Kirk-Othmer Encyclopedia of Chemical Technology.

VITAMIN B_{12}

Vitamin B_{12} [68-19-9] (1, 2) is the generic name for a closely related group of substances of microbial origin. Although the last of the vitamins to be characterized, its history is a long one, dating from 1824 when Combe (3) proposed the relationship of pernicious anemia, a disease characterized by defective (megoblastic) red blood cell formation, to disorders of the digestive system. Additional study of pernicious anemia, in particular the work of Addison (4), continued for over 100 years before Minot and Murphy (5) reported that a diet containing large quantities of raw liver restored the normal level of red blood cells in patients with pernicious anemia. This clinical breakthrough was based on the findings of Whipple and Robscheit-Robbins (6) that liver was of benefit in regeneration of blood in anemic dogs. For this work, Whipple, Minot, and Murphy were awarded the Nobel Prize in medicine and physiology in 1934.

In 1929, Castle (7) tied the work of Combe and Addison with that of Whipple, Minot, and Murphy by proposing that both an extrinsic factor and an intrinsic factor are involved in the control of pernicious anemia. The extrinsic factor, from food, is vitamin B_{12}. The intrinsic factor is a specific B_{12}-binding protein secreted by the stomach. This protein is required for vitamin B_{12} absorption.

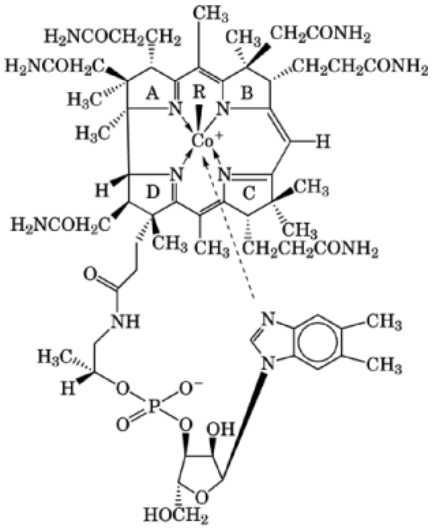
Work for the next 20 years focused on purification of the extrinsic factor from liver. The work was slow and tedious, because fractionation was guided by tests on pernicious anemia patients. The discovery (8) that *Lactobacillus lactis Dorner* requires liver extracts for growth greatly accelerated the isolation process. In 1948, groups at Merck (9) in the United States and Glaxo (10) in England reported isolation of vitamin B_{12} (cyanocobalamin) as a crystalline, red pigment. The clinical efficacy of this material in the treatment of pernicious anemia was rapidly established (11).

Parallel to the activities in the treatment of pernicious anemia were observations in the 1930s that most farm animals had a requirement for an unknown factor beyond the vitamins then known. The lack of this factor became apparent, eg, when chicks or pigs fed a diet with only vegetable protein evidenced slow growth rate and high mortality. It became apparent that the required factor, termed animal protein factor, was present in animal sources such as meat and tissue extracts, milk, whey, and cow manure. Subsequent to its isolation, it was rapidly shown that vitamin B_{12} is the same as animal protein factor.

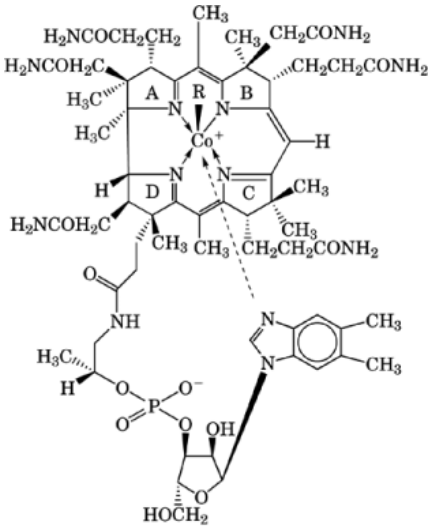
After its separation from liver extracts, vitamin B_{12} was also isolated from cultures of *Streptomyces aureofaciens* (12). All vitamin B_{12} sold commercially is produced by microbial fermentation.

1. Structure

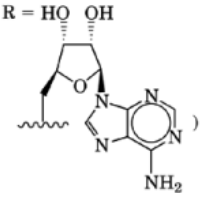
The structure of the first isolated vitamin B_{12}, cyanocobalamin [68-19-9] **(1a)** is known to occur only sporadically, at best, in biological systems. Its isolation was an artifact resulting, probably, from use of charcoal containing cyanide in the purification process.



(**1a**, R = CN)



(**1b**, R = HO OH



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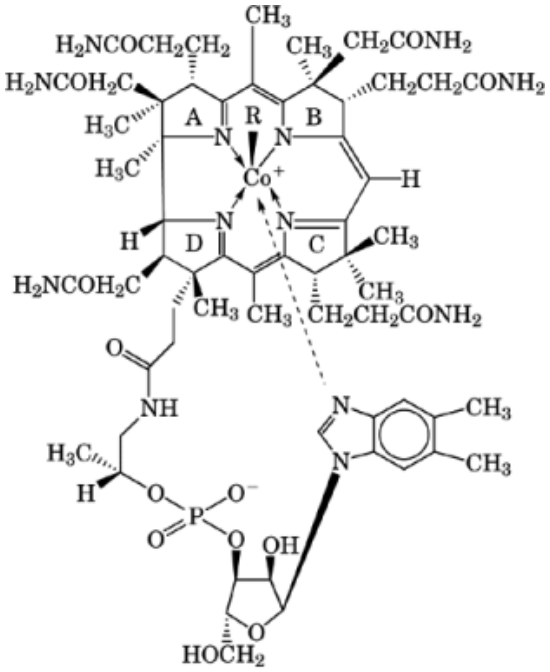
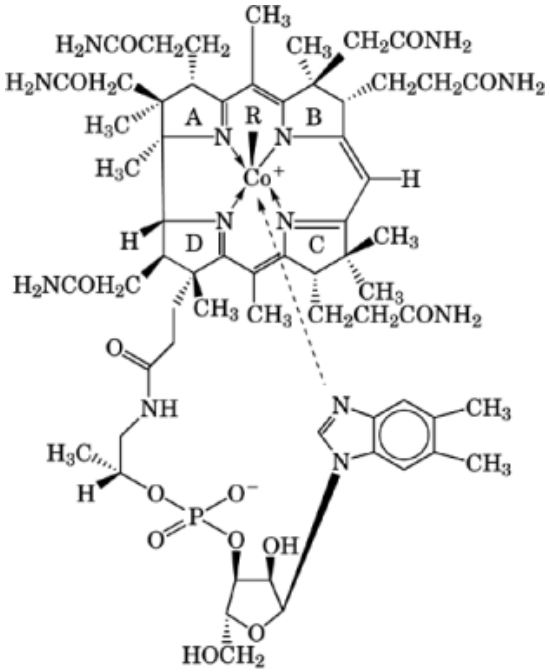
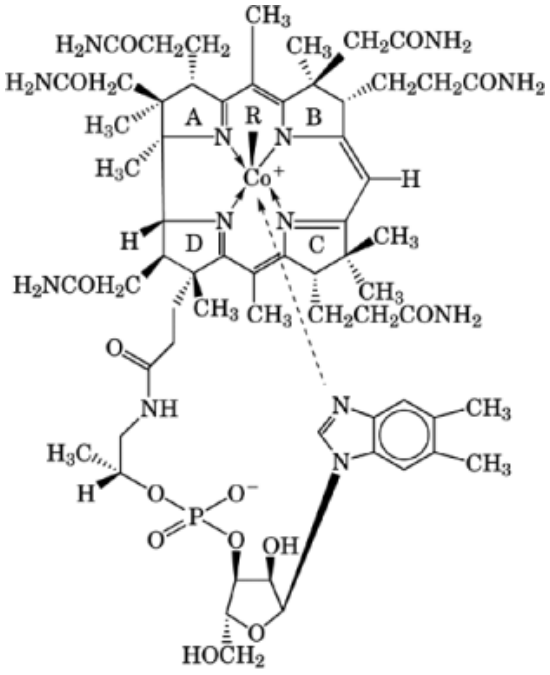


