The Photolysis of Methylcobalamin*

H. P. C. Hogenkamp

ABSTRACT: The photodecomposition of methylcobalamin has been studied in aqueous solution at different oxygen concentrations. In the presence of excess oxygen methylcobalamin is photolyzed at a rapid rate to yield aquocobalamin and formaldehyde as the major products. On the other hand, photolysis in the presence of only a trace of oxygen is slow and yields vitamin B_{12r}, formaldehyde, methane, and ethane. The yield of methanol is small and is not affected to an appreciable extent by the oxygen concentration. At limiting oxygen concentrations many side reactions take place which probably lead to modification of the corrin nucleus. The major secondary reactions involving the methyl radical appear to be reaction with oxygen to yield a methyl peroxide radical or formaldehyde and a hydroxyl radical.

The very rapid oxidation of vitamin B_{12r}, during photolysis of methylcobalamin in the presence of oxygen, has been attributed to the reaction of vitamin B_{12r} with either a hydroxyl radical or an alkyl peroxide radical. Ethane does not appear to be a product of radical coupling. Both methane and ethane are probably formed *via* hydrogen or methyl abstraction from the corrin ring by a methyl radical.

The corrinoids containing a carbon–cobalt covalent bond are decomposed by exposure to visible light. Photolysis of coenzyme B_{12} (5′-deoxyadenosylcobalamin) in the presence of oxygen results in the formation of aquocobalamin, adenosine-5′-aldehyde (adenine-9-β-D-*ribo*-pentofuranosyldialdose), and 8,5′-cyclic-adenosine. During the photolytic cleavage of the organometallic bond of coenzyme B_{12} approximately 0.75 mole of oxygen is consumed/mole of coenzyme B_{12} photolyzed. If the photolytic cleavage is carried out in the absence of oxygen, vitamin B_{12r}[1] and 8,5′-cyclic-adenosine are the major products (Hogenkamp, 1964).

The alkylcobalamins are very rapidly decomposed by light in the presence of oxygen. Wagner and Bernhauer (1964) isolated formaldehyde as the dimedon derivative in nearly quantitative yield as a product of photolysis of methylcobalamin, while Hogenkamp (1964) showed the production of formaldehyde in 84% yield by the chromotropic acid method. Weissbach *et al.* (1963) isolated radioactive methanol and formaldehyde in 10 and 20% yields, respectively, after photodecomposition of ^{14}C-methylcobalamin. In the absence of oxygen (10^{-6} mm) the alkylcobalamins are stable in light of moderate intensity, but in the presence of a small amount of air (10^{-4} mm) the alkylcobalamins are photolyzed, giving vitamin B_{12r} and a mixture of olefins and paraffins (Dolphin *et al.*, 1963). If the photolytic decomposition of methylcobalamin is carried out in the absence of oxygen, but in the presence of a thiol, such as homocysteine or cysteine, the methyl radical is trapped to yield methionine or *S*-methylcysteine, respectively (Johnson *et al.*, 1963b). These observations suggest that the photolytic cleavage of coenzyme B_{12} and the alkylcobalamins involves the homolytic fission of the carbon–cobalt bond with the production of vitamin B_{12r} and a 5′-deoxyadenosyl or alkyl radical. Pratt (1964) reported that the rate of oxidation of vitamin B_{12r} by air is smaller than the rate of photolysis of methylcobalamin in the presence of air. These results indicate that vitamin B_{12r} reacts with an oxidizing agent formed during the photolysis of methylcobalamin in the presence of oxygen. A methyl peroxide radical was suggested as the oxidizing agent. Very little is known, however, about the secondary reactions involving the radicals. The present study was undertaken to determine the fate of the alkyl radicals as a function of oxygen concentration. A series of reactions is proposed to account for the formation of the photolysis products under various conditions.

Experimental Procedures

Materials. Vitamin B_{12} was purchased from Sigma Chemical Co. and ^{14}C-methyl bromide, specific activity approximately 1 μcurie/μmole, was obtained from Merck Sharp and Dohme of Canada, Ltd. Methylcobalamin and ^{14}C-methylcobalamin were prepared by treating methyl iodide or ^{14}C-methyl bromide with hydridocobalamin; the desired products were purified by chromatography and crystallized from aqueous acetone (Hogenkamp and Oikawa, 1964). Formaldehyde was prepared from hexamethylenetetramine as described by MacFayden (1945).

