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Role of Peripheral Side Chains of Vitamin B_{12} Coenzymes in the Reaction Catalyzed by Dioldehydrase†

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ABSTRACT: Isomers of adenosylcobalamin in which one of the three amide groups of the propionamide side chain of the corrin ring was converted to $-COOH$, $-COOCH_3$, or $-CONHCH_3$ were tested for coenzyme activity with dioldehydrase. The coenzyme activity of these nine isomers ranged from 86 to 7% that of the normal coenzyme. The rate-limiting step with the monocarboxylic acid analogues was the same as with the normal coenzyme as indicated by a 12-fold deuterium isotope effect on the maximal velocity of the reaction with [1,1-^{2}H]-1,2-propanediol. With the *b* site modifications of the coenzyme, the maximal velocities decrease in the order $-COO^-$ $\gg$ $CONHCH_3^-$, $COOCH_3$ suggesting inhibition by steric effects, while at the diagonal *e* site the maximal velocities decrease in the order $-CONHCH_3$, $-COO^-$, $-COOCH_3$ suggesting the importance of H-bond donation from coenzyme to enzyme. The binding of the adenosyl and cyano forms of the analogues to apoenzyme was reversible, although the hydroxo forms bind essentially irreversibly. This is in contrast to the adenosylcobalamin and cyanocobalamin which bind essentially irreversibly. In the presence of propanediol, the *e*-carboxylic acid, *e*-methyl ester, and *b*- and *e*-methylamide coenzymes brought about irreversible inactivation of the enzyme. Optical and EPR spectroscopy demonstrated that except for the complexes with the inactivating analogues, the holoenzymes of the other analogues generate an organic radical and cob(II)alamin during catalysis, whose steady-state concentrations correlated with their activity. The concentrations of the cob(II)alamin species and the radical species are equal. EPR spectra of the complexes with the four inactivating analogues show the accumulation of a disproportionally higher concentration of cob(II)alamin relative to the organic radical. These findings indicate the quenching of the organic radical intermediates in side reactions presumably due to their improper positioning. All of the four binary complexes of the inactivating analogues with the apoenzyme were relatively stable in the absence of substrate. In contrast, the complexes with the normal coenzyme and with the analogues which do not inactivate are unstable in the absence of substrate, presumably due to reaction of the radical species with O_2. This stability of the inactivating complexes suggests insufficient activation of the coenzyme, i.e., lower levels of the dissociated form. Since the modifications of the propionamide side chains used do not affect the intrinsic chemical properties of the coenzyme, all of the observed structural and kinetic effects result from alterations of the coenzyme–protein interaction. The present data thus indicate that the interactions of the coenzyme propionamide side chains with the apoprotein facilitate the homolytic cleavage of the C–Co bond and contribute to the stabilization of the radical intermediates.

Dioldehydrase (DL-1,2-propanediol hydrolyase, EC 4.2.1.28), an enzyme which requires a B_{12} coenzyme (adenosylcobalamin), catalyzes the conversion of several glycols to corresponding aldehydes, for instance, D- or L-1,2-propanediol to propionaldehyde and 1,2-ethanediol to acetaldehyde. A mechanism has been proposed for this reaction (see Scheme I) which is accepted by many, although not all, investigators (Schrauzer, 1971; Corey et al., 1977). An early event in the proposed mechanism is the homolytic cleavage of the C–Co bond of the coenzymes. How does the enzyme catalyze this cleavage? Consideration of the structural features of the coenzyme suggested a possible answer to this question. There are six amide groups, three propionamide groups, and three acetamide groups around the periphery of the corrin ring of the coenzyme. The possibility was considered that these amide groups interact through hydrogen bonds with amide groups of the protein. This interaction could lead to distortion of the corrin ring and possibly facilitate C–Co bond cleavage. These considerations led us to examine the role of the peripheral amide groups in the catalytic process. We prepared analogues of adenosylcobalamin in which each one of the propionamide groups was individually replaced by either $-COOH$, $-COOCH_3$, or $-CONHCH_3$. We investigated the interaction of these nine analogues with dioldehydrase. Our results are reported here.

Previously, some of the above-mentioned analogues have been prepared and some properties reported. It was reported

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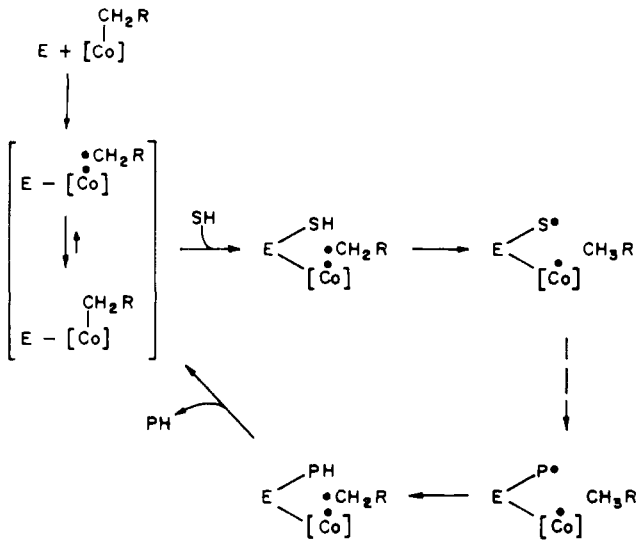
Scheme I: Mechanism of Action of Vitamin B_{12} Coenzyme[a]



[a] RCH_2–, 5′-deoxyadenosyl; [Co], cobalamin; SH, substrate; PH, product.

that monocarboxylic acid coenzyme is inactive as coenzyme in the ribonucleotide reductase system (Morely et al., 1968; Yamada & Hogenkamp, 1972) and partially active in the dioldehydrase (Tamao et al., 1970) and glycerol dehydrase (Yakusheva et al., 1977) systems. Some amide and ester analogues have also been reported (Morley et al., 1968; Yakusheva et al., 1977; Rapp & Hildebrand, 1972) but, again, no detailed investigation of their coenzymic properties was carried out. It has also been reported that the di- and tricarboxylic acid analogues of adenosylcobalamin are inactive as coenzymes with dioldehydrase (Tamao et al., 1970).

Experimental Procedures

Materials. Crystalline cyanocobalamin and adenosylcobalamin were a gift from Glaxo Laboratories Ltd., Greenford, England. β-NADH ($2Na^+$) and yeast alcohol dehydrogenase (322 units/mg of protein) were obtained from Sigma. All other chemicals were reagent grade commercial products and were used without further purification. The apoenzyme of dioldehydrase was purified by a modification[1] of a published procedure (Abeles, 1966) from extracts of *Klebsiella pneumoniae* (formerly *Aerobacter aerogenase*) ATCC 8724 grown without aeration in a glycerol–1,2-propanediol medium (Abeles, 1966). An essentially homogeneous preparation having specific activity more than 70 units/mg of protein was used in this study, and enzyme concentration was calculated on the basis of a molecular weight of 230 000[1] (Essenberg et al., 1971). The substrate-free apoenzyme was obtained by dialyzing apoenzyme overnight against 1000 volumes of 0.05 M potassium phosphate buffer (pH 8.0).

Dioldehydrase Assay. The activity of dioldehydrase was determined either by the 3-methyl-2-benzothiazolinone hydrazone (MBTH) method of Toraya et al. (1977) or by a coupled reaction in which the propionaldehyde formed by the dioldehydrase reaction is reduced to 1-propanol in the presence of added excess β-NADH and alcohol dehydrogenase. The decrease in the absorbance of NADH at 340 nm per unit time is a measure of the dioldehydrase activity. The usual assay mixture was composed of an appropriate amount of dioldehydrase, 0.2 M 1,2-propanediol, 38 μg (about 12 units) of yeast alcohol dehydrogenase, 0.2 mM NADH, 0.04 M potassium phosphate buffer (pH 8.0), and 10 μM cobalamin coenzymes in a total volume of 1.0 mL. The reaction was carried out at 37 °C in a cuvette with a 1.0-cm light path and started by adding coenzyme to the remainder of the reaction mixture which was preincubated at 37 °C for about 10 min. One unit is defined as the amount of enzyme activity catalyzing the formation of 1 μmol of propionaldehyde/min under the standard conditions. For routine assays, the amount of enzyme to be assayed is kept between 0.001 and 0.05 unit.