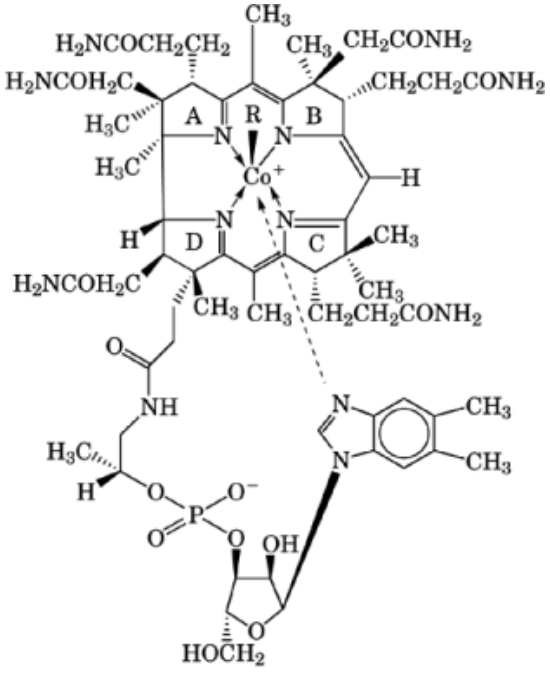
(**1c**, R = CH_3)



(**1d**, R = OH)



(**1e**, R = NO_2)



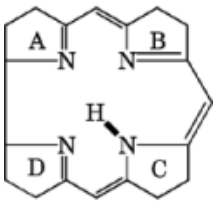
(**1f**, R = OH_2Cl)

It is recognized that there are several important forms of vitamin B_{12}. The active coenzyme forms are adenosylcobalamin [13870-90-1] (coenzyme B_{12} **(1b)**) and methylcobalamin [13422-55-4] **(1c)**. These, along with hydroxocobalamin [13422-51-0] (vitamin B_{12a} **(1d)**), are the forms found in humans and other animals. Other forms of interest are nitrocobalamin (vitamin B_{12c} **(1e)**) and aquacobalamine chloride [13422-52-1] (vitamin B_{12b} **(1f)**). The primary commercial form is cyanocobalamin, due to its ease of isolation and purification as well as its stability.

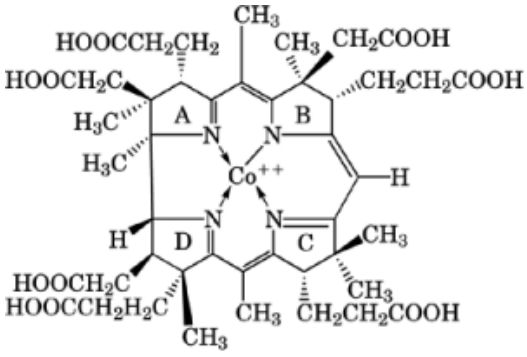
The IUPAC-IUB Commission on Biochemical Nomenclature (13) recommends that the term vitamin B_{12} be used as the generic descriptor for all corrinoids exhibiting qualitatively the biological activity of cyanocobalamin. However, because of its commercial importance, cyanocobalamin is used interchangeably with vitamin B_{12} herein.

Determination of the structure of vitamin B_{12} was a slow, tedious process with the tools available in the 1940s. Despite extensive information gathered by degradation studies, the structures of cyanocobalamin and its coenzyme forms were only established beginning in 1955 by x-ray crystallography (14–16). The value of this work in the study of natural products was recognized by the award to Hodgkin of the Nobel Prize in chemistry in 1964.

Vitamin B_{12} belongs to the class of molecules known as corrins. The core corrin structure **(2)** consists of four linked, partially saturated pyrrole rings. Corrin is a truncated form (no CH_2 between rings A and D) of the more common porphyrin skeleton. The corrin ring in vitamin B_{12} is substituted in a highly regio- and stereospecific manner with eight methyl groups, three acetic acid chains, and four propionic acid chains, as in cobyrinic acid **(3)**. The six conjugated double bonds of the corrin system give octahedral complexes with a number of metals. However, only complexes with cobalt exhibit vitamin B_{12} activity.

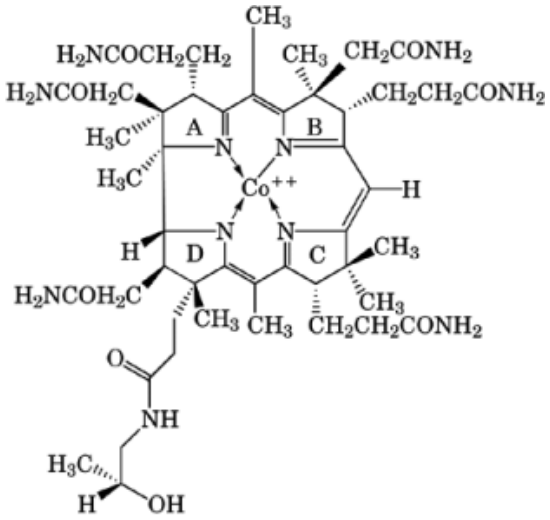


(2)



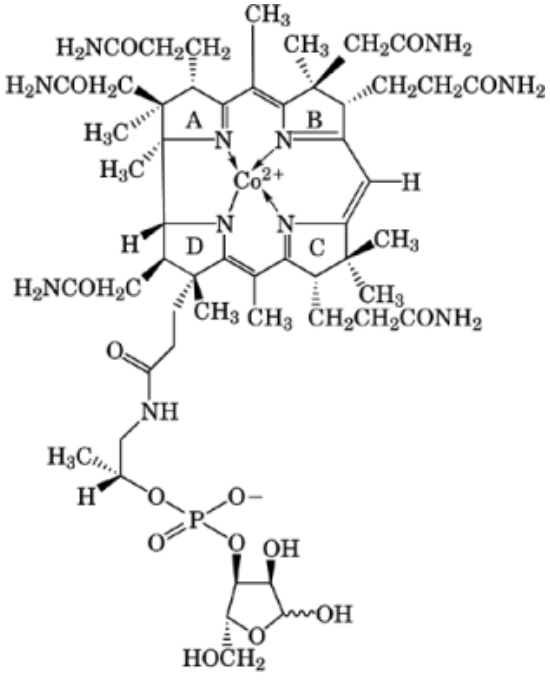
(3)

Cobrynic acid in which the propionic acid is amidated with 1-amino-2-propanol is known as cobinic acid, whereas the compound in which all other acids are primary amides is cobinamide **(4)**.



(4)

Cobinic acid and cobinamide, which are linked to ribose 3-phosphate, are known as cobamic acid and cobamide **(5)**, respectively.



(5)

The cobalt corrin complex is octahedral. The four nitrogens of the corrin ring occupy the four equatorial ligand positions in a virtually planar arrangement. The nucleotide formed from cobamide and

5,6-dimethylbenzimidazole provides a fifth ligand in the alpha, axial position of vitamin B_{12}. The group occupying the sixth, beta-position can vary substantially, as noted in structure **(1)**, and dictates the compound name. Thus, when the ligand is cyanide, the compound name is cyanocobalamin; when methyl, methylcobalamin, etc.

Vitamin B_{12} exists as a neutral complex. The beta-ligand and one nitrogen atom in the corrin ring each contribute a negative charge to Co(III). The nucleotide phosphate contributes the final negative charge. The structure of vitamin B_{12} is highly organized and compact, as dictated by charge neutralization and the ligand sphere of the cobalt atom.

Many analogues of vitamin B_{12} are known. These occur either naturally or are obtained by synthesis, degradation of the vitamin, or directed biosynthesis. Most of these compounds have little or no biological activity in animals. However, many have significant microbiological activity. Among the analogues found are those in which the 5,6-dimethylbenzimidazole is replaced by another (or no) base. These include 2-methyl adenine (Factor A), no base (Factor B), guanine (Factor C), 5-hydroxybenzimidazole (Factor III), 5-methoxybenzimidazole (Factor III_m) and adenine (pseudovitamin B_{12}) (17–20).

