N-23, C-14, C-15, C-16, and N-24. Horizontal scale represents distances between intercepts of projections of atomic positions on a cylinder, radius 3.5 Å., possessing a vertical axis through the metal atom.

from it the β-positions stand out clearly. The system is most buckled about C-1–C-19, but all the β-carbons are twisted out of the plane: rings B and C have a conformation resembling the C_2 conformation of cyclopentane (205), while A and D appear to approximate more closely to the C_s(envelope) arrangement.

IV. Analogs

Variations on the vitamin B_{12} structure involving the axial ligands are remarkably common; those involving the replacement of cyanide by other inorganic ligands will be considered in section V-A, while the many natural analogs containing modified nucleotide moieties, and the various coenzyme forms, will be considered in outline here. The chemistry of many of these compounds has not been examined in detail, and most have been formulated by analogy. Thus the position of the phosphate–ribose linkage and the configuration of the glycosylamine depends generally on analogy and apparent structural requirements rather than direct chemical or physical demonstration. Many of the analogs have had their gross composition checked by biosynthesis, but this procedure does not necessarily prove structural detail.

No natural modification of the corrinoid system itself has yet been reported.

A. Natural analogs wthout a nucleotide

Aquocobyric acid cyanide (Cobyric acid, Factor V_{1a}) is one of a group of acidic factors (the Factors V) which have been isolated from sewage sludge (27) and which have also been detected (25) in fermentations of *P. shermanii*. Two series (one with, and one without, the aminopropanol residue) have been recognized, and electrophoresis indicates mono- to pentacarboxylic acids are present. These acids are presumably on the biosynthetic pathway, although their formation, in part, by hydrolysis has not been excluded.

Two monocarboxylic acids of this group have been obtained and one, cobyric acid (factor V_{1a}), has become important. The other, Factor V_{1b}, is a cobinic acid pentamide. Crystals of cobyric acid aquocyanide carboxylate (or hydroxocyanide) have been obtained (40). The compound did not contain aminopropanol or nucleotide; the position of the carboxyl group at *f* thus suggested was confirmed by converting cobyric acid to cobinamide and the vitamin by chemical methods (section VIII-A).

The crystalline material has been examined (in its mother liquors) by X-ray crystallographic analysis; in this case it proved possible to solve the structure by making use of the anomalous scattering of X-rays by the cobalt atom (which was quite pronounced over a large number of reflections) and calculating phase angles from an accurate measurement of intensity differences between hkl and *hkl* reflections (100, 171). The measurement of Fhkl and *Fhkl* for nearly 4000 reflections was carried out. This elegant operation gave an essentially complete three-dimensional solution almost in one move and led, moreover, to a more precise determination of molecular structure (see Fig. 7 and 8) than had previously been possible. Three-quarters of the hydrogen atoms appeared as definite peaks of 0.5–1 e/Å.[3]

Another series of acidic factors isolated from *P. shermanii* is nucleotide-containing, and the mono-, di-, and tricarboxylic acids have been separated on cellulose ion-exchangers (25). Some of these compounds are presumably identical with the products of the mild hydrolysis of the vitamin. The major monocarboxylic acid on removal of the nucleotide gives a substance with the properties of Factor V_{1b}.

Aquocobinamide cyanide (cobinamide, Factor B) is essentially vitamin B_{12} without the nucleotide. The replacement of the nucleotide by a coordinated water

molecule requires that the system become a monoacidic base [pK_a' 6.8 (5)]. In aqueous potassium cyanide two cyanide ligands give a neutral species (XXXIV, Table IV), and the absorption spectrum above 300 mμ is then similar to that of dicyanocobalamin. In contrast to the dicyanide of the vitamin, cobinamide dicyanide is stable in weakly acidic media. When the dicyanide in aqueous solution at pH 2.5 is concentrated, the aquocyanide is formed. The second cyanide group may be removed by reduction (35).

Cobinamide was originally found (128) in rumen contents (along with Factor A) in the first demonstration of the multiple nature of the vitamin, and it has been obtained widely from microorganisms. A more convenient source is from vitamin B_{12} under conditions which favor ester rather than amide hydrolysis (section V-F-2). The action of acid on cobinamide apparently produces a different compound (Factor 1a) which has, however, electrophoretic properties similar to those of cobinamide (140). The monocarboxylic acids resulting from the acid hydrolysis of cobinamide have been reconverted to cobinamide by amidation using the mixed anhydride method (5); a similar route (with D*g*-1-aminopropan-2-ol) leads from cobyric acid to cobinamide (section VIII–A).

Cobamide dicyanide is the vitamin without the 5,6-dimethylbenziminazole fragment. It has been reported in an amorphous form from sewage sludge (105). The identification rests on (i) electrophoretic properties; (ii) the identity of the absorption spectrum with that of cobinamide; (iii) cleavage with cerous hydroxide to give cobinamide; (iv) estimation of cobalt, phosphorus, and ribose. The nature of the ribose ring is unknown.

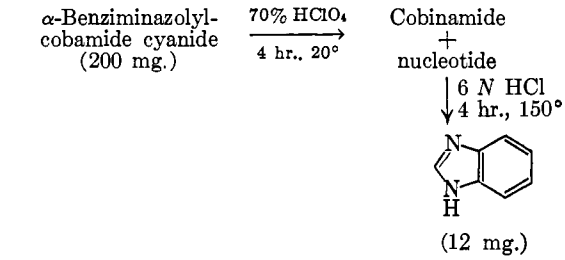
This compound could, in fact, arise by the mild hydrolysis of the adenylcobamide coenzyme and has been so obtained *in vitro* (282).

Cobinamide phosphate dicyanide (Factor B phosphate).—A compound which appears to be the phosphate ester of the alcohol cobinamide has been reported in *N. rugosa* fermentations, although the structural evidence is at present not complete, and isolation of the compound appears to be difficult (8, 54). It is formed by enzymic hydrolysis of P^1-guanosine-5′P^2-cobinamide pyrophosphate, but does not contain guanine.

It behaves as an acidic substance, and although essentially neutral at pH 2.7, migrates in the presence of cyanide as a monoanion at pH 6.2 and as a dianion at pH 7.5 (XLII, XLIII, XLIV, Table IV). Titration (39) indicates a pK_2 of 6.86, in the region expected for a secondary phosphoryl dissociation. The synthesis of this phoshate from cobyric acid (mixed anhydride route) and cobinamide (β-cyanoethylphosphate route) has been reported (section VIII-A).

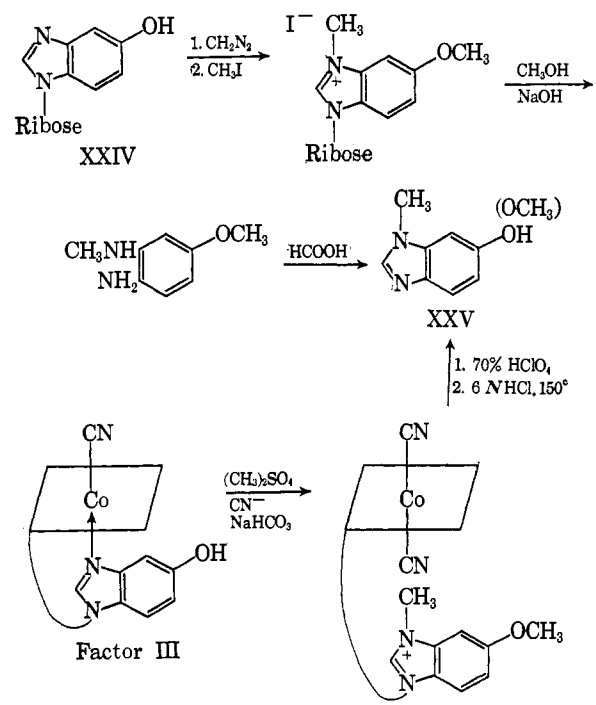
B. NATURAL ANALOGS POSSESSING A NUCLEOTIDE

α-Benziminazolylcobamide cyanide and α-(5-methylbenziminazolyl)cobamide cyanide analogs were in fact available biosynthetically before they were discovered in a fermentation sludge (139). Each was hydrolyzed to the corresponding nucleotide (identified spectroscopically) and base.



The orientation of the methylbenziminazole had already been determined with the biosynthetic analog (138), which on methylation and hydrolysis gave 1,6-dimethylbenziminazole.

α-(5-Hydroxybenziminazolyl)cobamide cyanide (Factor III) has been extensively investigated. It is a neutral crystalline substance, but on electrophoresis in basic solution behaves as an acid (phenolic group). On hydrolysis many of the features of the hydrolysis of the vitamin are reproduced, although complications arise owing to the instability of the hydroxybenziminazole under vigorous conditions (140, 143, 256). Methylation has shown that the base is present as the 5-(and not 6-)hydroxy-1-ribofuranosylbenziminazole unit. One proof (261) involved the methylation of the nucleoside (XXIV), the other the methylation of the factor itself (134). Both routes gave 6-hydroxy-1-methylbenziminazole (XXV), in the former case as the methyl ether.



Synthetical evidence (261) has been presented which suggests that the nucleoside is the α-D-ribofuranoside.

Alkylation at both N-3 and oxygen requires the presence of base and cyanide; the dialkylated products are basic at pH 2.7 (*cf.* XL, Table IV). In the absence of cyanide etherification predominates, and as expected, the products do not show the pH-dependent spectrum observed for the parent factor (153).

α-Aden-7-ylcobamide cyanide (ψ-vitamin B_{12}), in contrast to the benziminazole derivatives, is cleaved to the corresponding base under mild conditions; thus *N* HCl at 100° gives adenine, identified chromatographically and spectroscopically (70, 110). Smooth cleavage with cerous hydroxide (135) furnishes cobinamide, phosphate, and a crystalline nucleoside having a spectrum resembling that of 7-methyladenine rather than that of 9-methyladenine. On the basis of these observations together with the nitrogen analysis, the hydrolysis to 1 mole each of ribose and adenine, and the consumption of 1 mole of periodate, the nucleoside is formulated as 7-D-ribofuranosyladenine; presumably the normal 9-substitution found in adenosine would not allow coordination of N-7 with cobalt due to the steric influence of the 6-amino group. It is assumed that the α-glycosylamine linkage is required to fit the molecular structure (169).

α-(2-Methyladen-7-yl)cobamide cyanide (Factor A), was discovered at the same time as cobinamide and was later obtained crystalline; the base which has been identified (69, 111) as 2-methyladenine (spectra, paper chromatography) had not been previously found in nature. The corresponding nucleoside (136) has been obtained crystalline (as the trihydrate), and by methods similar to those applied to the adenyl derivative it has been shown to be 2-methyl-7-D-ribofuranosyladenine.

A partial synthesis of this factor has been effected (147).

α-Hypoxanth-7-ylcobamide cyanide (Factor G) *and α-(2-methylhypoxanth 7-yl)cobamide cyanide* (Factor H) have been isolated in small amounts from feces (69). These neutral factors probably have the structures indicated, since Factor G, which has been shown to contain hypoxanthine in the nucleotide, can be obtained by the action of nitrous acid on α-aden-7-ylcobamide cyanide. In an analogous fashion Factor H contains 2-methylhypoxanthine, and is obtained on deamination of α-(2-methyladen-7-yl)cobamide cyanide (69, 247).