Other Analytical Procedures. Protein concentration was determined either by the method of Lowry et al. (1951) using crystalline bovine serum albumin as the standard or by measurement of the absorbance of 278 nm. An extinction coefficient of 5.27 for 10 mg of dioldehydrase/mL and for a 1-cm light path (Toraya et al., 1977) was used for the latter method.

The concentration of corrinoids was determined spectrophotometrically based on either ϵ_{367} 30.4×10^3 M^{-1} cm^{-1} for dicyanocobalamin (Barker et al., 1960), ϵ_{361} 28.06×10^3 M^{-1} cm^{-1} for cyanocobalamin (Friedrich, 1964), ϵ_{351} 26.5×10^3 M^{-1} cm^{-1} for aquacobalamin (Firth et al., 1967), or ϵ_{525} 7.6×10^3 M^{-1} cm^{-1} for adenosylcobalamin (Morley et al., 1968).

Optical spectra were obtained on a Beckman Model 25 recording spectrophotometer. X-band electron paramagnetic resonance (EPR) spectra were taken with a Varian E-4 spectrometer at microwave frequencies between 9.140 and 9.146 GHz. Sample preparation and EPR measurments were carried out essentially as described before (Finlay et al., 1973; Valinsky et al., 1974). Spectra were recorded at an incident microwave power of 5 mW with the sample kept at −155 ± 5 °C in the cavity. Detailed experimental conditions are described in legends to Table V and Figure 4. The absorption spectrum obtained by single integration of the net first derivative spectrum with a Fabritek Instrument 1070 computer was printed out and photocopied. After an appropriate base line was drawn, the peaks were cut out and weighed accurately on a Mettler balance. Concentrations of unpaired spins were determined to ±25% by comparing the relative areas with that of copper(II)–EDTA as an intensity standard. The concentrations of an organic radical and cob(II)alamin were calculated by integration over doublet and cobalt(II) signals, respectively.

Desalting of Corrinoids by Amberlite XAD-2 Chromatography. For the purpose of desalting a small amount of corrinoids, Amberlite XAD-2 chromatography was routinely employed. A dilute solution of corrinoids containing inorganic salts was applied to a column of Amberlite XAD-2 (Mallinckrodt, 20–50 mesh), and the column was washed with water to remove salts. The corrinoids adsorbed on this nonionic polymeric adsorbent were then eluted with 10–20% *tert*-butyl alcohol. The corrinoid-containing fractions were pooled and evaporated to dryness. The column can be used repeatedly by washing with 50% *tert*-butyl alcohol containing 2% acetic acid. This procedure is particularly useful for concentrating and desalting a very dilute solution of corrinoids in a high yield.

Preparation of Cyanocobalaminmonocarboxylic Acid. Cyanocobalamin was hydrolyzed by a modification of the method of Bernhauer et al. (1966). Cyanocobalamin (1.0 g, 700 μmol) was dissolved in 1 L of 0.8 M phosphoric acid and heated at 70–72 °C for 3 h under a hydrogen atmosphere. The mixture was then cooled in an ice–water bath, and the corrinoids were desalted by phenol extraction and evaporated to dryness. The residue was dissolved in a small amount of water

[1] Poznanskaja, A., Tanizawa, K., Soda, K., Toraya, T., and Fukui, S., manuscript in preparation.

Table I: Chromatographic and Electrophoretic Behaviors of the Analogues Used in This Study

cobalamin[e]	$R_{CN\text{-}Cbl}$ in thin-layer chromatography[a] solvent A	solvent B	solvent C	$R_{CN\text{-}Cbl}$ in chromatography[b] cellulose	DEAE-cellulose	retention time in high-pressure LC (min)[c]	effective charge in paper electrophoresis[d] pH 2.7	pH 7.0
CN-Cbl	1	1	1	1	1	10.2	0	0
CN-Cbl(*b*-OH)	1.47	1.37	1.13	1.32	0.96	16.5	0	−
CN-Cbl(*e*-OH)	1.16	1.37	1.09	1.35	0.78	14.2	0	−
CN-Cbl(*d*-OH)	0.93	1.37	1.06	1.28	0.66	17.3	0	−
CN-Cbl(*b*-OCH_3)	2.77						0	0
CN-Cbl(*e*-OCH_3)	2.77						0	0
CN-Cbl(*d*-OCH_3)	1.77						0	0
CN-Cbl(*b*-$NHCH_3$)	1.11						0	0
CN-Cbl(*e*-$NHCH_3$)	1.10						0	0
CN-Cbl(*d*-$NHCH_3$)	1.07						0	0
OH-Cbl	0.12	0.03	0.01			3.3	+	δ+
AdoCbl	0.73± 0.03						+	0
AdoCbl(*b*-OH)	1.05						+	−
AdoCbl(*e*-OH)	0.89						+	−
AdoCbl(*d*-OH)	0.78						+	−
AdoCbl(*b*-OCH_3)	1.85						+	0
AdoCbl(*e*-OCH_3)	1.92						+	0
AdoCbl(*d*-OCH_3)	1.19						+	0
AdoCbl(*b*-$NHCH_3$)	0.73						+	0
AdoCbl(*e*-$NHCH_3$)	0.73						+	0
AdoCbl(*d*-$NHCH_3$)	0.64						+	0

[a] On Anasil of precoated silica gel plates. Solvent A, 2-butanol–acetic acid–water (127:1:50 v/v); solvent B, 1-butanol–2-propanol–water (10:7:10); solvent C, 2-propanol–NH_4OH (28%)–water (7:1:2 v/v). [b] On Whatman 3MM paper and on Whatman DE 81 in water-saturated 2-butanol containing 1% acetic acid. [c] On a μBondapak C_{18} column (3.9 mm i.d. × 30 cm) with water–methanol–acetic acid (79:20:1) at a flow rate of 2.0 mL/min. [d] In 0.5 N acetic acid (pH 2.7) and 0.01 M potassium phosphate buffer (pH 7.0) at a voltage gradient of about 11 V/cm. [e] Abbreviations used: AdoCbl, adenosylcobalamin or *Co*α-[α-(5,6-dimethylbenzimidazolyl)]-*Co*β-adenosylcobamide; AdoCbl(*b*-, *d*-, or *e*-OH), adenosylcobalamin-*b*-, -*d*-, or -*e*-carboxylic acid; AdoCbl(*b*-, *d*-, or *e*-OCH_3), adenosylcobalamin-*b*-, -*d*-, or *e*-methyl ester; AdoCbl(*b*-, *d*-, or *e*-$NHCH_3$), adenosylcobalamin-*b*-, -*d*-, or -*e*-methylamide.

and applied to a column (3.5 × 42 cm) of DEAE-cellulose (acetate form). The column was washed thoroughly with water to remove the unreacted cyanocobalamin and other nonanionic corrinoids and then developed with 0.05% acetic acid. Monocarboxylic acids were eluted in two major bands: the first fraction (eluate 1) contains one cyanocobalamin-monocarboxylic acid isomer (I) and the second fraction (eluate 2), a prominent band, contains two monocarboxylic acid isomers (II and III). These fractions were desalted by phenol extraction, evaporated to dryness, and taken up in a small amount of water.

Separation of Cyanocobalaminmonocarboxylic Acid Isomers by High-Pressure Liquid Chromatography. Since both eluates 1 and 2 turned out on paper chromatography to contain some other corrinoid monocarboxylic acids (with and without nucleotide), isomers I–III were separated from these minor impurities by high-pressure liquid chromatography. A Waters Associate liquid chromatography apparatus, which is equipped with a UV detector, was used throughout. By chromatography of the concentrated sample of eluate 1 through a column (3.9 mm i.d. × 30 cm or 7.8 mm i.d. × 30 cm) of μBondapak C_{18} with water–methanol–acetic acid (79:20:1) at a flow rate of 2 mL/min, isomer I was purified to homogeneity from eluate 1. Isomers II and III were isolated from eluate 2, II and III being eluted in this order, under the same operation conditions. These isomers were desalted by phenol extraction and evaporated to dryness. They proved completely pure by criteria of thin-layer chromatography and paper electrophoresis (yields of I–III were 24, 42, and 26 μmol, respectively).