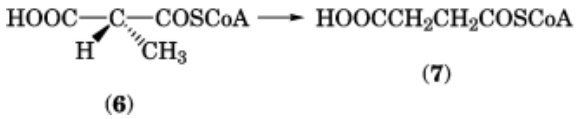
2. Occurrence

For many years, it was thought that the occurrence of vitamin B_{12} was limited to animal tissues and bacteria, with all of the material originating from bacterial sources. More recently, the presence of vitamin B_{12} and/or vitamin B_{12}-like activity at low levels in plants has been recognized (21–24). Nonetheless, the largest portion of the vitamin B_{12} requirement is met by consumption of animal tissues and from microorganisms in the animal's digestive tract. Herbivorous animals satisfy their vitamin B_{12} needs by absorption of material produced by rumenal or intestinal flora. In humans and carnivorous/omnivorous animals, intestinal production of vitamin B_{12} occurs but at an insufficient level. As a result, vitamin B_{12} levels are maintained by consumption of foods rich in vitamin B_{12} such as liver, heart, and kidney. Egg yolk and some fish and shellfish are also good sources. Vitamin B_{12} supplements, both oral and parenteral, are also available. Dietary sources of foodstuffs are provided in Table 1 (25–27).

3. Biochemical Functions

Methylcobalamin and adenosylcobalamin are the two coenzyme forms of vitamin B_{12} in animals and humans. Each is involved in the catalysis of a specific transformation. In humans, it appears that there are only two enzymes requiring vitamin B_{12} as an essential coenzyme although many other, particularly bacterial enzyme systems also require a vitamin B_{12} coenzyme (2, 28).

Adenosylcobalamin (coenzyme B_{12}) is required in a number of rearrangement reactions; that occurring in humans is the methylmalonyl-CoA mutase-mediated conversion of (*R*)-methylmalonyl-CoA **(6)** to succinyl-CoA **(7)** (eq. **1**). The mechanism of this reaction is poorly understood, although probably free radical in nature (29). The reaction is involved in the catabolism of valine and isoleucine. In bacterial systems, adenosylcobalamin drives many 1,2-migrations of the type exemplified by equation **1** (30).



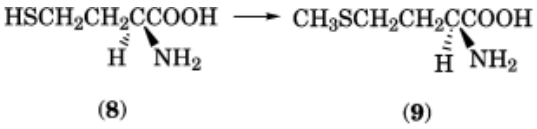
(6) (7) (1)

Methylcobalamin is involved in a critically important physiological transformation, namely the methylation of homocysteine **(8)** to methionine **(9)** (eq. **2**) catalyzed by N^5-methyltetrahydrofolate homocysteine

Table 1. Dietary Sources of Vitamin B_{12}

>50 μg/100 g	5–50 μg/100 g	<5 μg/100 g
liver	liver	beef (lean)
lamb	rabbit	lamb
beef	chicken	pork
calf	kidney	chicken
pork	rabbit	egg (whole)
kidney	beef	cheese
lamb	fish	american
brain	sardines	swiss
beef	salmon	milk (cow)
	herring	fish
	heart	cod
	beef	flounder
	rabbit	haddock
	chicken	sole
	egg yolk	halibut
	clams	swordfish
	oysters	tuna
	crabs	mackerel
		lobster
		scallop
		shrimp
		green vegetables

methyltransferase. The reaction sequence involves transfer of a methyl group first from N^5-methyltetrahydrofolate to cobalamin (yielding methylcobalamin) and thence to homocysteine. Once again, the intimate details of the reaction are not well known (31). Demethylation of tetrahydrofolate to tetrahydrofolic acid is a step in the formation of thymidine phosphate, in turn required for DNA synthesis. In the absence of the enzyme, excess RNA builds up in red blood cells.



(8) (9) (2)

Homocysteine has been identified as an independent risk factor for atherosclerosis (32) and thus metabolic control over homocysteine levels has major health implications.

3.1. Deficiency

Macrocytic anemia, megaloblastic anemia, and neurological symptoms characterize vitamin B_{12} deficiency. Alterations in hematopoiesis occur because of the high requirement for vitamin B_{12} for normal DNA replication necessary to sustain the rapid turnover of the erythrocytes. Abnormal DNA replication secondary to vitamin B_{12} deficiency produces a defect in the nuclear maturational process of committed hematopoietic stem cells. As a result, the erythrocytes are either morphologically abnormal or die during development.

Neurological symptoms result from demyelination of the spinal cord and are potentially irreversible. The symptoms and signs characteristic of a vitamin B_{12} deficiency include paresthesis of the hands and feet, decreased deep-tendon reflexes, unsteadiness, and potential psychiatric problems such as moodiness,

Table 2. The 1989 RDA for Vitamin B_{12}

Group	Age, yr	Vitamin B_{12}, μg
RDA		
infants	0–0.5	0.3
	0.5–1.0	0.5
children	1–3	0.7
	4–6	1.0
	7–10	1.4
males	11 – 51+	2.0
females	11 – 51+	2.0
	pregnant	2.2
	lactating	2.6
U.S. RDI (daily value)		
infants and children <4 yr		3.0
adults and children >4 yr		6.0
pregnant or lactating women		8.0

hallucinations, delusions, and psychosis. Neuropsychiatric disorders sometimes develop independently of the anemia, particularly in elderly patients. Visual loss may develop as a result of optic atrophy.

Clinical manifestation of vitamin B_{12} deficiency is usually a result of absence of the gastric absorptive (intrinsic) factor. Dietary deficiency of vitamin B_{12} is uncommon and may take 20 to 30 years to develop, even in healthy adults who follow a strict vegetarian regimen. An effective enterohepatic recycling of the vitamin plus small amounts from bacterial sources and other contaminants greatly minimizes the risk of a complete dietary deficiency. Individuals who have a defect in vitamin B_{12} absorption, however, may develop a deficiency within three to seven years.

Dietary deficiency in the absence of absorption defects can be effectively reversed with oral supplementation of 1 μm of vitamin B_{12} daily. If deficiency is related to a defect in vitamin absorption, daily doses of 1 μg administered subcutaneously or intramuscularly are effective (33). However, a single intramuscular dose of 100 μg of cobalamin once per month is adequate in patients with chronic gastric or ileal damage. Larger doses are generally rapidly cleared from the plasma into the urine and are not effective unless the patient demonstrates poor vitamin retention.

3.2. Requirement

A daily intake of 1 μg should cover the daily loss of vitamin and maintain an adequate body pool. The RDA (34), however, has been established at 2 μg/day to cover metabolic variation among individuals and to ensure normal serum concentrations and adequate pool sizes (Table 2).

Smaller pool sizes with normal serum B_{12} levels may be maintained with dietary intakes below 1 μg. However, more substantial pool sizes are considered advantageous as protection against the development of pernicious anemia, which may occur in advanced age; achlorhydria becomes more common after age 60, resulting in compromised absorption of vitamin B_{12}.

In general, maternal stores of vitamin B_{12} are considered adequate to meet the demands of pregnancy.

3.3. Safety

No toxicity has been associated with acute or chronic intakes of vitamin B_{12} in doses of 100 and 1 mg, respectively. Vitamin B_{12} absorption is both limited and affected by vitamin status. Therefore, absorption is reduced with improved status, lessening the risk of toxicity.

Cobalamin should be administered parenterally by the intramuscular or subcutaneous route. Isolated cases of anaphylaxis have been reported with intravenous administration.

4. Metabolism

4.1. Absorption

An absorption mechanism capable of handling between 1.5 to 3.0 μg of vitamin B_{12} is responsible for most of the intestinal absorption. This mechanism includes the binding of vitamin B_{12} to a specific transport protein (intrinsic factor) and the presence of specific membrane-bound receptors on the ileal cell surface in the small intestine. In addition, there is a second mechanism for the absorption of vitamin B_{12} by diffusion. This mechanism can provide a physiologically significant source of the vitamin when delivered in pharmacological dosages.

Food vitamin B_{12} appears to bind to a salivary transport protein referred to as the R-protein, R-binder, or haptocorrin. In the stomach, R-protein and the intrinsic factor competitively bind the vitamin. Release from the R-protein occurs in the small intestine by the action of pancreatic proteases, leading to specific binding to the intrinsic factor. The resultant complex is transported to the ileum where it is bound to a cell surface receptor and enters the intestinal cell. The vitamin B_{12} is then freed from the intrinsic factor and bound to transcobalamin II in the enterocyte. The resulting complex enters the portal circulation.

