RECENT ADVANCES IN THE CHEMISTRY OF VITAMIN B_{12} AND VITAMIN B_{12} MODEL COMPOUNDS: REDUCTIVE COBALT–CARBON BOND CLEAVAGE REACTIONS

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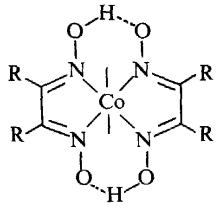
ABSTRACT

The cobalt–carbon bond in alkylcobaloximes and in alkylcobalt derivatives of related chelates is reductively cleaved by thiols in mildly acidic medium, or by carbon monoxide, dithionite and stannite in alkaline solution. The reductants interact initially by *trans* attack of the cobalt atom, followed by the rate-determining cleavage of the Co–C bond. Cobalt-bound methyl groups are converted into methyl carbanions, or species with the reactivity of methyl carbanions, which react with protons of the medium to form methane. In the presence of CO_2 detectable amounts of acetic acid are formed, in accord with this mechanism. The reductive cleavage of organocobalt complexes, derived from vitamin B_{12} or vitamin B_{12} model compounds, by thiols is correlated with available enzymological evidence on ribonucleotide reductase of *Lactobacillus leichmannii*, microbial methane biosynthesis of *Methanobacillus omelianskii* and acetate biosynthesis by *Clostridium thermoaceticum*. All three enzymatic processes are envisaged to involve reductive Co–C bond cleavage reactions of coenzyme–substrate intermediates as part of the catalytic cycle.

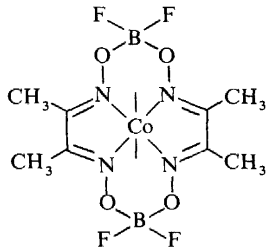
INTRODUCTION

A number of bacterial enzymes catalyse the reduction of substrates utilizing corrinoid coenzymes as cofactors, e.g. in ribonucleotide reductase of *Lactobacillus leichmannii*[1], methane biosynthesis by cell extracts of organisms such as *Methanosarcina barkeri*[2] and *Methanobacillus omelianskii*[3]. A probably related reaction is the synthesis of acetate from active methyl groups, as well as from methylcobalamin and carbon dioxide by extracts of *Clostridium thermoaceticum*[4]. In an attempt to devise functional non-enzymatic models of these reactions we have followed the hypothesis that corrin cofactors catalyse the reduction of these substrates by forming intermediate organocobalt compounds, which subsequently are reductively cleaved through interaction with biogenic reducing agents containing thiol groups. The fact that thiols or thioproteins are the reducing agents in ribonucleotide reductase is well established. However, the nature of the reducing agents interacting with the corrin cofactors in the remaining enzyme systems

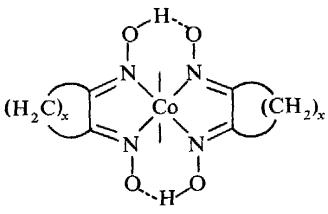
is as yet unknown. We have consequently first investigated the reductive cleavage of various alkylcobalt derivatives of vitamin B_{12} and of vitamin B_{12} model compounds (Chelates I–VII) by various thiols and dithiols. In the course



R = CH_3 (I) (Dmg)
R = C_6H_5 (II) (Dpg)
R = H (III) (Gly)

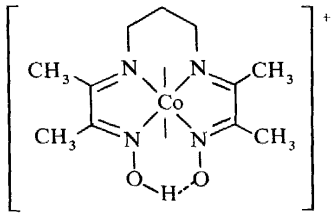


(IV) ($DmgBF_2$)



(V)

(x = 6, CHD)



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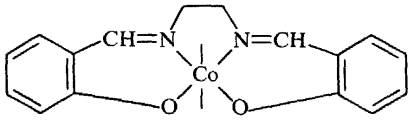


(VII)

(Sch.B.II)

of this work additional reducing agents capable of cleaving the cobalt–carbon bond were discovered, including alkaline carbon monoxide, dithionite, and stannite. The general features of these reactions will be outlined in the following and will be correlated with the enzymatic corrin dependent reductases. Individual examples of reductive Co—C bond cleavage reactions of alkylcobalamins and -cobaloximes by thiols have already been described[5], but have not yet been discussed in detail. It has also been reported that methylcobalamin undergoes reductive Co—C bond cleavage with Pt/H_2[6]. However, we shall confine ourselves in this paper to Co—C bond cleavage reactions in homogeneous systems.

THE REDUCTIVE COBALT–CARBON BOND CLEAVAGE BY THIOLS AND DITHIOLS

General characteristics of the reaction

Neutral or slightly acidic solutions of methylcobalamin or of methylcobaloximes remain unchanged in the dark at room temperature for many hours. Even at 65°C, these methylcobalt compounds are still essentially stable, giving rise only to traces of methane and ethane as the products of thermally induced Co—C bond homolysis. The addition of dithioerythritol (DTE), for example, to such solutions causes CH_4 evolution, the rate of which depends on the concentration of thiol, buffer, pH and the nature of the axial base components. For example, methyl(aquo)cobaloxime in pH 4.6 sodium acetate buffer with DTE releases half of the cobalt-bound methyl groups as methane in 39 hours at 65°C (*Figure 1*). Under these conditions the

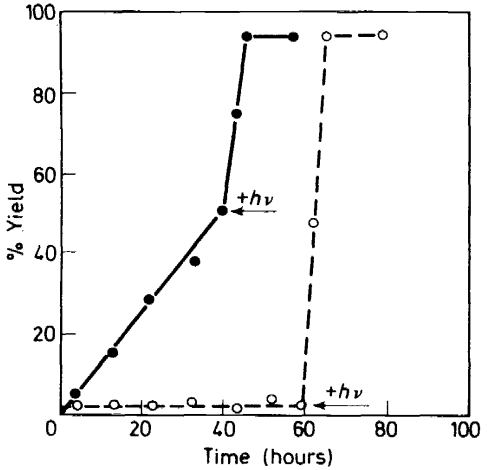


Figure 1. Methane yield as a function of time from methyl(aquo)cobaloxime (1.67 mM) with (———) and without (– – – –) DTE (0.05M) at 65°C in pH 4.6 sodium acetate buffer (0.167 M). Arrow indicates beginning of photolysis. Ethylene glycol, glycerol, butyl methyl sulphide or pyridine (0.01 M) instead of DTE is the same as plot (– – – –) without DTE

reductive Co—C bond cleavage is the main reaction; no methylated derivatives of the thiols are formed, and the remaining cobalt-bound methyl groups after 39 hours of reaction at 65°C may be converted quantitatively to methane upon subsequent photolysis. Methylcobalamin similarly releases half of the cobalt-bound methyl groups with DTE at pH 2.0 in 210 hours (*Figure 2*). Again, the remaining cobalt-bound methyl groups are released on photolysis, affording a recovery of over 95 per cent of CH_4 combined from both reductive cleavage and photolysis. No methylated thiols are produced under these conditions. The prolonged reaction of either methyl(aquo)-cobaloxime or methylcobalamin with DTE in mildly acidic solution in the