Each isomer of cyanocobalaminmonocarboxylic acid gives the same absorption spectrum as that of cyanocobalamin and shows the mobility corresponding to one net negative charge on paper electrophoresis at pH 7.0. However, as shown in Table I, the three isomers were distinguishable from each other in several chromatographic systems: (1) thin-layer chromatography on silica gel; (2) paper chromatography on Whatman 3MM and DEAE-cellulose paper (DE-81); (3) high-pressure liquid chromatography. By comparing the chromatographic data with those of Bernhauer et al. (1966, 1968) and Yamada and Hogenkamp (1972), we tentatively identified isomers I–III as cyanocobalamin-*b*-, -*e*-, and -*d*-carboxylic acid, respectively. The nomenclature for the designation of carboxyl groups is standard nomenclature (see Babior, 1975a,b).

Synthesis of Cyanocobalamin Monomethyl Ester. Cyanocobalaminmonocarboxylic acid isomers (10 μmol) were dissolved in 5 mL of anhydrous methanol and evaporated to dryness in order to remove water. This procedure was repeated twice. The residue was then dried in the vacuum desiccator for 3 h. The residue was then dissolved in 4 mL of anhydrous dimethylformamide. To this solution was added 0.08 mL of redistilled triethylamine, and the mixture was stoppered and cooled to −10 °C in a methanol–ice bath. A 10% solution of ethyl chloroformate (0.5 mL) in anhydrous dimethylformamide was added by an injection through a serum cap, and the mixture was kept on ice for 10 min. Anhydrous methanol (1 mL) was then added to the system by an injection, and the reaction vessel was removed from the ice bath and kept at room temperature for 30 min. After the vessel was heated at 50 °C for 5 min, 40 mL of water was added and the corrinoid products were desalted by Amberlite XAD-2 column chromatography. The crude products were taken up in a small volume of water and passed through a column of P-cellulose (adjusted to pH 3.1 with KOH) to remove nucleotide-lacking corrinoid impurities. The effluent was concentrated to a small volume by evaporation, treated with a trace of KCN, and then passed through a column of DEAE-cellulose (acetate form). Nucleotide-lacking corrinoids, still present, were adsorbed at

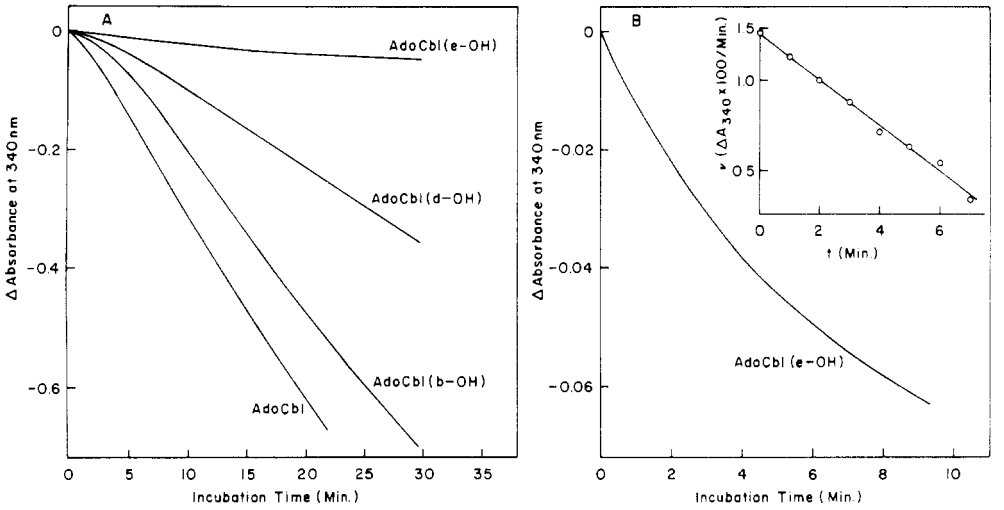


FIGURE 1: Time course of the dioldehydrase reaction with adenosylcobalamin and its monocarboxylic acid analogues as coenzymes. The coupled assay method was employed. (A) 0.006 unit of apoenzyme was used. The reaction was started by adding each coenzyme to a final concentration of 10 μM. (B) To shorten the lag time, 0.6 unit of the substrate-free apoenzyme was incubated at 37 °C for 20 min with 50 μM adenosylcobalamin-*e*-carboxylic acid in 1.0 mL of 0.05 M potassium phosphate buffer (pH 8.0). An aliquot of 0.1 mL was withdrawn from the mixture and added to the preincubated mixture (0.9 mL) of the remaining reactants. Inset: the kinetics of inactivation of the apoenzyme–adenosylcobalamin-*e*-carboxylic acid complex.

a top of the column in a purple band as the dicyano form. The corrinoid in the effluent was electrically neutral at pH 7.0 and 2.7 and proved pure as judged by thin-layer chromatography and paper electrophoresis. The spectrum of each monomethyl ester isomer is essentially identical with that of cyanocobalamin. The yield was approximately 20–30% theoretical.

Synthesis of *Cyanocobalaminmonomethylamide.* Each monocarboxylic acid isomer (10 μmol) was dissolved in 2 mL of water and added to a solution of methylamine hydrochloride (0.67 g) in 3 mL of water, which was previously adjusted to pH 5–6 with KOH. After the pH of the mixture was readjusted to 5–6, 100 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added in 20-mg aliquots at 1-min intervals. Gentle stirring was continued at room temperature. After 30 and 60 min, an additional 20 mg of the carbodiimide was added, and the mixture was stirred overnight. To the mixture were then added 50 mL of water and a trace amount of KCN, and the corrinoids were desalted by Amberlite XAD-2 chromatography. The crude products were taken up in a small volume of water and passed through columns of P-cellulose (adjusted to pH 3.1 with KOH) and DEAE-cellulose (acetate form) to remove any acylurea derivatives formed by a side reaction and unreacted monocarboxylic acid, respectively. The corrinoid in the effluent was neutral at pH 7.0 and 2.7 and proved pure by thin-layer chromatographic and paper electrophoretic criteria. Each monomethylamide isomer gives essentially the same spectrum as that of cyanocobalamin. The yield was 50–80% theoretical.

Conversion to Adenosyl and Hydroxo Forms. Each isomer of cyanocobalaminmonocarboxylic acid, monomethyl ester, and monomethylamide (7–10 μmol) in 3 mL of water was reduced either with 25 mg of $NaBH_4$ (monocarboxylic acid and monomethylamide) or with NH_4Cl (10%)–zinc powder (0.3 g) (monomethyl ester). 5′-Iodo-5′-deoxyadenosine (25 mg) in 3 mL of dimethylformamide was added to the system through a serum cap. After 20–30 min, crude corrinoid products were desalted by phenol extraction and applied to a column of P-cellulose (adjusted to pH 3.1 with KOH). The column was thoroughly washed with water to remove the unreacted cyano form of the side-chain analogues. The adenosyl form of monocarboxylic acid isomers was purified by developing the column with 0.1 M potassium acetate buffer (pH 3.8). The slowest moving corrinoid was the desirable product. The adenosyl form of monomethyl ester and monomethylamide isomers was purified by eluting with 0.2 M KCl, followed by desalting with an Amberlite XAD-2 column and passing through a column of P-cellulose (adjusted to pH 6.0 with KOH). In the case of adenosylcobalamin monomethyl ester, the purified product was finally passed through a DEAE-cellulose (acetate form) column to remove a trace amount of monocarboxylic acid impurity formed during the conversion and purification procedures. The electrophoretic mobilities of the coenzymes, thus obtained, were as expected theoretically and are summarized in Table I, together with their chromatographic behaviors. The spectrum of each isomer of the side-chain analogues was essentially identical with that of adenosylcobalamin.

The hydroxo form of the derivatives was readily obtained by photolysis of the adenosyl form of the corresponding analogues with a 250-W tungsten bulb from a distance of 15 cm for 10–20 min.

