5′-Peroxyadenosine and 5′-Peroxyadenosylcobalamin as Intermediates in the Aerobic Photolysis of Adenosylcobalamin[†]

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ABSTRACT: The photolysis of adenosylcobalamin (coenzyme B_{12}) results in homolytic cleavage of the Co−C5′ bond, forming cob(II)alamin and the 5′-deoxyadenosyl radical. In the presence of molecular oxygen, it has been proposed that the primary reaction is interception of the 5′-deoxyadenosyl radical by O_2 to form adenosine-5′-aldehyde as the product (Hogenkamp, H. P. C., Ladd, J. N., and Barker, H. A. (1962) *J. Biol. Chem. 237*, 1950−1952). 5′-Peroxyadenosine is here found to be the initial nucleoside product of this reaction and found to decompose to adenosine-5′-aldehyde. Evidence indicates that 5′-peroxyadenosine arises from the hydrolysis of 5′-peroxyadenosylcobalamin, with the formation of cob(III)alamin. 5′-Peroxyadenosine undergoes further decomposition to adenosine-5′-aldehyde as the major final product of aerobic photolysis as well as to adenosine and adenine as minor products. In a cobalamin-dependent process, 5′-peroxyadenosine becomes re-ligated to cob(III)alamin to form 5′-peroxyadenosyl-cobalamin, which quickly decomposes to adenosine-5′-aldehyde and cob(III)alamin. This is supported by spectrophotometric observations of both rapidly photolyzed adenosylcobalamin and the reaction of cob(III)alamin with excess 5′-peroxyadenosine. 5′-Peroxyadenosine also slowly undergoes cobalamin-independent decomposition to adenosine-5′-aldehyde and the minor products adenosine and adenine. The present study provides a detailed description of the products initially formed when aqueous, homolytically cleaved adenosylcobalamin reacts with molecular oxygen and provides a detailed description of the behavior of those products subsequent to photolysis.

Enzymes dependent upon the vitamin B_{12}-coenzyme adenosylcobalamin[1] catalyze intriguingly diverse chemical reactions, including carbon skeleton rearrangements, reductive heteroatom eliminations, and intramolecular 1,2-migrations of heteroatomic substituents, and they proceed by mechanisms involving organic free radicals as intermediates (*1*, *2*). Adenosylcobalamin incorporates a cobalt ion in an octahedral arrangement with the pyrroline and pyrrolidine groups of a corrin ring system making up the equatorial ligands, the nitrogen from a dimethylbenzimidazole moiety as the lower axial ligand, and a 5′-deoxadenosyl moiety

forming a cobalt−carbon (Co−C5′) bond in the sixth, upper axial ligand position (*3*).

The key to the function of adenosylcobalamin in enzymatic catalysis is the reversible homolytic scission of the Co−C5′ bond, generating cob(II)alamin and a transiently formed 5′-deoxyadenosyl radical (*4*−*9*). The bond dissociation energy of the Co−C5′ bond is 30−33 kcal mol^{-1}, and in aqueous solution at 25 °C, thermolysis proceeds quite slowly with a rate constant on the order of 10^{-9} s^{-1}, corresponding to a half-life of 22 years (*10*−*15*). The rate of Co−C5′ homolysis is drastically increased at enzymatic sites, proceeding with a rate enhancement of approximately 10^{12} (*12*, *16*). The mechanism employed to enhance the rate of thermal dissociation enzymatically is poorly understood.

The nonenzymatic, photolytic decomposition of adenosylcobalamin, also resulting in the formation of cob(II)alamin and the 5′-deoxyadenosyl radical (*17*−*19*), has been regarded as a model system for the enzymatic, thermal decomposition of the coenzyme. Anaerobically, cob(II)alamin and 5′-deoxy-5′,8-cycloadenosine are formed, whereas aerobically cob(III)alamin and adenosine-5′-aldehyde are reported as the major products of photolysis (*20*, *21*).

The present study investigates the oxidized nucleoside products derived from the aerobic photodissociation of the Co−C5′ bond in adenosylcobalamin. We study this process in an atmosphere of 100% O_2 to suppress the anaerobic process. In addition to adenosine-5′-aldehyde and cob(III)-alamin, we find that interception of cob(II)alamin and the 5′-deoxyadenosine radical by O_2 leads initially to 5′-

[1] Abbreviations: Adenosylcobalamin, 5′-deoxyadenosylcobalamin; Ado, adenosine; cob(III)alamin, hydroxocobalamin and/or aquocobal-amin; 5′-deoxyadenosyl radical, 5′-deoxyadenosine-5′-yl; DMSO, dimethyl sulfoxide; EPPS, *N*-[2-hydroxyethyl]piperazine-*N*′-3-propane-sulfonic acid; ESI, electrospray ionization; FOX, ferrous oxidation in xylenol orange assay; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography-mass spectrometry; MES, 2-[*N*-mor-pholino]ethanesulfonic acid; NMR, nuclear magnetic resonance; RT, retention time; TOF, time-of-flight.

peroxyadenosine and cob(III)alamin as well as minor amounts of adenine and adenosine. Furthermore, it was determined that 5′-peroxyadenosine decomposes to adenosine-5′-aldehyde and that this process is catalyzed by cob(III)alamin. The results shed further light on the aerobic photolysis of adenosylcobalamin and also create another foundation to explore the behavior of the coenzyme in its enzymatic process.

MATERIALS AND METHODS

Materials. Adenine, adenosine, 5′-deoxyadenosine, adenosylcobalamin, hydroxocobalamin hydrochloride (cob(III)-alamin), EPPS, and MES were purchased from Sigma. Monobasic phosphate buffer was purchased from Fisher. Oxygen gas (99.5%) was purchased from Aldrich. Columns used in HPLC analysis were filled with Altima-HP C_{18} reverse phase resin, purchased pre-packed from Alltech. The column used in LC/MS analysis was filled with Inertsil C_{18} reverse phase resin (GL Sciences), packed in-house. The FOX assay was performed using reagents from the Peroxoquant kit (Pierce).

Photolysis of Adenosylcobalamin in Oxygen Equilibrated Water. A steady stream of oxygen gas was bubbled through 4 mL of water for 30 min. Oxygenated water (990 μL) was placed in a glass test tube 1 cm in diameter and made to be 100 μM adenosylcobalamin with the addition of 10 μL of a 10 mM solution. Oxygen was again bubbled through the solution for 5 min in the dark. The sample was irradiated for 4 min with a 100 W flood lamp placed approximately 1 cm from the test tube, with continued oxygen sparging for the duration of the photolysis.

After photolysis, 100 μL aliquots were placed in small-volume auto-sampler target vials and sealed with airtight caps. A standard HPLC method was employed to separate and quantify the nucleoside products. Sample vials were loaded into the HPLC auto-sampler, and injections were scheduled at indicated times. All procedures were performed under dim lighting.

