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Vitamin B_{12}

Biochemical studies elucidate the role of this complex molecule in diverse metabolic processes.

Thressa C. Stadtman

In 1948 the isolation of crystalline vitamin B_{12} was announced simultaneously by research teams working at two of the world's large pharmaceutical concerns, Merck in the United States and Glaxo in England (*1*). Elucidation of the complete structure of this red, cobalt-containing substance culminated seven more years of intensive work which included the brilliant x-ray analysis of the crystalline vitamin by Hodgkin and associates (*2*), as well as the efforts of many others, on the chemical characterization and biological assay of numerous fragments of the complicated molecule. Among the many excellent accounts of this phase of investigations on the chemistry and nutritional aspects of the vitamin B_{12} class of compounds is the series of monographs entitled *Vitamin* B_{12} by E. Lester Smith, in particular the third revision (*3*).

The next dramatic development in vitamin B_{12} research, from the standpoint of the biologist and chemist alike, was the discovery by Barker and coworkers (*4*, *5*) of the biologically active forms (coenzyme forms) of the B_{12} vitamins. Discovery of the coenzyme derivatives was an outgrowth of Barker's effort to elucidate the mechanism by which a little known anaerobic bacterium, *Clostridium tetanomorphum*, was able to ferment glutamate. He demonstrated that the first step in this fermentation involved cleavage of the α,β-carbon-carbon bond of glutamate and rearrangement of the carbon skeleton to form the branched chain isomer β-methylaspartic acid. This led ultimately to the discovery that the isomerization reaction is catalyzed by a specific mutase and that a light sensitive derivative of vitamin B_{12}, coenzyme B_{12}, is an obligatory cocatalyst in the reaction. The circumstances that led to the discovery of B_{12} coenzyme thus illustrate how a biochemical problem, initiated from one standpoint, may take an unexpected direction of even more general and perhaps greater significance.

Although the coenzyme derivatives are, in fact, the more abundant naturally occurring forms of B_{12} in most organisms, their existence was overlooked in the earlier investigations because of the rapidity with which they are decomposed by visible light. Treatment with either acid or cyanide ion also increases their rate of decomposition; both were used in most of the earlier procedures devised for the isolation of vitamin B_{12} from natural sources. In particular, cyanide was widely used because the cyano derivative of the vitamins proved to be much more stable (*6*).

What particularly excited the chemist as the structure of the light-labile B_{12} coenzymes (Fig. 1) was unraveled was the finding that these substances contain a deoxyadenosine moiety covalently linked, through the 5′-carbon atom, to the cobalt in the corrin ring of the vitamin (*7*). This finding represented the first known naturally occurring substance containing carbon covalently bonded to cobalt; and moreover, the existence of a stable alkylcobalt compound of any kind was demonstrated for the first time.

It is not within the scope of the following discussion to consider in detail the chemistry of the vitamin B_{12}

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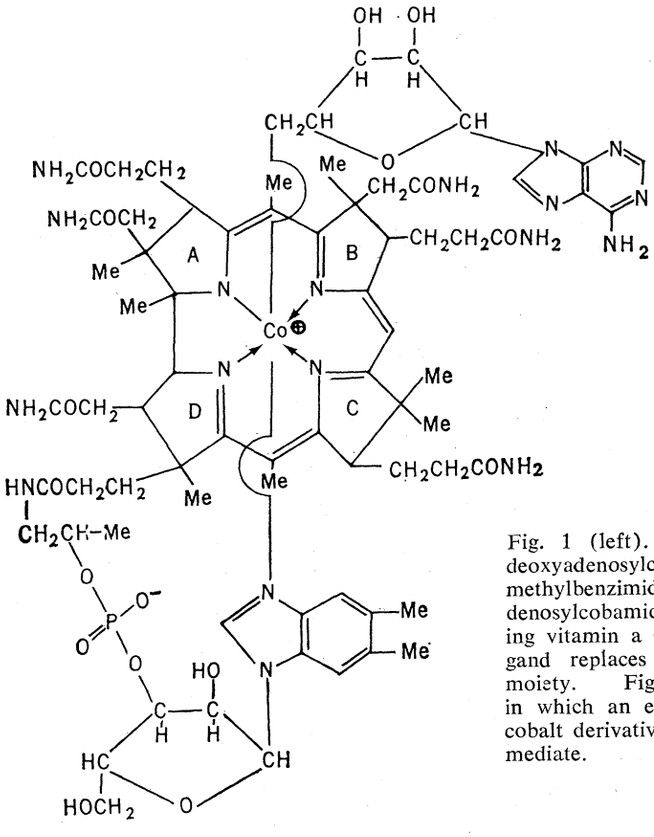


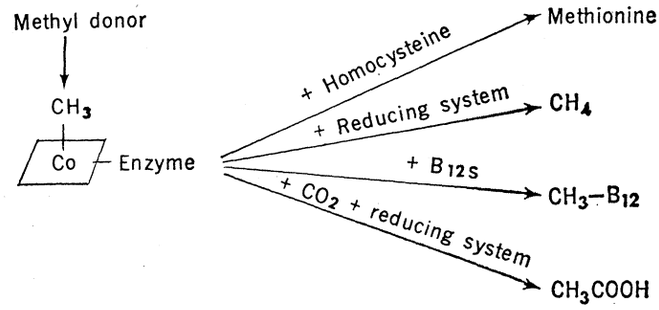


Fig. 1 (left). B_{12} coenzyme or 5′-deoxyadenosylcobalamin or α-(5,6-dimethylbenzimidazolyl)-Co-5′-deoxyadenosylcobamide. In the corresponding vitamin a CN^-, OH^-, or H_2O ligand replaces the 5′-deoxyadenosyl moiety. Fig. 2 (above). Reactions in which an enzyme bound methylcobalt derivative is a presumed intermediate.

class of compounds and their organocobalt derivatives (coenzyme forms of B_{12} compounds). In recent years, numerous analogs of the coenzymes have been prepared and partially characterized by the organic chemists; a great many cobalt-containing model compounds that mimic the vitamins in some of their properties have been synthesized and studied, and substantial progress toward the total synthesis of the vitamin B_{12} molecule itself has been achieved (*8*). Bonnett (*9*), Bernhauer *et al.* (*10*), Wagner (*11*), Schrauzer (*12*), and Hill *et al.* (*13*) have written detailed and comprehensive reviews of the chemistry of this group of complex compounds, insofar as it is now understood.

