THE MECHANISM OF ADENOSYLCOBALAMIN-DEPENDENT REACTIONS*

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INTRODUCTION

Among the cofactors employed in biochemical catalysis is a unique series of compounds, all cobalt-containing derivatives of corrin, a partially reduced tetrapyrrole macrocycle. These compounds, collectively called the cobamides, are noteworthy for their chemical nature, since they constitute the only biological organometallics hitherto discovered. They are even more remarkable for the reactions in which they participate as catalysts. Among these reactions, and typical of the group, are certain carbon skeleton rearrangements for which no precedent exists in organic chemistry.

The very unusual features of these cobamide-requiring reactions have represented a challenge to chemists and biochemists to discover their mechanisms, and many have taken up that challenge. As a result, the past 15 years has witnessed a considerable advance in the comprehension of cobamide-dependent reactions at a mechanistic level. Nevertheless, conceptual gaps of major proportions still remain to be filled. Indeed, certain aspects of these reaction mechanisms are almost as obscure now as they were before the cofactors were discovered.

The cobamide-dependent reactions can be divided into two classes: those that require a methylcobamide as cofactor and those that require an adenosylcobamide. It is the purpose of this article to survey the latter class of reactions from the point of view of catalytic mechanisms. It will become apparent in this survey that certain mechanistic features are on reasonably solid experimental footing, while others are highly speculative. Areas of controversy will also become evident. In speculative and controversial areas, we will summarize the arguments pro and con and make our own views explicit.

CHEMISTRY[1]

Structure

The cobamides are all derivatives of *corrin*, a nitrogen-containing macrocycle whose

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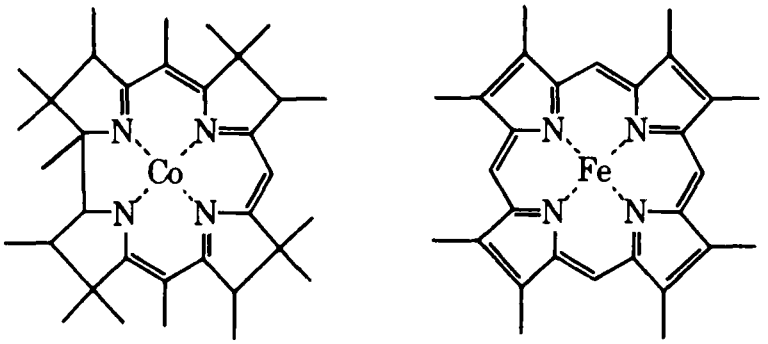


FIGURE 1. Corrin and porphyrin. The lines extending from the periphery of the macrocycles represent alkyl or acyl substituent groups. Strictly speaking, the terms "porphyrin" and "corrin" refer to the metal-free macrocycles. For purposes of emphasis, however, the structures are shown containing metals usually associated with them under biological conditions.

structure is shown in Figure 1 (left). This macrocycle is related structurally to prophyrin (Figure 1, right), the familiar tetrapyrrole found in the prosthetic groups of the cytochromes and the chlorophylls. Close examination, however, reveals two important chemical distinctions between these two ring systems:

1. While porphyrin is fully aromatic, corrin is highly reduced. The corrin ring contains only 6 double bonds, arranged as a linearly conjugated system involving 12 of the 15 atoms comprising the inner margin of the macrocycle. Extension of this double bond system by further oxidation is prevented by strategically placed substituents.
2. The corrin ring is contracted compared with its porphyrin counterpart. In porphyrin, each pair of pyrrole rings is separated by a methyne bridge. In corrin, however, the A and D rings are not methyne-interrupted, but are joined directly to each other by a bond between the α-positions (see Figure 1, the two left-hand rings of the corrin structure). Thus, in its inner margin the corrin macrocycle contains one less atom than the porphyrin.

The *cobamides* are corrins which are further characterized by the following structural features:

1. All contain an atom of cobalt. The cobalt is held tightly in the center of the macrocycle by coordination to the four nitrogen atoms, as shown in Figure 2. All attempts to remove this cobalt reversibly from the corrin ring have met with failure;[2,3] the metal can only be released by the destruction of the ring.
2. All cobamides display the same distinctive pattern of ring substitution. The substituents are methyl and carboxamide residues, and they are disposed according to the pattern shown in Figure 2. Stereochemically, the propionamide residues all lie on one side of the macrocycle — the "α" side — while the acetamide residues all lie on the other (β) side.
3. Six of the seven peripheral carboxamide residues are simple primary amides ($-CONH_2$). The propionamide on the C ring, however, has a much more complex structure. The carboxyl group in this substituent is amidated with (R)-1-amino-2-propanol, which in turn is attached by a phosphodiester bridge to the 3′-hydroxyl group of a ribonucleoside. Many such ribonucleosides have been reported as con-

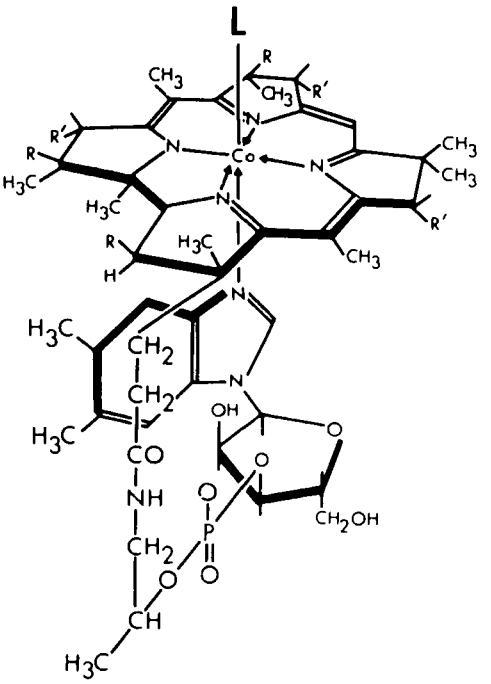


FIGURE 2. Cobalamins. R = CH_2CONH_2, R′ = $CH_2CH_2CONH_2$.

stituents of cobamides from various sources. These differ in the identity of the heterocyclic base, as well as in the stereochemistry of the bond between the base and the sugar. The cobamide isolated from mammalian tissues contains 5,6-dimethylbenzimidazole as the heterocycle. This cobamide, the only one which is functional in mammals, is called cobalamin and is the one illustrated in Figure 2. Other common cobamides, isolated mainly from bacteria, contain benzimidazole or adenine as the heterocycle. The heterocycle may be coordinated to the α position of the cobalt through a ring nitrogen, as shown in Figure 2, or it may not, depending on conditions.

Cobamide chemistry and biochemistry deals largely with the properties of the cobalt atom in the center of the ring. This atom has the capacity to coordinate up to six surrounding ligands. Four of these coordination positions are occupied by the four nitrogens of the corrin ring. A fifth position, the α position, is usually occupied either by the side-chain heterocycle or by solvent; it may, however, be unoccupied. The group occupying the sixth position (usually designated the β position) is extremely variable, and much of the rich chemistry of the cobamides has to do with reactions involving this position of the cobalt coordination sphere. In biological systems, the β ligand is almost invariably one of the following four groups: $-CN$, $-OH$, or the alkyl groups $-CH_3$ or 5′-deoxyadenosyl. Of these four, the two alkyl ligands are the ones found in the coenzymatically active cobamides. Methylcobamide-dependent reactions include the synthesis of methionine from homocysteine and N^5-methyltetrahydrofolate,[4,5] as well as the production of methane[6,7] and acetic acid[8,9] by certain anaerobic microorganisms. These will not be discussed here. The adenosylcobamide-dependent reactions include a group of extremely unusual rearrangements as well as a remarkable reduction reaction. In this article, the mechanism of the adenosylcobamide-dependent reactions will be surveyed in detail.

Chemical Properties

When placed in a very strong ligand field, such as is provided by the corrin macrocycle or other unsaturated nitrogen-containing polydentate ligands (e. g., dimethylglyoxime, bis(acetylacetone)ethylenediamine (BAE), bis(salicylaldehyde)-ethylenediamine (SALEN)), cobalt acquires a number of extraordinary chemical properties,[10] several of which are very important in terms of the mechanism of adenosylcobamide-dependent reactions. Before discussing the reactions themselves, it is useful to review those chemical properties which are pertinent to the biochemical function of the adenosylcobamides. These properties will be discussed specifically in terms of the chemistry of cobalamin (5,6-dimethylbenzimidazolylcobamide), since this is the cobamide with which most of the chemical studies were carried out. The chemistry to be described, however, almost certainly applies generally to all cobamides.

Redox Properties

The cobalt atom in cobalamin displays three stable oxidation states, corresponding formally to Co(III), Co(II), and Co(I). In cobalamins off the shelf, such as OH-Cbl or AdoCbl, the metal is in the trivalent state. Potentiometric titrations showed that the two other oxidation states were produced by sequential one-electron reductions of Co(III)-containing cobalamins.[11,12] Very recently, accurate potentials for the redox couples have been obtained at a variety of pH values by cyclic voltammetry.[13,14] These studies showed that for the physiologically important reduction of OH-Cbl at neutral pH, the two redox potentials were

$$\text{OH-Cbl} \xrightarrow{-0.04\ \text{mV}} \text{Cbl(II)} \xrightarrow{-0.85\ \text{mV}} \text{Cbl(I)}$$

Other cob(III)alamins, such as CN-Cbl, showed different redox behavior.[13,15]

Cob(II)alamin (containing Co(II)) is a well-studied species which can be clearly distinguished from both OH-Cbl and alkyl cobalamins (in the figure, AdoCbl) by optical spectroscopy (Figure 3). It can be produced not only potentiometrically, but chemically as well. Typical reducing agents which have been used for the production of cob(II)alamin are thiols[16] and carbon monoxide.[17] In addition, cob(II)alamin is produced by the photolysis of alkyl cobalamins, a topic which will be discussed further below. The compound is stable in water under anaerobic conditions, but oxidizes slowly in the presence of air ($t_{1/2}$ about 20 min) to OH-Cbl.[18] It contains a single unpaired electron which is localized to the $3d_{z^2}$ orbital of the cobalt[19] — that is, the orbital which points in the direction of the β ligand (although cob(II)alamin is generally regarded as five-coordinate, the β position being unoccupied).[20] Because of this unpaired electron, cob(II)alamin shows a very characteristic EPR spectrum,[19,20] a property which has been of great importance for the study of AdoCbl-dependent reactions. The configuration of the EPR spectrum depends on whether the heterocyclic base in the α position is coordinated to the cobalt or not. The so-called "base on" cob(II)alamin gives a spectrum with axial symmetry ($g_{\parallel} = 2.004$; $g_{\perp} = 2.32$) in which the $g_{\perp}$ peaks show hyperfine structure due to the interaction of the electron with the nucleus of the α-linked N atom (Figure 4, top), while the "base off" cob(II)alamin gives a much more rhombic signal showing no hyperfine splitting (Figure 4, bottom).

Cob(I)alamin, whose spectrum is shown in Figure 5, is formed chemically by the use of sufficiently powerful reducing agents (e. g., BH_4^-, or zinc in NH_4Cl) employed under anaerobic conditions.[21-24] It is itself a powerful reducing agent, as indicated by its redox potential. It not only reacts rapidly with oxygen, but also reduces protons to hydrogen gas.[12] It is therefore unstable in water even under anaerobic conditions. It is also an exceedingly potent nucleophile,[25] a property which has been exploited exten-

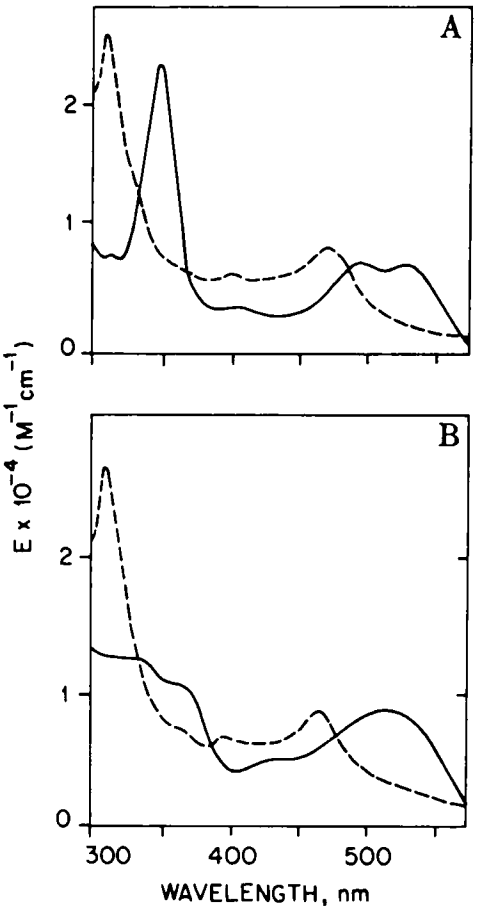


FIGURE 3. Comparison of the spectra of OH-Cbl, AdoCbl and cob(II)alamin. (A) Top: OH-Cbl and cob(II)alamin, (B) bottom: AdoCbl and cob(II)-alamin. In both panels, the cob(II)alamin spectrum is shown as the dashed line (----).

sively for synthetic purposes. This cobalamin contains a pair of unshared electrons in the $3d_{z^2}$ orbital, bringing the number of d electrons to 8. As is typical of other d^8 transition metal ions, the preferred coordination number for the cobalt of cob(I)alamin is 4,[10,25] so that both of the axial coordination positions are ordinarily vacant in this compound. Under sufficiently acid conditions, however, the metal will protonate; the pK for the cob(I)alamin·H^+ ⇌ cob(I)alamin + H^+ acid base pair has recently been determined to be 1.0.[13]

Alkyl Cobalamins

If one property of the cobamides had to be singled out as the chemical characteristic of greatest biological significance, it would probably be their ability to form a carbon-metal bond which is stable to air and water. This property, which was a great surprise to contemporary workers interested in the problem, became strikingly apparent when X-ray crystallography of a coenzymatically active cobamide disclosed an adenosyl group attached to the cobalt by a σ bond between the metal and the 5′-carbon of the nucleoside.[26] The discovery that a cobamide coenzyme was alkylated on the cobalt atom led immediately to the intensive study of the chemistry of organocobamides.

A synthetic route to these compounds was soon reported by Johnson and co-work-

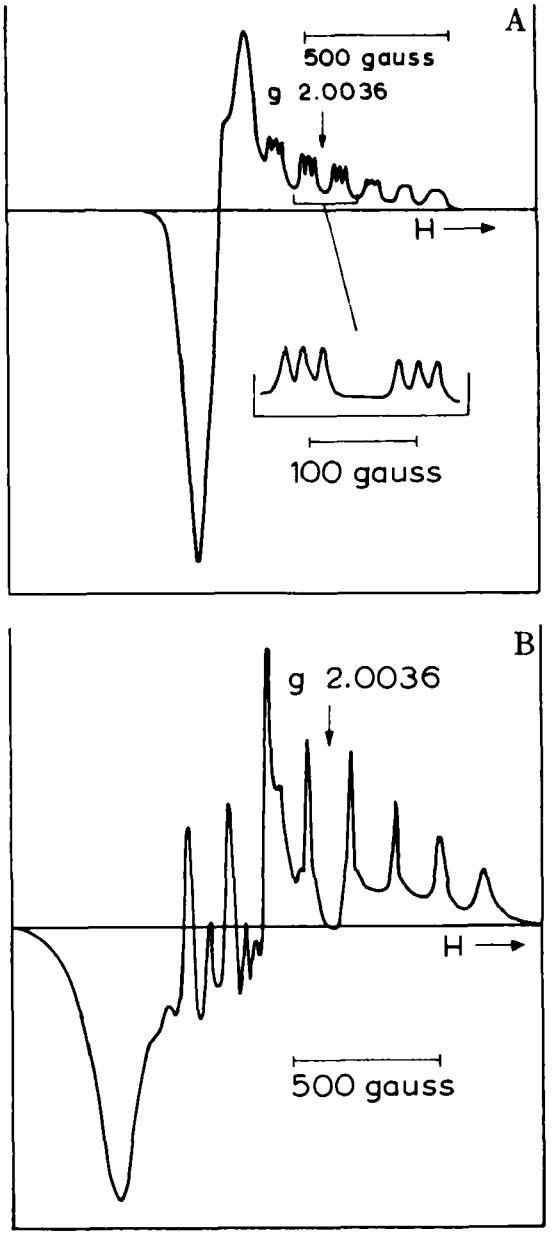


FIGURE 4. EPR signals of cob(II)alamin. (A) Top: "base on" (pH 7.0), (B) bottom: "base off" (pH 0).

ers[27] and by Müller and Müller.[22] These two groups discovered the extraordinary nucleophilicity of cob(I)amides, and they and others[28] used this discovery to synthesize a large number of alkyl cobamides by nucleophilic

$$Co(I) + RX \rightarrow Co\text{-}R + X^-$$

displacement reactions, or by nucleophilic additions across suitably polarized double bonds:

$$Co(I) + CH_2{=}CH\text{-}CO\text{-}R \rightarrow Co\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}R$$

$$Co(I) + HC{\equiv}CR \rightarrow Co\text{-}CH{=}CHR$$

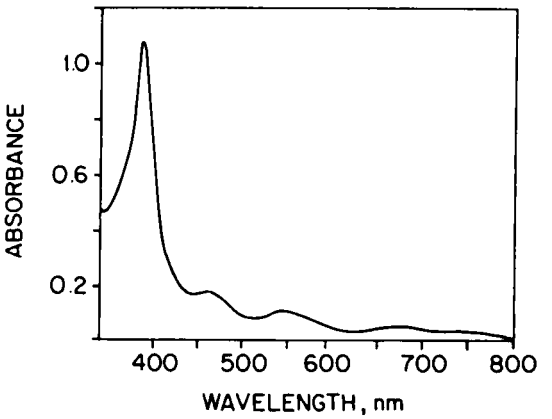


FIGURE 5. Spectrum of cob(I)alamin.

Although this is by far the most commonly used synthetic route to the organocobamides, they have also been synthesized from cob(III)amides, using as the alkyl donor a Grignard reagent[29] or an electron-rich olefin,[30] the latter a

$$OH-Cbl + RMgX \rightarrow R-Cbl$$

reaction which, as will be seen below, has attracted considerable recent attention as a model for certain AdoCbl-dependent rearrangements:

$$OH-Cbl + CH_2=CH-O-R \rightarrow Cbl-CH_2-CHO + ROH$$

Finally, an example has been reported of the synthesis of an organocobalamin by the addition of a free radical to cob(II)alamin:[31]

$$Co(II) + R\cdot \rightarrow R-Co$$

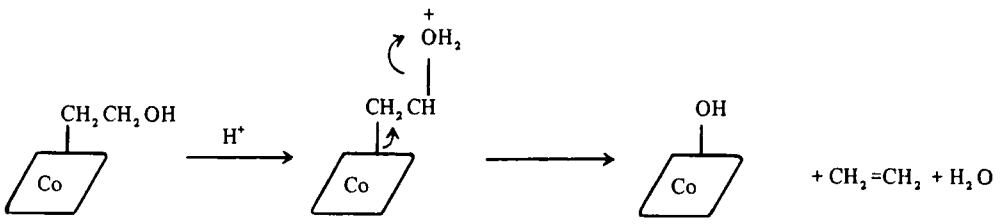
Of the hundreds of alkyl cobamides whose syntheses have been reported to date, virtually all are primary; that is, the cobalt is attached to a carbon which possesses at most one additional substituent ($Co-CH_2-R$).* A few secondary cobamides have been prepared, including the 2-butyl and cyclohexyl compounds.[33] These are only moderately stable, and then only in the "base off" form, possibly because displacement of the metal from the plane of the ring is required to stabilize the secondary cobamides. Tertiary cobamides have not been synthesized to date. These results are not surprising, since space-filling models show that the region around the β coordination position is seriously crowded by the substituents of the corrin ring.

Cleavage of the carbon-cobalt bond can occur by heterolysis or homolysis. Heterolysis in turn can be subdivided into those reactions which give rise to Co(III)-containing cobamides, and those which form cob(I)amides. A general category of heterolytic reactions which yield Co(III)-containing products are the reactions of polarizable cations such as Hg^{++} Cd^{++} and As^{+++} with alkyl cobamides.[34] In these reactions, electrophilic displacement at the cobalt-linked carbon atom results in the alkylation of the attacking cation with concomitant production of hydroxocobamide:

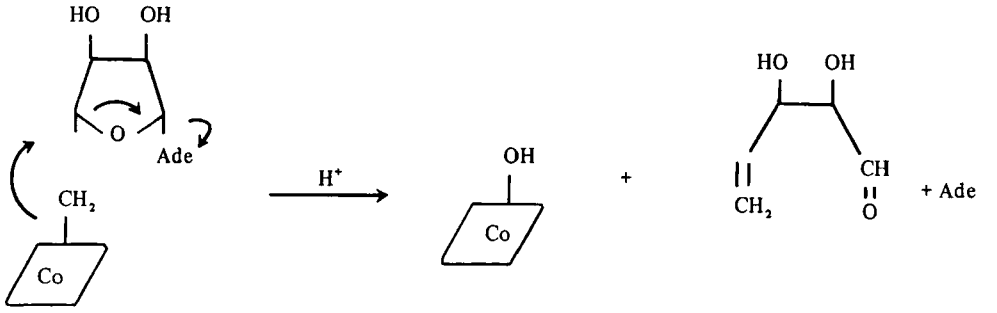
$$Hg^{++} + CH_3-Cbl \rightarrow CH_3-Hg^{+} + OH-Cbl$$

* Halomethylcobamides substituted with up to three halogen atoms (e. g., trichloromethyl cobalamin) have been prepared by Wood and associates.[32] The halogen substituents do not add much bulk to the alkyl group, however, so that sterically the halogenated alkyl group is almost equivalent to an unsubstituted $-CH_3$ group.

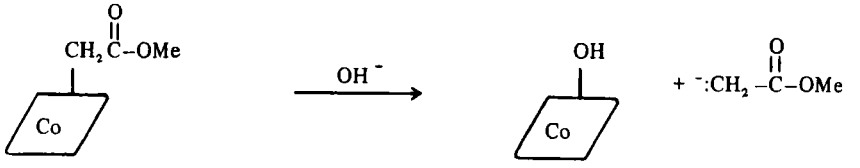
For other heterolytic reactions, an alkyl group with a special functionality is required. For example, OH−Cbl is formed by the acid-catalyzed heterolysis of β-hydroxyethylcobalamin.[35]



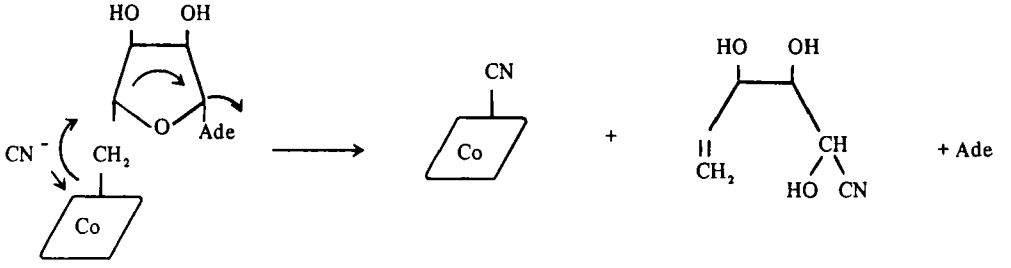
Adenosylcobalamin is similarly cleaved under acid conditions to generate OH−Cbl:[36]



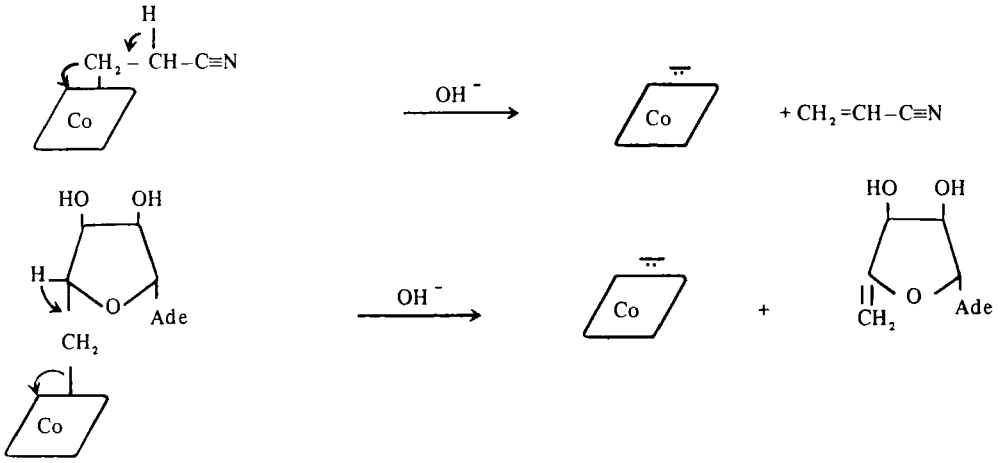
Under basic conditions, appropriately substituted alkyl cobamides react to form cob(III)amides with the departure of a carbanion:[35]



CN^- can also effect heterolysis to produce Co(III)-containing cobamides. An example is the cyanolysis of adenosylcobalamin:[37]



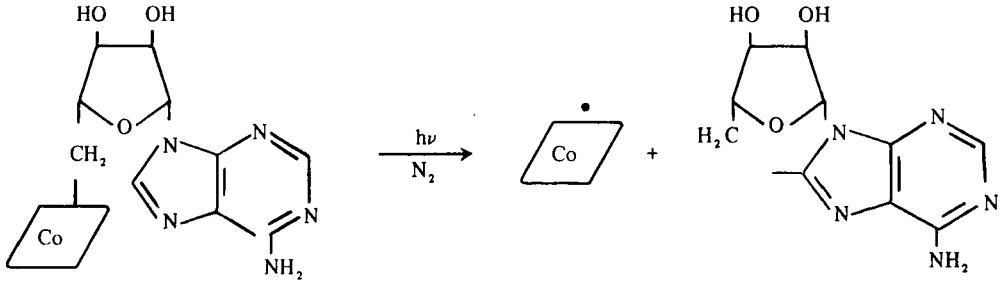
Heterolysis to produce cob(I)alamin generally occurs by β elimination, as in the following examples:[38,39]



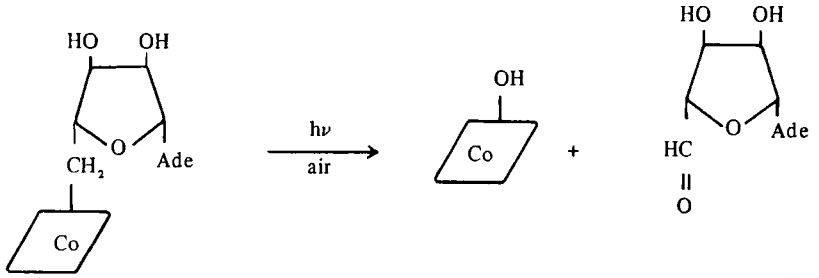
The above examples show that hetereolysis of the carbon-cobalt bond is a common and important class of reactions involving alkyl cobamides. According to current evidence, however, the reaction which is relevant to the adenosylcobamide-dependent reactions is the homolysis of the carbon-cobalt bond. Facile homolysis of this bond is a property common to all alkyl cobamides. It is usually induced by exposure of the cobamide to light,[35,37,40] but also occurs when solutions of such compounds are heated.[41] For the light-induced reaction, the quantum yield has been found to be roughly constant with wave-length over the visible region of the spectrum, ranging between 0.05 and 0.3, depending on the compound.[40] The initial products of photolysis are the cob(II)amide and a free radical:

$$\text{Alkyl cobamide} \overset{h\nu}{\rightleftharpoons} \text{cob(II)amide} + \text{R}\cdot$$

These then undergo secondary reactions to yield the final product. Because they are formed in secondary reactions, the final products will vary depending on the conditions under which the photolysis takes place. For instance, the photolysis of adenosylcobalamin under anaerobic conditions yields cob(II)alamin and 5′,8-cycloadenosine:[42]



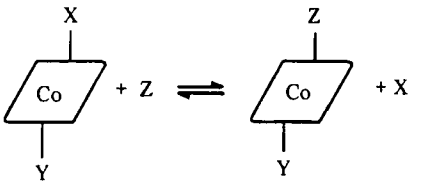
Aerobically, however, the products are OH–Cbl and adenosine-5′-aldehyde.[43] Very little 5′,8-cycloadenosine is formed under these conditions:



An important unsolved chemical problem which is pertinent to the mechanism of catalysis in AdoCbl-dependent reactions concerns the strength of the carbon-cobalt bond in alkyl cobamides. On the basis of the wavelength of the light which is able to cleave the bond photolytically, it can be concluded that the bond energy cannot exceed 50 kcal (roughly the strength of a Br–Br bond). In fact, it is likely that the bond energy is much lower than this value, since in one case (that of ethylcobalamin), homolysis has been induced by mild heating (60°C),[41] a process which introduces much less energy into the cobamide than would be imparted by the absorption of a 50-kcal photon. An accurate measurement of the activation energy for the thermolysis of the carbon-cobalt bond would be a valuable contribution to cobamide chemistry.

