the text. K_0 was calculated from experiments in the absence of inhibitor. Simplifying these equations gives eq 6 which was used to calculate K_I values.

$$K_I = \frac{2\left[[A_{t_i}] - [H_{b_i}] - \frac{[H_{b_i}]}{K_0[H_{f_i}]}\right]}{\frac{[H_{b_t}]}{K_0[H_{f_t}]}\left[[I_f] - [A_{t_i}] + [H_{b_i}] + \frac{[H_{b_i}]}{K_0[H_{f_i}]}\right]} \quad (6)$$

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Polarography of Cobalamins and Cobinamides*

H. P. C. Hogenkamp and S. Holmes

ABSTRACT: The polarographic behavior of several cobalamins and cobinamides provides additional evidence indicating that the nature of the ligands bound to cobalt in the upper and lower coordination positions profoundly influences the reactivity of the cobalt atom. In the cobalamin series, the first wave half-potential is determined by the nucleophilicity of the upper ligand. An increase in the nucleophilic character of this ligand causes a shift of the polarographic wave to a higher potential.

If this upper ligand is a strong nucleophile, such as ^-OH, ^-CN, or $^-CH_3$, the polarographic wave represents a reduction by two electrons; on the other hand, the polarogram of aquocobalamin shows two distinct waves, each corresponding to a one-electron reduction. In the cobinamide series, the half-wave potential of the two waves is also determined by the nucleophilicity of both ligands in the upper and lower coordination position. The equilibrium between cyanoaquocobinamide and aquocyanocobinamide is established at a rate which is slow enough that each isomer is reduced separately at the dropping mercury electrode. The polarographic behavior of the cobinamides and the cobalamins indicates that even in the base-off position, the 5,6-dimethylbenzimidazole nucleotide greatly influences the electronic character of the cobalt atom, and thus the differences in reactivity between cobalamins and cobinamides cannot be due solely to steric factors.

Two different types of reactions in which a corrinoid participates as a coenzyme have been described. 5′-Deoxyadenosylcobalamin is required in reactions involving the transfer of hydrogen, while methylcobalamin is involved in methyl group transfer reactions (Hogenkamp, 1968). The evidence accumulated thus far suggests that during some of these transfer reactions, the carbon–cobalt bond of the coenzyme is cleaved heterolytically to an electrophilic organic moiety (5′-deoxyadenosyl cation or methylcarbonium ion) and the powerful nucleophile, cob(I)alamin.[1]

Such a heterolytic cleavage of suitable alkylcobalamins has been reported by Barnett *et al.* (1966) who found that cyanoethylcobalamin and 2(methoxycarbonyl)ethylcobalamin undergo a base-catalyzed E_2 elimination reaction in which the electron pair of the carbon–cobalt bond remains with the

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: cob(I)alamin, reduced aquocobalamin containing monovalent cobalt; cob(II)alamin, reduced aquocobalamin containing divalent cobalt; the same designation is used for the cobinamides; S.C.E, saturated calomel electrode.

cobalt atom, and by Bernhauer and Irion (1964) who showed that acylcobalamins are decomposed by nucleophiles such as hydroxylamine to cob(I)alamin and the corresponding acylhydroxamic acid.

It has also been shown that the electronegativity of the carbon atom attached to the cobalt profoundly affects the coordinate linkage between the cobalt atom and the nucleotide base (Smith *et al.*, 1964; Dolphin *et al.*, 1964; Hogenkamp, 1966).

Conversely, the coordinated base greatly influences the reactivity of the metal and of the organometallic linkage. Brodie (1969) has recently demonstrated that a strong ligand in the lower axial position[2] pulls the cobalt atom into the plane of the corrin ring and thus sterically hinders the reaction with secondary alkyl halides. In contrast, in the cobinamides lacking this strong ligand in the lower position, the cobalt atom is located above the plane of the corrin ring and reaction with secondary alkyl halides is possible.

In an attempt to obtain more information about the reactivity of the organometallic linkage and of the cobalt atom, we have studied the polarographic behavior of a series of cobalamins and cobinamides (Figure 1). The polarographic characteristics of cyanocobalamin, aquocobalamin, cob(II)alamin, and cob(I)alamin have been studied earlier by Diehl *et al.* (1950), Jaselskis and Diehl (1954), and Tacket *et al.* (1963), respectively, while the first half-wave potentials of a few alkylcobalamins have been reported by Müller and Müller (1962) and by Bernhauer *et al.* (1964).

In this communication, we have extended these earlier studies and have also studied the polarographic behavior of some cobinamides. The effect of ligands on the polarographic characteristics of cobinamides has also been investigated.

