Spin Labeling of Vitamin B_{12}*

T. Buckman,† F. Scott Kennedy,‡ and J. M. Wood

ABSTRACT: Several new paramagnetic (Co^{III}) corrinoids have been synthesized by the reaction of piperidine nitroxide derivatives with cobamides and cobinamides. The synthesis of the Co–Cσ bond was effected by the reaction of 4-bromoacetamido-2,2,6,6-tetramethylpiperidine-*N*-oxyl with strongly nucleophilic Cob(I)$^-$alamin(B_{12}-s). The corresponding cobinamide was prepared by basic hydrolysis of this nitroxalkylcobamide. A variety of cobinamides were shown to react with nitroxide derivatives by the displacement of water from the 6th coordination position of the cobalt atom, allowing the nitroxide group itself to coordinate to the cobalt. Modifications of the electron spin resonance spectra of nitroxide radicals which result from association with B_{12} are discussed. Electron spin resonance has been used to study the kinetics of the homolytic cleavage of Co–C bond for both cobamide and cobinamide derivatives. These spin-labeled cobamides and cobinamides are of interest as probes in order to study the effect of the environment on binding of vitamin B_{12} in enzymes. The compounds which contain nitroxide as a coordinate ligand represent the first example of a stable, well-characterized, nitroxide–transition metal complex.

The unique chemistry of the Co–Cσ bond in corrinoids (Bonnett, 1963) and more recently in cobaloximes (Schrauzer, 1968) has been the subject of intensive research largely due to its significance in vitamin B_{12}–enzyme mechanisms (Barker, 1967). Such alkyl derivatives of cobalt are photolabile undergoing homolytic cleavage to give a paramagnetic Co^{II} species and alkyl radicals (Hogenkamp, 1966; Schrauzer *et al.*, 1968). Heterolytic cleavage mechanisms have been observed also (Barrett *et al.*, 1966). Reduction of some alkylcobalamins yields Cob(I)alamin[1] and a carbanion. Treatment of some alkylcobalamins with strong nucleophiles such as cyanide ion results in the slow displacement of the alkyl group as a carbanion (Hogenkamp, 1968). Examples of cleavage to give carbonium ions have thus far not been reported.

The majority of B_{12}–enzymes studied to date contain 5′-deoxyadenosyl as the functional alkyl substituent. The mechanisms of catalysis by enzymes containing this coenzyme are the subject of considerable speculation, and while it is generally agreed that breaking of the Co–C bond occurs during catalysis it has not been possible to demonstrate whether this cleavage is homolytic or heterolytic (Hogenkamp, 1968; Babior and Gould, 1969; Hamilton *et al.*, 1969).

Alkylcobalamin derivatives are prepared by the reaction of alkyl halides with Cob(I)alamin. This reaction suggested a way in which an extension of the spin-labeling technique developed in the laboratory of McConnell could be brought to bear on the problem of B_{12}–enzyme mechanisms. The theory and application of this technique have been reviewed recently by Hamilton and McConnell (1968). Basically the technique involves covalent attachment of stable nitroxide free radicals to proteins, or nucleic acids, and the use of the motion sensitive electron spin resonance spectra of these radical labels to observe local changes in conformation of the polymer. It occurred to us that alkyl halides containing a nitroxide group, such as 4-bromoacetamido-2,2,6,6-tetramethylpiperidine-*N*-oxyl could be used as a reagent to react with nucleophilic Cob(I)alamin (Ogawa *et al.*, 1968). Details of this reaction and the hydrolysis of the resulting nitroxalkylcobalamin to nitroxalkylcobinamide[2] are presented in Scheme I. Removal of benzimidazole from cobamides by basic hydrolysis results in the formation of cobinamides which have water coordinated as the lower axial ligand. We have found that 2,2,6,6-tetramethylpiperidine-*N*-oxyl will displace water from the lower axial ligand to give a new class of cobalt complexes in which the nitroxide group serves as a ligand (Scheme II). The electron spin resonance spectra of both of these new types of complexes have been investigated in detail, and the nitroxalkyl derivatives have been used to study the kinetics of the homolytic cleavage of the Co–C bond by light (Scheme III).