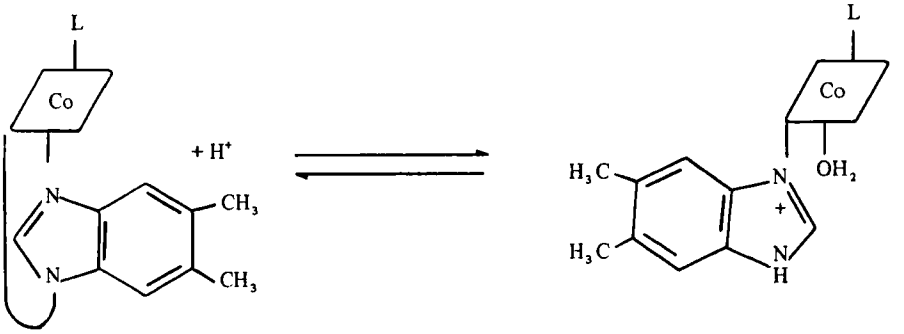
Ligand Exchange

An important group of reactions involving cobamides is the exchange of electronegative α or β ligands. Many electronegative ligands have been



shown to participate in such reactions.[44-46] These include groups in which the coordination to the metal is through a nitrogen ($-NH_3$, $-N_3$), oxygen ($-OH_2^+$, $-OR$), or sulfur atom ($-SH$, $-SO_3^-$). Cyanide also constitutes such a ligand, even though it is attached to the cobalt by its carbon atom.

An unusually well-studied class of such reactions is the displacement of the heterocyclic base from the α coordination position by a molecule of solvent. This occurs on acidification of the cobamide solution[47] and results from the protonation of the heterocycle, as illustrated here for a cobalamin:



For a given cobamide, the pK for the "base-on" to "base-off" equilibrium depends on the electron density associated with the metal, a parameter which in turn depends on the nature of the trans (i. e., β) ligand. The greater the electron-withdrawing power of the β ligand, the lower the pK of the transition. Thus, the pK for the replacement of an α-coordinated heterocycle by water is several units higher for alkyl cobamides than for the corresponding aqua cobamides (see Table 1).

The replacement of the α-coordinated heterocycle by water is associated with a characteristic change in the spectrum of the cobamide (Figure 6). The new spectrum is very different from the spectrum of the "base-on" cobamide, but resembles rather closely that of the paramagnetic Co(II)-containing cobamide.

TABLE 1

The pK for the Displacement of 5,6-Dimethylbenzimidazole by Water in a Series of Cobalamins

β Ligand	pK_a
H_2O	−2.4
CN^-	0.1
$HC \equiv C-$	0.7
$H_2C = CH-$	2.4
CH_3-	2.7
$CH_3CH_2CH_2-$	3.8
5′-deoxyadenosyl−	3.5

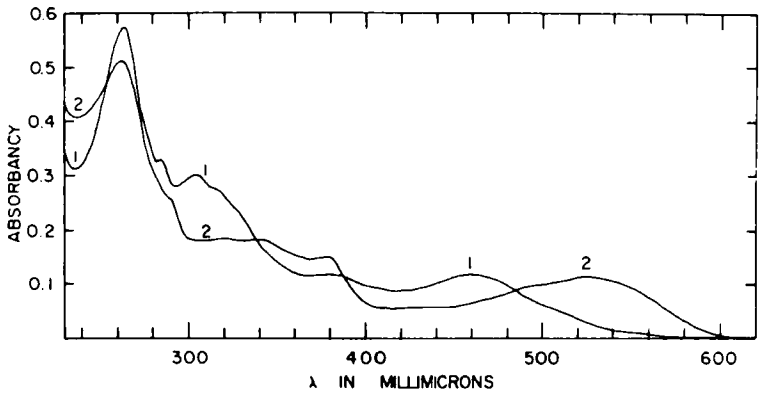


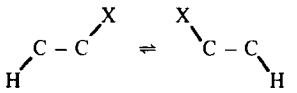
FIGURE 6. Spectrum of "base off" (pH 2.0; curve 1) and "base on" (pH 7.0; curve 2) AdoCbl.

ADENOSYLCOBAMIDE-DEPENDENT REACTIONS

Adenosylcobamides serve as cofactors for two types of enzyme-catalyzed reactions: a group of rearrangements and a reduction reaction in which a hydroxyl group is replaced by a hydrogen atom. While these two reaction types appear to be very different, the role played by the cofactor in the two cases will be seen to be similar.

Adenosylcobamide-Dependent Rearrangements

Adenosylcobamide-dependent rearrangements are all reactions in which a hydrogen atom moves from one carbon to an adjacent one in exchange for a substituent group X which migrates in the opposite direction:

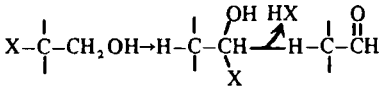


To date, ten such rearrangements have been reported (Table 2). They are conveniently classified into three groups. The first group consists of reactions in which vicinal diols or amino alcohols are converted to carbonyl compounds. Though these reactions are formally equivalent to dehydrations or deaminations, the evidence indicates that they proceed via 1,2 migration rather than β elimination:

TABLE 2

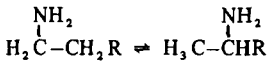
Adenosylcobalamin-Dependent Rearrangements

Enzyme	Reaction	Group X
Glutamate mutase	L-Glutamate → L-*threo*-β-methylaspartate	$-CH(NH_2)COOH$
Methylmalonyl-CoA mutase	L-Methylmalonyl-CoA → Succinyl-CoA	$-COSCoA$
α-Methyleneglutarate mutase	α-Methyleneglutarate → Methyl itaconate	$-C(=CH_2)COOH$
Diol dehydrase	Ethylene glycol → acetaldehyde 1,2-Propanediol → propionaldehyde	$-OH$
Glycerol dehydrase	Glycerol → β-hydroxypropionaldehyde	$-OH$
Ethanolamine ammonia lyase	Ethanolamine → acetaldehyde + NH_4^+ 2-Aminopropanol → propionaldehyde + NH_4^+	$-NH_2$
β-Lysine mutase	L-β-Lysine (L-3,6-diaminohexanoic acid) → 3,5-diaminohexanoic acid	$-NH_2$
α-Lysine mutase	D-Lysine → 2,5-diaminohexanoic acid	$-NH_2$
Ornithine mutase	D-Ornithine → 2,4-diaminopentanoic acid	$-NH_2$
Leucine aminomutase	L-Leucine → 3-aminoisocaproic acid	$-NH_2$



X=−OH,−NH_2

The second group is a series of reactions in which an interconversion between a primary and a secondary amine takes place through the migration of an $-NH_2$ group:



The third group, perhaps the most interesting of all, consists of reactions in which the migrating group is a bulky carbon-containing fragment.

Oxo-Forming Reactions

Diol Dehydrase

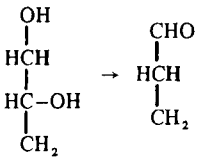
Diol dehydrase, an enzyme present in *Aerobacter aerogenes*, catalyzes the formation of oxo compounds from glycols. The substrates originally reported to be acted on by the enzyme were ethylene glycol and propylene glycol,[48] which were converted to acetaldehyde and propionaldehyde, respectively. Recent investigations have shown, however, that the enzyme is capable of dehydrating a remarkably broad range of substrates (see Table 3).[49-51] Of particular interest is the fact that diol dehydrase is able to convert 2,3-butanediol to methyl ethyl ketone, a finding of considerable mechanistic significance, as will be discussed below.

The stereochemistry of the diol dehydrase-catalyzed dehydration of propylene glycol is largely worked out.[52-54] Early kinetic studies[52] disclosed the surprising fact that diol dehydrase was able to act on both (R)- and (S)-propylene glycol with almost equal facility. Using substrates stereospecifically deuterated on the 1-carbon, it was possible to show that (R)-propylene glycol rearranged by migration of the *pro*-(R) hydrogen, while the *pro*-(S)-hydrogen migrated during the rearrangement of (S)-propylene glycol. With both substrates, the carbon which received the migrating hydrogen inverted its configuration. When the two stereoisomers of [1-^{18}O] propylene glycol were

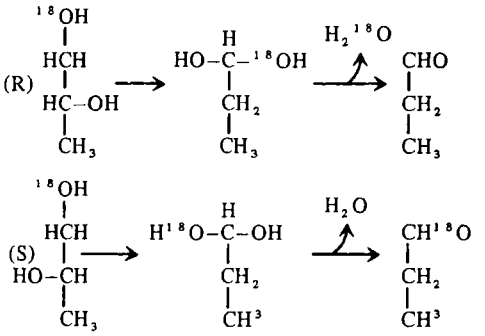
TABLE 3

Substrates for Diol Dehydrase

1,2-Ethanediol
1,2-Propanediol (R- and S-)
1,2-Butanediol
2,3-Butanediol
Glycerol (R- and S-)
2-Methyl-1,2-propanediol
2-Fluoro-1,2-propanediol



studied, the labeled oxygen was found to have been completely retained in propionaldehyde formed from the (S)-isomer, but was lost altogether when the (R)-isomer was the substrate.[55] It is clear from this observation that



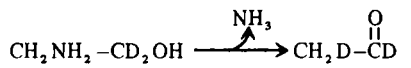
propionaldehyde formation in this reaction involves the migration of the oxygen atom followed by the elimination of water from the gem-diol, rather than the β elimination of water with subsequent tautomerization of the resulting enol. Furthermore, it can be concluded that the dehydration of the gem-diol is enzyme-catalyzed and not a spontaneous reaction occurring after the release of the gem-diol from the enzyme. If the latter were the case, the propionaldehyde from both enantiomers would have been expected to contain half the starting quantity of $^{18}0$.

Ethanolamine Ammonia Lyase

Ethanolamine ammonia lyase catalyzes the formation of acetaldehyde and propionaldehyde from ethanolamine and propanolamine (2-aminopropanol), respectively.[56,57] The enzyme appears to be able to act on both enantiomers of propanolamine, resembling diol dehydrase in this respect.* Unlike diol dehydrase, however, the range of substrates acted on by ethanolamine ammonia lyase appears to be restricted to the three listed (ethanolamine, and (R)- and (S)-propanolamine), at least on the basis of present information.

As is true with most adenosylcobamide-dependent reactions, the migration of hydrogen during ethanolamine ammonia lyase-catalyzed deaminations has been demonstrated directly by isotopic substitution.[58] Direct evidence is lacking,

* Krouwer, J. S. and Babior, B. M., unpublished.

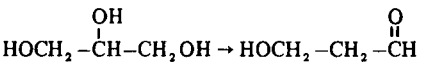


however, that the $-NH_2$ group migrates to produce a 1-amino alcohol during the course of the reaction. The possibility that the immediate product of the enzyme-catalyzed reaction is an aldimine produced by consecutive $-NH_2$ migration and dehydration which undergoes spontaneous hydrolysis upon release into the medium has been ruled out by experiments showing that the oxygen of ethanolamine is retained in the product.[58] The only other studies published to date bearing on this point have shown that NH_3 is necessary for the enzyme-catalyzed exchange of hydrogen between the cofactor and the product aldehydes.[59] This hydrogen exchange is a partial reaction of great catalytic significance, as will be seen. For the purposes of the present discussion, however, the important point is that this reaction requires NH_3. While this finding is consistent with the participation of a 1-amino alcohol in reactions catalyzed by ethanolamine ammonia lyase, it actually constitutes proof only for a catalytically significant association of some sort between NH_3 and the enzyme. Thus, the statement that reactions catalyzed by ethanolamine ammonia lyase involve $-NH_2$ group migration is based primarily on analogy with other adenosylcobamide-dependent rearrangements and is only weakly buttressed by experiment.

Stereochemical studies with this enzyme, though far from complete, have been unusually informative. With ^3H-substituted ethanolamine, it was shown that the enzyme distinguished stereochemically between the *pro*-(R) and *pro*-(S) hydrogens on the carbinol carbon, selecting one of the two to migrate.[58] It is not known which of the two meso hydrogens is the migrating atom. More interesting is the stereochemical fate of the amino carbon, which is converted to a methyl group in the product. The stereochemistry at this atom was examined by a double label technique which used ethanolamine chirally labeled with deuterium and tritium on the amino carbon. Stereospecific transfer of hydrogen to this carbon would yield a chiral methyl group (−CHDT) whose configuration would depend on whether the hydrogen migration took place with retention or inversion at the recipient carbon. To the surprise of the investigators, the analysis revealed the methyl group to be optically inactive.[60] Hydrogen migration therefore resulted in the recemization of the carbon atom at the migration terminus, an event which is quite unusual in enzyme-catalyzed reactions.

Glycerol Dehydrase

Of the adenosylcobamide-dependent rearrangements which give rise to carbonyl compounds, that catalyzed by glycerol dehydrase[61,62] has been the least studied from a mechanistic point of view. To date, the only compound shown to be a substrate for this enzyme is glycerol, which is converted to β-hydroxypropionaldehyde. The reaction probably involves an exchange of

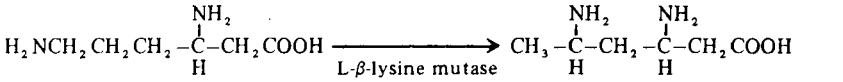


places between a hydrogen and a hydroxyl group, but studies to demonstrate this with isotopically labeled substrates have not been carried out. Similarly, there have been no studies on the stereochemistry of this reaction.

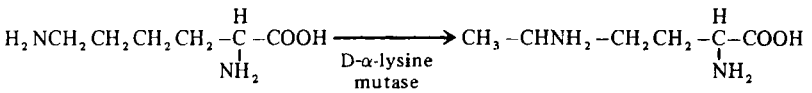
Interconversion between Amines

Lysine and Ornithine Mutases

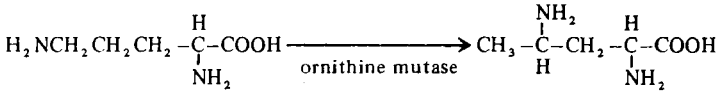
This group of mutases comprises three closely related adenosylcobamide-requiring enzymes which catalyze rearrangements involving amino acids additionally substituted in the ω position with $-NH_2$. The three enzymes are L-β-lysine mutase, D-α-lysine mutase, and D-ornithine mutase, and they catalyze the following reactions:[63-72]



L-β-Lysine — L-Erythro-3,5-diaminohexanoic acid (63–66)



D-α-lysine — D-2,5-Diaminohexanoic acid (67,68) (The configuration of the 4-amino group is not established.)



D-Ornithine — D-Threo-2,4-diaminovaleric acid (69–72)

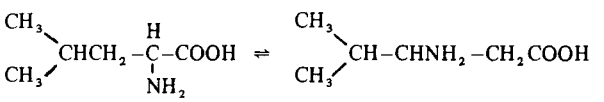
These enzymes all show a high degree of specificity for their substrates and are inhibited by closely related substrate analogues.[72,73]

Unlike the ethanolamine ammonia lyase reaction, in which the direct experimental evidence supporting $-NH_2$ migration is weak, the reactions catalyzed by lysine and ornithine mutase clearly proceed by way of amino group migration. The migration is probably intramolecular, although experiments to exclude the possibility that an $-NH_2$ can be transferred between molecules have not been performed. However, it has been shown, at least with β-lysine mutase, that amino group migration occurs without the participation of free NH_4^+.[74]

A fact of considerable interest regarding these enzymes is that in addition to adenosylcobamide, they all require pyridoxal phosphate for catalytic activity.[64,72,73,75] The role of pyridoxal phosphate in other enzymatic reactions involving amines is well understood, and its participation here raises interesting questions regarding the mechanism of amino group migration in these reactions which will be dealt with subsequently at greater length. It is worth noting here, however, that β-lysine mutase catalyzes a slow pyridoxal phosphate-dependent exchange of hydrogen between the solvent and the 6 carbon of its substrate,[67] an observation which strongly suggests the enzyme-dependent formation of a Schiff base between the pyridoxal and the terminal amino group of the substrate.

Leucine Aminomutase

Leucine aminomutase, the most recently discovered adenosylcobamide-requiring enzyme, catalyzes the interconversion of α- and β-leucine.[76,77]



L-α-leucine ⇌ β-leucine

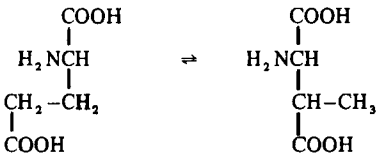
This reaction, about which little is known mechanistically, since it was only discovered in 1976, shows a remarkably wide distribution among living organisms. While most other adenosylcobamide-dependent reactions are restricted to unicellular organisms, the leucine aminomutase reaction has been demonstrated in both higher animals and plants. The discovery of an adenosylcobamide-requiring enzyme in the plant kingdom was a remarkable event, since plants had universally been considered to require no cobamides for their metabolism.

Carbon Skeleton Rearrangements

Glutamate Mutase

Glutamate mutase, which has been studied in detail by Barker and his associates, was the first enzyme for which a cobamide requirement was demonstrated.[78] Although the cobamides, under the names of "antipernicious anemia principle," "extrinsic factor," and later "vitamin B_{12}", had been known since 1926,[79] a biological function for this family of compounds was not discovered until 1958, when Barker and associates found that they were necessary for the glutamate mutase reaction. Moreover, it was the same group of workers, studying the cobamide requirement for the enzyme, who discovered the coenzymatically active forms of the cobamides, with their light-sensitive carbon-cobalt bonds.[78]

Glutamate mutase catalyzes the interconversion of L-glutamic acid and β-methyl aspartic acid.[80-82] In this reaction, the migrating X group

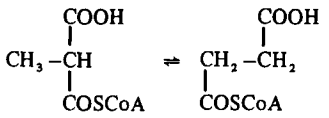


is a glycyl residue, which is transferred between carbons without exchange with free glycine, acrylate, propionate, or α-ketoglutarate.[83] Unlike diol dehydrase, but like the aminomutases, glutamate mutase is highly specific for its substrates;[81,82] none of a number of compounds related chemically to glutamate or β-methylasparate are acted on by the enzyme.

The stereospecificity of the glutamate mutase reaction is also high, a rule which is obeyed in most enzyme-catalyzed reactions but is violated frequently, as discussed above, by adenosylcobamide-requiring enzymes. L-glutamate is converted exclusively to L-*threo*-methylaspartate and vice versa.[80,83] Stereospecificity extends to hydrogen migration as well, since it has been shown that the transfer of hydrogen from the methyl to the methylene carbon during the rearrangement of methylaspartate to glutamate results in the inversion of configuration of the carbon atom which receives the migrating hydrogen atom.[84,85]

Methylmalonyl CoA Mutase

Methylmalonyl CoA mutase catalyzes the interconversion of methylmalonyl CoA and succinyl CoA, a reaction which involves exchange of places between a hydrogen atom and the bulky −COSCoA



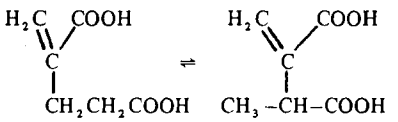
group.[86-92] Methylmalonyl CoA mutase is one of only two adenosylcobamide-requiring enzymes which have been found in mammalian tissues[93] (the other is leucine aminomutase). In mammals, the methylmalonyl CoA mutase reaction is a key metabolic step in the pathway which catabolizes propionyl CoA,[91] a compound whose accumulation is thought to be responsible for the neurological manifestations of cobamide deficiency in humans.[94,95]

Like glutamate mutase, methylmalonyl CoA mutase shows a high degree of stereospecificity. With regard to the substrate, the enzyme acts on L-methylmalonyl CoA, but has no effect on the D-enantiomer.[96] Hydrogen migration is also stereospecific. When methylmalonyl CoA is converted to succinyl CoA, the transfer of hydrogen

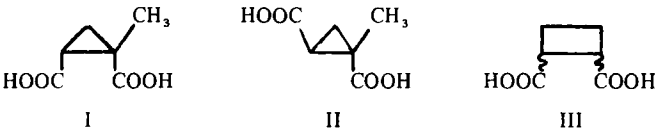
occurs with retention of configuration at the carbon terminus;[96,97] this stereochemical outcome is the opposite of that seen in the glutamate mutase reaction. Experiments with ethylmalonyl CoA, an alternative substrate which is rearranged by the mutase to α-methylsuccinyl CoA, have shown that the −CoSCoA group, too, migrates with retention of configuration at the migration terminus.[98]

α-Methyleneglutarate Mutase

This enzyme, the most recent of the carbon skeleton mutases to be discovered, catalyzes the interconversion between α-methylene glutarate and methyl itaconate.[99-101] In this reaction, the migrating group



is an acrylyl residue (−C (=CH_2)COO^-). A number of cyclic dicarboxylic acids, examined because of mechanistic considerations, have been shown not to be substrates for the enzyme. These include 1-methyl-1,2-*cis*- (I) and 1-methyl-1,2-*trans*-cyclopropanedicarboxylate (II), and 1,2-cyclobutanedicarboxylate (III), as well as malate, succinate, glutaconate, mesaconate, and itaconate.[102]



Nothing is known about the stereochemistry of this reaction.