Analytical Methods. A standard method was developed to separate and quantify the nucleoside products of adenosylcobalamin photolysis using a Beckman Coulter System Gold HPLC apparatus equipped with a model 128 diode array detector. Reaction products were separated on a 4.6 × 250 mm Altima-HP analytical C_{18} reverse phase column. Sample vials were loaded into the auto-sampler, and 50 μL injections were scheduled. Separation proceeded by using the following elution program: flow 1 mL min^{-1}, isocratic 100% H_2O for 5 min; ramp to 95% H_2O/5% CH_3CN over 1 min, isocratic 95% H_2O/5% CH_3CN for 25 min; and ramp to 70% H_2O/30% CH_3CN over 10 min, isocratic 70% H_2O/ 30% CH_3CN for 10 min. If the sample contained cobamides, the column was reconditioned with multiple washes, cycling from 5% to 95% CH_3CN at 25 min per cycle. Nucleoside product concentrations were determined by integration of total peak area using the Beckman Coulter 32 Karat software (version 7.0.1048) from the absorbance output measured at 260 nm and compared to a standard curve.

A standard curve was created by using known concentrations of adenine and adenosine and running them by the standard HPLC method. Integration of total peak area from the absorbance output of the HPLC diode array detector at

260 nm was used to relate total absorbance to moles of standard in a 50 μL injection. The standard curve used to determine the concentration of any experimental peak was chosen by the proximity of elution time to the standard. The adenine curve was used for early peaks, and the adenosine curve was used for later peaks.

Mass spectral analysis was performed using an Agilent LC/MSD ESI-TOF mass spectrometer in the positive ion mode. A reaction sample was prepared as described (see Photolysis of Adenosylcobalamin in Oxygen Equilibrated Water) and separated on a 2.1 × 200 mm Inertsil C_{18} reverse phase column in line with the ESI-TOF detector. Separation was performed isocratically at 95% H_2O/5% CH_3CN. Peaks were analyzed for m/z by the detector as they emerged. Mass spectrometry was done at the Mass Spectrometry Facility (Biotechnology Center, University of Wisconsin—Madison).

^{1}H NMR analysis was performed on an Avance DMX-600 NMR spectrometer (Bruker Biospin Corp) using a CryoProbe operated at 600.13 MHz or an Avance DMX-400 NMR spectrometer (Bruker Biospin Corp) operated at 400.13 MHz. This work was performed at the National Magnetic Resonance Facility at Madison.

Peroxides were detected by ferrous oxidation in xylenol orange (FOX assay) (*22*) in samples from reverse phase HPLC purification (see Preparation of 5′-Peroxyadenosine for Kinetic Analysis).

Absorption spectra were recorded on a Cary 50 UV/vis spectrophotometer using the Cary WinUV software in the scanning kinetics mode.

Characterization of Nucleosides. Photolysis of adenosylcobalamin in oxygen equilibrated water produced four major non-cobamide products. Of these four compounds, two constituted the bulk of the products observed and were named nucleoside A and nucleoside B for their order of elution upon C_{18} reverse phase HPLC. Both products contained an adenine moiety, as determined spectrophotometrically from the absorbance output of the HPLC detector (λ_{max} 206 and 259 nm; λ_{min} 227 nm).

Samples of photolyzed adenosylcobalamin containing enough material for NMR analysis of nucleoside B were prepared and separated on a 10 × 250 mm Altima-HP analytical C_{18} reverse phase column. In a small test tube, 9 mg of adenosylcobalamin was dissolved in 2 mL of oxygen equilibrated water. Oxygen gas was bubbled through the solution for 5 min in the dark. The sample was irradiated for two and a half minutes with a 100 W flood lamp placed approximately 1 cm from the test tube, with continued oxygen sparging for the duration of the photolysis. The sample was quickly cooled and immediately injected into a 2 mL loop on the HPLC apparatus. Separation proceeded with a flow rate of 5 mL min^{-1}, isocratically at 95% H_2O/ 5% CH_3OH. After elution of the nucleoside, successive washes with 95% CH_3OH removed remaining cob(III)alamin. Fractions containing nucleoside B from 6 repetitions were pooled and dried by rotary evaporation at 30 °C. The sample was further dried on a lyophilizer and resuspended in DMSO-d_6: ^{1}H NMR (600 Mhz, DMSO-d_6) δ-values 4.06 (dd, 1H), 4.11 (d, 1H), 4.15 (d, 1H), 4.16 (dd, 1H), 4.63 (d, 1H), 5.32 (d, 1H), 5.52 (d, 1H), 5.89 (d, 1H), 7.28 (brs, 2H), 8.15 (s, 1H), 8.32 (s, 1H), 11.92 (s, 1H). The mass of nucleoside B was 283.099 amu. The spectroscopic analysis indicated that nucleoside B is 5′-peroxyadenosine.

Samples of photolyzed adenosylcobalamin with enough material for NMR analysis of nucleoside A were prepared as described above. The photolysis mixture was stored overnight in the dark to allow for conversion of nucleoside B into nucleoside A and separated on a 10×250 mm Altima-HP analytical C_{18} reverse phase column. Samples were injected into a 2 mL loop on the HPLC apparatus. Separation proceeded with a flow rate of 5 mL min^{-1}, isocratically at 95% H_2O/5% CH_3CN. After elution of the nucleoside, successive washes with 95% CH_3CN removed remaining cob(III)alamin. Fractions containing nucleoside A from 3 repetitions were pooled and dried by rotary evaporation at 30 °C. The sample was further dried on a lyophilizer and resuspended in DMSO-d_6. Hydrated species: ^{1}H NMR (400 Mhz, DMSO-d_6) δ-values 3.82 (d, 1H), 4.14 (d, 1H), 4.64 (d, 1H), 4.70 (d, 1H), 5.18 (d, 1H), 5.75 (d, 1H), 5.88 (d, 1H), 6.31 (d, 2H), 7.33 (brs, 2H), 8.13 (s, 1H), 8.31 (s, 1H). Dehydrated species: ^{1}H NMR (400 Mhz, DMSO-d_6) δ-values 4.12 (d, 1H), 4.44 (d, 1H), 4.92 (d, 1H), 5.36 (d, 1H), 5.80 (d, 1H), 6.04 (d, 1H), 7.35 (brs, 2H), 8.14 (s, 1H), 8.40 (s, 1H), 9.75 (s, 1H). The mass of nucleoside A dissolved in water was identified as 283.099 amu. The spectroscopic analysis indicated that nucleoside A is adenosine-5′-aldehyde.

Samples of photolyzed adenosylcobalamin were co-injected with commercial standards. Only two standards, adenine and adenosine, co-migrated with minor peaks found in the photolysis reaction mixture. The masses of these two peaks were determined to be 135.062 and 267.104 amu, confirming their identities as adenine and adenosine, respectively.

Anaerobic Decomposition of Nucleoside Products Derived from the Aerobic Photolysis of Adenosylcobalamin. An experiment was performed to monitor the decomposition of nucleoside B (5′-peroxyadenosine) in an anaerobic solution. A sample was prepared as described (see Photolysis of Adenosylcobalamin in Oxygen Saturated Water). Immediately after irradiation, the sample was placed in a glass cuvette sealed with a stopcock, where it was subjected to partial evacuation for 20 s and flushed with argon. This procedure was repeated six times. The sample was sealed in a cuvette under a blanket of argon and passed into an anaerobic glove box. Several 100 μL aliquots were placed in small-volume auto-sampler target vials and sealed with airtight caps. These samples were brought out of the glove box and subjected to the standard HPLC method at indicated times. A similar experiment was repeated wherein the sample remained aerobic for the duration of the incubation.

