Synthesis and Catalytic Activity of Spin-Labeled Cobinamide Coenzymes*

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ABSTRACT: In order to facilitate the study of the mechanism of action of corrin-dependent enzymes, the preparation of active spin-labeled cobinamides was undertaken. The synthesis of these compounds has been accomplished by the coordination of a piperidine-*N*-oxyl stable free radical to the cobalt atom of cobinamides through the oxygen atom of the *N*-oxyl functional group. By this procedure spin-labeled derivatives of methylcobinamide (MCN) and 5′-deoxyadenosylcobinamide (DACN) are obtained in good yield. The synthesis of DACN was accomplished as a result of our development of a new procedure for the synthesis of 5′-deoxyadensylcobinamide (DAC) in good yields. The structure of these derivatives has been rigorously defined by 220-MHz proton nuclear magnetic resonance, by electron spin resonance, by circular dichroism, and by the determination of pK_a values for displacement of the nitroxide ligand. Dissociation constants for these coordination complexes have been determined for the pH at which enzyme assays were performed (*i.e.*, 7.5). In the enzymatic experiments 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl 5′-deoxyadensylcobinamide (DACN) was found to replace 5′-deoxyadenosylcobalamin (DMBC) in ethanolamine ammonia-lyase. Electron spin resonance has been used to demonstrate that homolytic cleavage of the Co–C σ bond occurs when ethanolamine is added to the spin-labeled enzyme–DACN complex. Control experiments involving photolysis of the spin-labeled corrinoid have been performed to help confirm this mechanism. Homolysis of the Co–C σ bond also occurs when acetaldehyde and ammonium ions are added to the enzyme–DACN complex. This experiment suggests that partial reversibility of this deamination reaction can take place.

Buckman *et al.* (1969) reported the synthesis of both covalent and coordination complexes of piperidine-*N*-oxyl derivatives with corrinoids. The syntheses of spin-labeled corrinoids are of great potential interest because of the possibilities which these molecules offer in the study of corrin-dependent enzymes. Our synthesis of a new group of alkylcobinamides to which a spin label is attached provides the first opportunity to determine the mechanism of enzymatic Co–C σ bond cleavage by using catalytic amounts of enzyme under normal assay conditions. Experiments with ethanolamine–ammonia-lyase and ribonucleotide reductase suggest substrate-dependent homolytic cleavate of the Co–C bond (Babior and Gould, 1969; Hamilton *et al.*, 1969). However, these experiments rely on substrate-dependent appearance of a low-spin Co^{II} esr signal. For these experiments large amounts of enzymes are required and the esr spectra are recorded at 77°K. Although the appearance of a low-spin Co^{II} signal qualitatively suggests a homolysis mechanism, this approach makes kinetic measurements difficult and quantitation of this reaction virtually impossible. The use of DACN[1] in place of DMBC affords the opportunity to use nitroxide esr to quantitatively monitor the valency of the cobalt atom during catalysis. Scheme I demonstrates the electronic state of the cobalt atom of DACN if addition of substrate leads to Co–C bond cleavage, either homolytic or heterolytic.

In this communication the synthesis of an active spin-labeled 5′-deoxyadenosylcobinamide analog has permitted evidence to be obtained concerning the mechanism of action of ethanolamine–ammonia-lyase.

Materials and Methods

Enzyme Assays. Ethanolamine–ammonia-lyase was assayed by the method of Kaplan and Stadtman (1968). K_M values of DMBC, DAC, and DACN were determined by adding variable amounts of each coenzyme to reaction mixtures containing 0.1 μmole of NADH, 10 μmoles of ethanolamine at pH 7.5, and 5 units of yeast alcohol dehydrogenase, in 1.0 ml of 0.05 M potassium phosphate buffer (pH 7.5). Reaction rates were determined at 340 nm upon the addition of 3 μg of pure ethanolamine–ammonia-lyase apoenzyme.

For the esr experiments 7.6 mg of ethanolamine–ammonia-lyase apoenzyme was reconstituted with 20 μl of DACN (1.4 μmoles/ml). These conditions theoretically fill every B_{12} binding site on the apoenzyme (Babior and Li, 1969). To ensure binding, this mixture was stirred for 20 min at 4°. This reconstituted holoenzyme was divided into equal aliquots. One aliquot was used to determine the maximum intensity of the nitroxide esr spectrum, and the second aliquot was used for enzymatic experiments using different concentrations of ethanolamine or deuterated ethanolamine (C_1 deuterated) as