The incorporation of these paramagnetic (Co^{III}) corrinoids into apoenzymes to form reconstituted holoenzymes has been successful for both a B_{12}–protein involved in methane formation (Wood and Wolfe, 1966) and for ethanolamine deaminase (Kaplan and Stadtman, 1968). Examination of electron spin resonance spectra of these enzymes furnishes information on the environment of the B_{12} derivative in the enzyme, such as the effect of binding of substrates and inhibitors on the conformation of active sites. The introduction of nitroxalkylcobalamins into proteins represents a relatively small perturbation on the over-all coenzyme structure, and it is possible that in some cases enzymatic activity may be retained in the spin-labeled holoenzyme.

The synthesis of cobinamide derivatives in which nitroxide displaces water at the 6th coordination position on the cobalt atom should provide a sensitive means to determine pK_a values of amino acid residues which displace nitroxide when

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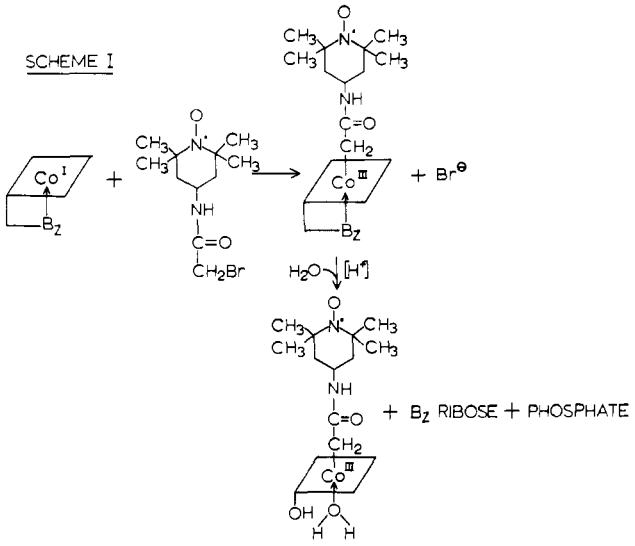
[1] Cob(I)alamin (B_{12}-s) is Co^{+}5,6-dimethylbenzimidazolylcobamide.

[2] Nitroxalkylcobamide is 4-acetamido-2,2,6,6-tetramethylpiperidine-*N*-oxyl-Co^{3+}-5,6-dimethylbenzimidazolylcobamide.

SCHEME I



B_{12} binds in apoenzymes. This group of compounds represents the first example of a stable, well-characterized, nitroxide–transition metal complex, and permits the use of spin-labeled derivatives in enzymes where the B_{12} analog retains 5′-deoxyadenosyl as the upper axial ligand.

The synthesis of spin-labeled coenzyme analogs has an advantage over the previous methods of reacting the labeling reagent with the intact protein, in that it allows precise control over the site of the labeling reaction.

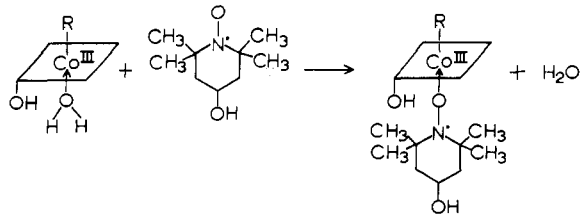
Preliminary experiments directed toward forming nitroxide complexes of other transition metal containing prosthetic groups are in progress, and details of this study in addition to enzyme studies with these paramagnetic (Co^{III}) corrinoids are forthcoming.

Materials and Methods

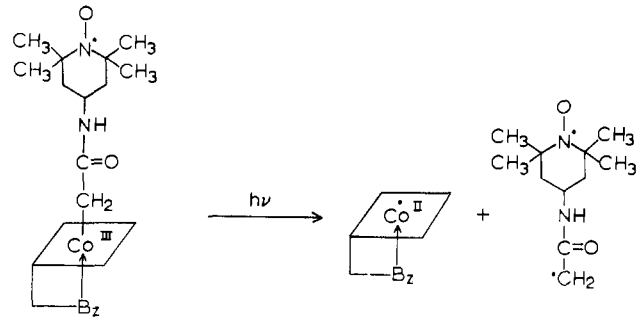
Chemicals and supplies were obtained from commercial sources. Vitamin B_{12} was purchased from the Sigma Chemical Co., St. Louis, Mo. Electron spin resonance measurements were made with a Varian V-4502 electron spin resonance spectrometer fitted with the Varian V-4531 multipurpose cavity. *g* value comparisons were made with a dual cavity accessory. Ultraviolet visible spectra were recorded on a Cary Model 14 spectrophotometer.