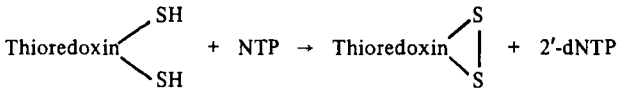
Ribonucleotide Reductase

Ribonucleotide reductase catalyzes the conversion of ribonucleotide phosphates to 2′-deoxyribonucleotide phosphates. This reaction, which is in the metabolic pathway responsible for providing the building blocks used in the synthesis of DNA, is obviously indispensible for all life. It is not surprising, then, that this enzyme is distributed ubiquitously in living organisms. In most species, including mammals, the enzyme is an iron-containing protein which requires no cofactor and which uses ribonucleotide diphosphates as substrates.[103-105] However, in certain unicellular organisms, including the flagellate *Euglena gracilis* as well as a number of bacteria, ribonucleotide reduction is accomplished by an iron-free adenosylcobamide-requiring enzyme[106,107] which uses the nucleoside triphosphates as substrates.[108-112]

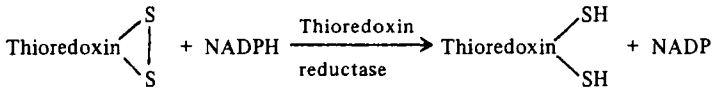
The most extensively studied of the cobamide-requiring ribonucleotide reductases is the enzyme from *Lactobacillus leichmannii*, whose properties have been investigated in detail both in crude and more recently in pure preparations. This enzyme catalyzes the reduction of all four ribonucleotide triphosphates[109,111,113] to the corresponding deoxyribonucleotide triphosphates. It also shows some activity with ribonucleotide diphosphates. With the triphosphates, the rates of reduction differ widely among the specific nucleotides; for instance, the conversion of ATP to dATP is over ten times as fast as the conversion of CTP to dCTP in a system containing only enzyme, substrate, and reducing agent. Rates of substrate consumption, however, vary in an exceedingly complex fashion with changes in the nucleotide composition and Mg^{++} concentration in the reaction mixture.[109,111,113] If dATP is added to the reaction mixture, for example, the ratio for the reaction rate with ATP vs. the rate with CTP changes from a value of 10:1 favoring ATP to a figure of 6:1 favoring CTP.[111] All the deoxynucleotide products exert this type of effect on the activity of ribonucleotide reductase, the exact

pattern of rate alterations being characteristic for each individual dNTP. The ribonucleotide substrates also alter the activity of ribonucleotide reductase, although their effects are smaller in magnitude than those of the dNTP products. These subtle alterations in the activity of ribonucleotide reductase by nucleotide and deoxynucleotide triphosphate, the purpose of which is certainly to regulate the tissue concentrations of these essential nucleic acid precursors, appear to be accomplished through the allosteric binding of the regulating nucleotide at a location remote from the active site of the enzyme. Ribonucleotide reductase thus appears to bind nucleoside triphosphates at two locations: the catalytic site, where the reduction reaction takes place, and the regulatory site, through which are mediated the effects of nucleoside substrates and products on the catalytic properties of the enzyme.[111]

The electron donor for the ribonucleotide reductase reaction is thioredoxin, a small protein found in both bacterial and mammalian systems which is used as the reducing agent by both the cobamide-requiring and the iron-requiring enzymes.[114-116]* Electron donation by this protein involves the oxidation of a pair of −SH groups to a disulfide:[117]



Oxidized thioredoxin is then reduced back to the dithiol by NADPH in a reaction which is catalyzed by a flavoenzyme, thioredoxin reductase:[115,118-120]



In the test tube, the thioredoxin-thioredoxin reductase system can be replaced by a number of dithiol compounds, including such low-molecular-weight dithiols as dihydrolipoic acid, dithiothreitol, and dithioerythritol.[110,117,121] For the sake of convenience, these are the reductants usually employed in studies on ribonucleotide reductase.

The course of the reaction has been studied with ^{18}O-labeled ATP. This study, which was carried out with 2′-^{18}O- and 3′-^{18}O-labeled substrate, showed that during the reduction of the nucleotide the 2′ oxygen was entirely lost and the 3′ oxygen completely retained, but that in the absence of a reducing agent, neither of the two oxygens exchanged with water.[122] Stereochemical analysis of the product showed that the hydrogen added to the 2′ carbon during the reduction reaction was found exclusively in the position formerly occupied by the 2′ hydroxyl group.[123-126] In the ribonucleotide reductase reaction, then, the 2′ hydroxyl group is replaced by hydrogen with retention of configuration at the 2′ carbon atom.

THE MECHANISM OF ADENOSYLCOBAMIDE-DEPENDENT REACTIONS

All adenosylcobamide-dependent reactions involve the displacement by hydrogen of another group, designated group X, whose structure varies from reaction to reaction. In the rearrangements, the displacing hydrogen originates from an adjacent carbon of

* A T7 phage-resistant mutant of *E. coli* with an otherwise normal phenotype has recently been shown to be devoid of thioredoxin.[116a] Ribonucleotide reduction in this mutant is accomplished at the expense of reduced glutathione and is catalyzed by a hitherto unrecognized enzyme designated glutaredoxin. Glutaredoxin has subsequently been found in wild-type *E. coli*. There are thus at least two enzymes, thioredoxin and glutaredoxin, which are able to catalyze the reduction of ribonucleotides to deoxyribonucleotides. Which of these two reductases is physiologically more important for DNA synthesis remains to be established.

the substrate molecule, and the displaced group migrates to the carbon from which the displacing hydrogen departed. The net result is an exchange of places between the hydrogen and group X. In the ribonucleotide reductase reaction, the displacing hydrogen originates from the reducing agent and the displaced group (in this case, −OH) is released into the medium.

In analyzing the mechanism of adenosylcobamide-dependent reactions, it is useful to consider hydrogen transfer and group X displacement separately. In the discussion to follow, it will become clear that enzymological studies over the past 10 years have succeeded in providing an explanation, at least in outline, for the mechanism of hydrogen transfer in these reactions. The mechanism of group X displacement, however, is still a puzzle.

Hydrogen Transfer

The initial pieces of evidence regarding the mechanism of hydrogen transfer in adenosylcobamide-dependent reactions were obtained through studies of the rearrangements. The chemical formulas for these rearrangements show that they involve a formal transfer of hydrogen from one carbon atom to an adjacent one. Experiments with isotopically labeled substrates suggested that this was true in a literal sense as well. Substrates in which the migrating hydrogen atom was replaced with deuterium or tritium retained the label, which was found in the expected location in the product.[58,72,91,100,127-130] Moreover, there was no exchange between the migrating hydrogen atom and solvent protons, as established from experiments with isotopically labeled water. The natural conclusion drawn from these experiments was that hydrogen transfer in adenosylcobamide-dependent rearrangements took place by intramolecular migration. Further, it was widely assumed that the hydrogen migrated as a hydride ion, an assumption based on the failure of product to incorporate protons from the solvent; Iodice and Barker, however, pointed out that in fact no conclusions could be drawn from the solvent exchange data regarding the nature of the migrating species.[129]

This was the general view until 1966, when a series of experiments carried out with diol dehydrase[131] disclosed that the previously held notions regarding hydrogen transfer were only partially correct and provided the key to understanding the mechanism of this process. In this series of experiments, the ability of diol dehydrase to act on both ethylene glycol and propylene glycol was exploited to study the fate of the migrating hydrogen atom. In the crucial experiment, diol dehydrase was incubated with a mixture of unlabeled ethylene glycol plus propylene glycol in which the migrating hydrogen was labeled with tritium. Analysis of label in the products (Table 4) showed that tritium was present in both propionaldehyde and acetaldehyde, even though only the three-carbon substrate had initially been labeled. This could only have come about by the transfer of tritium from one molecule to another during the course of the reaction. This experiment showed that hydrogen transfer was *inter*molecular, at least in part, instead of exclusively *intra*molecular, as originally thought.

The Cofactor as Intermediate Hydrogen Carrier

One way in which the transfer of hydrogen between molecules could be accomplished by an adenosylcobamide-dependent enzyme would be for the enzyme-cofactor complex to play the role of intermediate hydrogen carrier, accepting the migrating hydrogen atom from one substrate molecule and then donating it at some later time to product derived from a second substrate molecule. According to this mechanism, substrate labeled with tritium in the position of the migrating hydrogen atom should transfer label to the enzyme-cofactor complex during the course of the reaction. This possibility was initially examined with the diol dehydrase system,[132-134] using AdoCbl

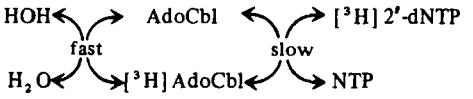
TABLE 4

Intramolecular Hydrogen Transfer in the Reaction Catalyzed by Diol Dehydrase

Starting material	^3H (% of total) in product	
	Acetaldehyde	Propionaldehyde
1,2-Propanediol-1-T plus ethylene glycol	30	62
1,2-Propanediol-1-T plus acetaldehyde	0	100

as cofactor and [1-^{3}H]propylene glycol as the labeled substrate. The experiment showed that during the course of the reaction, tritium was transferred from the substrate to the cobalt-linked carbon atom (hereafter referred to as the 5′ carbon) of AdoCbl, and that cofactor labeled in this manner was subsequently able to transfer tritium to the reaction product. These results strongly suggested that the cofactor served as an intermediate hydrogen carrier in adenosylcobamide-requiring reactions.

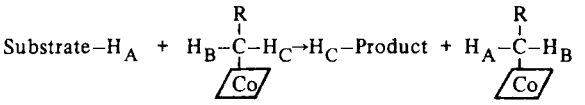
These results were soon confirmed with other adenosylcobamide-dependent enzymes.[67,100,135-145] In the case of the mutases, the transfers of hydrogen occurred between substrate, cofactor, and product; solvent did not participate. In those reactions in which the overall rearrangement is reversible, tritium originally in the cofactor exchanged into both the substrate and the product. In the irreversible rearrangements — the diol dehydrase-catalyzed reactions and the deamination of ethanolamine* by ethanolamine ammonia lyase — tritium was transferred from substrate to cofactor, but could not be transferred back. Exchange did take place, however, between the cofactor and the reaction products when these were incubated together with the respective enzymes in the absence of substrate.[59,133] In contrast to the rearrangements, solvent protons participated in the hydrogen transfer reactions involving ribonucleotide reductase.[144] When ribonucleotides were reduced in the presence of ^3H_2O, tritium was incorporated into both the coenzyme and the product. Even in the absence of a reducible substrate, exchange between ^3H_2O and coenzyme took place as long as dihydrolipoic acid and a suitable allosteric effector were present. When tritiated cofactor was used, only the solvent became labeled during ribonucleotide reduction; the product contained no tritium. This labeling pattern can be explained by invoking an exchange reaction between solvent protons and the 5′ position of the cofactor which is rapid compared with the rate of reduction of NTP to dNTP, so that when tritiated cofactor was used in the reaction, the dilution of isotope on the 5′ position by exchange with solvent was complete before significant amounts of dNTP were produced. With some of the enzymes, the tritium was shown to be attached to the 5′



carbon of the cofactor, usually by demonstrating that the enzyme in question exchanged tritium in and out of the same position which is labeled in the diol dehydrase reaction.[57,100,133,136,142] With other enzymes, the location of the substrate-derived tritium has not been established, but the likelihood is overwhelming that in these instances, too, the label is in the 5′ position.

* The deamination of propanolamine by this enzyme is slightly reversible.[57]

To explore further the mechanism of hydrogen migration in adenosylcobamide-requiring reactions, detailed studies were carried out on the hydrogen transfer reactions catalyzed by diol dehydrase. Two points emerged from these studies. First, the transfer of tritium both from substrate to cofactor and from cofactor to product were catalytically competent processes;[146] that is, they occurred at a rate consistent with their participation as steps in the catalysis of glycol dehydration. Second, during catalysis the migrating hydrogen equilibrates with the two 5′ hydrogen atoms of the cofactor before being transferred to the product. Equilibration of these three hydrogen atoms during catalysis was initially suspected from the finding that both the *pro*-R and *pro*-S 5′-H atoms were transferred to product during the reaction.[132,134] The involvement of both enantiomeric hydrogen atoms was unexpected for an enzyme-catalyzed reaction and suggested that hydrogen exchange in this system involved either obligatory transfer to the product molecule of a hydrogen atom distinct from the one abstracted from the substrate (the so-called merry-go-round mechanism),



The merry - go - round

or equilibration between substrate and cofactor hydrogen atoms as described above. The choice between these two mechanisms was made possible by the demonstration that there was always a finite chance that a given product molecule would receive the hydrogen which was attached to it before rearrangement.[132,146] This observation ruled out the merry-go-round mechanism and left equilibration as the likely explanation for the participation of both enantiomeric 5′-H atoms in the diol dehydrase reaction. Finally, an ingenious series of experiments involving comparisons between the transfer of tritium from [5′-^{3}H]AdoCbl to product derived from deuterated as opposed to undeuterated substrate established that at least three hydrogens were involved in the equilibration between the substrate and the cofactor.[146]

Studies with other enzymes have confirmed that the mechanism of hydrogen transfer involves equilibration between the migrating hydrogen of the substrate and the two 5′-H atoms of the coenzyme. With glutamate mutase[141] and methylmalonyl CoA mutase,[140] enzymes which catalyze reversible adenosylcobamide-dependent rearrangements, experiments were performed in which the deuterium content of the products obtained from defined mixtures of undeuterated and specifically deuterated substrates were compared with the values predicted on the basis of various hydrogen transfer models, including both merry-go-round and equilibration mechanisms, taking into consideration the deuterium isotope effects. In both cases, the experimental figures agreed most closely with an equilibration model involving three hydrogen atoms. With ribonucleotide reductase, in which the substrate originally carrying the migrating hydrogen is a sulfhydryl compound, all the tritium in a preparation of AdoCbl labeled equally in the two 5′ positions was lost to solvent during the dihydrolipoate-dependent exchange reaction,[142] suggesting that both 5′-H atoms participate in the reaction. Supporting this notion was the additional finding[145] that exchange of hydrogen in the opposite direction (i. e., from ^3H_2O to AdoCbl) did not reach completion until the coenzyme had acquired 1.4 (i. e., > 1) g-atoms of hydrogen per mol.

The conclusion from these studies is that hydrogen transfer in adenosylcobamide-dependent reactions involves the coenzyme as an intermediate hydrogen carrier. The migrating hydrogen is transferred from the substrate to the 5′-carbon of the coenzyme, where it equilibrates with the two hydrogen atoms already attached to the 5′-carbon. After equilibration, hydrogen is returned from the cofactor to the final product.

5′-Deoxyadenosine

The transfer of hydrogen from substrate to cofactor which was shown to occur during catalysis indicates that adenosylcobamide-requiring enzymes must be able to alter the cofactor in such a way as to provide a place on the 5′ carbon to which a substrate hydrogen atom can become attached. Enzyme-catalyzed cleavage of the carbon-cobalt bond would clearly furnish such a place. Moreover, a substrate hydrogen atom transferred to this carbon would become equivalent to the two 5′-H atoms originally on the 5′ carbon of the intact coenzyme, as required by the experimental observations.

The compound formed by such a hydrogen transfer would be 5′-deoxyadenosine. The participation of this compound in adenosylcobamide-dependent reactions was first proposed on theoretical grounds by Ingraham in 1964.[147] Evidence in support of this proposal was obtained two years later in a study on the reaction between diol dehydrase, AdoCbl and the substrate analog, glycolaldehyde.[148] In this study, it was shown that the addition of glycolaldehyde to a mixture of enzyme and AdoCbl led to the rapid destruction of the cofactor by cleavage at the C-Co bond. The products resulting from the cleavage of the cofactor were identified as 5′-deoxyadenosine and a then uncharacterized cobalamin derivative, while the glycoladehyde was oxidized to glyoxylic acid. The third hydrogen in the methyl group of 5′-deoxyadenosine was found to have originated with glycolaldehyde.

Subsequent studies have shown that the production of 5′-deoxyadenosine from AdoCbl by adenosylcobamide-requiring enzymes in the presence of appropriate substrates or substrate analogues is a very general reaction.[59,75,148-155] A well studied example is the cleavage of AdoCbl by ethanolamine ammonia lyase in the presence of ethylene glycol.[149] Cleavage under these conditions is quite rapid ($k = 0.2\ sec^{-1}$) and gives rise to stoichiometric amounts of acetaldehyde, 5′-deoxyadenosine and an unidentified cobamide which decomposes to OH—Cbl on denaturation of the enzyme. In this reaction, too, the third hydrogen in the methyl group of 5′-deoxyadenosine originated with the substrate analogue. Other mutases, including methylmalonyl CoA mutase and L-β-lysine mutase, have also been shown to generate 5′-deoxyadenosine from AdoCbl in the presence of appropriate substrates or analogues (see Table 5 for a list of enzyme-analogue systems in which the production of 5′-deoxyadenosine from AdoCbl has been demonstrated). Ribonucleotide reductase also cleaves AdoCbl to give rise to 5′-deoxyadenosine. With this enzyme, requirements for cleavage are a suitable thiol (the hydrogen-donating substrate) and an effector nucleotide capable of activating the enzyme by binding to the allosteric site.[153,154]

TABLE 5

Formation of 5′-Deoxyadenosine from AdoCbl by Enzymes which Catalyze Adenosylcobamide-Dependent Rearrangements

Enzyme	Substrate or analogue with which 5′-deoxyadenosine formation has been demonstrated
Diol dehydrase	Glycolaldehyde
	Chloroacetaldehyde
Methylmalonyl CoA mutase	Malonyl CoA
	Succinyl CoA
L-β-Lysine mutase	L-Erythro-3,5-diaminohexanoate
Ethanolamine ammonia-lyase	Ethylene glycol
	Acetaldehyde
	Ethanolamine
	2-Aminopropanol

TABLE 6

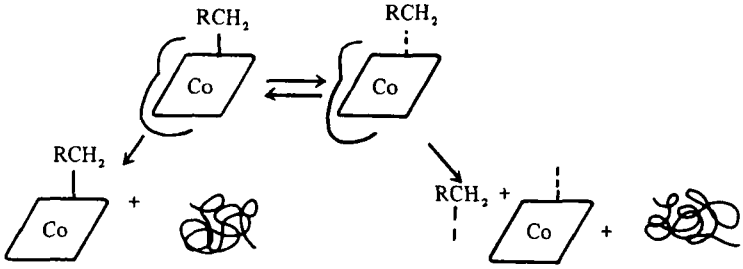
Fraction of Cofactor in the Form of 5′-Deoxyadenosine as a Function of the Denaturing Agent

Substrate	Denaturing agent	Fraction as 5′-deoxyadenosine (%)
Ethanolamine	Hot 1-propanol	3.6
	Tetrahydrofuran	1.6
Propanolamine	Trichloroacetic acid	86.9
	Heat	89.3
	Ethanol	12.7

The fact that adenosylcobamide-requiring enzymes are able to cleave AdoCbl to form 5′-deoxyadenosine, a reaction which in some cases has been shown to involve the transfer of hydrogen from a substrate analogue to the 5′ carbon, strongly suggests that 5′-deoxyadenosine participates in the catalysis of hydrogen transfer by these enzymes. Clouding this interpretation, however, is the fact that the reactions discussed so far all generate 5′-deoxyadenosine by an irreversible path. On the basis of the irreversibility of these reactions, it can be argued that 5′-deoxyadenosine is actually the product of an abortive pathway which leads to inactivation of the catalytic species by destruction of the cofactor, and has nothing to do with the catalysis of hydrogen transfer *per se.*

To settle this question, a number of studies have been carried out to try to show that 5′-deoxyadenosine can be formed reversibly from AdoCbl by adenosylcobamide-requiring enzymes. Most of these studies have involved attempts to demonstrate an enzyme-catalyzed exchange between AdoCbl and 5′-deoxyadenosine. Such an exchange has never been shown, indicating that free 5′-deoxyadenosine is not formed reversibly from AdoCbl. Using another approach, however, it has been possible to show that enzyme-bound 5′-deoxyadenosine can be formed reversibly from AdoCbl.

This approach involved an examination of the status of the carbon-cobalt bond of AdoCbl bound to ethanolamine ammonia lyase. The first evidence for the utility of such an approach was the observation that when ethanolamine ammonia-lyase was engaged in the catalysis of ethanolamine deamination, a small fraction of the cofactor released from the enzyme by denaturation was in a form in which the carbon-cobalt bond was broken.[150] Identification of the adenosyl portion of this form of the cofactor revealed it to be 5′-deoxyadenosine. The evidence that carbon-cobalt bond cleavage under these conditions was reversible consists of the finding that the extent of cleavage varied with denaturing agent (Table 6). This sort of variation is expected if (a) the cofactor is tightly bound to the enzyme, (b) the enzyme-cofactor complex consists of a mixture of two equilibrating forms, one containing intact cofactor and the other containing cofactor in which the C-Co bond is dissociated, and (c) there is a difference in the susceptibility of the two forms of the enzyme-cofactor complex to inactivation by a given denaturing agent. For example, if the two forms are equally susceptible to denaturing agent A, but the form with the intact cofactor is more susceptible than the form with the dissociated cofactor to denaturing agent B, then treatment with agent A will release intact and broken cofactor in ratios proportional to their equilibrium distribution, while treatment with agent B will release a disproportionate amount of intact cofactor as the equilibrium is pulled to the left by the differential depletion of the form of the enzyme-cofactor complex



containing intact adenosylcobamide. In contrast, if cleavage is irreversible, the proportion of intact vs. broken cofactor should be the same for similar incubation conditions regardless of the identity of the denaturing agent. These experiments thus provide evidence for the reversible formation of 5′-deoxyadenosine from AdoCbl by an adenosylcobamide-requiring enzyme engaged in catalysis.

Inspection of the data presented in Table 6 shows that while the evidence for reversibility is suggestive, the numbers are not convincing. The differences between the percentages of broken cofactor released by the two denaturing agents were highly significant by statistical analysis ($p < 0.0001$), but the magnitude of cleavage in the presence of ethanolamine was quite small with both denaturing agents. Much more persuasive evidence was provided through the use of 2-aminopropanol.[155] As mentioned before, this compound is a true substrate for ethanolamine ammonia lyase, and in addition promotes irreversible cleavage of the carbon-cobalt bond, a reaction which takes several minutes to reach completion. In accord with the relatively slow rate of irreversible carbon-cobalt bond cleavage, a reaction mixture containing enzyme, AdoCbl, and propanolamine treated with ethanol at 15 sec released only 12% of the cofactor in a form in which the carbon-cobalt bond was broken (Table 6). Treatment of a similar reaction mixture at 15 sec with trichloroacetic acid or heat, however, released 90% of the cofactor in the dissociated form. These results indicate that reversible cleavage of the carbon-cobalt bond of AdoCbl occurs when propanolamine is used as substrate in the ethanolamine ammonia lyase reaction, and that in the steady state, most of the enzyme-bound cofactor is in the form in which the C-Co bond is dissociated. The adenosyl portion of the dissociated cofactor was identified by both chromatography and chemical degradation as 5′-deoxyadenosine. Through the use of deuterium-labeled propanolamine and AdoCbl, it was possible to show that the third hydrogen on the methyl group of this 5′-deoxyadenosine was derived from the substrate.[156]

Taken together, the results of the denaturation experiments with the two substrates suggested that in steady state, the extent of cleavage of the C-Co bond of the AdoCbl at the active site of the catalyzing enzyme was much smaller with ethanolamine than with propanolamine. If this were true, and if C-Co cleavage were truly reversible, then the addition of excess ethanolamine to a reaction mixture containing enzyme, AdoCbl, and propanolamine should cause the extent of cleavage of enzyme-bound cofactor to fall from the very high value seen with propanolamine to the much lower figure characteristic of ethanolamine. To examine this possibility, the following experiment was performed.[155] Two reaction mixtures were prepared, each containing ethanolamine ammonia-lyase, AdoCbl labeled with ^{14}C in the adenosyl portion, and 2-aminopropanol labeled with ^{3}H in the position of the migrating hydrogen atom. At 15 sec, one incubation was terminated with trichloroacetic acid, while the other received a large quantity of ethanolamine. This second incubation was terminated 1 min later with trichloroacetic acid. Analysis of the radioactive material obtained from the incubation terminated at 15 sec showed that most of the cofactor was in the dissociated form (shown by the fact that 85% of the ^{14}C was in 5′-deoxyadenosine and only 15% in intact AdoCbl) as expected, and that tritium from the substrate was present in 5′-deoxyadenosine and AdoCbl at the ratio predicted on the assumption that the dissociated

TABLE 7

Reversible Formation of 5′-Deoxyadenosine

Compound	Counts (% of total)			
	TCA only		Ethanolamine, then TCA	
	^3H	^{14}C	^3H	^{14}C
5′-Deoxyadenosine	91	83	3	11
Adenosylcobalamin	9	17	3	89
Acetaldehyde	—	—	94	—

and intact forms of the cofactor are in rapid equilibrium (Table 7). When the labeled material from the ethanolamine-treated reaction mixture was analyzed, however, most of the ^{14}C was found in intact AdoCbl, indicating that the C-Co bond, which was mostly dissociated before ethanolamine was added, had largely reformed following the addition of the second substrate. Moreover, the tritium which had been present in both forms of the cofactor was almost entirely washed out into acetaldehyde, the product arising from the deamination of ethanolamine. These results show not only that the C-Co bond of the cofactor was reconstituted by the change in substrate, but also that the cofactor so formed was catalytically active, in that it was able to transfer ^3H into the product during the deamination of the new substrate.

The foregoing experiments leave little doubt that 5′-deoxyadenosine participates in the catalysis of hydrogen transfer by ethanolamine ammonia lyase. From the observations concerning 5′-deoxyadenosine which have been made with other adenosylcobamide-requiring enzymes, and by analogy with hydrogen transfer by ethanolamine ammonia lyase, it seems likely that 5′-deoxyadenosine is an intermediate in all adenosylcobamide-dependent hydrogen transfer reactions.

Species with Unpaired Electrons

It is clear from the preceding discussion that catalysis by adenosylcobamide-dependent enzymes involves the reversible cleavage of the C-Co bond of the cofactor. Chemical investigations have disclosed many routes by which the C-Co bond can be cleaved, any one of which could in principle be involved in the enzyme-catalyzed reactions. Enzymological studies, however, indicate that C-Co cleavage occurs by homolysis, and that the transfer of hydrogen from the substrate to the adenosyl residue of the cofactor takes place by a free radical mechanism.

Evidence for the intermediacy of species with unpaired electrons was first reported in 1969.[157-159] Studies prior to that date had shown that during catalysis, diol dehydrase[147,160] and ethanolamine ammonia-lyase[161] underwent spectral changes consistent with the occurrence of C-Co bond homolysis, but the similarities in optical spectra among cobamide derivatives of diverse structure precluded an unambiguous interpretation of those results. In 1969, however, it was shown by EPR spectroscopy that unpaired electrons are generated by a system containing ethanolamine, AdoCbl, and ethanolamine ammonia lyase.[157] Although the signals observed in those experiments were small (the total spin concentration was 5% that of the active sites), broad, and poorly resolved, they were interpreted as evidence for the participation of species with unpaired electrons in the ethanolamine ammonia-lyase reaction. If substrate was omitted from the system, another signal was observed, smaller and with different characteristics. The appearance of this signal, together with later observations made with radioactively labeled AdoCbl, suggested that even in the absence of substrate, ethanol-

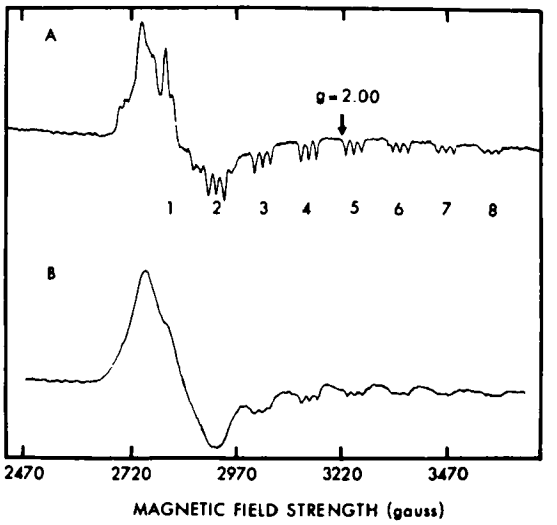


FIGURE 7. EPR spectra of cob(II)alamin formed from AdoCbl by ribonucleotide reductase.