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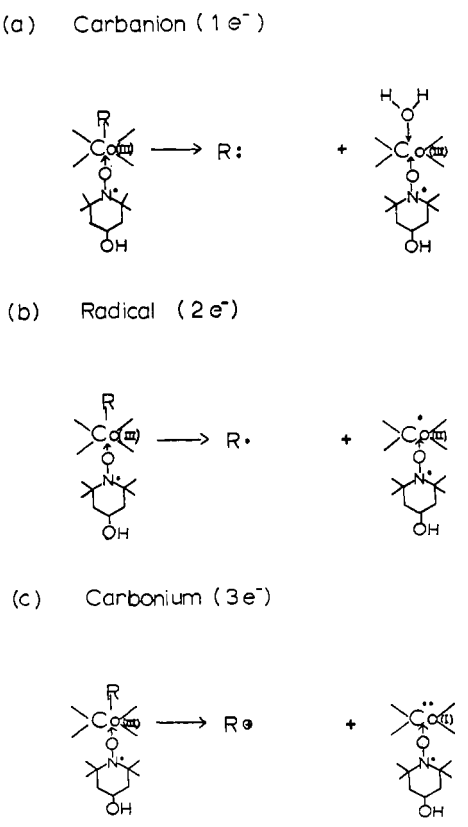
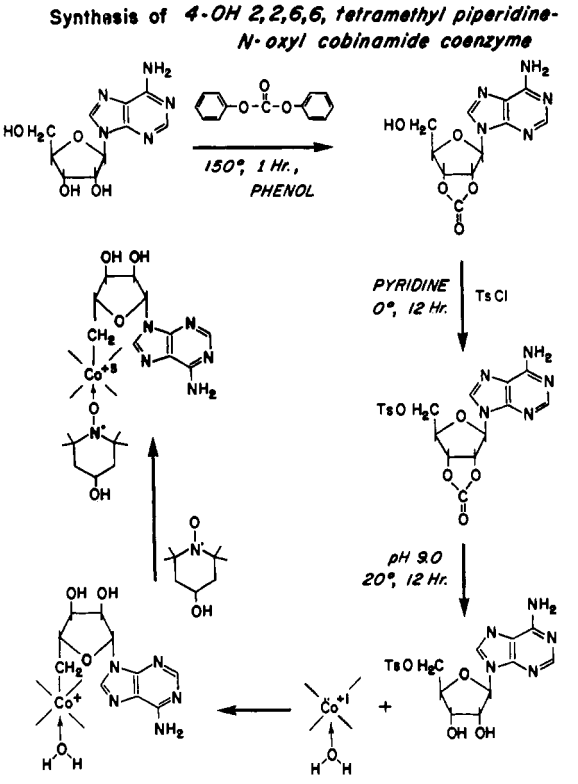
[1] Abbreviations used are: DMBC, 5′-deoxyadenosylcobalamin; DAC, 5′-deoxyadenosylcobinamide; DACN, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl 5′-deoxyadenosylcobinamide; MCN, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl methylcobinamide; MC, methylcobinamide.

SCHEME I



SCHEME II



substrates. Other experiments were performed using similar holoenzyme preparations except that the reaction products acetaldehyde and ammonia were added instead of ethanolamine.

Removal of DACN from the Ethanolamine Ammonia-lyase–DACN Complex. Ethanolamine–ammonia-lyase binds DMBC only weakly (Stadtman, 1971), and DACN was shown to be easily removed from the holoenzyme with other proteins which bind corrinoids. In order to determine whether 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl remains coordinated to DAC during catalysis it is necessary to remove DACN from the holoenzyme after conversion of the substrate into product. Since acetaldehyde was shown to cause homolysis of the Co–C bond in the enzyme–DACN complex it was also necessary to remove residual acetaldehyde from the vicinity of the active site. This was accomplished by the addition of alcohol dehydrogenase and NADH. Equilibrium dialysis experiments with yeast alcohol dehydrogenase shows that this enzyme binds 40 moles of either DMBC or DACN per mole of enzyme. Bovine serum albumin possesses analogous B_{12} binding properties (Taylor and Hanna, 1970). Consequently, the addition of alcohol dehydrogenase and NADH not only removed the residual acetaldehyde from the active site, but also removed DACN from the holoenzyme. Under normal spectrophotometric assay conditions scavenging of the enzyme–DACN complex is not evident since reactions are always performed in the presence of a large excess of coenzyme.

Chemical and Analytical Methods. The procedure used for the synthesis of DACN is outlined in Scheme II. The synthesis of intermediates was performed as follows.

SYNTHESIS OF 5′-TOSYLADENOSINE. Adenosine 2′:3′-cyclic carbonate (2.0 g; 0.647 mmole) was dissolved in 10 ml of anhydrous pyridine and cooled to 0°. Then 1.8 g (0.874 mmole) of *p*-toluenesulfonyl chloride was added. The mixture was shaken until all solids were dissolved. The resulting reddish solution was left at 0° overnight. Then the mixture was added to 400 ml of anhydrous diethyl ether. The ether was decanted. Adenosine 2′:3′-cyclic carbonate was washed with several aliquots of ether to remove remaining pyridine. The precipitate was recrystallized from ethanol–ether before being subjected to nmr analysis. 5′-Tosyladenosine 2′:3′-cyclic carbonate (2 g) was dissolved in 50 ml of water–pyridine (4:1, v/v), and the pH was raised to 9.0 with 1 N NaOH. Complete hydrolysis of the carbonate occurred upon standing overnight at room temperature. This hydrolysate turned yellow when the reaction was complete. The resulting 5′-tosyladenosine was left in solution and kept at 4° for further use.

SYNTHESIS OF DAC. MC was synthesized by the method outlined by Penley *et al.* (1970). Diaquocobinamide was synthesized by aerobic photolysis of MC. Diaquocobinamide (200 mg) was dissolved in 50 ml of water. This solution was degassed with argon and reduced with 100 mg of sodium borohydride. A tenfold excess of 5′-tosyladenosine was allowed to react with reduced cobinamide for 4 hr. The resulting DAC was extracted with phenol by the method of Johnson *et al.* (1963), and purified by chromatography on phosphocellulose which was generated by the method of Lezius and Barker (1965). Yields of 40% were routinely obtained.

SYNTHESIS OF DACN. A 20-fold excess of 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl was added to 50 mg of DAC dissolved in ethanol (4.0 ml), and ethyl orthoformate (1.0 ml) was added as a dehydrating agent. This mixture was stirred in the dark for 3 days at 4° to ensure coordination of the spin label. Dehydration to give 5-coordinate cobalt is a slow reaction, and so the formation of the spin-labeled complex is

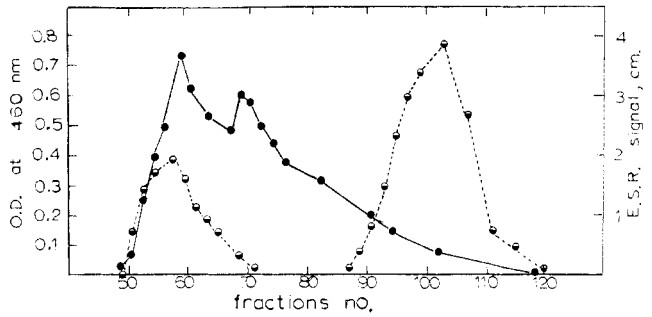


FIGURE 1: Elution of MCN from Sephadex G-10. (●) OD at 460 nm. (◒) Intensity of nitroxide esr signal. Fractions 50–60 = MCN, fractions 65–75 = MC, and fractions 90–110 = 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl.