With regard to the medical aspects of vitamin B_{12} metabolism, it has been known for a long time that in humans certain types of pernicious anemias can be cured by inclusion of relatively high amounts of vitamin B_{12} in the diet. Although the normal intestinal flora synthesize appreciable amounts of vitamin B_{12}, parenteral supplements may also be necessary, particularly if the ability of certain individuals to absorb vitamin B_{12} is limited. The chemical nature of the lesion which is expressed clinically as pernicious anemia is unknown. In some cases of B_{12} deficiency, severe neurological symptoms develop. As each new B_{12}-dependent type of reaction is discovered, attempts are made to see whether a defect in this type of process can explain the clinical symptoms observed. At present there is no definite evidence that any of the known reactions is at fault.

Since the discovery of the coenzyme forms of the B_{12} class of compounds, there has been a rapid advance both in the detection of new types of chemical reactions catalyzed by B_{12} coenzymes and also in elucidation of the mechanism of action [see "Symposium on B_{12} coenzymes" (*14*) and reviews by Barker (*15*) and Hogenkamp (*16*)]. In this article some of the more recent aspects of the biochemistry of the B_{12} vitamins and coenzymes are emphasized.

Reactions Dependent on Vitamin B_{12} or on B_{12} Coenzyme

The specific biochemical reactions in which some form of a B_{12} compound participates as a catalyst comprise two groups; (i) those that require the derivatives commonly designated cobamide coenzymes or B_{12} coenzymes that contain a 5′-deoxyadenosine moiety covalently bonded to the cobalt atom and (ii) those that utilize a B_{12} compound in its vitamin form (for example, a form lacking a ligand covalently bound to the cobalt atom). This subdivision undoubtedly is an operational rather than a definitive one in view of the fact that the protein-bound cobamide cofactors required for the reactions of the second group (ii) may, in their normal active forms in the cell, also exist chiefly as alkyl cobalt derivatives. As will be discussed later, if this is so, the fundamental differences between the two groups would be the nature of the alkyl group covalently bonded to the cobalt atom of the particular B_{12} catalyst and the type of exchange process between the alkyl group and the substrate during the catalytic reaction. The entire alkyl group might exchange with the substrate in some instances and only certain of its hydrogens in others.

Enzyme systems containing a vitamin B_{12} protein component. Three overall reactions are known that require a protein-bound B_{12} vitamin as an essential catalyst (Table 1). One of these, and the one studied for many years by a number of investigators in several different laboratories, involves the transfer of a methyl group from N^5-methyltetrahydrofolate to the amino acid homocysteine to form methionine, an essential building block of a great many proteins. In certain strains of *Escherichia coli* and in the livers of various animals (pig, sheep, chicken) it has been established that for this methyl transfer reaction, catalyzed by methionine synthetase, a B_{12} protein is utilized as intermediate carrier of the methyl group (Fig. 2). In yeasts and higher plants that apparently do not contain members of the vitamin B_{12} class of compounds, methionine also is synthesized; but in these biological systems the methyl transfer to homocysteine involves a different, but not yet understood, mechanism. It is interesting that one particular mutant strain of *E. coli* possesses

both the vitamin B_{12}–dependent and the independent methyl transferase reactions leading to the synthesis of methionine from N^5-methyltetrahydrofolate and homocysteine (*17*).

The biosynthesis of methane from various types of methyl donor compounds by the methane bacteria also requires the participation of a red B_{12} protein. Several lines of evidence (*14*) suggest that the mechanism involves transfer of the methyl group to the cobalt atom of the B_{12} compound on the red protein to form an enzyme-bound methyl-cobalt derivative followed by a reductive cleavage to yield the hydrocarbon methane (Fig. 2). In the analogous chemical reaction methyl-B_{12} is reductively cleaved by hydrogen and a platinum catalyst to yield methane according to reaction 1 (*18*).

$$CH_3 - B_{12} + \tfrac{1}{2}\ H_2 \longrightarrow CH_4 + B_{12r} \quad (1)$$

In the enzyme-catalyzed reactions, molecular hydrogen and hydrogenase, or other low potential systems such as pyruvate dehydrogenase, serve as ultimate sources of reducing equivalents necessary for the final step of methane biosynthesis. Prior to the discovery of the alkyl-cobalt derivatives of the B_{12} class of vitamins there were no known methyl compounds that could reasonably be expected to undergo reductive cleavage in a biological system to yield methane. Final decision as to whether the postulated methyl-cobalt derivative of the red protein is indeed the normal intermediate awaits availability of substrate amounts of the pure protein in order that the chemical and enzymic properties of its methylated derivative can be ascertained.

In at least two species of methane bacteria the chromophore of the red protein required for methane biosynthesis is a B_{12} vitamin containing a 5-hydroxybenzimidazole moiety as the base constituent of the nucleotide-cobalt ligand. This member of the B_{12} group was isolated from sewage sludge by Bernhauer and Friedrich (*19*) and termed factor III. In the B_{12} derivatives produced by many bacteria, a 5,6-dimethylbenzimidazole moiety is attached to the cobalt on the under side of the corrin ring (see Fig. 1); however, in the methane bacteria thus far examined, this moiety is replaced by 5-hydroxybenzimidazole. Whether this 5-hydroxybenzimidazole moiety has any special significance in the methane-forming reaction per se is unknown. Since the same cobalt-containing corrin ring structure which is presumed to be the functional portion of the molecule in the methyl group reactions of Table 1 is common to all of the B_{12} vitamins, variations in other portions of the vitamin molecule should be related more to matters of enzyme specificity than to any fundamental difference in chemical mechanism.