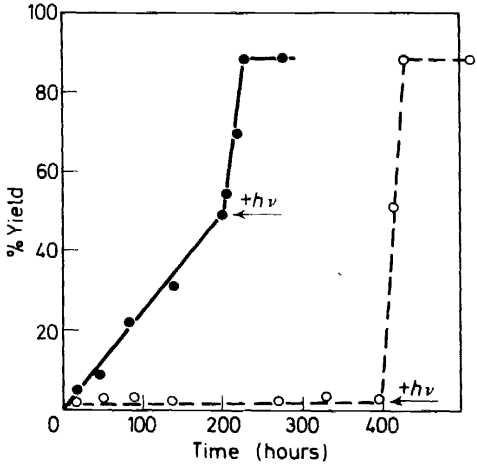


Figure 2. Methane yield as a function of time from methylcobalamin (1.67 mM) with (———) and without (– – – –) DTE (0.05 M) at 65°C in pH 2.0 sodium phosphate buffer (0.167 M). Arrow indicates beginning of photolysis. Ethylene glycol, glycerol, butyl methyl sulphide or pyridine (0.01 M) instead of DTE is the same as plot (– – – –) without DTE

dark at 65°C, finally leads to virtually total conversion of cobalt-bound methyl groups to CH_4. Dithiols such as DTE are as a rule more reactive reducing agents than monothiols, as follows from the data compiled in *Table 1*.

Methyl(aquo)cobaloxime is converted to mercaptocobaloxime in this reaction. Similarly, methyl(pyridine)cobaloxime reacts with n-butylmercaptan in pH 4.6 sodium acetate buffer to afford methane and n-butylmercapto(pyridine)cobaloxime. The overall reaction of methylcobaloximes with thiols in mildly acidic medium thus is as shown in equation 1.

$$\begin{array}{c} CH_3 \\ | \\ (Co) \\ \uparrow \\ B \end{array} + RSH \rightarrow \begin{array}{c} SR \\ | \\ (Co) \\ \uparrow \\ B \end{array} + CH_4 \qquad (1)$$

Table 1. Relative rates of demethylation of methyl(aquo)cobaloxime* by various thiols and dithiols at 65°C in pH 4.6 sodium acetate buffer†

Thiol	Relative rate
None	0.01
1-Thioglycerol	0.24
1,3-Dimercapto-2-propanol	0.49
2,3-Dimercapto-1-propanol	0.69
Dithioerythritol (DTE)	1.00

* 1.67 mM
† 0.167 M

The corresponding reductive demethylation of methylcobalamin may be formulated analogously, except that mercaptocobalamins are as such not detected. Instead, they decompose to form vitamin B_{12r} and the equivalent amount of disulphide. The reaction of methyl(aquo)cobaloxime with thiols affords methane optimally between pH 4 and 7 (*Figure 3*), methylcobalamin

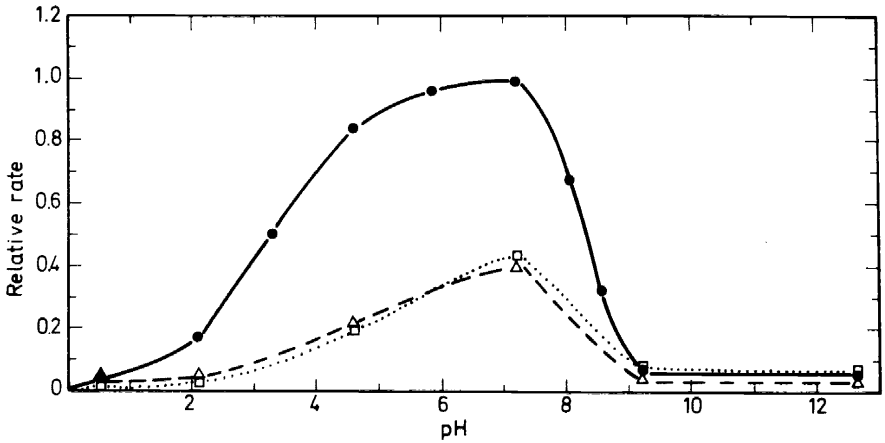


Figure 3. Relative rates of methane formation from methyl(aquo)cobaloxime (1.0 mM) by DTE (———), mercaptoethanol (– – – –), and thioglycerol (· · · ·) at 65°C as a function of pH. Reaction conditions: 0.2 M sodium acetate buffer, 0.05 M thiol

at pH $\approx$ 2. In alkaline medium the methane production is very slow; under these conditions methylthioethers are formed, as evidenced by the analysis of the volatile reaction products by means of gas–liquid chromatography and mass spectroscopy (using n-butanethiol as the thiol component). The dependence of the reductive demethylation on the nature and concentration of buffer is indicated by the fact that the rate of methane production reaches a maximum in 0.2 M pH 7.5 phosphate or tris-buffer. However, the concentration-rate profiles are different for both buffers (*Figure 4*). With dihydrolipoic acid as reducing agent the maximum rates of methane evolution are seen at 0.05 M tris, and 0.1 M sodium phosphate buffer (both at pH 7.5), respectively. In all cases the rate of methane production eventually decreases at higher buffer concentrations. The effect of the buffers is attributed to a combination of specific and non-specific salt effects influencing the dissociation of the free and cobaloxime-coordinated thiol. The demethylation reaction is assumed to involve *trans* attack of the cobaloxime or cobalamin cobalt atom by the thiol as the first step, followed by the rate-determining cleavage of the Co—C bond (equation 2):

$$\begin{array}{l} CH_3 \\ | \\ (Co) \\ \uparrow \\ B \end{array} + RSH \underset{}{\overset{\pm B}{\rightleftharpoons}} \begin{array}{l} CH_3 \\ | \\ (Co) \\ \uparrow \\ RSH \end{array} \overset{\pm H^+}{\rightleftharpoons} \begin{array}{l} CH_3 \\ | \\ (Co) \\ \uparrow \\ RS^- \end{array} \xrightarrow{\text{rate}} [CH_3^-] \xrightarrow{+H^+} CH_4 \qquad (2)$$