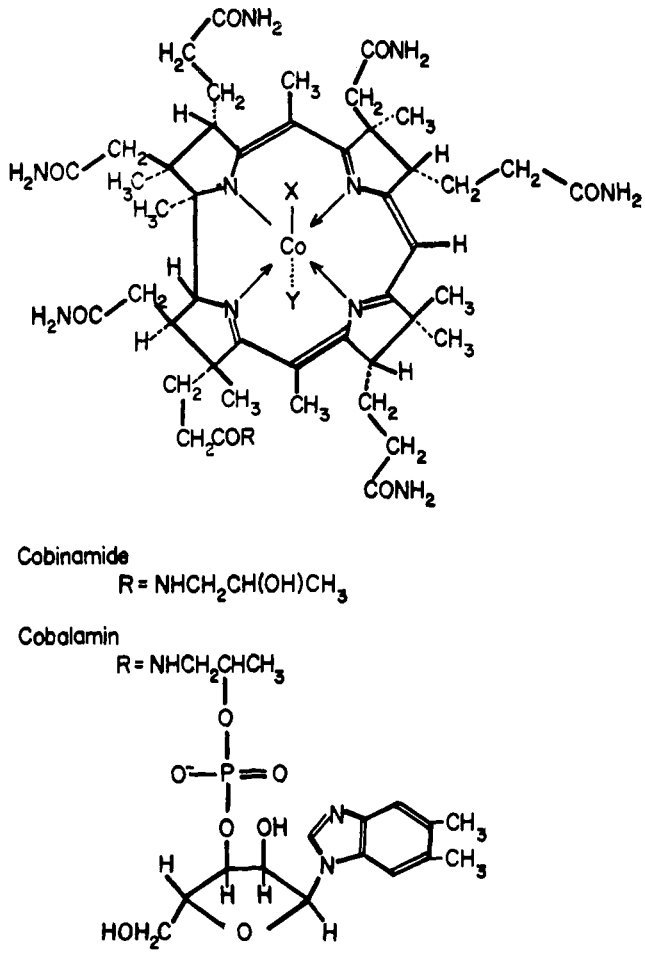


FIGURE 1: The structural formulas of cobalamins and cobinamides.

Experimental Procedure

Materials and Methods. Cyanocobalamin was purchased from Sigma Chemical Co. Other corrinoids were prepared from cyanocobalamin by published procedures: aquocobalamin, Hogenkamp and Rush (1968); diaquocobinamide, alkylcobinamides, Pailes and Hogenkamp (1968); (cyanoaquo)cobinamide,[3] Friedrich and Bernhauer (1956); alkylcobalamins, Hogenkamp *et al.* (1965); 5′-deoxyadenosylcobalamin, Hogenkamp and Pailes (1968). Other chemicals were obtained from commercial sources.

The purity of the corrinoids was established by spectral analysis and by paper chromatography in three solvent systems. Paper chromatography was performed by the descending technique on Whatman No. 40 paper. The following solvent systems were used: solvent I, 2-butanol–acetic acid–water (100:1:50); solvent II, 1-butanol–2-propanol–acetic acid–water (100:70:1:100); solvent III, 2-propanol–ammonium hydroxide–water (7:1:2). Diaquocobinamide and (cyanoaquo)cobinamide show extensive streaking in these solvent systems (Friedrich, 1966), but in the presence of 0.1 M KCN both preparations were converted into chromatographically homogeneous dicyanocobinamide.

Absorption spectra were recorded with a Cary Model 15 spectrophotometer; other visible and ultraviolet spectral measurements were made with a Zeiss PMQII spectrophotometer.

Concentrations of the corrinoids were determined from the absorbance and the molar extinction coefficient of their α band: cyanocobalamin, ϵ_{550} 8.6 $\times$ 10^3 M^{-1} cm^{-1}; aquocobalamin, ϵ_{525} 8.7 $\times$ 10^3 M^{-1} cm^{-1}; diaquocobinamide, ϵ_{515} 9.2 $\times$ 10^3 M^{-1} cm^{-1}; cyanoaquocobinamide, ϵ_{530} 7.6 $\times$ 10^3 M^{-1} cm^{-1}; methylcobalamin, ϵ_{519} 8.7 $\times$ 10^3 M^{-1} cm^{-1}; ethylcobalamin, ϵ_{509} 8.8 $\times$ 10^3 M^{-1} cm^{-1}; *n*-propylcobalamin, ϵ_{508} 8.7 $\times$ 10^3 M^{-1} cm^{-1}; vinylcobalamin, ϵ_{520} 9.3 $\times$ 10^3 M^{-1} cm^{-1}; hydroxyethylcobalamin, ϵ_{520} 8.2 $\times$ 10^3 M^{-1} cm^{-1}; methoxyethylcobalamin, ϵ_{521} 8.3 $\times$ 10^3 M^{-1} cm^{-1}; carboxymethylcobalamin, ϵ_{523} 7.3 $\times$ 10^3 M^{-1} cm^{-1}; methoxycarbonylmethylcobalamin, ϵ_{524} 6.2 $\times$ 10^3 M^{-1} cm^{-1}; aminoethylcobalamin, ϵ_{520} 8.1 $\times$ 10^3 M^{-1} cm^{-1}; deoxyadenosylcobalamin, ϵ_{522} 8.0 $\times$ 10^3 M^{-1} cm^{-1}; methylcobinamide, ϵ_{462} 10.7 $\times$ 10^3 M^{-1} cm^{-1}; *n*-propylcobinamide, ϵ_{440} 9.2 $\times$ 10^3 M^{-1} cm^{-1}. These molar extinction coefficients are based on ϵ_{368} 30.8 $\times$ 10^3 M^{-1} cm^{-1} (Friedrich, 1964) for dicyanocobalamin. The cobalamins were converted into dicyanocobalamin by photolysis in the presence of 0.1 M KCN. The extinction coefficient at 368 mμ for dicyanocobinamide has been shown