TABLE I: $R_{B_{12}}$ of Derivatives.[a]

R_F of Cyanocobalamin:	I 0.417	II 0.372	III 0.445	IV 0.526
		$R_{B_{12}}$		
CN	1.00	1.00	1.00	1.00
DMBC	0.86	0.85	0.95	0.91
DAC	0.95	1.03	0.97	0.91
DACN	0.93	1.01	0.93	0.91
MC	1.19	1.42	1.25	1.16
MCN	1.12	1.38	1.11	1.16

[a] For solvent, see text.

slow. After 3 days this solution was lyophilized and the excess uncoordinated nitroxide was extracted with ether until the ether layer was colorless. This procedure was followed by Soxhlet solid–liquid ether extraction for 48 hr to ensure removal of all uncoordinated nitroxide.

SYNTHESIS OF MCN. MCN was prepared by a procedure identical with DACN except that MC was used in place of DAC.

DISSOCIATION CONSTANTS FOR DACN AND MCN. Dissociation constants DACN and MCN were determined by chromatography on Sephadex G-10. A 2.27×10^{-3} M solution of DACN (0.5 ml) was taken after the ethyl orthoformate step and washed with ether. This solution was then applied to a 1.5×22 cm Sephadex G-10 column which had been equilibrated with 0.05 M potassium phosphate buffer (pH 7.4). By this procedure DACN and MCN are readily separated from unreacted DAC, MC, and free 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl. The concentration of coordinated nitroxide was determined by the intensity of the nitroxide esr signal, and the concentration of cobinamide was determined at 460 nm. Figure 1 shows a typical elution profile for this separation procedure. Both DACN and MCN represent 1:1 complexes between spin label and corresponding cobinamide. Dissociation constants for these coordination complexes were MCN (K_D 4×10^{-6} M) and DACN (K_D 2×10^{-5} M).

Chromatographic Methods. Cobinamides were purified on phosphocellulose columns by the following procedure. Lyophilized product (200 mg) from phenol extraction was dissolved in a minimal amount of 0.01 M acetic acid and applied to a 30×120 mm phosphocellulose column which had been previously equilibrated with 0.025 M acetate buffer (pH 4.1). Alkylcobinamides were separated from other more polar contaminants by elution with 0.01 M acetic acid followed by 0.025 M sodium acetate buffer (pH 4.1). Each eluted band was reextracted with phenol and lyophilized. The purity of all products was ascertained by thin-layer chromatography.

Thin-Layer Chromatography. Chromatograms were run on Eastman chromagram cellulose sheets (6065 cellulose) in the following solvent systems: solvent I, *sec*-butyl alcohol–H_2O (9.5:4, v/v); solvent II, *sec*-butyl alcohol–HOAc–H_2O (100:1:50, v/v); solvent III, *sec*-butyl alcohol–0.88 M ammonia–H_2O (9.5:0.68:4, v/v); solvent IV, *n*-butyl alcohol–HOAc–H_2O (4:1:5, v/v, upper layer).

The R_F values of derivatives were expressed in Table I in terms of $R_{B_{12}}$ as defined by Firth *et al.* (1968). The R_F value of cyanocobalamin is given as a reference in each case. In each solvent except the basic solvent (III), the nitroxide ligand is displaced from DACN and MCN, respectively, to give DAC and MC.

Spectroscopic Methods. Uv–visible spectra of derivatives were recorded with a Beckman DB-G spectrophotometer. Molar extinction coefficients of derivatives and other spectrophotometric measurements were done with a Carl Zeiss PMQ II spectrophotometer. Circular dichroism experiments were conducted on a JASCO ORD, uv, CD 5 spectrophotometer.

^{1}H nmr spectra of adenosine 2′:3′-cyclic carbonate and 5′-tosyladenosine were recorded with a Varian A-60. ^{1}H nmr spectra of B_{12} derivatives were recorded with a Varian HR-220 operating at 220 MHz.

Esr studies at 25° were done with a quartz liquid sample cell on a Varian V-4502 epr spectrometer operating at X band.

Materials. Adenosine 2′:3′-cyclic carbonate was prepared by the method of Hampton and Nichols (1966). 4-Hydroxy-2,2,6,6,-tetramethylpiperidine-*N*-oxyl was prepared by the method of Buckman *et al.* (1969).

Cyanocobalamin was obtained from Sigma, St. Louis, Mo. Diphenyl carbonate was obtained from Aldrich Chemical Co. *p*-Toluenesulfonyl chloride was vacuum distilled and recrystallized from benzene–hexane. Pyridine was distilled over BaO and stored over KOH. Ethanolamine–ammonia-lyase was prepared by the method of Kaplan and Stadtman (1968).