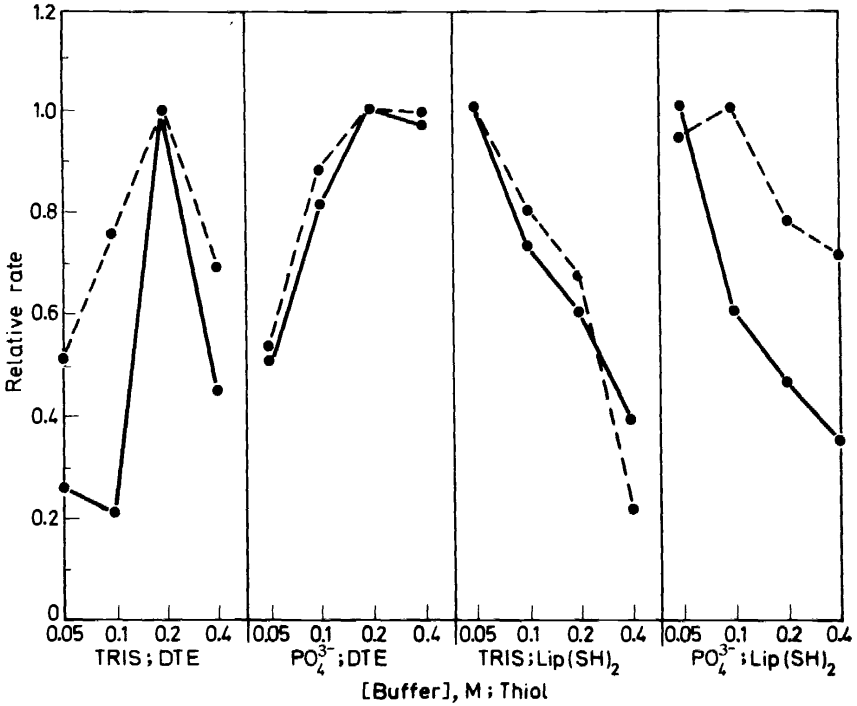


Figure 4. Relative rate profiles of buffer concentration dependences for the non-enzymatic demethylation of methyl(aquo)cobaloxime (———) and for ribonucleotide reductase catalysed deoxyribonucleotide formation[20] (– – –) using DTE or dihydrolipoic acid, Lip(SH)$_2$. Reaction conditions for cobaloxime: 65°C for 48 hours, pH 7.5 sodium phosphate or tris-(trishydroxymethylaminomethane) buffer, 2.0 mM methyl(aquo)cobaloxime, 0.17 M thiol

The mercaptocobaloxime formed in the reaction recombines with the base component B according to equation 3:

$$\begin{array}{ccc} \text{SR} & & \text{SR} \\ | & & | \\ \text{(Co)} & + \text{B} \rightarrow & \text{(Co)} + \text{H}_2\text{O} \\ \uparrow & & \uparrow \\ \text{HOH} & & \text{B} \end{array} \qquad (3)$$

In equation 2 the initial product of the reductive Co—C bond cleavage reaction is assumed to be a methyl carbanion or species with the reactivity of a methyl carbanion. Evidence for this reactive intermediate was obtained previously[7] by conducting the reductive demethylation in the presence of CO_2. Under these conditions, detectable amounts of acetic acid are produced (equation 4):

$$\begin{array}{c} \text{CH}_3 \\ | \\ \text{(Co)} \rightarrow [\text{CH}_3^-] \xrightarrow{\text{CO}_2} \text{CH}_3\text{CO}_2^- \\ \uparrow \\ \text{RS}^- \end{array} \qquad (4)$$

The reaction of equation 4 was initially demonstrated with methylcobaloxime and 1,4-butanedithiol[7]. In the meantime we have also obtained affirmative evidence for the production of acetate from methylcobalamin and CO_2 in the presence of DTE as the reducing agent. These reactions will be discussed later in relation to the mechanism of the corrin-dependent acetate biosynthesis.

Inhibition of reductive demethylation by bases

A crucial feature of the mechanism of reductive demethylation is the assumed competition of the mercaptan with the axial base B, for the cobaloxime–cobalt coordination site. This mechanism is supported by the observed dependence of the rate of methane production on the nature of the axial base component B, which decreases in the order of $H_2O > C_6H_5NH_2 >$ py > benzimidazole > $C_6H_{11}NC \approx (n\text{-}C_4H_9)_3P$, a sequence clearly parallelling the increase of π- and d_π-electron accepting character of the axial bases (*Table 2*). The demethylation of methylcobinamide proceeds at a faster rate than that of methylcobalamin for similar reasons (*Table 3*).

Table 2. Rates of alkane formation from alkylcobalt complexes by DTE at 65°C in pH 4.6 acetate buffer (0.17 M)

In-plane ligand*	Axial base†	R	Rate constant $k_1(\times 10^6)$‡
Corrin	H_2O	CH_3	1.4
Corrin	H_2O	C_2H_5	0.17
Corrin	5,6-Dmbz	CH_3	0.65
Corrin	5,6-Dmbz	C_2H_5	0.15
Dmg	H_2O	CH_3	4.9
Dmg	H_2O	C_2H_5	0.01
Dmg	H_2O	$n\text{-}C_3H_7$	0.05
Dmg	H_2O	$i\text{-}C_3H_7$	0.70
Dmg	pyr	CH_3	2.10
Dmg	Bzim	CH_3	0.36
Dmg	$C_6H_{11}NC$	CH_3	0.27
Dmg	$P(C_6H_5)_3$	CH_3	0.27
Dmg	$P(n\text{-}C_4H_9)_3$	CH_3	slow
Dmg	$C_6H_5NH_2$	CH_3	4.4
Dpg	H_2O	CH_3	4.3
Gly	pyr	CH_3	0.26
$DmgBF_2$	pyr	CH_3	0.31
Sch.B.I	H_2O	CH_3	1.96
Sch.B.II	H_2O	CH_3	3.4

* Dmg = dimethylglyoxime; Dpg = diphenylglyoxime; Gly = glyoxime; $DmgBF_2$ = ligand (IV); Sch.B.I = bis(biacetylmonoxime)propylenediimine (ligand VI); Sch.B.II = bis(salicylaldehyde)ethylenediimine (ligand VII)

† 5,6-Dmbz = 5,6-dimethylbenzimidazole; Bzim = benzimidazole; pyr = pyridine

‡ Rate constants are pseudo first-order rate constants in s^{-1}. Initial concentration of cobalt complex: 0.001 M; of thiol, 0.05 M

Confirmation of the formation of an initial thiol adduct of methylcobaloxime was obtained by studying the inhibition of the methane production by bases such as pyridine. The rate of methane production decreases linearly

Table 3. Relative rates of methane formation from methylcobalamin and methylcobinamide with n-butylmercaptan as the reducing agent, at 65°C as a function of pH, under argon (in 0.17 M sodium phosphate buffers)*

pH	Relative rates of methane formation from Methylcobalamin	Methylcobinamide
2.0	0.82	1.00
4.6	0.36	0.58
7.0	0.10	0.33
12.0	0.02	0.05

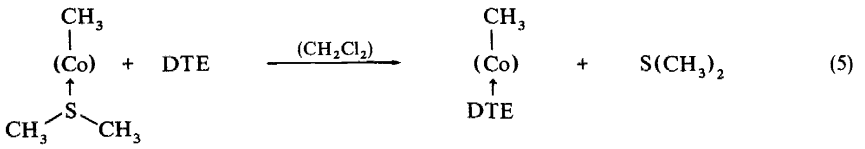
* Initial concentration of methylcorrins: 1.0×10^{-4} M

Table 4. Relative rates of reductive demethylation of methyl(aquo)cobaloxime by DTE at 65°C in the presence of pyridine or 2,6-lutidine*

Molar ratio of cobaloxime to added base	Relative rates of methane formation Pyridine	2,6-lutidine
1:0	1.00	1.00
1:1	0.71	0.97
1:3	0.48	0.96
1:10	0.11	0.55

* Reaction conditions: pH 4.6 sodium acetate buffer (0.13 M); Methyl(aquo)cobaloxime (initial concentration), 0.7×10^{-3} M; DTE, 0.17 M; pyridine or lutidine added in amounts indicated

as a function of $[py]^{-1}$ (*Table 4*). When 2,6-lutidine was employed instead of pyridine the observed inhibition under otherwise identical conditions was much smaller, in accord with the diminished tendency of this sterically hindered pyridine derivative to form adducts with cobaloximes. We have also prepared an adduct of methylcobaloxime with DTE by the reaction given in equation 5:



(5)

The DTE adduct of methylcobaloxime forms orange crystals and is stable on storage. Decomposition occurs in aqueous solution and is accompanied by a slow formation of methane. The ability of methylcobaloximes to form adducts with thiols has also since been observed by other workers[8].