[2] The lower axial position (Y) refers to the propionamide side of the corrin ring, while the upper axial position (X) refers to the acetamide side of the ring.

[3] Denotes the two isomeric cyanoaquo forms of cobinamide—one with cyanide ion in the upper and water in the lower coordination position, and the other with water in the upper and cyanide ion in the lower coordination position.

TABLE I: Polarographic Characteristics of Cyano- and Aquocobalamin.

Cobalamin	pH	First Wave $E_{1/2}$ Volts *vs.* S.C.E.	First Wave n	Second Wave $E_{1/2}$ Volts *vs.* S.C.E.	Second Wave n
Cyano-cobalamin	12.4	−1.14	1.85		
Cyano-cobalamin	12.4[a]	−1.33	1.93		
Cyano-cobalamin	13.5	−1.07	1.75		
Aquo-cobalamin	2.4	~+0.03[b]	~0.53	−0.75	1.38
Aquo-cobalamin	7.1	−0.03	0.93	−1.07	0.92
Aquo-cobalamin	12.4	−1.07	2.04		

[a] In 0.1 M potassium cyanide. [b] This wave is ill defined; the half-wave potential and diffusion current are only approximate.

TABLE II: First Half-Wave Potential of Some Co-alkylcobalamins.

Nature of the Alkyl Group	$E_{1/2}$ Volts *vs.* S.C.E.	Nature of the Alkyl Group	pH	$E_{1/2}$ Volts *vs.* S.C.E.
CH_3	−1.39	$NH_2CH_2CH_2$	12.4	−1.35
CH_3CH_2	−1.37		7.5	−1.56
$CH_3CH_2CH_2$	−1.37	$HOOCCH_2$	12.4	−1.40
$HOCH_2CH_2$	−1.39		4.9	−0.84
$CH_3OCH_2CH_2$	−1.38	CH_3OOCCH_2	12.4	−1.14
$CH_2{=}CH$	−1.53			
Deoxyadenosyl	−1.37			

to be equal to that of dicyanocobalamin (Firth *et al.*, 1967b). The alkylcobinamides were also converted into dicyanocobinamide by photolysis in the presence of 0.1 M KCN.

Determination of Diffusion Coefficients. The diffusion coefficients of aquocobalamin and diaquocobinamide were determined with the dual-chambered diffusion cell described by Stokes (1950). Glass stirrers were replaced by Teflon stirrers rotated synchronously at 60 rpm. The cell was thermostated in a water bath at 25 ± 0.5°. The cell constant (β = 0.0611 ± 0.0019) was determined with 0.1 N KCl using $D = 1.873 \times 10^{-5}$ cm^2 sec^{-1} as the diffusion coefficient for 0.1 N KCl at 25° (Stokes, 1951), and is the average of six determinations (Bull, 1964). The diffusion coefficients for aquocobalamin ($D = 4.05 \pm 0.36 \times 10^{-6}$ cm^2 sec^{-1}) and for diaquocobinamide ($D = 4.54 \pm 0.14 \times 10^{-6}$ cm^2 sec^{-1}) were determined in triplicate. The corrinoid, dissolved in 0.02 N KCl, was allowed to diffuse against 0.02 N KCl for approximately 5 days. Initial concentration in the lower chamber and final concentrations in the upper and lower chambers were measured spectrophotometrically. The initial concentration of the corrinoid used in the diffusion cell was similar to that used in the polarograph. For cyanocobalamin, $D = 4.46 \times 10^{-6}$ cm^2 sec^{-1} determined by Diehl *et al.* (1950) was used.