4.2. Transport

Transcobalamin II delivers the absorbed vitamin B_{12} to cells and is the primary plasma vitamin B_{12}-binding transport protein. It is found in plasma, spinal fluid, semen, and extracellular fluid. Many cells, including the bone marrow, reticulocytes, and the placenta, contain surface receptor sites for the transcobalamin II–cobalamin complex.

Other plasma vitamin B_{12} proteins, transcobalamines I and III, appear to have primarily a storage function and only a lesser role in transport.

4.3. Tissue Uptake and Storage

Cell surface receptors take up the transcobalamin II–cobalamin complex, which is internalized into endosomes. The complex is dissociated and the transcobalamin II released. The mechanism by which cobalamin leaves the endosome is uncertain.

The liver is the principal site of vitamin B_{12} storage, containing between 50 and 99% of the total body pool. The average total body pool is estimated to range between 2.0 and 5.0 mg. Higher storage values reported probably reflect noncobalamin analogues as well as cobalamin. The main storage form of vitamin B_{12} appears to be coenzyme B_{12}. The primary circulating form in the plasma appears to be methylcobalamin.

4.4. Metabolism and Mobilization

On entry of vitamin B_{12} into the cell, considerable metabolism of the vitamin takes place. Co(III)cobalamin is reduced to Co(I)cobalamin, which is either methylated to form methylcobalamin or converted to adenosylcobalamin (coenzyme B_{12}). The methylation requires methyl tetrahydrofolate.

Approximately 0.05 to 0.2% of vitamin B_{12} stores are turned over daily, amounting to 0.5–8.0 μg, depending on the body pool size. The half-life of the body pool is estimated to be between 480 and 1360 days with a daily loss of vitamin B_{12} of about 1 μg. Consequently, the daily minimum requirement for vitamin B_{12} is 1 μg. Three micrograms (3.0 μg) vitamin B_{12} are excreted in the bile each day, but an efficient enterohepatic

Table 3. Physical and Chemical Properties of Vitamin B_{12} (Cyanocobalamin)[a]

Property	Characteristic
appearance	crystalline
color	dark red
mol wt	1355.42
empirical formula	$C_{63}H_{88}CoN_{14}O_{14}P$
mp, °C	darkens 210–220; does not melt <300
specific rotation	$\alpha_{656}^{23} = -59 \pm 9°$ (diluted aqueous)
uv absorption	278,361,550 nm (water)
pH (aq)[b]	neutral
solubility %	
H_2O	1.25
alcohol	2.03
acetone	insoluble
ether	insoluble

[a]Properties given for the anhydrous compound.
[b]Vitamin B_{12} exhibits maximum stability between pH 4.5 and 5.0.

circulation salvages the vitamin from the bile and other intestinal secretions. This effective recycling of the vitamin contributes to the long half-life. Absence of the intrinsic factor interrupts the enterohepatic circulation. Vitamin B_{12} is not catabolized by the body and is, therefore, excreted unchanged. About one-half of the vitamin is excreted in the urine and the other half in the bile.

5. Properties

Table 3 lists some of the physical and chemical properties of vitamin B_{12} (1, 35). Crystalline vitamin B_{12} is stable in air and is not affected by moisture. The anhydrous compound, however, is very hygroscopic, and when exposed to moist air may absorb about 12% of water.

Aqueous solutions of vitamin B_{12} at pH 4.0 to 7.0 show no decomposition during extended storage at 25°C. For optimum stability at elevated temperatures, solutions should be adjusted to pH 4.0 to 4.5. Aqueous solutions in this pH range may be autoclaved for 20 min at 120°C without significant decomposition.

5.1. Redox Reactions

Critical to the function of cobalamins as enzyme cofactors is the ability of the cobalt atom to exist in the Co(III) and Co(I) oxidation states. Chemically, Co(II) forms are also known. Each oxidation state has different ligand-accepting abilities. The chemical **(1a, 1d–1f)**and coenzyme **(1b,1c)**forms of cobalamin are trivalent. These compounds are readily reduced to Co(II) and Co(I)cobalamins. The redox potentials for the aquacobalamin to Co(II)cobalamin and Co(II)cobalamin to Co(I)cobalamin couples are -0.04 and -0.85 V, respectively (36). Chemical reduction of aquacobalamin to Co(II)cobalamin is effected by thiols or carbon monoxide. Cobalt(II)cobalamin contains a single unpaired electron in the $3d_{z2}$ orbital of the cobalt ion; one of the two axial positions is unoccupied and thus this material is five-coordinate. Chemically, Co(I)cobalamin is obtained with strong reducing agents, eg, $NaBH_4$ or zinc and NH_4Cl. It is a powerful reducing agent, and reacts rapidly with oxygen and reduces protons to hydrogen. It is, therefore, unstable in aqueous acid solution, less so in neutral or basic aqueous solution. It is frequently used for the preparation of organocobalamins, eg, adenosylcobalamin and methylcobalamin. Co(I)cobalamin contains two electrons in the $3d_{z2}$ orbital. It has been postulated that the coordination number for the cobalt is four and that both axial ligands are vacant (37).

5.2. Exchange of Axial Ligands

Many ligand-exchange reactions involve groups in which the coordination to the metal is through nitrogen (NH_3,N^-_3), oxygen (H_2O,OH^-), sulfur (SH^-,SO^-_{23}), halogen, or carbon (CN^-,CH^-_3). Important reactions involve displacement of the heterocyclic base from the alpha-coordination position by a solvent, usually water. This displacement occurs in acidic solution and results from the protonation of the heterocyclic base. The protonation is associated with a characteristic change in the spectrum. The pK_a for the base-on/base-off equilibrium depends on the nature of the beta-ligand. Displacement of H_2O, adenosyl, or methyl from cobalt by cyanide has also been studied. In the presence of cyanide ion, aquacobalamin and adenosylcobalamin are converted to cyanocobalamin. In contrast, methylcobalamin and other alkyl corrinoids are stable in the presence of 0.1 *M* cyanide in the dark (37, 38). The equilibrium, aquacobalamin $\rightleftharpoons$ hydroxocobalamin $+H^+$ ($pK_a = 6.9 - 7.8$), is not a pure ligand exchange but only a ligand modification. The cobalt-bound water is acidic and reversibly loses a proton at neutral pH.

5.3. Chemical Reactions

A wealth of information exists on the chemistry of vitamin B_{12} (1, 39, 40). Much of this chemistry was established during the studies leading to structure determination, partial synthesis, and the total synthesis of vitamin B_{12}. Vitamin B_{12} is slowly decomposed by ultraviolet or strong visible light. By controlled irradiation with visible light, cyanide may be selectively liberated from cyanocobalamin without destruction of the cobalamin structure, but long exposure to light results in complete inactivation of the vitamin. Vitamin B_{12} is also inactivated by treatment with strong acids or bases.

One development involves the use of vitamin B_{12} to catalyze chemical, in addition to biochemical processes. Vitamin B_{12} derivatives and B_{12} model compounds (41, 42) catalyze the electrochemical reduction of alkyl halides and formation of C–C bonds (43, 44), as well as the zinc–acetic acid-promoted reduction of nitriles (45), alpha, beta-unsaturated nitriles (46), alpha, beta-unsaturated carbonyl derivatives and esters (47, 48), and olefins (49). It is assumed that these reactions proceed through intermediates containing a Co–C bond which is then reductively cleaved.

