The Mechanism of Action of Ethanolamine Deaminase. V. The Photolysis of Enzyme-Bound Alkylcobalamins*

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ABSTRACT: The photolysis of complexes between various alkylcobalamins and ethanolamine deaminase was studied by optical and electron spin resonance spectroscopy. In some cases, the products of photolysis of the free and enzyme-bound cobalamins were characterized. The alkylcobalamins used in these studies were methylcobalamin, β-hydroxyethylcobalamin, δ-(9-adenyl)butylcobalamin, 5′-deoxyinosylcobalamin, and 5′-deoxyadenosylcobalamin. The optical spectra of the enzyme–alkylcobalamin complexes are similar to the spectra of the corresponding free cobalamin derivatives. After photolysis, the spectra of the samples containing enzyme-bound β-hydroxyethylcobalamin, 5′-deoxyinosylcobalamin, or 5′-deoxyadenosylcobalamin resemble the spectrum of hydroxocobalamin while the spectrum of the enzyme–methylcobalamin complex becomes similar to that of cob(II)alamin and the spectrum of the enzyme–δ-(9-adenyl)butylcobalamin complex is unchanged. The electron paramagnetic resonance spectrum of the unphotolyzed enzyme–5′-deoxyadenosylcobalamin complex displays signals resembling those obtained by a brief anerobic photolysis of 5′-deoxyadenosylcobalamin. Electron paramagnetic resonance spectra of the other unphotolyzed complexes show no signal. After irradiation, the β-hydroxyethylcobalamin, 5′-deoxyinosylcobalamin, and 5′-deoxyadenosylcobalamin complexes show no change, while signals resembling those of anerobically photolyzed 5′-deoxyadenosylcobalamin are exhibited by the methylcobalamin complex. Brief irradiation of the enzyme–δ-(9-adenyl)butylcabalamin complex leads to no change in its electron paramagnetic resonance spectrum, but prolonged irradiation with intense light leads to the appearance of signals similar to those seen in the photolyzed methylcobalamin complex. The major products arising from the organic fragments generated by the photolysis of enzyme-bound methylcobalamin and β-hydroxyethylcobalamin are formaldehyde and acetaldehyde, respectively. However, little of these compounds is produced by the photolysis of the free cobamides. The corrinoid moiety appears to be unaltered by the photolytic process. The implications of these findings are discussed in terms of the possibility that the mechanism of action of coenzyme B12 requiring enzymes might involve a free-radical rearrangement.

A well-known property of alkylcobalamins is their susceptibility to photolytic destruction. Light has been shown to induce a homolytic cleavage of the carbon–cobalt bond of these compounds, with the production of B12r,[1] a paramagnetic species in which the cobalt has a valence of +2, and an organic free radical (Bernhauer *et al.*, 1964). If the photolysis is conducted anaerobically, the B12r produced by the photolysis of alkylcobalamins is stable and can be observed by optical and electron paramagnetic resonance spectrometry (Hogenkamp *et al.*, 1963).

The fate of the organic radical has been studied with two compounds, DMBC and MeB12. With DMBC, photolysis in air is followed by the oxidation of the deoxyadenosyl radical to adenosine-5′-aldehyde, while anaerobic photolysis leads to the formation of 8,5′-cycloadenosine (Hogenkamp *et al.*, 1962; Hogenkamp, 1963). After aerobic photolysis of MeB12, the methyl fragment is recovered primarily as formaldehyde, although a small fraction is converted into low molecular weight hydrocarbons. As the oxygen tension is decreased, the fraction of methyl fragments recovered as formaldehyde diminishes; in the complete absence of oxygen MeB12 appears to be stable to light (Hogenkamp, 1966).

Recent work with ethanolamine deaminase, a coenzyme B12 requiring enzyme which catalyzes the conversion of ethanolamine into acetaldehyde and ammonia, has shown that the aerobic photolysis of a mixture of enzyme, ethanolamine, and MeB12 leads to the formation of a stable complex between enzyme and ethanolamine (Babior, 1969). This complex does not form if MeB12 is replaced by OHB12, nor does it appear after the photolysis of a mixture of enzyme, ethanolamine, and EtB12. These findings imply that the course of photolysis of at least one of these two alkylcobalamins is altered in the presence of enzyme. An investigation was therefore conducted on the photolysis of a variety of enzyme–alkyl B12 complexes. The results of this investigation are the subject of the present report.

Materials and Methods

Materials. Ethanolamine deaminase was purified and resolved of bound cobamide by the method of Kaplan and Stadtman (1968a). For calculations of concentration, the equivalent weight was taken as 260,000 (Kaplan and Stadtman, 1968b; Babior and Li, 1969). DMBC, IB12, and BB12 were the generous gifts of Professor H. P. C. Hogenkamp; unlabeled and ^{14}C-labeled MeB12 were kindly provided by J. Michael Poston; unlabeled and ^{14}C-labeled EtB12 were prepared as previously described (Babior and Li, 1969). Radioac-

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[1] MeB12, methylcobalamin; EtB12, β-hydroxyethylcobalamin; BB12, δ-(9-adenyl)butylcobalamin; IB12, 5′-deoxyinosylcobalamin; DMBC, 5′-deoxyadenosylcobalamin; B12r, cob(II)alamin.