Polarographic Measurements. Polarograms were recorded using a Sargent Model XV polarograph. A saturated calomel electrode with an agar–potassium chloride salt bridge was used as reference electrode. The polarograph cell, maintained at 25°, contained 10 ml of the solution to be analyzed. Each solution contained 0.02 M KCl, approximately 0.5 mM corrinoid, and 0.01% gelatin as maximum suppressor. When required, the pH of this solution was adjusted with 1 N KOH or 1 N HCl. Before each determination, nitrogen saturated with water was bubbled through the solution contained in the cell for 5 min. The polarogram was recorded from 0 to 2.0 V with a current sensitivity setting at 0.020 μA/mm. When the stoichiometry of the reduction was required, the mercury electrode drop time (t) and mass (m) were measured after each polarogram. The stoichiometry of the reduction was calculated from the diffusion current by applying the Ilkovic equation (Kolthoff and Lingane, 1941)

$$I_D = 605nD^{1/2}cm^{2/3}t^{1/6}$$

where n denotes the number of electrons required in the reduction, I_D is the diffusion current in microamperes, and c is the concentration of the corrinoid in millimoles per liter. For each corrinoid, the polarographic measurements were repeated at least three times. The half-wave potentials determined from the polarograms did not vary more than 0.01 V between experiments.

Results

Polarography of Cobalamins. The polarographic characteristics of cyano- and aquocobalamin are presented in Table I. The polarogram obtained for cyanocobalamin is virtually identical with that reported before (Jaselskis and Diehl, 1954). In the presence of 0.1 M potassium cyanide, the two-electron wave of cyanocobalamin shifts in half-wave potential from −1.14 to −1.33 V *vs.* S.C.E., whereas at high pH values, this wave shifts down from −1.14 to −1.07 V *vs.* S.C.E. In the pH range from pH 1 to 12.5, the two-electron wave of cyanocobalamin is independent of pH.

The polarographic behavior of aquocobalamin is more complex. At neutrality, the polarogram of aquocobalamin shows two one-electron waves at −0.03 and −1.07 V *vs.* S.C.E., indicating that the extra wave at −0.55 V *vs.* S.C.E. obtained by Jaselskis and Diehl (1954) was indeed due to an impurity in their preparation. At higher pH values where hydroxycobalamin is the predominant species, the polarogram is identical with that of cyanocobalamin at pH 13.5. In acid solution (pH 2.4) where aquocobalamin is the predominant species, two waves at approximately +0.03 and −0.75 V *vs.* S.C.E. are evident; however, the first wave is ill defined and consequently the stoichiometry of the reduction is only approximate.

The polarograms of cyano- or hydroxycobalamin are not affected by the following ligands (concentration 0.1 M): imidazole, methylimidazole, histidine, benzimidazole, pyri-

TABLE III: Polarographic Characteristics of Some Cobinamides.

Ligand[a]			First Wave		Second Wave	
X	Y	pH	$E_{1/2}$ Volts *vs.* S.C.E.	n^b	$E_{1/2}$ Volts *vs.* S.C.E.	n
H_2O	H_2O	12.4	−0.75	1.27	−1.02	1.10
H_2O	H_2O	6.2	−0.74	1.52		
Im	Im	12.4	−0.81	2.09		
CN, H_2O^c	H_2O, CN	12.4	−0.75	1.45	−1.18	0.98
CN, H_2O	H_2O, CN	6.3	−0.73	0.92	−1.79	1.40
CN, Im	Im, CN	12.4	−1.01	2.25		
CN	CN	12.4	−1.18	1.94		
CH_3	H_2O	12.4	−1.17	0.94	−1.44	1.09
CH_3	H_2O	6.7	−1.17	1.04	~−1.47[d]	
CH_3	Im	12.4	−1.19	0.92	−1.43	1.10
CH_3	CN	12.4	−1.25	1.01	−1.36	1.13
$CH_3CH_2CH_2$	H_2O	12.4	−1.24	0.70	−1.52	1.30
$CH_3CH_2CH_2$	H_2O	5.9	−1.26	1.61		
$CH_3CH_2CH_2$	CN	12.4	−1.26	1.23	−1.48	1.30

[a] The ligand in the upper axial coordination site (the acetamide side of the corrin ring) is defined as ligand X, and the ligand in the lower coordination site (the propionamide side of the ring) is defined as ligand Y. All ligands were tested at 0.1 M concentration. [b] The diffusion constants of all cobinamides were assumed to be the same as that of diaquocobinamide. [c] Denotes the presence of both isomeric cobinamides: cyanoaquocobinamide and aquocyanocobinamide. See footnote 3. [d] This wave is ill defined; the half-wave potential is only approximate.

dine, piperidine, methionine, lysine, and ammonia. However, in the presence of 0.1 M potassium cyanide, the first half-wave potential of both cobalamins changes to that of dicyanocobalamin.