Results

Characterization and Properties of DACN and MCN. The uv–visible spectra of DAC and MC are identical with those reported by Bernhauer and Müller (1961). These spectra are similar to those of DACN and MCN, respectively. However, the spectra of the spin-labeled coenzymes display pH-dependent changes which are not observed in the spectra of cobinamides. At pH 7.0 MCN has a λ_{max} at 310 nm which shifts to 301 nm upon acidification to pH 2.0. Also, DACN has λ_{max} values at 260 and 267 nm at pH 2.0 which shift to 265 nm at pH 7.0. The above spectral changes were used to determine pK_a values for the displacement of the nitroxide from the sixth coordination position of the cobalt atom (Hogenkamp *et al.*, 1965). For MCN the pK_a for displacement of nitroxide was 4.9, and for DACN, 6.3.

Molar extinction coefficients of these spin-labeled derivatives were determined in 0.05 M phosphate buffer (pH 7.0). Exhaustive photolysis of DACN and MCN was used since reference to the extinction coefficient of the aquocobinamide product could be made. These molar extinction coefficients are

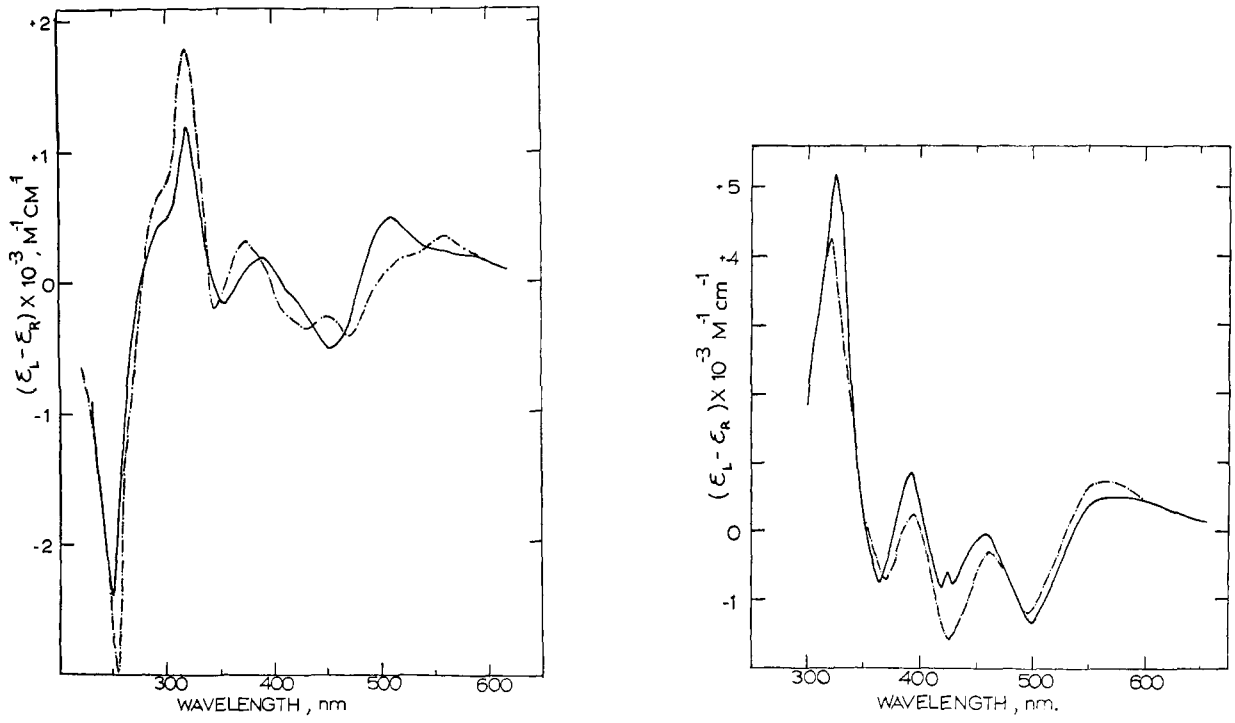


FIGURE 2: CD spectra of (a, left) MCN (—) and MC + 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (- - -) at 3×10^{-5} M in ethanol and (b, right) DACN (—) and DAC + 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (- - -) at 2×10^{-4} M in ethanol.

determined at each λ_{max} and compared to those of DMBC (Table II).

The CD spectra of DACN and MCN were compared to those of solutions of equal concentrations of DAC and MC to which an equimolar concentration of 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl was added just prior to running the spectra. A marked difference is observed for the CD spectra of the coordination complexes MCN and DACN compared to the spectra of the dissociated complexes MC and DAC plus free nitroxide (Figure 2).

Nmr Studies. Nmr was used to confirm the structure of intermediates in the synthesis of DACN. Both adenosine 2′:3′-cyclic carbonate and 5′-tosyladenosine 2′:3′-cyclic carbonate were hygroscopic. Adenosine 2′:3′-cyclic carbonate recrystallized from ethanol, and 5′-tosyladenosine 2′:3′-cyclic carbonate was recrystallized from ethanol–ether and both were kept in a vacuum desiccator over KOH pellets. The chemical shifts of these two intermediates in D_2O are expressed in Table III using sodium 3-(trimethylsilyl)-1-propanesulfonate as an internal reference.

Assignments of δ values for $C_6NH_2C_1'HC_5'H$ are straightforward. Assignments of $C_3''H$ and $C_5''H$ *vs.* $C_2''H$ and $C_6''H$ on 5′-tosyladenosine 2′:3′-cyclic carbonate are arbitrary. The C_2H and C_8H on adenine are distinguishable by exchanging the C_8H with D_2O (Shelton and Clark, 1967). A spectrum of adenosine 2′:3′-cyclic carbonate recorded in Me_2SO-d_6 resulted in the disappearance of the signal at 8.29. The assignment of $C_2'H$ and $C_3'H$ was done by resolving the multiplets from 4.0 to 4.6 with a Varian HA-100 spectrom-

TABLE II: Molar Extinction Coefficients $\times 10^3$ in 0.05 M Phosphate Buffer (pH 7.0).