Preparation of Nitroxide Reagents. 4-Bromoacetamido-2,2,6,6-tetramethylpiperidine-*N*-oxyl was prepared by a method similar to that of Ogawa *et al.* (1968), mp 123–124°.

SCHEME II



SCHEME III



Anal. Calcd for ($C_{11}H_{20}N_2$): C, 45.2; H, 6.85; N, 9.59. Found: C, 46.0; H, 6.79; N, 9.44. 4-Hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (a) and 2,2,6,6-tetramethylpiperidine-*N*-oxyl (b) were prepared from the corresponding amine by oxidation according to the method of Briere *et al.* (1965). *Anal.* (a) Calcd for ($C_9H_{18}NO_2$): C, 62.76; H, 10.53; N, 8.13. Found: C, 63.20; H, 10.43; N, 8.42. (b) Calcd for ($C_9H_{18}NO$): C, 69.18; H, 11.61; N, 8.97. Found: C, 69.49; H, 11.53; N, 8.87.

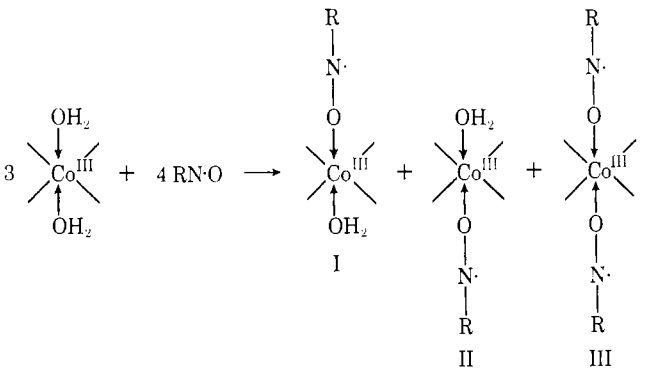
Preparation of Nitroxalkylcorrinoids. Aquocobalamin (20 mg) was reduced electrolytically to Cob(I)alamin at pH 8.0 in 50 ml of 0.05 M sodium borate buffer. A solution of 4-bromoacetamido-2,2,6,6-tetramethylpiperidine-*N*-oxyl (20 mg) in 3.0 ml of ethanol was purged with N_2 and injected into the solution of Cob(I)alamin after shutting off the potential. The reaction mixture was stirred for 20 min before being opened to the atmosphere, neutralized with 1 N acetic acid, and phenol was extracted by the method of Johnson *et al.* (1963). Concentrated samples were purified by chromatography on phosphocellulose. (Wood *et al.*, 1968). Nitroxalkyl derivatives were eluted from this column with 0.01 N acetic acid. The eluate was adjusted to pH 7.0 with 6 N ammonium hydroxide and lyophilized. Yields of this nitroxalkylcobamide were rather low (7–10%), probably because of the bulky nature of the 4-bromoacetamido-2,2,6,6-tetramethylpiperidine-*N*-oxyl causing steric problems. All above operations were conducted in subdued light.

Monocyanocobinamide (factor B) was prepared from cyanocobalamin by catalytic basic hydrolysis (Bonnett, 1963). Cyanocobalamin (500 mg) was added to a N_2-purged solution composed of 60 ml of 0.3 M ceric nitrate and 50 ml of 1 N sodium hydroxide at 95°. The mixture was kept boiling on a water bath for 2.5 hr, cooled, and filtered through Celite. The pH of this solution was adjusted to pH 7.0 with 6 N acetic acid, and after phenol extraction the aqueous phase was lyophilized. This lyophilized product was dissolved in 3.0 ml of water and applied to a Bio-Gel P10 column (1.5 × 100 cm). The column was eluted with water at a flow rate of 0.5 ml/hr, and the extinction of each 2.0-ml fraction was determined at 260 nm. By this procedure benzimidazole–ribose was separated from the corrinoid fraction, and cobinamide was eluted as the tail fractions of the corrinoid hydrolysate. Factor B was found to be pure as adjudged by thin-layer chromatography on Eastman Chromogram 6065 sheets in 1-butanol–acetic acid–water (4:1:5, v/v). λ_{max} values of 577, 533, 500, 366, 303, 275, and 262 nm in 0.1 M phosphate