Influence of the in-plane ligand systems

The rate of reductive dealkylation of alkylcobalt complexes by thiols also depends on the nature of the in-plane ligand. With water in the axial position the Co—C bond cleavage of the methyl derivatives decreases in the sequence

Sch.B.I ≫ Dmg ≈ Dpg > Sch.B.II > Corrin (for identification of these ligands see formulae I–VII), with DTE as the reducing thiol, at 65°C in pH 4.6 acetate buffer (*Table 2*). This sequence is not readily interpreted in terms of a single constitutional factor. The overriding influence appears to be the effective charge on cobalt, since the net charge on the cobalt atom is likely to be more strongly positive in the methyl derivative of Sch.B.I than in either the methyl derivatives of Sch.B.II or of the corrin. The pyridine adduct of the methylcobalt derivative of chelate (IV) is more resistant to reductive demethylation even though the two inductively electron-attracting BF_2 groups *increase* the positive charge on cobalt. This anomalous behaviour is attributed to the greater stability of the coordinate cobalt–pyridine bond. The corresponding aquo complex is indeed more susceptible to reductive cleavage than methyl(aquo)cobaloxime under the same conditions. All in all, the influence of the in-plane ligands represents only a minor factor, since the observed rates of demethylation of the methyl(aquo)derivatives of chelates (I–VII) all lie within the same order of magnitude (*Table 2*).

Reductive cleavage of higher alkylcobalt derivatives

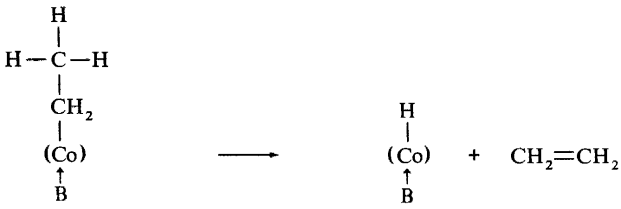
Higher alkylcobaloximes are dealkylated by DTE (at 65°C and pH 4.6) more slowly than the methyl derivatives, yielding alkanes and alkenes (*Tables 2* and *5*). Alkanes are only formed in the presence of DTE; alkenes are formed both in the presence and absence of DTE. The ratio of alkene to alkane increases in the presence of pyridine, since pyridine prevents the

Table 5. Alkene to alkane ratios for reductive and photolytic dealkylation of alkylcobaloximes at 65°C, in the presence of DTE, with and without excess of pyridine*

Alkyl group	Reaction			
	reductive		photolytic	
	−Pyridine	+Pyridine	−Pyridine	+Pyridine
C_2H_5	2.3	3.8	0.07	0.07
n-C_3H_7	2.4	3.9	0.04	0.04
i-C_3H_7	7.2	7.9	0.09	0.09

* Initial concentrations of cobaloximes: 1.0×10^{-3} M; [pyridine] = 1.0×10^{-2} M

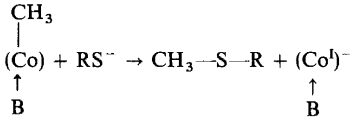
reductive cleavage reaction by blocking the axial cobaloxime–cobalt coordination site. The alkene–alkane ratio remains unchanged during photolysis of methylcobaloxime in the presence of both DTE and pyridine (*Table 5*). This indicates that the DTE promoted Co—C bond cleavage *in the dark* does not involve free ethyl radicals, since otherwise the behaviour should be the same or similar to that of the photolysis reaction. The sensitivity of the alkane production to pyridine is consistent with a mechanism analogous to equation 2; the thermal alkene production, on the other hand is best formulated as a hydride elimination:



(6)

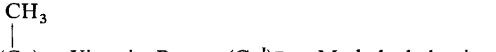
METHYL GROUP TRANSFER REACTIONS

In the course of the present studies the possibility of methyl group transfer reactions was also considered. We have previously shown[9] that methylcobaloxime reacts with mercaptide ions by nucleophilic attack to form methylthioethers (equation 7)*.



(7)

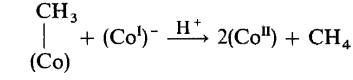
The ability of mercaptide ions to displace cobalt-bound methyl groups promoted us to study the analogous reaction with the vitamin B_{12s} nucleophile, using the methylcobalt derivatives of chelates (I–VII). In accord with the reaction equation 8, a rapid transfer of methyl groups to vitamin B_{12s} was observed.



(8)

This reaction is readily reversible; similarly, methyl transfer processes occur in solutions of methylcobaloximes and of other alkylcobalt chelates in the presence of the cobaloxime(I) nucleophile. Methylrhodoxime, on the other hand, does not transfer its methyl group to either vitamin B_{12s} or cobaloxime(I)[11]. Evidently, in this case the equilibrium favours the methylrhodium derivative, owing to the greater thermodynamic stability of the Rh—C relative to the Co—C bond.

Solutions of methylcobaloxime react with the cobaloxime(I) nucleophile also by cleaving the Co—C bond, i.e. according to equation 9.



(9)

This reaction was first reported in 1966 and represents another example of a reductive Co—C bond cleavage[12]. The $(Co^{I})^{-}$ nucleophile is assumed in this case to interact with the methylcobaloxime carbon atom by *trans* attack.

* It is of interest to note that reactions of equations 2 and 7 have been overlooked by several workers[8,10]. In analogy with equation 7 methionine was synthesized on a preparative scale from methylcobaloxime and homocysteine at pH 10. Reaction of methyl(aquo)cobaloxime with CH_3S^- in methanolic NaOH similarly afforded $(CH_3)_2S$ in 40 per cent yield[9].

Thus, thiols and the Co^{I} nucleophiles react with alkylcobaloximes in an essentially identical manner. The methyl group transfer reactions discussed thus far involve methyl carbonium ion species rather than methyl radicals or methyl carbanions. Methylcobaloximes do not react with Co^{II} derivatives of cobaloximes or with vitamin B_{12r} as such. However, the photolysis of mixtures of methylcobaloxime with vitamin B_{12r} afforded detectable yields of methylcobalamin[13], thus providing an example for a methyl radical transfer reaction (equation 10).

$$\begin{matrix} CH_3 \\ | \\ (Co) \end{matrix} + h\nu \rightleftharpoons (Co^{II}) + CH_3^{\bullet} \xrightarrow{+\text{Vitamin } B_{12r}} \text{Methylcobalamin} \qquad (10)$$

It is important to point out that the transfer of methyl radicals does not occur unless the original Co—C bond is cleaved homolytically, i.e. by photolysis or thermolysis (examples for thermally induced methyl radical transfer reactions in the corrin series have been reported by Friedrich and Moskophidis[14]).