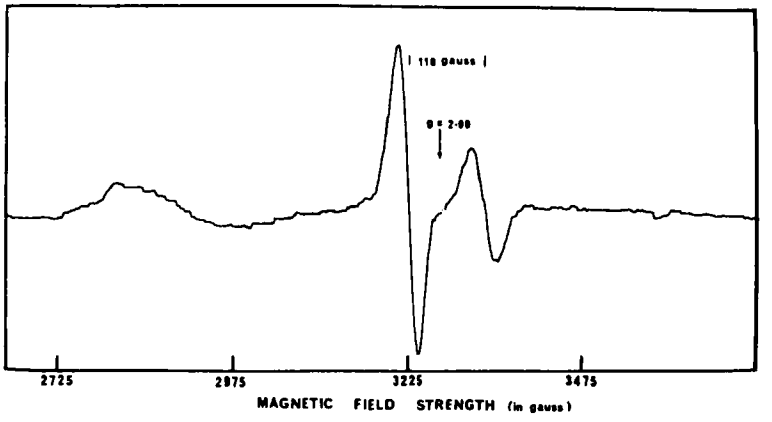


FIGURE 8. EPR spectrum of the second paramagnetic species produced by ribonucleotide reductase.

amine-ammonia lyase homolytically splits the C-Co bond of bound cofactor, albeit to a very limited extent.

EPR signals with ribonucleotide reductase were also reported in 1969. The first report[158] described a signal clearly due to cob(II)alamin which appeared gradually in a system containing enzyme, thiol, and dGTP (an allosteric effector) (Figure 7). The second[159] reported a different type of signal consisting of a broad peak assigned to cob(II)alamin and an unassigned doublet at higher field, whose appearance required reducible substrate (Figure 8). Because the signals appeared so slowly in the reaction mixture, they were attributed to products formed during a side reaction and were thought to be without direct catalytic significance.

The other adenosylcobamide-requiring enzymes which have been shown to generate paramagnetic species during catalysis are diol dehydrase and glycerol dehydrase. The signals in both cases were similar to those observed with ribonucleotide reductase, consisting of a broad peak assigned to cob(II)alamin and a higher field doublet. EPR signals have thus been demonstrated with all of the enzymes which catalyze oxo-form-

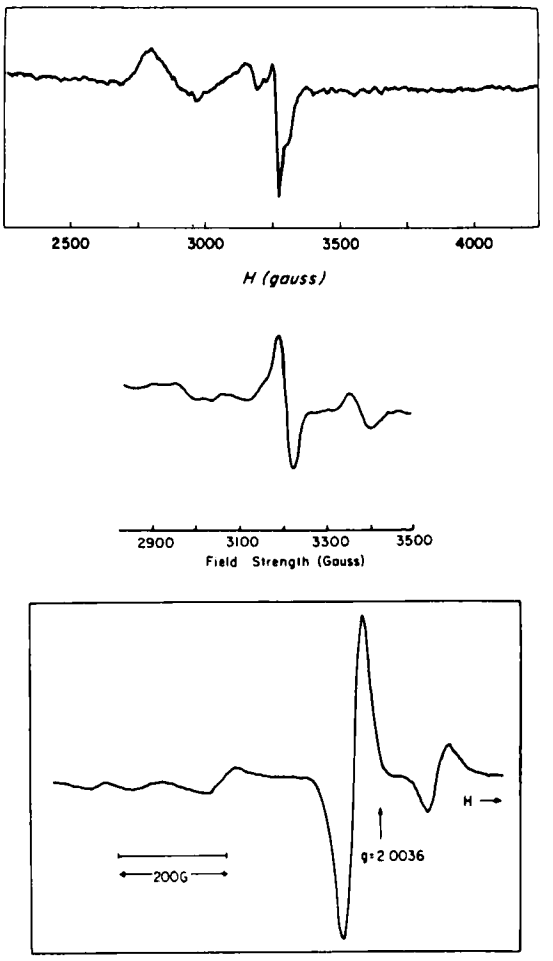


FIGURE 9. EPR signals observed upon incubation of ethanolamine ammonia lyase (top), glycerol dehydrase (middle), and dioldehydrase (bottom) with AdoCbl and their respective substrates.

ing reactions (Figure 9),[157,162-164] as well as with ribonucleotide reductase. To date, however, no reports have appeared describing the formation of paramagnetic species by other adenosylcobamide-dependent mutases.

The nature and behavior of the paramagnetic species generated during the ethanolamine ammonia lyase reaction have been extensively studied by EPR spectroscopy.[165] While the EPR signal obtained with ethanolamine as substrate was quite small, a very large signal was seen when propanolamine was used as substrate, an observation in accord with the extent of cleavage of the C-Co bond in steady state as determined from other experiments (see previous section). This signal, shown in Figure 10, was found to correspond to > 1.3 spins per active site, indicating that at least 65% of the enzyme-bound cofactor was in the dissociated form. Rapid freeze experiments showed that the paramagnetic species were generated at a rate of 7 $sec.^{-1}$ This rate was substantially faster than the overall rate of 1 to 2 sec^{-1} which was measured for the conversion of propanolamine to propionaldehyde and NH_3, indicating that the formation of the paramagnetic species was a kinetically competent process. In its configuration, the signal obtained with the ethanolamine ammonia lyase-propanolamine system was typical of those obtained with mutases, with a broad low-field peak (g = 2.34) which by

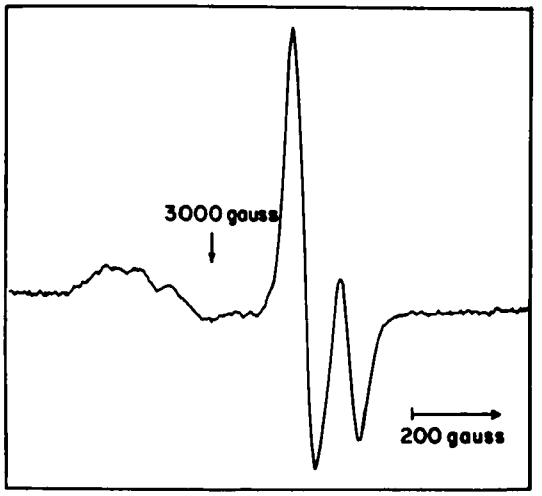
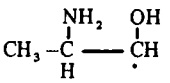


FIGURE 10. EPR spectrum of the ethanolamine ammonia lyase·AdoCbl complex in the presence of propanolamine.

its width, fine structure, and saturation properties could be assigned to enzyme-bound cob(II)alamin, and a narrower high-field doublet. Through the use of isotopically labeled substrates (Figure 11), it was possible to identify the species responsible for the doublet signal as the 2-aminopropanol-l-yl radical:



The paramagnetic species obtained with ethanolamine as substrate were also studied, though not as thoroughly.[162] This investigation confirmed the original observation that the signal corresponded to only a small concentration of electrons (0.04 spins per active site in steady state). A broad cob(II)alamin signal was seen, together with a narrow free radical signal consisting of only one peak rather than the usual two. The width of free radical peak, which represented substantially fewer spins than were represented by the cob(II)alamin signal, was not altered when deuterated substrate was used. It was tentatively proposed that the species responsible for the narrow free radical peak in this spectrum was the carbonylmethyl radical ($\cdot CH_2CHO$), but the evidence for this identification is very scanty. Also to be explained is the reason why the concentration of free radical is so much lower than the cob(II)alamin concentration, as indicated by the relative sizes of the two signals.

A further peculiarity of the EPR signal generated by the ethanolamine ammonia lyase-ethanolamine system is pointed out by the results of a study performed by Hollaway et al. using rapid spectrophotometric techniques.[166] In this study, the changes in the spectrum of the enzyme·AdoCbl complex on addition of substrate were determined as a function of time. With propanolamine as substrate, it was observed that the spectrum of the enzyme·cofactor complex changed from that of AdoCbl to that of cob(II)alamin at a rate of 3 $sec.^{-1}$ This finding is in agreement with the results of previously obtained EPR and optical spectra using this substrate. In contrast are the results obtained with ethanolamine. The visible spectrum seen when ethanolamine was used as substrate (Figure 12) suggests that with this substrate, too, most of the enzyme-bound cobamide is present as cob(II)alamin in steady state. However, the results discussed previously, which were obtained by isotopic and EPR techniques, indicated that

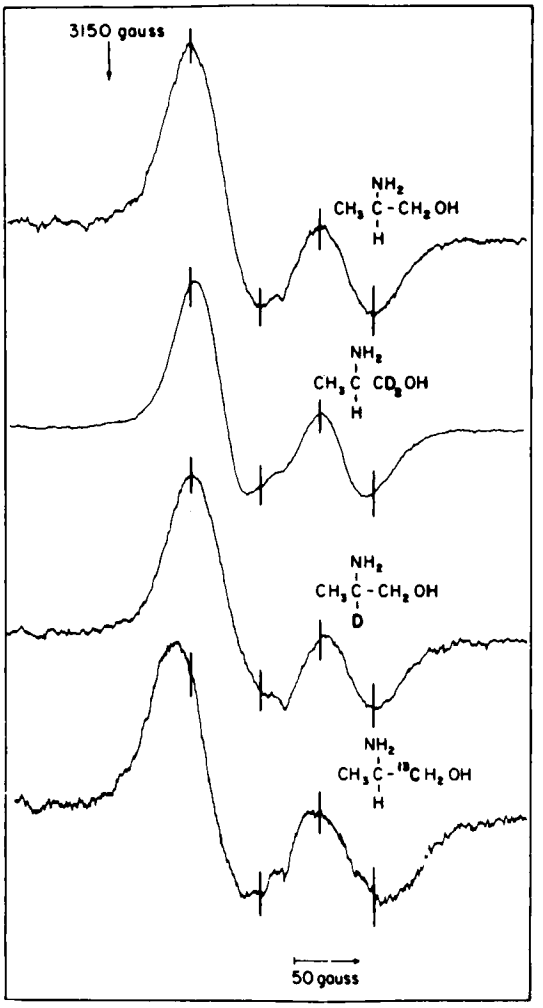


FIGURE 11. Effect of isotopic labeling of propanolamine on the free radical component of the signal shown in Figure 10.

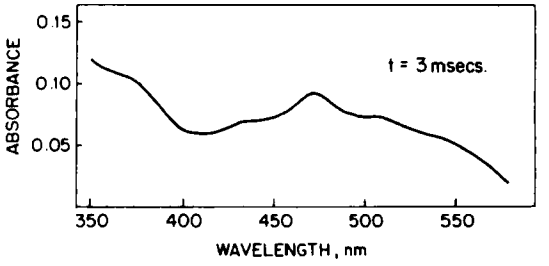


FIGURE 12. Visible spectrum seen 3 msec after adding ethanolamine to the ethanolamine ammonia lyase·AdoCbl complex.

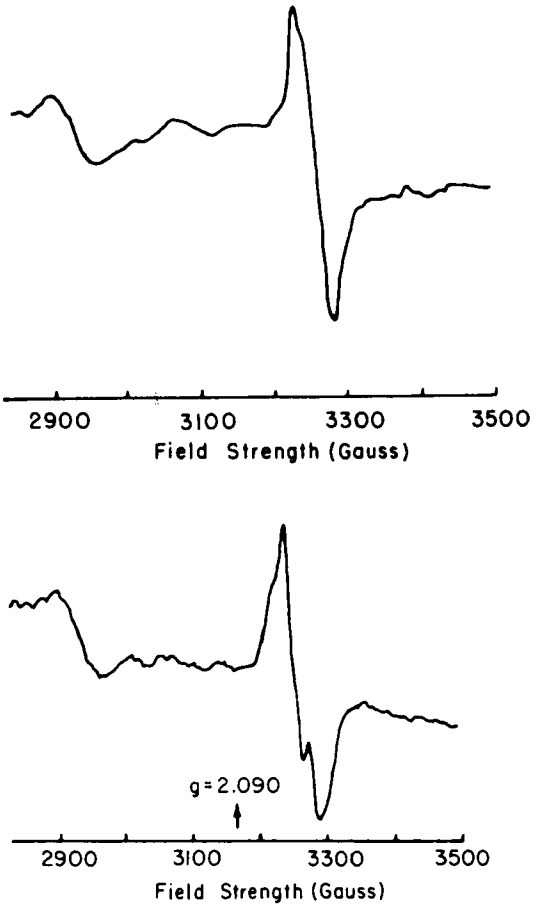


FIGURE 13. EPR signals observed upon incubation of diol dehydrase and AdoCbl with glycolaldehyde (above) and chloroacetaldehyde (below).

the extent of cleavage of the C-Co bond in steady state appeared to be less than 10% when ethanolamine was the substrate. The discrepancy between the results obtained with optical spectroscopy on the one hand and with EPR spectroscopy and isotopic techniques on the other remains to be accounted for.

Diol dehydrase has also been thoroughly studied by EPR spectroscopy. The signal generated during the catalysis of propionaldehyde dehydration was found to appear at a catalytically competent rate and when fully expressed accounted for 25% of the enzyme-bound cofactor.[163,167] EPR signals were also observed with chloroacetaldehyde and glycolaldehyde, two substrate analogues which promote C-Co cleavage by diol dehydrase (Figure 13). In both cases, the signal consisted of a broad cob(II)alamin peak and a narrow free radical signal. Complete homolysis of the enzyme-bound cofactor appeared to occur with each of these analogues, since the EPR signals represented two spins per active site in both cases. The narrow free radical signal obtained in the presence of chloroacetaldehyde was broadened significantly when ^{13}C-labeled analogue was used, indicating that the radical itself was derived from the analogue. It should be noted that by these experiments the previously unidentified cobamide product of the reaction between AdoCbl, diol dehydrase, and glycoldehyde was established to be cob(II)alamin.

Ribonucleotide reductase has been found to generate several types of EPR signals,

depending on the reaction conditions. Incubation of the enzyme with AdoCbl and a thiol in the presence of allosteric effector, a set of conditions under which cleavage of the cofactor with 5′-deoxyadenosine production has been shown to take place, produced cob(II)alamin, identified by its optical and EPR spectrum.[158] In this reaction, cob(II)alamin was produced catalytically, since the enzyme, which remained fully active during the course of the reaction, was able to split several AdoCbl molecules per active site. Under these conditions, there was no evidence for the appearance of a second EPR-absorbing species. Such a species did become evident, however, when a substrate nucleoside (as opposed to an allosteric effector) was present in the reaction mixture.[159,168] The signal corresponding to this second species was a narrow doublet resembling those seen with the mutases whose characteristics depended in subtle ways on the particular ribonucleotide used as the substrate. The identity of the species responsible for this doublet signal is not known.

Kinetic studies have been carried out on the two EPR signals generated by ribonucleotide reductase.[168] Both in the presence and absence of a reducible nucleotide triphosphate, the rate of appearance of the cob(II)alamin signal is slow with respect to the catalytic rate, excluding the species responsible for this signal as a participant in the catalytic reaction. The free radical doublet signal, which as mentioned above is only seen when the substrate is present, also arises too slowly to be associated with a process involved in catalysis. In addition, the kinetics of the free radical signal are very different from those of the cob(II)alamin signal, obscuring the relationship between the reactions responsible for radical formation on one hand and cob(II)alamin production on the other.

The initial EPR studies on the ribonucleotide reductase reaction thus demonstrate a process which generates cob(II)alamin and a free radical by the homolytic cleavage of the C-Co bond of the cofactor, but is otherwise rather poorly understood. Probably the most salient feature of this process is that it is very slow compared with the rate of ribonucleotide reduction by the enzyme, ruling out its participation in the catalytic mechanism. The generation of cob(II)alamin by ribonucleotide reductase was also demonstrated by optical spectroscopy, which showed that a reaction mixture containing AdoCbl, enzyme, thiol, and effector nucleotide (but no substrate) displayed a cob(II)alamin-like spectrum with a prominent peak at 475 nm, rather than the spectrum of the original cofactor.[169] Contrary to expectations, however, the cob(II)alamin spectrum was fully expressed only 30 sec after initiating the incubation (Figure 14), whereas the cob(II)alamin peak in the EPR spectrum required more than an hour to reach maximum size. Subsequent measurements showed that the rate of appearance of the optical cob(II)alamin spectrum was 40 $sec.^{-1}$ A further property of this system was that cooling the reaction mixture to 5°C caused the optical spectrum to transform to that of AdoCbl, while rewarming resulted in the reappearance of the cob(II)alamin spectrum. Partial transformation of the cob(II)alamin spectrum to that of AdoCbl is also seen on addition of substrate nucleotides to the reaction mixture, the rate and extent of transformation varying from substrate to substrate. If substrate nucleotides were present at the start of the reaction, both of these spectral events were observed. An initial transformation of the AdoCbl spectrum to that of cob(II)alamin, which took place at a rate independent of the identity of the substrate and equal to that seen in the absence of substrate, was followed by a partial return of the AdoCbl spectrum at a rate and to an extent which varied from substrate to substrate (Figure 15). These findings suggest that AdoCbl, when bound to ribonucleotide reductase in the presence of dihydrolipoate and an allosteric effector, undergoes a rapid and reversible reaction in which enzyme-bound AdoCbl achieves equilibrium with a form in which the C-Co bond has been ruptured homolytically, exactly analogous to the reaction which takes place when the AdoCbl·ethanolamine ammonia lyase complex is exposed to propano-

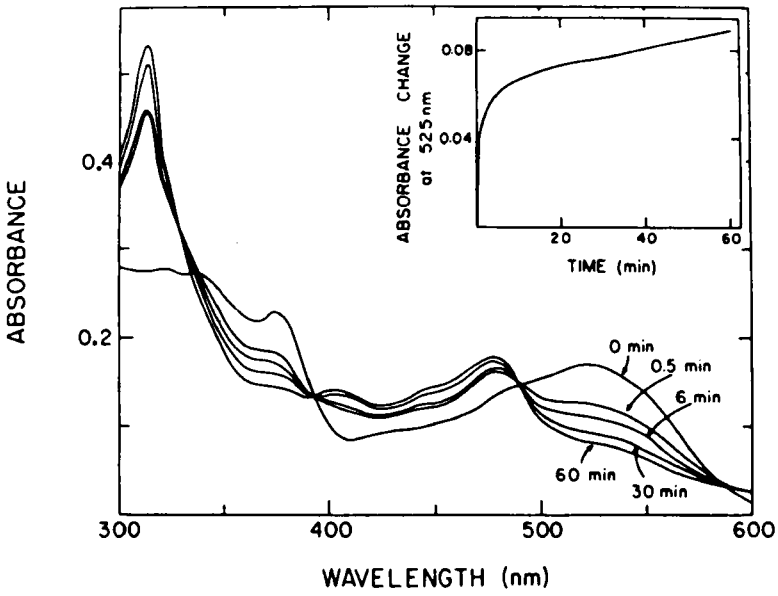


FIGURE 14. Rapid spectral change occurring on addition of AdoCbl to a ribonucleotide reductase-containing reaction mixture.

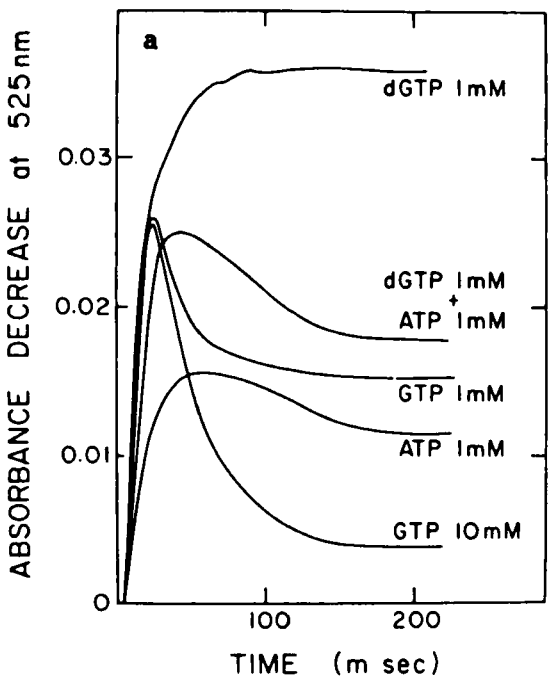
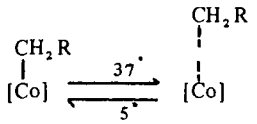


FIGURE 15. Absorbance changes associated with the catalytic activity of ribonucleotide reductase.

lamine. The data further suggest that the equilibrium position is affected by temperature, shifting in the direction of intact AdoCbl as the temperature is lowered, as well as by the presence of reducible substrate.



The effect of temperature on the equilibrium suggested an explanation for the discrepancy between the results obtained by optical spectroscopy, which showed extensive C-Co cleavage very soon after mixing AdoCbl with enzyme, and those obtained by EPR spectroscopy, which showed very little cob(II)alamin at early times. It seemed possible that the chilling necessary to prepare a sample for EPR spectroscopy cooled the sample at a rate slow enough so that by the time the sample was frozen, the C-Co bond of enzyme-bound cofactor had reformed, owing to the temperature-dependent shift in the equilibrium between the intact and dissociated forms of the cofactor. EPR spectroscopy was therefore performed on a sample in which the cofactor was trapped in its high-temperature equilibrium position by a rapid freezing technique.[170] This experiment disclosed an EPR signal of a type which had not been observed before with adenosylcobamide-requiring enzymes (Figure 16). The signal consisted of a broad and rather symmetrical peak with superimposed fine structure which in the absence of substrate represented 0.6 electrons per active site. An identical signal was observed when reducible substrate was present in the reaction mixture along with enzyme, cofactor, thiol, and effector. The properties of the signal were not altered by the use of isotopically labeled AdoCbl or by performing the incubation in D_2O instead of H_2O. Both in the absence and presence of reducible substrate, the kinetics of appearance and disappearance of this signal were identical to those of the early changes in the optical spectrum of the enzyme-bound cofactor, leaving little doubt that the same species was responsible for both the EPR and optical spectra. It was concluded that this species was enzyme-bound cob(II)alamin. Its EPR spectrum, however, is very different from that of either free cob(II)alamin, the enzyme-bound cob(II)alamin which appears during catalysis by the mutases, or the enzyme-bound cob(II)alamin which is formed irreversibly by prolonged incubation of the same ribonucleotide reductase system in which the signal under discussion is generated, suggesting that the environment around the rapidly formed cob(II)alamin is in some respect unique. A free radical signal was

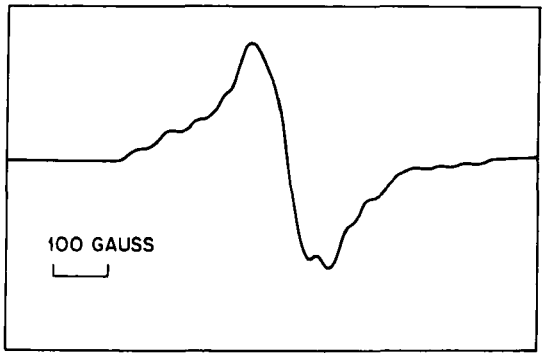


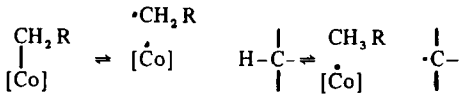
FIGURE 16. EPR spectrum of a ribonucleotide reductase-containing reaction mixture frozen quickly in isopentane at 130K.

not observed in this system, a puzzling feature since such a species must have been produced in the homolytic process which generated the cob(II)alamin.

Though all the EPR spectra seen with adenosylcobamide-requiring enzymes have been attributed to the presence of cob(II)alamin plus a free radical (in the case of some of the ribonucleotide reductase spectra, cob(II)alamin alone) at the active site of the enzyme, the configurations of the spectra are rather exceptional. Apart from the slowly developing signal observed when ribonucleotide reductase plus AdoCbl is incubated with thiol and effector, the spectra ascribed to cob(II)alamin are very different in envelope configuration and fine structure from that of free cob(II)alamin, although they resemble the latter in g value and width. The free radical signals are even more unusual, consisting as they do of asymmetrical doublets whose peak widths are 30 G or more, rather than the narrow singlets which constitute typical carbon radical signals. These unusual features have been explained by successful attempts to model the experimentally obtained spectra by computer. The properties of the spectra containing the free radical doublet have been shown by these theoretical modeling studies to result from an interaction between the unpaired electron on the free radical and that on the nearby cob(II)alamin moiety.[171,172] These studies have also permitted estimates to be made of the separation between the two unpaired electrons. To be consistent with the observed EPR spectra, this separation cannot be less than 5 Å, a long distance in molecular terms. A detailed analysis of the ribonucleotide reductase doublet,[172] generated to be sure by a species which is not catalytically significant, showed the separation in this system to be 9.9 Å. The distance between the two paramagnetic species is of some interest in terms of the mechanism for group X transfer, as will be discussed below.

The EPR spectrum of the *catalytically competent* species in the ribonucleotide reductase reaction was also analyzed in detail.[173] This analysis suggested that the spectrum was generated by a mixture of two forms of cob(II)alamin, both presumably bound to the active site of the enzyme. Comparison of the spectrum with those of model Co(II) complexes further suggested that the enzyme-bound cob(II)alamin complexes are not 5-coordinate, but rather 6-coordinate, the sixth ligand being an electron donor disposed so as to place the cobalt in a highly asymmetric octahedral field.