buffer (pH 7.0) were obtained. Nitroxalkylcobinamide was synthesized and purified by alkylation of controlled potential reduced factor B with 4-bromoacetamido-2,2,6,6-tetramethylpiperidine-*N*-oxyl under conditions identical with those used for the synthesis of nitroxalkylcobalamin.

Preparation of Coordinate Nitroxide Corrinoids. Aquo-Co^{III}-nitroxylcobinamide was prepared by standing a tenfold excess of either 2,2,6,6-tetramethylpiperidine-*N*-oxyl or 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl with 10.0 mg of diaquocobinamide at room temperature for 10 days in 20 ml of ethanol. Diaquocobinamide was synthesized by exhaustive photolysis of methylcobinamide aerobically. Methylcobinamide was synthesized and purified by the method described by Wood *et al.* (1966). Aquo-Co^{III}-nitroxycobinamide was crystallized by addition of excess acetone, followed by a 2-day growth period at 4°. The crystals (7.0 mg) were centrifuged and Soxhlet extracted for 48 hr with diethyl ether at a rate of 5 ml/min. When the colorless ether layer, dried over $CaSO_4$, was evaporated to dryness, no residue remained. The coordinate complex was stored at −15° over $CaSO_4$. The ultraviolet–visible spectrum of this derivative is presented in Figure 1, and indicates that water was not displaced from the upper axial ligand.

The possibility of forming two isomers of this coordinate derivative cannot be ruled out. In addition a dinitroxyl derivative could be formed, *i.e.*



Product I is not formed since repeated attempts to displace water from aquocobalamin with nitroxide derivatives have failed. Also space-filling models indicate that steric hindrance would ensue from the four methyl groups on these reagents. Product III is not formed because dinitroxyl compounds give a complex five-line electron spin resonance spectrum (Osiecki and Ullman, 1968).

Photolysis Experiments. Derivatives were dissolved in H_2O to a concentration of 10^{-4} M with respect to nitroxide, and the electron spin resonance spectra were recorded. The spectrometer was then centered on the high-field line maximum with increased gain and modulation. With the recorder on, the sample was irradiated with a 750-W tungsten filament lamp from a distance of 70 cm. During photolysis the samples were cooled by a constant stream of N_2. In experiments where methylcobalamin was photolyzed in the presence of free nitroxide, the samples were removed from the cavity and irradiated at 10 cm with a 750-W tungsten light for 20 sec. Methane and ethane were assayed by the gas chromatographic technique described by Wolin *et al.* (1963).

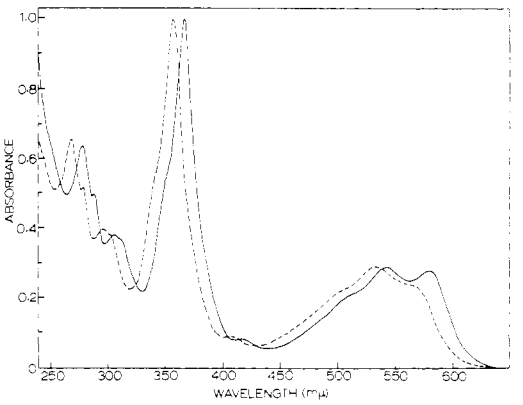


FIGURE 1: Ultraviolet–visible spectra of aquo-4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxylcobinamide at 25°, pH 7.0 (-----), and the spectrum of this derivative following treatment with CN at 25°, pH 9.0 (———).