Table 1. Reactions requiring vitamin forms of B_{12} compounds. B_{12x} indicates that the oxidation state of the cobalt atom is uncertain; the product may be B_{12r} in which the cobalt atom is in the +2 oxidation state. THFA, tetrahydrofolic acid.

Reaction catalyzed	Enzyme system	Distribution of enzyme	References
CH_3–THFA + $HSCH_2CH_2CH(NH_2)COOH \rightarrow$ $CH_3SCH_2CH_2CH(NH_2)COOH$ + THFA	Methionine synthetase	Bacteria Mammals	(*17*, *58*) (*59*)
$CH_3OH + H_2 \rightarrow CH_4 + H_2O$ CH_3–$B_{12} + H_2 \rightarrow CH_4 + B_{12x}$	Methane synthetase	Methane bacteria	(*20*, *21*)
$CH_3OH + B_{12s} \rightarrow CH_3$–$B_{12}$	Methyl transferase	Methane bacteria	(*22*)
$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O$ $CH_3B_{12} + CO_2 \rightarrow CH_3COOH + B_{12x}$	Acetate synthetase	Bacteria	(*26*, *27*)

Compounds that serve as methyl donors for methane biosynthesis in the enzyme systems in vitro are methanol, acetate, N^5-methyltetrahydrofolate, synthetic methyl-B_{12}, and one-carbon (C_1) compounds that can be reduced enzymically to the level of a methyl group —for example, carbon dioxide, formate, and formaldehyde (*20*).

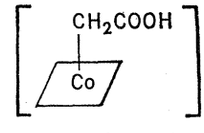
The overall reduction of methyl alcohol to methane by *Methanosarcina barkeri* involves the transfer of the methyl group of the alcohol to an enzyme-bound intermediate that can be trapped in vitro by its reaction with added B_{12s} (reduced B_{12}) to form free methyl-B_{12} (Table 1). The same purified red protein that is required for methane synthesis is needed for this methyl transfer reaction and several lines of evidence (*14*, *21–23*) suggest that in each instance an enzyme-bound methyl-cobalt derivative is an intermediate (Fig. 2). In the normal overall reaction the methyl group is reductively cleaved to yield methane, whereas in the absence of the reductase it can transfer to free B_{12s} in solution. Transfer of the methyl moiety from the cobalt of the enzyme-bound B_{12} compound to the cobalt atom of B_{12s} would be analogous superficially to the transfer of biotin-enzyme-bound carbon dioxide to free biotin by certain biotin-containing enzymes (*24*). In both reactions a high concentration of the free vitamin is needed to serve as trapping agent for the enzyme-bound intermediate. In the case of the biotin-dependent carboxylation system, the free biotin-CO_2 derivative and the enzyme-bound derivative eventually were shown to be chemically equivalent (*25*). In such instances the elucidation of a biochemical mechanism is greatly facilitated by the ability of an enzyme to use as substrate a free analog of its protein-bound cofactor and thereby to synthesize appreciable amounts of a compound closely resembling the normal transient intermediate bound to the enzyme.

Another strictly anaerobic process in which acetate rather than methane is the final fermentation product also appears to require a B_{12} vitamin as an essential catalyst. Whereas the methane bacteria are able to grow on a mixture of carbon dioxide and hydrogen which they reduce to methane (reaction 2), *Clostridium aceticum* satisfies its energy requirements for growth by converting a mixture of hydrogen and carbon dioxide to acetic acid (reaction 3).

$$4\ H_2 + CO_2 \longrightarrow CH_4 + 2\ H_2O \quad (2)$$

$$4\ H_2 + 2\ CO_2 \longrightarrow CH_3COOH + 2\ H_2O \quad (3)$$

In extracts of *Clostridium thermoaceticum* and *Clostridium sticklandii*, organisms which also synthesize acetate from carbon dioxide, methyl-B_{12} serves as an efficient source of the methyl moiety of acetate (Fig. 2). Furthermore carbon dioxide is reduced to the level of a methyl derivative that can be trapped as methyl-B_{12} (*26*, *27*). A cobalt-carboxymethyl derivative



has been suggested as the final intermediate in acetate synthesis in these systems since (i) the cobalt-carboxymethyl bond is readily cleaved to yield acetate by a wide variety of reducing agents and (ii) small amounts of radio-

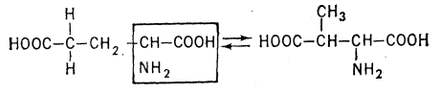
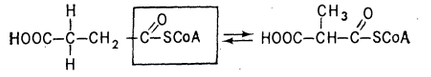
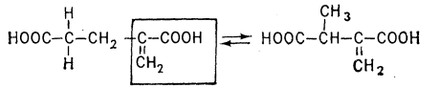
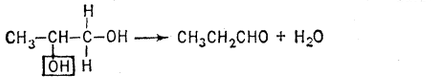
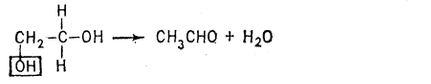
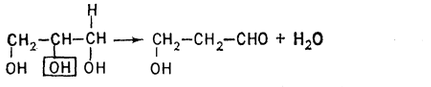
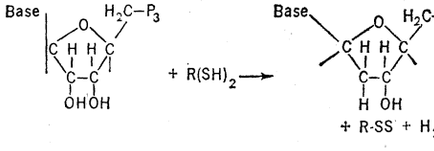
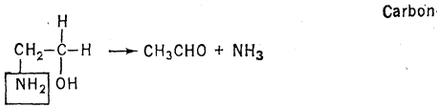
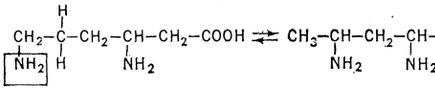
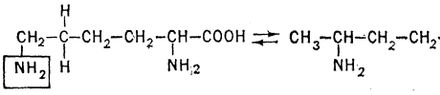
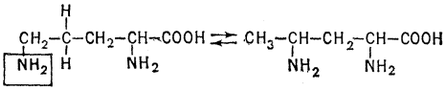
active cobalt-carboxymethyl corrinoids were isolated from *C. thermoaceticum* after administration of highly radioactive carbon dioxide (*27*).