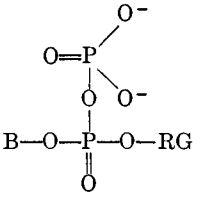
Cleavage of *α-(2-methylthioaden-7-yl)cobamide cyanide* (137) in the presence of cerous hydroxide gave cobinamide and a nucleoside which consumed 1 mole of periodate and which on mild acid hydrolysis gave D-ribose and 2-methylthioadenine—the first natural sulfur-containing purine. As supporting evidence for the location of the ribose residue at N-7, the spectroscopic differences between the nucleoside and the N-9 substituted system have been cited; a direct comparison with a 7-alkyl-2-methylthioadenine has not been reported.

The electron-withdrawing effect of the 2-substituent in this case, as in the hypoxanthyl derivatives, results in a lowering of basicity, and the factor is essentially neutral on paper electrophoresis at pH 3. The action of nitrous acid gives the crystalline deaminated analog.

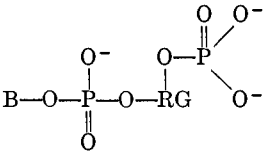
A substance which presumably has the structure of *α-guan-7-ylcobamide cyanide* has been obtained crystalline from a sewage digest (144). Careful acid treatment (section V-F-2) gave cobinamide and the crystalline nucleotide, which differed in spectral characteristics from guanosine, but closely resembled 7-methylguanine in this respect. Hydrolysis of the nucleotide gave 1 mole each of ribose, phosphate, and guanine.

P^1-Guanosine-5′P^2-(cobinamide dicyanide) pyrophosphate (Factor C) is a second guanine-containing factor associated with cobinamide phosphate and has been isolated from *Nocardia* fermentations (8). This acidic factor differs from all the analogs containing heterocyclic bases so far described in that the guanine appears to be unable to coordinate with the cobalt atom. This factor has not been obtained crystalline, and degradation evidence must be interpreted with caution, but it appears to contain cobalt, phosphorus, ribose, and guanine in the ratio 1:2:1:1, respectively. Enzymatic hydrolysis (pyrophosphatase activity) gave a substance which resembled (spectrophotometrically) 9-methylguanine rather than 7-methylguanine. As has been argued before, N-7 of guanosine would be unlikely to coordinate with the metal atom; thus the spectrum of the factor in water resembles that of the aquocyanide form of cobinamide rather than that of the vitamin itself.

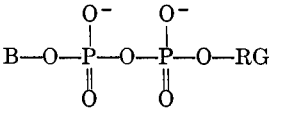
A second product of the enzymatic hydrolysis was indistinguishable from cobinamide phosphate. Acceptance of these conclusions leads to three likely structures, represented at XXVI–XXVIII (R = ribose, G = guanine).



XXVI

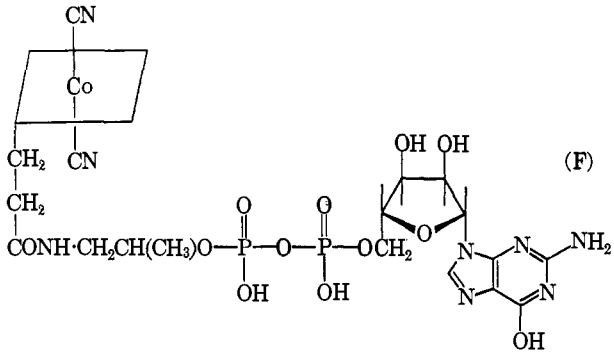


XXVII



XXVIII

Of these XXVI and XXVII were excluded, respectively, by the observations that inorganic phosphate was not formed on mild acid hydrolysis nor with phospho-



XXIX

monoesterase. That the ribose was phosphorylated at C-5′ was inferred from Dische's color reaction (112), and structure XXIX was proposed for the dicyanide. While this structure requires confirmation, preferably with a crystalline material, it does bear a striking analogy to other natural pyrophosphates and also accords with its apparent function as a biological precursor, which function had been suspected of Factor C (104, 127, 129) for some years (section VII).

The formulation has been considerably strengthened by a partial synthesis (section VIII-A) which, however, started with cobinamide containing the racemic aminoalcohol. Condensation (carbodiimide) of the phosphate of this with guanosine 5′-phosphate gave a 54% yield of an amorphous red powder which had the same chromatographic properties as the natural material (37). It seems likely that other natural derivatives of this type may occur.

C. NATURAL COENZYME ANALOGS

These could in principle differ in the 5′-deoxynucleoside or in the nucleotide; so far only 5′-deoxyadenosyl derivatives have been obtained from natural sources.

α-Adenylcobamide coenzyme was the first coenzyme form to be recognized (13), and its chemical investigation followed a broadly similar course to that described for the vitamin coenzyme. It is of interest that under very mild conditions of hydrolysis (0.07 *N* HCl, 10 min. at 85°) the adenine of the nucleotide is preferentially removed; treatment of the yellow product with cyanide gives a further mole of adenine and the purple cobamide dicyanide (282). In contrast to the vitamin coenzyme, which is red in neutral solution (but turns yellow in acid solution) the adenyl coenzyme is yellow over a range of pH values and in this respect resembles cobinamide coenzyme (see section V-A-4).

Cobinamide coenzyme has been detected in fermentations of *P. shermanii* to which cobalt salts but no heterocyclic bases have been added (29, 244). It was originally thought to contain 2 moles of adenine, but this has not been substantiated (244). It may also be obtained by the treatment of vitamin B_{12} coenzyme with cerous hydroxide in the absence of cyanide ions (30). Evidence has also been presented for a coenzyme form of cobyric acid (233), while a considerable number of natural analogs remain unidentified (222).

D. BIOSYNTHETIC ANALOGS

A large number of analogs (222), including coenzymes (32, 273) have been prepared by biosynthetic procedures, and this has, in fact, been a useful way of confirming the structures of certain analogs. One of these, from 5,6-dichlorobenziminazole, featured in the X-ray work (203). Coenzyme analogs based on chemically modified corrinoid nuclei (dehydrovitamin B_{12} (section V-D-2) and chlorodehydrovitamin B_{12} (section V-E)) have been reported (278).

V. CHEMICAL REACTIONS

A. REPLACEMENT OF AXIAL LIGANDS

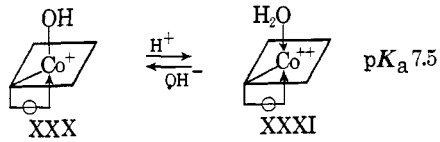
In this section ligand reactions other than processes involving metal–alkyl bonds will be considered; it is convenient to deal with the latter after reduction has been discussed. Considerable variation in the nucleotide ligand has already been met (section IV).

1. Displacement of Cyanide; Aquocobalamin

The cyanide group is firmly bound to the cobalt atom; massive doses of the vitamin do not produce symptoms of cyanide poisoning, and indeed aquocobalamin has some effect in experimental cases of this condition (102). The displacement of cyanide in the presence of excess thiocyanate does occur in alkaline solution; the equilibrium favors the cyanide form, however (224). The stability of this linkage has led to the use of aquocobalamin as an intermediate in the preparation of modified cobalamins with inorganic ligands other than cyanide. Aquocobalamin has been obtained from natural sources (3, 248) and was, indeed, the first modification to be so found. It can be prepared from the vitamin by reduction (section V-B-1) or, better, by photolysis—processes which presumably involve homolysis of the Co–CN bond rather than direct displacement. The photolytic transformation has been observed on paper chromatograms where ~10% conversion can occur under ordinary working conditions in the absence of hydrogen cyanide (288). In a sealed system the reaction is reversed in the dark (20), so in preparative work the hydrogen cyanide is removed in a stream of inert gas (184).

Aquocobalamin titrates (225) as a weak base (pK_a 7.5) and in aqueous solution an equilibrium between the hydroxo form (XXX) and the aquo form (XXXI) is associated (93) with a pronounced dependence of the spectrum on pH (Table V), in notable contrast to the behavior of the vitamin. An alternative proposal

(216) involving rupture of the N-3–Co linkage in alkali would accord with the lability of aquocobalamin toward cerous hydroxide (30) but seems less likely, since no fine structure at 288 mμ (associated with the noncoordinated benziminazole) is observed (21).



XXX XXXI

2. Other Ligands

The reaction of aquocobalamin with acids yields a series of compounds, some of which may be regarded as salts of aquocobalamin, at least in aqueous solution. Among the crystalline products are the chloride, bromide, sulfate, cyanate, nitrite (201), thiocyanate (75), and selenocyanate (61). These derivatives are dark red crystalline solids with physical properties similar to those of the vitamin, but the ligands in certain of them, *e.g.*, the chloro and sulfato compounds, are much less firmly bound. This is illustrated in the cobyrinyl series by the detectability of free chloride ion in aqueous solution (48) and by the Co–Cl bond length in the crystalline hexacarboxylic acid chloride cyanide (2.40 Å., *cf.* sum of ionic radii 2.38 Å.; sum of covalent radii 2.21. Å) (177). In other cases, for example, thiocyanate, the ligand appears to be more firmly bound (224, 286). The sulfite derivative is presumed to have a Co–S bond (168), although the question of concomitant reduction in the corrinoid system remains open. Unlike aquocobalamin salts this compound has a strong band in the 320 mμ region; on oxidation it gives aquocobalamin sulfate (201). At pH 0 the solution is yellow (rather than red) (section V-A-4) and a cobaltous complex appears to be present under these conditions, although the red form is diamagnetic (168). It is curious that this substance occasionally appears as an artifact (222) in isolation work (presumably arising from residual sulfite in the paper); this can lead to confusion since the spectrum bears some resemblance to that of the vitamin B_{12} coenzyme. A sulfito derivative of cobinamide has also been reported as a yellow solution (35, 168).

Another type of derivative is formed with certain bases; these compounds have generally been detected spectroscopically, but not isolated. The ammonia complex is a crystalline solid, however (93). Various pyridines (168, 262) have been examined, and the γ-picolino complex appears to react further in the dark (250), although no products have been isolated. Among the common amino acids only histidine readily forms a complex, as do its derivatives histamine, carnosine, and histidylhistidine (18, 93). Methyl isocyanide forms a complex with cobinamide (150). The variation in the interaction of the ligand with the cobalt

TABLE II

VARIATION OF POSITION OF γ-BAND WITH LIGAND IN α-(5,6-DIMETHYLBENZIMINAZOLYL)COBAMIDE DERIVATIVES

Ligand replacing CN	pH	γ-band (mμ)	Ref.
H_2O	1	350	168
Aquocobalamin salts	~7	352	201
NH_3	7	356	168
–OH	12	357	168
Iminazole	4	358	168
Pyridine	7	360	168
–CN	7	361	201

TABLE III

SPECTRA IN THE COBINAMIDE SERIES

Ligand	Ligand	$\lambda_{max.}$ mμ γ	β	α	Ref.
H_2O	–OH(H_2O?)	348	492	517	35
H_2O	–CN	355	498	531	150
Iminazole	–CN	362	520	550	150
–OH	–CN	362	520	553	150
NH_3	–CN	362	520	555	150
$-N_3$	–CN	363	522	552	150
CH_3NC	–CN	368	540	580	150
–CN	–CN	368	541	581	150

atom and the corrin chromophore is reflected in the pronounced shift observed in the main band positions of the electronic spectrum; the shift to longer wave lengths roughly parallels the increasing σ-donor and π-acceptor character of the ligand (168) (Table II). A similar series (150) has been observed for cobinamide (Table III). It should be emphasized that many of the derivatives in Tables II and III have been observed only in solution.