Results

Derivatives. Alkyl corrinoid derivatives containing nitroxide groups were prepared by reacting 4-bromoacetamido-2,2,6,6-tetramethylpiperidine-*N*-oxyl with an excess of Cob(I)alamin. The paramagnetic (Co^{III}) nitroxalkylcobalamin product was purified by phenol extraction (Johnson *et al.*, 1963), followed by chromatography on phosphocellulose. Basic hydrolysis of this paramagnetic (Co^{III})nitroxalkylcobalamin gave the corresponding yellow (Co^{III})nitroxalkylcobinamide derivative. Details of the preparation of these derivatives are shown in Scheme I. These products were identified by their ultraviolet–visible spectra (Figure 2). The absorption spectrum of the nitroxalkylcobalamin is similar to those recorded for typical alkylcobalamins (Firth *et al.*, 1967). This derivative shows λ_{max} values at 525, 357, and 329 nm with relative extinctions of 1.1, 1.2, and 0.65, respectively. Spectral details of nitroxalkylcobinamide provide λ_{max} values at 455, 428, 360, and 325 nm with relative extinctions of 1.4, 1.0, 0.61, and 0.62, respectively, Absolute extinction coefficients were ϵ 9.1×10^3 at 535 nm for the cobamide and the same at 455 nm for the cobinamide in 0.1 M phosphate buffer (pH 7.0). Addition of cyanide ion to nitroxalkylcobinamide derivatives

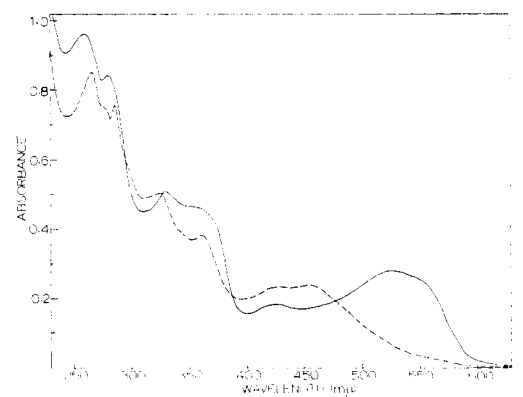


FIGURE 2: Ultraviolet–visible spectra of the nitroxalkylcobamide (———) and the nitroxalkylcobinamide (-----) at 26°, pH 7.0.

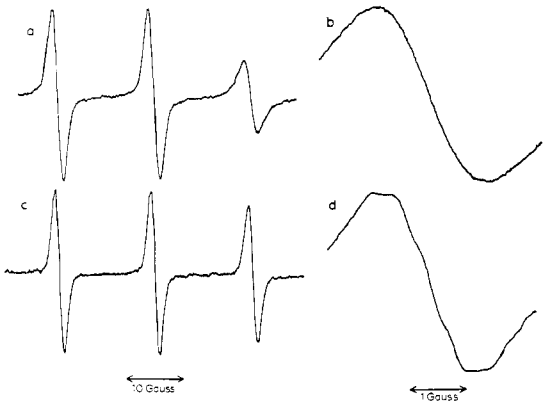


FIGURE 3: Electron paramagnetic resonance spectra of the nitroxalkylcobamide before and after photolysis. (a) Derivative before photolysis; scan rate 10 gauss/min. (b) Center line of derivative before photolysis; scan rate 1 gauss/min. (c) Photolysis product from derivative; scan rate 10 gauss/min. (d) Center line of c; scan rate 1 gauss/min.

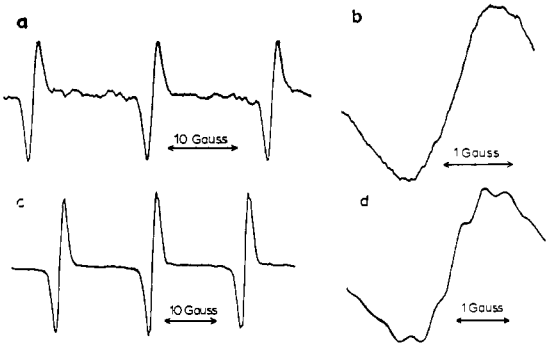


FIGURE 4: Electron paramagnetic resonance spectra of aquo-4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxylcobinamide before and after treatment with CN^-. (a) Spectrum of derivative in H_2O; scan rate 10 gauss/min. (b) Center line of derivative; scan rate 1 gauss/min. (c) Spectrum of cyanolysis mixture showing the free nitroxide; scan rate of 10 gauss/min. (d) Center line of free nitroxide following cyanolysis; scan rate 1 gauss/min.

in the presence of base yielded the corresponding dicyano derivatives.