Results

Coenzymic Action of B_{12} Coenzyme Analogues in the Dioldehydrase Reaction. Three series of B_{12} coenzyme analogues were prepared in which either the *b*-, *d*-, or *e*-propionamide group was modified. In each case, a single group was modified. The following modifications were made. (1) One of the propionamide groups was hydrolyzed to the carboxylic acid. (2) One of the propionamide groups was converted to the methyl ester. (3) One of the propionamide groups was replaced by the methylamide. A total of nine coenzymes were prepared and tested for coenzyme activity with dioldehydrase. Typical examples of the time courses of the reaction with the adenosylcobalaminmonocarboxylic acid isomers as coenzymes are illustrated in Figure 1. The *b*- and *d*-carboxylic acid isomers showed relatively high coenzyme activity. The reaction rates with these isomers were essentially linear for at least 15 min after constant velocity was attained. In that respect, they resemble the normal coenzyme (AdoCbl).[2] In contrast, the *e*-carboxylic acid isomer serves as a very poor

[2] Abbreviation used: AdoCbl, adenosylcobalamin.

Table II: Coenzyme Activity and Kinetic Parameters of Adenosylcobalamin and Its Side-Chain Analogues

coenzyme	app K_m (μM)	k_{cat}[a] [s^{-1} (%)]	k_{inact}[b] (min^{-1})	(k_{cat}/k_{inact}) $\times 10^{-4}$
AdoCbl	0.28	337[c] (100)	0.014	144
AdoCbl(*b*-OH)	2.3	290 (86)		
AdoCbl(*e*-OH)	4.5	37 (11)	0.173	1.28
AdoCbl(*d*-OH)	8.0	222 (66)		
AdoCbl(*b*-OCH_3)	3.1	47 (14)		
AdoCbl(*e*-OCH_3)	1.9	24 (7)	0.109	1.32
AdoCbl(*d*-OCH_3)[d]		47 (14)[e]		
AdoCbl(*b*-$NHCH_3$)	2.1	54 (16)	0.064	5.06
AdoCbl(*e*-$NHCH_3$)	0.94	139 (41)	0.083	10.0
AdoCbl(*d*-$NHCH_3$)	1.5	145 (43)		

[a] Calculated from the maximum velocity. [b] Calculated from the time course of the reaction with the analogue complexes which were formed by preincubating 0.5–0.6 unit of apoenzyme with 50 μM each analogue at 37 °C for 20 min in the absence of substrate. In the case of adenosylcobalamin, no preincubation was carried out because of the lack of significant log period with 15 μM coenzyme. [c] The value is from Toraya et al. (1976). [d] The sample of adenosylcobalamin-*d*-methyl ester is contaminated with a minor impurity. [e] Relative activity obtained at a concentration of 10 μM.

coenzyme. Furthermore, the time course of the reaction was not linear, as shown in Figure 1B. Eventually, aldehyde production ceased completely. At this point, the activity could not be restored by addition of adenosylcobalamin. This indicates that the *e* isomer brings about the irreversible inactivation. Inactivation proceeded according to the first-order kinetics with a rate constant of inactivation (k_{inact}) of 0.17 min^{-1} (Figure 1B, inset).

Table II summarizes the kinetic parameters of the coenzyme analogues in terms of apparent K_m and k_{cat} values. In the series of monocarboxylic acid coenzymes, the coenzyme activity is clearly dependent on the position of the carboxyl group and decreases in the order of $b > d > e$. The same order of reactivity of monocarboxylic acid isomers has been reported by other investigators (Tamao et al., 1970). The three monomethyl ester coenzymes are much less active, although their apparent K_m values are similar to those of the monocarboxylic acid coenzymes. Again as with the monocarboxylic acid analogues, the *e* isomer shows least reactivity. The coenzyme activity of monomethylamide coenzyme does not follow the above-mentioned order. In contrast to the carboxylic acid and ester analogues, the *e* isomer shows relatively high activity, whereas the *b* isomer shows low activity. The *e*-ethylamide coenzyme was reported to be 19% as active as the normal coenzyme, while the *b*-ethylamide is inactive (Rapp & Hildebrand, 1972). These findings are consistent with our results with methylamide analogues. The fact that the apparent K_m value for *e*-methylamide coenzyme is low also suggests that the methylamide group of the *e* side chain interacts with the apoenzyme more favorably than does the carboxyl or the methyl ester group.

It was stated above that the holoenzyme formed with the *e*-carboxylic acid analogue gradually lost catalytic activity. A similar inactivation phenomenon was also observed with the other *e* analogues, as well as with *b*-methylamide. The rates of inactivation for these analogues are given in Table II. Analogues for which k_{inact} is not given in Table II do not cause significant inactivation.

Binding of Analogues to Apoenzyme. The binding of cobalamins to the apodioldehydrase is known to be very tight and essentially irreversible. When hydroxocobalamin is added to the preformed enzyme–adenosylcobalamin complex, no

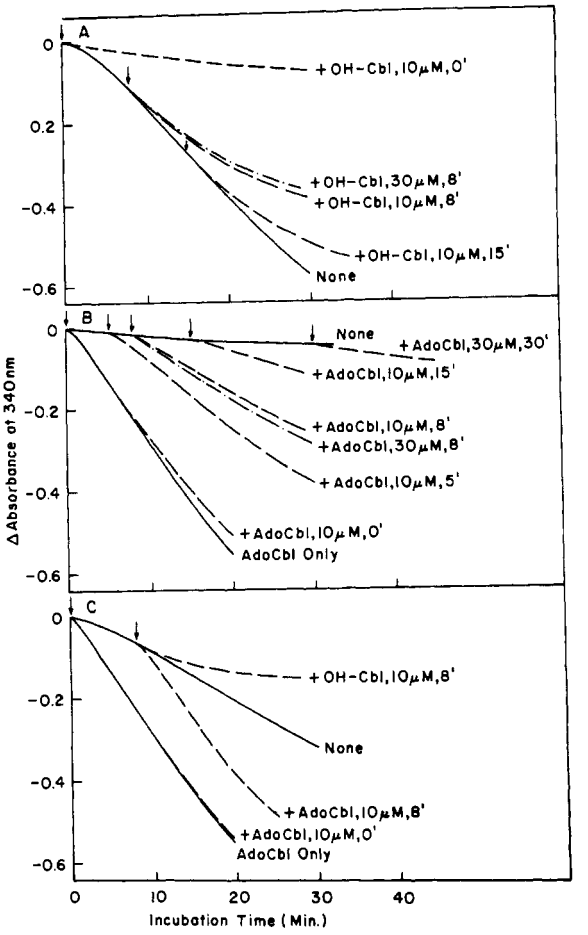


FIGURE 2: Displacement of the enzyme-bound adenosylcobalaminmonocarboxylic acid analogues by hydroxocobalamin and adenosylcobalamin. The coupled assay method was employed. The reaction was started by adding 10 nmol of each coenzyme to the usual reaction mixture (containing 0.005 unit of apoenzyme in a total volume of 0.9 mL. After the time indicated by the arrows, the indicated amount of hydroxocobalamin or adenosylcobalamin in 0.1 mL was added to the system. Change in absorbance was corrected for dilution. (A) Adenosylcobalamin-*b*-carboxylic acid, (B) adenosylcobalamin-*e*-carboxylic acid, and (C) adenosylcobalamin-*d*-carboxylic acid.

inhibition is observed. Conversely, when adenosylcobalamin is added to the enzyme–hydroxocobalamin or enzyme–cyanocobalamin complex, no catalytic activity is observed. Experiments were carried out to establish whether similar irreversible binding is observed with the modified coenzymes. A typical experiment with *b*- and *d*-carboxylic acid coenzymes is shown in Figure 2A,C. Hydroxocobalamin was added to the reacting systems containing *b*- and *d*-carboxylic acid coenzymes. Hydroxocobalamin was added after 8 min of incubation. (By this time all the apoenzyme can be considered to be converted to the corresponding holoenzyme, since the constant maximum velocity was attained.) Addition of hydroxocobalamin reduced the rate of aldehyde formation and eventually totally inhibits aldehyde formation. This observation demonstrates that the bound *b* and *d* isomers were displaced by the hydroxocobalamin added. The rate of displacement of the *d* isomer ($t_{1/2}$ = 5.8 min) was faster than that of *b* isomer ($t_{1/2}$ = 15 min) as expected from their apparent K_m values. The experiments with hydroxocobalamin show that the modified coenzyme can be displaced from the apoenzyme. It would therefore be expected that addition of adenosylcobalamin to the carboxylic acid coenzyme–enzyme complexes should displace the modified coenzyme and therefore lead to an increased rate of reaction. The data in Figure 2 show that