Synthesis of coenzyme or deoxyadenosyl derivatives of B_{12} compounds. As noted above, the free vitamin is not the catalytically active form of B_{12} utilized by many organisms. Conversion of the vitamin form of a B_{12} compound to its coenzyme form (Fig. 1) is catalyzed by an enzyme system, B_{12} coenzyme synthetase, present in a wide variety of microorganisms (*28*). The vitamin after first undergoing reduction, presumably to the B_{12s} or Co^{1+} form, then reacts with a deoxyadenosyl moiety derived from adenosine triphosphate (ATP) as shown in reaction 4. Thus ATP serves as the biological alkylating agent in this reaction. The chemist, on the other hand, often

$$\underset{\text{(ATP)}}{\text{adenine-ribose-P-O-P-O-P}} + B_{12s} \longrightarrow B_{12}\text{ coenzyme} + \underset{\text{(tripolyphosphate)}}{\text{P-O-P-O-P}} \quad (4)$$

uses 2′,3′-isopropylidineadenosine-5′-tosylate as the alkylating agent to react with B_{12s} and then later hydrolyzes off the isopropylidine blocking groups to generate the coenzyme in the form shown in Fig. 1. In the enzyme-catalyzed overall reaction some microorganisms utilize a system consisting of a reduced pyridine nucleotide, such as reduced nicotinamide adenine dinucleotide (DPNH), which serves as electron donor to a flavoprotein which in turn reduces a small sulfhydryl protein to its dithiol form and the latter then reduces the B_{12} vitamin. For the reactions in vitro this reductase portion of the enzyme system can be omitted if chemically prepared B_{12s} is added; then the "adenosylating enzyme" component and ATP achieve the alkylation step shown in reaction 4 (*29*).

Table 2. B_{12} coenzyme—dependent reactions. In each reaction the migrating group that is replaced by a hydrogen is shown enclosed in a rectangle.

Reaction catalyzed	Enzyme	Distribution of enzyme	References
Carbon-carbon bond cleavage			
	Glutamate mutase	Bacteria	(4, 60)
	Methylmalonyl-CoA mutase	Bacteria, Mammals	(31,32, 45)
	α-Methylene-glutarate mutase	Bacteria	(61)
Carbon-oxygen bond cleavage			
	Diol dehydrase	Bacteria	(62)
	Diol dehydrase		
	Glycerol dehydrase	Bacteria	(39,63)
	Ribonucleotide reductase	Bacteria, Euglena	(64, 65)
Carbon-nitrogen bond cleavage			
	Ethanolamine deaminase	Bacteria	(55, 66)
	L-β-Lysine mutase	Bacteria	(67)
	D-α-Lysine mutase	Bacteria	(68)
	Ornithine mutase	Bacteria	(34, 69)

B_{12} coenzyme–dependent systems. Ten different biochemical reactions that require a B_{12} coenzyme as catalyst have so far been discovered (Table 2). These reactions involve (i) carbon-carbon bond cleavage and rearrangement of the carbon skeleton, (ii) elimination of water or ammonia, (iii) migration of amino groups, and in one case (iv) a net reduction of the substrate (ribonucleotide reduction to deoxyribonucleotide). In general, these reactions involve replacement of a group attached to one carbon atom of the molecule with a hydrogen from an adjacent carbon atom and, in a sense, can be viewed as internal oxidation-reduction reactions. The exception already noted is the net reduction of the ribose moiety of ribonucleotides to a deoxyribose moiety in a reaction where a dithiol protein is used as the external reducing agent. In all cases so far examined the B_{12} coenzyme serves as the intermediate carrier of the hydrogen that migrates, and this is the unifying feature of these seemingly quite different chemical reactions.

Some properties of B_{12} coenzyme–dependent enzymes. The enzymes that catalyze the reactions listed in Table 2 are of two general types. There are those that appear to consist of similar subunits having sulfhydryl groups and cobamide binding sites on the same subunit (Table 3), and there are others that are made up of two dissimilar protein moieties (Table 4). In the latter group one of the two protein moieties binds the cobamide and the other is a sulfhydryl protein; both are required for catalysis of the overall reactions. In general, the larger of the two proteins binds the B_{12} compound, and the sulfhydryl protein is considerably smaller; the exception is glycerol dehydrase where the reverse is the case. A spe-

cific effect of the low-molecular-weight sulfhydryl protein component of the glutamate mutase system is to increase the affinity of the cobamide binding protein moiety for B_{12} coenzyme (*30*). The methylmalonyl coenzyme A mutase of animal origin has sulfhydryl groups that are relatively inaccessible to organic mercurials and to alkylating agents until the tightly bound B_{12} coenzyme is removed (*31*), and therefore it is possible that sulfhydryl groups of this enzyme also are important in binding the coenzyme. Once the B_{12} coenzyme is removed, the enzyme from liver is extremely sensitive to mercurials and alkylating reagents whereas the methylmalonyl coenzyme A mutase of *Propionibacterium shermanii* is inhibited only slightly by reagents that combine with sulfhydryl groups. If the conclusion is correct that the bacterial methylmalonyl coenzyme A mutase is not a sulfhydryl enzyme (*32*), then the generalization cannot be made that all B_{12} coenzyme–dependent enzymes contain one or more sulfhydryl groups that are essential for catalytic activity. A list of enzymes that are either dependent on B_{12} coenzyme or vitamin B_{12} and that actually require the addition of a mercaptan reducing agent for maximal catalytic activity is given in Table 5. Presumably essential exposed sulfhydryl groups on these enzymes become oxidized during isolation and handling and must be reduced again by the added mercaptans before they can function catalytically. The specific role or roles played by sulfhydryl groups in the various catalytic reactions is not known and at the present is only a matter of conjecture.