Purification of 5′-Peroxyadenosine for Kinetic Analysis. Nucleoside B, provisionally identified as 5′-peroxyadenosine, was separated from cobamides and other oxidized nucleoside products on an analytical C_{18} reverse phase column. Water was equilibrated with oxygen gas. Into 1 mL of oxygen equilibrated water, 4.4 mg of adenosylcobalamin was dissolved, and oxygen was bubbled through the solution for 5 min in the dark. The sample was irradiated for 4 min with a 100 W flood lamp placed approximately 1 cm from the test tube, and sparging with oxygen was continued. The reaction mixture (50 μL) was loaded onto a 4.6×250 mm Altima-HP C_{18} reverse phase column and subjected to standard HPLC method. The column was visibly overloaded

but still provided sufficient separation from all other products of the photolysis.

Determination of Rates of 5′-Peroxyadenosine Decomposition under Various Conditions. Experiments were performed to observe the rates of decomposition of 5′-peroxyadenosine at 20 °C, 60 °C, and in the presence of 200 μM cob(III)-alamin. Purified 5′-peroxyadenosine, from the analytical C_{18} reverse phase column (see Preparation of 5′-Peroxyadenosine for Kinetic Analysis), was adjusted to either pH 5.8 or 8.4 by the addition 1 M phosphate at an appropriate pH to a final concentration of 20 mM. At selected intervals over 24 h at 20 °C, samples were withdrawn, sealed in small-volume target vials, and subjected to the standard HPLC method at periodic times over 24 h. Rates in the presence of 200 μM cob(III)alamin were measured in the same way except for the addition of cob(III)alamin.

For rate measurements at 60 °C, solutions of 5′-peroxy-adenosine were prepared in a manner identical to that at room temperature but placed in a 60 °C water bath. Approximately every 45 min, a 100 μL aliquot was removed and placed in the 250 μL small-volume insert for the auto-sampler target vial. The insert was then transferred into an auto-sampler target vial that contained 1.5 mL of water at room temperature. After 4 min, the sample was run using the standard HPLC method at the indicated times.

An experiment was performed in which 200 μM adenosylcobalamin was photolyzed, and the rate of decomposition of unpurified 5′-peroxyadenosine was observed over 20 h. The reaction mixture was prepared as described above (see Photolysis of Adenosylcobalamin in Oxygen Equilibrated Water) but included the addition of 20 μL of 10 mM adenosylcobalamin instead of 10 μL.

Absorption Spectroscopy in the Photolysis of Adenosyl-cobalamin. A stream of oxygen gas was bubbled through water for 30 min, and 1 mL was placed in a glass test tube 1 cm in diameter and brought to 80 μM adenosylcobalamin. Oxygen bubbling continued through the solution for 5 min in the dark. The sample was irradiated for 45 s with a 100 W flood lamp placed approximately 1 cm from the test tube, with continued oxygen sparging for the duration of photolysis. Immediately after photolysis, the sample was adjusted to pH 8.5 in 10 mM EPPS and placed in the spectrophotometer, where absorption spectra were periodically acquired over 20 h.

A complementary sample was prepared containing 80 μM cob(III)alamin, 10 mM EPPS at pH 8.5, and 400 μM 5′-peroxyadenosine. Immediately after the addition of 5′-peroxyadenosine, the sample was placed in the spectrophotometer where absorption spectra were acquired periodically over 1 h. In this and the preceding experiment, the absorption spectrum of 80 μM cob(III)alamin in 10 mM EPPS at pH 8.5 was obtained for comparison with the recorded spectra of test solutions.

RESULTS

Photolysis of Adenosylcobalamin in O_2-Saturated Water. Photolysis products in O_2-saturated water are cob(III)alamin, identified spectrophotometrically, and products derived from the 5′-deoxyadenosyl moiety. Figure 1 shows the HPLC elution profile of the adenine nucleosides. The two major products of aerobic photolysis are designated nucleosides A
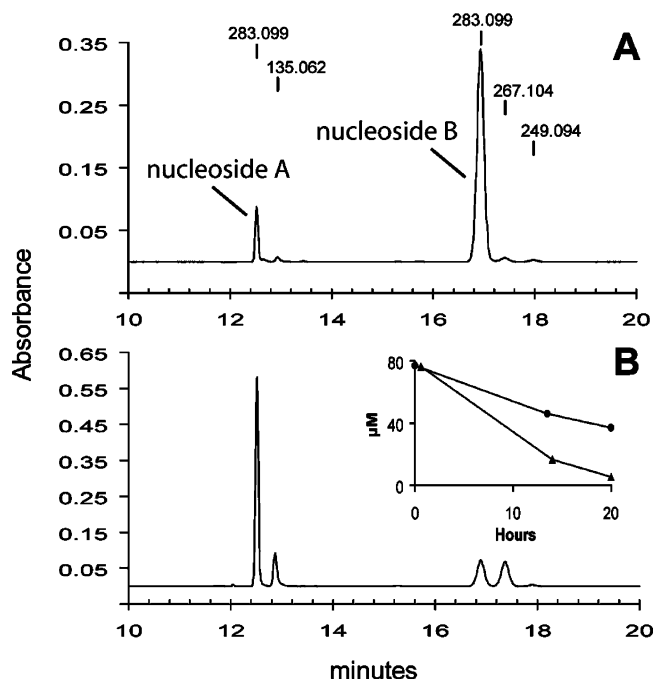
FIGURE 1: HPLC elution profile of aerobically photolyzed adenosylcobalamin. Adenosylcobalamin (100 μM) was irradiated with a 100 W flood lamp for 4 min and subjected to reverse phase chromatography. (A) Chromatography immediately after photolysis. Above each peak is the mass as acquired by ESI-TOF mass spectrometry. (B) Sample made anaerobic immediately after photolysis and aged 14 h before chromatography. The compounds identified are RT 12.6 min, adenosine-5'-aldehyde hydrate; RT 12.9 min, adenine; RT 16.9 min, 5'-peroxyadenosine; and RT 17.4 min, adenosine. (Inset) Decomposition of 5'-peroxyadenosine under aerobic ($\bullet-\bullet$, 2 μM/h) and anaerobic ($\blacktriangle-\blacktriangle$, 4 μM/h) conditions in the presence of cob(III)alamin from photolysis.

and B, named for the order of elution upon C_{18} reverse phase chromatography. Other products are detectable amounts of adenine (135 amu) and adenosine (267 amu), identified by spectrophotometry (λ_{max} 206 and 259 nm; λ_{min} 227 nm) by chromatographic co-migration with standards and by mass analysis through LC/MS (Figure 1).

Figure 1A is the reverse phase HPLC elution profile of adenine products immediately after photolysis, showing a significant amount of nucleoside A, traces of adenine and adenosine, and the major product nucleoside B. With the passage of time after photolysis, HPLC analysis shows increasing amounts of nucleoside A, adenine, and adenosine and decreasing amounts of nucleoside B, which is clearly decomposing. Figure 1B is the reverse phase HPLC profile 14 h after photolysis, when most of the nucleoside B is decomposed, and the amount of nucleoside A is dramatically increased, with lesser increases in adenine and adenosine. Clearly, nucleoside B gradually decomposes, mainly to nucleoside A but also to smaller amounts of adenine and adenosine.