The transfer of methylcarbanions to Co^{III} derivatives of the chelates is also not normally observed. Thus, there is no evidence for reaction according to equation 11.

$$\begin{matrix} CH_3 \\ | \\ (Co) \end{matrix} + \text{Vitamin } B_{12a} \rightarrow (Co^{III}) + \text{Methylcobalamin} \qquad (11)$$

However, chelate (VI) forms a neutral dimethyl derivative (in contrast to cobaloximes and corrins, which only form monoalkylated derivatives) in which one methyl group has sufficient carbanionic reactivity to methylate vitamin B_{12a} (equation 12)[15].

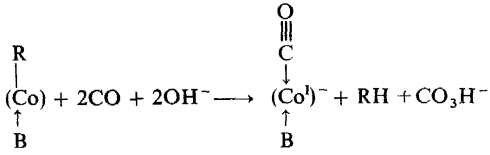
$$\begin{matrix} CH_3 \\ | \\ [Co] \\ | \\ CH_3 \end{matrix} + \text{Vitamin } B_{12a} \rightarrow \begin{matrix} CH_3 \\ | \\ [Co]^{+} \end{matrix} + \text{Methylcobalamin} \qquad (12)$$

([Co] = Chelate VI)

Under reducing conditions, methyl group transfer reactions in the corrin and cobaloxime series are favoured only in the presence of the Co^{I} nucleophiles and involve methyl carbonium ions as the species undergoing transfer.

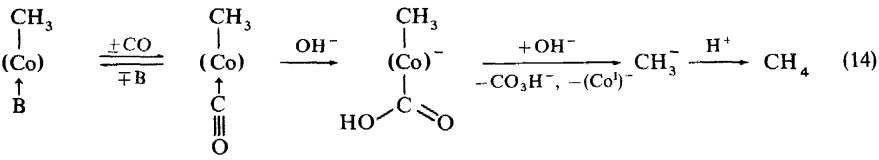
REDUCTIVE Co—C BOND CLEAVAGE BY ALKALINE CARBON MONOXIDE AND OTHER REDUCING AGENTS

Alkylcobaloximes react with carbon monoxide in alkaline, aqueous or alcoholic solutions to yield alkane, carbonate and intensely coloured CO adducts of the Co^{I} nucleophiles (equation 13)[16].



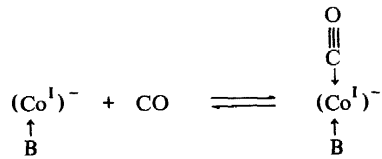
(13)

The reaction is first order in alkylcobaloxime and CO, and second order in $[OH^-]$. The dealkylation rates also depend on the nature of the axial bases, decreasing in the order of ligands $PhNH_2 > py > Sb(Ph)_3 > As(Ph)_3 > P(Ph)_3 >$ Benzimidazole $> C_6H_{11}NC > P(n\text{-}C_4H_9)_3$. The overall rates of dealkylation are much faster than those with thiols as reductants (factor of approximately 10^4). Alkaline carbon monoxide evidently is a stronger reducing agent, since higher alkylcobalt derivatives are reductively cleaved to form alkanes in much higher proportion relative to alkenes than with thiols. For example, ethyl(aquo)cobaloxime affords ethane and essentially no ethylene, n- and i-propylcobaloxime propane and propylene in the ratios of 99 :1 and 75 :25, respectively. However, as in the reduction with thiols, higher alkylcobaloximes are reduced at slower rates than the methyl derivatives, and the dealkylation is inhibited by pyridine. The mechanism of Co—C bond cleavage by alkaline carbon monoxide may thus be formulated according to equation 14.



(14)

In the presence of excess CO, adducts[17] of the Co^I nucleophile with CO are formed which are recognized on the basis of their characteristic optical absorption spectra (equation 15).



(15)

The reaction of equation 14 applies for the CO-dealkylations of other alkylcobaloximes as well as for the reactions with alkylcobalamins. Methylcobinamide is dealkylated at a rate corresponding to approximately 2/3 of that of methyl(aquo)cobaloxime. Methylcobalamin, however, is dealkylated only very slowly (2 per cent of the rate of methylcobinamide), reflecting the inhibiting effect of the axially coordinated 5,6-dimethylbenzimidazole in the cobalamin derivative. Selected rates of dealkylation of alkylcobalt derivatives of chelates (I–VII) and of corrins are compiled in *Table 6*. As

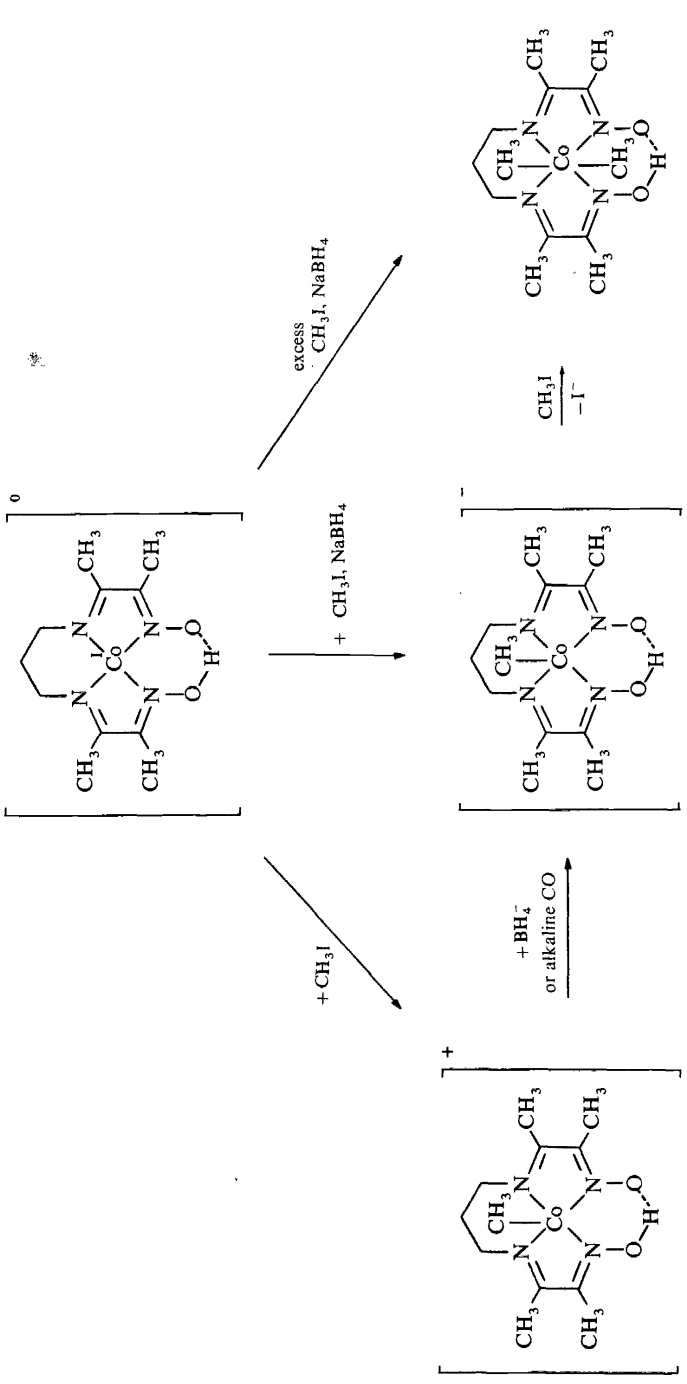
in the reduction with thiols, the in-plane ligand also influences the rate of dealkylation to some extent, the rates decreasing in the order, CHD > Dmg > Corrin > Dpg > $DmgBF_2$ > Gly > Sch.B.II. In this sequence Sch.B.I is missing because of its tendency to undergo disproporthionation with formation of the neutral dimethyl derivative. The reactions observed are summarized in *Scheme 1.*