Photolysis Technique. In the photolysis experiments 1 or 2 ml of an unbuffered aqueous solution of methylcobalamin in a 15-ml flask, sealed with a rubber serum cap, was exposed to a 375-w Photo-EBR General

* From the Department of Biochemistry, University of Iowa, Iowa City, Iowa. *Received September 13, 1965.* Supported by Public Health Service Research Grant AM-08627 from the National Institutes of Health.

[1] Vitamin B_{12r} denotes reduced vitamin B_{12} containing divalent cobalt.

Electric light at a distance of 20 cm. The reaction flasks were cooled by a stream of cold air; during illumination for 1 hr the temperature of the solution rose to approximately 35°. When oxygen was removed from the methylcobalamin solution prior to photolysis, a hypodermic needle was inserted through the serum stopper and connected to a water aspirator and a tank of argon, both controlled by a two-way stopcock. The vessel was evacuated (30 mm) for 5 min and then filled with argon, this procedure was repeated 10 times, and the vessel was finally filled with argon at a slightly positive pressure. When the air above the methylcobalamin solution was replaced with argon, without prolonged evacuation, an oxygen concentration of approximately 0.26 μmole/ml of solution was obtained (Loomis, 1928).

Analyses. Radioactivity was measured in a Packard Model 3003 Tri-Carb liquid scintillation spectrometer. Aqueous samples were counted in the scintillation solvent of Bray (1960); counting efficiency 62.5%. Other samples were dissolved in a scintillation fluid containing 5 g of 2,5-diphenyloxazole (PPO)[2] and 0.3 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl-POPOP)/l. of toluene (Hayes, 1963); counting efficiency 79%.

The identity of the gases, formed during photolysis, was determined with a Beckman GC-2 gas chromatograph which contained a silica gel column connected to a hydrogen flame detector (Wolin *et al.*, 1963). Gas samples were removed from the reaction vessels through the serum cap by means of a hypodermic needle attached to a syringe. Total radioactivity of the gas phase was determined by removing 1.0 ml of the gas and bubbling it very slowly through 15 ml of the toluene scintillation fluid. The radioactivity of the individual gases was determined with a Packard gas chromatograph, containing a silica gel column. From this column a portion of the sample was directed *via* a stream splitter to a hydrogen flame ionization detector; another portion of the sample was directed to a Packard combustion furnace from which it entered a Tri-Carb liquid scintillation spectrometer (efficiency 0.93%).

For formaldehyde measurements a 0.1-ml aliquot of the photolysis reaction mixture was added to 25 ml of formaldehyde solution (60 μmoles/ml, pH 4.6). The formaldehyde was isolated as the dimedon derivative and was crystallized twice from aqueous ethanol. A sample of the dimedon derivative was then used for determining radioactivity.

Methanol was measured by dissolving a 0.1-ml aliquot of the reaction mixture in 5 ml of pyridine to which exactly 0.20 ml of anhydrous methanol was added as a carrier. Five grams of *p*-nitrobenzoyl chloride dissolved in 50 ml of methylene chloride was slowly added and allowed to react for 12 hr. After this time the excess *p*-nitrobenzoyl chloride was destroyed by the addition of a chip of ice. The reaction mixture was extracted with four 100-ml volumes of saturated sodium bicarbonate solution and two 100-ml volumes of 2 N sulfuric acid, and washed twice with 100 ml of water. The solution was dried over sodium sulfate and evaporated to dryness under reduced pressure. The dry residue was extracted with hot 95% ethanol; on cooling, methyl *p*-nitrobenzoate crystallized from the extract; a fraction of the dry crystalline derivative was used for radioactivity measurements.