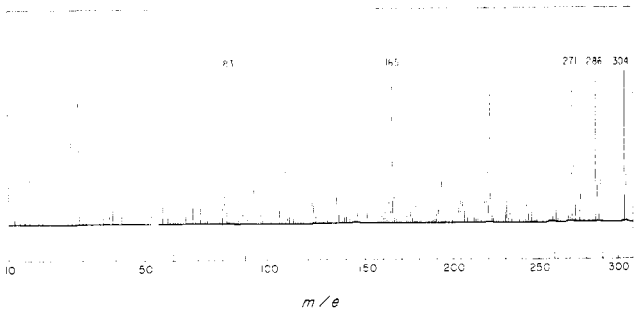

FIGURE 1: Mass spectrum of the product of the reaction between dimedon and glycolaldehyde.

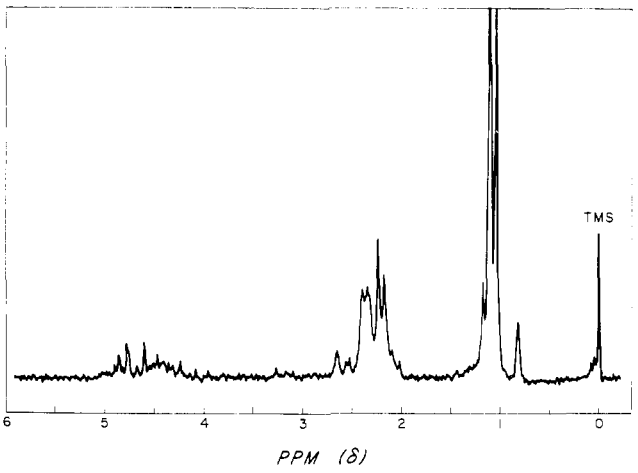

FIGURE 2: Nuclear magnetic resonance spectrum of the product of the reaction between dimedon and glycolaldehyde. The solvent was $CDCl_3$. The peak labeled TMS is due to the tetramethylsilane used as the internal standard.

tive ethylene bromohydrin (ethylene bromohydrin-1,2-^{14}C, 4 mCi/mmole) was obtained from New England Nuclear. Other chemicals were reagent grade, and were used without further purification.

Optical and Electron Paramagnetic Resonance Spectroscopy. Optical spectra were obtained on a Cary Model 15 recording spectrophotometer, using 1-ml quartz cells with a 1-cm light path. Electron paramagnetic resonance spectra shown in Figures 5, 6, and 8 were obtained on a Varian 4500 X-band electron paramagnetic resonance spectrometer with 100-kHz modulation. These spectra were all recorded at the same gain. Those shown in Figure 7 were taken on a Varian E-3 instrument. All electron paramagnetic resonance spectra were taken at 77°K.

Labeling of Enzyme by the Photolysis of Radioactive Alkylcobalamins. Incubations were conducted in 2-ml glass centrifuge tubes as described in Table I. After terminating the incubation with 0.1 ml of 6% (w/v) trichloroacetic acid, the reaction mixture was centrifuged at 25,000*g* for 2 min. The supernatant was transferred to a scintillation vial containing 10 ml of Bray's solution (Bray, 1960) and the precipitate was washed with approximately 0.2 ml of 3% (w/v) trichloroacetic acid containing 5 mM ethanolamine. After centrifugation at 25,000*g* for 2 min the washings were transferred to a second scintillation vial containing Bray's solution. The precipitate was washed once again, transferring the washings to a third vial for counting, and then was dissolved in 0.2 ml of NCS (Nuclear-Chicago). The solution of protein was counted in 10 ml of Bray's solution containing 0.1 ml of 2 N acetic acid. Radioactivity was determined in a Nuclear-Chicago Mark I liquid scintillation counter.

Chromatography on Amberlite CG-50. Incubations were conducted as described in Table II. The reaction was terminated by diluting the incubation mixture to 0.9 ml with water and immediately adding 0.1 ml of 6% (w/v) trichloroacetic acid. The mixture was then applied to a 0.5-ml column of Amberlite CG-50 (H^+), collecting the effluent in a receiver cooled in ice. Following passage of the reaction mixture, the column was washed with 1 ml of water; the pooled effluent and water wash constituted the first fraction. The cobalamin was then eluted from the column with 2 ml of 2 N NH_4OH. Aliquots of the two fractions were then added to Bray's solution, and the radioactivity was determined by liquid scintillation counting.

Dimedon Adducts. To aliquots of the first fractions obtained from Amberlite CG-50 chromatography were added known amounts of unlabeled aldehyde as described in Table III. Dimedon adducts were then prepared as described by Lee and Abeles (Lee and Abeles, 1963). All the formaldehyde and acetaldehyde adducts melted in the ranges of 186–188 and 137–139°, respectively (lit. mp (Hodgman, 1960) 189° for the formaldehyde adduct and 139° for the acetaldehyde adduct).