The first half-wave potentials of a series of Co-alkylcobalamins are presented in Table II. For the simple alkylcobalamins (R = CH_3, CH_3CH_2, $CH_3CH_2CH_2$, $HOCH_2CH_2$, $CH_3OCH_2CH_2$) and 5′-deoxyadenosylcobalamin, the first wave involves a two-electron reduction and is independent of pH (pH 4.0–12.4). On the other hand, the polarograms of aminoethyl- and carboxymethylcobalamin are affected by ionization of the functional group.

Polarography of Cobinamides. The polarographic characteristics of a series of cobinamides are shown in Table III. The cobinamides with identical axial ligands show rather simple polarographic behavior. The two-electron wave of diaquocobinamide shifts in half-wave potential from −0.74 to −0.81 V and to −1.18 V *vs.* S.C.E. when both ligands are replaced by imidazole or cyanide ion, respectively. The polarograms of cobinamides with dissimilar axial ligands, such as cyanoaquocobinamides, show two waves. In the presence of imidazole, the two waves of [cyanoaquo]cobinamide with half-wave potentials of −0.75 and −1.18 V *vs.* S.C.E. are converted into one two-electron wave with a half-wave potential of −1.01 V *vs.* S.C.E. The conversion of the two isomeric cyanoaquo forms of cobinamide into dicyanocobinamide in the presence of potassium cyanide can be readily determined by measuring the decrease of the first wave ($E_{1/2}$ = −0.76 V *vs.* S.C.E.) or the increase of the second wave ($E_{1/2}$ = −1.18 V *vs.* S.C.E.).

The polarogram of methylaquocobinamide shows two one-electron waves with half-wave potentials at −1.17 and −1.44 V *vs.* S.C.E. Displacement of the lower ligand (H_2O) by imidazole or cyanide ion causes a shift of the first half-wave potential to −1.19 and −1.25 V *vs.* S.C.E., respectively. The first half-wave potential of propylaquocobinamide is not affected by these ligands at the same concentration (0.1 M).

Discussion

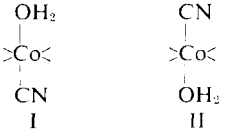
Polarography of Cobalamins. Comparison of the polarographic behavior of the cobalamins shown in Tables I and II indicates that the first half-wave potential is determined by the character of the axial ligand. This half-wave potential increases from −0.03 V *vs.* S.C.E. for aquocobalamin to −1.39 V *vs.* S.C.E. for methylcobalamin. This increase in $E_{1/2}$ parallels the increase in nucleophilicity of the ligand, (H_2O < ^-OH < ^-CN < $^-CH_3$), and indicates that the electron affinity of the central cobalt atom is in part determined by the nucleophilicity of the leaving group. Furthermore, if this leaving group is a strong nucleophile, such as ^-OH, ^-CN, or $^-CH_3$, the polarogram shows only one two-electron wave because the high potential required to remove the ligand is sufficient to accomplish the two-electron reduction of the cobalt atom to the monovalent state. The finding that at high pH values the polarogram of cyanocobalamin is identical with that of hydroxycobalamin suggests that at pH 13.5, the cyanide ion is displaced by the hydroxyl ion to an appreciable extent. The two waves obtained for aquocobalamin at pH 7.1 do not represent a two-electron reduction of hydroxycobalamin (hydroxycobalamin is the conjugate base of aquocobalamin, pK_a = 7.5), but rather two one-electron reductions of aquocobalamin. The equilibrium between aquocobalamin and hydroxycobalamin is established ex-

tremely rapidly and thus only the more readily reduced aquocobalamin is converted into cob(I)alamin in two separate reduction steps (Müller, 1960). At low pH, the stoichiometry of the reduction of aquocobalamin is ambiguous; however, it is evident that the second wave half-potential (−0.75 V *vs.* S.C.E.) is considerably lower than that of aquocobalamin at pH 7.1 (−1.07 V *vs.* S.C.E.). This observation is interpreted to indicate that at pH 2.4, cob(II)alamin with the dimethylbenzimidazole moiety protonated and replaced by water as the axial ligand in the lower coordination position (base-off cob(II)alamin) is reduced at the dropping mercury electrode. Since the pK_a of this protonation is approximately 2.5 (Firth *et al.*, 1967a), a considerable concentration of the base-off cobalamin would be present at pH 2.4. If, on the other hand, the 5,6-dimethylbenzimidazole moiety is replaced by a stronger nucleophile, such as cyanide ion, the half-wave potential is increased from −1.14 V for cyanocobalamin to −1.33 V *vs.* S.C.E. for dicyanocobalamin. Thus, these results indicate the nucleophilic character of both the upper and the lower axial ligand determines the polarographic behavior of the corrinoids. This dependence is also very evident in the results shown in Table II. Whereas the first wave half-potential of the simple Co-alkylcobalamins is quite high (−1.38 V *vs.* S.C.E.) because the alkylcarbanions are very strong nucleophiles and thus poor leaving groups, this potential is lowered if the leaving group contains an electron-withdrawing function and if the carbanion is stabilized by resonance. Thus, the first wave half-potential of methoxycarbonylmethylcobalamin is much lower than that of methylcobalamin (−1.14 and −1.39 V *vs.* S.C.E., respectively). Furthermore, the first wave half-potential of carboxymethylcobalamin at low pH, where the carboxyl function is protonated, is even lower because the leaving group lacks the electron-donating effect of the methyl group of the ester. At pH values where the carboxyl function is ionized, the first wave half-potential is identical with that of the simple alkylcobalamins. These results are in accord with earlier studies concerning the decomposition of alkylcobalamins by cyanide (Hogenkamp, 1966) and indicate that both the reduction of the cobalt atom and the nucleophilic displacement of the upper ligand of the corrinoids are affected in the same way by the nature of both axial ligands.