Max. (nm)	DMBC	DAC	DACN	MC	MCN
260	32.2				
265		34.1	34.0	23.8	28.1
306		20.2	20.3		
310				22.9	23.1
345	12.1				
358	11.8				
380	8.67	8.25	8.12	9.5	9.5
460		8.63	8.65	11.1	11.5
525	7.34				

TABLE III: Nmr Assignments for Adenosine 2′:3′-Cyclic Carbonate and 5′-Tosyladenosine 5′:3′-Cyclic Carbonate.

	Chemical Shifts (δ)	
	Adenosine 2′:3′-Cyclic Carbonate	5′-Tosyladenosine 2′:3′-Cyclic Carbonate
C_6NH_2	7.28[a]	
C_2H	8.06	8.31
C_8H	8.29	8.45
$C_1'H$	6.06	6.11
$C_2'H$	4.58	4.50
$C_3'H$	4.42	4.31
$C_4'H$	4.61[a]	
$C_5'H$	4.02	3.95
$C_4''CH_3$		2.33
$C_3''H$ and $C_5''H$		7.23
$C_2''H$ and $C_6''H$		7.63

[a] In Me_2SO-d_6 with Me_2SO as reference.

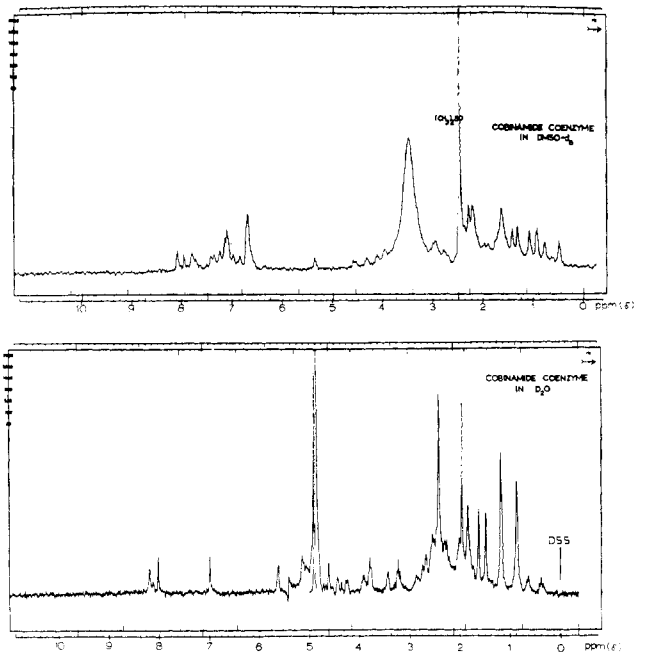


FIGURE 3: 220-MHz ^1H nmr spectra of DAC in (a, top) Me_2SO-d_6 and (b, bottom) D_2O.

eter. This spectrum yielded a triplet and a quartet. The triplet has J values of 3.6 and 5 Hz at low and high field, respectively. The quartet resembles two doublets having J values = 3.5 and 3.0 Hz between them. Since $C_2'H$ is trans to $C_1'H$, it should have a J value less than that of $C_1'H$, which is 5.5 Hz. $C_4'H$ should be a multiplet, and $C_3'H$ a quartet. When a spectrum was run in Me_2SO-d_6 a multiplet was observed at 4.61. Therefore, $C_4'H$ must be obscured by the contaminating water in D_2O.

Figure 3 shows a 220-MHz nmr spectrum of DAC in Me_2SO-d_6. The vinyl region is complicated with many amide protons. When the spectrum was recorded in D_2O (Figure 3) all but the resonances at 5.63, 7.00, 8,00, and 8.18 disappear. When these spectra are compared to that of MC in D_2O, where it is observed that only the vinyl proton at 7.0 (C_{10}-corrin ring proton) is present, it is clear that the resonances at 8.00 and 8.18 must be the C_2H and the C_8H of the adenine moiety of DAC (Penley *et al.*, 1970). It is observed that the $C_1'H$ of ribose is shifted upfield. This proton has not yet been assigned, but its upfield shift could be because of its proximity to the

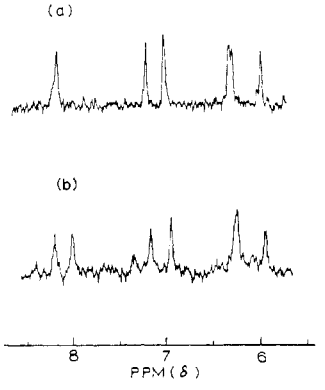


FIGURE 4: 220-MHz ^1H nmr spectra of DMBC in D_2O (a) with adenine C_8 deuterated (b) with adenine C protonated.