6. Analysis

6.1. Specifications

Cyanocobalamin is the commercial form of vitamin B_{12}. It is sold under the following tradenames (35):

Anacobin	Ducobee
Antipernicin	Duodecibin
Bedoce	Embiol
Bedodeka	Emociclina
Bedoz	Eritrone
Behepan	Erycytol
Berubi	Erythrotin
Berubigen	Euhaemon
Betalin-12	Fresmin
Betolvex	Hemo-B-Doze
Bevatine-12	Hemomin
Bevidox	Hepagon
Bexii	Hepavis
Bexil	Hepcovite
Biocobalamine	Hydroxamin
Biocres	Hydroxobase
Bitevan	Macrabin
B-Telve	Megabion (Indian)
B-Twelv	Megalovel
Byladoce	Milbedoce
Claretin-12	Millevit
Cobalin	Nagravon
Cobamin	Normocytin
Cobamine	Peraemon
Cobione	Pernaevit
Covit	Pernipur
Crystamin	Plecyamin
Cycobemin	Poyamin
Cycolamin	Redamina
Cykobeminet	Redisol
Cytacon	Rhodacryst
Cytamen	Rubesol
Cytobion	Rubivitan
Distivit (B_{12} peptide)	Rubramin
Dobetin	Rubripca
Docemine	Rubrocitol
Docibin	Sytobex
Docigram	Vitalt
Docivit	Vibisone
Dodecabee	Virubra
Dodecavite	Vitarubin
Dodex	Vita-Rubra
	Vitral

Specifications are found in the *Codex* for food use (50) and in the USP (51) for pharmaceutical use.

6.2. Analytical Methodology

Vitamin B_{12} can be determined by microbiological, radioisotope dilution, spectrophotometric, chemical, or biological methods employing animals (52–54). Microbiological assays involve the extraction and stabilization of vitamin B_{12} from the food, feed, or pharmaceutical matrix prior to assay. The official method of the AOAC (55) accomplishes this by autoclaving samples in a phosphate–citric acid buffer containing metabisulfite, whereas the British Analytical Methods Committee (56) recommends extraction with aqueous cyanide solution at pH

4.6–5.0 in a boiling water bath. The AOAC extraction procedure was found to yield somewhat higher results than the British Analytical Methods Committee procedure (57).

The AOAC assay is based on the graded growth of the bacterium *Lactobacillus leichmannii ATCC 7830* in a medium containing all required growth factors but vitamin B_{12}. Results are obtained by determining the transmittance of the sample and standard tubes after 16–24 h incubation at 30–40°C or by titrating the acid produced after 72 h incubation. The British Analytical Methods Committee assay employs the protozoan *Ochromonas malhamensis ATCC 11532* in an assay based on the graded growth of the protozoan. The assay incubation period varies from 3–6 days and degree of growth is measured turbidimetrically. Although the *L. leichmannii* assay is claimed to be less specific than the *O. malhamensis* assay it has several advantages over the latter. The AOAC procedure has a shorter incubation period and the assay is set up in test tubes, whereas the *O. malhamensis* assay is set up in 25-mL micro-Fernbach flasks, the assay medium is less complex, and samples whose turbidity or color would interfere in a turbidimetric assay can be analyzed by the titrimetric assay. Derivatives of deoxynucleic acid that stimulate the growth of *L. leichmannii* in the absence of vitamin B_{12} can be measured after destroying the vitamin B_{12} by autoclaving the sample at pH 11–12 and determining the residual activity. The bacteria *Lactobacillus lactis Dorner ATCC 8000* and *Escherichia coli ATCC 10799* and *14169* as well as the protozoan *Euglena gracilis ATCC 12716* have been used also for the determination of vitamin B_{12}.

Radioisotope dilution assays are based on the principle of competition between radioactive labeled (^{57}Co) vitamin B_{12} and cobalamins extracted from matrices for binding sites on the intrinsic factor (a glycoprotein). Binding is in proportion to the concentration of the radioactive and nonradioactive B_{12} with the concentration of intrinsic factor as the limiting factor. Free cobalamins are separated from those bound on the intrinsic factor by absorption onto treated charcoal and the amount of free-labeled vitamin B_{12} is determined. Vitamin B_{12} content of the sample is determined from a standard curve. Results obtained by a radioisotopic dilution method are very similar to those obtained by the AOAC microbiological assay (58).

Spectrophotometric determination at 550 nm is relatively insensitive and is useful for the determination of vitamin B_{12} in high potency products such as premixes. Thin-layer chromatography and open-column chromatography have been applied to both the direct assay of cobalamins and to the fractionation and removal of interfering substances from sample extracts prior to microbiological or radioassay. Atomic absorption spectrophotometry of cobalt has been proposed for the determination of vitamin B_{12} in dry feeds. Chemical methods based on the estimation of cyanide or the presence of 5,6-dimethylbenzimidazole in the vitamin B_{12} molecule have not been widely used.

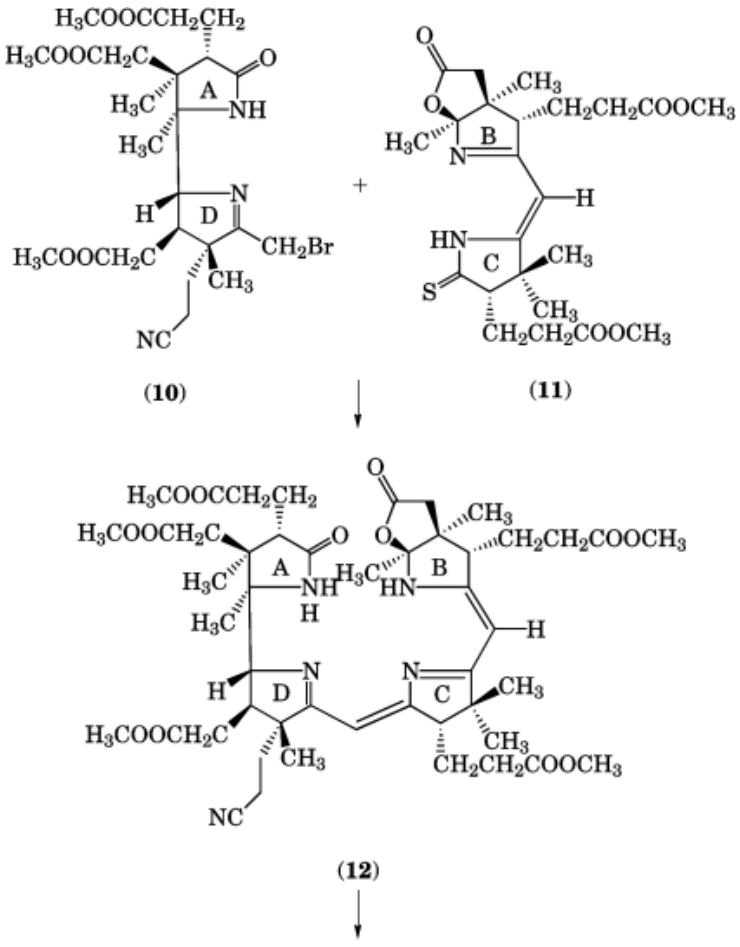
Various aspects of the chromatography of vitamin B_{12} and related corrinoids have been reviewed (59). A high performance liquid chromatographic (hplc) method is reported to require a sample containing 20–100 μg cyanocobalamin and is suitable for premixes, raw material, and pharmaceutical products (60).

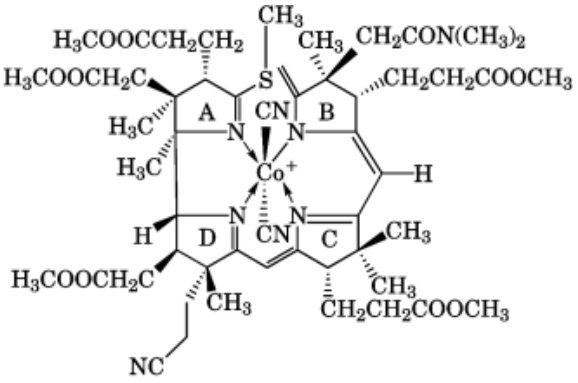
Bioassays are based on the growth response of vitamin-depleted rats or chicks to graded amounts of vitamin B_{12} added in the diet. These assays are not specific for vitamin B_{12} because factors, other than vitamin B_{12} present in biological materials, produce a growth response. Because coenzyme B_{12}, a primary form of natural vitamin B_{12}, is light sensitive, assays should be carried out in subdued light.