Mass spectral analysis of nucleoside A indicates a mass of 283.099 amu (Figure 1), which is in agreement with the identification of this peak as the hydrate of adenosine-5'-aldehyde (20). In addition, sodium borohydride reduction of adenosine-5'-aldehyde would be expected to yield adenosine, and adenosine is produced when 0.5 mL of 80 μM nucleoside A is reduced with 2 mg of NaBH$_4$ for 30 min. Co-injection of the resultant reaction mixture with standards in reverse phase chromatography shows that the reduced nucleoside is
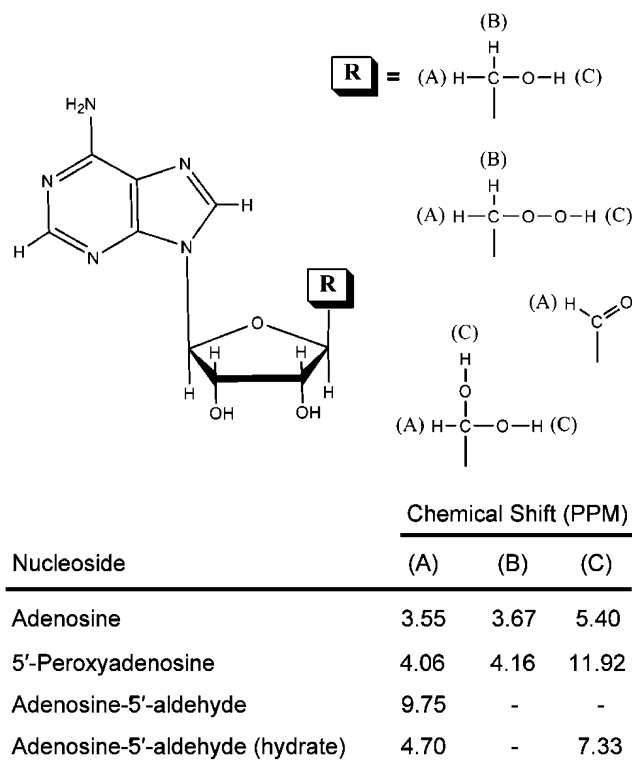

Nucleoside	Chemical Shift (PPM)		
	(A)	(B)	(C)
Adenosine	3.55	3.67	5.40
5'-Peroxyadenosine	4.06	4.16	11.92
Adenosine-5'-aldehyde	9.75	-	-
Adenosine-5'-aldehyde (hydrate)	4.70	-	7.33

FIGURE 2: Structures of nucleoside products from the aerobic photolysis of adenosylcobalamin. The ^{1}H NMR chemical shifts for each R group labeled in the structure are in the table.

identical to adenosine, as reported previously (20). ^{1}H NMR spectroscopy performed on adenosine-5'-aldehyde (nucleoside A) in DMSO-d_6 shows a clear signal for a single proton at 9.75, diagnostic of an aldehydic proton. A comparison of NMR chemical shifts for the C5'(H), C5'(OH), and C5'-(OOH) protons in nucleoside products derived from the aerobic photolysis of adenosylcobalamin is presented in Figure 2.

Identification of Nucleoside B. Nucleoside B is unstable and decomposes over time under a variety of conditions. The major decomposition product of nucleoside B is nucleoside A, adenosine-5'-aldehyde, together with minor but detectable amounts of adenosine and adenine (Figure 1B). This decomposition is inhibited by the presence of oxygen and progresses at a faster velocity when the solution is made anaerobic (Figure 1B, inset).

Nucleoside B has a mass identical to that of the hydrate of adenosine-5'-aldehyde (Figure 1). The most obvious isomer of the hydrate is 5'-peroxyadenosine, a reasonable structure, taking into consideration its observed decomposition to adenosine-5'-aldehyde. ^{1}H NMR analysis of nucleoside B in DMSO-d_6 is consistent with 5'-peroxyadenosine and shows that the 5'-peroxyadenosine hydroperoxy proton resonates far downfield at 11.92 ppm relative to the 5'-hydroxy proton of adenosine at 5.40 ppm (21) (Figure 2). This is somewhat further downfield than expected for a hydroperoxide proton but can be explained by an unusually low pK_a for this substituent and/or participation in internal hydrogen bonding. In addition, the two C5'-protons in nucleoside B appear downfield compared to those in adenosine (Figure 2). This behavior would be expected for a transformation of the C5'-substituent from an alcohol to a hydroperoxide.

Table 1: Initial Rate of Formation of Adenosine and Adenosine-5′-aldehyde from the Decomposition of 5′-Peroxyadenosine[a]

	pH 5.8		pH 8.4	
condition[b]	Ado-5′-CHO (μM min^{-1})	Ado (μM min^{-1})	Ado-5′-CHO (μM min^{-1})	Ado (μM min^{-1})
20 °C	7×10^{-4}	1×10^{-4}	3×10^{-4}	4×10^{-4}
60 °C	1.3×10^{-2}	1.2×10^{-3}	2.1×10^{-3}	7.5×10^{-3}
Cbl(III), 20 °C	2.9×10^{-2}	5×10^{-4}	3.2×10^{-2}	1.9×10^{-3}

[a] All reactions were run with chromatographically purified 5′-peroxyadenosine at approximately 45 μM. [b] 5′-Peroxyadenosine was incubated at 20 °C (room temperature), at 60 °C, and in the presence of 200 μM cob(III)alamin at 20 °C (room temperature).

An assay was performed in which purified nucleoside B gave a rapid and strong positive response to ferrous oxidation in xylenol orange (FOX assay) (22). In this assay, 25 mM Fe^{2+} in 2.5 M sulfuric acid is oxidized to Fe^{3+} by the hydroperoxide. Xylenol orange then forms a complex with Fe^{3+}, giving a purple product with a maximum absorbance at 560 nm. Sorbitol is included in this reaction mixture to increase assay sensitivity. No other compounds tested, adenosine or a variety of carboxylic acids, gave a positive result in this assay.

Initial work with 5′-peroxyadenosine was performed in a buffer system comprising either EPPS or MES, both of which are tertiary amine-containing sulfonic acids. The properties of 5′-peroxyadenosine were obscured when work in these buffers at 60 °C yielded unexpectedly high yields of adenosine as a cobalamin-independent decomposition product, compared to those of unbuffered solutions or in phosphate buffer. It is known that amines and hydroperoxides can react to form N-oxides and the corresponding alcohol. This result further supports the identity of nucleoside B as 5′-peroxyadenosine.

A very minor product of aerobic photolysis is a peak that elutes with the highest retention time in our chromatographic system. The mass of this peak is 249.094 amu, in agreement with previous work identifying 5′-deoxy-8,5′-cycloadenosine as the product of anaerobic photolysis of adenosylcobalamin (21). The present photolysis experiments in pure O_2 are intended to minimize the yield of this product.

One of the two major nucleoside products of photolysis of adenosylcobalamin has been reported to be adenosine-5′-carboxylic acid (24). We did not find this compound as a photolytic product of adenosylcobalamin.