All the cobalamin derivatives mentioned so far are reconverted into vitamin B_{12} in the presence of cyanide ions, although the reaction with sulfitocobalamin appears to be complex (168). The products of the reactions of vitamin B_{12} with hydrogen sulfide (201) and with hydrogen fluoride (204) have not been fully examined, but on treatment with cyanide they also appear to revert to the vitamin.

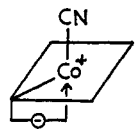
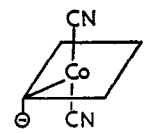
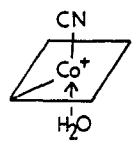
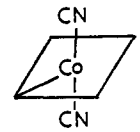
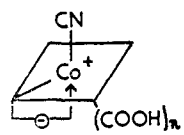
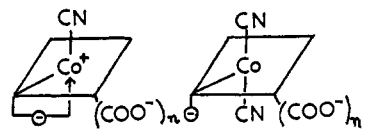
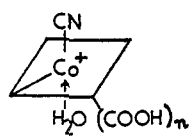
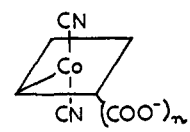
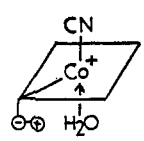
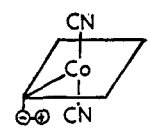
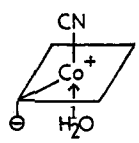
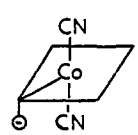
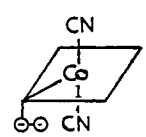
There is some evidence that aquocobalamin (but not the vitamin) is able to take up oxygen reversibly (189, 208). A binuclear complex containing 1 mole of oxygen has been suggested, and a similar concept has been put forward to explain (114) the low optical rotatory dispersion curve obtained on oxidizing the reduced vitamin.

3. The Dicyanide Complex

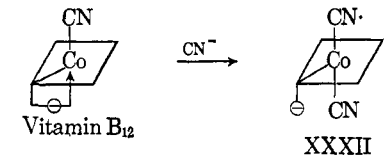
When potassium cyanide is added to a solution of vitamin B_{12}, the red solution becomes purple at a measurable rate [the coenzyme is converted into the same product more slowly (section V-C)]. Although the reaction is complete only when excess cyanide is present, only one cyanide group is introduced (89). The dicyanide XXXII thus formed migrates as a monoanion

TABLE IV

ELECTROPHORETIC BEHAVIOR OF VITAMIN B_{12} AND ITS DERIVATIVES (HCN PRESENT)

Compound	Acid pH 2-3	Neutral	Alkaline pH 10-11
1. Vitamin B_{12} and other essentially neutral factors	 neutral		 XXXII monoanion
2. Cobinamide	 XXXIII monocation		 XXXIV neutral
3. Carboxylic acids retaining the nucleotide	 XXXV neutral		 XXXVI anion n^- XXXVII anion$(n+1)^-$
4. Carboxylic acids without the nucleotide	 XXXVIII monocation		 XXXIX anion n^-
5. α-(3,5,6-Trimethylbenzimidazolyl)-cobamide	 XL monocation		 XLI neutral
6. Cobinamide phosphate	 XLII neutral	 XLIII monoanion (pH 6.2)	 XLIV dianion

(*cf.* Table IV); it has not been reported as a crystalline salt and is not stable in neutral or acid solution.



XXXII

The evidence strongly suggests that in basic solution the cyanide ion displaces the nucleotide. Thus (i) the small peak at 288 mμ appears and is associated with the free N-3 position of the 5,6-dimethylbenziminazole in basic solution (93); (ii) the spectra of cobinamide and vitamin B_{12} are very similar above 300 mμ in potassium cyanide solution; (iii) various cobalti complexes of benziminazole are cleaved by cyanide ions, while analogous studies with cobaltiporphyrins have been made (101, 232). Certain chemical reactions which take place readily only in the presence of cyanide accord with the conclusion that the nucleotide is extruded. Thus, hydrolysis with hot aqueous potassium cyanide (5) gives a series of acids none of which retains the nucleotide. Again, several members of the group (section IV-B) are hydrolyzed to cobinamide by buffered cerous hydroxide, but rather slowly (vitamin B_{12} especially) unless cyanide is present, when the reaction proceeds more readily, since one linkage is already broken. On the basis of these experiments it appears that the lability of the base–cobalt bond increases in the series vitamin B_{12} < α-(5-hydroxybenziminazolyl)-cobamide cyanide < adenylcobamide cyanide (135) (see also section V-A-4). Cyanide ions also have a decisive influence on the course of alkylation. Thus methyl sulfate in alkaline buffer methylates vitamin B_{12} at N-3 of the benziminazole system, provided that cyanide ions are present (134). The amorphous product, which on hydrolysis gives 1,5,6-trimethylbenziminazole, is neutral in the dicyanide form since a quaternary ammonium ion has been generated (XLI, Table IV).

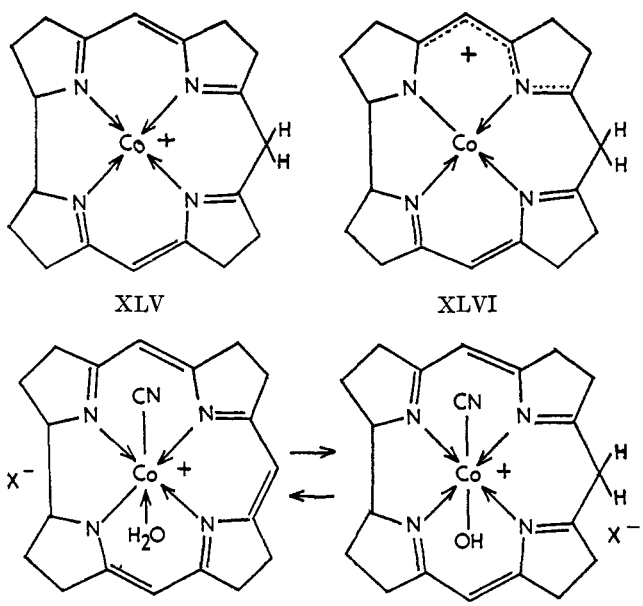
Except where special conditions operate (*e.g.*, the N-methylated product above or P^1-guanosine-5′P^2-cobinamide pyrophosphate) the purple dicyanides of those members of the vitamin B_{12} group retaining the nucleotide are stable only in alkaline solution (5). When the nucleotide is absent, however, the dicyanides are generally stable in neutral solution, and crystallization is possible in certain instances (*e.g.*, penta- and hexacarboxylic acids) from solutions containing excess hydrogen cyanide (48). The dicyanides are not stable in acid and, in this respect, resemble the cobaltiporphyrin dicyanides (232). These concepts allow a fairly extensive interpretation (Table IV) of the complex electrophoretic data if the somewhat higher mobility of the nucleotide-free compounds is taken into consideration (5, 48).

4. The Effect of Acid on Ligands and Chromophore

In addition to catalyzing the hydrolysis of amide, ester, and glycosylamine linkages (section V-F) acid

conditions may also cause the displacement of ligands and, probably, the protonation of the chromophore. These reactions have in general been observed spectroscopically, and conclusions are not in all cases beyond dispute. Thus the spectrum of vitamin B_{12} changes little as the acid concentration (HCl) is increased to 0.15 *M*, but above this concentration fine structure assigned to noncoordinated 5,6-dimethylbenziminazole appears, and it is concluded that the base is displaced (150). Other workers have suggested that the nucleotide is still coordinated to the metal in concentrated sulfuric acid (168).

Under conditions of high acidity [*e.g.*, concentrated H_2SO_4, HF (204)] a yellow solution is formed in which the γ-band no longer dominates the spectrum. It has been suggested that the red-yellow change involves a reversible protonation of the chromophore of the vitamin (168), although partly-irreversible changes in strong acid have been observed (23) and the concomitant formation of cobinamide would not be unexpected (section V-F-2). Protonation could conceivably occur at C-5, C-10, or C-15; protonation at C-10 is perhaps favored since the chromophore is known to be slightly deformed about this position (sections III-C and V-E). The postulated cationic species has been represented as XLV; presumably contributing structures such as XLVI should also be considered. An interpretation of the spectrum of cobinamide (in which, for example, the γ-band is less intense than usual) in terms of tautomerism between structures such as XLVII and XLVIII has been advanced (168).

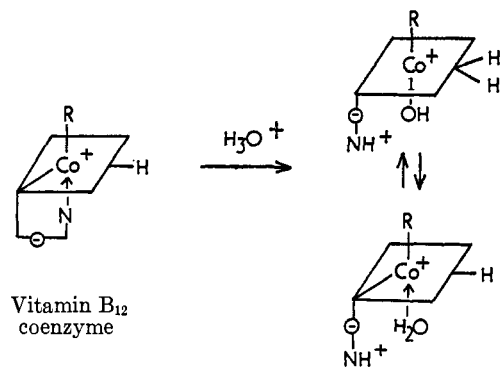


XLV XLVI

XLVII XLVIII

A red (522 mμ) → yellow (458 mμ) color change is also observed on acidification of vitamin B_{12} coenzyme. This change (pK 3.5) occurs much more readily than with the vitamin (pK <-1), and in the case of the adenylcobamide coenzyme even the neutral solution is yellow. Although the visual change is similar to that observed with the vitamin, the changes in the actual absorption curves in the two cases are quite different, since the vitamin and coenzyme spectra are themselves quite distinct, and clearly more than one phenomenon may be involved here. Two explanations for the color change in the coenzyme series have been advanced. One interpretation (213) suggests that it is caused by the cleavage of the nucleotide–cobalt bond, and would thus be analogous to the hypsochromic shift observed in replacing iminazole by water in the cobalt coordination sphere (*cf.* Tables II, III). The following evidence supports this conclusion: (i) the yellow adenylcobamide coenzyme loses the nucleotidic adenine on mild acid hydrolysis; the product, and also the cobinamide coenzyme, which cannot have a coordinated base, have spectra similar to that of the adenyl coenzyme above 300 mμ; (ii) in the acidic yellowish solution of vitamin B_{12} coenzyme (the spectrum of which also resembles that of adenylcobamide coenzyme above 300 mμ) there appears a minor peak at 284 mμ (noncoordinated, protonated 5,6-dimethylbenziminazole); (iii) the pK_a of 5,6-dimethylbenziminazole in the coenzyme situation (~3.5) is much higher than that in the vitamin (~0). This indicates a weaker Co–N-3 bond in the coenzyme which is reasonable in view of the labilizing *trans* effect which might be expected of the alkyl ligand (84, 251). It is of interest that the Co–N-3 bond in the 2′-phosphate isomer of vitamin B_{12} appears to be more labile than that in the vitamin, presumably for steric reasons (146).

Another interpretation (168) extends this by suggesting that in acid solution a tautomeric system is set



Vitamin B_{12} coenzyme

L

XLIX

up; one tautomer, XLIX, is that already visualized, but the other, L, in which protonation has occurred in the chromophore, predominates.