When both nitroxalkylcobamide and nitroxalkylcobinamide derivatives were photolyzed under aerobic conditions aquocobalamin and diaquocobinamide were obtained, respectively. No attempt was made to isolate free nitroxide-containing photolysis products. The release of these ligands was followed by changes in the nitroxide electron spin resonance hyperfine line widths resulting from changes in the rotational averaging of g value and hyperfine splitting anisotropies.

Cobinamide derivatives which contained 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl or 2,2,6,6-tetramethylpiperidine-*N*-oxyl in the 6th coordination position on the cobalt atom were prepared by stirring the nitroxide reagent with diaquocobinamide in absolute ethanol. The resulting coordinate complexes were crystallized from aqueous acetone followed by Soxhlet extraction of these crystals with diethyl ether to remove any uncoordinated free nitroxide reagent. Spectral details support that only water from the lower axial ligand is displaced to give a γ band at 361 nm (ϵ 9.3 $\times$ 10^{-3} at 535 nm in 0.1 M phosphate buffer, pH 7.0). Treatment of this aquo(Co^{III})nitroxyl coordinate derivative with cyanide ion in base shifts the λ_{max} of the γ band from 361 to 367 nm to give a typical dicyanocobinamide spectrum (Figure 1). In order to confirm that nitroxide is coordinated to the cobalt atom and not an occluded impurity, the aquonitroxide coordinate derivative was subjected to gel filtration on Bio-Gel P10. The elution profile of this derivative from the gel filtration showed that B_{12} absorbance at 361 nm coincided with the electron spin resonance signal due to the nitroxide. The electron spin resonance intensity was arbitrarily defined as the peak-to-peak height in centimeters for the central first derivative peak of the frozen sample using constant gain and modulation amplitude.

Electron Spin Resonance Spectra. The electron spin resonance spectra of both the nitroxalkylcobamide and nitroxalkylcobinamide derivatives were recorded in aqueous solution at 25°, and are very similar (Figure 3a). Both compounds show a_N values of 17.2 gauss compared with 17.0 gauss for free 4-bromoacetamido-2,2,6,6-tetramethylpiperidine-*N*-oxyl. There is no shift in the isotropic g value relative to free nitroxide. Line widths for the nitroxalkyl corrinoids for low-, center-, and high-field hyperfine splitting are 1.87, 1.87, and 2.20 gauss, respectively. When free nitroxide is dissolved in a medium of high viscosity similar broadening of the high-field line has been observed as a result of incomplete motional averaging of g value and hyperfine splitting anisotropies. Attachment of nitroxide to a large molecule, such as B_{12} (mol wt 1357) which has a slow tumbling rate in solution, is expected to give the same effect providing the motion of the radical remains roughly isotropic. Spectra which are very similar to Figure 3a have been reported for spin-labeled polylysine and for proteins which are labeled on the surface. An expression for the calculation of a rotational correlation time for nitroxide derivatives using hyperfine splitting line widths was derived by Stone *et al.* (1965). Using this expression a correlation time of 0.15 nsec was calculated for both the nitroxalkylcobamide and nitroxalkylcobinamide (Figure 3a). A simple calculation based on Stokes' law, which assumes vitamin B_{12} to be a rigid anhydrous sphere, gave a rotational correlation time of 1 nsec. The lower value obtained from the electron spin resonance spectra seems reasonable since the nitroxide ligand almost certainly has some residual motion relative to the corrin ring. An additional hyperfine splitting of 0.4 gauss for protons on the four methyl groups on 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl could be resolved (Figure 4d). This value is in good agreement with the value of 0.43 obtained by nuclear magnetic resonance studies (Kreilick, 1967). This coupling could not be resolved in the nitroxalkylcorrinoid derivatives, but was observed when these derivatives were photolyzed to give free nitroxide (Figure 3d). Motional broadening in the B_{12} derivatives is probably sufficient to result in loss of this resolution.