Table 6. Rates of dealkylation of alkylcobalt chelates by alkaline CO at 27°C in 0.5 N methanolic NaOH; [CO] = saturated in CH_3OH at 1 atm

In-plane ligand*	Axial base*	R	Products of reduction†	Product ratio	$k(\times 10^2)$‡
Corrin	H_2O	CH_3	CH_4		0.35
Corrin	H_2O	C_2H_5	C_2H_6, (C_2H_4)	99:1	0.055
Corrin	H_2O	C_3H_7	C_3H_8, (C_3H_6)	75:25	0.052
Corrin	5,6-Dmbz	CH_3	CH_4		0.007
Corrin	5,6-Dmbz	C_2H_5	C_2H_6, (C_2H_4)	99:1	0.0004
Dmg	H_2O	CH_3	CH_4		0.59
Dmg	H_2O	C_2H_5	C_2H_6		0.034
Dmg	H_2O	n-C_3H_7	C_3H_8 (C_3H_6)	99:1	0.022
Dmg	H_2O	i-C_3H_7	C_3H_8 (C_3H_6)	75:25	0.053
Dmg	pyr	CH_3	CH_4		0.60
Dmg	pyr	C_2H_5	C_2H_6		0.019
Dmg	pyr	n-C_3H_7	C_3H_8 (C_3H_6)	99:1	0.039
Dmg	pyr	i-C_3H_7	C_3H_8 (C_3H_6)	78:23	0.024
Dmg	pyr	CH_2—C_6H_5	CH_3—C_6H_5		3.58
Dmg	pyr	CF_3	CF_3H		0.01
Dmg	Bzim	CH_3	CH_4		0.22
Dmg	$C_6H_{11}NC$	CH_3	CH_4		0.15
Dmg	$P(C_6H_5)_3$	CH_3	CH_4		0.35
Dmg	$P(C_4H_9)_3$	CH_3	CH_4		0.01
Dmg	$As(C_6H_5)_3$	CH_3	CH_4		0.48
Dmg	$Sb(C_6H_5)_3$	CH_3	CH_4		0.56
Dmg	Ph-NH_2	CH_3	CH_4		0.71
Dpg	H_2O	CH_3	CH_4		0.08
CHD	pyr	CH_3	CH_4		1.0
Gly	pyr	CH_3	CH_4		≈0.01
$DmgBF_2$	pyr	CH_3	CH_4		0.01
Sch.B.I	H_2O	CH_3	CH_4		1.2
Sch.B.II	H_2O	CH_3	CH_4		0.0005

* Notation as in *Table 2*
† Minor product in parentheses
‡ Pseudo first-order rate constants, s^{-1}, ±5 per cent under conditions specified above

The Co—C bond in alkylcobalt complexes is also reductively cleaved by alkaline dithionite and by stannite. The latter is the weakest reducing agent of those studied thus far, operating only in very strongly alkaline medium (e.g. 3 M NaOH). Analysis of the alkane–olefin product ratio of the reduction of alkylcobaloximes indicates that alkaline carbon monoxide is the most and DTE the least powerful reducing agent in this series (*Table 7*). Although numerical data will not be given for dithionite or stannite reductions, the


excess
CH_3I, $NaBH_4$
$+ CH_3I$, $NaBH_4$
$+ CH_3I$
$+ BH_4^-$
or alkaline CO
$\frac{CH_3I}{-I^-}$

Scheme 1.

results clearly indicate that the mechanisms of both reactions incorporate *trans* attack of the reducing agent at the cobaloxime cobalt atom prior to Co—C bond cleavage.

Table 7. Alkane–olefin ratios in the reduction of alkylcobaloximes with various reducing agents

Alkyl residue	Alkaline CO*	$S_2O_4^{2-}$†	SnO_2^{2-}‡	DTE§	Products
C_2H_5	100:0	99:1	60:40	43:57	C_2H_6, C_2H_4
n-C_3H_7	99:1	99:1	55:45	42:58	C_3H_8, C_3H_6
i-C_3H_7	78:23	55:45	20:80	14:86	C_3H_8, C_3H_6

* At 27°C in 0.5 M methanolic NaOH, [CO] = saturated in CH_3OH at 1 atm
† At 27°C in 0.58 M aqueous NaOH; [$Na_2S_2O_4$] initially 0.0030 M
‡ At 27°C in 3 M aqueous NaOH; [Stannite] initially 0.22 M
§ At 65°C in 0.2 M aqueous pH 4.6 sodium acetate buffer; [DTE] initially 0.05 M

COMPARISON WITH ENZYMATIC REACTIONS

Ribonucleotide reductase

In the ribonucleotide reductase of *Lactobacillus leichmannii*, dithiols such as dihydrolipoic acid, DTE or 1,3-dithioglycerol serve as the electron donors for the coenzyme B_{12}-dependent reduction of ribonucleotides to deoxyribonucleotides[18]. The reaction is schematically represented in equation 16 (Ad-B_{12} denotes 5′-deoxyadensoylcobamide coenzyme, $R(SH)_2$ and $R\begin{smallmatrix}S\\|\\S\end{smallmatrix}$ the reduced and oxidized forms of the dithiols, respectively).

$$GTP + R(SH)_2 \xrightarrow[\text{Ad-}B_{12}]{\text{ribonucleotide reductase}} d\text{—}GTP + R\begin{smallmatrix}S\\|\\S\end{smallmatrix} + H_2O \qquad (16)$$