B. REDUCTION

The chemistry of the reduction of vitamin B_{12} is still poorly defined, even though the reduction products have acquired a considerable importance. A variety of reducing agents (24), including cysteine in alkali, lithium or sodium borohydride, zinc dust in aqueous

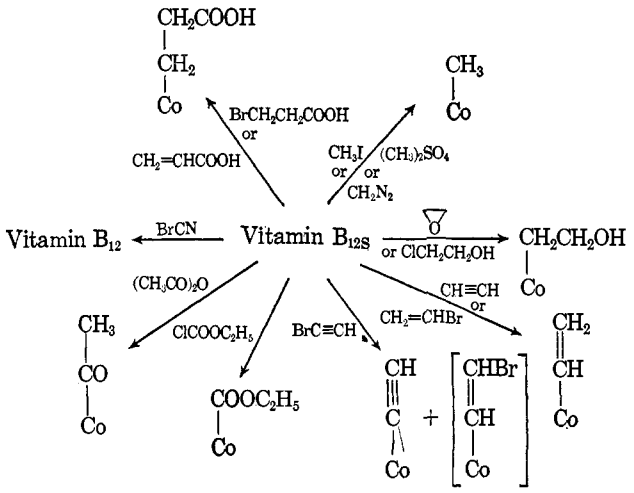


Fig. 9.—Organocobalt compounds from vitamin B_{12s}.

ammonium chloride (257), hypophosphorous acid (55), and catalytic reduction, have been used. While continued catalytic reduction leads to colorless compounds, intermediate brown and gray-green stages are distinguishable in certain cases.

1. *Removal of Cyanide; Vitamin B_{12r}*

One of the functions of reduction is to remove the cyanide ligand as hydrogen cyanide [chemical reduction (55)] or as methylamine [catalytic reduction (116, 258)]. The other product of reduction, and the final one (24) with chromous acetate at pH 5, is a brown solution containing "vitamin B_{12r}." This has a spectrum (Table V) with no strong γ-band, and may, therefore, have suffered reduction in the conjugated system. Aerial oxidation, which appears to be complex (116), furnishes aquocobalamin (198). That vitamin B_{12r} contains divalent cobalt is suggested by titrimetric and polarographic evidence (108, 188). Other workers have observed vitamin B_{12r} (reduction with stannous chloride) to be diamagnetic and conclude that it contains Co(I), for which there is some titrimetric evidence (168). However, vitamin B_{12r} (catalytic reduction) gives an electron spin resonance spectrum (182) of at least six lines (Co^{59} spin $^7/_2$ requires eight lines) consistent with a low-spin cobaltous state, and resembling the spectrum of cobaltous phthalocyanine. More work is needed to clarify this situation, but on balance the divalent state is indicated at present.

Although vigorous reduction of vitamin B_{12} does remove cobalt, attempts to exchange the metal atom of vitamin B_{12r} with $Co^{60}Cl_2$ proved unsuccessful (109). It is also reported that vitamin B_{12r} is converted by cyanide to dicyanocobalamin even in the absence of oxygen (108, 168); vitamin B_{12s} does not show this behavior, but on acidification in the absence of oxygen apparently gives the brown reduction product (168). In general aquocobalamin is more readily reduced than the vitamin, and, remarkably, appears to be hydrogenated to B_{12r} without the addition of catalyst (188); this homogeneous hydrogenation has an analogy in the reduction of pentacyanocobaltate (II) ion by molecular hydrogen (206).

2. *Vitamin B_{12s}; Organocobalt Derivatives*

On continued reduction, and preferably with excess chromous acetate at pH 9.5 (24, 51), zinc and ammonium chloride, or with sodium borohydride, the red → brown color change is followed by the appearance of a gray-green color; the substance(s) responsible for this will be designated vitamin B_{12s} (168). The solution, which has a strong ultraviolet band at ~385 mμ (Table V), can still be reoxidized to aquocobalamin, but when treated with alkylating agents (192, 226, 237, 238) immediately becomes red, yielding compounds formulated as cobalt alkyls on the basis of their relationship to the coenzyme together with spectroscopic and chemical evidence, among which it has not been possible, in many cases, to include chemical analysis. When the alkylating agent is 2′, 3′-isopropylidene-5′-tosyladenosine, a partial synthesis of the vitamin B_{12} coenzyme is effected (section VIII-A). With alkyl halides and sulfates the corresponding alkyls are formed; the crystalline C_1 to C_{10} *n*-alkyl derivatives have been obtained. Acylating agents react analogously. Diazomethane also gives the methyl compound, as does S-adenosylmethionine iodide (148). Bulky alkyl groups (*e.g.*, from *t*-butyl bromide) do not give derivatives, nor does reaction with phenyl bromide occur, but bromocyanogen gives vitamin B_{12} directly. Ethylene oxide and tetrahydrofuran rings are both opened to give the ω-hydroxyalkyl derivatives. Some similar reactions have been reported for the corresponding reduced form of cobinamide; here an interesting stereospecificity emerges, since it appears that alkylation occurs on that side of the molecule bearing the acetamide groups (*cf.* the partial synthesis of the cobinamide coenzyme, section VIII-A).

Addition reactions also occur. With alkenes addition proceeds only if the double bond is activated by an adjacent electron-withdrawing group and is apparently also controlled by a steric requirement which limits branching on the carbon adjacent to the metal atom. Alkynes require no activation, but the steric limitation still applies (Fig. 9).

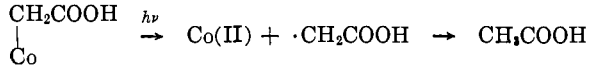
C. REACTIONS OF THE ORGANOCOBALT DERIVATIVES, INCLUDING THE VITAMIN B_{12} COENZYME

The compounds resulting from the alkylation of vitamin B_{12s} are remarkably stable; thus, although $CH_3Co(CO)_4$ decomposes at −35° (167), the methyl coenzyme analog is stable at room temperature in the absence of light. The absorption spectra of the alkyl compounds resemble that of the coenzyme; in the

TABLE V
SOME DERIVATIVES OF VITAMIN B_{12}

Systematic name	Other names	State	Light absorption: pH	Light absorption: λ_{max} mμ	Ref.
α-(5,6-Dimethylbenziminazolyl)-cobamide hydroxide (aquocobalamin)	B_{12a}, B_{12b}, B_{12d}	Cryst.	2	274, 351, 408, 522	93
			10	278, 358, 418, 535	93
			~7	270–277, 352.5, 530	201
α-(5,6-Dimethylbenziminazolyl)-cobamide nitrite (nitritocobalamin)	B_{12c}	Cryst.	~7	255–275, 354, 530	201
	Sulfitocobalamin	Solution	*N* HCl	284, 317, 419	168
			3.3 *M* KOH	289, 310.5, 364	168
Ammine-α-(5,6-dimethylbenziminazolyl)cobamide	Ammonia cobalichrome	Cryst.	2	275, 355, 411, 536	93
			10	276, 357, 412, 541	93
	Vitamin B_{12r}	Solution		312.5, 405, 473	24, 108
	Vitamin B_{12s} "gray-green reduction product"	Solution		385, 460, 554, 680, 800	51, 168
8-Amino-α-(5,6-dimethylbenziminazolyl)cobamic acid *abdeg*-pentaamide *c*-lactam cyanide	Dehydrovitamin B_{12}	Cryst.	~7	278, 304, 320, 359, 408, 517, 548	46
8-Amino-10-chloro-α-(5,6-dimethylbenziminazolyl)cobamic acid *abdeg*-pentamide-*c* lactam cyanide	Chlorodehydro vitamin B_{12}	Cryst.	~7	282, 288, 365, 417, 548, 580	46
			0.1 *N* KCN	289, 303, 316, 369, 424, 568, 609	46
8-Hydroxy-α-(5,6-dimethylbenziminazolyl)cobamic acid *abdeg*-pentamide *c*-lactone cyanide	Vitamin B_{12} lactone	Cryst.	~7	278–9, 302–6, 320, 359–360, 407–409, 524–525, 552	46
8-Amino-cobyrinic acid *c*-lactam chloride cyanide	Hexacarboxylic acid chloride cyanide	Cryst.	~7	317, 350, 380–381, 402, 497, 528	48

alkenyl and alkynyl derivatives the spectrum assumes a form intermediate between those of the vitamin and the coenzyme. Presumably perpendicular conjugation (120) through the d-orbitals is involved here. The compounds are light-sensitive, and under anaerobic conditions give vitamin B_{12r} [spectrum, polarogram (30, 208)] and other products which suggest that homolysis is involved. This interpretation is in accord with the e.s.r. spectrum of the irradiated coenzyme which resembles that of vitamin B_{12r}, but which also shows evidence of the formation of an organic free radical (182). An analogy with the photolysis of $Co(NH_3)_5I^{2+}$, which gives Co(II) and iodine, is apparent (1). Thus irradiation of the carboxymethyl coenzyme analog gives acetic acid (192). Similarly



anaerobic photolysis of vitamin B_{12} coenzyme gives vitamin B_{12r} and an adenine nucleoside (180, 192) which analyzes for $C_{10}H_{11}N_5O_3$ and which has been provisionally formulated as XVII. It absorbs at 260 mμ (acid) and 263 mμ (neutral and basic solution) and consumes 1 mole of periodate. Acid hydrolysis does not liberate adenine but gives an aldehyde; further reactions of this accord with the proposed structure as indicated in Fig. 10.

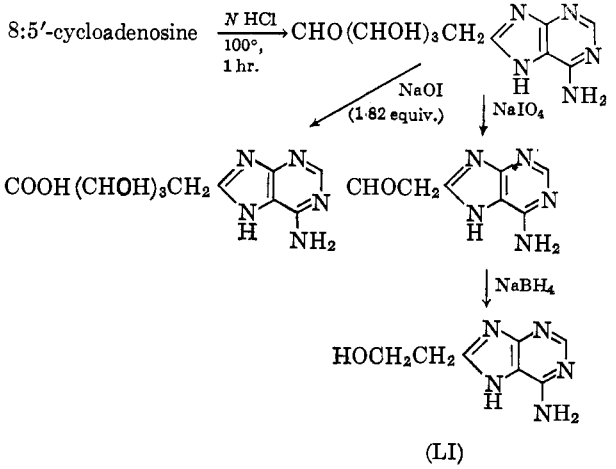
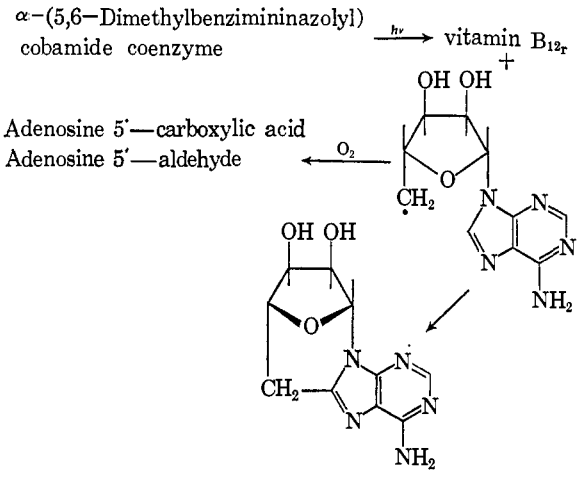


Fig. 10.—Some transformations of the adenine nucleoside formed on anaerobic irradiation of vitamin B_{12} coenzyme.