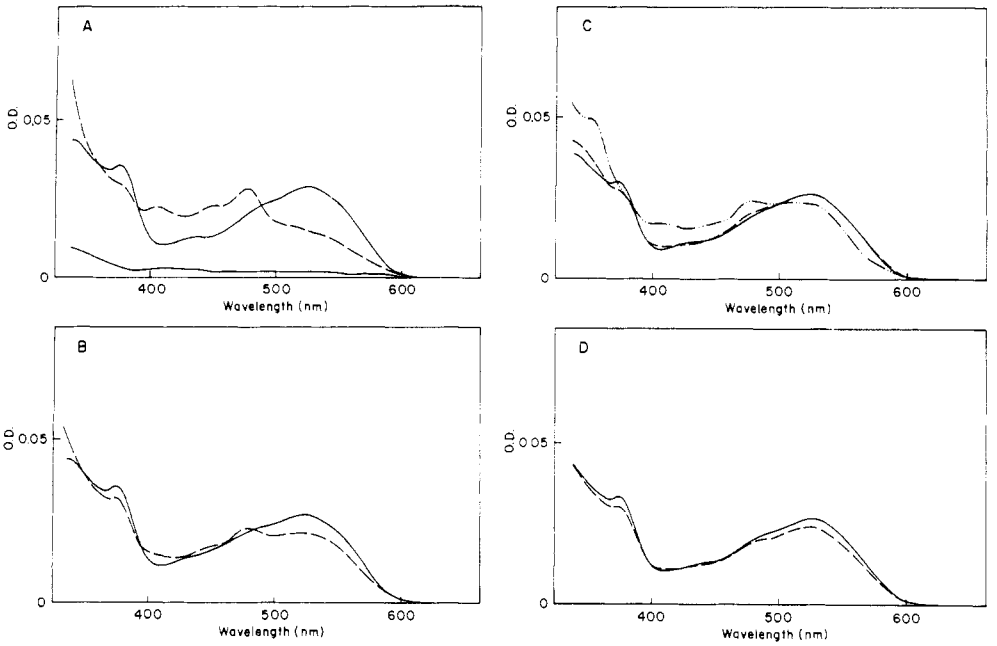


FIGURE 3: Optical spectra of adenosylcobalamin and its analogue–apoenzyme complexes in the presence of substrate. Apoenzyme (80 units, 4 nmol) was preincubated at 30 °C for 10 min in 0.05 M potassium phosphate buffer (pH 8.0) containing 1.1 M 1,2-propanediol. To the mixture was added 4 nmol of each coenzyme. Final volume 1.2 mL, 30 °C. Spectra were taken at the time indicated. Spectra of the apoenzyme (bottom spectrum in A) and each free coenzyme at the same concentrations were measured as controls (solid lines). (A) Apoenzyme–adenosylcobalamin complex (---, 10 min), (B) apoenzyme–adenosylcobalamin-*b*-carboxylic acid complex (---, 10 and 30 min), (C) apoenzyme–adenosylcobalamin-*e*-carboxylic acid complex (---, 10 min; ·—, 90 min), (D) apoenzyme–adenosylcobalamin-*d*-carboxylic acid complex (---, 10 and 30 min).

this is the case. Addition of adenosylcobalamin to the enzyme–*d*-carboxylic acid complex leads to an increase in the rate of aldehyde production. The rate of aldehyde production becomes identical with that found when adenosylcobalamin is initially present. With the *e*-carboxylic acid analogue, a rate increase is also observed, but the rate obtained after addition of adenosylcobalamin is less than that seen when adenosylcobalamin is added at $t = 0$ (Figure 2B). Furthermore, when adenosylcobalamin is added at later stages in the reaction, less activity is regained. We attribute this to the gradual inactivation of the enzyme–3-carboxylic acid complex, a phenomenon which was discussed above.

Experiments were also done with the other coenzyme analogues to determine if the analogues could be displaced from the apoenzyme. In these experiments adenosylcobalamin was added to the modified coenzyme–enzyme complexes at various times after the rate of aldehyde production was linear. The change in rate of aldehyde production was then determined. Total displacement of the coenzyme analogue should lead to a rate of aldehyde production equivalent to that observed when adenosylcobalamin alone is added to the apoenzyme. The results obtained are summarized in Table III. It is obvious that with several of the analogues, nearly total displacement occurs. With *e*-methylamide coenzyme, no displacement is seen. In some other cases, the amount of displacement observed decreases with time. We believe that a major contributing factor to this decrease is inactivation of the enzyme–coenzyme complex, although other, as yet underestimated, factors may be involved. These experiments establish that some of the coenzyme analogue–enzyme complexes are dissociable. In that respect, they differ significantly from the normal coenzyme where no dissociation occurs. The side-chain amide group must therefore make a very significant contribution to the formation of the complex between coenzyme and apoenzyme.

In clear contrast to the adenosyl form, none of the bound hydroxo forms of the monocarboxylic acid analogues were

Table III: Displacement of the Bound Side-Chain Coenzyme Analogues by Adenosylcobalamin[a]

bound analogue	% app displacement by AdoCbl when added at 5 min	8 min	15 min	30 min
AdoCbl(*b*-OH)		70	53	
AdoCbl(*e*-OH)	50	33	14	3
AdoCbl(*d*-OH)		86	65	
AdoCbl(*b*-OCH_3)		72		50
AdoCbl(*e*-OCH_3)		64		36
AdoCbl(*d*-OCH_3)		61		51
AdoCbl(*b*-$NHCH_3$)		64		
AdoCbl(*e*-$NHCH_3$)		2		
AdoCbl(*d*-$NHCH_3$)		22		

[a] The experimental conditions were identical with those described in the legend to Figure 2. The value of percent apparent displacement by AdoCbl was calculated by comparing the maximum slope obtained after the addition of adenosylcobalamin with the slope obtained with each analogue alone and adenosylcobalamin alone.

appreciably displaceable by adenosylcobalamin. On the other hand, the bound cyano form of the analogues was significantly displaced even by an equal concentration (10 μM) of adenosylcobalamin in a time-dependent manner. Fifteen minutes after addition of adenosylcobalamin, the *b*-, *e*-, and *d*-carboxylic acid isomers of cyanocobalamin were displaced 21, 13, and 30%, respectively. Under identical conditions, cyanocobalamin was displaced less than 2%. These data indicate that the cyano form of the analogues also forms a reversible complex with the apoenzyme, but the complex formed with the hydroxo form is an irreversible one.