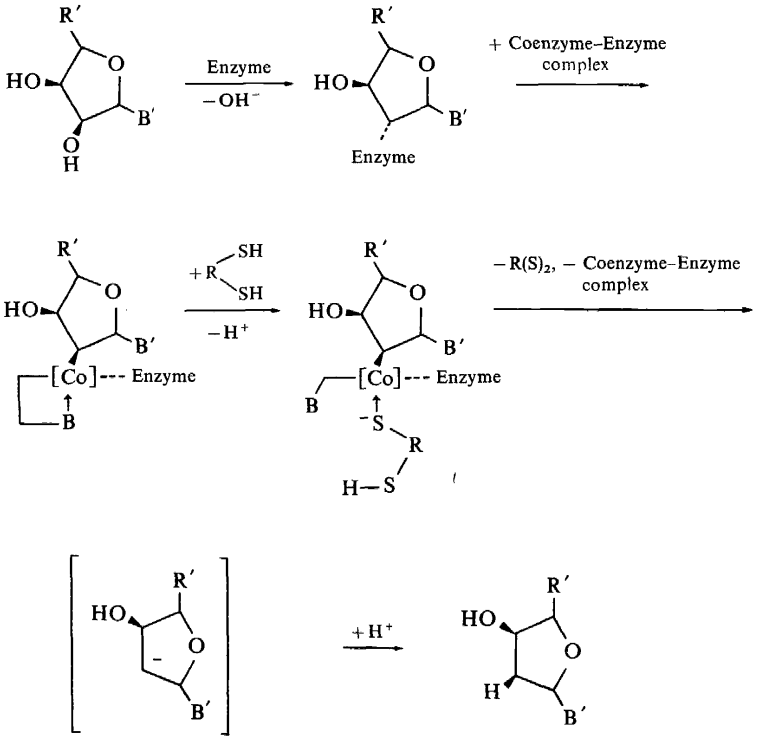
Monothiols such as mercaptoethanol, cysteine or glutathione are considerably less reactive than 1,4- or 1,3-dithiols[18]. Studies with coenzyme B_{12} labelled with tritium in the 5′-position of the deoxyadenosyl moiety indicate that the reductase catalyses the exchange of hydrogen from the 5′-position with water[18], and that vitamin B_{12r}, identified by its e.s.r. spectrum, slowly accumulates during the enzymatic reaction[19]. It has been proposed that the reduction of the substrates occurs with participation of the 5′-protons of the deoxyadenosyl moiety of coenzyme B_{12}. Although the details of such a reaction have not been given, it has been suggested that 5′-deoxyadenosine functions as the donor of the hydrogen atom. Chemically, such a mechanism cannot be accepted, even though it appears to be in accord with the available experimental evidence.

As was outlined in the Introduction, the present investigation was carried out with the ultimate aim to provide experimental data comparable with enzymological results. All available evidence derived from the previous studies on organocobalamins and the corresponding vitamin B_{12} model compounds suggested that if coenzyme B_{12} was to be a catalyst of ribonucleotide reduction it could excercise its catalytic action only by initially

cleaving the cobalt–carbon bond and by forming intermediate organocobalt compounds with the substrates or their derivatives. It therefore appeared natural to compare the results of the reductive Co—C bond model experiments with the behaviour of ribonucleotide reductase, even though both types of reaction seemed to have nothing in common, except that they led to the formation of a new C—H bond. At first, parallels were noted in the reactivity sequence of thiols and dithiols of ribonucleotide reductase and in the rate of methane production from methylcobaloxime. We subsequently became aware of recent work on the dependence of ribonucleotide reductase activity on the buffer concentration[20]. For both tris- and sodium phosphate buffer, the activity of ribonucleotide reductase reaches a maximum at 0.20 M. This was interpreted[20] to suggest a chaotropic effect of the buffer constituents on the enzyme in the vicinity of the active site. However, the data compiled in *Figure 4* demonstrate an astonishing similarity of the dependence of ribonucleotide reductase activity and the rate of the non-enzymatic methane production on the nature and concentration of buffers. It thus becomes apparent that the buffer effects observed with ribonucleotide reductase may be reproduced by non-enzymatic model systems composed of thiol, and an alkylcobaloxime. Since the cleavage of the Co—C bond is rate-determining in the model reaction and this reaction is preceded by a pre-equilibrium involving the coordination of the thiol to the cobaloxime cobalt atom, it must be concluded that the reductive cleavage of a Co—C bond between coenzyme and substrate is also rate-determining in ribonucleotide reductase, and that the thiols or dithiols interact with the coenzyme–substrate intermediate by *trans* attack.

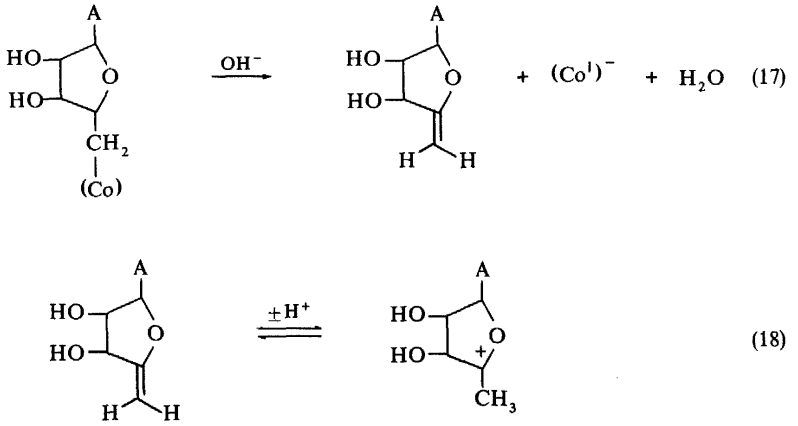
Essential steps of the catalytic conversion of ribonucleotides into deoxyribonucleotides accordingly consist of the alkylation of the cobalt atom of the coenzyme and the reductive dealkylation of the resulting coenzyme–substrate complex. The alkylation step is likely to involve the powerfully nucleophilic Co^{I} derivative of the corrin. It should be mentioned, however, that the Co^{I} nucleophile is incapable as such of displacing normal carbon-bound hydroxyl groups. It is assumed that the substrate nucleoside is specifically activated at the 2′-position on the interaction with the enzyme. In *Scheme 2* essential steps of the catalytic reactions of ribonucleotide reductase are summarized.

The mechanism of ribonucleotide reduction in *Scheme 2* does not yet specify the essential activation step involving the coenzyme (5′-deoxyadenosylcobamide coenzyme as such is clearly incapable of any catalytic action prior to the cleavage of the Co—C bond), nor does it account for the equilibration of the 5′-protons of the coenzyme with those of the solvent during the reaction. Deoxyadenosylcobalamin undergoes facile Co—C bond cleavage in alkaline medium via β-elimination to yield 4′,5′-didehydro-5′-deoxyadenosine and the Co^{I} corrin nucleophile[21]. We have previously offered this reaction as a possible mechanism of coenzyme activation as it reversibly leads to the formation of the powerful Co^{I} nucleophile without the requirement of a reducing agent[21]. The 4′,5′-didehydro-5′-deoxyadenosine is expected to remain attached to the enzyme, and is envisaged to react with the Co^{I} nucleophile and a proton to regenerate the coenzyme after completion of the reaction. Furthermore it must be expected that the olefinic nucleoside is at

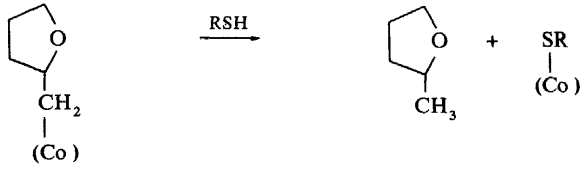


Scheme 2.

equilibrium with protonated forms. The three 5′-protons in the resulting ion (equation 18) are equivalent and labile with respect to exchange with the solvent[21].