Transformation of 5′-Peroxyadenosine into Adenosine-5′-aldehyde. 5′-Peroxyadenosine decomposes to adenosine-5′-aldehyde under a variety of conditions. In order to study this reaction, larger amounts of 5′-peroxyadenosine were purified on a C$_{18}$ reverse phase HPLC column, eluted isocratically at 5% CH$_3$CN in water. The purified compound was divided and incubated at two temperatures and at pH 5.8 and 8.4. The initial rates of production of adenosine-5′-aldehyde and adenosine were monitored by reverse phase HPLC (Table 1). Adenine production was also observed, but the rate was too slow for accurate measurement by the HPLC assay.

In the cobalamin-independent reactions, the rate of appearance of decomposition products increases approximately 10- to 20-fold at both pH values with a 40 °C increase in temperature (Table 1). Adenosine-5′-aldehyde and adenosine production are slightly sensitive to pH, both exhibiting small

differences in rates of formation at the two pH values. The trend for the change in the initial rate of formation due to pH is observed at 20 and 60 °C for both products. Interestingly, adenosine-5′-aldehyde production is 6-fold slower at the higher pH at 60 °C, while adenosine production is accelerated by the same magnitude at the higher pH (Table 1). The most striking result of the pH study is the remarkable insensitivity to pH: the rates decrease or increase 2- to 6-fold with the 400-fold increase in hydroxide concentration between pH 5.8 and 8.4.

When 5′-peroxyadenosine decomposition is monitored within the photolysis reaction mixture, which includes cob-(III)alamin, adenosine-5′-aldehyde formation is much faster than that in the transformation of the purified nucleoside. Addition of authentic cob(III)alamin to the purified 5′-peroxyadenosine reproduces these faster rates, as documented in Table 1.

The rate of formation of adenosine-5′-aldehyde and adenosine from 5′-peroxyadenosine incubated with cob(III)-alamin showed even less dependence on pH than in the absence of cob(III)alamin. At the two pH values studied, the appearance of adenosine-5′-aldehyde in the cobalamin-dependent process was similar at pH 5.8 and 8.4. In the formation of adenosine, the magnitude of rate increase between pH 5.8 and 8.4 was the same for the cobalamin-dependent and -independent processes. The production of adenosine-5′-aldehyde was never seen to be reversible under any conditions studied. Increasing the ionic strength to 0.5 elicited a very small decrease in rate (<10%) at 20 °C, an effect inconsistent with a polar transition state.

Absorption Spectrum of an Intermediate in the Photolysis of Adenosylcobalamin. Figure 3 shows the changes in the absorption spectra associated with the photolysis of adenosylcobalamin and the subsequent cobalamin-dependent decomposition of free 5′-peroxyadenosine. Figure 3A shows the initial absorption spectrum and its decay of 80 μM adenosylcobalamin at pH 8.5 after exposure to a short period of photolysis. Over time, the initial cob(III)alamin-related spectrum decays, nearly quantitatively, to that of cob(III)-alamin (- - -).[2] The decay occurs in a biphasic manner, with a rapid change over ca. 30 min and a subsequent slower decay over a period of nearly 20 h (Figure 3A, inset). The spectral change and the biphasic time course indicate the formation and decay of an intermediate.

In a second experiment, 80 μM cob(III)alamin at pH 8.5 was incubated with 400 μM 5′-peroxyadenosine. Figure 3B shows the evolution of the cob(III)alamin absorption spectrum (- - -) to one similar to that of the apparent intermediate initially observed following the photolysis of adenosyl-cobalamin. The maximal change in the absorption spectrum occurs at approximately 25 min. At lower pH values, the spectral change occurs to a much smaller extent (data not shown). The observed spectral changes implicate the formation of a complex between cob(III)alamin and 5′-peroxy-adenosine that is similar or identical to that initially generated by aerobic photolysis of adenosylcobalamin.

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[2] The pK_a for cob(III)alamin is 7.8. Hydroxocobalamin and aquo-cobalamin in aqueous solution have different absorption spectra.
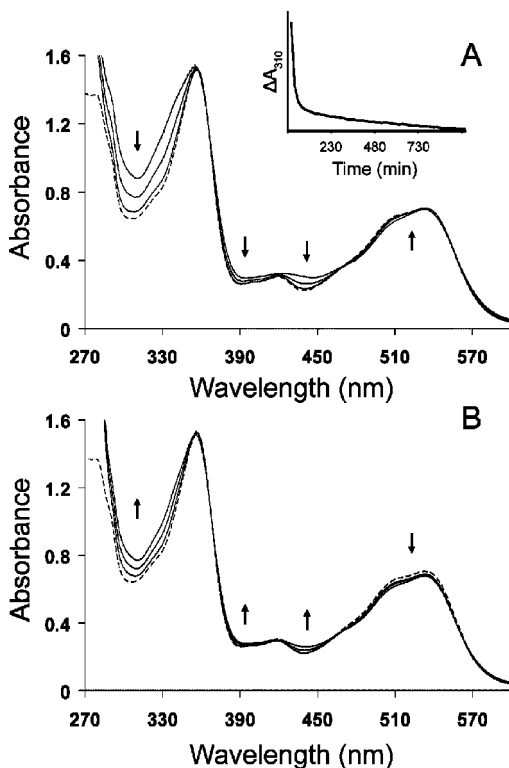
Scheme 1

FIGURE 3: Changes to the absorption spectra of cob(III)alamin after the photolysis of adenosylcobalamin and from the addition of 5′-peroxyadenosine. (A) Photolyzed adenosylcobalamin (80 μM) held for 20 h at pH 8.5. The initial spectrum decays to that of cob(III)-alamin (---). (Inset) Biphasic decay of reaction intermediate monitored spectrophotometrically at 312 nm. (B) Cob(III)alamin in the presence (80 μM) of 400 μM 5′-peroxyadenosine held 25 min at pH 8.5. The initial absorption spectrum of 80 μM cob(III)-alamin at pH 8.5 is (---).

DISCUSSION

Photolysis of Adenosylcobalamin. Exposure to visible light decomposes adenosylcobalamin (*25*), producing a radical pair (*17−19*). Photodissociation serves as a model for the thermal homolysis of the Co−C5′ bond in adenosylcobalamin, and Scheme 1 illustrates the proposed relationship between the photolytic and thermolytic pathways (*17, 25−30*). Photolysis begins with activation of the Co−C5′ bond and proceeds through excited states [A] and [B] displaying absorption spectra evolving from cob(III)alamin-like to cob(II)alamin-like species and leading to an intermediate [I], which either re-forms adenosylcobalamin or relaxes to a geminate radical pair [Ado• + Cob(II)alamin], similar to that formed upon thermolysis of the Co−C5′ bond. The radical pair is the intersection of the two pathways. In aqueous solution, the radical pair react either by recombination or escape from the solvent cage and quenching. Anaerobically, cob(II)alamin and 5′-deoxy-8,5′-cycloadenosine are formed (*21*). Aerobi-cally, the major reported products are adenosine-5′-aldehyde and cob(III)alamin (*20*).