Whereas the formaldehyde and acetaldehyde adducts precipitated immediately upon acidification of the reaction mixture, the compound formed with glycolaldehyde crystallized only slowly from the reaction mixture at pH 3, although crystals appeared more rapidly (but still not immediately) when the mixture was acidified further. The crystals isolated from the glycolaldehyde–dimedon mixture melted with decomposition over a range of 220–230°. Since a search of the literature revealed no previous report of the reaction between glycolaldehyde and dimedon, this material was characterized further. The elemental composition was carbon, 71.09%, hydrogen, 7.98%, and oxygen, 21.22%, and mass spectroscopy showed the molecule ion to be at *m/e* 304 (Figure 1); these results are in accord with the formula $C_{18}H_{24}O_4$, equal to the formula expected for the ordinary dimedon adduct minus a molecule of water. Mass spectroscopy also showed prominent peaks at M − 28 (286), M − 34 (271) and 83, as have been observed previously with dimedon alkylated in the 2 position (Maquestiau and Lejeune, 1967). In addition, a large peak is seen at *m/e* 165, the location expected for a fragment produced by the loss of a dimedon radical from the molecule ion of a dimedon–glycolaldehyde adduct which has lost the elements of water (Vanderwalle *et al.*, 1967). Nuclear magnetic resonance spectroscopy (Figure 2) showed three closely coupled

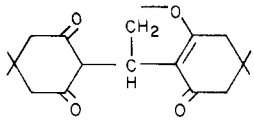

FIGURE 3: Structure assigned to the product of the reaction between dimedon and glycoladehyde.

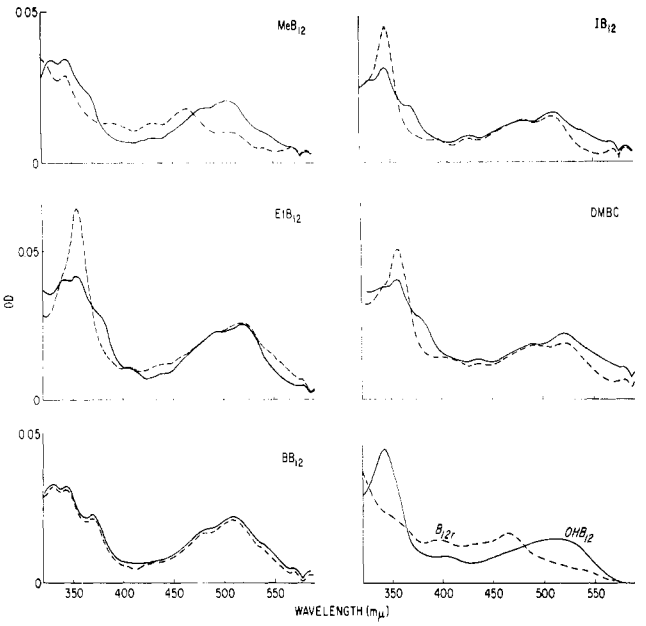

FIGURE 4: Spectra of enzyme–alkyl B_{12} complexes before and after photolysis. Samples contained 1.1 nmoles of ethanolamine deaminase, 2.2 nmoles of cobalamin, 1 μmole of ethanolamine (or in the case of DMBC, 1 μmole of 1-amino-2-propanol), and 10 μmoles of potassium phosphate buffer (pH 7.4) in a total volume of 1.0 ml. (———) Spectrum before photolysis; (– – – –) spectrum after photolysis. For comparison, the spectra of hydroxocobalamin (25 μM) and B_{12r} (25 μM) are also shown.

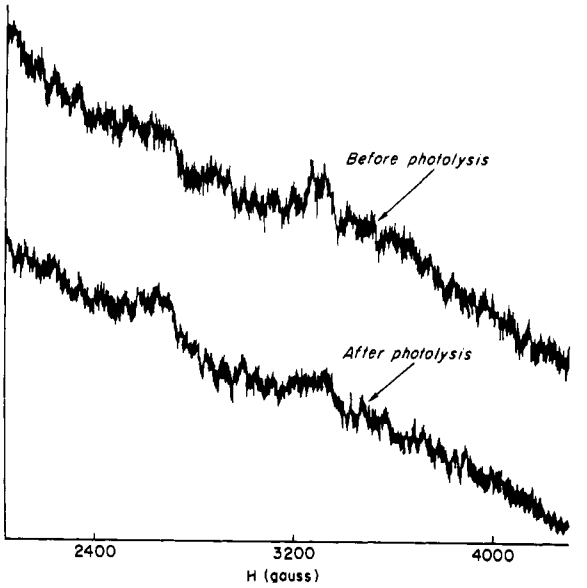

FIGURE 5: Electron paramagnetic resonance spectroscopy of the enzyme–DMBC complex before and after photolysis. The sample, containing 5.9 nmoles of ethanolamine deaminase, 12 nmoles of DMBC, and 1.2 μmoles of potassium phosphate buffer (pH 7.4) in a total volume of 0.2 ml, was placed in a quartz sample tube, frozen in liquid nitrogen (77°K), and subjected to electron paramagnetic resonance spectroscopy. All operations were conducted in dim light. The sample was then thawed, irradiated for 10 min with light from a 100-W tungsten lamp at a distance of 10 cm, refrozen in liquid nitrogen, and the spectrum was repeated. Finally, the sample was again thawed, allowed to stand at room temperature in ambient light for 1 hr, then frozen as before and subjected to electron paramagnetic resonance spectroscopy; the spectrum, not shown, was essentially identical with the spectrum observed immediately after photolysis.

protons in the region of δ 3.5–5.0 as well as eight protons at δ 2.0–2.5 and twelve at δ 1.0–1.5. Failure to observe an additional proton was attributed to line broadening due to keto-enol tautomerization. On the basis of these results, the compound was assigned the structure shown in Figure 3. This structure was chosen, rather than a structure in which the dimedon residues were linked by a $-C(=CH_2)-$ or $=C(CH_3)-$ group, because of the appearance of three nonequivalent protons in the nuclear magnetic resonance spectrum as well as the absence of a vinyl proton signal in the infrared spectrum.