We favour this mechanism over all others as it is strongly supported by relevant model experiments, in spite of the fact that hitherto neither the Co^{I} nucleophile nor 4',5'-didehydro-5'-deoxyadenosine were detected in the ribonucleotide reductase holoenzyme under various conditions. The stationary concentration of the enormously reactive Co^{I} nucleophile of vitamin B_{12} is expected to be very low, and if the cobalt–carbon bond of the coenzyme is regenerated upon the completion of the reaction it may well be impossible to ever detect any free Co^{I} nucleophile in the enzymatic system. It has recently been shown that 5'-deoxyadenosine and vitamin B_{12r} are formed in ribonucleotide reductase preparations in the absence of substrate[22]. The 5'-deoxyadenosine is more likely to be a product of the reductive Co—C bond cleavage of the coenzyme than a genuine catalytic intermediate of the enzymatic reaction. Since 5'-deoxyadenosine has also been isolated from the preparations of ethanolamine deaminase holoenzyme[23], a mechanism of ethanolamine deaminase action was proposed which invokes 5'-deoxyadenosine as part of the catalytic cycle[23]. In view of the inertness of the 5'-protons of 5'-deoxyadenosine, hydrogen could be removed from the 5'-position only by a specific H-abstraction mechanism involving free radical intermediates. Such a process would appear chemically and energetically improbable if not impossible and is not supported by model reactions. We therefore consider the appearance of 5'-deoxyadenosine in ribonucleotide reductase, or ethanolamine deaminase preparations, as due to abortive side-reactions occurring preferentially under non-optimal conditions. The presence of mercapto groups in the vicinity of the active site of ribonucleotide reductase and their oxidation–reduction during the enzymatic catalysis of ribonucleotide reduction has been demonstrated[24]. Conceivably, these or other mercapto groups may act as the reducing agents in the conversion of the coenzyme to 5'-deoxyadenosine. A model reaction for this process is available. Thus, 2-methylene(tetrahydrofurfuryl)pyridinecobaloxime is reductively cleaved on heating with 1-thioglycerol to afford 2-methyltetrahydrofuran; thermal decomposition of this complex in the absence of thiol yields methylenetetrahydrofuran exclusively (equation 19):



(19)

The same reaction has since been verified with coenzyme B_{12} itself.

Methane and acetate biosynthesis

Cell extracts of methane-producing bacteria such as *Methanosarcina barkeri* and of *Methanobacillus omelianskii* convert the methyl group of added methylcobalamin to methane[25]. Cell extracts of *M. omelianskii* also demethylate methylcobaloximes in the presence of catalytic amounts of ATP and of a corrin[26]. The reason for the ATP requirement, which is catalytic rather than stoichiometric[27], is as yet unknown. The demethylation does not occur in the absence of added corrin cofactor. The relative rates

of methane evolution from the model compounds show a dependence on the axial bases which strongly resembles that observed in the thiol promoted reductive cleavage experiments[5]. It thus appears that the cell extracts contain a biogenic reducing agent capable of cleaving the Co—C bond by a mechanism involving initial *trans* attack. Although the nature of this reducing agent is as yet unknown, it is possible that it is a thiol, dithiol or thioprotein. We have carefully considered the possibility of a mechanism of cobaloxime demethylation which involves the transfer of the cobaloxime methyl group to the corrin cofactor prior to the reductive Co—C bond cleavage. However, we were unable to support this mechanism in appropriately designed model systems. Although it is possible to transfer the methyl group of the cobaloxime substrates to vitamin B_{12s}, this reaction does not depend on the axial bases to a degree similar with the available enzymological evidence. We can also eliminate the possibility that the corrin cofactor required for enzymatic demethylation of the cobaloximes functions as the actual agent effecting the reductive Co—C bond cleavage, since this reaction, though reproducible in model systems, is comparatively slow, particularly in the presence of thiols. To account for the corrin requirement of the enzymatic methylcobaloxime demethylation, we assume that the corrin participates indirectly, being required for the conversion of the enzyme into its active form. Once activated, the biogenic reducing agent demethylates the cobaloximes by a mechanism similar to equation 2. The overall catalytic process is represented in equation 20 (axial base is not shown).

$$\underset{(\mathrm{Co})}{\overset{\mathrm{CH_3}}{|}} \xrightarrow[\text{[ATP, corrin, cell extract]}]{+e^- \text{ (biogenic reducing agent)}} CH_3^- \xrightarrow{H^+} CH_4 \qquad (20)$$

A further complicating feature of methane biosynthesis by *M. omelianskii* is indicated by the isolation of an incompletely characterized cofactor ('Coenzyme M') from the cell extracts, which, if removed by dialysis, prevents methane production[28]. This cofactor is partially methylated under certain reaction conditions, the methylated form gives rise to methane if added back to the dialysed cell extracts. The biochemical relevance of these findings depends on the further purification and characterization of 'Coenzyme M'.

The formation[4] of acetate from methylcobalamin and CO_2 by cell-free extracts of *Clostridium thermoaceticum* is likely to represent the terminal stage of acetate biosynthesis by this organism. The demonstrated formation of acetate from methylcobaloxime[7] or methylcobalamin, in the presence of thiols and CO_2, provides a plausible model for this process. As in methane biosynthesis, the nature of the biogenic reducing agent interacting with the methyl corrin remains to be identified. The process is thus represented schematically in terms of equation 21.

$$\underset{[\mathrm{Co}]}{\overset{\mathrm{CH_3}}{|}} \xrightarrow[\text{cell extract}]{+e^- \text{ (biogenic reducing agent)}} (CH_3^-)\text{–Enzyme} \xrightarrow{+CO_2} CH_3CO_2^- \qquad (21)$$

In equation 21 '(CH_3^-)–Enzyme' implies the formation of an enzyme-bound methyl carbanion or species with the reactivity of a methylcarbanion, which

is carboxylated by CO_2; conceivably, the latter reaction occurs in a locally aprotic environment of the enzyme. Methylcobalamin has occasionally been considered a kind of 'biological Grignard Reagent'[29]. It should be emphasized, however, that the methyl group of methylcobalamin exhibits carbanionic reactivity only with certain electrophilic reagents, e.g. Hg^{2+}, and that it does not react with CO_2 except under conditions leading to reductive Co—C bond cleavage. Acetate biosynthesis *in vitro* requires anaerobic conditions and is inhibited by thiol blocking reagents[30]. It is therefore likely that the biogenic reducing agent is a thioprotein or related thioredoxin system.

In summary, the present work indicates that corrin-dependent reductases involve the formation and reductive cleavage of organocobalt intermediates as an essential feature of the catalytic reaction, and that this also applies to corrin-dependent acetate biosynthesis[31].

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