The very high first wave half-potential of vinylcobalamin (−1.53 V *vs.* S.C.E.) suggests that the carbon–cobalt bond in this cobalamin is stronger than the carbon–cobalt bond of the simple alkylcobalamins. This extra bond strength may be the result of π bonding between the axial ligand and the metal. On the other hand, the high half-wave potential of the protonated from of aminoethylcobalamin (−1.56 V *vs.* S.C.E.) is probably due to shielding of the cobalt atom by the positively charged amino function.

Polarography of Cobinamides. The dependence of the polarographic behavior of the corrinoids on both axial ligands is also evident from the polarograms of several cobinamides, shown in Table III. As was the case for the cobalamins, the half-wave potential of the cobinamides increases with increasing nucleophilicity of the ligand. The first half-wave potential of the simple cobinamides containing the same ligand in both the upper and the lower position increases from −0.75 V *vs.* S.C.E. for diaquocobinamide to −0.81 V *vs.* S.C.E. for diimidazolecobinamide and to −1.18 V *vs.* S.C.E. for dicyanocobinamide. The absence in the polarogram of diaquocobinamide of the first wave with a very low potential ($E_{1/2}$ = −0.03 V) characteristic of aquocobalamin indicates that the absence of the nucleotide has drastically altered the electron affinity of the cobalt atom. At neutrality, the cobalt atom of aquocobalamin is reduced more readily ($E_{1/2}$ = −0.03 V) than that of diaquocobinamide ($E_{1/2}$ = −0.74 V) to the divalent state; on the other hand, reduction of cob(II)alamin to cob(I)alamin requires a higher potential ($E_{1/2}$ = −1.07 V) than reduction of cob(II)inamide to cob(I)inamide ($E_{1/2}$ = −0.74 V). X-ray analysis of cyanocobalamin, 5′-deoxyadenosylcobalamin, and cobyric acid has shown that the distortion of the corrin nucleus that is found in the cobalamins is quite different from the distortion found in cobyric acid and presumably also in the cobinamides (Hodgkin, 1965a,b). Brodie (1969) has suggested that the inability of cob(I)alamin to react with secondary alkylhalides is due primarily to steric factors, since in the cobalamin series the nucleotide base tends to pull the cobalt atom into the plane of the corrin ring and thus renders it inaccessible to the bulky secondary alkylhalides. However, the large difference in the half-wave potentials of aquocobalamin and diaquocobinamide points to an appreciable difference in the electronegativity of the cobalt atom of the two corrins, indicating that the difference in reactivity between the cobinamides and the cobalamins is not only due to steric hindrance, but also due to differences in the electronic configuration of the metal.

The polarogram obtained for the two cyanoaquo forms of cobinamide at pH 12.4 shows two waves ($E_{1/2}$ = −0.75 and −1.18 V *vs.* S.C.E.). While the half-wave potential of the first wave is identical with that of diaquocobinamide, the half-wave potential of the second wave is higher than that of diaquocobinamide at pH 12.4. Friedrich (1966) has shown that two isomeric cyanoaquo forms of cobinamide—namely, cyanoaquocobinamide (II) and aquocyanocobinamide (I) are present in solution and that these two isomers are interconvertible at room temperature. In a later publication, Friedrich *et al.* (1967) demonstrated that the equilibrium between these two forms is established quite slowly because the two isomers can be separated by paper chromatography at 3°. If this is indeed the case, then the two waves of the polarogram each represent a two-electron reduction of one isomer followed by a two-electron reduction of the other isomer. Since the polarography of the cobalamins showed that the ligand in the upper position has the predominant effect on the half-wave potential, the first wave probably corresponds to the reduction of cobinamide with water in the upper and cyanide ion in the lower coordination position (I), while the second wave corresponds to the isomer with



cyanide ion in the upper position (II). If it is assumed that both isomers have identical diffusion constants, then the relative heights of the two waves in the polarogram provide a measure of the concentration of each isomer present under the experimental conditions. Thus, at pH 12.4 approximately