7. Synthesis

The achievements in total synthesis of organic compounds in recent years are perhaps nowhere better illustrated than in the synthesis of vitamin B_{12} by the groups of Woodward (61–63) and Eschenmoser (64–69) in a collaborative effort (70, 71). The work has been reviewed (1, 72, 73). The real value of the synthesis lies in the synthetic methodology developed in the course of the work. During the synthesis of the A–D fragment, the Woodward-Hoffmann rules for the conservation of orbital symmetry (74) were developed. For this work, Hoffmann shared the Nobel Prize in chemistry in 1981 with Fukui. Woodward was awarded the Nobel Prize in

chemistry in 1965 for the synthesis and structure elucidation of natural products, including the work leading up to the synthesis of vitamin B_{12}. Thus, the history of vitamin B_{12} is intimately connected with the awarding of four Nobel Prizes to date.





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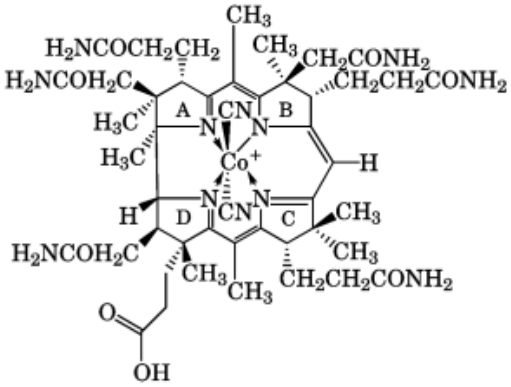
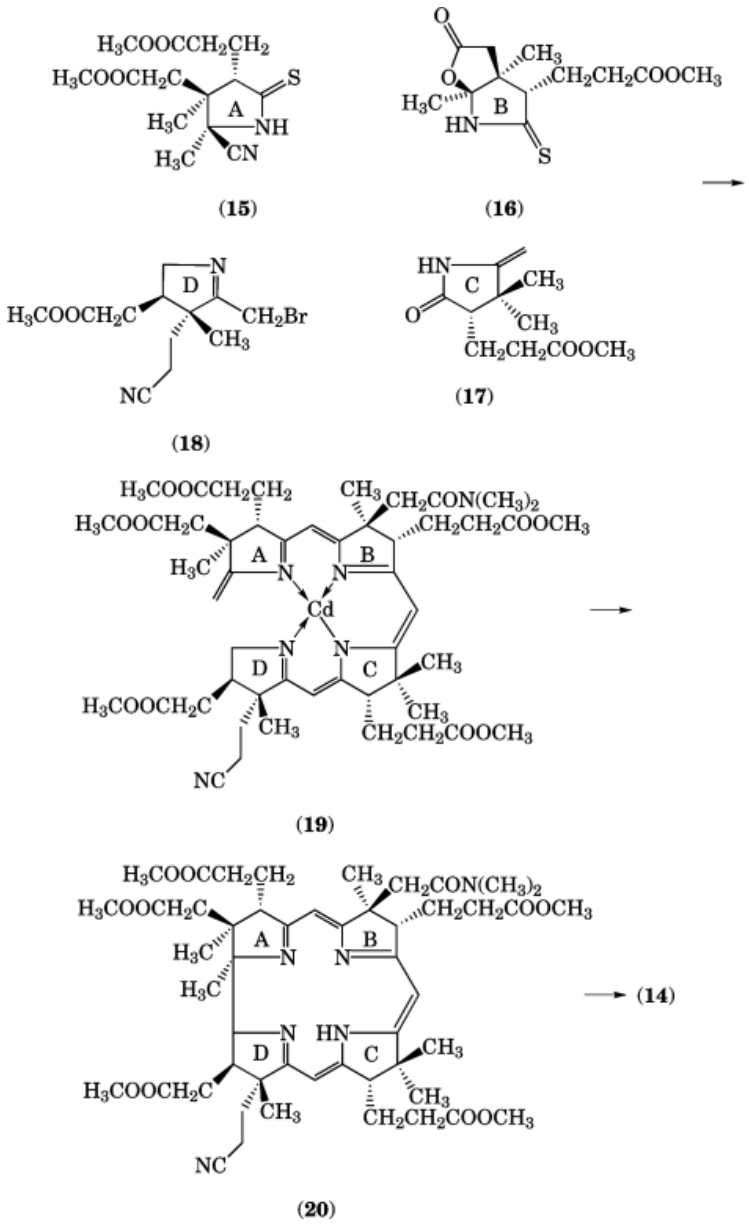


(14)

The core of the first synthesis of vitamin B_{12} involved condensation of the A–D ring fragment **(10)** with the B–C fragment **(11)**. The former compound was obtained by the Harvard group in ca 35 steps, including a classical resolution, from 3-methoxyaniline and camphor.

The synthesis of the second fragment **(11)** by the group at the ETH in Zurich occurred in 18 steps from camphorquinone (ring C) and *trans*-3-methyl-4-oxopentenoic acid (ring D). Base-catalyzed alkylation of **(11)** with **(10)** yielded a thioiminoether that underwent sulfide contraction upon treatment with acid to give the tetrapyrrole **(12)**. Manipulation of substituents and introduction of the cobalt atom gave complex **(13)**. The stereo-organizing template effect of the complex allowed base-catalyzed cyclization to complete the corrin ring system. Functional group exchange and addition of the final methyl group then yielded cobyric acid **(14)**. This material had previously been converted to vitamin B_{12} (75) and thus its obtention by total synthesis completed the synthesis.



A second synthesis of cobyric acid **(14)** involves photochemical ring closure of an A–D secocorrinoid. Thus, the Diels-Alder reaction between butadiene and *trans*-3-methyl-4-oxopentenoic acid was used as starting point for all four ring A–D synthons **(15–18)**. These were combined in the order B + C ⟶ BC + D ⟶ BCD + A ⟶ ABCD. The resultant cadmium complex **(19)** was photocyclized in buffered acetic acid to give the metal-free corrinoid **(20)**. A number of steps were involved in converting this material to cobyric acid **(14)**.

The total syntheses have yielded cobyric acid and thence cyanocobalamin. Routes to other cobalamins, eg, methylcobalamin and adenosylcobalamin, are known (76–79). One approach to such compounds involves

the oxidative addition of the appropriate alkyl halide (eg, CH_3I to give methylcobalamin) or tosylate (eg, 5′-*p*-tosyladenosine to yield adenosylcobalamine) to cobalt(I)alamine.

The complexity of the vitamin B_{12} molecule makes it extremely unlikely that total synthesis will ever be employed for preparation of commercial quantities.

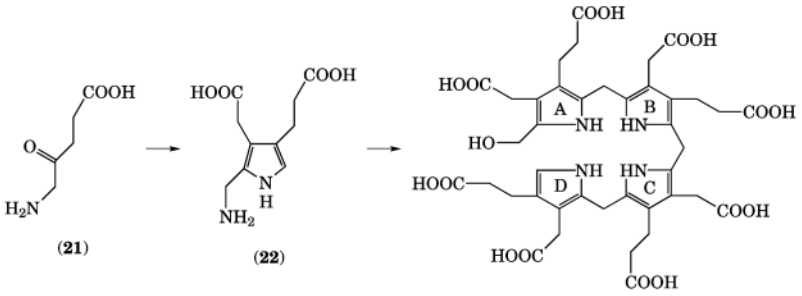
8. Biosynthesis

The study of the biosynthesis of vitamin B_{12} is a saga whose resolution, due primarily to Battersby (80–83) and Scott (84, 85), required an effort on the same magnitude as the total synthesis. It was only when recent molecular biology tools became available to complement enzymology, isotopic labeling, chemical synthesis, and spectroscopy that solution of this problem became possible.

The biosynthesis of vitamin B_{12} in the species *Pseudomonas dentrificans* has received the most attention due to its importance as a commercial source of the vitamin. The 22 genes involved have been identified and the sequence of the resulting proteins described (83). The sequence is initiated from 5-aminolevulinic acid **(21)** which is biosynthesized from succinyl-CoA **(7)** and glycine. Condensation of two molecules of 5-aminolevulinic acid yields porphobilinogen **(22)**, which is tetramerized as a linear, head-to-tail arrangement to give hydroxymethylbilane **(23)**. Ring closure and rearrangement (formally on exchange of acetate and propionate substituents in ring C) gives uroporphyrinogen III (uro'gen III) **(24)**. Uro'gen III is a key biosynthetic branch point, leading not only to vitamin B_{12} but also to chlorophyll and heme.