Formic acid was determined by the enzymic method of Rabinowitz and Pricer (1957). To estimate the total amount of organic acids formed, 0.5 ml of the photolysis reaction mixture was extracted five times with 2 ml of ethyl ether, and the aqueous solution was then acidified to pH 1 with 1 N hydrochloric acid and again extracted four times with 1 ml of ethyl ether. The second ether extracts were added to a solution of 0.1 M sodium bicarbonate and the ether was evaporated. The aqueous solution was made up to 10 ml with water; 0.1-ml aliquots were used to determine radioactivity.

For the determination of carbon dioxide 252 mg of sodium bicarbonate was added to 0.5 ml of the photolysis reaction mixture, 1 ml of 1 M barium chloride and 1 ml of 1 M ammonium chloride were added, and barium carbonate was isolated by filtration. The filter pad was washed with ethanol and dried. A known amount of dry barium carbonate was converted to potassium carbonate using a Warburg vessel with 0.5 ml of 2 N H_2SO_4 in the side arm, 0.5 ml of 6 N KOH in the center well, and barium carbonate suspended in 1 ml of water in the main compartment; after addition of the acid, the vessel was shaken for approximately 15 min. A 0.05-ml aliquot of the center well was used for measuring radioactivity.

Another 0.5-ml aliquot of the photolysis reaction was applied to a 5 × 0.5 cm column of Dowex 50, 2% cross linked, 200–400 mesh, pH 3, in the sodium form. The column was washed with 10 ml of water and treated with 10 ml of 0.1 M sodium acetate, pH 6.4, to remove unphotolyzed methylcobalamin. Hydroxocobalamin and corrinoids with similar charge were eluted with 0.1 N ammonium hydroxide, the eluate was made up to 10 ml with water, and 0.1-ml aliquots were used for measuring radioactivity.

Visible and ultraviolet spectral measurements were made with a Zeiss PMQ II spectrophotometer. Methylcobalamin concentration was determined from its absorbance and the molar extinction coefficient of 7.6 × 10^3 M^{-1} cm^{-1} at 525 mμ (Johnson *et al.*, 1963a). Hydroxocobalamin concentration was determined from its absorbance in 0.1 N ammonium hydroxide and the molar extinction of 9.3 × 10^3 M^{-1} cm^{-1} at 535 mμ (H. P. C. Hogenkamp and J. E. Rush, 1965, unpublished data).

Results

Effect of Oxygen Concentration on the Photolysis Products. The results of a series of experiments shown in Table I indicate that the oxygen concentration during the photolytic cleavage of the carbon–cobalt bond profoundly affects the nature of the photolysis products.

[2] Abbreviations used: PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

TABLE I: Effect of Oxygen Concentration on the Products of the Photolysis of Methylcobalamin.[a]

	Expt No.				
	1	2	3	4	5
			Oxygen Concn (μmole)		
Photolysis Products	Excess[b]	0.52	0.26	Trace	Excess
Gaseous products	0.008 (1.5)[c]	0.041 (7.7)	0.050 (9.4)	0.095 (18)	
Products in solution	0.46 (87)	0.40 (76)	0.36 (68)	0.27 (51)	
Formaldehyde	0.40 (76)	0.33 (62)	0.25 (47)	0.12 (23)	
Methanol	0.010 (1.8)	0.0054 (1.0)	0.0049 (0.9)	0.0049 (0.9)	
Corrinoids[d]	0.002 (0.4)	0.018 (3.2)	0.022 (4.2)	0.033 (6.2)	
Organic acids[e]					0.010 (1.9)
CO_2					0.0007 (0.13)
Recovery	0.468 (88)	0.441 (83)	0.410 (77)	0.365 (69)	

[a] Values are given in microcuries. Each reaction mixture contained 0.57 μmole of methylcobalamin, specific activity 0.93 μcurie/μmole. Reaction 1, 3, 4, and 5 contained 1 ml and reaction 2 contained 2 ml of methylcobalamin solution. [b] Photolyzed in the presence of air. [c] The figures in parentheses denote the percentage of the radioactivity recovered (0.53 μcurie = 100%). [d] This fraction contains all the corrinoids which behave like hydroxycobalamin on a Dowex 50 column. [e] This fraction contains approximately 50% formic acid.