Optical Spectroscopic Study. To obtain information regarding the mechanism of the reaction with the side-chain analogues, the optical spectra of the complex of dioldehydrase with each coenzyme analogue in the presence of substrate ("reacting complex") were compared with that of the corresponding free analogue. As illustrated in Figure 3, the

Table IV: Optical Spectrometric Determination of Extent of Cleavage of Carbon-Cobalt Bond[a]

coenzyme	act. (at 10 min)[b]	% conversion to cob(II)alamin: short-time incubation 3–4 min	short-time incubation 10 min	long-time incubation 30 min	long-time incubation 90 min	long-time incubation 120 min
AdoCbl	32.4	43	46			
AdoCbl(*b*-OH)	11.0	13	17	18		
AdoCbl(*e*-OH)	0.8	10	12	16	24	
AdoCbl(*d*-OH)	4.4	2	6	5		
AdoCbl(*b*-OCH_3)	0.4	<1	1	2	<1	<1
AdoCbl(*e*-OCH_3)	0.3	<1	3	7	9	11
AdoCbl(*b*-$NHCH_3$)	2.5	8	10	11	13	14
AdoCbl(*e*-$NHCH_3$)	7.3	9	10	14	20	25
AdoCbl(*d*-$NHCH_3$)	5.9	7	7	7	7	5

[a] The experimental conditions were identical with those described in the legend to Figure 3. The percentage of conversion of cob(II)alamin was calculated on the basis of ϵ_{473} 9.2 × 10^3 M^{-1} cm^{-1} for cob(II)alamin (Pratt, 1972). [b] Activity obtained by 10-min incubation, micromoles of propionaldehyde formed per minute at 30 °C. The value was obtained by withdrawing 5-μL aliquots from the cuvette at 5- and 10-min intervals, followed by measurement of the amount of propionaldehyde in them by the MBTH method. If all the enzyme present in the cuvette (80 units) is converted to functional holoenzyme, 53.8 μmol/min of activity should be expected at 30 °C.

complexes with *b*- and *d*-carboxylic acid coenzymes showed spectral changes similar to those observed with the regular holoenzyme; the absorption at 525 nm decreased and a new peak or shoulder appeared at 478 nm. The shape of the spectra did not change significantly with time, as long as the substrate (1,2-propanediol) was present in the system. From the spectra, the steady-state concentration of cob(II)alamin (B_{12r}) during catalysis can be calculated. Table IV summarizes the percentage of coenzyme converted to cob(II)alamin during the steady-state catalysis. Except for the complexes with *e*-carboxylic acid, *e*-methyl ester, and *b*-methylamide coenzymes, the concentration of cob(II)alamin at short incubation times, that is, in the steady state, closely correlated with the activity in the system; the extent of cleavage of the carbon–cobalt bond of the complexes is proportional to their catalytic activity. In the case of adenosylcobalamin, the degree of the formation of the functional holoenzyme after 10-min incubation was estimated from its activity to be approximately 60% of the total amount of the enzyme (80 units) in the system. Therefore, it can be concluded that about 77% of the functional holoenzyme exists in a dissociated state and 23% in an associated state with respect to the carbon–cobalt linkage.

The complexes with *e*-carboxylic acid, *e*-methyl ester, and *b*- and *e*-methylamide coenzymes, all of which undergo inactivation at substantial rates, also showed similar spectral changes. In these cases, however, the extent of the cob(II)alamin formation was much more than expected from their activity and increased gradually with time (Figure 3C and Table IV). Hence, the cob(II)alamin accumulated to an abnormally high concentration in these systems may be derived not only from the intermediate but also from the inactivated species.

Electron Paramagnetic Resonance. In the discussion of the optical spectra, we have assumed that the spectral changes observed are due to the formation of cob(II)alamin. More convincing evidence for the formation of cob(II)alamin is provided by EPR spectra. Therefore, the EPR spectra obtained in the presence of the various coenzyme analogues were examined. As shown in Figure 4A–C, the reacting complexes

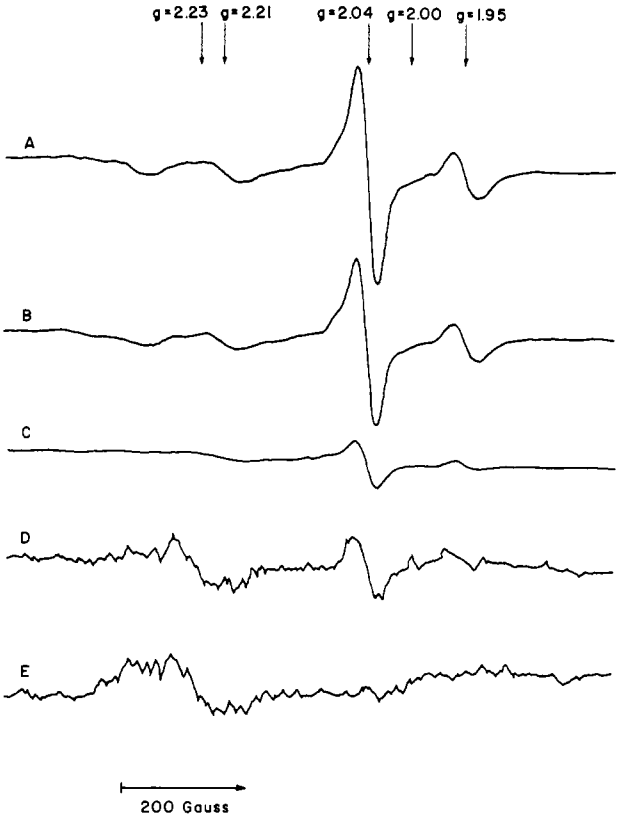


FIGURE 4: EPR spectra of adenosylcobalamin and its analogue–apoenzyme complexes in the presence of substrate. Apoenzyme (80 units, 4 nmol) in 0.12 mL of 0.05 M potassium phosphate buffer (pH 8.0) containing 2% 1,2-propanediol as a stabilizer was degassed in a quartz EPR tube (3 × 246 mm) with argon. An excess amount (29–32 nmol) of each coenzyme was added through a Hamilton syringe, and the mixture was incubated at 30 °C for 5 min and then at 0 °C for 1 min prior to the addition of substrate. 1,2-Propanediol was added to a final concentration of about 1.2 M; 45 s later, the mixture (0.18–0.20 mL) was rapidly frozen in a cold isopentane bath (−160 °C) ("short-time incubation", A–D). An EPR spectrum was also measured with the sample which was incubated at 30 °C for an additional 30 min after the addition of substrate ("long-time incubation", E). Recording conditions for the EPR spectra: scan length, 1000 G; scan time, 8 min; time constant, 3.0 s; modulation frequency, 100 KHz; modulation amplitude, 10 G; microwave frequency, 9.142–9.148; microwave power, 5 mW; temperature, −155 ± 5 °C; receiver gain, 1.25 × 10^3 for A–C and 10 × 10^3 for D and E. (A) Apoenzyme–adenosylcobalamin complex, (B) apoenzyme–adenosylcobalamin-*b*-carboxylic acid complex, (C) apoenzyme–adenosylcobalamin-*d*-carboxylic acid complex, and (D and E) apoenzyme–adenosylcobalamin-*e*-carboxylic acid complex.

with *b*- and *d*-carboxylic acid coenzymes gave X-band EPR spectra similar to those obtained with the regular holoenzyme: an asymmetric doublet signal in the high field with *g* values of 1.95 and 2.04 which is assigned to a single organic free radical and a broad resonance in the low field with *g* values of 2.2–2.4 (a prominent resonance, $g = 2.21$) which is attributed to low-spin cobalt(II) B_{12r} (Finlay et al., 1973; Valinsky et al., 1974; Schepler et al., 1975). Moreover, these two analogue complexes and the regular holoenzyme showed essentially the same characteristics of the doublet resonance [line widths of the doublet, 41–43 and 34–36 G, respectively; the coupling, 160–165 G; ratio of relative amplitudes (L/H), 5.1–6.6]. Figure 4D,E also shows the spectra obtained with the *e*-carboxylic acid analogue after short-time and long-time incubation. This analogue leads to inactivation. The spectrum in the low-field region [prominent resonance at $g = 2.23$–2.24 attributed to cob(II)alamin] is qualitatively different from and somewhat narrower than that seen with noninactivating

Table V: EPR Spectrometric Determination of Concentrations of the Organic Radical and Cob(II)alamin[a]

	spin concn (mol/mol of active site)				
	short-time incubation			long-time incubation	
coenzyme	organic	Co(II)	act.[b]	organic	Co(II)
AdoCbl	0.58	0.50	25.8		
AdoCbl (equiv)[c]	0.37	0.36			
AdoCbl(*b*-OH)	0.33	0.29			
AdoCbl(*b*-OH) (equiv)[c]	0.18	0.19			
AdoCbl(*e*-OH)	0.02	0.07		<0.01	0.09
AdoCbl(*d*-OH)	0.11	0.11	7.2		
AdoCbl(*b*-OCH_3)	0.01	<0.01		<0.01	0.01
AdoCbl(*e*-OCH_3)	0.01	<0.01		<0.01	0.03
AdoCbl(*d*-OCH_3)	0.01	0.02			
AdoCbl(*b*-$NHCH_3$)	0.01	0.04		<0.01	0.11
AdoCbl(*e*-$NHCH_3$)	0.02	0.03	3.5	<0.01	0.10
AdoCbl(*d*-$NHCH_3$)	0.04	0.04			