Additional cofactors and activators of B_{12}-dependent enzymes. In addition to a mercaptan reducing agent and a monovalent cation which are required by many of the B_{12}-dependent enzymes, several also require for full activity one or more of the following: a divalent cation, pyridoxal phosphate, pyruvate, ATP, and *S*-adenosylmethionine. Ribonucleotide reductase, the α- and β- lysine mutases, and possibly ornithine mutase require a divalent metal ion for maximum catalytic activity. For these enzymes magnesium and manganese are about equally effective. Pyridoxal phosphate is specifically required for D-α-lysine mutase activity; a number of other carbonyl compounds including pyruvate fail to replace pyridoxal phosphate. In the presence of this cofactor the cobamide protein moiety of D-α-lysine mutase reacts with lysine and

Table 3. Enzymes with cobamide binding sites and sulfhydryl groups on the same subunits.

Enzyme	Source	Molecular weight	B_{12} coenzyme–bound		References
			Moles	Type of binding	
Methylmalonyl-CoA mutase	Sheep liver	165,000	2 per mole or 1 per subunit*	Strong	(*31*)
Methylmalonyl-CoA mutase	*Propionibacterium shermanii*	56,000		Weak	(*32*)
Diol dehydrase	*Aerobacter aerogenes*				(*62*)
Ethanolamine deaminase	*Clostridium* sp.	520,000	2 per mole	Weak	(*66*)
α-Methyleneglutarate mutase	*Clostridium barkeri*	200,000†		Weak	(*41*)
Ribonucleotide reductase	*Lactobacillus leichmannii*	70,000 to 110,000			(*65*)

* Subunit of 80,000 molecular weight. † Approximate.

catalyzes an exchange of a proton of the carbon-6 methylene group with a proton of the solvent (*33*). This exchange reaction, which occurs in the absence of the sulfhydryl protein moiety, may be related to the amino group migration catalyzed by the mutase complex. If so, the role of pyridoxal phosphate in the overall mutase reaction may be to form a derivative with the amino group that migrates from carbon 6 to carbon 5 of the lysine molecule (Table 2). The pyridoxal phosphate requirement for the ornithine mutase reaction (*34*) also could be directly concerned with migration of the terminal amino group. L-β-Lysine mutase, which is activated by pyruvate and not by pyridoxal phosphate, may utilize the keto acid in an analogous fashion. The possibility that pyridoxal phosphate might be required for migration of the glycine moiety in the reaction catalyzed by glutamate mutase (Table 2) was excluded by the findings that pyridoxal phosphate is not present in the purified enzyme, nor is its addition required (*35*).

The same enzymes that exhibit divalent metal ion requirements also require ATP for maximum activity—for example, ribonucleotide reductase, β-lysine mutase, and D-α-lysine mutase. Ribonucleotide reductase and β-lysine mutase also are activated by 2′-deoxy-ATP. The specific effect of ATP on D-α-lysine mutase is to increase the affinity of the enzyme for its substrate, lysine (*35a*). The phosphonic acid analogs, β,γ-methylene-ATP and α,β-methylene-ATP, which cannot be cleaved to yield orthophosphate and adenosine diphosphate (ADP) or pyrophosphate and adenosine monophosphate (AMP), respectively, replace ATP as activator. Hence it is concluded that ATP binds to D-α-lysine mutase and activates it without being cleaved in the process. Apparently it is important to the bacterial cell to increase the activity of this enzyme when concentrations of ATP increase.

In early studies, methionine synthetase activity was observed to be increased by ATP addition, but upon purification of the enzyme system it was found that *S*-adenosylmethionine is the substance actually required (*36*). In the crude enzyme preparations *S*-adenosylmethionine synthetase generated the activator from ATP and methionine. This is not the case with

Table 4. Enzyme systems consisting of a cobamide protein moiety and a dissimilar sulfhydryl protein moiety.

Enzyme system	Cobamide protein moiety		Sulfhydryl protein moiety		References
	Molecular weight	Affinity of protein for B_{12} coenzyme	Molecular weight	No. of sulfhydryl groups	
Glutamate mutase	128,000	Weak	17,000	6 to 7	(*30, 35*)
Glycerol dehydrase*	22,000		240,000	≥ 1	(*39*)
L-β-Lysine mutase	160,000†	Strong	60,000†	≥ 1	(*67*)
D-α-Lysine mutase	160,000†	Strong	60,000†	≥ 1	(*35a*)
Ornithine mutase	160,000†		60,000†		(*35a*)
Methionine synthetase	125,000	Strong	3,000	≥ 1	(*70*)

* The apoenzyme complex of the reassociated protein moieties binds B_{12} coenzyme. † Approximate values estimated from gel filtration studies.

the ATP activated α- and β-lysine mutases. Furthermore from recent studies (*37*) it is known that the phosphonic acid analogs of ATP are inhibitors of *S*-adenosylmethionine synthetase and have little if any ability to replace ATP as a source of the adenosyl moiety of the product.

Methane biosynthesis from a variety of methyl group donors and methyl-B_{12} formation from methanol (Fig. 2) also are ATP-dependent processes. Preliminary experiments indicate the β,γ-methylene phosphonic acid analog of ATP is just as effective an activator as ATP for methanol reduction to methane by extracts of *Methanosarcina barkeri* (*38*). This suggests that ATP may not be consumed during the reaction but is merely required as an activating ligand for one of the protein catalysts.

Enzymes that require monovalent cations for activity. Several of the B_{12} coenzyme–dependent enzymes of Table 2 exhibit greatly increased catalytic activity when certain monovalent cations are present. These enzymes show the same general patterns of specificity that the enzymologist has come to expect for most monovalent cation–dependent enzymes. The cations that are the most effective activators are potassium (K^+), ammonium ion (NH_4^+), rubidium (Rb^+), and thallium (Tl^+); those that are less effective or sometimes even inhibitory are sodium (Na^+), cesium (Cs^+), and lithium (Li^+). The B_{12} coenzyme–dependent enzymes that are known to require a monovalent cation and the specific cations that have been tested with each are listed in Table 6. Unlike the other enzymes in the list, β-lysine mutase is activated only slightly by ammonium ion in the absence of other inorganic monovalent cations; in the presence of potassium ion, for example, ammonium ion is a potent inhibitor.