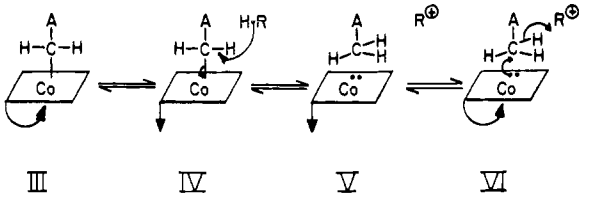
60% of the cobinamide is present as isomer I, while at pH 6.3, this isomer constitutes only 40% of the total cobinamide concentration. A similar dependence on pH has been shown by Friedrich *et al.* (1967) for the two cyanoaquo isomers of the tetracarboxylic acid derived from cobyric acid.

In the presence of cyanide, the two-wave polarogram is converted into a two-electron wave with a half-wave potential of −1.18 V *vs.* S.C.E. This conversion into dicyanocobinamide can be readily followed by measuring the increase in the diffusion current at −1.18 V or by the decrease at −0.75 V *vs.* S.C.E. It should be noted that the half-wave potential of this two-electron wave of dicyanocobinamide is lower than that of dicyanocobalamin, indicating that even in the off-base position, the nucleotide side chain has a significant effect on the electronegativity of the cobalt atom. It is conceivable that in the "base-off" position the phosphate function of the nucleotide is able to approach the cobalt atom and thus decrease its electrophilic character. In the presence of 0.1 M imidazole, the two waves of the cyanoaquo forms of cobinamide change to one two-electron wave with a half-wave potential of −1.01 V *vs.* S.C.E. This half-wave potential is between the half-wave potentials of diimidazolecobinamide and dicyanocobinamide, suggesting that only one isomer, presumably with imidazole in the upper and cyanide ion in the lower position, is reduced at the mercury electrode. This suggestion implies that the equilibrium between the two imidazole cyanide isomers of cobinamide is established fast enough so that only one wave corresponding to the more readily reduced isomer is obtained.

The half-wave potentials of the Co-alkylcobinamides tested are higher than that of diaquocobinamide reflecting the basicity of the alkyl anions, but they are lower than those of the corresponding cobalamins because they lack the strong nucleophile in the lower axial position. It is evident that an increase in the nucleophilic nature of the ligand in this position increases the half-wave potential of the first wave. In the presence of a weak nucleophile, imidazole, the first wave half-potential of methylaquocobinamide shifts from −1.17 to −1.19 V *vs.* S.C.E., whereas in the presence of a stronger nucleophile, cyanide ion, the half-wave potential shifts to −1.25 V *vs.* S.C.E. The half-wave potential of *n*-propylcobinamide is not affected by these ligands at the same concentration. This observation is in accord with earlier findings that the formation constant of the *n*-propylcobinamide–imidazole complex is two orders of magnitude smaller than that of the methylcobinamide–imidazole complex (Pailes and Hogenkamp, 1968) and thus these ligands do not bind to the cobalt atom to an appreciable extent.

Possible Implications for the Mechanism of Action of Corrinoids. The results presented above provide additional evidence indicating that changes in the nature of the lower axial ligand profoundly alter the character of the central cobalt atom and the polarization of the organometallic bond. Hodgkin (1965a,b) and Brodie (1969) have shown that this lower ligand also affects the geometry of the corrin ring. It has been suggested by Brodie (1969) that the coordinate linkage between cobalt and the 5,6-dimethylbenzimidazole moiety of the cobalamin coenzyme is opened in those enzymic reactions thought to involve transfer of a hydride ion (propanedioldehydrase and ribonucleotide reductase) or transfer of a methyl carbonium ion (N^5-methyltetrahydrofolate–homocysteine transmethylase), but that the coordinate