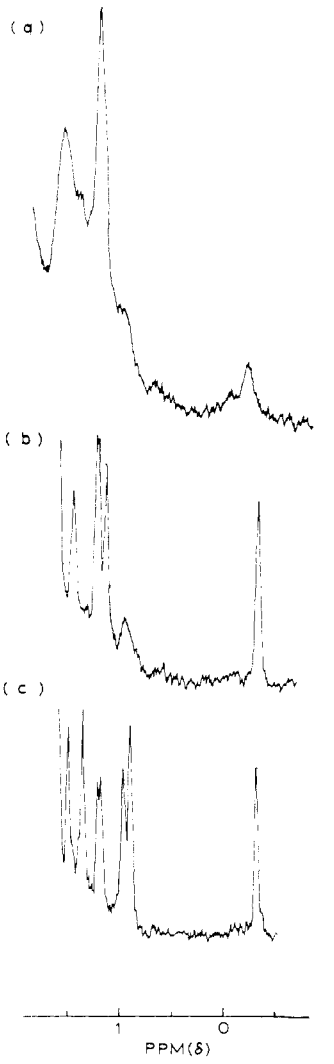
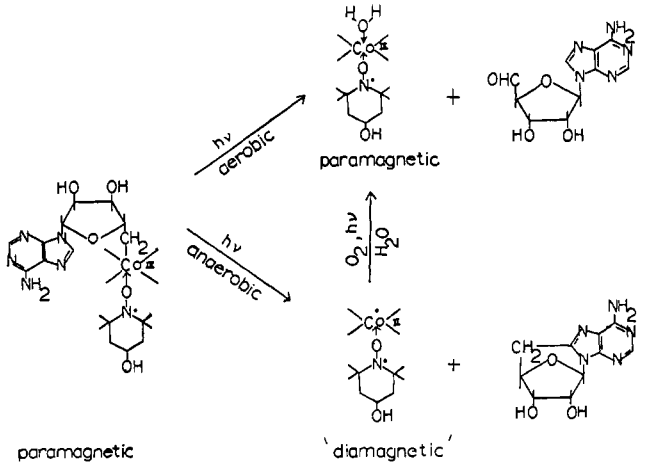


FIGURE 5: 220-MHz ^1H nmr spectra in D_2O of (a) MCN, (b) MC, and (c) methylcobalamin at pH 1.2.

adenine ring. This proton could only be influenced by this aromatic ring current if the purine lies perpendicular to the corrin ring. If the adenine moiety lies parallel to the corrin ring, as is observed in the crystalline form of DMBC (Lenhert and Hodgkin, 1961), then this $C_1'H$ of ribose would not be influenced and should not be shifted. This observation suggests that a significant conformational change occurs in corrinoid coenzymes when they are dissolved in water, and the movement of the adenine from a horizontal to a perpendicular position uncovers the 5′-CH_2 protons of the deoxyadenosyl moiety. Further proof for this conformational change is demonstrated by our assignment of the C_2H and C_8H of the adenine moiety of DMBC. We have synthesized C_8D DMBC by using the method described by Shelton and Clark (1967), and this deuterated DMBC allows the designation of C_8 at 8.00 and C_2 at 8.18 (Figure 4). The positions of these two resonances are reversed in free adenosine.

A comparison of the 220-MHz spectra of DMBC and DAC shows that a single resonance at 0.4 in DMBC is replaced by two resonances at 0.38 and 0.63 in DAC. These resonances show that the nonequivalence of the 5′-CH_2 protons of the deoxyadenosyl moiety is attributed to incomplete environmental averaging despite fast rotation about the Co–C bond. The deoxyadenosyl moiety restricts the rotation which con-

SCHEME III



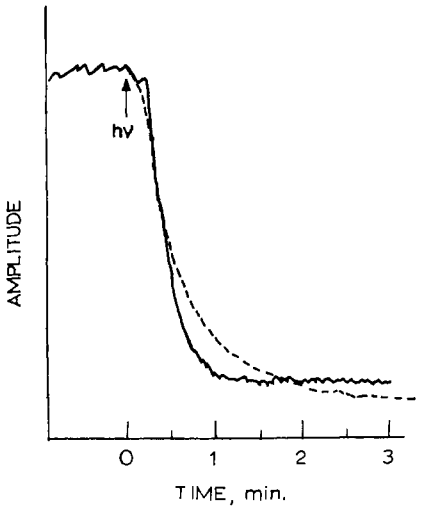


FIGURE 6: Kinetics of homolysis of DACN (——) and MCN (---) 10^{-3} M when subjected to anaerobic photolysis at 23°.

sequently leads to a fixed rotomer configuration for this 5′-CH_2 group. When a spectrum of DAC was run in Me_2SO-d_6, the water signal is observed to be much broader than that in D_2O. This broad signal is due to the water molecule coordinated to the cobalt exchanging very slowly (Figure 3a).

Nmr 220-MHz studies with DACN and MCN pose difficulties because these coordination complexes are paramagnetic. However, good resolution for the methyl group on the cobalt atom of MCN was obtained. This methyl resonance is both contact shifted and broadened by the nitroxide (Figure 5). These data prove that the spin label is coordinated to the cobalt atom. Nmr 220-MHz experiments in which free nitroxide was mixed with methylcobinamide immediately prior to running the spectrum broadened this methyl resonance, but did not contact shift it downfield.

Esr Studies. The esr spectra of DACN and MCN are similar to that of the uncoordinated nitroxide. However, the lines are broadened in the coordinated derivatives. When the nitroxide is coordinated to MC the line is broadened from 1.3 to 1.8 G. In the case of DAC the line is broadened from 1.3 to 2.0 G. Hyperfine structure (hfs), due to coupling of the unpaired electron with protons of the four methyl groups, could not be resolved in the coordinate nitroxide complexes. But, for the free nitroxide hfs of 0.4 G for these protons could be resolved. When an aqueous solution of either DACN or MCN was photolysed aerobically there was virtually no change in the esr spectra. However, when a deoxygenated solution was photolyzed, the esr spectrum disappeared. The disappearance of the esr signal can be conveniently monitored by setting the magnetic field at the maximum of the center field line, illuminating the esr cavity, and following the decrease in the intensity of the signal with time (Figure 6). The experimental conditions used for this procedure were identical with those reported previously by Buckman *et al.* (1969). When the photolyzed solution was exposed to air, 60% of the original signal returned for photolysis experiments with DACN. With MCN a 100% recovery of the signal was routinely obtained when the photolysis reaction products were oxidized. Photolysis of MCN under anerobic conditions gave a mixture of methane and ethane and a corrinoid species with either zero or two unpaired electrons. In the case of DACN the anerobic photolysis products were 8:5′-cyclic adenosine and a corrinoid with zero or two unpaired electrons (Hogenkamp, 1964; Pratt, 1964) (Scheme III).