XVII 8,5′-cycloadenosine

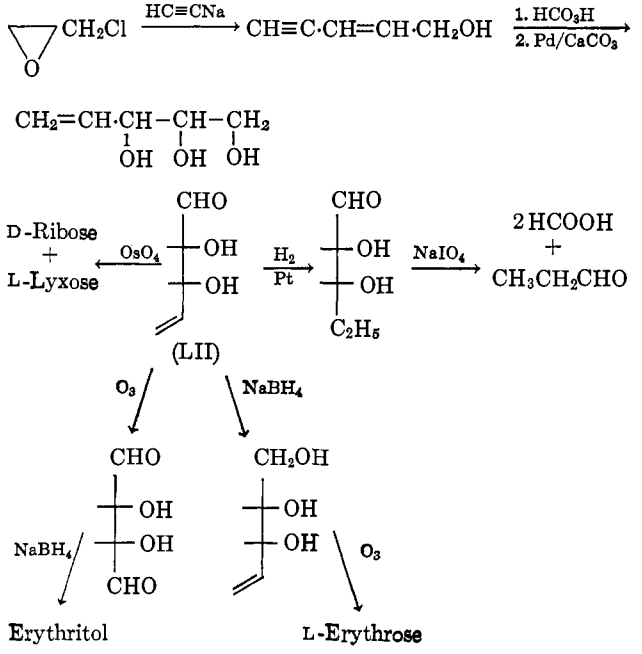


Fig. 11.—Synthetical and degradative reactions associated with the formulation of D-*erythro*-2,3-dihydroxypent-4-enal (LII).

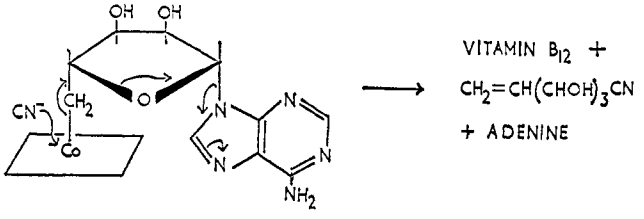


Fig. 12.—Cleavage of the coenzyme with cyanide ions.

LI has a spectrum similar to that of 8-ethyladenine, but it has not, as yet, been possible to reduce it to this compound.

In the case of the coenzymes the alkyl ligand is fairly readily displaced under acid conditions to give aquocobalamin, adenine, and D-*erythro*-2,3-dihydroxypent-4-enal (LII) which has been identified (181) on the basis of the reactions shown in Fig. 11, and by the synthesis (195) of its (racemic) reduction product (Fig. 11). Acid treatment of the ethoxycarbonyl analog also generates aquocobalamin (238).

With cyanide ions the dicyanide form of the vitamin is formed; adenine and the cyanohydrin(s) of the unsaturated sugar are the other cleavage products. The reaction has been envisaged as an SN2 displacement (195) and appears to proceed more slowly (or not at all) with the simple alkyl analogs (237) than with the coenzyme itself, presumably because in the latter case a concerted cleavage is possible which does not require a carbanion leaving group. This process (Fig. 12) has some analogy in the alkaline hydrolysis of sulfonium nucleosides (130).

The methyl coenzyme analog has become of especial interest (155) since in an enzymic system it methylates homocysteine. An *in vitro* demonstration of this methyl transfer has also been made (196); the methyl coenzyme analog when photolyzed under reducing conditions ($NaBH_4$) methylates homocysteine to give methionine in 58% yield as the dinitrophenyl derivative. Similarly the vitamin B_{12} coenzyme gives S-adenosylhomocysteine, an observation which may be significant in the problem of the generation of active methionine (252).

Several questions remain unsettled in this area.

(*i*) *The Nature of Vitamin* B_{12r}.—The evidence for divalent cobalt in this compound has been fairly widely accepted. The spectrum differs markedly from that of the vitamin, but the shift in the major absorption (361 mμ → 312 mμ) could perhaps be accounted for by the removal of cyanide and nucleotide ligands, and the valence change [*cf.* (191) in the presence of platinum–hydrogen, etioporphyrin I pyridinobromocobalt(III) (417 mμ) → cobaltous etioporphyrin I (392 mμ)]; an interruption in the conjugated system has not been excluded, however.

(*ii*) *The Unsaturated System in the Coenzyme.*—Arguments for the reduction of the chromophore are stimulated by the absence in both red and yellow forms of the strong γ-band (ε ~ 30,000) in the 360-mμ region which, though it does not closely resemble the Soret band of the porphyrins in either intensity or position, is reasonably ascribable to the unsaturated system (113) interacting with the metal atom. Reduced forms with a tetrahedral C-10 position have been suggested (286), and it has been observed that reactions which involve position 8 [lactam and lactone formation (sectionsV-D-2, V-E)] do not appear to occur with the coenzyme (278). This has been suggested as evidence for the reduction of the 9–22 bond. Reaction with cyanide must then involve a dehydrogenation step, although oxygen does not appear to affect the reaction (31). Moreover the reactivity of position 8 is known to depend on the nature of the coordination sphere (46). This leads to the suggestion that the nature of the axial ligands may have a more important influence on the spectra in this series than has hitherto been recognized, in particular when the distinction is between ligands which have available p-orbitals for interaction with the corrinoid system and ligands which have not. The intermediate character (192, 238) of the spectra of the vinyl and ethoxycarbonyl coenzyme analogs is significant here.

(*iii*) *The Nature of Vitamin* B_{12s}.—It has been suggested (168, 192, 237) that the gray-green reduction product is a cobalt hydride complex. The formation of $Co^{III}(CN)_5H^{3-}$ by the reduction of $Co^{II}(CN)_5^{3-}$ with aqueous borohydride (206) is an interesting analogy. Moreover, certain of the additions to alkenes also find analogies in the first step of the hydroformylation reaction (158) and in the addition of $Mn(CO)_5H$ to

fluoralkenes (274). The reaction with diazomethane recalls the process (124)

$$\pi\text{-}C_5H_5W(CO)_3H + CH_2N_2 \rightarrow \pi\text{-}C_5H_5W(CO)_3CH_3 + N_2$$

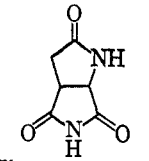
The reactions in Fig. 9 are typical of a nucleophilic species, and it seems likely that the nucleophilic reagent may be Na^+ [corrinoid]$^-$ (which can be regarded as a Co(I) complex), probably in equilibrium with the hydrido complex. It is pertinent that the reaction is generally carried out in basic solution, and that certain cobalt hydrides are known to be acidic; $HCo(CO)_4$ is as strong as a mineral acid, for example (166). At any event the search for the Co–H linkage should be possible by n.m.r. techniques, where a peak in the region of 20 τ might be expected (152), although an appreciable ring-current would cause further shielding.

The occurrence of a strong band at 385 mμ in the spectrum of vitamin B_{12s} is difficult to reconcile with an interruption of the conjugated system (24). The fine structure in the 285-mμ region suggests that the nucleotide is no longer coordinated in vitamin B_{12s}; presumably it and the propionamide groups [*cf.* the partial synthesis of cobinamide coenzyme (238)] may still exert a steric influence which leads to the correct Co–alkyl bond on partial synthesis of the vitamin B_{12} coenzyme (section VIII-A).

D. OXIDATION

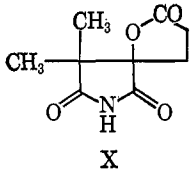
1. Vigorous Oxidation

The part that vigorous oxidation played in the formulation of the vitamin has already been considered (section II) and it is interesting to note that no fragments have been obtained which can definitely be assigned to rings A and D. Permanganate oxidation of the esters of the mixed pigment acids gives nine nitrogen-free acids, six of which have been identified as acetic, oxalic, succinic, methylsuccinic, dimethylmalonic, and α,α-dimethyl-β-carboxyadipic acids (149, 258). Oxidation with hydrogen peroxide furnishes oxamic acid (47, 81). Chromic acid oxidation has furnished the succinimides X (from the acid-hydrolyzed vitamin, the hexacarboxylic acid, and the vitamin itself), XI (from the acid-hydrolyzed vitamin and the hexacarboxylic acid), XII (from the vitamin), and XIV (from the hexacarboxylic acid and dehydrovitamin B_{12}). Of these only XII and XIV were demonstrably optically active, the latter substance requiring examination at 365 mμ (86). The structure of XIV is established (86) on the basis of analysis, infrared and ultraviolet spectra, titration data, and methylation experiments. The isomeric glutarimide structure is explicitly excluded on pK data and infrared evidence, and the compound bears the expected similarities to the synthetic lactam–imide LIII. The formation of the

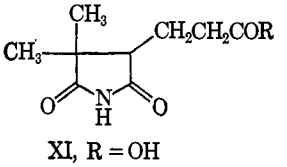


LIII

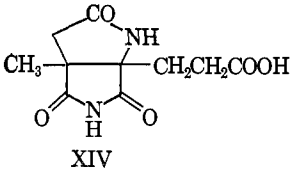
spirolactone in X is of interest; it is not detected on chromic acid oxidation of imides XI and XII. Presumably then, the lactone is formed before the disruption of the corrinoid system; cleavage reactions compete with lactone formation and hence the lactone is not the only product derived from ring C. Lactone formation unattended by cleavage has been observed (on ring B) under mild conditions (section V-E) and possibly the present reaction is analogous mechanistically.



X



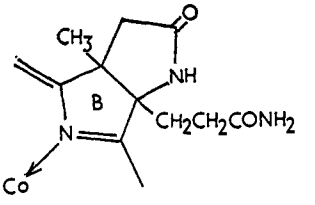
XI, R = OH
XII, R = NH_2



XIV

2. Mild Oxidation

It was observed at an early stage that preparations of vitamin B_{12} were stabilized in the presence of ascorbic acid and other reducing agents (222). In fact, aeration of vitamin B_{12} for a short time in hot alkaline solution gives a crystalline neutral product, which resembles the vitamin in physical properties but has a very low microbiological activity (46, 218). This has been called dehydrovitamin B_{12} and has been formulated (46) as 8-amino-α-(5,6-dimethylbenziminazolyl)cobamic acid *abdeg*-pentamide *c*-lactam LIV (partial structure). It is pertinent that alkaline hydrolysis of vitamin B_{12} gives a series of acids different from that obtained under acid conditions, and that the monocarboxylic acids obtained by such an alkaline hydrolysis give a microbiologically inactive product on reamidation. Vigorous acid hydrolysis of dehydrovitamin B_{12} fails to reveal a heptacarboxylic acid, while vigorous alkaline hydrolysis gives a mixture of penta- and hexacarboxylic acids which appears to be the same as that obtained by



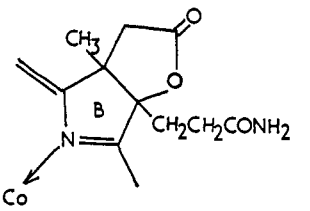
LIV (partial structure: remainder as in vitamin B_{12})

similar treatment of the vitamin. Infrared evidence is consistent with a lactam structure, and a fused lactam is indicated since the dehydrovitamin undoubtedly represents a stage on the way to the hexacarboxylic acid, which is known to have a *cis*-7,8 fused lactam structure from the X-ray evidence. In addition chromic acid oxidation furnishes the lactam–imide XIV. Removal of the nucleotide gives 8-aminocobinic acid *abdeg*-pentamide *c*-lactam which may also be prepared by the aerial oxidation in alkali of cobinamide itself.

The mechanism of the formation of dehydrovitamin B_{12} must involve an oxidation step since in the absence of air, or in the presence of a reducing agent such as mercaptoacetate, the vitamin is partially protected. When the tervalent cobalt complex is stabilized by the presence of cyanide ions no reaction occurs; this may be compared to the inability of hemoglobin to transfer oxygen in the presence of cyanide. That the cobalt atom is involved, and is in fact reduced during the reaction, is suggested by the formation of a brownish red color associated with the Co(II) complex (section V-B-1); a one-electron oxidation generating a radical at C-8 which subsequently reacts with acetamide group *c* is possible. Alkaline conditions appear to favor lactam generation, while the two examples of lactone formation (*cf.* section V-E and section V-D-1) both occur under acid conditions. Similar behavior has been observed during the cyclization of amides in simpler systems (97, 266).