These experiments indicate that species with unpaired electrons are catalytic intermediates in adenosylcobamide-dependent reactions. In the case of the mutases, the participating species have been identified as cob(II)alamin and a free radical derived from the substrate, suggesting a mechanism for hydrogen transfer involving homolysis of the C-Co bond followed by the reversible transfer of hydrogen between substrate (or product) and the resulting 5′-deoxyadenosyl radical:



The situation is less clear with regard to ribonucleotide reductase, since with this enzyme only a cob(II)alamin signal has been identified to date. The fact that substrate (i. e., a thiol) is necessary for the generation of this signal suggests the involvement of thiol groups in the formation of the paramagnetic species. This notion is further supported by evidence to be discussed later indicating that hydrogen from water (i. e., hydrogen from the reducing agent, which is in equilibrium with water protons) is transferred to the 5′-carbon of the cofactor by way of enzymatic −SH groups. This evidence suggests a mechanism for hydrogen transfer analogous to that operating in the mutases, except that the hydrogen-donating element is not the substrate molecule, but a thiol group at the active site of the enzyme:

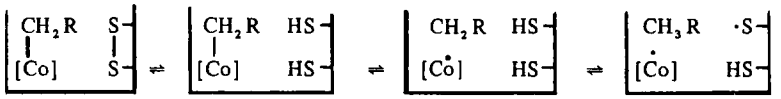
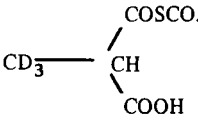
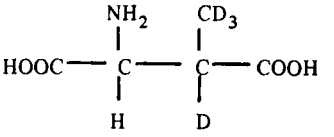


TABLE 8

Overall Deuterium Isotope Effects for Some Adenosylcobamide-Dependent Rearrangements

Enzyme	Deuterated substrate	k_H/k_D
Diol dehydrase	$HOCD_2CD_2OH$	6.4
	$CH_3CHOHCD_2OH$	10
Ethanolamine ammonia lyase	$NH_2CH_2CD_2OH$	7.4
Methylmalonyl CoA mutase		3.5
Glutamate mutase		6.6

Other Aspects of Hydrogen Transfer

Isotope Effects

With several of the adenosylcobamide-requiring enzymes, studies with isotopically labeled substrate have shown that the rate-limiting step in the catalytic mechanism involves hydrogen transfer.[52,53,58,140,141,146,174,175] Deuterium isotope effects for the overall reaction ranging between 3.5 and 10 have been measured with four of the mutating enzymes (Table 8). These values are those expected for primary isotope effects and indicate that a hydrogen abstraction constitutes the slow step in these reactions. With ribonucleotide reductase, the second step of the rapid two-step process observed spectrophotometrically when ribonucleotide reduction begins (see Figure 16) showed a deuterium isotope effect of 2.2, also a primary isotope effect, when AdoCbl was replaced by [5′,5′-D_2] AdoCbl. Whether or not this step is rate limiting is not known, since the deuterium isotope effect for the overall reaction has not yet been measured.

What cannot be established by measurements of the overall isotope effect is which of the sequential hydrogen transfer steps, substrate to cofactor or cofactor to product, is rate-limiting for a given enzyme. With diol dehydrase[146] and ethanolamine ammonia lyase,[175] enzymes which catalyze irreversible reactions, it was possible to measure the isotope effects for the two individual hydrogen transfer steps because back-exchange of hydrogen from cofactor to substrate (and under the conditions of the experiments, from product to cofactor as well), which would complicate the calculation of those kinetic isotope effects, did not take place. The tritium isotope effect for the first hydrogen transfer step was measured by comparing the rate of loss of ^3H from a pool of tritiated substrate with the rate at which the substrate itself was consumed in the enzyme-catalyzed reaction, while that for the second hydrogen transfer step was determined from the rate of transfer of ^3H from [5′-^3H] AdoCbl into product. The deuterium isotope effect for the second step was calculated by comparing the tritium isotope effects obtained with deuterated as opposed to undeuterated substrate, starting with ^3H-labeled cofactor as mentioned above. The deuterium isotope effect for the transfer from substrate to cofactor could not be measured.

The isotope effects obtained in these experiments are shown in Table 9. For the first step, the tritium isotope effects for the two reactions are well within the range expected for a primary isotope effect. Assuming the Swain relationship to hold,[176] the deuterium isotope effects for the diol dehydrase and ethanolamine ammonia lyase reactions can be calculated to be 8 and 2.9, respectively. The deuterium isotope effects for the transfer of hydrogen from cofactor to product (the second step) are as shown in the table. Comparison of these isotope effects with the deuterium isotope effects for the overall

TABLE 9

Isotope Effects for the Individual Hydrogen Transfer Steps in Certain Adenosylcobamide-Dependent Rearrangements

	Isotope effect		
	Substrate → cofactor	Cofactor → product	
Enzyme	k_H/k_T	k_H/k_D	k_H/k_T
Diol dehydrase	20	28	125
Ethanolamine ammonia lyase	4.7	7.3	160

reactions showed that the transfer of hydrogen from substrate to cofactor is rate limiting for the diol dehydrase reaction, while the slow step in the ethanolamine ammonia lyase reaction is the transfer from cofactor to product.

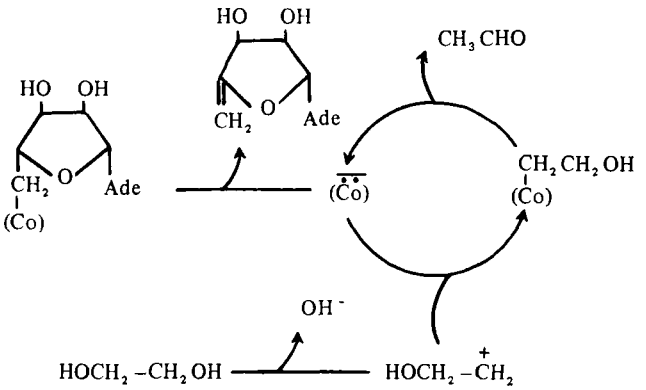
The surprising feature of these experiments was the size of the tritium isotope effect for the second step of the reaction (it should be noted that these figures already incorporate the statistical correction necessary because of the equivalence of the three 5′ hydrogen atoms on 5′-deoxyadenosine). This is particularly striking for the ethanolamine ammonia lyase reaction, in which the tritium isotope effect is almost an order of magnitude larger than that calculated from the deuterium isotope effect by the Swain relationship. The origin of this anomalously high tritium isotope effect has not been elucidated experimentally. One possible explanation involves hydrogen transfer by tunneling, a process which has been reported to result in unusual relationships between deuterium and tritium isotope effects,[177] but the normal heat of activation seen with ethanolamine ammonia lyase[175] excludes this interpretation, at least for that reaction. Another possibility is the failure of the three 5′ hydrogens on enzyme-bound 5′-deoxyadenosine to become fully equivalent during the interval between the two hydrogen transfer steps. This is exceedingly unlikely, because it would require that the association between 5′-deoxyadenosine and the enzyme causes the rate of rotation about the 4′-C—5′-C bond to fall from its usual value of about 10^{11} to 10^{12} sec^{-1}* to a rate close to that of the second hydrogen transfer step in the catalytic mechanism (the latter is equal to the turnover number of the enzyme, or approximately 100 sec^{-1} [179]), an unprecedented example of steric hindrance of a methyl group rotation. Moreover, evidence against this possibility has been provided by the demonstration that the rotation of the methyl group of 5′-deoxyadenosine in a ternary complex of nucleoside, CN-Cbl, and ethanolamine ammonia lyase exceeds 10^9 $sec.^{-1}$ [180] The most likely explanation, albeit one which has not yet been tested experimentally, is that there exists a hitherto unrecognized pool of enzyme-bound hydrogens which are in communication with the mobile hydrogens on the substrate and product. These enzyme-bound hydrogens either equilibrate with the 5′-hydrogens of the cofactor, a process which would affect the statistical factor to be used in calculating the tritium isotope effect, or they provide an alternative pathway which permits the transfer of hydrogen from substrate to product without involving the 5′-hydrogens of the cofactor.

An Alternative Mechanism

The evidence cited above favors a mechanism for hydrogen transfer in adenosylcobamide-dependent reactions in which hydrogen is transferred from substrate to product by way of the 5′ position of the cofactor, the site for the hydrogen atom being generated by C-Co bond homolysis. Schrauzer and associates have postulated an alternative mechanism for hydrogen transfer in which neither C-Co bond homolysis nor the transfer of hydrogen to and from the 5′ position of the cofactor takes place.[39]

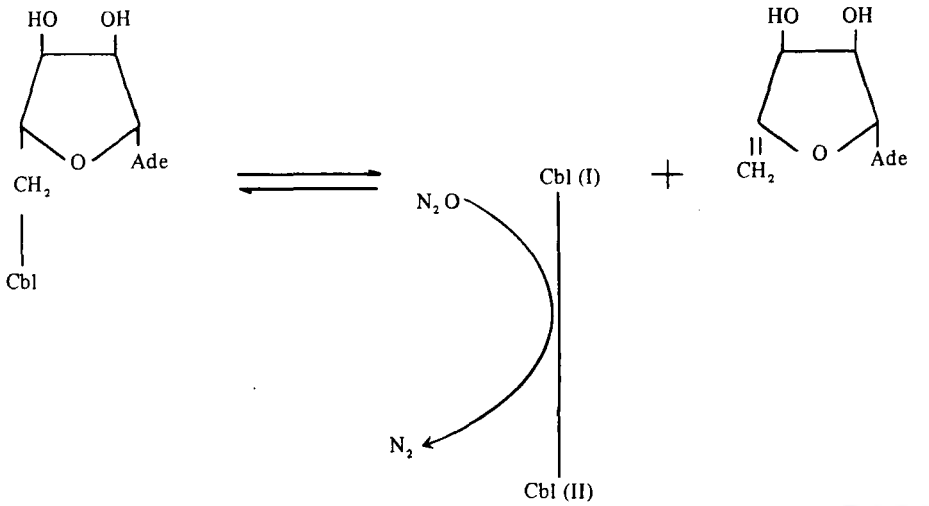
* Expected from dielectric relaxation times of substituted methyl groups.[178]

The alternative mechanism, which is largely based on studies with model compounds (see below), is as follows:



Catalysis begins with heterolysis of the C-Co bond to give cob(I)amide and 4′,5′-anhydroadenosine. The enzyme-catalyzed removal of a nucleophilic leaving group from the substrate (in the example shown, ethylene glycol) then produces a 2-hydroxyethyl carbonium ion which combines with the cob(I)amide at the active site to yield β-hydroxyethylcobalamide. After the alcoholic proton of the new organocobamide is removed by a basic group on the enzyme, a hydride ion shift occurs, producing acetaldehyde by the nucleophilic displacement of cob(I)amide, which is thereby made available to participate in another cycle of catalysis. During all of this activity, the 4′,5′-adenosine merely waits as an innocent bystander; transfer of hydrogen into the 5′ position of the cofactor is explained by a fortuitous exchange reaction of no catalytic significance which is postulated to take place between the methyl protons of the product (acetaldehyde) and the 5′ hydrogens of 4′,5′-anhydroadenosine.

Although most of the evidence concerning this mechanism has come from chemical studies, there are a few biochemical experiments which have been claimed to support it.[161-164] Some[161,162] have to do with the effect of N_2O on adenosylcobamide-dependent reactions. They are based on the fact that N_2O, generally an unreactive species, rapidly oxidizes cob(I)amides to cob(II)amides. If the alternative mechanism is correct, an experiment in which an adenosylcobamide-dependent reaction is carried out under N_2O should result in the irreversible cleavage of the C-Co bond with the production of 4′,5′-anhydroadenosine due to N_2O-mediated oxidation of enzyme-bound cob(I)alamin (provided that the reagent reaches the active site of the catalyzing enzyme):



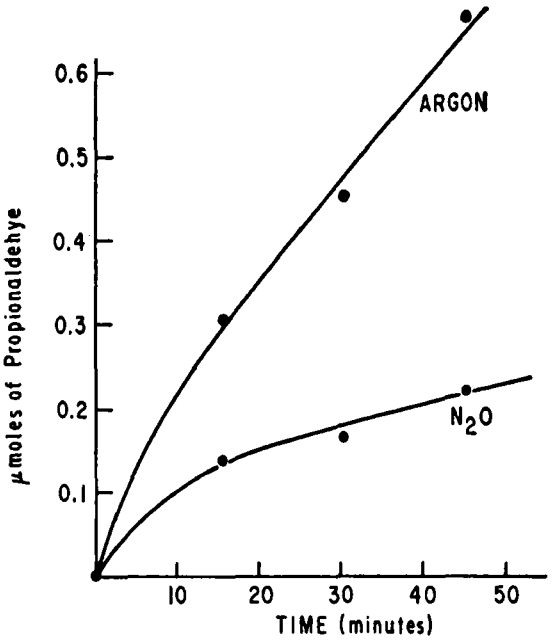


FIGURE 17. Inhibition of propionaldehyde formation by diol dehydrase with N_2O.

This prediction was tested in a study which showed a reduction in the rate of conversion of propanediol to propionaldehyde when the diol dehydrase reaction was conducted under an atmosphere of N_2O (Figure 17)[181]. The authors claimed that this study supported the alternative mechanism. Critics pointed out, however, that the phenomenon observed was inhibition, not the progressive inactivation of the catalyst that would be expected if N_2O were acting according to postulate (i. e., destroying the cofactor). The proponents of the alternative mechanism replied that inactivation was in fact taking place, but that only that portion of cofactor bound to N_2O-sensitive enzyme was being destroyed.[182] They said that the residual activity actually represented the full activity of that fraction of the enzyme-cofactor complex which was not susceptible to N_2O inactivation. In our opinion, the proponents of the alternative mechanism seem to be invoking a disturbing number of *ad hoc* postulates to enable the data to conform to their hypothesis. This same group has recently published reports to the effect that the incubation of ethanolamine ammonia lyase[183] or diol dehydrase[184] with AdoCbl yields 4′,5′-anhydroadenosine. There are several problems with those studies, including the identification of the nucleoside and the stoichiometry of the reaction (a particularly serious problem).

It is evident that the support provided for the alternative mechanism by the N_2O experiments is highly debatable. Evidence against the alternative mechanism is its failure to explain the very large body of experimental data discussed at length in the foregoing sections. To cite a few examples of this failure, the reversible homolytic cleavage of the majority of the enzyme-bound cofactor during catalysis by ethanolamine ammonia lyase and ribonucleotide reductase is not accounted for by the alternative mechanism, nor does it explain the generation of substrate radical at catalytically competent rates by diol dehydrase and ethanolamine ammonia lyase. The notion that the incorporation of hydrogen into adenosylcobamide results from a catalytically irrelevant exchange reaction between product and cofactor has been conclusively ruled out by experiments with diol dehydrase which showed that during the dehydration of tritiated

propanediol, the specific activity of tritium in the cofactor far exceeded that of the product, a finding which is impossible to reconcile with a mechanism in which the label has to be incorporated into product before it can be transferred to the cofactor.[185]

Thus, the evidence favoring the alternative mechanism seems dubious, while that against it appears incontestible. In our opinion, the likelihood that the alternative mechanism is correct is exceedingly remote.

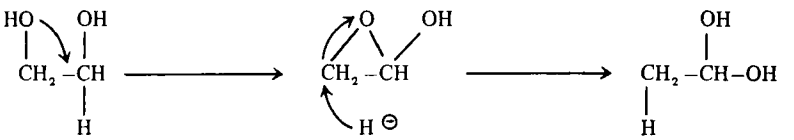
The Transfer of Group X in the Rearrangements

While a reasonably complete picture of hydrogen atom transfer has emerged from recent studies, the process by which group X is transferred remains a mystery. Biochemical studies have disclosed little concerning group X transfer by the mutases and nothing pertaining to ribonucleotide reduction. In the absence of enzymological data, workers in the field have been forced to fall back on model reactions to try to acquire some understanding of this process. Many model reactions based on a wide variety of chemical principles have been proposed to explain group X transfer. The ensuing section will deal largely with these model reactions. It will be seen that most can explain group X transfer in at least some of the adenosylcobamide-dependent reactions. What their relevance is to the enzyme-catalyzed processes remains to be seen.

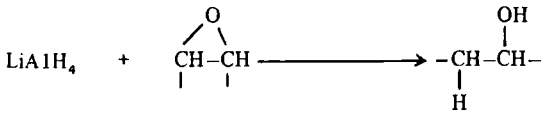
Mutase Models

Reactions Involving Epoxides

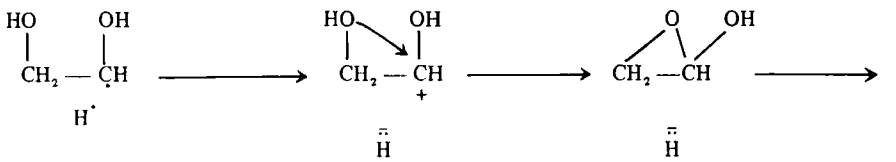
An epoxide has been proposed as an intermediate in the reaction catalyzed by diol dehydrase.[186] According to this proposal, the epoxide is formed by the displacement of a hydride ion by a hydroxyl group on the neighboring carbon atom. The 1,1-dihydroxyethane is produced by the readdition of the hydride to the epoxide, opening the ring in the opposite sense:



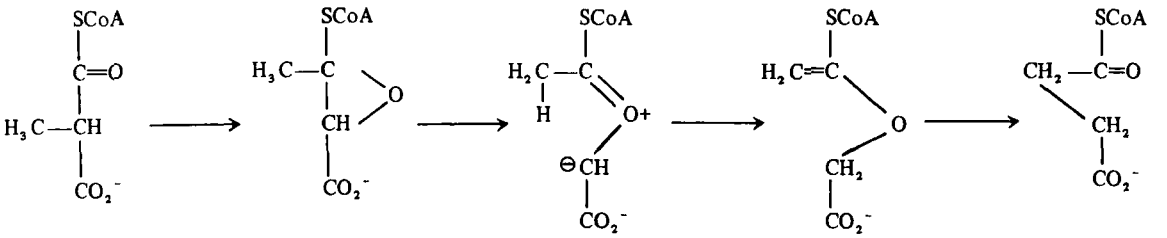
In agreement with this proposal is the previously discussed ^{18}O-labeling data, which showed that in the enzyme-catalyzed reaction, the hydroxyl group underwent migration rather than elimination. In addition, the reduction of epoxides to alcohols by hydride-containing species is a well-known organic reaction:[187]



Against this proposal is the demonstration that the enzyme-catalyzed hydrogen abstraction gives rise to radical, not ionic, species. However, the transfer of an electron from the substrate radical to the hydrogen atom to generate the rearranging species is still a possibility:

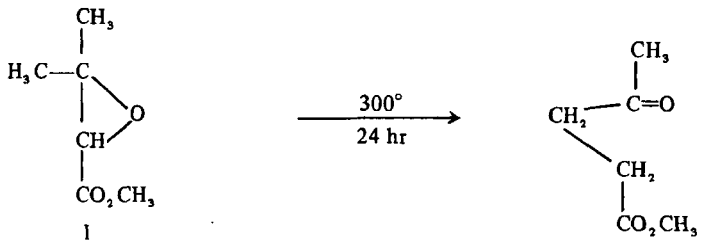


Dowd and Nakagawa have proposed a much more complicated mechanism to explain the rearrangement of methylmalonyl CoA to succinyl CoA.[188] In this mechanism, rearrangements of the thioester group occurs before hydrogen transfer. Specifically, the thioester oxygen atom forms an epoxide, with



concomitant shift of the methyl group. This is followed by the spontaneous opening of the epoxide ring to an oxygen ylide, which abstracts a proton from the methyl group to give a vinyl ether. This species then undergoes a 1,3-shift of an acetate fragment to yield the product.

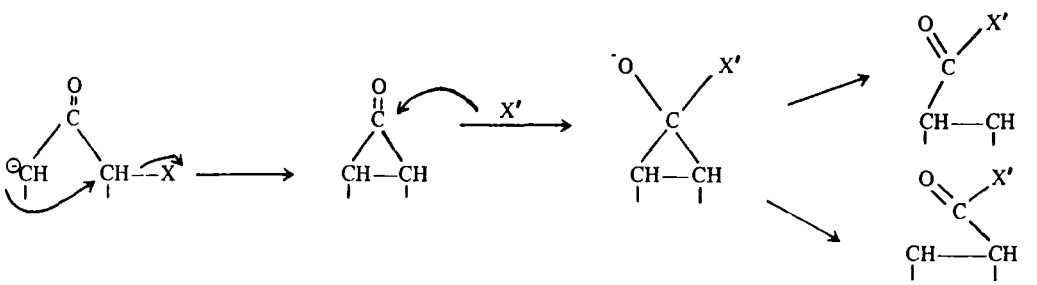
As a model for the steps occurring after formation of the hypothetical epoxide, Dowd studied the pyrolysis of epoxide I, a compound similar to the postulated epoxide intermediate, though lacking the thioester group. It was found that treatment of epoxide I for 24 hr at 300° gave rise to methyl 4-ketovalerate (26% yield as determined by gas-liquid chromatography). Other products



identified included methanol, acetone, methyl 2-ketoisovalerate, methyl 2-hydroxy-3-methylbut-3-enoate, and tar. Although methyl 4-ketovalerate is the product stipulated by Dowd's mechanism, there is little basis to assume that the formation of this product occurred uniquely by the proposed sequence of steps. Rather, the complex product mixture (which includes among other things fragmentation products such as acetone and methanol) and the extreme reaction conditions point to a very complicated and poorly defined process.

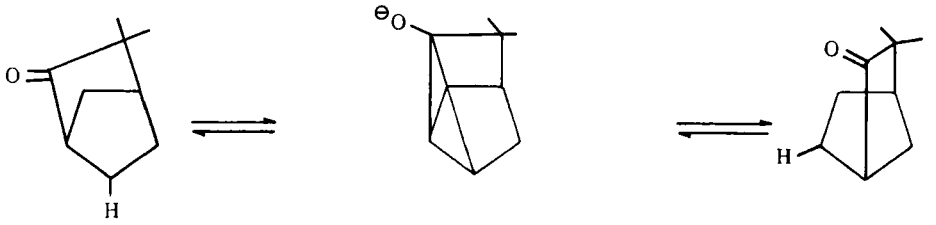
Favorskii-Like Rearrangements and the Methylmalonyl CoA Mutase Reaction

The Favorskii rearrangement of a ketone involves the displacement of an α-leaving group by an α' carbanion to form an intermediate cyclopropanone, followed by ring opening via nucleophilic (usually solvolytic) attack on the carbonyl group to form products whose structures depend on the sense in which the ring is opened. A number of workers have proposed models for the methyl

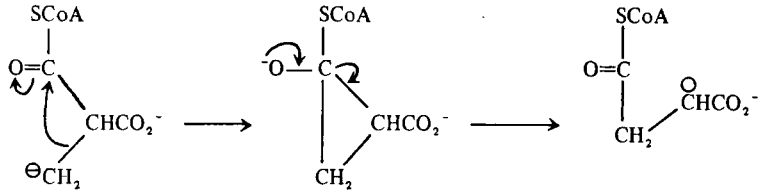


malonyl CoA mutase reaction which involve cyclopropanolate anion intermediates similar to those thought to participate in the Favorskii rearrangement.

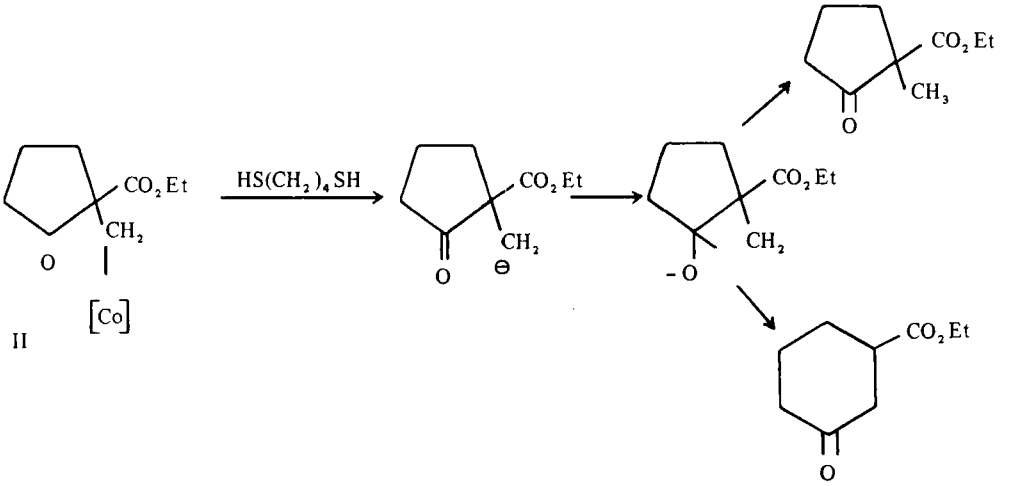
The racemization of camphenilone, a reaction studied by Nickon and Lambert,[189] was the basis for the first such proposal, which was made by Ingraham in 1964.[147] The camphenilone racemization was postulated to occur by way of enantiomeric homoenolate ions which could equilibrate via a cyclopropanolate anion intermediate.



Applying this model to the enzyme-catalyzed reaction, Ingraham proposed that the homoenolate ion of methylmalonyl CoA could equilibrate via a cyclopropanolate anion with the carbonion of succinyl CoA:



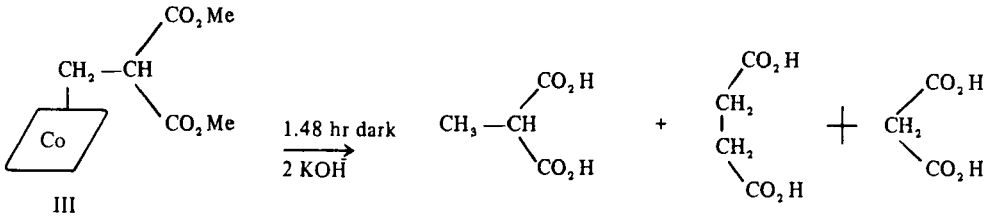
An additional model for this proposal was provided by Lowe and Ingraham.[190] They synthesized cobaloxime II with an axial ligand containing functional groups similar to those of methylmalonyl CoA. This cobaloxime, upon treatment with butanedithiol (which splits the C-Co bond heterolytically), afforded a very small amount of ethyl 3-ketocyclohexane carboxylate, the compound expected from the Favorskii-like rearrangement of the homoenolate anion produced by the cleavage of cobaloxime II.