Products from the Photolysis of Adenosylcobalamin. Scheme 2 outlines a minimal mechanism for the major reactions of the geminate radical pair resulting from ho-molytic cleavage of the Co−C5′ bond in air. Molecular oxygen intercepts the alkyl radical within the radical pair to form an alkylperoxy radical. This reaction occurs at the diffusional limit for the methyl radical (4.7×10^9 M^{-1} s^{-1})
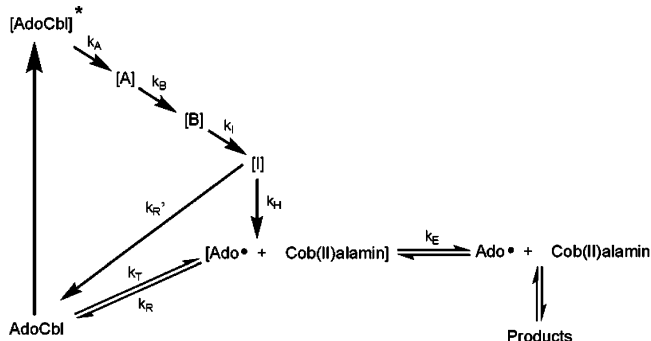
(*31*), much faster than the reaction of O_2 with cob(II)alamin ($t_{1/2} = 21$ min) (*32*). Formation of the methylperoxy radical is proposed to be the major secondary reaction in the photolysis of alkylcobalamins (*33*). Reaction of cob(II)alamin with the methylperoxy radical leads to concomitant electron transfer from Co^{2+} and ligation to the upper-axial position of cob(III)alamin, leading directly to 5-peroxyadenosylco-balamin.

Four lines of evidence implicate 5′-peroxyadenosylcobal-amin as an intermediate in the formation of adenosine-5′-aldehyde: (a) In photolysis, a small amount of adenosine-5′-aldehyde appears in a burst (Figure 1A), followed by much slower accumulation over hours, implicating an intermediate in the mechanism. (b) Hydroxocobalamin accelerates the decomposition of purified 5′-peroxyadenosine (Table 1). It is difficult to imagine how this could occur except through complexation, and the obvious complex is one with 5′-peroxyadenosine ligated to cob(III)alamin in the upper axial position. (c) The cob(III)alamin-dependent decomposition is slower in the presence of O_2 than that in its absence (Figure 1B, inset), which we take to indicate competition of O_2 with 5′-peroxyadenosine for binding to cob(III)alamin. (d) The transient absorption spectra in Figure 3 clearly show the formation and decomposition of an intermediate. The elec-tronic spectral evidence is very powerful because of the well-known effects of axial ligands on the electronic spectra (*38*). Taken together, the evidence implicates an intermediate, and the simplest and most reasonable structure is 5′-peroxy-adenosylcobalamin.

Precedents for the mechanism in Scheme 2 have been published. On the basis of the production of cob(III)alamin, formaldehyde, and hydroxide ion in the photolysis of methylcobalamin, and the behavior of alkyl radicals in the presence of O_2 (*34, 35*), the methyl peroxy radical was proposed as an intermediate (*33*). Moreover, the methyl peroxy radical reacts rapidly with the model compound, Co-([14]tetraeneN$_4$)$^{2+}$ (*36*). Kinetic analysis monitoring absor-bance changes at 470 nm was attributed to the loss of cob(II)alamin due to the formation of this alkylperoxoco-balamin. Rate constants were 2.4×10^9 M^{-1} s^{-1} for methylcobalamin (*37*) and 3×10^9 M^{-1} s^{-1} for adenosyl-cobalamin (*18*). Spectrophotometric evidence for the initial formation of 5′-peroxyadenosylcobalamin is provided in Figure 3. Spectral differences for cob(III)alamin derivatives in the main absorption bands (i.e., α, 535 nm; β, 510 nm; and γ, 360 nm) of the spectrum arising from differences in the upper axial ligand of cob(III)alamin have been primarily attributed to differences in the sum of the σ-donor strength of the axial ligands (*38*).
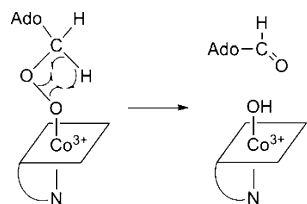
Scheme 2
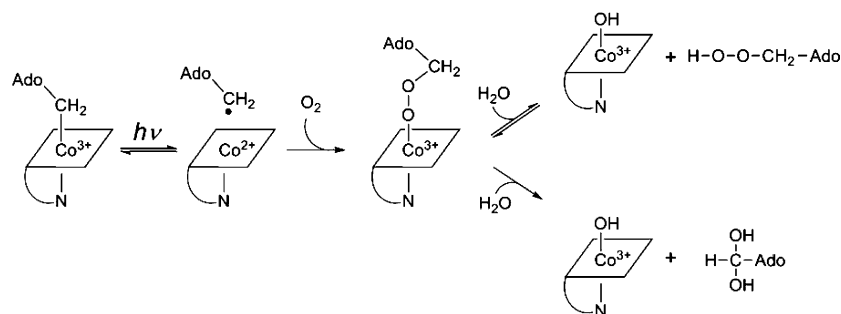
Scheme 3
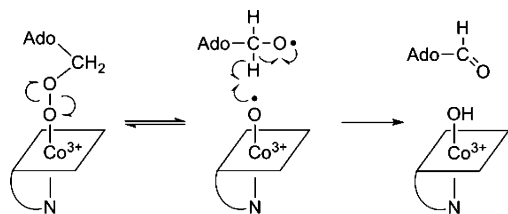
Scheme 4

Two major products from the aerobic photolysis of adenosylcobalamin likely arise from decomposition of 5′-peroxyadenosylcobalamin (Scheme 2). First, free 5′-peroxyadenosine can appear by substitution of water or hydroxide ion into the upper axial position of 5′-peroxyadenosylcobalamin. This allows for the reversible association of 5′-peroxyadenosine with cob(III)alamin. 5′-Peroxyadenosine accounts for 89% of the nucleoside products immediately following photolysis. Second, 5′-peroxyadenosylcobalamin decomposes to adenosine-5′-aldehyde and cob(III)alamin in an apparently irreversible reaction.

Adenosine-5′-aldehyde likely results from homolytic scission of the O—O bond in the hydroperoxide. In the 5′-peroxyadenosylcobalamin intermediate, cleavage of this bond generates an alkoxyl radical and an oxocobalamin radical species. Abstraction of a C5′-hydrogen atom by the oxocobalamin radical in a cage reaction would yield both the aldehyde and cob(III)alamin (Scheme 3). Alternatively, a concerted reaction involving 5′-peroxyadenosylcobalamin could lead directly to adenosine-5′-aldehyde through a 4-membered cyclic transition state (Scheme 4). The aldehyde initially accounts for 9% of the nucleoside products in photolysis, one-tenth of the initial yield of 5′-peroxyadenosine. Small amounts of adenosine and adenine are also observed, each accounting for <2% of the nucleoside products that arise by unknown mechanisms.
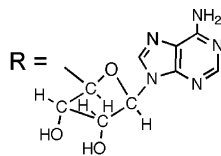
On the basis of available information, a polar reaction mechanism for the production of adenosine-5′-aldehyde from 5′-peroxyadenosylcobalamin appears unlikely. Such a mechanism would involve base catalysis in the abstraction of a proton from the 5′-methylene group, either by a base in solution or intramolecularly by the peroxyanion ligated to