The mode of action of the monovalent cation activators of these, as well as of many other enzymes, is still obscure. Glycerol dehydrase tends to dissociate into subunits in the absence of monovalent cations; addition of potassium or ammonium ions promotes reassociation of the subunits and formation of the active enzyme complex (*39*). Similar observations have been made with a few other enzymes. Perhaps the macrocyclic polyethers which complex with monovalent cations (*40*), under some conditions to form a crystalline complex believed to consist of a "sandwich" of two moles of polyether and one of cation, may serve as models of how monovalent cations influence the interaction of protein subunits.

Table 5. Cobamide-dependent reactions that require added mercaptan reducing agents.

B_{12} coenzyme dependent
Glutamate mutase
L-β-Lysine mutase
D-α-Lysine mutase
α-Methyleneglutarate mutase
Ornithine mutase
Ribonucleotide reductase (requires a dimercaptan as electron donor)
Vitamin B_{12} dependent
Methionine synthetase

Bacterial methylmalonyl-coenzyme A mutase (*32*) and α-methyleneglutarate mutase (*41*) are two B_{12} coenzyme–dependent enzymes that apparently do not require monovalent cations for activity. The latter, fully active in a reaction mixture containing tris(hydroxymethyl)aminomethane as the only cation, was unaffected by the addition of K^+, NH_4^+, Rb^+, Na^+, or Li^+ ion. The activities of ribonucleotide reductase and glutamate mutase usually are measured in reaction mixtures containing monovalent cations that are used to neutralize the acidic substrates, and it is not certain whether these enzymes are equally active in the absence of inorganic monovalent cations.

B_{12} metabolism of C. sticklandii. *Clostridium sticklandii*, an anaerobic bacterium which ferments amino acids, affords an interesting example of an organism that synthesizes large amounts of B_{12} compounds, particularly B_{12} coenzyme, and utilizes this compound as catalyst for many of its metabolic transformations. The list of reactions in Table 7, which undoubtedly is far from complete, includes only those that are definitely established to be B_{12}-linked. Several red proteins of unknown catalytic function that contain B_{12} compounds as chromophores have been accumulated as by-products during the isolation of other enzymes from this microorganism. These red proteins may catalyze some of the other reactions listed in Tables 1 and 2 or other reactions not yet known. Like several other clostridia, *C. sticklandii* normally synthesizes the form of B_{12} containing adenine as the basic component (that is, pseudo-B_{12}); however if benzimidazole or dimethylbenzimidazole is furnished, the enzyme system also is able to incorporate these bases (*42, 43*). Under certain cultural conditions the level of B_{12} compounds synthesized by this microorganism increases in proportion to the amount of formate and carbon dioxide available for the B_{12}-dependent synthesis of acetate (*44*).

Mechanism of reactions mediated by B_{12} coenzyme. It is clear that B_{12} coenzyme acts as intermediate carrier of the migrating hydrogen, at least in seven (Table 8) of the ten known reactions (Table 2), but whether the species abstracted from the substrate is a proton (H^+), a hydride ion (H^-), or a radical ($H\cdot$) is still uncertain. Whereas, at one time it was considered, at least in a formal sense, that the B_{12} coenzyme probably served as a hydride ion carrier, there now is some tendency among workers in the field to favor an earlier view (*45*) that the reactions involve a radical mechanism and, therefore, a $H\cdot$ is the species abstracted. It must be confessed that this tentative conclusion is based almost as much on numerous failures to obtain data in support of a hydride ion mechanism as any really direct proof of radical formation. In general, the signals indicative of radical formation detected in electron spin resonance spectra (*46*), although they correlate qualitatively with catalysis of the B_{12} coenzyme–dependent reactions, are either quantitatively very small or are seen best in reactions with model substances which may or may not be comparable to the biochemical processes in question.

As to the specific site on the B_{12} coenzyme involved in its hydrogen-carrying function, the careful experiments of Frey, Essenberg, and Abeles (*47*) have shown this to be the 5′ carbon of the 5′-deoxyadenosyl moiety covalently bonded to the cobalt (Fig. 3).

In the intact coenzyme the two hydrogens on this 5′ carbon are not sterically equivalent, but, in fact, both are transferred apparently without discrimination and therefore, during the catalytic process, they must somehow become equivalent. One of the earlier suggested ways that this might be accomplished involved rupture of the carbon-cobalt bond so that a third hydrogen could be accepted at the 5′-carbon position. Abstraction and transfer to the product of any one of these *three equivalent hydrogens* followed by the remaking of the carbon-cobalt bond would ready the coenzyme molecule for the next catalytic sequence of the reaction. At each turn of the cycle there would thus be a

chance that a hydrogen already present on the coenzyme might be transferred rather than the one just added, and this would explain the observations (*47, 48*) that with some substrates interas well as intramolecular hydrogen transfers can occur. If the reaction mechanism does involve a transient cleavage of the carbon-cobalt bond and the addition of an extra hydrogen (*49*), the resulting 5′-deoxyadenosine moiety must remain so tightly bound to the enzyme protein that it is unable to exchange with free added 5′-deoxyadenosine. The free compound, labeled in the 5′-methyl group with radioactive hydrogen (tritium), was tested in a few of the B_{12} coenzyme–dependent systems but in no case was any transfer of tritium to reactants observed (*50*).

One hypothesis (*49, 51*) to explain the nature of the primary attack on the coenzyme molecule that would precede cleavage of the carbon-cobalt bond required a preliminary protonation of the ribosyl oxygen of the deoxyadenosyl moiety of the coenzyme—perhaps by an acidic group on the enzyme—and then the resulting complex could act as intermediate acceptor for the hydrogen abstracted from the various substrates (Fig. 4). However, the demonstration that an analog containing a bridge methylene group instead of this ribosyl oxygen also is active as a coenzyme for the overall hydrogen transfer process (*51*) shows that the ribosyl oxygen is not essential for catalytic activity.