Isolation and Chromatography of Cyanocorrinoids. Incubations were carried out in 1-ml glass centrifuge tubes as described below. After terminating the reactions with 0.1 ml of 6% (w/v) trichloroacetic acid and removing precipitated protein by centrifugation, the corrinoids were extracted from the reaction mixture with approximately 0.05 ml of 80% phenol–water. The phenol extract was washed with 0.5 ml of water, adding more phenol as necessary to form a two-phase solution. The extract was then dissolved in 0.5–0.7 ml of water and 10 μl of 1.4 M KCN was added to convert the corrinoids into the cyano derivatives. Extraction of the corrinoids back into phenol (*ca.* 0.05 ml) yielded a solution with the characteristic violet color of dicyanocobalamin. The dicyano derivatives were converted into the monocyano compounds by washing the phenol extract with 0.1 ml of 1 N HCl. The phenolic solution of corrinoids was then applied to a plate of silica gel F684 (Merck) and subjected to chromatography, using *sec*-butyl alcohol–water–concentrated ammonium hydroxide (100:36:14, v/v) as the developing solvent (Morley *et al.*, 1968).

Results

Spectra. The derivatives studied included MeB12, EtB12, BB12, IB12, and DMBC. Spectra of the complexes[2] between enzyme and each of these derivatives were taken before and after photolysis. In these experiments, enzyme, buffer, and ethanolamine (or in the case of DMBC, 1-aminopropanol-2, an inhibitor competitive with ethanolamine) were added to the cuvet, and the spectrum was recorded in the absence of cobalamin. The B12 derivative was then added, taking care to perform the addition in dim light and the spectrum was again recorded. Finally, the cuvet containing the enzyme–B12 complex was irradiated for 6 min at a distance of 10 cm with the light from a 100-W tungsten lamp, after which the spectrum of the photolyzed complex was recorded. The experiment was conducted entirely in air. In the case of MeB12, the spectrum was taken several times after photolysis, the final spectrum being recorded 50 min after the end of the period of photolysis.

[2] A previous study has shown that all of the cobalamins used in the present investigation affect the activity of ethanolamine deaminase–DMBC is a cofactor, while each of the other four compounds is a powerful inhibitor (Babior, 1969). Thus, each of the compounds forms a complex with the enzyme. Estimates based on the results of that study indicate that except in the cases of the spectrophotometric experiments with DMBC and IB12, in which about half the cobalamin was bound to enzyme, over 90% of the cobalamin in the reaction mixtures was in the form of the enzyme–cobalamin complex.

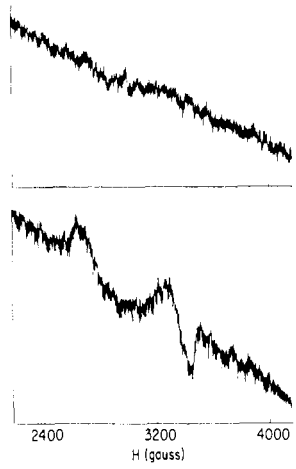

FIGURE 6: Electron paramagnetic resonance spectroscopy of the enzyme–MeB_{12} complex before and after photolysis. The composition of the sample was as described in Figure 5, except that MeB_{12} was used instead of DMBC. The experiment was conducted exactly as described in Figure 5. Above, before photolysis; below, after photolysis.

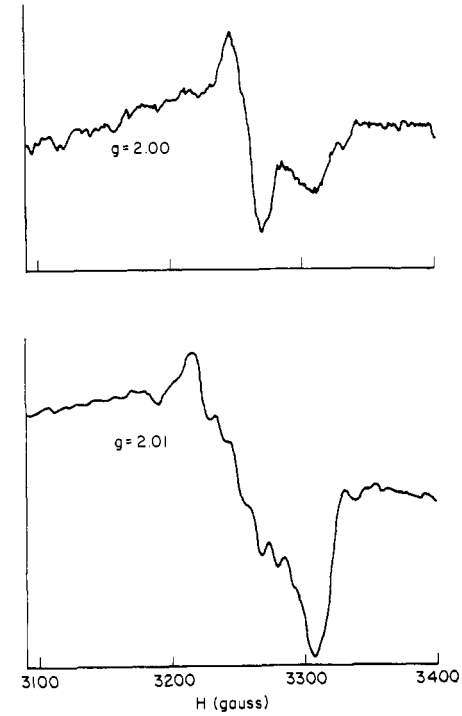

FIGURE 7: The signal at $g = 2.00$ produced by the photolysis of the enzyme–MeB_{12} complex at higher resolution. The samples were placed in quartz sample tubes, irradiated for 10 min with light from a 100-W tungsten lamp at a distance of 10 cm, and subjected to electron paramagnetic resonance spectroscopy. Above, electron paramagnetic resonance spectrum of a dilute sample. The sample contained 3 nmoles of ethanolamine deaminase, 6 mmoles of MeB_{12}, and 2 μmoles of potassium phosphate buffer (pH 7.4) in a total volume of 0.26 ml. Gain = 4×. Below, electron paramagnetic resonance spectrum of a concentrated sample. The sample contained 12.4 nmoles of ethanolamine deaminase, 25 nmoles of MeB_{12}, and 2 μmoles of potassium phosphate buffer (pH 7.4) in a total volume of 0.23 ml. Gain = 1×.