The migration of alkoxycarbonyl groups is the subject of a recent review by Acheson.[191] Although most of these reactions have not been considered in terms of cobamide-dependent rearrangements, many would seem to be appropriate as models for the methylmalonyl CoA mutase reaction.

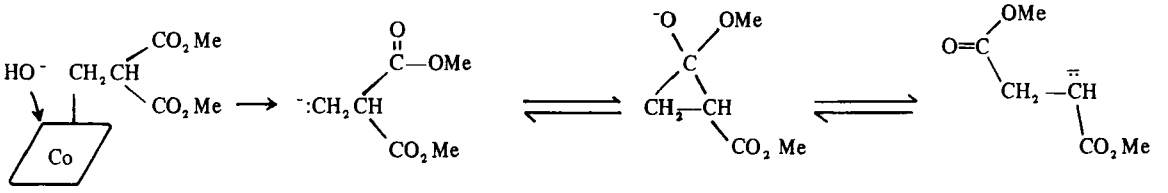
Recently, Dowd, the author of a previously discussed mechanism, proposed another model for methylmalonyl CoA mutase, namely, the decomposition of cobalamin III.[192]

Unable to obtain the pure material, he had to work with the reaction mixture used to prepare the compound

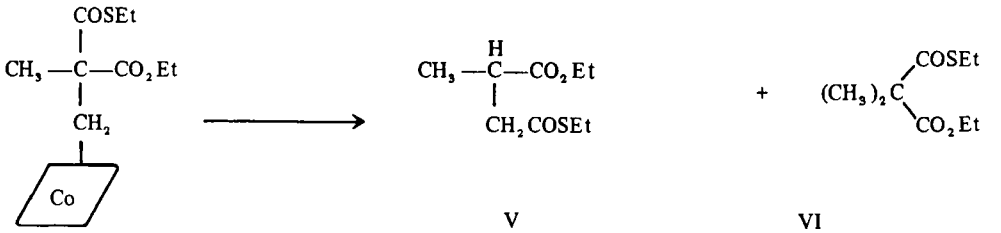


III

(containing hydroxocobalamin, $NaBH_4$, and the alkylating agent [bromomethylmalonate diethyl ester], in addition to the alkyl cobalamin). Upon storage in the dark for 48 hr followed by basic hydrolysis, this mixture yielded 13.6% methylmalonic acid, 3.7% succinic acid, and 18% malonic acid. Malonic acid, but not succinic acid, was formed in a control reaction in which $Co(NO_3)_2$ was substituted for hydroxocobalamin. It appears from these results that rearrangement of the alkyl group took place during the course of the model reaction. While no specific pathway was proposed to account for the products arising from the decomposition of cobalamin III, these experiments were regarded as evidence that a C-Co bond formed between substrate and cobalamin played a crucial role in the reaction catalyzed by methylmalonyl CoA mutase. No evidence was provided regarding the ionic state of the carbon atom of the rearranging fragment, nor was there direct evidence that the cobalamin portion participated actively in the rearrangement mechanism (i. e., that the true rearranging species was an organocobalamin as opposed to an alkyl fragment released by C-Co bond cleavage). The answers to these questions await further experimentation. One point which might be made is the similarity of this model to that of Lowe and Ingraham. Thus, as with Lowe and Ingraham's cobalamin, perhaps Dowd's compound undergoes heterolytic cleavage to yield a homoenolate ion, which rearranges by a Favorskii-like reaction, with C-Co bond cleavage initiated by attack of hydroxyl instead of butanedithiol:



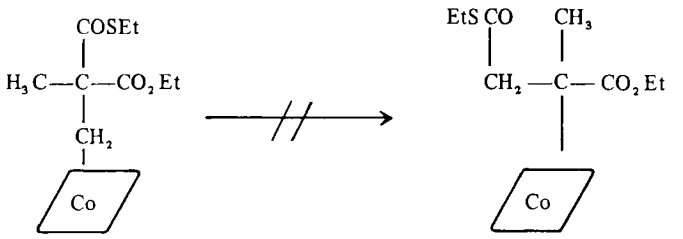
Scott and Kang put forward another model for methylmalonyl CoA mutase.[193] They prepared cobalamin IV, which like the actual substrate, contains a thioester group. Like the cobalamin prepared by Dowd and coworkers, this compound could only be partially purified. A crude mixture containing the model cobalamin (identified spectroscopically), together with other constituents employed in the alkylation reaction, was allowed to stand in the



IV V VI

dark for one day. Ether extraction of this mixture gave the methylsuccinate ester V (70.3%, based on hydroxocobalamin) and the dimethylmalonate ester VI (16.7%), together with unreacted alkylating agent. It is evident that the alkyl group on the model cobalamin underwent rearrangement. The position of the methyl group in the methylsuccinate ester was proof that the rearrangement occurred by migration of the thioester group rather than the ester group, as seen in the methylmalonyl CoA mutase reaction.

While the mechanism of this rearrangement has not been precisely defined, the reaction provides a crucial insight into methylmalonyl CoA mutase models. It can be concluded that this reaction has to involve the rearrangement of a free alkyl fragment, not an organocobalamin. The present reaction cannot proceed by way of a rearranging organocobalamin because the product of such a rearrangement would contain a bond between the cobalt and a tertiary carbon atom, a structure which, as discussed before, is extremely implausible on steric grounds.



The fact that the rearrangement in this model occurs without producing an intermediate rearranged organocobalamin suggests that in related model reactions the cobalamins may not themselves rearrange, but may only serve to generate intermediates (e. g., Lowe and Ingraham's model), which then rearrange without the further involvement of cobalt.

Radical Rearrangements

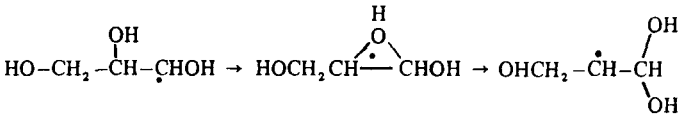
The discovery that free radicals participate in the hydrogen transfer step of adenosylcobamide-requiring enzymes raises the possibility that free radicals might also participate in the migration of group X.

Eggerer et al. were the first to propose a radical pathway to rationalize a cobamide-dependent rearrangement — in their case, thioester group migration in the methylmalonyl CoA mutase reaction.[194] Their proposal, made shortly after the discovery of adenosylcobamides and before the radical nature of the hydrogen transfer was known, was based on a reaction described many years earlier by Urry and Kharasch.[195] Those workers found that in the presence of $CoCl_2$ and a Grignard reagent, 2-methyl-2-phenylpropyl chloride rearranged to form products in which the phenyl group had migrated to an adjacent carbon atom. The proposed mechanism involved the abstraction of a chlorine atom by univalent Co^{+} produced when Co^{++} reacted with phenylmagnesium bromide. The resulting primary free radical then isomerized to the more stable tertiary free radical by migration of a phenyl group. Subsequently, other radicals containing

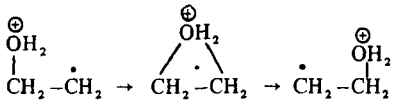
$$\phi Me_2\ C{-}CH_2Cl \rightarrow \phi MeC\dot{C}H_2 \rightarrow Me\dot{C}CH_2\phi \rightarrow \text{Products}$$

an adjacent phenyl group have been reported to undergo phenyl group migration.[196]

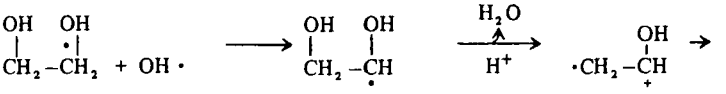
The closely related diol dehydrase and glycerol dehydrase reactions have also been modeled by free radical reactions. Cockle et al., having confirmed the role of free radicals in adenosylcobamide-dependent rearrangements by demonstrating the appearance of an EPR signal during catalysis by glycerol dehydrase, proposed that −OH migration in this reaction occurred by way of an epoxide radical transition state:[164]



Molecular orbital calculations by Golding and Radom suggested that this was unlikely, since the lowest energy path for hydroxyl group migration appeared to involve free hydroxyl radical plus ethylene.[197,198] However, these authors calculated that when the hydroxyl group was protonated, hydroxyl group migration by way of an epoxide cation radical intermediate required only 8 kcal/mol of activation energy, making this path attractive for this and other adenosylcobamide-requiring reactions.

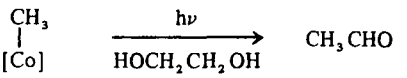


More recently, Golding sought experimental evidence to support his calculations. He has pointed out that the attack of hydroxyl radical on ethylene glycol affords acetaldehyde. This particular reaction has been studied in detail by Walling and Johnson, who postulated that it proceeds by the mechanism shown in the scheme below, which involves a cation radical formed under acidic conditions by the elimination of OH^- from the 1,2-ethanediol-1-yl radical:[199]

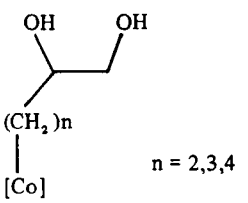


Golding wished to demonstrate that a similar reaction could be carried out using an alkyl rather than a hydroxyl radical as the attacking species. Such a reaction would be analogous to the enzyme-catalyzed dehydration of ethylene glycol, in which the 5′-deoxyadenos-5′-yl radical is the attacking species.

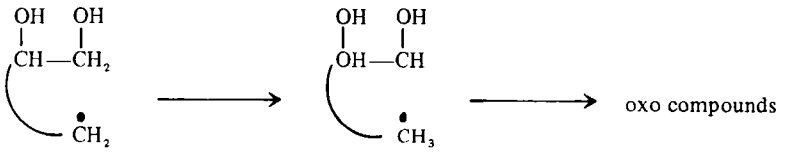
In a preliminary experiment designed to test this proposal, methyl radicals were generated by the photolysis of methylcobaloxime in the presence of a 250-fold excess of ethylene glycol, the aim being to form 1,2-ethanediol-l-yl radicals which would react further to produce acetaldehyde under the acidic reaction conditions employed in the experiment.[200] Only a trace amount



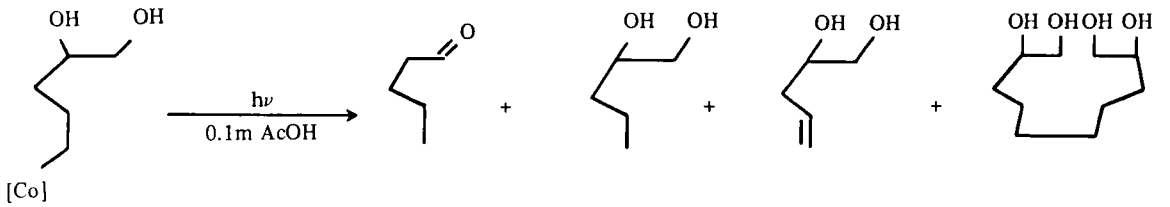
of acetaldehyde was obtained. The low yield of product was thought to be due to the low probability of intermolecular radical attack. To obviate this problem, a series of cobaloximes was prepared whose axial ligands consisted of alkyl chains of various lengths in which the last two carbon atoms were hydroxylated.[201,202]



It was hoped that in certain of these alkylcobaloximes, the radical produced by homolysis of the C-Co bond would rearrange to yield 1,2-dihydroxyalk-l-yl radicals by intramolecular hydrogen transfer and that these radicals

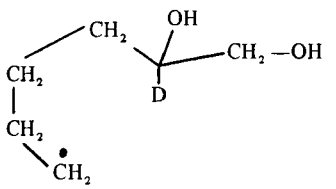


would react further to produce oxo compounds. Photolysis of the butyl cobaloxime produced no oxo compounds. This was the expected result, since the alkyl radical produced in this reaction is too short to rearrange by hydrogen abstraction via a cyclic transition state. In contrast, pentanal was found among the products formed by anaerobic photolysis of the pentylcobaloxime in the presence of 0.1 *M* acetic acid. No oxo products were detected when the reaction was conducted either under less acidic conditions (pH 3), or in the presence of oxygen. The



requirement for acid is in accord with Golding's proposal (discussed previously) that rearrangement requires protonation of the hydroxyl group. When the 1,1,5,5-tetradeuteropentyl derivative was photolyzed, the pentanal isolated from the reaction mixture was found to contain three D atoms in the terminal methyl group, as expected if an intramolecular 1,5-hydrogen transfer occurred during the course of this reaction.

A mixture of hexan-2-one and hexanal was produced by anaerobic photolysis of the hexyl derivative under acidic conditions. The product ratio (ketone to aldehyde = 3.7:1) demonstrated a preference for 1,5- over 1,6-hydrogen atom transfer. This ratio was reversed (ketone to aldehyde = 1:5) by deuterium substitution at the 5 position of the starting hexyl derivative. The reversal

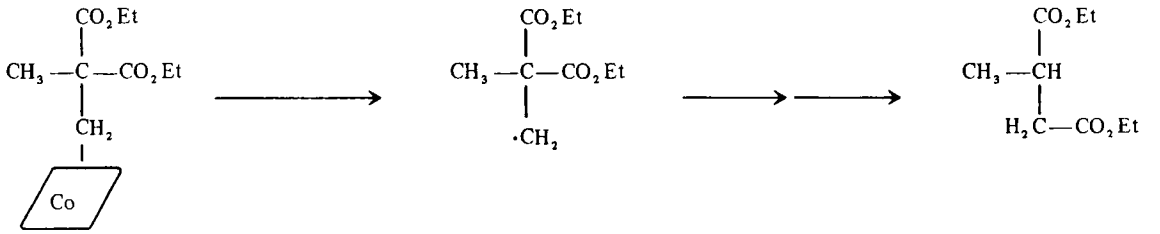


of the product ratio was thought to reflect a kinetic isotope effect which retarded the abstraction of the hydrogen in the 5 position, thereby favoring the competing abstraction of the 6-hydrogen atom.

These results support Golding's hypothesis and demonstrate that an alkyl radical as well as a hydroxyl radical can effect the conversion of 1,2-diols to aldehydes. On the basis of these model reactions, Golding has proposed that the hydroxyl group migration in the diol dehydrase reaction takes place by a radical rearrangement and the cobamide portion of the cofactor plays no role in this segment of the reaction. It is fair to point out, however, that while these model reactions do produce oxo compounds from vicinal diols by a radical mechanism, hydroxyl migration as seen in the enzyme-catalyzed reaction has so far not been demonstrated. Until evidence for such an event is provided, these models must be regarded as imperfect representations of the diol dehydrase reaction.

A recent free radical model of the methylmalonyl CoA mutase reaction has been proposed by Bidlingmaier et al.[203] These workers reported that photolysis of cobalox-

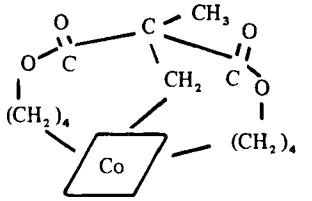
ime VII, or storage of the corresponding cobalamin, afforded methyl succinate, the rearrangement product. Interestingly, a small



VII

amount of acetic acid was needed in order to obtain consistent results (see the previous discussion of Golding's experiments). The authors interpreted these results by proposing homolysis of the cobalt-containing species to give a Co(II)-containing macrocycle and a carbon radical resembling that produced by the abstraction of a hydrogen from methylmalonyl CoA in the enzyme-catalyzed reaction. They suggested that this substrate-like radical rearranged to methylsuccinate by a mechanism which depended on the presence of the Co(II) macrocycle. As evidence supporting the participation of the Co(II) macrocycle in the rearrangement mechanism, the authors cited a reaction in which the appropriate radical was generated by another method in the absence of cobalt and failed to rearrange.[204]

In a refinement of this model, Rétey et al. synthesized the capped cobaloxime VIII.[205] They reasoned that with this model, they would observe



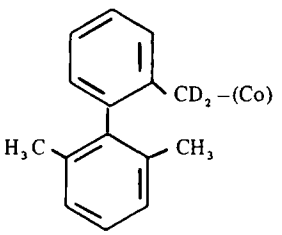
VIII

a higher yield of rearranged product because the caged structure would prevent the radical formed by photolysis from diffusing away from the Co(II) species postulated to catalyze the rearrangement. Photolysis of the capped cobaloxime followed by alkaline hydrolysis gave methylsuccinic acid as the exclusive product. Additional evidence provided to support the catalytic role of the Co(II)-containing macrocycle in this rearrangement was the observation that bromomethyl methyl malonate, the alkylating agent used to prepare the model cobaloxime, yielded unrearranged methylmalonate as the sole product of its reaction with tri-*n*-butyltin hydride, a reagent which produces free radicals from alkyl halides.[206] On the other hand, tri-*n*-butyltin hydride is known to reduce free radicals exceedingly rapidly, so this observation might merely indicate that the dimethylmalonyl free radical was reduced by a second molecule of tributyltin before it could rearrange.

A role for the Co(II) macrocycle in the radical rearrangement thus remains to be documented. Rather than indicating a requirement for the Co(II) of the macrocycle, the high yield of rearranged product obtained by photolysis of the capped cobaloxime could just as easily be explained by the ability of the bulky caged structure to protect the radical from side reactions by steric means. In considering this reaction as a model of the methylmalonyl CoA-catalyzed rearrangement, the absence of any dimethylmalonate as a product is somewhat troubling, since the enzyme-catalyzed reaction is reversible. In spite of these objections, however, this model displays a very compelling

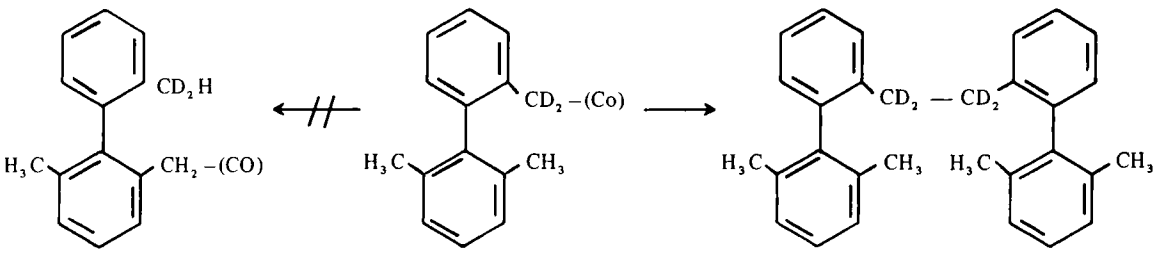
feature; namely, a radical close in structure to the substrate radical rearranges to product.

Experiments to demonstrate radical rearrangements have been carried out by Breslow and Khanna, using cobamides themselves, as well as the chemically related cobaloximes, as the sources of the radicals.[207] Their experiments involved homolysis of an alkyl cobalamin or cobaloxime to generate a radical which could rearrange to a new radical by intramolecular hydrogen transfer. In their initial experiments, the alkyl cobalamin and cobaloxime each contained the same deuterated aromatic axial ligand (IX). Both these compounds were

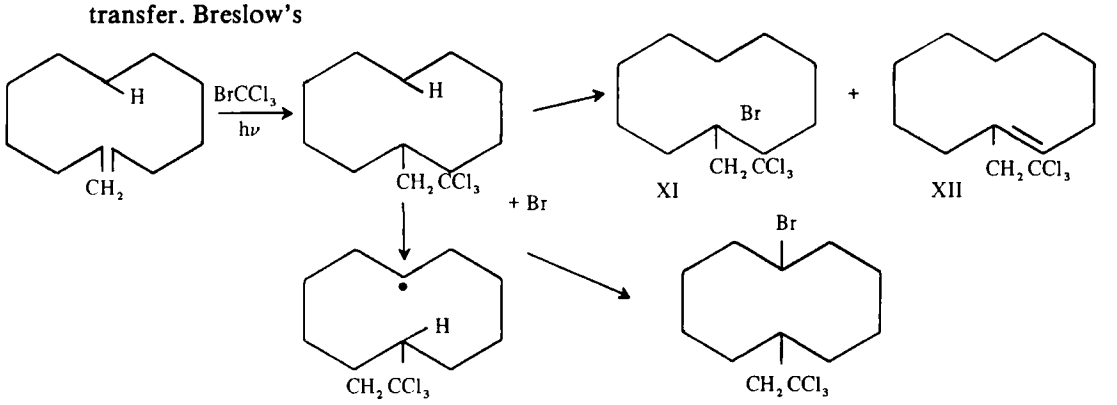


IX

treated so as to homolyze the C-Co bond. The rationale was that the resulting benzylic radical would carry out a 1,5 abstraction of hydrogen from the methyl group on the 2,5-dimethylphenyl substituent, and then would recombine with the Co(II) macrocycle to produce a rearranged organocobalt complex which would be detected by analysis of the product mixture. However, the radical produced by photolysis dimerized without yielding hydrogen transfer products:

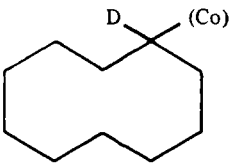


These workers then turned to the cyclodecyl system, for which intramolecular hydrogen transfer had already been demonstrated. Several years earlier, Fisch and Ourisson had investigated the reaction which took place when $BrCCl_3$ was photolyzed in the presence of methylenecyclodecane, conditions which led to the formation of the substituted cyclodecyl radical.[208] The products isolated from the reaction mixture included not only the tertiary bromide (XI) and the olefin derived therefrom (XII), but also a secondary bromide (XIII) which must have arisen by transannular hydrogen atom transfer. Breslow's

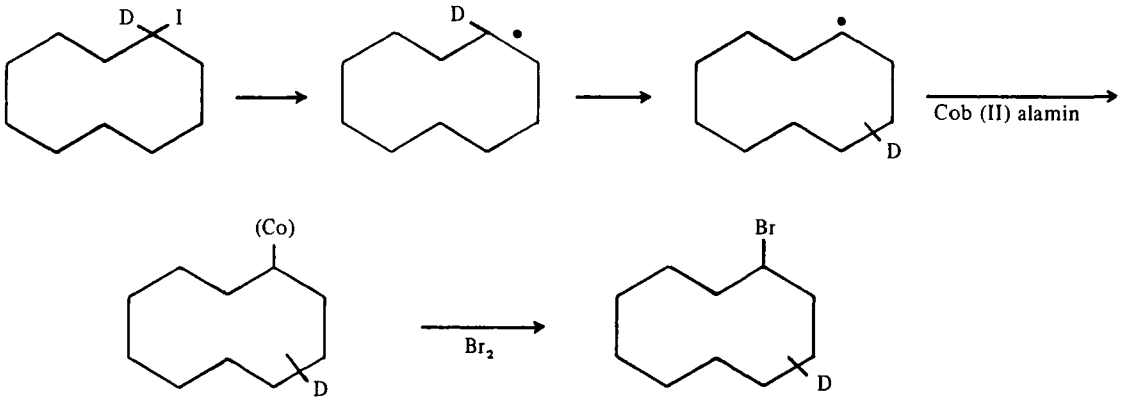


XIII

goal was to prepare [1-D] cyclodecylcobalamin and then to ascertain whether its photolysis would result in hydrogen transfer products. The [1-D] cyclodecyl



cobalamin was formed from the reaction of cyclodecyl iodide and cob(I)alamin. Cyclodecyl tosylate did not react with cob(I)alamin, an unexpected result if alkylation took place by the usual nucleophilic displacement, since tosylates are at least as reactive as iodides in this type of reaction. Because of the unreactivity of the tosyl derivative, Breslow and Khanna proposed that the alkylation of cob(I)alamin by cyclodecyl iodide did not involve nucleophilic displacement of I^- by the cobamide, but rather occurred by an electron transfer mechanism in which cyclodecyl iodide was oxidized by cob(I)alamin to produce cob(II)alamin and the cyclodecyl radical, which then coupled to form cyclodecylcobalamin. The formation of the cyclodecyl radical in the presence of cob(II)alamin was what the investigators had hoped to achieve by the photolysis of cyclodecylcobalamin, but had actually accomplished inadvertently during the synthesis of the alkyl cobamide. To determine whether the cyclodecyl radical had undergone rearrangement by hydrogen transfer before it combined with cob(II)alamin, the investigators cleaved the alkyl cobamide with bromine and measured the distribution of deuterium in the resulting cyclodecyl bromide. NMR

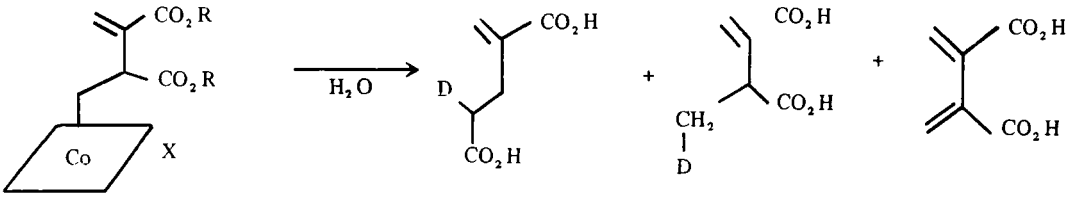


spectroscopy showed the deuterium to be distributed over several carbon atoms, indicating that the radical had in fact rearranged. Since the cyclodecylcobalamin itself spontaneously decomposed upon storage to products with the same deuterium distribution, the rearrangement had to have occurred during the alkylation reaction, not during the subsequent workup. The ability of radicals to alkylate cob(II)alamin had previously been demonstrated by Schrauzer,[209] while transannular hydrogen migration to cyclodecyl radicals had been shown by Fisch and Ourisson, as mentioned above. These authors have been able to combine both observations in one experiment, an interesting though not unexpected result.