Photolysis of nitroxalkyl corrinoids results in a decrease in the width of the three nitrogen hyperfine splitting as the nitroxide diradical is released from the cobalt atom. This spectral change has been used to study the kinetics of the homolytic cleavage of these derivatives with light. This was accomplished by setting the magnetic field on the maximum of the high-field line, illuminating the electron spin resonance cavity, and following the increase in height which accompa-

nies line narrowing as the photolysis proceeds. Kinetic experiments were conducted for both nitroxalkylcobamide (Figure 5) and nitroxalkylcobinamide derivatives. Both types of derivative show normal first-order dependence for release of the alkyl group. When nitroxalkylcobinamide was photolyzed under identical conditions, the homolytic cleavage was more rapid. This observation confirms the view of Pailes and Hogenkamp (1968), that electron withdrawal from the cobalt atom by water compared with benzimidazole weakens the Co–C bond.

When nitroxalkylcobinamide was photolyzed in very dilute solution the high-field line increased rapidly, followed by a slower decrease in height. When the electron spin resonance spectrum was recorded at this time, all three hyperfine splitting lines were uniformally broadened, and the spectrum was identical with that of derivatives in which free nitroxide displaced water from the lower axial ligand to form a coordinate complex.

It was shown in another set of experiments that the electron spin resonance spectrum of 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl could be abolished by coupling of this radical with methyl radicals generated by photolysis in the presence of an excess of methylcobalamin. 4-Hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl was dissolved in solutions containing equimolar, 10, 10^2, and 10^3 molar excess of methylcobalamin, and each solution was photolyzed. In the absence of methylcobalamin, light caused no change in the nitroxide electron spin resonance spectrum but in the presence of each concentration of methylcobalamin the electron spin resonance signal was completely abolished. In a similar series of reactions, conducted under H_2, it was found that methane and ethane formation from methylcobalamin was quenched by coupling molar excesses of nitroxide with methyl radicals generated by homolytic cleavage.

The electron spin resonance spectra of both the aquonitroxide and cyanonitroxide coordinate complexes were similar to those recorded for free nitroxide reagents except for a uniform broadening of the three hyperfine splitting lines (line width 1.99 gauss for complex *vs.* 1.33 gauss for the free ligand) (Figure 4a). These complexes showed no g value shifts, and a_N in water for the 4-hydroxynitroxide complex is 17.6 compared with 17.1 gauss for free 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl. No hyperfine coupling to methyl groups was resolved for these derivatives. When these coordinate nitroxide ligands were displaced with cyanide ion in base the hyperfine splitting lines narrowed as the nitroxide was released from the cobalt, and hyperfine coupling with protons on the four methyl groups could be resolved (Figure 4d). Generation of methyl radicals by photolysis of an excess of methylcobalamin in the presence of coordinate nitroxide cobinamide did not abolish the nitroxide electron spin resonance spectra as in the case of photolysis in the presence of free nitroxide, indicating no coupling between the methyl and nitroxide radicals. Instead methane and ethane were formed by hydrogen and methyl abstraction from the corrin ring and by methyl radical coupling.

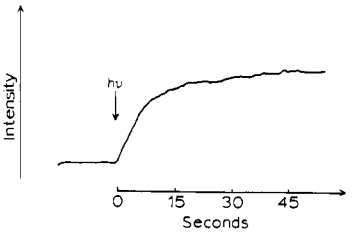


FIGURE 5: Photolysis kinetics of nitroxalkylcobamide. (a) Solution of 10^{-4} M nitroxalkylcobamide was photolyzed under aerobic conditions in the cavity with a 750-W tungsten bulb at a distance of 70 cm. The increase in the maximum of the high-field line is shown as a function of time.