As shown in Figure 4, photolysis of the complexes formed between the enzyme and EtB12, IB12, or DMBC gives rise to products which have spectra closely resembling the spectrum of hydroxocobalamin, although in the case of DMBC, the band in the region of 350 mμ is less intense compared with the rest of the spectral bands than is observed with hydroxocobalamin. In contrast, the spectrum of the photolyzed enzyme–MeB12 complex is not like the spectrum of hydroxocobalamin at all, but is very similar to the spectrum of B12r, the paramagnetic species produced by the anerobic photolysis of alkylcobalamins. This spectrum is unchanged after 50 min despite the fact that the entire experiment, including photolysis, was conducted in air. Differing from all these results is the finding that the spectrum of the irradiated enzyme–BB12 complex is identical with the spectrum of the unirradiated complex, indicating that BB12 is not photolyzed under these conditions when bound to ethanolamine deaminase.

The spectrum of a free alkylcobalamin is indistinguishable from that of the enzyme-bound compound. However, with the free cobalamins, the spectrum observed after photolysis under the conditions described above was in every case that of hydroxocobalamin.

Electron Paramagnetic Resonance. Because of the resemblance between the spectrum of the photolyzed MeB12–enzyme complex and the spectrum of B12r, electron paramagnetic resonance spectroscopy was performed on the complex before and after photolysis to establish whether a stable paramagnetic species was in fact produced or whether the similarity between the two spectra was merely coincidence. In addition, electron paramagnetic resonance spectroscopy was performed on the complexes formed with the other cobalamins, to determine whether the photolysis of any of these complexes leads to the formation of a stable paramagnetic species. As with the spectrophotometric studies, all these experiments were performed in air.

No paramagnetic resonance was detected in the unirradiated complexes of enzyme with MeB12, EtB12, BB12, or IB12. However, two small signals are observed with the unirradiated enzyme–DMBC complex (Figure 5). These signals, one in the vicinity of $g = 2.4$ and the other in the region of $g = 2.00$, are very similar to the signals obtained by Hogenkamp *et al.* upon brief photolysis of DMBC under anerobic conditions (Hogenkamp *et al.*, 1963). Following exposure to light, no signals were observed in the electron paramagnetic resonance spectra of the enzyme–EtB12 or the enzyme–IB12 complexes, nor was there a change in the electron paramagnetic resonance spectrum of the enzyme–DMBC complex (Figure 5). With the other complexes, however, new paramagnetic species appeared after irradiation.

Figure 6 shows the electron paramagnetic resonance spectrum of the enzyme–MeB12 complex before and after photolysis. The photolysis of this complex leads to the appearance of two large signals. It is noteworthy that these signals, like the signals of the enzyme–DMBC complex, closely resemble the signals generated by the anerobically photolyzed free coenzyme. No change in the spectrum is produced by allowing the photolyzed complex to stand for 1 hr in air at room temperature.

The signal in the region of $g = 2.00$ is shown at higher resolution in Figure 7. It is seen to consist of six peaks covering a span of 85 gauss (Figure 7, bottom). All the peaks display the

TABLE I: Labeling of Ethanolamine Deaminase by Photolysis of the Complex between Enzyme and Radioactive Alkylcobalamins.[a]

Cobalamin	Experimental Conditions			Radioactivity (cpm)			
	Atmosphere	Ethanolamine	Photolysis	Supernatant	1st Wash	2nd Wash	Precipitate
[^{14}C]MeB$_{12}$	Air	+	+	1262	41	6	82
	Air	−	+	1214	53	6	63
	N_2	+	+	177	17	8	80
	N_2	−	+	195	14	5	102
	Air	+	−	1869	97	12	32
	Air	−	−	1866	91	16	31
[^{14}C]EtB$_{12}$	Air	+	+	2386	87	8	8
	Air	−	+	2823	99	21	12
	N_2	+	+	1154	52	6	18
	N_2	−	+	1899	52	5	31

[a] Reaction mixtures contained 0.3 nmole of ethanolamine deaminase, 0.6 nmole of Co-[^{14}C]methylcobalamin (1.8 mCi/mmole) or Co-[1,2-^{14}C]3-hydroxyethylcobalamin (4.2 mCi/mmole), 0.5 μmole of potassium phosphate buffer (pH 7.4) and, where noted, 50 nmoles of ethanolamine, in a total volume of 0.03 ml. Incubations were conducted for 8 min at 23° in air (aerobic) or in an atmosphere of 100% N_2 (anerobic). Samples to be photolyzed were incubated for 2 min in the dark, and then were irradiated for 6 min with a 100-W tungsten lamp at a distance of 10 cm. Other samples were incubated in the dark for 8 min. The reactions were then terminated and the samples were treated as described in Materials and Methods.

same saturation behavior, suggesting that they represent hyperfine structure of a signal generated by a single species. This signal is stable for many hours in air at 3°. However, when the sample was diluted by a factor of 5 (final enzyme concentration 11 μM), the complex signal originally observed was replaced by a much simpler signal, consisting of a large peak 25 gauss in width and a smaller, broader satellite which saturated with the large peak and is separated from it by 50 gauss. A similar signal was produced by direct irradiation of the enzyme–MeB12 complex at a lower concentration (Figure 7, top). This signal too, was stable for hours in air at 3°. In contrast to the behavior of the signal at $g = 2.00$, the low-field signal is unchanged by dilution, except for an appropriate decrease in amplitude.