Rearrangement via Allylcarbinyl Ions

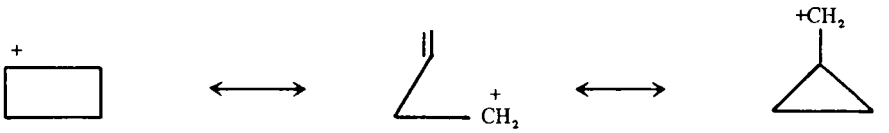
Dowd proposed a model for α-methyleneglutarate mutase which involved the decomposition of cobamide X, whose axial substituent was a diester of α-methyleneglutaric acid.[210,211] The observations on which Dowd based his conclusions were made with the 2-hydroxypyranyl ester, which could only be partially purified without decom-

position. This material, upon either irradiation (48 to 60 hr) or storage in the dark (168 to 200 hr), yielded α-methyleneglutarate (15%), the rearranged product, along with methylitaconic acid (7%) and butadiene-2,3-dicarboxylic acid (3.5%). When the reaction was



carried out in D_2O, deuterium was found to be incorporated in the products as shown. The position of deuterium was regarded as evidence for migration of the acrylate group rather than the esterified carboxyl group. It was emphasized that this was the first time that a derivative of a true substrate of a cobamide-dependent mutase had been rearranged in a cobamide-requiring reaction in the absence of the enzyme. Based on this model, Dowd assigned a key role in the enzyme-catalyzed reaction to the formation of a bond between cobalt and a substrate carbon (transalkylation) and suggested that the enzyme served no catalytic role apart from bringing the substrate and the coenzyme together.

The mechanism proposed for the rearrangement was an equilibration involving allylcarbinyl cations or their organometallic counterparts. Allylcarbinyl cations are homoallylic ions which are generally regarded as resonance hybrids of a category of carbonium ions whose other members include ions with three- and four-member



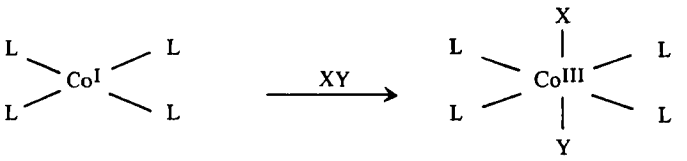
rings. Ordinarily, the products found in reactions thought to involve allylcarbinyl ions include derivatives of cyclopropane and cyclobutane. The failure to detect such products in the reaction described by Dowd renders the participation of allylcarbinyl ions somewhat unlikely, although it does not exclude them.

There are other problems with these experiments. Whereas the dipyranyl ester underwent rearrangement, the dimethylester was stable to the reaction conditions. Why the two esters should behave differently remains unexplained, although it may relate to the difference in the methods used in preparing the two cobamides, one of which (the dimethyl ester) was purified by phenol extraction, while the other was not. Furthermore, the structure of the dipyranyl ester cannot be regarded as firmly established. In fact, a mixture of alkyl cobamides is distinctly possible, since the synthetic method was ambiguous (alkylation of the precursor bromide by cob(II)alamin could occur either by displacement of Br^- or by Michael addition across the exo-methylene double bond), and the method for characterizing the product, optical spectroscopy, cannot distinguish between pure alkyl cobamides and mixtures of these compounds, most of which have very similar spectra.

These problems aside, the main question raised by this work is the following: can these results be explained without invoking organocobamides as specific requirements? In other words, could an appropriate alkyl fragment, generated by means not involving a cobalt-containing macrocycle, also undergo rearrangement to yield similar products? If so, the idea that organocobamides are obligatory participants in this model rearrangement (and, by extension, in the α-methyleneglutarate mutase reaction) would have to be reevaluated.

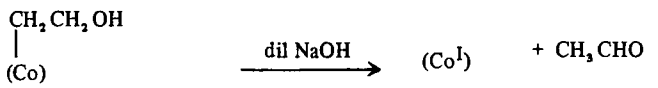
Oxidative Addition and Reductive Elimination

Certain transition metals, particularly those with nearly filled *d* shells (d^7-d^{10}), undergo a characteristic reaction in which the coordination number and the formal oxidation state of the metal both change by 2. If these values increase, the reaction is known as oxidative addition; the reverse reaction, leading to a decrease in these values, is called reductive elimination. A representation of this reaction is shown below, in which a hypothetical 4-coordinate Co(I) complex undergoes reversible oxidative addition to a 6-coordinate Co(III) complex. Oxidative addition reactions are the topic of a recent review by Halpern.[212]

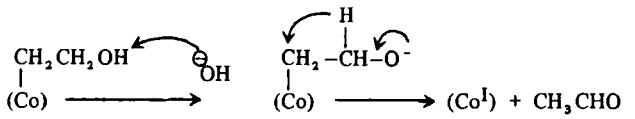


As discussed earlier, organocobalamins can undergo C-Co cleavage either homolytically or heterolytically. One type of heterolytic cleavage proposed as a model for adenosylcobamide-requiring reactions is a reductive elimination with rupture of the C-Co bond to yield cob(I)amide and an alkyl fragment.

The bulk of work on reductive elimination in cobamides has been carried out by Schrauzer and associates, who have proposed a mechanism for the diol dehydrase reaction based on this process.[213,214] They demonstrated that β-hydroxyalkylcobamides undergo base-catalyzed eliminations. An example is the elimination of acetaldehyde from β-hydroxyethylcobalamin:

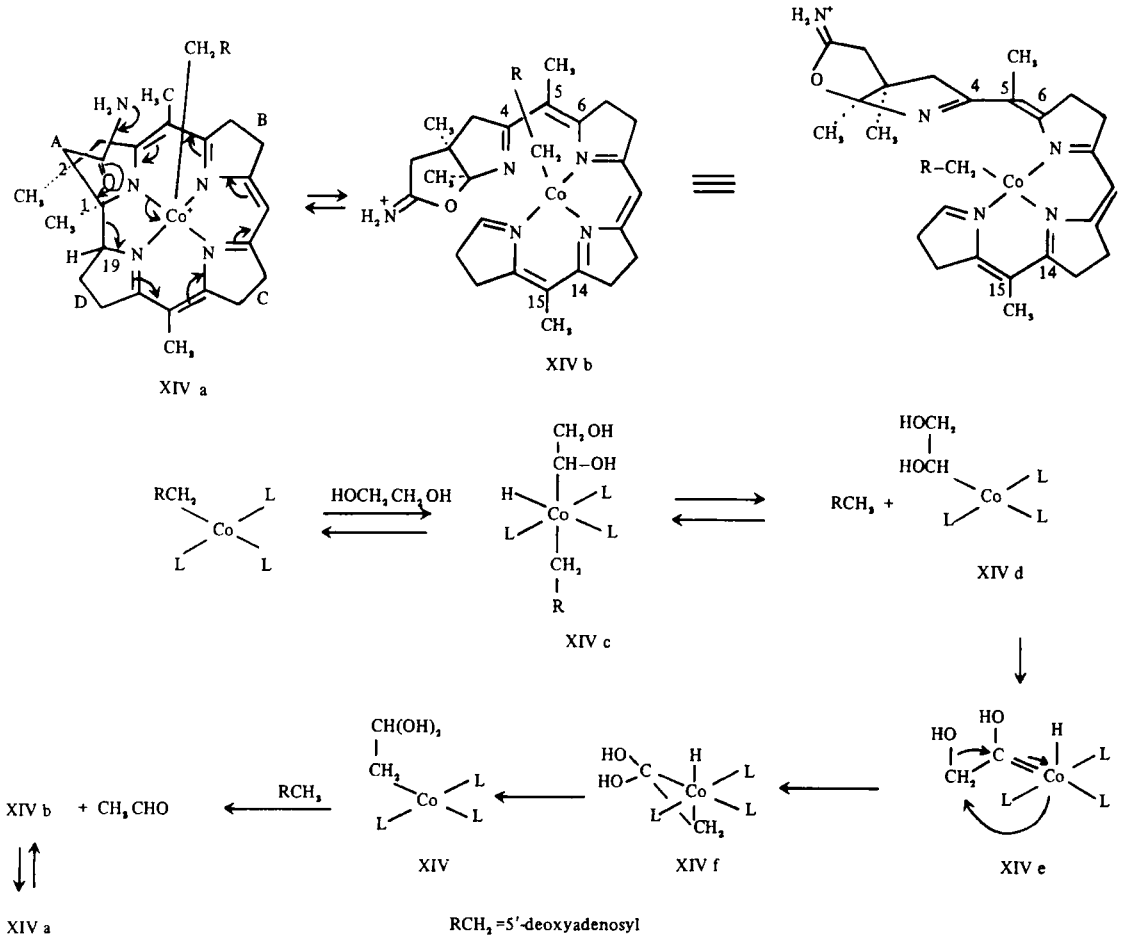


Similar reactions have been demonstrated with other β-hydroxyalkyl derivatives. However, a β-ethoxyalkylcobamide failed to eliminate, even in 50% NaOH. These findings led to the following proposed mechanism, which involves departure of cob(I)alamin accompanied by a hydride shift:



Although simple β-alkoxyalkylcobamides are stable to base-catalyzed elimination, certain more complex members of this class are able to undergo this reaction. In particular, adenosylcobalamin, a β-alkoxyalkyl derivative, undergoes reductive elimination in the presence of base. The reductive elimination of both adenosylcobalamin and β-hydroxyethylcobalamin form the basis for Schrauzer's proposed mechanism for the diol dehydrase reaction, which was discussed in detail in the section entitled An Alternative Mechanism.

Another mechanism involving reductive elimination has been proposed recently by Corey and associates.[215] This is a rather complex mechanism in which the rearrangement of substrate is accomplished by a series of reductive eliminations and oxidative additions involving a 4-coordinate Co(I) complex which is initially produced on the enzyme by the opening of the corrin ring. The details of the mechanism are shown below, using the diol dehydrase reaction as the specific example:

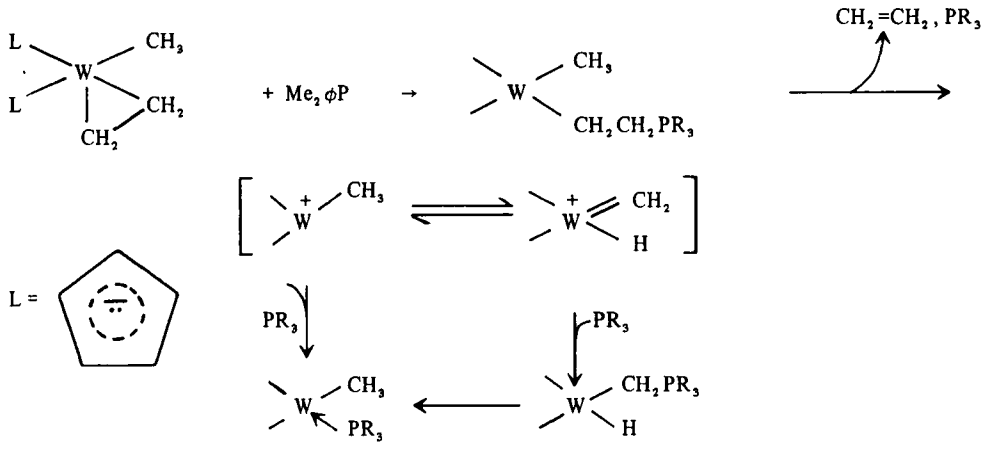


Its features are as follows. The cofactor is initially bound to the active site in a 5-coordinate "base-off" form (XIVa). The amide oxygen of the A-ring acetamide residue attacks the nearest α-carbon atom, opening the macrocycle by displacing the D-ring and itself bonding to that carbon to form a cyclic imino ester (iminolactone). Concomitant with the opening of the macrocycle, the nitrogen atom of the iminolactonized A-ring leaves the cobalt coordination sphere, generating a fundamentally altered species in which the cobalt, now in the d^8 Co(I) state, is coordinated to only 4 ligands: the three remaining macrocycle nitrogens and the deoxyadenosyl group (XIVb). It is this 4-coordinate Co(I) species which is postulated to be the active form of the coenzyme.

Catalysis now takes place by oxidative addition and reductive elimination reactions involving this active form. The original cobamide, containing the intact macrocycle, plays no further part in the reaction, serving only as a precursor of the active species. The substrate adds oxidatively to the 4-coordinate cofactor, giving a 6-coordinate species in which the migrating hydrogen has been transferred as a hydride ion to one of the equatorial coordination positions (in this discussion, the nitrogens are regarded as equatorially coordinated), while the axial positions contain the deoxyadenosyl fragment and the substrate fragment, both attached to the metal by C-Co σ-bonds (XIVc). Reductive elimination of the deoxyadenosyl moiety now takes place, giving rise to 5′-deoxyadenosine and a new 4-coordinate Co(I) complex in which the substrate fragment has replaced the deoxyadenosyl fragment (XIVd). It is in this step that the migrating hydrogen atom becomes incorporated into 5′-deoxyadenosine. The next step is an in-

ternal oxidative addition in which the hydrogen attached to the cobalt-linked carbon of the substrate fragment is transferred as a hydride ion to the cobalt coordination sphere, producing an organometallic carbene (XIVe). This rearranges with migration of the proximal −OH group to form a π-complex (XIVf), shown here in its cyclopropane-like resonance form. Internal reductive elimination with transfer of the hydride ion back to the alkyl fragment leads once again to a 4-coordinate Co(I) complex, this time, however, carrying the alkyl fragment in its rearranged form (XIVg). Readdition of 5′-deoxyadenosine followed by a final reductive elimination forms the final product, acetaldehyde, and regenerates the active form of the cofactor.

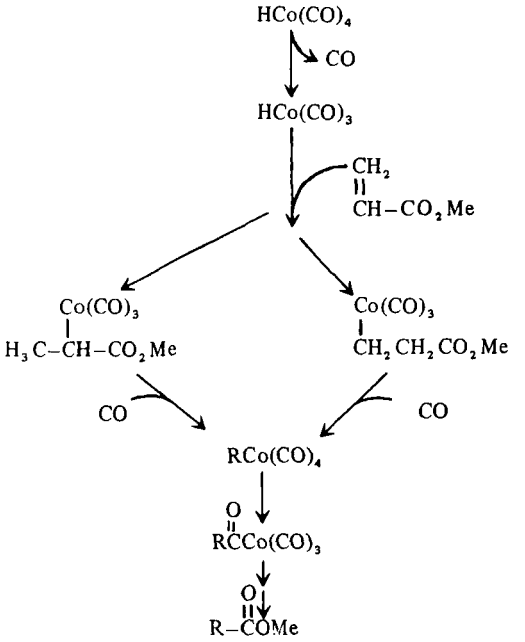
As mentioned above, oxidative addition and reductive elimination are characteristic reactions of transition metals. Precedents for the internal oxidative addition and isomerization reactions postulated in the above mechanism (the production of XIVe, XIVf, and XIVg) are provided by reactions involving alkyl tungsten complexes:[216,217]



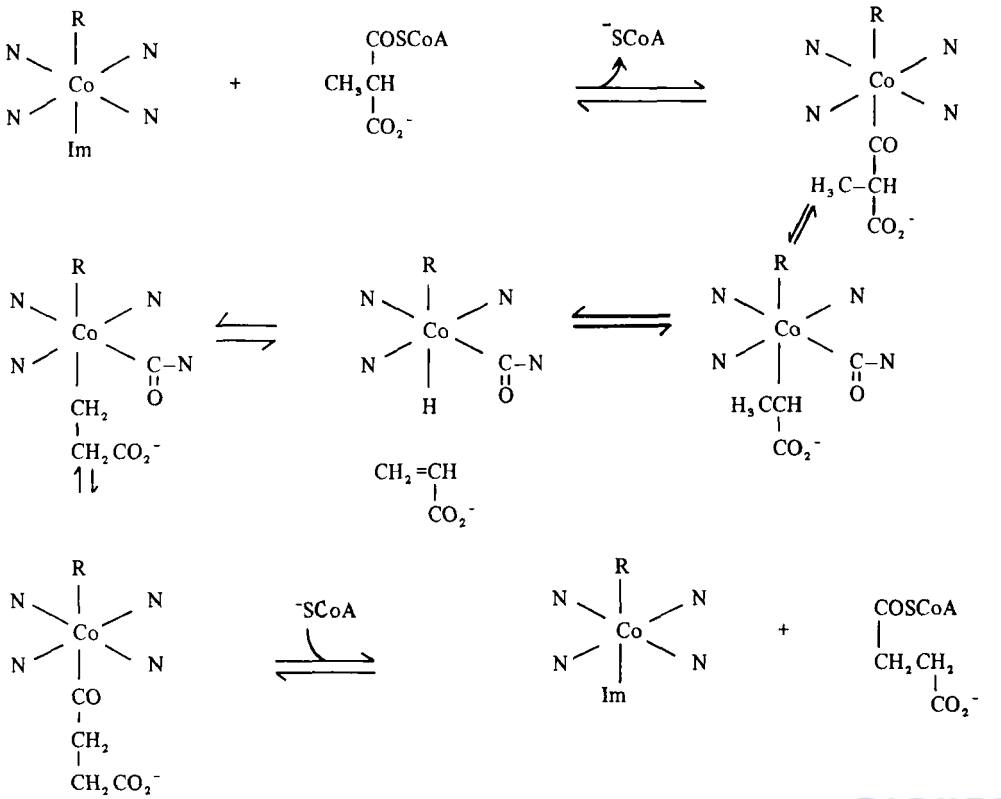
This proposal is thus consistent with known organometallic chemistry. It does not, however, explain all the observations made with enzymatic systems. In particular, it does not account for the participation of radicals in adenosylcobamide-requiring rearrangements, a feature for which abundant evidence exists. In addition, there is no evidence whatsoever to support the notion that the corrin macrocycle opens during catalysis, although to be fair it must be acknowledged that this possibility has never been considered before, so that biochemists working in the field have never looked specifically for such an event. On its face it seems to be a rather unlikely mechanism, but, like many other mechanisms proposed to explain the migration of group X in the mutase reactions, it has yet to be ruled out.

Migratory Insertion

Some time ago, Heck and Breslow[218] described a reaction which has been discussed by Whitlock as a model for the migration of the acyl CoA group in the methylmalonyl CoA mutase reaction.[219] In the model, methyl methacrylate was converted to a mixture of the methyl esters of succinic and methylmalonic acids by treatment with cobalt tetracarbonyl hydride and carbon monoxide followed by iodine in methanol. The mechanism proposed to account for this reaction involved spontaneous dissociation of cobalt tetracarbonyl hydride



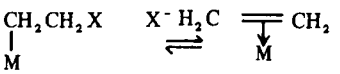
to form cobalt tricarbonyl hydride, which added across the double bond of methyl methacrylate to give a mixture of α- and β-alkylcobalt tricarbonyl complexes. Addition of carbon monoxide to this mixture gave the corresponding tetracarbonyl derivatives, which upon migratory insertion of carbon monoxide into the C-Co bond, followed by other steps, afforded the mixture of product esters. Based on this mechanism, Whitlock suggested that interconversion between methylmalonyl CoA and succinyl CoA might proceed by a similar sequence of steps, as shown below:



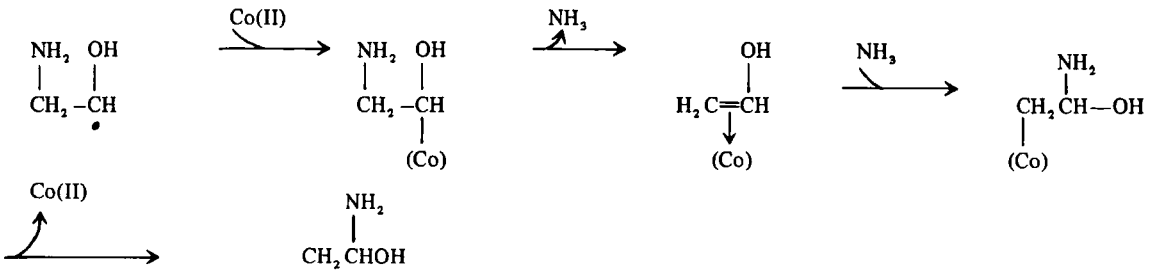
The first step features acylation of the cobalt by substrate, the reaction taking place on the α side (that is, the dimethylbenzimidazole side) of the corrin ring. Acylation is followed by a sequence which includes reversible migratory insertion of the acyl carbonyl between cobalt and one of the macrocycle nitrogen atoms and reversible elimination of propionate to yield a cobalt hydride and acrylic acid. The model reaction of Heck and Breslow provides a precedent for all of the steps in the mechanism proposed by Whitlock for the methylmalonyl CoA mutase reaction. However, this mechanism seems unlikely in view of the remarkable stability of the interaction between the cobalt and the corrin ring, which makes the displacement of one of the ring nitrogens from the cobalt coordination sphere difficult to conceive, and the recent evidence concerning the intermediacy of 5′-deoxyadenosine and paramagnetic species in adenosylcobamide-requiring reactions, evidence which to be sure was obtained long after Whitlock's proposal was published, but which nevertheless is not accounted for by the migratory insertion mechanism.

Sigma-Pi Rearrangements

Sigma-pi interconversions are typical reactions of organometallic compounds which possess a nucleophilic leaving group β- to the carbon-metal bond.[220] In these reactions, π complex formation is accomplished by the departure of the β-leaving group, together with its electrons, from the precursor σ complex. The σ complex can be regenerated by nucleophilic attack on the olefin. If the carbon atom which accepts the nucleophile is different



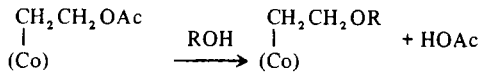
from the one originally carrying the leaving group, the net result will be the isomerization of the σ complex. The applicability of such a reaction to adenosylcobamide-dependent mutases is obvious, particularly in those cases which involve migration of an electronegative group (−OH or $-NH_2$). Alkylation of enzyme-bound cob(II)alamin by the substrate radical produced by abstraction the migrating hydrogen would yield an alkyl cobalamin which, in principle, could undergo a $\sigma \rightarrow \pi \rightarrow \sigma$ rearrangement to produce an alkyl cobalamin in which the migration of group X has taken place. Homolysis of this compound followed by a reversal of the hydrogen transfer steps would complete the reaction and yield the rearranged product. The essential steps are shown below for the ethanolamine ammonia-lyase reaction:



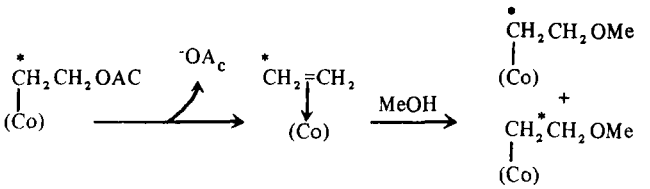
Recent studies with organocobamides and organocobaloximes have disclosed several model reactions whose mechanisms can best be rationalized by postulating $\sigma \rightarrow \pi$ interconversions. Schrauzer claims to have found π complexes in reactions between cob(I)alamin and electron-deficient olefins such as fumaronitrile, but since these claims are based on optical spectroscopic data only, they must be viewed with caution.[221] He also claims to have observed a cob(I)alamin-mediated interconversion between malon-

onitrile and fumaronitrile which he attributed to a $\sigma \rightarrow \pi$ rearrangement, but the evidence on which this conclusion was based is also weak.[221]

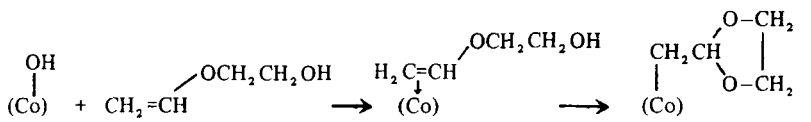
The first solid indication that organocobalt compounds could undergo $\sigma \rightarrow \pi$ rearrangements was the work of Golding and associates on the solvolysis of certain alkylcobaloximes.[222] They showed that β-acetoxyalkyl cobaloximes are converted to β-alkoxyalkyl cobaloximes by incubation at room temperature in alcohol at neutral pH. This reaction occurred by an intramolecular process—



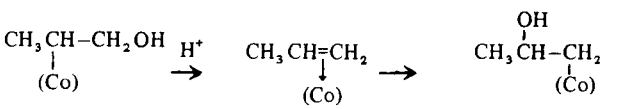
that is, the alkyl group was not released from the cobalt during the course of the reaction. Among the mechanisms proposed to explain this reaction was one involving a $\sigma \rightarrow \pi$ rearrangement. Evidence for this mechanism was later provided by the observation that β-acetoxyethyl cobaloxime labeled exclusively on the α-carbon atom with ^{13}C was solvolyzed in methanol to β-methoxyethyl cobaloxime with complete scrambling of the label between the α- and β-carbons.[223] This observation indicated that solvolysis proceeded by way of a symmetrical intermediate, supporting the idea that a π-complex between ethylene and cobaloxime participated in this reaction.



Up to that point, π complexes had been thought to arise by the reaction of a metal in a low valence state with an olefin substituted with electron withdrawing groups, the complex being stabilized by $d \rightarrow \pi^*$ back-bonding between the electron-rich metal and the electron-poor double bond of the olefin. However, the metal in Co(II)-containing complexes such as β-acetoxyethyl cobaloxime and adenosylcobalamin is relatively electron-poor, and as such would not be expected to form stable π complexes, at least according to the foregoing considerations. To explain this paradox, Silverman and Dolphin proposed that a Co(III)-containing π complex could be stabilized, provided the ligand coordinated to the electron-poor metal were an electron-rich olefin.[224] Consistent with this proposal, these workers found that hydroxocobalamin, a Co(III)-containing complex, could be alkylated by certain electron-rich olefins under mild conditions.[224] As shown below, these alkylations were conceived as proceeding by way



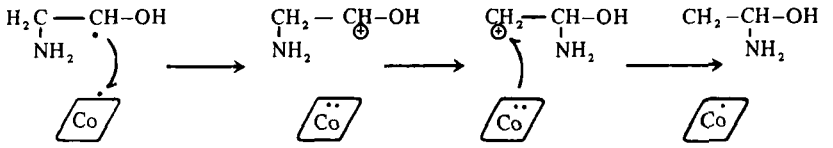
of an initial π complex which was subsequently converted to a σ complex by nucleophilic addition to one end of the double bond. A related reaction was discovered by Brown and Ingraham, who showed that 1-hydroxypropyl-2-cobaloxime could be converted to 2-hydroxypropyl-1-cobaloxime by dilute acid.[225] This reaction can also be envisioned as proceeding via a π complex.