The sequence to vitamin B_{12} proceeds by the introduction of the eight methyl groups, punctuated by ring contraction to the corrin, an oxidation and reduction, and a methyl migration. Thus, the first three methylations lead stereospecifically to precorrin-1 **(25)** and thence by precorrin-2 **(26)** to precorrin-3A (precorrin-3) **(27)**. Oxidation, ring contraction to the corrin, and introduction of the fourth methyl group give precorrin-4 **(28)**. Further methylation leads to precorrin-5 **(29)**. Loss of the acetyl group created by ring contraction as acetate occurs with methylation to give precorrin-6A (precorrin-6x) **(30)**, which is reduced to precorrin 6B (precorrin-6y) **(31)**. Decarboxylation of the ring C acetic acid subsequent to addition of the last two methyl groups (precorrin-8x) **(32)** and methyl migration gives hydrogenobyrinic acid **(33)**.

Introduction of the cobalt atom into the corrin ring is preceeded by conversion of hydrogenobyrinic acid to the diamide **(34)**. The resultant cobalt(II) complex **(35)** is reduced to the cobalt(I) complex **(36)** prior to adenosylation to adenosylcobyrinic acid *a,c*-diamide **(37)**. Four of the six remaining carboxylic acids are converted to primary amides (adenosylcobyric acid) **(38)** and the other amidated with (*R*)-1-amino-2-propanol to provide adenosylcobinamide **(39)**. Completion of the nucleotide loop involves conversion to the monophosphate followed by reaction with guanosyl triphosphate to give diphosphate **(40)**. Reaction with α-ribazole 5′-phosphate, derived biosynthetically in several steps from riboflavin, and dephosphorylation completes the synthesis.



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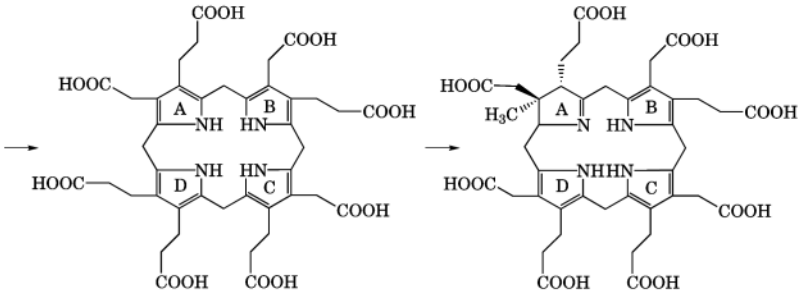

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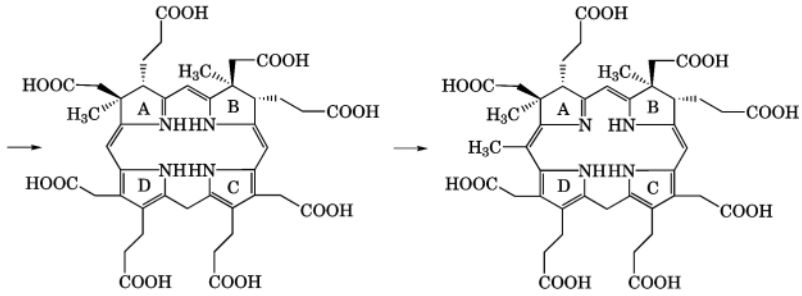

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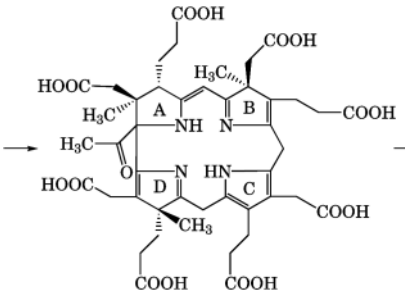

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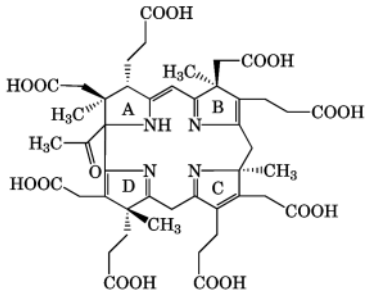

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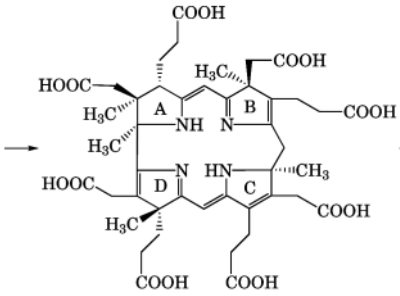

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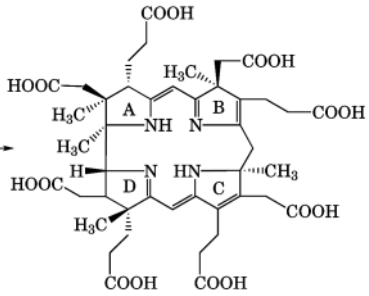

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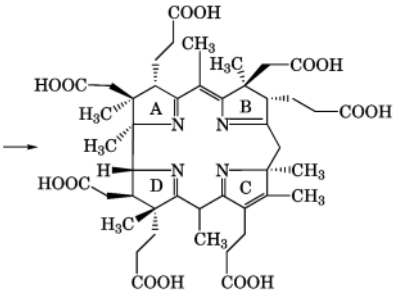

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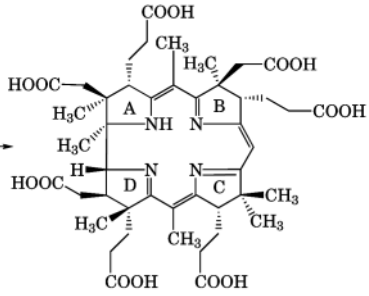

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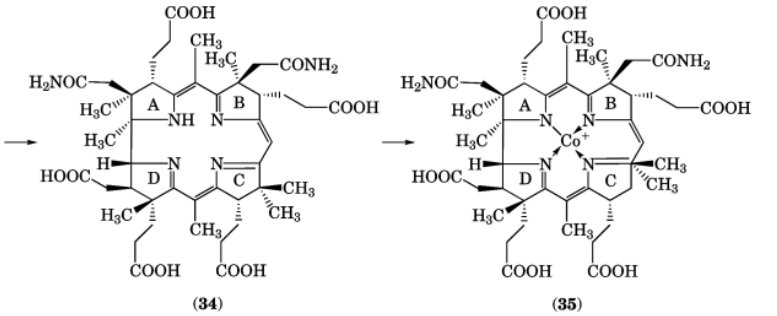

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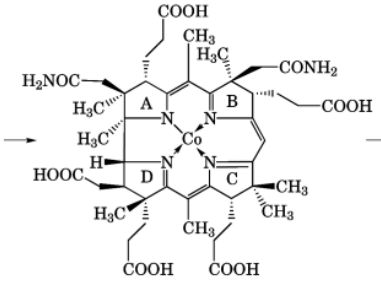

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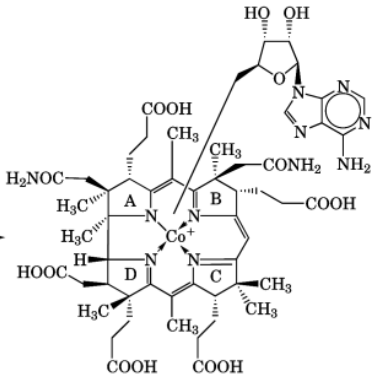

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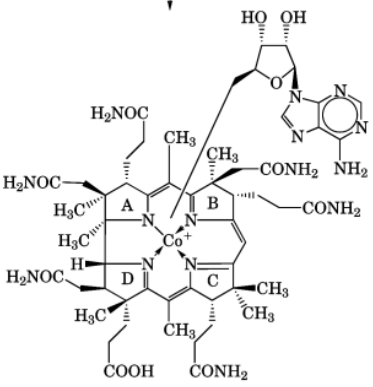