Again, then, one is returned to the original situation of having little or no positive experimental evidence concerning the nature of the primary reaction of enzyme and coenzyme with its substrate. Perhaps somewhere in the partial ionic character (*52*) of the carbon-cobalt bond of 5′-deoxyadenosyl-B_{12} lies the key to an understanding of the mechanism.

Occurrence of cobalt-free precursors of B_{12} compounds (cobalt-free corrinoids) in nature. Cobalt-free corrinoid compounds were first detected in certain sulfur and nonsulfur purple photosynthetic bacteria (*53*). These substances are orange-red in color and exhibit ultraviolet and visible absorption spectra much like those of the cobalt-containing vitamins. However, they differ in that they fail to react with cyanide in the manner characteristic of the vitamins, and they change color, from red to yellow, in mildly alkaline solution. At neutral *pH* the isolated cobalt-free corrinoids fail to take up cobalt, but in alkaline solution added cobaltous ion is rapidly inserted in the corrin ring to give a compound indistinguishable from vitamin B_{12}. It is presumed that in the cell at neutral *p*H an enzyme is responsible for addition of the cobalt atom to the otherwise complete corrin structure. Recently, a similar cobalt-free corrinoid compound was isolated from a nonphotosynthetic microorganism *Streptomyces olivaceus*, which had been grown in a cobalt-deficient medium (*54*). The yield of this corrinoid compound was appreciably increased if known metabolic precursors of the corrin ring structure also were included in the cobalt-deficient culture medium. In media containing normal concentrations of cobalt, *S. olivaceus* synthesized only cobalt-containing corrinoid compounds. This is in contrast to the photosynthetic bacteria originally studied, which accumulated cobalt-free corrinoid compounds even though there was an excess of cobalt in the culture medium (*53*).

General metabolic significance of B_{12}

Table 6. B_{12} coenzyme–dependent enzymes known to be activated by monovalent cations.

Enzyme	Monovalent cations that	
	Activate	Inhibit
Diol dehydrase	(K^+, NH_4^+, Tl^+, Rb^+) > (Cs^+, Na^+)	
Glycerol dehydrase	K^+, NH_4^+	
Ethanolamine deaminase	K^+, NH_4^+, Rb^+	Na^+, Li^+
L-β-Lysine mutase	K^+, Rb^+	NH_4^+; Na^+, Li^+ (slight)
D-α-Lysine mutase	K^+, NH_4^+, Rb^+	Na^+, Li^+

Table 7. Reactions catalyzed by *Clostridium sticklandii* that involve B_{12} compounds.

B_{12} coenzyme–dependent reactions

1. L-β-Lysine mutase
   L-3,6-Diaminohexanoate ⇋ 3,5-diaminohexanoate
2. D-α-Lysine mutase
   D-α-Lysine ⇋ 2,5-diaminohexanoate
3. Ornithine mutase
   Ornithine ⇋ 2,4-diaminopentanoate
4. Ribonucleotide reductase*
   Ribonucleotide triphosphate + $R(SH)_2$ → Deoxyribonucleotide triphosphate + R-SS

Other reactions involving B_{12}

5. Acetate synthetase ($C_1 + C_1$ → acetate)†
   Methyl-B_{12} + CO_2 + reducing system → acetate + B_{12x}
6. B_{12} coenzyme synthetase
   Vitamin B_{12} + reducing system + ATP → B_{12} coenzyme + tripolyphosphate

Synthesis of adenine and benzimidazole nucleotide precursors of B_{12} vitamins (42, 43)

7. Adenine (or benzimidazole) + nicotinate mononucleotide → adenine (or benzimidazole)-ribonucleotide-5′-P + nicotinate.

* Assayed by H. P. C. Hogenkamp; see (*16*). † Assayed by J. M. Poston and T. Price.

Table 8. Hydrogen transfer in B_{12} coenzyme–dependent reactions.

Reaction	Tritium transfer from substrate to coenzyme demonstrated	Tritium transfer demonstrated from 5′-position of deoxyadenosine moiety of coenzyme to		References
		Substrate	Product	
Diol dehydrase	+	*	+	(*47, 71*)
Methylmalonyl-coenzyme A mutase		+	+	(*71*)
Glutamate mutase	+	+	+	(*15*)
Ethanolamine deaminase	+	*	+	(*72*)
L-β-Lysine mutase	+	+	+	(*73*)
D-α-Lysine mutase	+	+	+	(*33*)
α-Methyleneglutarate mutase		+	+	(*74*)

* Overall reactions are not reversible.

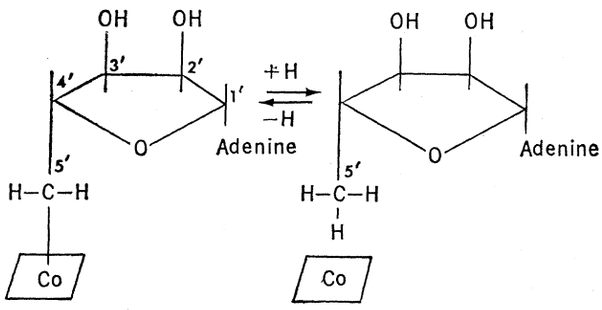


Fig. 3. Partial structure of B_{12} coenzyme showing the 5′-deoxyadenosyl moiety covalently linked through its 5′-methylene group to the cobalt atom. On the right is 5′-deoxyadenosine, which has a methyl group at the 5′-position.

coenzyme–dependent reactions. Several of the B_{12} coenzyme–dependent reactions are particularly important to the energy economy of the cell, at least for several of the bacterial species, in that these reactions rearrange the original substrate molecules to chemical forms that can more readily undergo the coupled oxidation-reduction reactions that furnish energy to the cell. Glutamate mutase and α-methyleneglutarate mutase catalyze rearrangements of the carbon skeleton of their respective substrates which finally, after hydration, undergo reverse aldol cleavage to yield pyruvate and acetate or pyruvate and propionate, respectively. Both pyruvate and one of its oxidation products, acetyl-coenzyme A serve as readily negotiable "biochemical currency" for many essential reactions of the organisms. In a similar fashion diol dehydrase and ethanolamine deaminase convert substrates that are not readily utilizable in the general metabolic pool to acetaldehyde, a common precursor of acetyl-coenzyme A. The amino group migrations that are catalyzed by the lysine and ornithine enzymes likewise prepare their respective substrates for attack by the usual types of oxidative deaminases after which the carbon skeletons then readily undergo thiolytic cleavage to form acetyl-coenzyme A, a key biochemical intermediate.