Clearly, the nitroxide esr signal disappears due to interaction of the unpaired spins. This probably results from a weak exchange interaction between the spins which alters the relaxation time to such an extent that the esr signal is broadened enough to escape detection. The alternative possibility, pairing of the electron on the cobalt with the one on the nitroxyl radical to form a covalent bond, is unlikely because of the symmetries of the orbitals of each of the two unpaired electrons (Kikuchi, 1969; Schrauzer and Lee, 1968).

During the anaerobic photolysis experiments the nitroxide remains coordinated to the Co^{II} atom, because no hyperfine coupling to methyl protons can be resolved when the amplitude and line width of the nitroxide esr signal is restored upon reoxidation of the Co^{II} species.

Since it was observed that only 60% of the N esr signal was restored upon oxidation of the anaerobic photolysis product of DACN, a spectrum was recorded at 77°K in order to ascertain whether low-spin Co^{II} was present in the photolysis reaction mixture. Exhaustive anaerobic photolysis of DACN gave a low-spin Co^{II} signal which was very similar to that reported for B_{12r} (Hogenkamp *et al.*, 1963) (Figure 7). A comparison of this signal to the signal obtained by anaerobic photolysis of an identical concentration of DAC showed that only 46% of the product was Co^{II}; therefore, the remaining 54% was diamagnetic. These data suggest that the 40% loss of the nitroxide esr signal occurs because under the conditions of photolysis a certain amount of irreversible reduction of the nitroxide takes place. This conclusion is supported by the

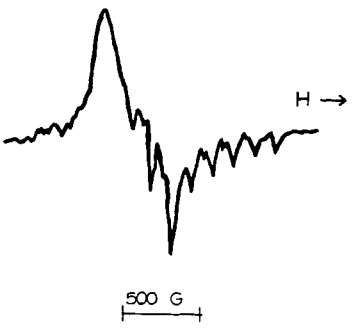


FIGURE 7: Co^{II} esr spectrum of anaerobic photolysed DACN (10^{-3} M) at 77°K.

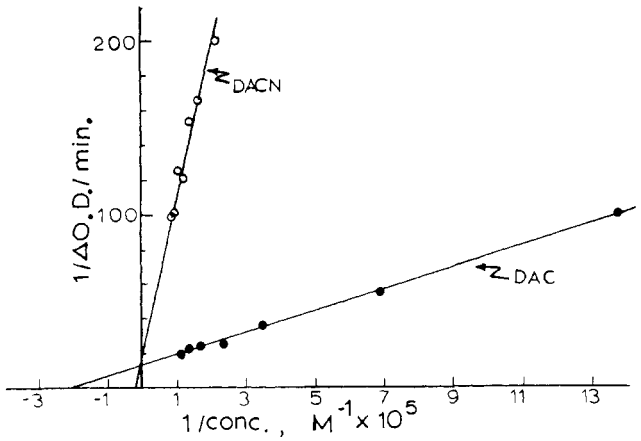


FIGURE 8: Lineweaver–Burk plot for ethanolamine–ammonia-lyase using DAC and DACN, respectively, as coenzymes.

observation that when methyl cobalamin is photolyzed in the presence of 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl the nitroxide esr signal disappears almost completely, but in contrast to the results obtained with MCN this esr signal cannot be restored upon oxidation of the resulting cob(II)alamin (B_{12r}). These differences in the quantitative recovery of the nitroxide esr spectra in MCN and DACN probably reflect the stability of nitroxide coordination at pH 7.5 in MCN compared to the weaker DACN complex.

Reactions with Ethanolamine–Ammonia-lyase. When DAC and DACN were used as coenzymes with ethanolamine–ammonia-lyase both of these corrinoids have the same V_{max}, but exhibit K_m values of 4.5×10^{-6} and 5.1×10^{-5} M, respectively (Figure 8). Neither of these coenzymes function as efficiently as DMBC which has a K_m of 1.5×10^{-6} M (Babior and Li, 1969).

When ethanolamine–ammonia-lyase apoenzyme was reconstituted with DACN to give active holoenzyme, and the esr signal examined, it was found that the nitroxide signal was broadened in the DACN–enzyme complex compared with the free DACN (*i.e.*, 2.2–2.0 G, respectively). The addition of 10 μmoles of ethanolamine to 3.8 mg of DACN–enzyme complex caused greater than 90% loss of the nitroxide esr spectrum. When this reaction had progressed to completion, a slow reappearance of the nitroxide esr signal occurred, but complete recovery to the original intensity did not occur in 30 min. The disappearance of the nitroxide esr signal observed upon the addition of substrate was so rapid that it was impossible to make kinetic measurements for this reaction. However, the use of deuterated ethanolamine (D_1) as a substrate with a less active enzyme preparation allows us to measure the kinetics of this reaction (Figure 9). These data show a rapid disappearance of the nitroxide esr spectrum which is dependent on the addition of deuterated ethanolamine. Also, the decrease in the intensity of this esr signal is dependent on the concentration of DACN added to the reaction mixture.