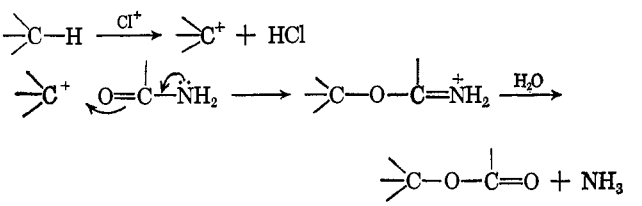
E. HALOGENATION

Whereas an excess of chlorine destroys vitamin B_{12} to give an almost colorless solution, controlled chlorination under mildly acidic conditions does not rupture the chromophore but proceeds first of all by an oxidative cyclization followed by a substitution reaction (46). One mole of chlorine or chloramine T at pH 4 gives a halogen-free crystalline substance which is also formed with bromine water and iodine in alkali, although in the latter case some dehydrovitamin is formed concomitantly. The product has an electronic absorption spectrum very similar to that of the vitamin, but shows a peak in the infrared at 1784 cm.$^{-1}$ suggestive of a γ-lactone. On electrophoresis the substance behaves as a lactone, and both the lactone and the hydroxy-acid forms have been isolated, and are interconvertible. Vigorous alkaline hydrolysis yields hexa- and hepta-carboxylic acid fractions. This suggests that lactam formation on ring B is not possible and an amide group is clearly involved since not only has ammonia been detected as a reaction product but the lactone contains one nitrogen atom less than the vitamin. That ring B is the reaction site is further supported by the observation that dehydrovitamin B_{12} does not give a lactone; that it is acetamide (*c*) and not propionamide (*d*)



LV (Partial structure, remainder as in vitamin B_{12}.)

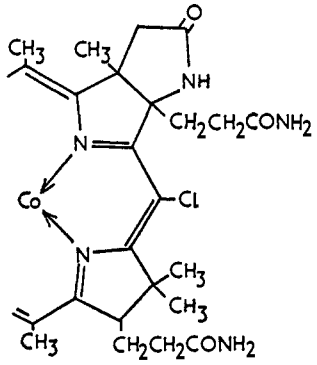
which is involved is suggested by the formation of a heptacarboxylic acid on alkaline hydrolysis, and by the action of halogen on the monocarboxylic acids derived from the vitamin. One of these is considered to have a free propionic acid group *d* (section V-F-3), but nevertheless they each give a lactonic acid on treatment with one equivalent of halogenating agent. The lactone is, therefore, formulated as 8-hydroxy-α-(5,6-dimethylbenziminazolyl)cobamic acid *abdeg*-pentamide *c*-lactone (LV). In agreement with this formulation the lactone is recovered after being submitted to the conditions used to prepare dehydrovitamin B_{12}. Controlled acid treatment removes the nucleotide and the major product behaves as the lactone derivative of cobinamide. The formation of the lactone presumably involves oxidation at the activated position 8 followed by the participation of the neighboring acetamide residue, a process which is analogous to the oxidative cyclization of tryptophanyl peptides with N-bromosuccinimide (214).



Lactone formation has also been observed on chromic acid oxidation (section V-D-1) and presumably follows a similar route; in this case a spirolactone is produced at C-13. This is the position in ring C corresponding to 8 in ring B; a fused ring system cannot be formed here since ring C bears no acetamide group.

Further halogenation (46, 117, 258) produces a substance with a royal blue dicyanide (λ_{max} 370, 572, and 612 mμ). Thus, the vitamin reacts with 2 moles of chlorinating agent to give an amorphous [or microcrystalline (258)] substance which behaves as a lactone. Dehydrovitamin B_{12} suffers a similar reaction (46) but requires only 1 mole of halogenating agent to effect the bathochromic shift, and the product, which is not a lactone, is crystalline and contains one atom of chlorine; its spectrum closely resembles that of the halogenated derivative of the vitamin. That the introduction of one chlorine atom is associated with a bathochromic shift suggests that substitution has occurred in the conjugated system, and a consideration of elec-

tron availability in the conjugated system [(*cf.* the C_{2v} tetrahydroporphyrin system (15)] leads to the conclusion that the most reasonable position for electrophilic substitution is C-10. Chlorodehydrovitamin B_{12} is therefore presumed to be 8-amino-10-chloro-α-(5,6-dimethylbenziminazolyl)cobamic acid *abdeg*-pentamide *c*-lactam (LVI). The reaction and the



LVI. 8-Amino-10-chloro-α-(5,6-dimethylbenziminazolyl)cobamic acid *abdeg*-pentamide *c*-lactam (partial structure, remainder as in vitamin B_{12}).

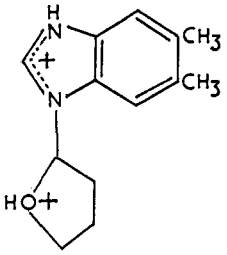
attendant bathochromic shift would thus be analogous to the bromination of 2,3-dihydro-5,7-dimethyl-2,3-cyclopentano-1,4-diazepine (325 mμ → 349 mμ) (228) and to the conversion (292) of chlorin p_6 dimethyl ester (498.3, 533.1, and 667.2 mμ) into the δ-chloro derivative (505.8, 541.1, and 681.8 mμ).

F. HYDROLYSIS

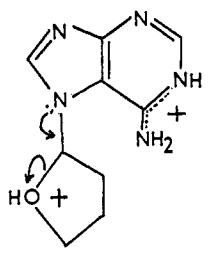
Hydrolysis in relation to axial ligands has already been considered [displacement of cyanide (section V-A-1), displacement of nucleotide (section V-A-4), cleavage of 5′-deoxyadenosyl group (section V-C)].

1. Glycosylamine Hydrolysis

In the benziminazolylcobamides the glycosylamine linkage is very resistant to hydrolysis—6 *N* HCl at 150° for several hours is required and these conditions destroy the free sugar. However, the purine analogs, and the 5′-deoxyadenosyl group of the coenzymes, resemble the common purine ribonucleotides and are cleaved much more readily. Thus, the nucleoside from ψ-vitamin B_{12} is hydrolyzed in 15 min. by 0.05 *N* HCl at 100° to D-ribose and adenine. This variation in lability has not been fully explained but is presumably related to the locus of the positive charge on protonation. Thus, the benziminazole system may be expected to exist in acid solution largely as the cation in an arrangement LVII which is unlikely to favor the ether cleavage process of a mechanism such as that proposed by Isbell (186). On the other hand, in the purine derivatives the initial protonation could lead to a situation in which the positive charge is confined to the six-membered ring and is still located in a meso-



LVII

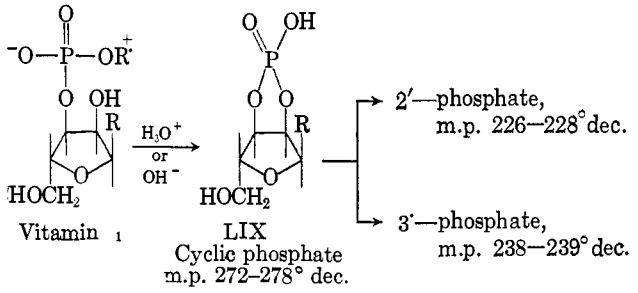


LVIII

meric system which would not markedly interfere with ether cleavage (LVIII-arrows).

2. Phosphate Hydrolysis

Two sets of conditions which will hydrolyze the phosphate ester preferentially, and so lead to cobinamide, have been established. The first employs conditions of high acidity. It is known (209, 269) that the rate of hydrolysis of amides by a bimolecular process reaches a maximum at an acid concentration of ~3 *M* (the precise position depends both on the acid and the amide) and then decreases as acidity increases, since the amide is at this stage in effect completely protonated, while the activity of water is falling. For weaker bases, such as esters, this point is generally not reached so readily; for example, the rate of hydrolysis of dimethyl phosphate increases with acid concentration in the range considered (77). Moreover in the present case participation of the C-2′ hydroxyl group will increase the rate of phosphate cleavage. Hence, carefully controlled conditions of high acidity (Fig. 13) furnish the nucleotide together with cobinamide (5). The vitamin is a monoalkyl 3′-ribonucleotide and as such (66, 68) hydrolyzes in acid or alkali to yield mixtures of the 2′- and 3′-phosphates *via* the cyclic phosphate (LIX).



Vitamin B_{12} LIX
Cyclic phosphate
m.p. 272–278° dec.

The 2′- and 3′-phosphates have been crystallized after separation by ion-exchange chromatography (44). The cyclic phosphate (obtained by the carbodiimide method (44)) has been obtained crystalline (133). On acid or alkaline treatment it gives a mixture of the two isomers. The 5′-phosphate has been obtained synthetically (133).

The second method for hydrolyzing the phosphate group employs a buffered suspension of cerous hydroxide generally in the presence of cyanide ions which assist the reaction by cleaving the Co–N-3 bond. Both phos-

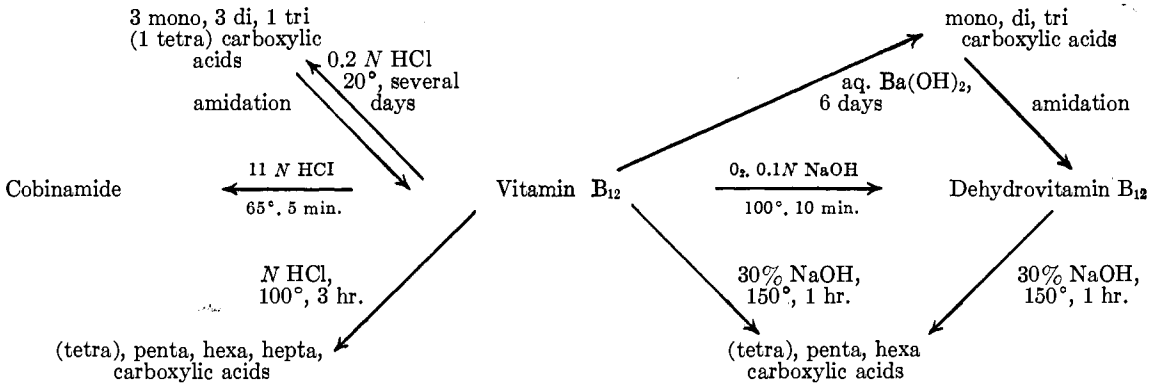


Fig. 13—Amide and ester hydrolysis in vitamin B_{12} (minor products in parentheses).

phate ester linkages are hydrolyzed and cobinamide, the nucleoside, and phosphate are produced cleanly (135, 140). This method makes use of the catalytic effect of rare earth hydroxides at pH ~8.5 on phosphate ester hydrolysis, a process which presumably has a cerous complex as an intermediate (79).

3. Amide Hydrolysis

The hydrolysis of the amide groups proceeds under acid or alkaline conditions; under acid conditions a series of acids possessing from one to seven carboxyl groups is formed, the lower members of which, formed under mild conditions, usually retain the nucleotide. Under alkaline conditions a similar series is formed, but no heptacarboxylic acid is produced. These acids presumably possess the fused lactam at ring B, as in dehydrovitamin B_{12} (Fig. 13) (5, 48).