The feasibility of enzyme-catalyzed $\sigma \rightarrow \pi$ rearrangements in adenosylcobamide-requiring systems is demonstrated by these model reactions. Evidence from biochemical systems for the occurrence of $\sigma \rightarrow \pi$ rearrangements remains to be obtained.

Substrate Radical Oxidation by Cob(II)alamin

Halpern has proposed a mechanism for ethanolamine ammonia lyase in which cob(II)alamin oxidizes the substrate radical to give a carbonium ion and cob(I)alamin.[226] After migration of the amino group,



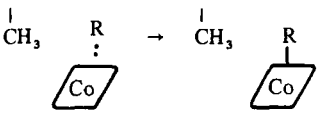
reversal of the previous electron transfer yields the product radical and regenerates cob(II)alamin. So far, no experimental evidence has been offered to support this proposal, although it is theoretically attractive. In considering this possibility, it must be borne in mind that cob(I)amides have been sought but never found as intermediates in adenosylcobamide-requiring reactions.

Results from Experiments with Enzymes — Evidence Against Transalkylation

The previous section has provided a survey of the various chemical model reactions upon which hypotheses have been based to explain group X migration. The abundance of models and their chemical diversity are striking. This plus the possibility that more than one mechanism may prevail for group X migration makes it exceedingly difficult to select from the models the one(s) most relevant to the enzyme-catalyzed reactions.

There is really only one criterion on which this selection can be based. This is that the model reaction must be compatible with evidence derived from experiments with enzymes. This criterion is in fact fulfilled by most of the models discussed above, probably because the evidence presently available concerning group X migration is scanty and for the most part negative. Most of the evidence has been obtained in experiments carried out to ascertain whether a particular compound is an intermediate in group X migration catalyzed by a given enzyme. The method used has been to determine whether the postulated intermediate is exchanged into substrate or product by the enzyme under study. The enzymes and intermediates that have been tested are listed in Table 10.[52,54,60,73-75,83,86,96,98,102,227-229] All of these experiments have failed, ruling out the participation of the listed compounds as free intermediates in the rearrangements, although of course no information is provided concerning their possible participation as enzyme-bound intermediates (cf., the situation with 5′-deoxyadenosine).

Many of the proposals discussed in the section on model reactions share a common feature; namely, the substrate radical alkylates the cob(II)amide



at the active site of the mutase (the transalkylation mechanism). This step seems quite reasonable on two grounds: first, because the two paramagnetic species are confined together at the active site, and second, because there are many precedents among the model reactions for rearrangement of the new alkyl cobamide formed in this reaction. Notwithstanding its attractiveness, however, the transalkylation hypothesis has for many years lacked experimental support. In particular, a cobamide transalkylated by either a true substrate or a substrate analogue has never been isolated from an enzymatic system, despite numerous experiments by several groups which have been carried

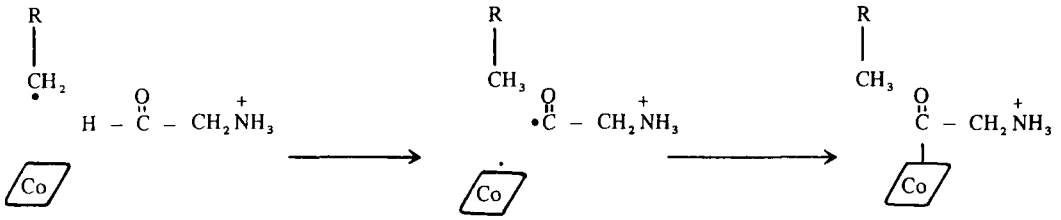
TABLE 10

Compounds Demonstrated not to be Free Intermediates in Adenosylcobamide-Dependent Rearrangements

Enzyme	Postulated intermediate
Glutamate mutase	Glycine
	Propionic acid
	Acrylic acid
	α-Ketoglutaric acid
Methylmalonyl CoA mutase	Propionic acid
	Acrylic acid
α-Methyleneglutarate mutase	Acrylic acid
	1-Methyl-1,2-*cis*-cyclopropanedicarboxylic acid
	1-Methyl-1,2-*trans*-cyrlopropanedicarboxylic acid
	1,2-Cyclobutanedicarboxylic acid
β-Lysine mutase	NH_4^+

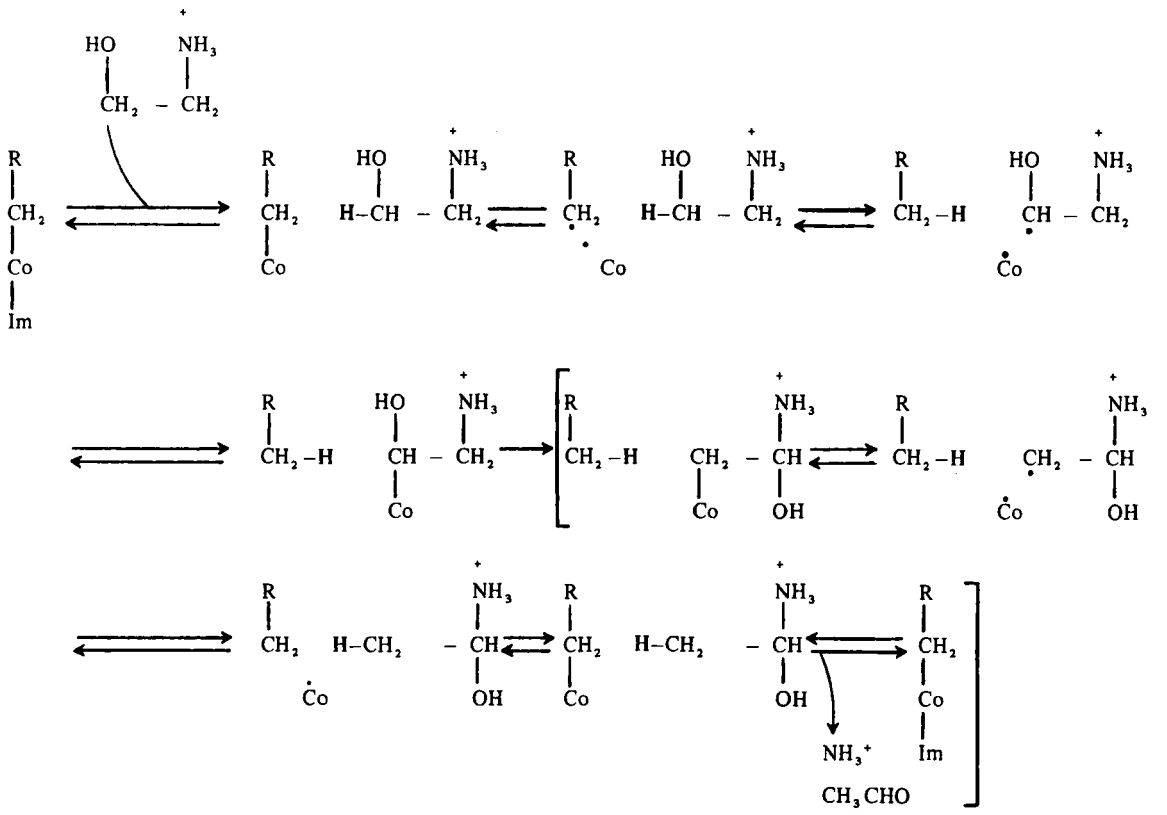
out with this aim in mind. While the foregoing constitutes a weak argument against the transalkylation hypothesis, the past 2 years have witnessed the appearance of evidence against this hypothesis which is much more substantial.

One piece of evidence has been obtained using the ethanolamine ammonia lyase system.[230] This evidence was based on the observation that this enzyme was able to cleave adenosylcobalamin in the presence of 2-aminoacetaldehyde. If the mechanism of this reaction resembled the catalytic mechanism (a likely supposition) and if the catalytic mechanism contained a transalkylation step, then transalkylation of cobalamin by an aminoacyl radical to form the known, stable glycylcobalamin should have occurred:



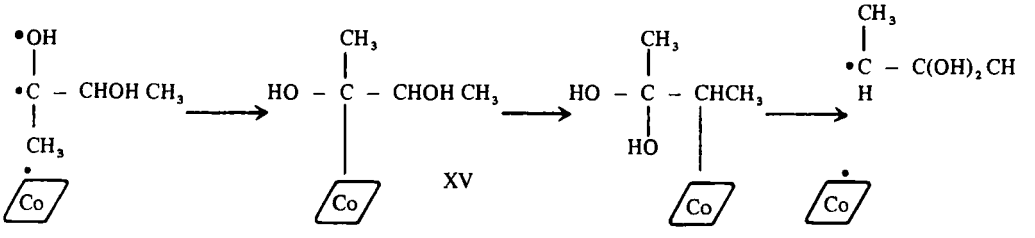
What was actually observed was the deamination of aminoacetaldehyde to acetic acid, accompanied by the oxidation of the adenosyl group of the cofactor to 4′-5′-anhydroadenosine together with the destruction of the corrin ring. Glycylcobalamin itself was stable to the enzyme, indicating that the products did not arise by the formation and subsequent degradation of glycylcobalamin. The substrate analogue was thus deaminated without transalkylation of the cobamide, a finding which suggests that the deamination of the true substrates could similarly take place without transalkylation.

Further evidence against transalkylation was obtained in a study of the interaction between ethanolamine ammonia lyase and the ammonia adduct of formylmethylcobalamin.[231] This cobalamin is in fact the rearranged transalkylation intermediate in the ethanolamine ammonia lyase reaction. The transalkylation hypothesis predicts that ethanolamine ammonia lyase would catalyze a reaction between 5′-deoxyadenosine and the ammonia adduct of formylmethylcobalamin to produce acetaldehyde, ammonia, and adenosylcobalamin. This reaction is actually the final segment of the hypothetical catalytic sequence for the deamination of ethanolamine by rearrangement of a transalkylated cobamide (see scheme):



This prediction was tested by looking for adenosylcobalamin production, using an assay capable of detecting the conversion of 0.0006% of the added formylmethylcobalamin to cofactor. Even with this extremely sensitive assay, no cofactor was detected. Although negative results of this kind cannot be interpreted unambiguously, this result is certainly consistent with the notion that transalkylation does not occur in the ethanolamine ammonia lyase reaction.

The strongest evidence against transalkylation has been provided by the recent studies on the substrate specificity of diol dehydrase.[50,51] As mentioned before, these studies showed that this enzyme is able to catalyze the conversion of 2,3-butanediol to methyl ethyl ketone. This mechanism of −OH group migration in this substrate according to the transalkylation hypothesis is shown below:



In this mechanism, the product of the initial transalkylation (XV) is of particular interest. In this intermediate, the cobalt is bonded to a carbon which is sterically equivalent to a tertiary carbon atom, since its other substituents consist of two carbons and an oxygen. Such a structure is so unlikely that the transalkylation mechanism is for practical purposes ruled out, at least for the dehydration of 2,3-butanediol by diol dehydrase.

In aggregate, the evidence presently available indicates that transalkylation of the

cobamide is probably not involved in at least those adenosylcobamide-dependent reactions in which oxo compounds are formed from glycols. Beyond this conclusion, the mechanism(s) for group X migration remain to be determined.

The Displacement of −OH in the Ribonucleotide Reductase Reaction

The mechanism of −OH displacement in the ribonucleotide reductase reaction is even less well understood than that of group X migration in the mutase reactions. Whereas a large number of model reactions provide a basis for constructing hypothetical mechanisms for group X migration, models for the ribonucleotide reductase reaction are completely lacking. Enzymological experiments show only that the −OH group is displaced directly by the incoming hydrogen atom, a finding which contributes little, if anything, to the understanding of the process. It is probably accurate to say that nothing at all is known about the mechanism by which ribonucleotide reductase catalyzes the displacement of −OH by −H.

THE ROLE OF THE ENZYME

The aim of most of the research on the mechanism of action of adenosylcobamide-dependent enzymes has been to understand how the cofactor participates in catalysis. There have been some studies, however, which have been concerned with the role of the enzyme in the catalytic process.

Binding of the Cofactor and the Homolysis of the C-Co Bond

One catalytic step for which the enzyme is obviously required is the homolytic cleavage of the C-Co bond of the cofactor. The rupture of this bond, which is weak to begin with, is likely to be accomplished by the application of a stretching force transmitted from the enzyme through the adenosyl residue and the corrin ring to the C-Co bond. Generally speaking, the energy required for such a distorting force is thought to originate from the energy released when the molecule to be distorted binds to the enzyme.[232-234] The binding reaction is a very exergonic process, and with suitable ligands (i. e., ligands which participate in catalysis, such as substrates and cofactors), a portion of the binding energy can be converted to a force which distorts the ligand and by this means facilitates the catalytic process.

With regard to adenosylcobamide-dependent enzymes, the existence of such a mechanism for facilitating C-Co bond cleavage would require that these enzymes bind both the adenosyl group and the corrin ring with considerable avidity. Considerable evidence for the binding of both these portions of the cofactor to the mutases as well as to ribonucleotide reductase has been obtained, largely through studies employing analogues of the adenosylcobamides.[48,57,59,61,62,70,91,102,113,153,154,174,179,227,229,232-261] Binding of the corrin ring has been demonstrated by experiments showing that a great number of corrinoids are powerful inhibitors of adenosylcobamide-requiring enzymes (both mutases and ribonucleotide reductases), displaying K_i values of the order of 1 μM or less. Though varying greatly in structure, particularly at the Coβ position, those corrinoids which have been shown to bind to adenosylcobamide-dependent enzymes have in common the feature that they are all fully amidated at the periphery of the ring. It is likely therefore that the binding of cobamides to enzymes takes place at least in part through interactions involving the peripheral carboxamide groups of the cofactor.

The adenine heterocycle has also been shown to bind to the active site of adenosylcobamide-dependent enzymes.[153,154,262] In some cases, this has been demonstrated by direct measurement of binding, while enzyme inhibition by adenosyl nucleosides,[167,249,262] or the effect of these nucleosides on the EPR spectra of enzyme·cobamide complexes,[168] have been used in other cases to infer an association

TABLE 11

Cofactor Activity of AdoCbl Analogues

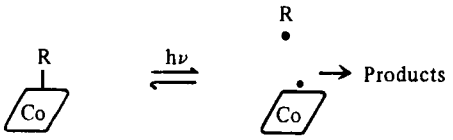
Active*	
AdoCbl	(AraAdo)Cbl
2′-dAdoCbl	(1-Ado)Cbl
3′-dAdoCbl	Aristeromycyl Cbl (bridge O of ribose replaced by $-CH_2-$)
(IproAdo)Cbl	Nebularyl Cbl (Ade replaced by Pur)
(N^6-MeAdo)Cbl	Tubericidyl Cbl (Ade replaced by 7-deazaAde)
Inactive or inhibitory	
UrdCbl	InoCbl
CydCbl	(Bza-ribosyl)Cbl
ThdCbl	(1-MeAdo)Cbl

* Reported to be active with one or more enzymes. Not all are active with every enzyme.

between the nucleoside and the enzyme. The most significant evidence, however, and that which pertains most directly to the binding of the heterocycle as opposed to the sugar, is that provided by studies carried out with a series of Coβ-nucleosidyl cobamides (Table 11). These studies showed that of all the compounds tested, only those in which adenine or a closely related compound was the heterocycle showed cofactor activity. The remainder were inhibitors which competed kinetically with AdoCbl. These findings indicate that in addition to a cobamide-binding site, adenosylcobamide-dependent enzymes possess an adenine-binding site, the occupancy of which is necessary for catalysis.

Occupation of the adenine site, however, is not the *sine qua non* for C-Co bond homolysis. This was shown in an important study carried out with AdoCbl analogues and ribonucleotide reductase in which a number of pyrimidyl nucleoside cobamides were found to be cleaved homolytically by the enzyme.[258] The common structural feature among the cobamides split by this enzyme was a β-furanosyl group connecting the cobalt to the heterocyclic base. The ribosyl moiety found in the natural cofactor is such a group, but several related groups which differ from the ribosyl moiety in their stereochemical configuration or the degree of oxygenation permit C-Co bond cleavage by ribonucleotide reductase. Similar observations have been made concerning cofactor activity of AdoCbl analogues. Compounds in which the D-ribosyl portion of the Co-linked adenosyl group has been replaced by 2′- and 3′-deoxy-D-ribose, 2′,3′-isopropylidene-D-ribose, L-ribose, D-arabinose, and a D-ribose analogue in which the ring oxygen was replaced by a $-CH_2$ group, have all displayed cofactor activity with one or another adenosylcobamide-requiring enzyme.[174,237,241,242,246,258,259] It seems then that the structure necessary for homolysis is a five-member ring attached to the Co by a $-CH_2-$ bridge, and that proper positioning of such a structure at the active site (the heterocycle undoubtedly plays an important part in this positioning) results in C-Co cleavage by the application of a stretching force to the C-Co bond. That this stretching force arises by the conversion of binding energy, as discussed above, is suggested by studies with adeninylalkyl cobamides, compounds in which the ribose has been replaced by a chain of methylene groups:[237,258,259]

With the correct n (usually, $n = 4$ or 5), these compounds are exceedingly powerful inhibitors of adenosylcobamide-dependent enzymes, K_i being an order of magnitude smaller than the K_m for the cofactors. It may be that with these analogues, the forces which are designed to stretch the C-Co bond merely change the conformation of the methylene bridge, a much less demanding task, so that more of the energy of interaction between the cobamide and the enzyme is expressed directly as binding energy (hence the low K_i) and less as deformation energy than in the case of the cofactors. Further evidence for this conclusion is the remarkable stability of the adeninylalkyl compounds toward photolysis when bound to adenosylcobamide-requiring enzymes, a degree of stability to be contrasted with the photolability of enzyme-bound nucleosidyl cobamides. Photolysis involves two separate events: the rupture of the scissile bond by light and the consumption of the light-generated fragments by secondary reactions. This second step competes with recombination, a process which regenerates the starting material, and is therefore not detected experimentally as a photolytic event:



It is likely that the C-Co bonds of both adeninylalkyl cobamides and nucleosidyl cobamides are dissociated by light when these compounds are bound to the enzyme, since both categories of cobamide photolyze readily in free solution. With the former, however, the methylene bridge must be held at the active site in a restricted conformation such that the free radical produced by C-Co bond photolysis remains close to the cob(II)alamin, and recombination is virtually the only reaction which the enzyme-bound species can undergo. With nucleosidyl cobamides, however, the stretching force applied by the enzyme ensures the separation of the radical and cob(II)alamin fragments as soon as the C-Co bond is broken by light, greatly reducing the probability of recombination and permitting photolysis to take place.

Besides weakening the C-Co bond of the cofactor, the enzyme must shelter the exceedingly unstable catalytic intermediates against degradation, particularly by oxygen. The major evidence for this, of course, is the high concentration of free radicals which some of the enzymes are able to maintain at the active site in steady state. The stability of the cob(II)alamin generated by the photolysis of enzyme-bound alkyl cobamides is another indication that adenosylcobamide-requiring enzymes can protect active site-bound species against oxidation. This protection, while highly effective, seems to be less than perfect. It is possible, for example, that the cob(II)alamin slowly but irreversibly generated at the active site of ribonucleotide reductase[159,168] appears because of the slow oxidation of a companion radical(s) formed in the catalytically significant C-Co homolysis reaction. The degradation of cofactor incubated with certain of the mutases in the absence of substrate is probably explained by the same mechanism,[148,263,264] particularly in view of data showing that the cofactor is stable in the presence of at least some of these enzymes under anerobic conditions.[148] Finally, it seems probable that the decline in activity which takes place during the rearrangement of certain substrates by some of the mutases[48,57,75] reflects, at least in part, the destruction of unstable intermediates by extraneous molecules (e.g., water or oxygen) which from time to time gain access to an imperfectly sealed active site.

The findings described above indicate that adenosylcobamide-requiring enzymes and adenosylcobamides associate by interactions involving at least three distinct regions of the cofactor: the corrin ring, the sugar of the Co-linked adenosyl group, and the nucleotide base of the same group. As a result of this association, the C-Co bond is labilized, while at the same time the active site is tightly closed to deny access to oxygen

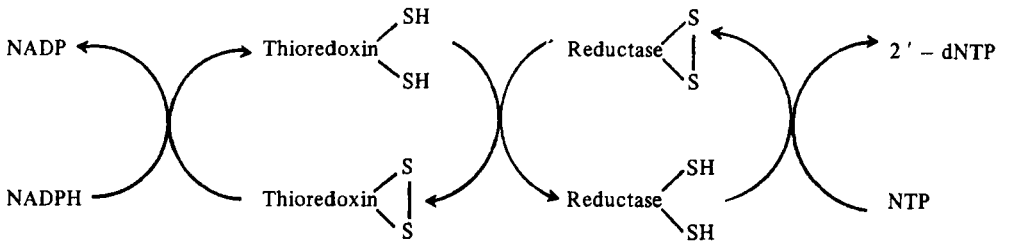
and other agents which could destroy the highly unstable catalytic intermediates generated during the reaction. With every enzyme the association with cofactor is thermodynamically strong. There is, however, considerable kinetic variability with regard to this association. With certain enzymes, cofactor once bound to the active site dissociates very slowly, if at all, unless the enzyme-cofactor complex is subjected to special conditions such as acid-ammonium sulfate treatment. This rather fixed association is shown by the observation that the enzymes carry cofactor with them through purification[56,72,73,75,229,240,265] and that in hydrogen exchange experiments only that fraction of added cofactor which initially associates with enzyme is able to exchange label with substrate or product.[67,175,266] Work with diol dehydrase by Fukui and associates has shown that, at least with that enzyme, K^+ is an important factor in the very stable enzyme-cofactor interaction.[235,267,268] With methyleneglutarate mutase[100] and ribonucleotide reductase,[142] however, enzyme-bound cofactor dissociates freely from the active site and exchanges readily with cofactor in solution. These enzymes are therefore obtained during purification as the cofactor-free apoenzyme, and hydrogen exchange reactions catalyzed by these enzymes result in the incorporation of tritium into all the cofactor in the reaction mixture, not just that fraction bound to the enzyme. An association between enzyme and cofactor which is freely reversible is thus compatible with catalysis. This is to be contrasted with the association between the enzyme and the 5′-deoxyadenosine generated during catalysis, an association which, almost certainly for important mechanistic reasons, is for practical purposes irreversible.

Sulfhydryl Groups

The importance of sulfhydryl groups for catalysis by adenosylcobamide-requiring enzymes has been amply demonstrated by many studies showing inhibition of such enzymes by −SH specific reagents.[48,56,61,62,72,73,102,228,229,236,238,240,263,265,268,269] Not all −SH specific reagents affect every enzyme, but (apart from the methylmalonyl CoA mutase of *Propionibacterium shermanii*) each enzyme tested to date is inhibited by at least one such reagent. In many cases, protection against −SH inhibitors is afforded by cofactor, as shown by the finding that the apoenzyme is much more sensitive to the inhibitor than the holoenzyme. It is of some interest in this regard that with the mutases, most of which are oligomeric enzymes composed of nonidentical subunits, the subunit to which the cofactor binds is different from the one which carries the critical −SH group. It is not known whether the protection by cofactor is due to a steric effect of the cofactor per se or to a conformational change leading to the burial of the critical −SH group.

The function of the −SH groups in the ribonucleotide reductase reaction has been clearly established.[270] Exposure of this enzyme to reduced thioredoxin or dihydrolipoic acid causes a reduction with the appearance of two −SH groups per molecule of enzyme. Incubation of the reduced enzyme with AdoCbl and ribonucleotide triphosphate leads to reoxidation with concomitant production of dNTP at a stoichiometry of 1 mol/mol of enzyme. With the enzyme, then, a pair of enzyme-bound −SH groups appear to undergo cyclic oxidation and reduction, transporting electrons from thioredoxin (or artificial exogenous reducing agents such as dihydrolipoate) to the nucleotide substrate:*

* Because of problems reconciling the rates of certain of the partial reactions with the overall catalytic rate, these observations were originally interpreted more conservatively. It is our belief that these problems are secondary matters related in part to tritium isotope effects and in part to the very complex regulatory properties of this enzyme. We are therefore willing to reinterpret this data, which we regard as convincing, in terms of the cyclic redox mechanism presented here.



In contrast to the ribonucleotide reductase reaction, nothing is known about the part played by −SH groups in the mutase reactions.

Miscellaneous Observations

Apart from the binding of cofactor to enzyme, the nature and importance of which is at least partially understood, and the function of −SH groups in ribonucleotide reductase, the details concerning the role of the enzyme in adenosylcobamide-requiring reactions are almost totally obscure. Here and there, though, there are a few observations which must represent important clues, if only they could be interpreted. What, for example, is the significance of the pyridoxal requirement in the aminomutase reactions,[64,72,73,75] and what, if anything, does it mean for other adenosylcobamide-requiring enzymes? Why are all but one of the mutases composed of two (in some cases, three) different kinds of subunits,[271] and why in many cases are the complete enzymes so large (e. g., ethanolamine ammonia lyase, possessing only two active sites,[179,237,260] is a 10- or 12-mer[272] constituted from two different types of subunits*)? Does the tyrosyl radical which seems to participate in catalysis by *E. coli* ribonucleotide reductase (an Fe-requiring enzyme)[273] say anything about the possibility of side-chain radicals in adenosylcobamide-requiring reactions? It is clear that despite the significant advances made in the past 15 years (or perhaps because of these advances) adenosylcobamide-requiring enzymes offer more questions than answers.

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