On exposure of the BB12–enzyme complex to light under the conditions used for the photolysis of the other complexes (see Figure 5), no signal is observed (Figure 8). However, photolysis of the complex in liquid nitrogen (77°K), using a 650-W quartz iodine lamp in a parabolic reflector at a distance of 15 cm, leads to the gradual appearance of a narrow peak in the region where a free-radical signal would appear. If the photolyzed complex is thawed and refrozen, this peak disappears. Further exposure of the frozen complex to light from the 650-W lamp causes the narrow peak to reappear, and in addition, a broad second signal resembling one of the signals of the photolyzed enzyme–MeB12 complex begins to emerge. Thawing this photolyzed complex again abolishes the narrow peak, which now is replaced by a smaller but broader signal resembling the second signal observed after photolysis of the MeB12–enzyme complex. Both of the broad signals are stable to thawing, as observed with the MeB12–enzyme and DMBC–enzyme complexes.

Fate of the Cobalt-Linked Alkyl Groups on Photolysis of the MeB12- and EtB12–Enzyme Complexes. Since the initial step in the light-induced cleavage of free alkylcobalamins involves the production of an alkyl radical, it is probable that a similar fragment is produced during the photolysis of the enzyme–B12 complex. Such a highly reactive fragment might be expected to react with nearby groups on the enzyme. If the fragment were radioactive, a means would thereby be provided for labeling amino acid residues in the vicinity of the active site.

To investigate this possibility, experiments were carried out using MeB12 and EtB12 labeled in the cobalt-linked alkyl groups. Enzyme–B12 complexes prepared with these derivatives were photolyzed, and the enzyme was subsequently precipitated with trichloroacetic acid and analyzed for radioactivity by liquid scintillation counting. The results, shown in Table I, indicate that under a wide variety of experimental conditions, no radioactivity is transferred to the enzyme from EtB12, while MeB12 only a small amount of the radioactivity appears in the enzyme after photolysis of the complex. The poor recoveries of radioactivity observed in the experiments involving the anaerobic photolysis of the MeB12–enzyme complexes can probably be attributed to the conversion of the methyl group to highly volatile low molecular weight hydrocarbons such as methane and ethane.

Since photolysis of these enzyme–B12 complexes result in little or no transfer of the organic fragments to the enzyme, the fragments must either have combined with the cobalamin group or been released as small molecules. Experiments were therefore conducted to determine the identity of the products ultimately arising from these fragments. In particular, the products arising from the photolysis of the enzyme–B12 complexes were compared with those formed upon photolysis of the free alkylcobalamin.

The experiments in Table II show that the photolysis of the enzyme–B12 complex does not lead to the combination of the organic fragment with the cobalamin residue. In these experi-

TABLE II: Labeling of the Cobalamin Moiety by Photolysis of the Complex between Enzyme and Radioactive Alkylcobalamins.[a]

Cobalamin	Experimental Conditions		Radioactivity Taken Up by Column (% of Total)
	Enzyme	Photolysis	
[^{14}C]MeB$_{12}$	+	−	92.2
	+	+	7.1
	−	−	91.9
	−	+	3.2
[^{14}C]EtB$_{12}$	+	−	98.1
	+	+	35.3
	+	+	6.0[b]
	−	−	93.8
	−	+	1.6

[a] The reaction mixtures contained 0.3 nmole of ethanolamine deaminase, 0.6 nmole of radioactive MeB$_{12}$ or EtB$_{12}$, 0.5 μmole of potassium phosphate buffer (pH 7.4), and 50 nmoles of ethanolamine in a total volume of 0.03 ml. Incubations were conducted for 8 min at 23°. Samples to be photolyzed were incubated for 2 min in the dark, and then irradiated for 6 min with a 100-W tungsten lamp at a distance of 10 cm. Other samples were incubated in the dark for 8 min. The reactions were then terminated and the samples were treated as described in Materials and Methods. As controls, identical experiments were carried out, except that enzyme was omitted from the reaction mixtures. [b] Irradiated for 18 min.