Mild acid hydrolysis (*e.g.*, 0.1 *N* HCl, room temperature, 65 hr.) produces three monobasic acids, three dibasic acids, one tribasic acid, and one tetrabasic acid; these have been separated and crystallized (with the exception of one dibasic acid). They all contain the nucleotide and the mono- to tribasic acids have been reconverted into the vitamin. More vigorous acid treatment gives mixtures of penta-, hexa-, and heptacarboxylic acids which have lost the nucleotide. These have not been obtained crystalline, and the heptacarboxylic acid cannot be obtained cleanly as a product of complete hydrolysis since it slowly reacts further under acid conditions (5). The nature of this change is not known but decarboxylation may be involved after the analogy of the acid-catalyzed decarboxylation of uroporphyrin to coproporphyrin (125).

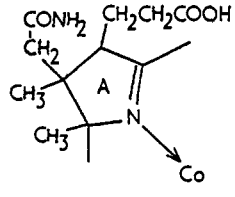
Alkaline hydrolysis of the vitamin furnishes another series of acids; the crystalline nucleotide-containing monobasic acids are chromatographically distinguishable (46) from the monocarboxylic acids produced by acid hydrolysis. Vigorous alkaline hydrolysis produces mainly penta- and hexacarboxylic acids, both nucleotide-free. The hexacarboxylic acid is featured as the chloride cyanide in the X-ray analysis; it has also been obtained crystalline as the dicyanide, as has the pentacarboxylic acid (48). The hexacarboxylic acid is homogeneous, and is unaffected by further alkaline treatment. This accords with the stability to alkali frequently observed with the lactam system (97, 266).

The general uniformity of the electronic absorption spectra of these acids indicates that the chromophore has not been affected. Racemization (281) might be expected to occur at certain β-positions (3, 8, 19, and notably 13) under strongly basic conditions, but the observation that the hexacarboxylic acid has the same configuration as the vitamin indicates that such a process is not favored.

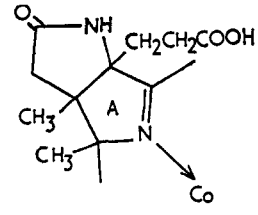
The course of this complex hydrolysis can be rationalized in a general way. The hydrolytic experiments (5) show that there are three labile amide groups which on hydrolysis give nucleotide-containing products, indicating that propionamide *f* is not involved. Propionamide *f* and another amide group appear to be somewhat more resistant, while under acid conditions two amide groups are particularly resistant to hydrolysis.

In vitamin B_{12} there are three acetamide groups and four propionamide groups, one of the latter being N-substituted. An apposite distinction between the two types is possible either in terms of participation of the unsaturated system (16, 215) in the hydrolytic process or as a consequence of steric control. The former process would probably not be favored since it requires a conformation in which the acyl groups are oriented toward the center of an already compact molecule. However, in terms of the steric effect of β-substituents compared with that of γ-substituents [*cf.* the rule of six (240)] a fairly complete rationalization is apparent (49). Thus the propionamides *b*, *d*, and *e* have no β-methyl groups and on steric grounds would be most labile to S_N2 reaction giving the three mono-, three di-, and one tricarboxylic acids observed. Although propionamide *f* possesses only a γ-methyl substituent, it is linked with a substituted ammonia which would be expected to have an additional steric effect [*cf.* the hindered alkyl acetates (241)]. Of the acetic acid residues, that at *g*, being on the "reversed" ring D, has

no methyl group at the β-position, whereas the remaining two acetamide groups at *a* and *c* both have β-methyl substituents and would be expected to hydrolyze most slowly. This rationalization is supported by kinetic measurements (49) on simple model systems (Fig. 14). It suggests that the "antivitamins" prepared from the monocarboxylic acids (219) are propionamide derivatives and that the pentacarboxylic acid from alkaline hydrolysis (the dicyanide of which analyzes as an N_8 compound (48)) is the acetamide derivative LX and/or the corresponding lactam LXI.



LX (remainder as in hexacarboxylic acid XV)



LXI (remainder as in hexacarboxylic acid XV)

Position 4 is in many respects equivalent to position 8 in ring B, and this readily undergoes cyclization to give the lactam; however, because of the steric effect of the methyl group at C-5 (not present at C-10) the cyclization on ring A is likely to be much slower.

Because of the intermediate ease of hydrolysis of amides *f* and *g*, a tetrabasic acid fraction is a minor product of mild or vigorous hydrolyses. The formation under mild conditions and in small yield of a nucleotide-containing tetracarboxylic acid is accounted for, as in the observation that no pentacarboxylic acid retaining the nucleotide is obtained in normal hydrolysis. In the presence of nitrous acid, which facilitates the cleavage of hindered amides, the complete range of acids with and without the nucleotide may be obtained in one operation (218). Under normal conditions, however, the steric factors appear to operate so as to reduce the number of isomeric acids which can be isolated.

G. MISCELLANEOUS

1. Pyrolysis

Vitamin B_{12} evolves ~1 mole of ammonia when heated to 180°; a 19% loss of weight at this stage is an indication of the extensive hydration already mentioned. At higher temperatures (~240°) more ammonia is released and extensive changes occur (239). Vigorous heating produces vapors which give the pine splinter test (65); total combustion leaves a lavender residue of cobalt phosphate.

2. Other Derivatives

A mercury derivative has been prepared containing three atoms of mercury per molecule (59). Sparingly soluble compounds are formed (156) between vitamin

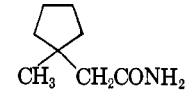
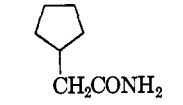
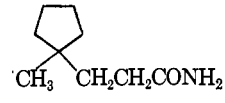
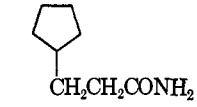
Amide	Model for positions	Relative rate of acid hydrolysis (0.05 *M*, 1.5 *N* HCl, 25% dioxan, 50°)
	2, 7	0.03
	18	0.35
	17	0.95
	3, 8, 13	1.0

Fig. 14.—Hydrolysis of model amides.

B_{12} and various complex inorganic acids including phosphotungstic, phosphomolybdic, auric, and platinic acids. Presumably in all these derivatives, as in the compound with xanthydrol (5), the amide groups are involved. Formaldehyde adducts have been reported (275).

In the presence of carbodiimide vitamin B_{12} reacts with β-cyanoethyl phosphate to give the 5′-β-cyanoethylphosphate derivative (277). On elimination of acrylonitrile the 5′-phosphate of vitamin B_{12} is formed; this behaves as a monobasic acid at pH 2.5, a dibasic acid at pH 7.5, and a tribasic acid in alkaline solution.

VI. THE INTERACTION OF VITAMIN B_{12} WITH PROTEINS

The combination of vitamin B_{12} with protein is of wide interest since Castle's intrinsic factor from gastric mucosa, which facilitates the absorption of the vitamin, appears to be a mucoprotein. The precise nature and mode of action of the intrinsic factor remain obscure in spite of much investigation. The coenzyme and the vitamin are bound by intrinsic factor preparations to the same degree; both are effective against pernicious anemia (222).

Many other proteins have been shown to form complexes with the vitamin, and natural complexes appear to occur fairly widely, although in some cases they may result as artifacts after the photolysis of the coenzyme (123). A complex from ox liver has been closely examined. It has a molecular weight of about 9000 and λ_{max} at 274, 350, and 500–523 mμ. The peptide appears to be a straight chain assemblage of 80 units (N terminus-histidine). The complex contains no adenine and no cyanide, but treatment with cyanide causes a bathochromic shift of the absorption bands, and the corrinoid portion formed on cleavage with trichloroacetic acid and cyanide is identical with vitamin B_{12} (159, 160, 161).

The mode of combination of protein with vitamin B_{12}

is of some chemical interest, since it is evident that there is more than one type of binding. One possibility, cobalichrome formation, is suggested by the observation that aquocobalamin forms complexes with proteins more readily than does vitamin B_{12} and there is some evidence that a histidyl residue may be involved since histidine is the only amino acid which readily coordinates (pH 4-5) with aquocobalamin (18). Such a compound would normally furnish vitamin B_{12} on treatment with cyanide. Another form of binding is required, however, since not only does histidine fail to react with vitamin B_{12}, but certain complexes apparently contain the cyanide ligand, but are not further affected by cyanide ions (151). Some studies on the effect of structural variation on protein binding have been made (151). Treatment of a protein with formaldehyde did not interfere with the reaction; treatment with 2,4-dinitrofluorobenzene did so, however. Variation of the corrinoid portion showed that whereas cobyrinic acid did not react, a tribasic acid did so, an observation which suggests that the acetamide groups may be important for binding processes. Since cobinamide was bound satisfactorily, the nucleotide is not necessarily involved. It has, indeed, been suggested that the protein may displace the nucleotide and form a new coordinate bond with cobalt (18, 19). At any event, it is likely that hydrogen-bonding between the protein molecule and the peripheral amide and alcohol groups is important as a secondary binding process. It is this hydrogen-bonding which is responsible for the tenacious hydration of the crystalline vitamin.

VII. BIOGENESIS

The biogenesis of the corrinoid system and its bearing on the biogenesis of porphyrins has occasioned much interest and speculation. The arrangement of the β-substituents suggests a close relationship to uroporphyrin III, and further suggests that methylation has occurred after the pyrrole rings have been joined together (but not necessarily closed) in the III type sequence (47).

It has indeed been shown that glycine and δ-aminolevulic acid are incorporated. δ-Aminolevulic acid-1,4-C^{14} gives a labeled vitamin in which the fraction of the activity of the amide carbons (obtained as CO_2 by Hofmann degradation) is close enough to that expected (6:15) to lead to the conclusion that this precursor follows a path similar to that found in porphyrin biosynthesis (94, 260). Labeled porphobilinogen is also incorporated (259). Certain reports suggest that some early modification of the porphyrin sequence may be required, although further experiments with labeled substrates are desirable. In certain systems, for example, producing both porphyrin and corrin, increase of δ-aminolevulic acid stimulates the formation of porphyrin but does not increase the production of vitamin B_{12} (9, 76), while when the biosynthesis of δ-aminolevulic acid is interrupted porphyrin synthesis is halted but corrinoid synthesis is unaffected (230).

Methionine is involved in the methylation step (57, 58). The nucleotide, unlike the corrinoid portion, is not labeled in biosynthetic experiments with methionine-$C^{14}H_3$. Oxidation of the labeled vitamin gives the imidic acid XI (section V-D-I) which has in the *gem*-dimethyl group one-sixth of the specific activity of the vitamin. Kuhn-Roth oxidation of the labeled corrinoid gives acetic acid labeled at the methyl group. That one of the methyl groups at C-12 is derived from δ-aminolevulic acid is shown by a two-stage oxidation following the incorporation of δ-aminolevulic acid -2,3-C^{14} in the absence of labeled methionine. The carboxyl carbon of the resulting acetic acid has twice the activity of the methyl group. That the C-1 methyl group corresponds to a meso-bridge is suggested by the observation that incorporation of δ-aminolevulic acid-5-C^{14}, followed by oxidation of the vitamin, gives acetic acid bearing some radioactivity in the methyl group.

The stage at which methylation occurs is not known, although the β-methylation of a 2*H*-pyrrole methiodide and the meso methylation of N,N-dimethyldipyrromethene salts (50) offer an interesting basis for further speculation. It is possible that incorporation of the cobalt atom occurs at an early stage—the cobalt-free macrocycle has yet to be recognized—and assists in the activation of the β-positions during methylation of a reduced intermediate (47). The direct linkage may be established as a consequence of the deformation of the system by the tetrahedral β-carbon atoms. An ingenious mechanism has been advanced to explain the reversal of ring D in both III-type porphyrins and the vitamin B_{12} group. It requires a final ring closure between rings C and D (96). In view of the ability of the porphyrinogen system to open and close again (231) the question of the incorporation of labeled uroporphyrinogen III into the corrinoid system remains of some interest.