[a] The experimental conditions were identical with those described in the legend to Figure 4. [b] Activity obtained by short-time incubation, micromoles of propionaldehyde formed per minute at 30 °C. The value was obtained in parallel experiments under conditions similar to those of EPR measurements; 5-μL aliquots were withdrawn at 1-min intervals from the reaction mixture, and the amount of propionaldehyde in them was determined by the MBTH method. If all the enzyme present in the EPR tube (80 units) is converted to functional holoenzyme, 53.8 μmol/min of activity should be expected at 30 °C. [c] An equivalent amount (4 nmol) of each coenzyme was added.

complexes. The type of spectrum seen in Figure 4D,E is observed with all inactivating coenzyme analogues. These results suggest that cobalt(II) of the coenzyme is in a different environment in the inactivating complexes than in the non-inactivating complexes. Table V summarizes the spin concentrations on the organic radical and the low-spin cobalt(II) which were generated by the complexes with coenzyme analogues by short- and long-time incubation at 30 °C with substrate. Qualitatively, the concentration of the radical formed from any coenzyme analogue is consistent with its reactivity as well as the results obtained from optical spectra. It must be kept in mind that the conditions, under which the three different experiments, i.e., activity assays, optical spectra, and EPR spectra were carried out, are different, and quantitative comparison in some cases may be invalid.

The data in Table V also give the relative concentration of the cobalt(II) signal to that of the organic radical. In the cases of the complexes with the analogues causing inactivation, i.e., *e*-carboxylic acid, *e*-methyl ester, and *b*- and *e*-methylamide coenzymes, the concentration of cob(II)alamin is more than that of the organic radical, while in the complexes with other coenzyme analogues, they are essentially equal. This imbalance is much more marked with the long-time incubated complexes of the inactivating analogues; cob(II)alamin accumulated with time, but the organic radical diminished almost completely. These observations suggest that the organic radical species normally present in the catalytically active enzyme–coenzyme complex is lost. The loss of these species leads to inactivation and also to the accumulation of a cob(II)alamin species. This species accumulates since the adenosyl radical with which it would normally combine to form the coenzyme is no longer available.

Oxygen Sensitivity of the Apoenzyme–Analogue Complexes in the Absence of Substrate. It is known that aerobic incubation of the regular holoenzyme in the absence of substrate leads to total inactivation (Lee & Abeles, 1963; Wagner et al., 1966). This inactivation process involving the irreversible

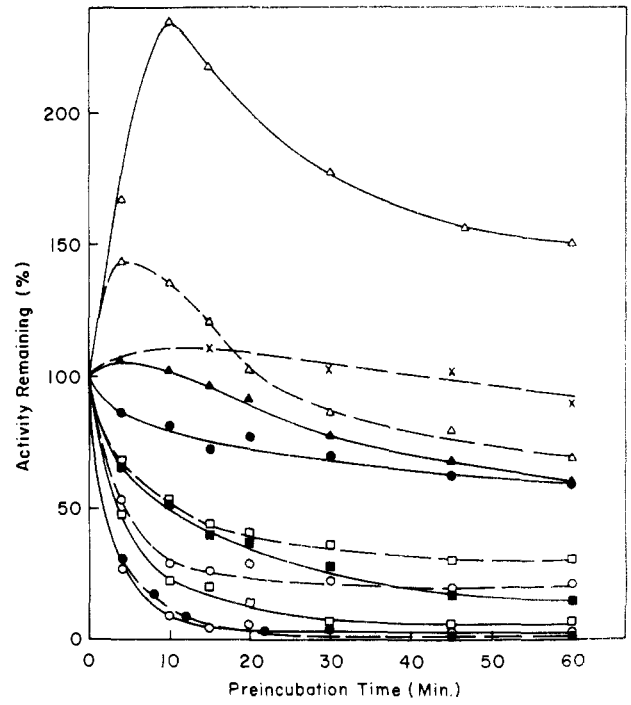


FIGURE 5: Oxygen inactivation of adenosylcobalamin and its analogue–apoenzyme complexes in the absence of substrate. The substrate-free apoenzyme (0.1–0.6 unit) was incubated at 37 °C with 50 μM each coenzyme in 1.0 mL of 0.05 M potassium phosphate buffer (pH 8.0). At the time indicated, 0.1-mL aliquots were withdrawn from the mixture and added to the preincubated mixture (0.9 mL) of the remaining reactants to determine the activity remaining. (x---x) Apoenzyme; (●---●) apoenzyme–adenosylcobalamin complex; (○-○) apoenzyme–adenosylcobalamin-*b*-carboxylic acid complex; (△—△) apoenzyme–adenosylcobalamin-*e*-carboxylic acid complex; (□—□) apoenzyme–adenosylcobalamin-*d*-carboxylic acid complex; (○---○) apoenzyme–adenosylcobalamin-*b*-methyl ester complex; (△---△) apoenzyme–adenosylcobalamin-*e*-methyl ester complex; (□---□) apoenzyme–adenosylcobalamin-*d*-methyl ester complex; (●—●) apoenzyme–adenosylcobalamin-*b*-methylamide complex; (▲—▲) apoenzyme–adenosylcobalamin-*e*-methylamide complex; (■—■) apoenzyme–adenosylcobalamin-*d*-methylamide complex.

cleavage of the carbon–cobalt bond of the bound coenzyme is believed to result from the reaction of the "activated" carbon–cobalt bond with oxygen. To get information concerning the degree of the activation of the carbon–cobalt bond in the complexes with the side-chain analogues, the stability of the analogue complexes in the absence of substrate was compared with that of the regular holoenzyme. As depicted in Figure 5, the complexes of enzyme with the four inactivating analogues, i.e., *e*-carboxylic acid, *e*-methyl ester, and *b*- and *e*-methylamide coenzymes, were much more stable in the absence of substrate than those with the other analogues. Presumably, they are resistant to oxygen inactivation. This result suggests that the carbon–cobalt bond in these complexes is not activated enough in the absence of substrate. This lack of activation appears to be related to their ability to inactivate the enzyme. It should also be noted that the enzyme–coenzyme analogue complexes which are rapidly inactivated in the presence of substrate are stable in the absence of substrate. This suggests that the species which becomes inactive is not the enzyme–coenzyme complex itself, but some species which is derived from the interaction of that complex with the substrate.

It has previously been shown (Wagner et al., 1966) that O_2 inactivation of the adenosylcobalamin–enzyme complex is accompanied by an increase in absorption at 360 nm, which indicates the formation of hydroxocobalamin. A similar

change at 360 nm was seen with the *d*-carboxylic acid analogue. On the other hand, with the *e*-carboxylic acid analogue where no inactivation is seen in the absence of the substrate, no change in absorbance at 360 nm was observed over a 90-min period.

^2H Isotope Effects. When [1,1-^{2}H]-1,2-propanediol is used as a substrate with dioldehydrase, a large deuterium isotope effect is seen, $V_{max}^H/V_{max}^D = 12$. A similar isotope effect was observed with the three carboxylic acid coenzyme analogues. These results indicate that breaking of the substrate carbon–hydrogen bond is rate determining with the coenzyme analogues as well as with the normal coenzyme.

Discussion

Scheme I shows a reaction sequence which has been proposed for dioldehydrase (Abeles & Dolphin, 1976) and ethanolamine ammonia-lyase (Babior, 1975a,b). Salient features of the mechanism are as follows. The interaction between enzyme and coenzyme leads to the breaking of the C–Co bond of the coenzyme to form cob(II)alamin and an adenosyl radical. In the absence of substrate, only a small fraction of the bound coenzyme is dissociated. Addition of substrate to the complex shifts the equilibrium so that a major fraction of the coenzyme is now present in the dissociated form. The adenosyl radical which results from the dissociation of the C–Co bond then abstracts a hydrogen atom from the substrate producing a substrate derived radical and 5′-deoxyadenosine. The substrate-derived radical rearranges to the product radical. For the purposes of this discussion, it is unnecessary to consider the details of this rearrangement. The product radical then acquires a hydrogen atom from 5′-deoxyadenosine. This leads to the formation of the final product and regeneration for the coenzyme.