Co(III). A base-catalyzed mechanism appears unlikely because of the absence of substantial rate enhancement at pH 8.4 relative to pH 5.8. Furthermore, high ionic strength would stabilize a polar transition state, but high ionic strength does not enhance the rate.
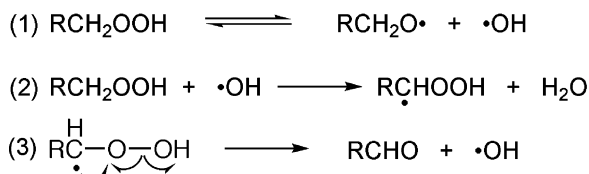
Cob(III)alamin-Catalyzed Decomposition of 5′-Peroxyadenosine. Catalysis of the isomerization of free 5′-peroxyadenosine to adenosine-5′-aldehyde by cob(III)alamin depends upon the reversible ligation of 5′-peroxyadenosine to cob(III)alamin to generate 5′-peroxyadenosylcobalamin (Cob(III)-OH + Ado-5′-OOH ⇌ Cob(III)-O—O-5′-Ado + H_2O). The catalytic effect of cob(III)alamin on the process of decomposition is likely brought about by the weakness of the O—O bond in the latter complex. Conceivably, participation of occupied nonbonding p-orbitals on the Co-coordinated oxygen atom interacting with the lowest unoccupied d orbitals in cob(III)alamin would weaken the peroxide bond, promoting homolysis and increasing the rate of aldehyde formation. Decomposition to adenosine-5′-aldehyde proceeds either by a caged radical mechanism as in Scheme 3 or by the concerted internal rearrangement in Scheme 4.

Cobalamin-Independent Decomposition of 5′-Peroxyadenosine. In the much slower, cobalamin-independent decomposition, 5′-peroxyadenosine also undergoes transformation into adenosine-5′-aldehyde as the major product or into adenosine as a minor product. Reactions 1—6 in Figure 4 provide a reasonable mechanistic rationale for the formation of these two products. The process likely occurs through a radical chain mechanism because of the apparent slightly autocatalytic production of both adenosine-5′-aldehyde (Figure S1, Supporting Information) and adenosine (data not shown). The reactions in Figure 4 leading to observed products are selected on the basis of the following criteria: radical coupling reactions are expected to be rare, as the short life-times of the radical intermediates would not allow them to accumulate. Abstraction of hydrogen atoms from the O—H bond of 5′-peroxyadenosine is not expected to contribute significantly to product formation because the O—H bond is stronger than the C—H bonds. Hydrogen abstractions from the C5′ of 5′-peroxyadenosine are selected because the C5′—H bonds are weaker, less sterically hindered, and provide a more stable radical product than cleavage of other C—H bonds. Products arising from radical abstraction of other hydrogens within the nucleoside are not observed. The rare chain-terminating radical couplings do not contribute significantly to the product profile.
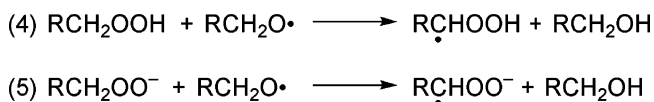
Reactions 1—3 in Figure 4 account for adenosine-5′-aldehyde formation, and reactions 4 and 5 account for

R =

Adenosine-5-aldehyde production

(1) $RCH_2OOH \rightleftharpoons RCH_2O\bullet + \bullet OH$

(2) $RCH_2OOH + \bullet OH \longrightarrow R\overset{\bullet}{C}HOOH + H_2O$

(3) $R\overset{\bullet}{C}\overset{H}{\underset{\bullet}{-}}O\overset{}{-}OH \longrightarrow RCHO + \bullet OH$

Adenosine production

(4) $RCH_2OOH + RCH_2O\bullet \longrightarrow R\overset{\bullet}{C}HOOH + RCH_2OH$

(5) $RCH_2OO^- + RCH_2O\bullet \longrightarrow R\overset{\bullet}{C}HOO^- + RCH_2OH$

Unproductive

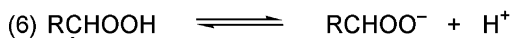(6) $R\overset{\bullet}{C}HOOH \rightleftharpoons R\overset{\bullet}{C}HOO^- + H^+$

FIGURE 4: Radical chain reactions leading to the cob(III)alamin-independent decomposition of 5-peroxyadenosine. Reactions 1−3 lead to the transformation of 5′-peroxyadenosine into adenosine-5′-aldehyde as the major product, and reactions 4 and 5 account for the production of smaller amounts of adenosine.

adenosine formation. Reaction 5 can explain the pH related increase in the rate of adenosine production under basic conditions. Fractional deprotonation of the alkyl hydroperoxide at the higher pH leads to the hydroperoxy anion, the reaction of which with the alkoxyl radical leads to adenosine and a C5′-centered radical in the hydroperoxy anion, which is stabilized through a dative effect from the electron-releasing oxyanion substituent. This product-stabilization would lead to an increase in the rate of reaction 5 relative to 4 and could account for the increase in the observed rate of appearance of adenosine (Table 1).

Reaction 6 in Figure 4 is unproductive and provides a rationale for the pH related decrease in the formation of aldehyde under basic conditions. Should the alkyl hydroperoxide species with the C5′-centered radical undergo fractional deprotonation to form the hydroperoxy anion, which is stabilized by the dative effect described above, it would lead to a dead-end regarding product accumulation.

The process leading to the formation of adenine is not known at this time. Adenine production was too slow for accurate rate measurements by the HPLC assay, either in the presence or absence of cob(III)alamin.

The identification of 5′-peroxyadenosine as the primary product of photoinduced homolysis of the Co−C5′ bond in the presence of O_2 sheds new light on the reactivity of adenosylcobalamin. The formation of the hydroperoxide and the cob(III)alamin-catalyzed decomposition of this compound provides chemical support for the existence of the 5′-peroxyadenosylcobalamin intermediate in the aerobic photolysis of adenosylcobalamin.