Discussion

The synthesis of nitroxalkylcobamides and nitroxalkylcobinamides has provided an ideal system to study the first-order kinetics of homolytic cleavage of the Co–C bond with light by using electron spin resonance spectroscopy. Previous studies in this area have been restricted to measurements of the appearance of end products which are formed by radical chain reactions which are initiated by homolytic cleavage. Experiments are in progress to determine the inductive effect of a variety of ligands, which displace water from the lower axial ligand of nitroxalkylcobinamides, on the rate of homolytic displacement of nitroxalkyl diradicals. Photolysis of nitroxalkylcobamides bound in B_{12}–enzymes causes the generation of carbon radicals which react with certain amino acid residues which are situated close to the active sites of these enzymes. Treatment of photolyzed enzymes with proteolytic enzymes provides spin-labeled peptides which can be detected by electron spin resonance, isolated, and sequenced.

Free nitroxide derivatives have been shown to be excellent radical scavengers. Photolysis of an equimolar mixture of methylcobalamin and 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl caused almost complete disappearance of the electron spin resonance spectrum. However, when the latter nitroxide derivative displaced water to coordinate as the lower axial ligand with cobinamide derivatives, no quenching of the electron spin resonance spectrum of this derivative occurred when methyl radicals were generated by photolysis of 100-fold excess of methylcobalamin. Failure of this coordinate derivative of B_{12} to react with methyl radicals generated by photolysis could be due to a number of factors. Space-filling models indicate that the nitroxide group would be sterically protected from attack in the complex by the corrin ring system. The unpaired electron on the nitrogen is probably stabilized by some degree of delocalization into the metal orbital system, although no hyperfine coupling to cobalt can be resolved to support this argument. The rate of the radical coupling reaction is almost certainly diffusion controlled, and the greatly increased size of the complex relative to the free nitroxide would increase the probability of methyl radicals reacting with solvent, dissolved oxygen, or one another before encountering a molecule of nitroxide. The fact that the nitroxide electron spin resonance spectrum is not abolished in the coordinate complexes supports the existence of these derivatives.

Since the nitroxide electron spin resonance spectrum is not abolished when the nitroxalkylcorrinoids are photolyzed, nitroxide–alkyl radical coupling is not favored under these conditions either. Intramolecular coupling would be prevented

by steric factors, and the alkyl radical is evidently too short-lived for a detectable amount of intermolecular coupling to occur in the dilute solutions used in these studies (Schrauzer, 1968). The strange electron spin resonance spectral changes observed upon photolysis of nitroxalkylcobinamide in very dilute solution, could be best explained by coordination of the nitroxide released upon photolysis to the open lower axial ligand of the cobalt atom. This reaction could occur by the alkyl end of diradical reacting with the corrin ring in such a manner that the nitroxide would be in a favorable position to displace water and coordinate to the cobalt.

Broadening of the nitrogen hyperfine splitting of the coordinate derivatives and the absence of a resolved methyl proton coupling may be the result of a small unresolved superhyperfine long-range coupling with the cobalt nucleus ($I = {}^{7}/_{2}$) in these derivatives. The slight increase in a_N observed for these complexes indicates an increased spin density on nitrogen. This is the expected result of donation of electrons to the metal orbital system through the nitroxide oxygen which would in turn increase the positive charge and thus the unpaired electron density on nitrogen. A similar effect on a_N is observed when the polarity of the solvent in which a free nitroxide is dissolved is increased.

The specific spin labeling of vitamin B_{12} coenzymes affords the unique opportunity to determine the nature of binding of this coenzyme in protein in addition to the effects of substrates and inhibitors on changes in conformation of these enzymes in the immediate vicinity of the catalytic site. The synthesis of a coenzyme derivative in which 5′-deoxyadenosyl carries a spin label could provide a system which retains catalytic activity, and could be used to follow the proposed breaking and joining of the Co–C bond which is predicted to occur during catalysis. The results of preliminary experiments indicate that analogous complexes can be prepared with heme-containing enzymes, and these derivatives should prove useful in the elucidation of these enzyme mechanisms.

Finally, spin-labeled derivatives of B_{12} should have unique advantages over B_{12} isotopes which have been used to study transport problems in clinical experiments. Tetramethylpiperidine-*N*-oxyl compounds in general are detectable in very dilute solutions, do not suffer from the hazards which isotopes offer to both operator and patient, and are physiologically inert (Cummings *et al.*, 1963).

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