Compared to photolysis in the presence of air (expt 1), more gaseous products are formed when oxygen is limited during the photolysis reaction. In contrast, in the absence of oxygen the yield of formaldehyde is greatly reduced (76% in air and 23% in the presence of only a trace of oxygen). The corrinoid fraction, which is eluted from a Dowex 50 column with 0.1 N ammonium hydroxide, contains a considerable amount of radioactivity if the photolysis is carried out in the absence of oxygen (expt 4). This fraction contains all corrinoids with a positive charge at pH 9, but primarily hydroxocobalamin. Methanol, formic acid, and carbon dioxide are minor products; the yield of methanol decreases if oxygen is limited during the photolysis. In agreement with the observations of Pratt (1964) oxygen markedly accelerates the rate of the photodecomposition of methylcobalamin. In expt 4 illumination for 60 min was required before the color of the solution had changed completely from red to brown, indicating the formation of vitamin B_{12r}. On the other hand, photolysis of an identical solution in the presence of air was completed in approximately 5 min ($k = 1.9 \times 10^2$ sec^{-1}) (Hogenkamp *et al.*, 1965). In expt 2 and 3 the methylcobalamin solutions were photolyzed for 30 min; the reddish brown color of the solutions indicated the presence of both vitamin B_{12r} and aquocobalamin. When air was admitted to reactions 2, 3, and 4 the color of the solution changed to red. The visible and ultraviolet absorption spectra of dilutions of these oxidized solutions were characteristic of those of aquocobalamin.

Identification of the Gaseous Products. If the photodecomposition of ^{14}C-methylcobalamin is carried out in the absence of oxygen, almost 20% of the label is present in the gaseous products. Radical coupling of two methyl radicals would yield ethane. To investigate the formation of this alkane 12.1 μmoles of methylcobalamin dissolved in 2 ml of water was photolyzed in the absence of oxygen. After illumination for approximately 1 hr with a 375-w Photo-EBR lamp, the color of the solution slowly changed from red to brown. After two additional hours of illumination, 0.4 ml of the gas phase was withdrawn and analyzed in a Beckman GC-2 gas chromatograph, standardized with methane and ethane. Two gases emerged from the silica gel column with retention times corresponding to the two known gases. Comparison of the peak heights with those of standard gas mixtures of methane in air and ethane in air indicated that 0.58 μmole of methane and 1.1 μmoles of ethane were formed from 12.1 μmoles of methylcobalamin. No ethylene, propane, or butane could be detected. If ethane is indeed formed by radical coupling of two methyl radicals, the specific activity of ethane should be twice as high as that of methane. To determine the radioactivity of the individual gases, 3.31 μmoles of ^{14}C-methylcobalamin dissolved in 1 ml of water was photolyzed in the absence of oxygen for 2 hr. After that time 0.4 ml of the gas phase was analyzed in a Packard gas chromatograph, as described in Experimental Procedures. Comparison of the peak height with those of standard gas mixtures of methane and ethane indicated that 0.25 μmole of methane and 0.40 μmole of ethane were produced. However, radioactivity measurements indicated that although the specific activity of methane (0.92 μcurie/μmole) was identical with that of the ^{14}C-methylcobalamin, the specific activity of ethane was much lower (0.50 μcurie/μmole).

Titration of the Hydroxyl Ions Released. During the photolytic reaction one of the primary photolysis products, vitamin B_{12r}, is oxidized to aquocobalamin. Concomitant with this one-electron oxidation, one

TABLE II: Effect of Oxygen Concentration on the Release of Hydroxyl Ions during Photolysis of Methylcobalamin.[a]

Methylcobalamin Used (μmoles)	Hydroxyl Ions Released (μequiv)
0.54	0.55
1.08	1.04
2.16	1.91
3.24	2.74

[a] Each reaction vessel contained methylcobalamin dissolved in 5 ml of water. (Oxygen content at 25° was approximately 1.3 μmoles.) Before photolysis the pH of the solution was adjusted to 4.0 with 0.0096 N HCl. The solution was then irradiated with a 375-w incandescent lamp for 5 sec and the pH was adjusted to 4.0 with 0.096 N HCl using a microburet. The procedure was repeated until no more acid was consumed.