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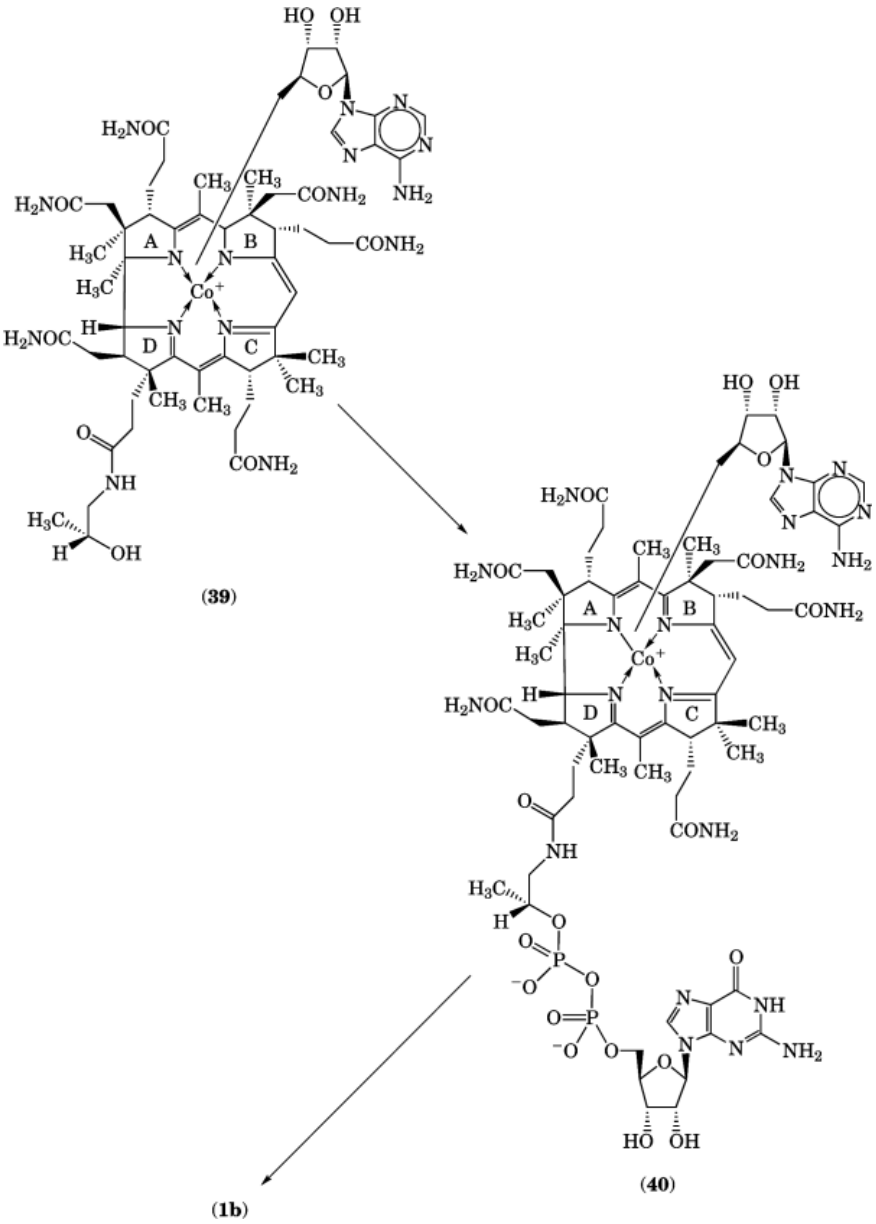

The biosynthetic sequence for other aerobic bacteria appears, where known, to be similar to that in *Pseudomonas dentrificans* although the genes involved, and thus the enzymes, exhibit differences.

In anaerobic (or more correctly, almost anaerobic or microaerophilic) bacteria such as *Propionibacterium shermanii*, a fundamental difference occurs in that the cobalt atom is introduced at a much earlier stage, possibly to precorrin-2 or precorrin-3A.

9. Manufacture

As noted above, all vitamin B_{12} is produced by microbial fermentation. A partial list of microorganisms that synthesize vitamin B_{12} under appropriate conditions follows. Most strains, in their wild state, produce less than 10 mg/L vitamin B_{12}, although a few approach 40 mg/L. The organisms are both aerobes and anaerobes. The carbon requirements in the fermentations are satisfied from sources as wide ranging as hydrocarbons, methanol, and glucose.

Arthrobacter hyalinus	*Nocardia rugosa*
Bacillus megaterium	*Propionibacterium arabinosum*
Butyribacterium rettgeri	*Propionibacterium freudenreichii*
Clostridium sticklandii	*Propionibacterium pentosaceum*
Clostridium tetanomorphum	*Propionibacterium peterssoni*
Clostridium thermoaceticum	*Propionibacterium shermanii*
Corynebacterium and Rhodopseudomonas	*Propionibacterium technicum*
	Propionibacterium vannielli
Crithidia fasciculata	*Protaminobacter ruber*
Methanobacterium arbophilicum	*Pseudomonas denitrificans*
Methanobacterium formicicum	*Rhizobium meliloti*
Methanobacterium ruminantium	*Rhodopseudomonas capsulata*
Methanobacterium thermoautotrophicum	*Rhodopseudomonas spheroides*
	Strigomonas oncopelti
Methanosarcina barkeri	*Streptomyces aureofaciens*
Micromonospora purpurea	Streptomyces griseus
Nocardia gardneri	*Streptomyces olivaceus*

9.1. Commercial Production

Vitamin B_{12}, as cyanocobalamin, is produced by several companies. The market is dominated, however, by two French firms, Rhône-Poulenc and, to a lesser extent, Roussel-Uclaf. Smaller amounts are produced in Japan by Nippon Petrochemical, in Hungary by Medimpex-Richter, and by minor producers in several other countries. Earlier manufacturers, particularly Merck (U.S.) and Glaxo (U.K.), have exited the market. Although estimates vary, it appears that ca 10,000 kg/yr of vitamin B_{12} is produced (1).

The process employed by Rhône-Poulenc for production of vitamin B_{12} has not been revealed. However, from a variety of sources (83, 86) it can be inferred that a *Pseudomonas dentrificans* producing over 200 mg/L is employed. The high production is the result of classical mutation as well as (possibly) genetic engineering.

A fermentation such as that of *Pseudomonas dentrificans* typically requires 3–6 days. A submerged culture is employed with glucose, cornsteep liquor and/or yeast extract, and a cobalt source (nitrate or chloride). Other minerals may be required for optimal growth. pH control at 6–7 is usually required and is achieved by ammonium or calcium salts. Under most conditions, adequate 5,6-dimethylbenzimidazole is produced in the fermentation. However, in some circumstances, supplementation may be required.

The fermentation product, which is primarily adenosylcobalamin, is retained to the largest extent within the cell. Centrifugation of the broth yields a sludge which, when dispersed in a minimum of water, water–alcohol, or water–acetone and heated, releases the vitamin into the solution. Addition of cyanide converts the cobalamins into cyanocobalamin which is then extracted from the filtered solution. Many procedures have been reported for this step, including adsorption on charcoal (87), bentonite (88), ion-exchange resins (89–91), or aluminum oxide (92). For elution, water, water–alcohol, organic bases, or hydrochloric acid are used. Chromatography on aluminum oxide and crystallization from methanol–acetone or water–acetone complete the process (93, 94).

9.2. Market Forms

Vitamin B_{12} is sold almost exclusively as cyanocobalamin. Approximately one-third of the material is for the human pharmaceutical market whereas two-thirds is used in the animal feed market, primarily for poultry and swine (see Feeds and feed additives). Modest growth in both markets has occurred in the period 1980–1995 and this trend is expected to continue.

In the human market, oral and parenteral dosage forms are prepared from the crystal. However, because of the extremely high potency, more dilute (0.1–10%) forms are available. These include dilutions with mannitol, triturations on dicalcium phosphate or resins, and spray-dried forms. Prices for these forms are driven by that of the crystal, which in early 1996 was ca $9.50/gram (95). Prices for the vitamin have risen during the first half of the 1990s. However, little growth in price beyond inflation is anticipated.

For animal feed use, vitamin B_{12} is usually provided in a diluted form on a carrier such as calcium carbonate and/or rice hulls. An earlier practice of using a spray-dried fermentation biomass in this application appears to be no longer used.

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JOHN W. SCOTT
Hoffmann-La Roche Inc.

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