Ethanolamine deaminase, α-methyleneglutarate mutase, and perhaps some of the other B_{12} coenzyme–linked enzymes of Table 2 are inducible and are not formed when the bacteria are grown on other fermentable substrates. For example, the *Clostridium* sp. that forms ethanolamine deaminase grows even better on choline which it cleaves to trimethylamine and acetaldehyde in a process that is not dependent on B_{12} (*55*). Cells cultured on choline contain very little ethanolamine deaminase. When *C. barkeri* grows on nicotinic acid, it produces a large amount of B_{12} coenzyme and elaborates a series of inducible enzymes (including α-methyleneglutarate mutase) that catalyze various intermediate steps of the fermentation (*41*). In contrast, cells cultured on glucose instead of nicotinate contain little, if any, of these enzymes and are low in B_{12} coenzyme.

With regard to the distribution in nature of the enzymes listed in Tables 1 and 2, little can be said. Presumably many of these same processes take place in higher green plants and other living organisms that seem not to contain B_{12} compounds. In such systems, some other catalyst performs the function of the cobalt-containing corrinoid compounds. The reduction of ribonucleotides to deoxyribonucleotides is catalyzed by B_{12}-independent as well as B_{12}-dependent enzyme systems. In *Escherichia coli*, ribonucleotide diphosphates are reduced to the corresponding deoxyribonucleotide diphosphates (*56*) by an enzyme system that resembles in many respects the B_{12} coenzyme–dependent one of *Lactobacillus leichmannii*. In both types of systems a dithiol protein serves as the immediate reducing agent, but the *E. coli* reductase contains a nonheme iron protein component and lacks B_{12} coenzyme. One of the questions investigators currently seek to answer is whether the iron-containing protein may perform the same function as the B_{12} coenzyme–linked protein of the other system.

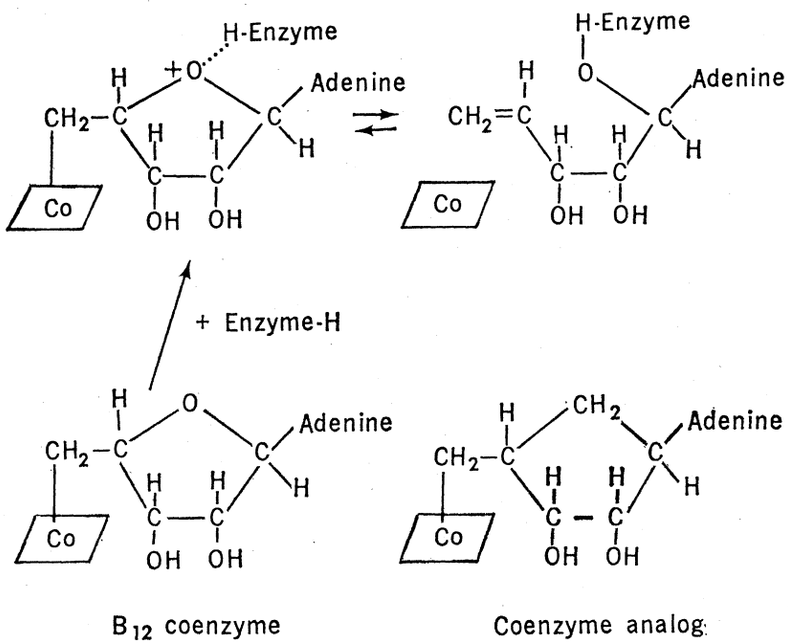


Fig. 4. Proposed mechanism of reaction of B_{12} coenzyme with an enzyme protein and structure of a methylene analog of the coenzyme used to test this hypothesis. See references (*49*) and (*51*).

Summary

In spite of the considerable progress made in recent years toward the understanding of the chemistry and biological function of the cobalt-containing B_{12} group of compounds, much of the information still is more descriptive than definitive in nature. In general terms, it is known that the free vitamin forms can function as methyl group carriers and that the 5′-deoxyadenosyl or coenzyme forms serve as hydrogen carriers; but the mechanism of these processes is not understood in detail. More systematic studies of the pure chemistry of these complex molecules containing a carbon-cobalt covalent bond are needed before the biochemist can interpret many of his observations on the enzyme-catalyzed reactions.

Even in relatively simple solutions it is difficult to ascertain the state of oxidation of several of the vitamin forms, and these problems are compounded when the reactive thiol compounds and complex proteins of the biological systems also are present. For example, both vitamin B_{12r} (the Co^{2+} form) and corresponding analogs are known to disproportionate in solution to B_{12s} (Co^{1+}) and B_{12a} (Co^{3+}) under a variety of mild conditions (*12*, *57*). This means that in the biological systems it is exceedingly difficult to ascertain the chemical nature of many B_{12} intermediates and reaction products. The role of the protein moiety of the various B_{12}-linked enzymes in the catalytic processes is little known as is, also, the mode of binding of the B_{12} derivative to the protein. These types of questions perhaps can be answered eventually by the crystallographers, whose art is becoming increasingly sophisticated.

Note added after preparation of manuscript. In contrast to the values given in Table 4 for the molecular weights of the two dissimilar protein moieties of glycerol dehydrase, a recent report (*57a*), gives a value of 188,000 for the molecular weight of a stable, catalytically inactive complex of 1 mole of hydroxocobalamin and 1 mole of the apoenzyme complex of glycerol dehydrase. The latter is presumed to contain one equivalent of each of the two dissimilar protein subunits. The original estimate of 240,000 as the molecular weight of the unstable sulfhydryl protein moiety (*39*) was undoubtedly made on partially aggregated material.

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