SCHEME I



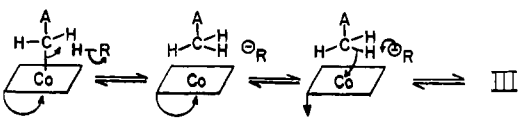
III IV V VI

linkage remains closed in those reactions involving proton abstraction (succinyl-CoA isomerase and glutamate mutase). Since all these reactions are transfer reactions in which the coenzyme functions alternatively as an acceptor and a donor, it seems reasonable to extend the mechanism proposed by Brodie (1969) by postulating that during these enzymic transformations the coordinate linkage is alternately opened and closed. Thus, in the reactions involving the transfer of a hydride, opening of the coordinate linkage would facilitate the heterolytic cleavage of the carbon–cobalt bond into an electrophilic 5′-deoxyadenosylcarbonium ion and a nucleophilic cob(I)inamide-like species (IV). After the 5′-deoxyadenosylcarbonium ion has accepted the hydride ion, transfer of this hydride ion would then be facilitated by increasing the nucleophilicity of the cobalt atom. Closing the coordinate linkage between the 5,6-dimethylbenzimidazole moiety and cobalt would increase the nucleophilicity of the metal (VI) (Scheme I). Recently, Fukui *et al.* (1969) have shown that Co-acetylcobalamin is decomposed by alkali to acetate and cob(I)alamin, but that Co-acetylcobinamide is decomposed to acetate and cob(I)inamide at a much faster rate. These results indicate that displacement of the 5,6-dimethylbenzimidazole moiety by a weaker nucleophile renders the carbon–cobalt bond more susceptible to a heterolytic cleavage in which the bonding electrons remain with the cobalt atom.

In reactions where a proton rather than a hydride ion seems to be transferred, heterolytic cleavage of the carbon–cobalt bond to a 5′-deoxyadenosylcarbanion and an electrophilic cob(III)alamin-like species would be the first event in the reaction. Coordination of the 5,6-dimethylbenzimidazole moiety with the cobalt atom would stabilize this intermediate (VII). After the 5′-deoxyadenosylcarbanion has accepted the proton, transfer to an acceptor molecule is facilitated by rendering the cobalt more electrophilic. Opening of the coordinate bond would increase the electrophilicity of the cobalt (IX); after transfer of a proton from this intermediate has occurred, the coordinate linkage is closed again (III) (Scheme II).

A similar series of reactions can be visualized for N^5-methyltetrahydrofolate–homocysteine transmethylase. This enzyme

SCHEME II



VII VIII IX

has been isolated in two different forms: the aquocobalamin–enzyme complex and the methylcobalamin–enzyme complex. Taylor and Weissbach (1969) have shown that incubation of the aquocobalamin enzyme complex (holoenzyme) with *S*-adenosylmethionine and $FMNH_2$ primes the holoenzyme for the methyl transfer reaction. This primed holoenzyme catalyzes the synthesis of approximately 15 moles of methionine per mole of enzyme before the latter becomes inactivated. In evacuated tubes or under nitrogen, this inactivation is slowed down so that 1 mole of enzyme is able to catalyze the synthesis of approximately 100 moles of methionine. These results can be interpreted as follows: In the aquocobalamin–enzyme complex, the pK_a of the coordinate bond between the 5,6-dimethylbenzimidazole moiety and cobalt is approximately −2.4, indicating that the base is bound very tightly to the metal. Methylation of the aquocobalamin–enzyme complex increases the pK_a of this coordinate bond from −2.4 to 2.7 and thus enables a histidine residue of the enzyme to effectively compete with the nucleotide moiety for the metal. This holoenzyme is the catalytic unit and alternates between an effective methylcarbonium ion acceptor and donor by closing and opening the histidine–cobalt coordinate linkage, respectively.

A histidine residue of the enzyme is implicated in this reaction sequence because the spectral properties and the photostability of N^5-methyltetrahydrofolate–homocysteine transmethylase suggest that the coordinate linkage between the nucleotide base and cobalt is broken (Taylor and Weissbach, 1967). Furthermore, model studies have shown that the methylcobinamide–imidazole complex has spectral properties similar to that of holotransmethylase and that the complex is more resistant to photodecomposition than methylcobalamin (Pailes and Hogenkamp, 1968).

Reaction of the highly reactive nucleophilic or electrophilic species of the coenzymes with the solvent is hindered because the ligand in the upper axial position of the cobalt is enclosed by nonpolar groups, such as the methyl and methylene groups of the corrin ring (Hodgkin, 1965a,b). Hamilton *et al.* (1969) have recently shown that when 5′-deoxyadenosylcobalamin is incubated with ribonucleotide reductase from *Lactobacillus leichmannii*, a nucleoside triphosphate and a suitable thiol, an electron spin resonance signal similar to that of cob(II)alamin is obtained. This paramagnetic species is formed very slowly and is probably derived from the strongly nucleophilic cob(I)alamin species which is either decomposed by the solvent (Tackett *et al.*, 1963) or by oxygen. Similarly, the active form of holotransmethylase containing cob(I)alamin may be slowly oxidized to an inactive form which contains a cobalamin with cobalt in the +2 or +3 state (Blakley, 1969).

Acknowledgments

We thank Drs. R. L. Dryer and C. D. Nordschow of the University of Iowa for the use of the polarograph and the diffusion cell, respectively.

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