The next intermediate which is at all clearly recognized is cobyric acid which is converted into cobinamide and vitamin B_{12} in *P. shermanii* fermentations (25). Related carboxylic acids (Factors V) have also been observed. The 1-aminopropan-2-ol which is incorporated to give cobinamide is derived from L-threonine (207), decarboxylation of which must, in some systems at least, occur prior to or on incorporation, since *P. shermanii* fermentations cannot effect the decarboxylation of cobyrinyl-*abcdeg*-hexamide *f*-N-threonine (36). Other compounds which are probably steps along the route are the phosphate of cobinamide and P^1-guanosine-5′P^2-cobinamide pyrophosphate. A reversed-labeling experiment (54) indicates that the phosphate group of cobinamide phosphate and

one of the phosphate groups of the pyrophosphate become incorporated (*N. rugosa*) into vitamin B_{12}. The pyrophosphate is considered to react with a suitable nucleoside, *e.g.*, 5,6-dimethyl-1-α-D-ribofuranosylbenziminazole to give vitamin B_{12} [*N. rugosa* and *P. shermanii* (7, 131)]. Other workers have suggested an alternative pathway involving the free base and cobamide-1′-phosphate (*P. shermanii*) (26, 103). Much, however, remains to be done in this area with labeled intermediates.

The *o*-xylene structure is unusual in nature. It was suggested at an early stage that this feature in both the 5,6-dimethylbenziminazole and riboflavin had a single biogenetic source (4), and it is likely that it is derived from diacetyl or its equivalent (42).

The vitamin coenzyme appears to be formed enzymatically by the incorporation of the adenosyl residue of adenosyltriphosphate into aquocobalamin in a reducing system [*cf.* the chemical synthesis (section VIII)]; adenosine, adenine, ribose, and ribose-5-phosphate are not individually involved (56, 246). This incorporation can occur at an earlier stage, and evidence for the occurrence of the 5′-deoxyadenosyl derivatives of cobyric acid (233) and cobinamide (29, 244) has been presented. There is also some direct evidence for the formation of neutral reduced intermediates in the enzymatic synthesis of cobinamide coenzyme (17).

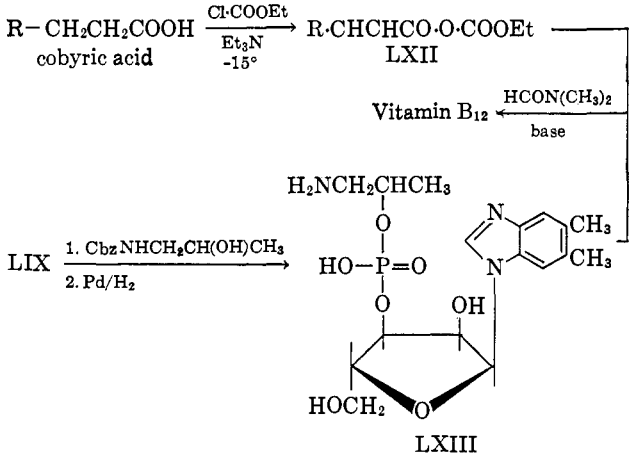
VIII. Synthetic Work

A. Partial Synthesis (132)

The amidation of the mono-, di-, and tricarboxylic acids derived from the acid hydrolysis of vitamin B_{12} regenerates the vitamin (5). The reaction of the intermediate mixed anhydrides with various amines gives substituted amides, some of which, notably the methylamides, are antimetabolites (222).

The isolation of cobyric acid from natural sources has made possible a more extensive partial synthesis (146), although some of the work has been carried out on such a small scale that the intermediates have not been fully characterized. The earlier mixed anhydride preparation when applied to cobyric acid gave LXII. The acid-catalyzed reaction of the 2′,3′-phosphate LIX with D-1-(benzyloxycarbonylamino)propan-2-ol, followed by hydrogenolysis, gave a mixture of the 2′- and 3′-phosphate diesters LXIII which were separated by paper chromatography. LXIII (2 mg.) was treated with LXII (from 2.6 mg. of cobyric acid) in dimethylformamide; crystalline vitamin B_{12} (1–2 mg.) was isolated and was identified on the basis of absorption spectrum, paper chromatography, electrophoresis, and microbiological activity.

When the mixed isomers of LXIII are used in this synthesis a second component is formed. This is presumably the isomer of vitamin B_{12} containing a 2′-



phosphate linkage. It is almost inactive microbiologically, and the N-3–Co bond is more labile than in the vitamin, probably because of steric factors. The preparation of isomeric 5′-phosphate along two routes has also been reported (Fig. 15) (133, 277). α-(5-Methoxybenziminazolyl)cobamide cyanide and α-(2-methyladen-7-yl)cobamide cyanide have been obtained by routes similar to that described for the vitamin (147).

An extensive series of variations has led to partial syntheses of many analogs, including some naturally-occurring ones. The method allows the construction of analogs containing unusual alkanolamine residues which are not otherwise accessible, some of which (*e.g.*, that containing 2-methyl-2-aminopropanol) have antivitamin activity (36, 38, 164). Reaction of the mixed anhydride LXII with D-*g*-1-aminopropane-2-ol gives cobinamide (90% yield) which is also formed (22.5%) by the amidation of cobyric acid using the carbodiimide method and in low yield by the direct amidation of methyl cobyrate (41).

Cobinamide phosphate has been reported from an analogous reaction (39), and has also been obtained directly from cobinamide (277) using the β-cyanoethyl phosphate route (270). In the presence of a carbodiimide it may be converted to a phosphoramide which phosphorylates adenosine-5′-phosphate to give P^1-adenosine-5′P^2-cobinamide pyrophosphate. This compound is obtained more conveniently from cobinamide phosphate and adenosine-5′-phosphate (Fig. 15); a similar route leads to the guanosine analog (37).

The coenzymes have also been obtained by partial syntheses (33, 192, 227) involving reactions similar to those discussed in section V-B-2. Vitamin B_{12s} displaces the tosylate ion from 2′,3′-isopropylidene-5′-tosyladenosine to give a crystalline product. This on removal of the isopropylidene group (mild acid) gives α-(5,6-dimethylbenziminazolyl)cobamide coenzyme, which in this case has been obtained in quantity and condition sufficient for elementary analysis. The 5′-deoxyuridyl, 5′-deoxyguanosyl, and 5′-deoxyinosyl analogs were also synthesized. The latter is

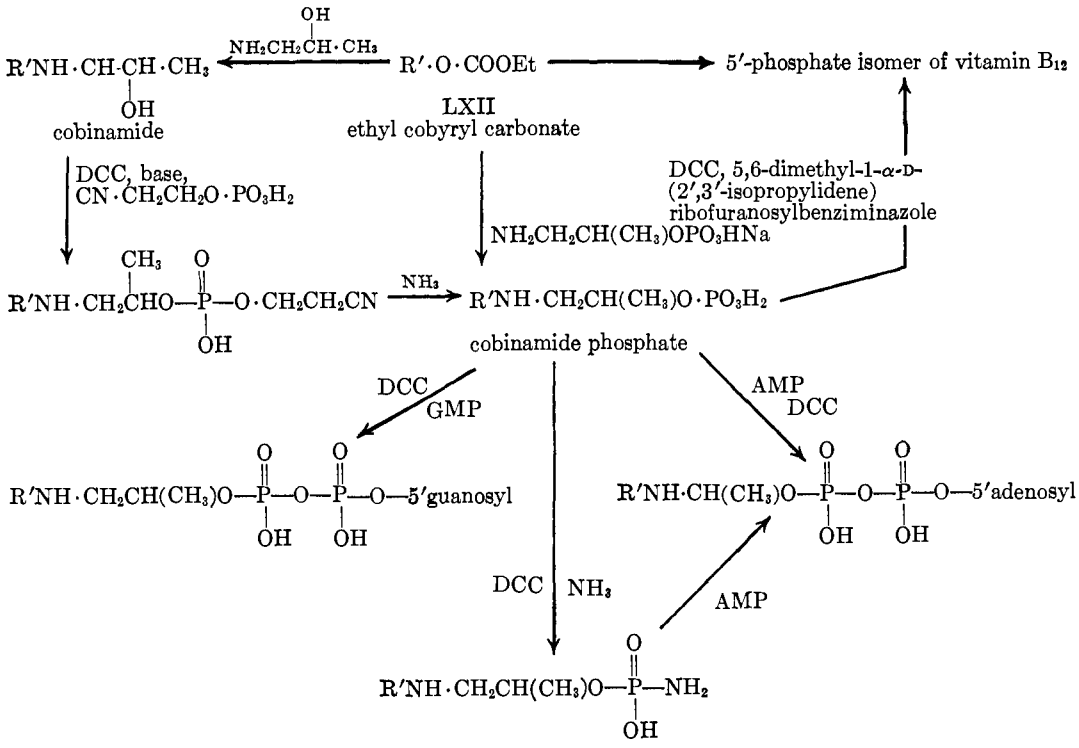


Fig. 15.—Some partial syntheses in the vitamin B_{12} series (R' = cobyryl, AMP = adenosine 5'-phosphate, GMP = guanosine 5'-phosphate, DCC = dicyclohexylcarbodiimide).

identical with the deamination product from vitamin B_{12} coenzyme (236, 238).

B. TOTAL SYNTHESIS

At present the simplest compound from which vitamin B_{12} has been obtained by chemical synthesis is cobyric acid, but cobyrinic acid contains all the essential novel features and may be regarded as the primary synthetic goal. There are nine asymmetric carbon atoms in cobyrinic acid, and the synthesis of this fragment clearly presents a challenge of considerable magnitude. The complexity of the stereochemical problem is emphasized in part by a comparison with the synthetical objectives in the syntheses of, say, chlorophyll (289), strychnine (291), and reserpine (290). These were, respectively, chlorin e_6 trimethyl ester, which has two asymmetric carbon atoms; isostrychnine I, which has four; and reserpine itself, which has six. Moreover, in the case of chlorophyll, for example, a discrete and voluminous body of information was available on the chemistry of pyrroles, dipyrromethanes, and porphyrins; a similar situation does not hold for the reduced pyrroles.

The tackling of the problem at the pyrrole level, thus utilizing the knowledge of pyrrole and porphyrin chemistry, seems unpromising, since it puts off stereochemical control to a later stage, when presumably, it will be more difficult to exercise. Although interest in cobalt porphyrins has been stimulated (191, 232, 279), and, indeed, a cobaltiporphyrin model of the vitamin complete with nucleotide loop and coordinated cyanide has been prepared (197), the pentadehydrocorrin system ("corrole") has not yet been described—although such a structure has been considered for a by-product from the preparation of cobaltous porphyrin (279). The 10-oxa, 10-aza-, and 10-thia- analogs are known however (190). An approach adopted by Todd and his colleagues is based on their discovery (72) of the 1-pyrroline 1-oxides. These are rather reactive compounds (45, 71) and methods for linking them by a meso bridge and by a direct α,α'-linkage have been presented. Furthermore, since the 3,4-positions of these compounds are reduced it is possible to introduce stereochemical features at the outset. Cornforth has outlined an elegant method of achieving certain of the stereochemical requirements using isoxazole intermediates (95), and the assimilation of these into the nitrone route would offer a promising approach to a formidable problem. The elegant development of an alternative route, which aims at a pentamethylcorrin derivative and which employs 2-alkoxy-1-pyrroline intermediates, has been described by Eschenmoser at the recent IUPAC meeting in London.

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