The experiments described here were carried out in order to determine what role the peripheral amide groups of the corrin ring play in the catalytic process. We prepared nine analogues of the coenzyme in which each of the three peripheral amide groups was chemically modified.

All modified coenzymes showed a lower rate of catalysis than the normal coenzyme. Reaction rates ranged from 86 to 7% that of the normal coenzyme. In all cases, there was a reduction in the steady-state concentrations of the cob(II)alamin intermediate as measured by optical and EPR spectroscopy. The reduction in the concentration of this intermediate was qualitatively correlated with the reduction in catalytic efficiency. Quantitative comparisons are difficult since the different measurements had to be carried out under different experimental conditions. The correlation between the intensity of the EPR signal produced by various modified coenzymes and their catalytic efficiency provides further support for the proposal that radical intermediates are involved in the reaction catalyzed by dioldehydrase. The data also indicate that interaction of each of the peripheral amide groups with the apoprotein contributes to the formation of a catalytically active holoenzyme since modification of any one group leads to reduced catalytic activity.

The data presented in this paper permit some speculation on the nature of the interactions between apoenzyme and each propionamide group of the coenzyme. When modified on the *b*-propionamide group, most of the activity is retained with the carboxylic acid coenzyme, whereas the activity is greatly diminished by conversion to the methyl ester or the methylamide coenzyme. It is reasonable to assume that steric effects are operative near the *b* site since the methyl ester and methylamide groups are bulkier than the carboxyl group. On the other hand, at the *e* site, the *e*-methylamide coenzyme is fairly active although the *e*-carboxylic acid and *e*-methyl ester analogues are very poor coenzymes. Hydrogen bond donation from the coenzyme to the apoenzyme is possible with the methylamide but not with the carboxylate or the methyl ester group. Based on these assumptions, it is suggested that hydrogen bonding is important for the interaction with the apoenzyme at the *e* side chain and that steric factors are more important for the interaction at the *b* side chain.

When the adenosylcobalamin–dioldehydrase complex is incubated in the absence of substrate, the complex is gradually inactivated and adenosylcobalamin is converted to hydroxocobalamin. We attribute this inactivation to reaction of O_2 with the radical species derived from adenosylcobalamin (Scheme I). Several of the coenzyme analogues also show O_2 inactivation. However, the *e*-carboxylic acid, the *e*-methyl ester, and the *e*- and *b*-methylamide analogues show little or possibly no inactivation. The O_2 inactivation with these compounds must be at least 100-fold slower than that observed with adenosylcobalamin. We believe the results indicate that the coenzyme analogues which show no O_2 inactivation undergo much less C–Co bond cleavage in the absence of substrate than adenosylcobalamin. This implies that interaction of the amide side chain of the coenzyme with the protein facilitates C–Co cleavage.

Adenosylcobalamin and the side-chain analogues also differ in the rate of dissociation of the apoenzyme–coenzyme complex. The enzyme–adenosylcobalamin complex as well as the enzyme–cyanocobalamin complex does not dissociate at a detectable rate ($t_{1/2}$ for dissociation > 1 h). Several of the coenzyme analogue–dioldehydrase complexes dissociate with $t_{1/2}$ = 6–15 min. These data again show the importance of protein–side-chain interaction in forming a tight enzyme–coenzyme complex.

All coenzyme analogues with the *e*-NH_2 group modified as well as the *b*-$NHCH_3$ coenzyme form a complex with dioldehydrase which becomes catalytically inactive in the presence of substrate. This inactivation is accompanied by a change in the optical spectrum which indicates a time-dependent increase in the cob(II)alamin species. A similar increase in the concentration of that species is seen in the EPR spectrum. The EPR spectra also show that concomitant with the increase in the cobalt(II) signal the signal due to the organic radical species decreases. We interpret the observations to indicate that inactivation occurs from one of the intermediates in which radical species are present. The inactivation involves quenching of one of the organic radical species present—S·, P·, or the adenosyl radical (Scheme I). Quenching could occur by reactions of the radical species with nearby groups possibly on the protein or the coenzyme. The EPR spectra of the inactivated systems are quite different from that of the catalytically active system, suggesting that the environment of cobalt(II) of the inactivated complexes is different from that of the normal intermediate. Probably, due to the lack of the organic radical partner, the coupling of the unpaired electron on the cobalt(II) with the organic radical disappears, resulting in a narrower and simpler low-field EPR signal. The *g* value of a prominent resonance of the signal is 2.23–2.24 which is very close to that of free cob(II)alamin. These observations are consistent with the explanation by Schepler et al. (1975) of the coupled EPR spectra observed in adenosylcobalamin-dependent rearrangements.

It is interesting to note that the same modifications of the coenzyme which result in insensitivity to O_2 in the absence of substrate also lead to rapid inactivation of the holoenzyme in the presence of substrate. It seems likely that these two

phenomena have a common basis, i.e., improper interaction between coenzyme and apoenzyme due to modification of the propionamide side chain.

It is striking that even slight modification of the large cobalamin molecule on one of the peripheral side chains of the corrin ring brings about such a dramatic change in coenzyme activity and in kinetic and binding properties. Studies have also been carried out in which modifications have been introduced in the adenosyl moiety of the coenzyme. These changes also lead to decreased catalytic efficiency and, in some cases, to lack of oxygen sensitivity (Toraya et al., 1977; Kerwar et al., 1970). It is unlikely that modification of the propionamide side chain alters the intrinsic chemical reactivity of the C–Co bond. Thus the effects on the catalytic properties of the coenzyme resulting from the modifications of the propionamide side chain are therefore not due to a change in mechanism but are most likely due to effects on the coenzyme–protein interaction resulting in a decrease in the concentrations of the reactive intermediates.

Evidence that a similar mechanism is involved with the normal coenzyme and the modified coenzymes is provided by the deuterium isotope effect observed when a substrate deuterated at C-1 is used. This isotope effect is of the same magnitude with both forms of the coenzyme.

The interpretations of the results which we have obtained rest on the assumption that the chemical transformations which were carried out did not result in modification of the corrin ring. Past experience with the chemical procedures used here suggests that this is not the case. We prepared the monocarboxylic acid derivatives by acid hydrolysis of cyanocobalamin and converted them to the methyl ester and methylamide analogues by the mixed anhydride method and the carbodiimide method, respectively. Armitage et al. (1953) have reported the conversion of monocarboxylic acid derivatives to cyanocobalamin by the mixed anhydride method. Yamada and Hogenkamp (1972) have shown that no products are formed by reaction of cyanocobalamin with diaminododecane in the presence of the carbodiimide. Thus, it seems unlikely that other parts of the cobalamin molecule than the side chain are modified by acid hydrolysis, the mixed anhydride reaction, or the carbodiimide reaction.

We interpret the results obtained here and by others to indicate that there are very specific interactions between the coenzyme and the apoenzyme which lead to a very "tight" or "rigid" complex. These interactions involve many of the functional groups of the coenzyme such as the propionamide side chains, the adenosyl moiety, the benzimidazole group, and probably also other functional groups. Formation of such a tight complex may facilitate several aspects of the catalytic process. The interaction of coenzyme and apoenzyme could bring about distortion of the corrin system which facilitates C–Co bond cleavage. These interactions could also greatly restrict the mobility of radical intermediates so that they cannot interact with other groups in the immediate environment. The modified coenzymes form a much "looser" complex. This is apparent from the fact that the modified coenzymes dissociate from the apoenzyme much more readily than the normal coenzyme. Formation of a "looser" complex makes the breaking of the C–Co bond energetically less favorable, resulting in a lower steady-state concentration of the dissociated form of the coenzyme, in lower catalytic activity, and, in some cases, in the absence of O_2 sensitivity of the enzyme–coenzyme complex. In those complexes in which no O_2 sensitivity is seen, inactivation occurs when the substrate is present, presumably because the intermediate radical species which ultimately form are less restricted and therefore subject to quenching by irrelevant side reactions with nearby groups.

Acknowledgments

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