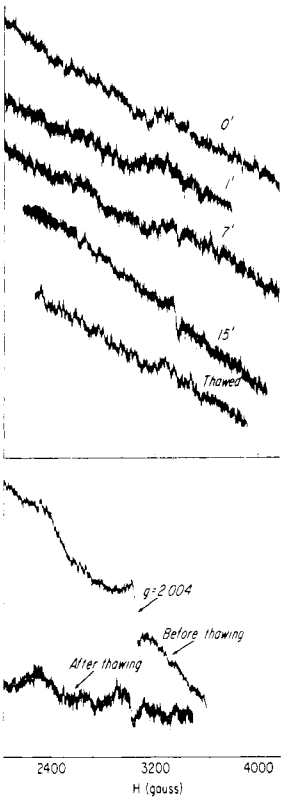

FIGURE 8: Electron paramagnetic resonance spectroscopy of the enzyme–BB$_{12}$ complex before and after photolysis. The sample contained 5.9 nmoles of ethanolamine deaminase, 12 nmoles of BB$_{12}$, and 1.2 μmoles of potassium phosphate buffer (pH 7.4) in a total volume of 0.2 ml. Above, generation of unpaired electrons by irradiation with a quartz-iodine lamp at 77°K. The sample was placed in a quartz tube, frozen in liquid nitrogen (77°K), and subjected to electron paramagnetic resonance spectroscopy. All operations were conducted in dim light. The sample was then thawed, irradiated for 10 min with light from a 100-W tungsten lamp at a distance of 10 cm, refrozen in liquid nitrogen, and the spectrum was repeated (top spectrum, labeled 0'). The frozen sample, cooled in liquid nitrogen, was then irradiated with light from a quartz-iodine lamp as described in the text. Electron paramagnetic resonance spectra were taken after 1-, 7-, and 15-min irradiation. The sample was then thawed, refrozen immediately, and a fifth spectrum was taken. Below, spectrum after prolonged photolysis with a quartz-iodine lamp at 77°K. After the fourth spectrum was taken, the sample was stored in the dark at −20° for 16 hr. It was then placed in liquid nitrogen and irradiated for an additional 20 min with the quartz-iodine lamp as described in the text, following which the electron paramagnetic resonance spectrum was again taken. The sample was then thawed, refrozen immediately, and a final spectrum was obtained.

ments, reaction mixtures containing labeled alkyl B12 together with various combinations of enzyme and ethanolamine were subjected to chromatography on Amberlite CG-50 before and after exposure to light. Under the conditions of the experiment, B12 derivatives are taken up by the column from the reaction mixture, and can only be eluted with base. If the radioactive fragment produced by photolysis had combined with the cobalamin group, the radioactivity would have been taken up by the column both before and after photolysis. The results however, demonstrate that in every case the radioactivity was only taken up from the unphotolyzed reaction mixture.

These experiments also show that the binding of EtB12 to enzyme appears to decrease the susceptibility of this cobalamin to photolysis (compare rows 6 and 9, Table II). This may be compared with the effect on the photolysis of BB12 produced by binding of this cobalamin to enzyme; BB12 appears to be quite stable to light when bound to ethanolamine deaminase, though it is rapidly photolyzed when free (*cf.* Figure 4).

The identity of the radioactive species released by the photolysis of labeled MeB12 and EtB12 both in the presence and absence of enzyme was investigated by adding various nonradioactive carrier compounds to aliquots of the acidic (first) fractions obtained in the experiments reported in Table II, preparing crystalline derivatives of these carrier compounds and recrystallizing the derivatives to constant specific activity. Formaldehyde was used as carrier with MeB12, and formaldehyde, acetaldehyde, and glycolaldehyde were used with EtB12. The results (Table III) show that while formaldehyde is the major radioactive product arising from the photolysis of the enzyme–MeB12 complex, it is only a minor fraction of the products of photolysis of free MeB12.[3] Similarly, acetaldehyde is the major product of photolysis of the enzyme–EtB12 complex, while relatively little of it appears on photolysis of the

[3] The difference between these results and those obtained by Hogenkamp (1966) can probably be explained by the variation in the reaction conditions employed in the two sets of experiments.

TABLE III: Identity of the Compounds Released by the Photolysis of Free and Enzyme-Bound MeB_{12} and EtB_{12}.[a]

Cobalamin	Experimental Conditions: Carrier	Enzyme	Ethanolamine	Specific Activity: Initial	After Recrystzn: 1st	2nd	3rd	Fraction of Radioactivity Cocrystallizing with Derivative (%)
[^{14}C]MeB_{12}	Formaldehyde[b]	+	+	2.7	2.0	1.9		72
	Formaldehyde	+	+	4.1	3.1	3.1		76
	Formaldehyde	+	−	4.1	2.7	2.6		65
	Formaldehyde[b]	−	+	3.0	0.4	0.5		15
	Formaldehyde	−	+	4.7	1.0	1.4		26
	Formaldehyde	−	−	4.3	0.7	0.7		16
[^{14}C]EtB_{12}	Acetaldehyde	+	+	5.2	4.3	4.4	4.1	82
	Acetaldehyde	+	+	6.0	5.2	5.2	5.0	86
	Acetaldehyde	+	−	5.9	4.7	4.8	4.6	80
	Acetaldehyde	−	+	9.1	2.6	2.4	2.3	27
	Acetaldehyde	−	−	7.5	2.6	2.5	2.4	33
	Glycolaldehyde	+	+	5.6	0.9	0.4	0.3	6
	Glycolaldehyde	+	+	6.4	1.1	0.6	0.5	9
	Glycolaldehyde	+	−	6.3	0.8	0.4	0.4	6
	Glycolaldehyde	−	+	9.8	1.0	0.8	0.7	8
	Glycolaldehyde	−	−	8.1	1.3	1.3	0.7	12
	Formaldehyde	−	−	9.8	1.2	1.2	1.3	13

[a] The dimedon derivatives were prepared and crystallized as described in Materials and Methods. With carrier formaldehyde, the volume of the aliquot used in the identification was 1.5 ml, and with acetaldehyde and glycolaldehyde the volume was 0.75 ml; in every case, the total volume of the first fraction was 2 ml. Except where noted, the amount of carrier formaldehyde added to the aliquot was 40 mg; the amounts of acetaldehyde and glycolaldehyde were 39 and 50 mg, respectively. [b] The amount of formaldehyde used in these experiments was 60 mg.

free cobalamin. Only small amounts of formaldehyde and glycolaldehyde were produced by the photolysis of EtB12.