SUPPORTING INFORMATION AVAILABLE

Figure S1, showing the progress curves for the transformation of 5′-peroxyadenosine into adenosine-5′-aldehyde under various conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Dolphin, D., Ed. (1982) In *B₁₂*, Vol. 1 and 2, Wiley-Interscience, New York.
2. Banerjee, R., Ed. (1999) In *Chemistry and Biochemistry of B₁₂*, Wiley-Interscience, New York.
3. Ouyang, L., Rulis, P., Ching, W. Y., Nardin, G., and Randaccio, L. (2004) Accurate redetermination of the X-ray structure and electronic bonding in adenosylcobalamin, *Inorg. Chem. 43,* 1235−1241.
4. Babior, B. M. (1970) The mechanism of action of ethanolamine deaminase. VI. Ethylene glycol, a quasi-substrate for ethanolamine deaminase, *J. Biol. Chem. 245*, 1755−1766.
5. Finlay, T. H., Valinsky, J. E., Mildvan, A. S., and Abeles, R. H. (1973) Electron spin resonance studies with dioldehydrase; evidence for radical intermediates in reactions catalyzed by coenzyme B₁₂, *J. Biol. Chem. 248*, 1285−1290.
6. Orme-Johnson, W. H., Beinert, H., and Blakley, R. L. (1974) Cobamides and ribonucleotide reduction. XII. The electron paramagnetic resonance spectrum of "active coenzyme B₁₂", *J. Biol. Chem. 24*, 2338−2343.
7. Babior, B. M. (1975) Mechanism of cobalamin-dependent rearrangements, *Acc. Chem. Res. 8*, 376−384.
8. Abeles, R. H., and Dolphin, D. H. (1976) The vitamin B₁₂ coenzyme, *Acc. Chem. Res. 9*, 114−120.
9. Banerjee, R. (2003) Radical carbon skeleton rearrangements: catalysis by coenzyme B₁₂-dependent mutases, *Chem. Rev. 103*, 2083−2094.
10. Halpern, J., Kim, S. H., and Leung, T. W. (1984) Cobalt-carbon bond dissociation energy of coenzyme B₁₂, *J. Am. Chem. Soc. 106*, 8317−8319.
11. Finke, R. G., and Hay, B. P. (1984) Thermolysis of adenosylcobalamin: a product, kinetic, and cobalt-carbon (C5′) bond dissociation energy study, *Inorg. Chem. 23*, 3041−3043.
12. Hay, B. P., and Finke, R. G. (1986) Thermolysis of the cobalt-carbon bond of adenosylcobalamin. 2. Products, kinetics, and cobalt-carbon bond dissociation energy in aqueous solution, *J. Am. Chem. Soc. 108*, 4820−4829.
13. Hay, B. P., and Finke, R. G. (1987) Thermolysis of the cobalt-carbon bond in adenosylcorrins. 3. Quantification of the axial base effect in adenosylcobalamin by the synthesis and thermolysis of axial base-free adenosylcobinamide. Insights into the energetics of enzyme-assisted cobalt-carbon bond homolysis, *J. Am. Chem. Soc. 109*, 8012−8018.
14. Hay, B. P., and Finke, R. G. (1988) Thermolysis of the Co−C bond in adenosylcobalamin (coenzyme B₁₂)−IV. Products, kinetics and Co−C bond dissociation energy studies in ethylene glycol, *Polyhedron 7*, 1469−1481.
15. Garr, C. D., and Finke, R. G. (1993) Adocobalamin (AdoCbl or coenzyme B₁₂) cobalt-carbon bond homolysis radical-cage effects: product, kinetic, mechanistic, and cage efficiency factor (Fc) studies, plus the possibility that coenzyme B₁₂-dependent enzymes function as "ultimate radical cages" and "ultimate radical traps" , *Inorg. Chem. 32*, 4414−4421.
16. Marsh, E. N. G., and Ballou, D. P. (1998) Coupling of cobalt-carbon bond homolysis and hydrogen atom abstraction in adenosylcobalamin-dependent glutamate mutase, *Biochemistry 37*, 11864−11872.
17. Walker, L. A., II, Shiang, J. J., Anderson, N. A., Pullen, S. H., and Sension, R. J. (1998) Time-resolved spectroscopic studies of

B$_{12}$ coenzymes: the photolysis and geminate recombination of adenosylcobalamin, *J. Am. Chem. Soc. 120*, 7286−7292.

18. Endicott, J. F., and Netzel, T. L. (1979) Early events and transient chemistry in the photohomolysis of alkylcobalamins, *J. Am. Chem. Soc. 101*, 4000−4002.

19. Hogenkamp, H. P. C. (1982) In *B$_{12}$* (Dolphin, D., Ed.) Vol. 1, pp 295−323, Wiley-Interscience, New York.

20. Hogenkamp, H. P. C., Ladd, J. N., and Barker, H. A. (1962) The identification of a nucleoside derived from coenzyme B$_{12}$, *J. Biol. Chem. 237*, 1950−1952.

21. Hogenkamp, H. P. C. (1963) A cyclic nucleoside derived from coenzyme B$_{12}$, *J. Biol. Chem. 238*, 477−480.

22. Jiang, Z. -Y., Woollard, A. C. S., and Wolff, S. P. (1990) Hydrogen peroxide production during experimental protein glycation, *FEBS Lett. 268*, 69−71.

23. Pouchert, C. J., and Behnke, J. (1993) *The Aldrich Library of ^{13}C and ^{1}H FT-NMR Spectra*, Vol. 3, pp 222, Sigma-Aldrich, Milwaukee, WI.

24. Johnson, A. W., and Shaw, N. (1962) Some reactions of the vitamin B$_{12}$ coenzyme, *J. Chem. Soc.* 4608−4614.

25. Chen, E., and Chance, M. R. (1990) Nanosecond transient absorption spectroscopy of coenzyme B$_{12}$. Quantum yields and spectral dynamics, *J. Biol. Chem. 265*, 12987−12994.

26. Chagovitz, A. M., and Grissom, C. B. (1993) Magnetic field effects in adenosylcob(III)alamin photolysis: relevance to B$_{12}$ enzymes, *J. Am. Chem. Soc. 115*, 12152−12157.

27. Brown, K. L. (2005) Chemistry and enzymology of vitamin B$_{12}$, *Chem. Rev. 105*, 2075−2150.

28. Chen, E., and Chance, M. R. (1993) Continuous-wave quantum yields of various cobalamins are influenced by competition between geminate recombination and cage escape, *Biochemistry 32*, 1480−1487.

29. Cole, A. G., Yoder, L. M., Shiang, J. J., Anderson, N. A., Walker, L. A., II., Banaszak, Holl, M. M., and Sension, R. J. (2002) Time-

30. Yoder, L. M., Cole, A. G., Walker, L. A., II, and Sension, R. J. (2001) Time-resolved spectroscopic studies of B$_{12}$ coenzymes: influence of solvent on the photolysis of adenosylcobalamin, *J. Phys. Chem. B 105*, 12180−12188.

31. Thomas, J. K. (1967) Pulse radiolysis of aqueous solutions of methyl iodide and methyl bromide. The reactions of iodine atoms and methyl radicals in water, *J. Phys. Chem. 71*, 1919−1925.

32. Pratt, J. M. (1964) The chemistry of vitamin B$_{12}$. Part II. Photochemical reactions, *J. Chem. Soc.* 5154−5160.

33. Hogenkamp, H. P. C. (1966) The photolysis of methylcobalamin, *Biochemistry 5*, 417−422.

34. Heicklen, J., and Johnston, H. S. (1962) Photochemical oxidations. II. Methyl iodide, *J. Am. Chem. Soc. 84*, 4030−4039.

35. Heicklen, J., and Johnston, H. S. (1962) Photochemical oxidations. I. Ethyl iodide, *J. Am. Chem. Soc. 84*, 4394−4403.

36. Mok, C. Y., and Endicott, J. F. (1978) The photochemistry of organocobalt complexes containing tetraaza macrocyclic ligands. Cobalt-methyl homolysis and the nature of the cobalt-carbon bond, *J. Am. Chem. Soc. 100*, 123−129.

37. Endicott, J. F., and Ferraudi, G. J. (1977) A flash photolytic investigation of low energy homolytic processes in methylcobalamin, *J. Am. Chem. Soc. 99*, 243−245.

38. Pratt, J. M. (1999) The Roles of Co, Corrin, and Protein. I. Co-Ligand Bonding and the Trans Effect, in *Chemistry and Biochemistry of B$_{12}$* (Banerjee, R., Ed.) pp 73−112, Wiley-Interscience, New York.

resolved spectroscopic studies of B$_{12}$ coenzymes: a comparison of the primary photolysis mechanism in methyl-, ethyl-, *n*-propyl-, and 5′-deoxyadenosylcobalamin, *J. Am. Chem. Soc. 124*, 434−441.