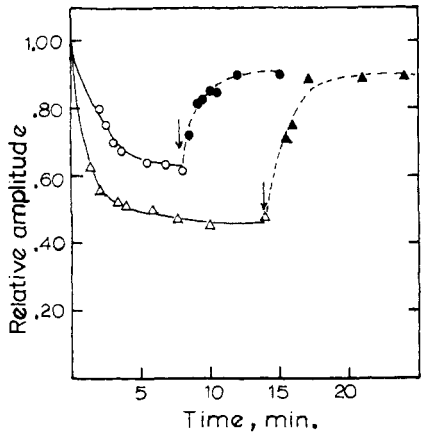


FIGURE 9: Decrease in the intensity of the nitroxide esr signal upon addition of deuterated ethanolamine to DACN–ethanolamine–ammonia-lyase. Ethanolamine–ammonia-lyase (3.8 mg) was used in each of these experiments and holoenzyme was reconstituted with 2.0 moles of DACN/DACN binding site (Δ) and 2.75 moles of DACN/DACN binding site (○). Reactions were started by adding 100 μmoles of deuterated ethanolamine. DACN was removed from the DACN–enzyme complex with alcohol dehydrogenase (5 units) as described in Methods (↓).

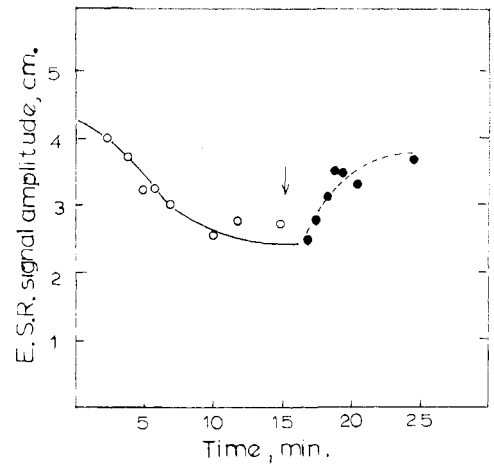


FIGURE 10: Decrease in the intensity of the nitroxide esr signal upon addition of acetaldehyde (100 μmoles) and ammonia (100 μmoles) to 3.8 mg of a 1:1 DACN–enzyme complex. Some homolysis occurs, but the DACN can be removed from the DACN–enzyme complex upon the addition of alcohol dehydrogenase (5 units) (↓).

Removal of DACN from ethanolamine–ammonia-lyase (Methods) gives the quantitative reappearance of the nitroxide esr signal (Figure 9). This experiment proves that homolysis of the Co–C σ bond occurs upon the addition of ethanolamine to the enzyme, and control experiments show that the total recovery of the nitroxide esr signal is dependent on the nitroxide remaining coordinated to the Co^{2+} species during catalysis. Control experiments were performed in which ethanolamine–ammonia-lyase apoenzyme was reconstituted with DMBC, and when this holoenzyme catalyzed the deamination of ethanolamine in the presence of free 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl, no change in the nitroxide esr spectrum was observed, and no inhibition of this reaction occurred with free nitroxide. When acetaldehyde and ammonium ions were added to the DACN–enzyme complex, slow partial homolysis of the Co–C bond occurred, indicating some reversibility for this reaction (Figure 10). Removal of DACN from the DACN–enzyme complex caused the nitroxide esr signal to return to its original intensity.

Discussion

The synthesis of active spin-labeled corrin coenzymes provides a unique opportunity to study the valency of the cobalt atom during catalysis in corrin-dependent enzyme systems. The use of nitroxide esr allows these reactions to be studied

in very dilute solutions under the normal experimental conditions similar to those which would be used for a spectrophotometric assay. If homolytic cleavage occurs on the addition of substrate to the corrin–enzyme, then the nitroxide esr signal disappears during catalysis, but reappears once again when the substrate is utilized and when the product is removed from the vicinity of the active site. If heterolytic cleavage occurs to give a carbanion and a Co^{III} corrin then no change is observed in the nitroxide esr signal. In the case of heterolytic cleavage to give a carbonium ion and a Co^{I} corrin, the nitroxide is displaced as a ligand from the cobalt (Brodie and Poe, 1971) and the free nitroxide is reduced by the Co^{I} corrin causing irreversible loss of the nitroxide esr signal. We have shown that in ethanolamine–ammonia-lyase homolysis of the Co–C bond occurs on the addition of ethanolamine. The fact that the signal disappears almost completely indicates that almost all of the enzyme is participating in the process leading to homolysis. However, it cannot be decided from these experiments whether the extensive loss of signal is due to quantitative homolysis of the C–Co bond or to a rapid equilibration between [enzyme–intact DACN] and [enzyme–dissociated DACN] complexes. This experiment confirms the observation of Babior and Gould (1969), and proves that this intramolecular hydrogen-transfer reaction involves a radical mechanism as was suggest initially by Eggerer *et al.* (1960). Our experimental data provide support for the radical mechanism previously proposed for ethanolamine–ammonia-lyase (Babior, 1970).

The structure of DACN was proved by uv–visible spectra, CD, nmr, esr, and by pK_a and K_D determinations for the displacement of the nitroxide. Our nmr study of DAC in D_2O and Me_2SO-d_6 provides useful information on the conformation of this coenzyme in solution. The spectrum in Me_2SO-d_6 shows that the water is coordinated in the sixth coordination position on the cobalt atom, and this water exchanges very slowly in this solvent, Also, we have been able to assign the 5′-CH_2 protons, $C_{10}H$ of the corrin ring, and C_2- and C_8H of the adenine moiety. The upfield shift of the C_1′-ribose proton probably takes place because in solution this proton comes under the influence of the ring current from the adenine moiety.

Space-filling molecular models show that this situation would only occur if the adenine is perpendicular to the corrin ring and not parallel to the ring as determined in the case of crystalline DMBC (Lenhert and Hodgkin, 1961).

Another interesting observation from this study is that even in the DACN–ethanolamine–ammonia-lyase complex, no significant immobilization of the nitroxide ligand occurs. Free rotation of this axial ligand must be allowed at the catalytic site of this enzyme. Previous studies in our laboratory using nitroxalkylcobalamins indicates that corrinoids bind close to the surface of ethanolamine–ammonia-lyase (Kennedy, 1969).

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