Fate of the Cobalamin Residue. To determine whether the photolysis of the enzyme–alkyl B12 complex produces a change in the cobalamin moiety, experiments were conducted in which corrinoids were recovered from irradiated alkylcobalamins or enzyme–B12 complexes and subjected to thin-layer chromatography. Reaction mixtures containing 1.5 nmoles of ethanolamine deaminase, 3.0 nmoles of alkylcobalamin, and 1 μmole of potassium phosphate buffer (pH 7.4) in a total volume of 0.1 ml were incubated in the dark at room temperature for 2 min, then exposed for 10 min to light from a 100-W tungsten lamp at a distance of 10 cm. The incubations were then terminated and the corrinoids were isolated and converted into the cyano derivatives as described in Materials and Methods. Control experiments were also performed in which enzyme was omitted. Three alkylcobalamins were used in these experiments: MeB12, EtB12, and DMBC. In every case, the corrinoid recovered from the reaction mixture migrated as a single spot with an R_F identical with that of cyanocobalamin within the limits of experimental error.

Discussion

The foregoing experiments show that there are remarkable differences between the course of photolysis of free alkylcobalamins and that of the same compounds when they are bound to ethanolamine deaminase. Equally striking are the differences among the various enzyme–B12 complexes; while the aerobic photolysis of the free cobalamin always gives rise to hydroxocobalamin, exposure of enzyme–B12 complexes to light leads in some cases to the production of species giving electron paramagnetic resonance signals, in other cases to species giving no signal, while in one case no change is observed unless extreme conditions are used.

In each case in which electron paramagnetic resonance signals were observed, these signals were very similar to those generated by the anerobic photolysis of DMBC (Hogenkamp *et al.*, 1963). This process, as mentioned before, gives rise to B12r. The enzyme thus appears to be capable of stabilizing the reduced cobalamin initially produced in the photolytic reaction, a species which is ordinarily oxidized fairly rapidly in the presence of air (Bernhauer *et al.*, 1964). Included among the causes to be considered for this stabilization are a special steric relationship between the reduced cobalamin and the enzyme, excluding oxygen from the vicinity of the unpaired electron, or a change in the redox potential of the B12r–hydroxocobalamin couple induced by binding to the enzyme. In any case, the fact that the enzyme is able to stabilize this form of B12 once again raises the possibility that this and other coen-

zyme B12 dependent reactions may proceed by a free-radical mechanism (Eggerer *et al.*, 1960).[4]

The change in the characteristics of the high-field signal which occurs on dilution of the photolyzed enzyme–MeB12 complex clearly indicate that the environment of the unpaired electron responsible for this signal has undergone a substantial change. The width and position of the signal in the dilute complex suggests that the signal may represent a free radical, with little delocalization of the electron over nearby magnetic nuclei. Anisotropy could account for the low, broad signal seen at slightly higher field strength.

The failure of enzyme-bound BB12 to be affected by light under conditions in which all the other complexes were photolyzed can probably be attributed to a situation in which the carbon–cobalt bond is actually cleaved by light, but the two fragments, bound tightly to the enzyme and thereby constrained to remain in each other's vicinity, recombine immediately with the result that there is no net loss of BB12. The alternative explanation that the enzyme prevents the homolytic cleavage of the carbon–cobalt bond by light is difficult to reconcile with the appearance of the free-radical signal upon photolysis of the frozen complex with the quartz-iodine lamp (Figure 8) and the absorption of light by the complex which is observed at wavelengths which will destroy free BB12, both of which suggest that the carbon–cobalt bond in the complex is cleaved by light.

On the photolysis of enzyme-bound MeB12 and EtB12, the cobalt-bound alkyl groups give rise primarily to formaldehyde and acetaldehyde, respectively. In contrast, relatively little of these compounds appear among the products of photolysis of free alkylcobalamins. It is therefore clear that there are significant differences between the products of photolysis of the free and enzyme-bound alkylcobalamins.

Formaldehyde is a compound which might be expected to be formed by oxidation of the methyl radical released by the photolysis of MeB12. However, a mechanism for the production of acetaldehyde is more difficult to formulate, particularly in view of the observation that hydroxocobalamin is also produced in this reaction while B12r is produced by the photolysis of enzyme-bound MeB12. The mechanism of this reaction will be the subject of further studies.

[4] In support of this hypothesis, it has subsequently been shown that an electron paramagnetic resonance signal is generated in the dark by the enzyme–DMBC complex in the presence of substrate (Babior and Gould, 1969).

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