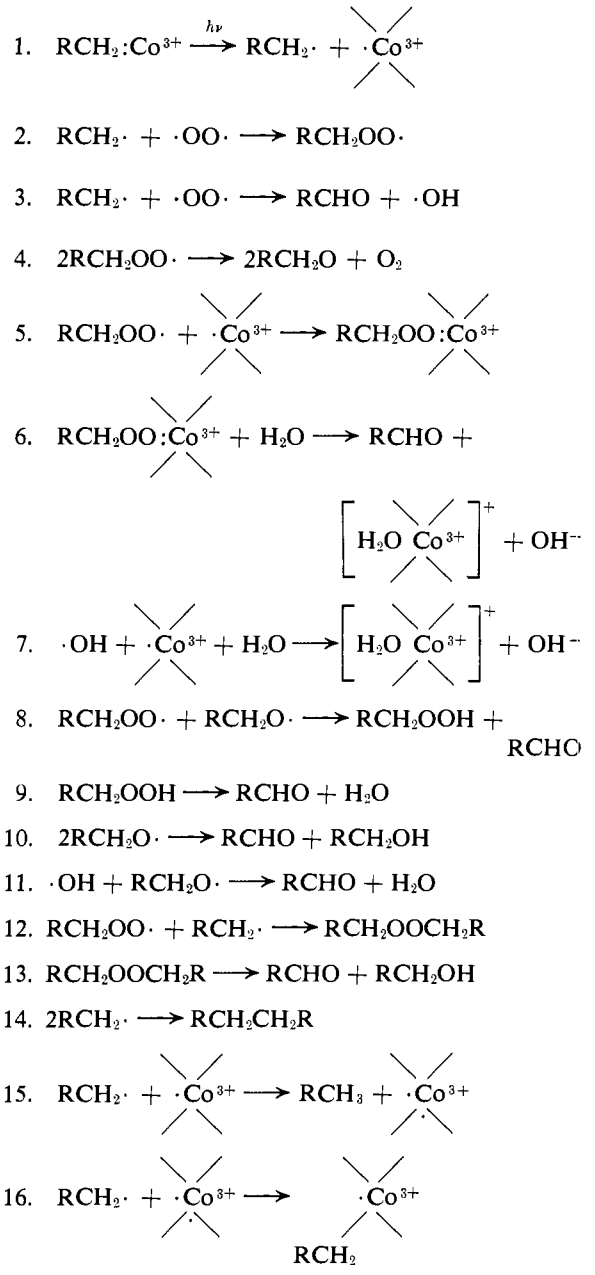
hydroxyl ion would have to be produced to maintain electrical balance. The data presented in Table II indicate that for every mole of methylcobalamin photolyzed a mole of hydroxyl ions is formed, when at least an equimolar amount of oxygen is available in solution. At limiting oxygen concentrations the yield of hydroxyl ions is lower.

Discussion

It seems reasonable that the primary reaction in the photodecomposition of the corrinoids, containing a carbon–cobalt bond, involves the homolytic cleavage of this linkage with the formation of vitamin B_{12r} and a free radical (Pratt, 1964; Hogenkamp *et al.*, 1963). If this is the case, then several products would be expected to be formed *via* the reactions of these primary products with other components of the reaction system. In Scheme I are presented the primary reaction and several secondary reactions involving both vitamin B_{12r} and the free radical.

When oxygen is available during the photolysis, the alkyl free radical can react with oxygen to yield an alkyl peroxide radical (reaction 2) or a hydroxyl radical and an aldehyde (reaction 3). Compared to the rate of reaction of the alkyl radical with oxygen, oxidation of vitamin B_{12r} by oxygen is very slow ($t_{1/2}$ = 21 min) (Pratt, 1964). As a result, most of the alkyl radicals will react with oxygen to form either peroxide radicals (reaction 2) or aldehydes and hydroxyl radicals (reaction 3), before an appreciable number of vitamin B_{12r} molecules are oxidized by oxygen to aquocobalamin. Reactions 2 and 3 are probably the initial reactions and reactions 4–13 indicate the products expected to follow the two initial reactions. Reactions 5–7 depict the

SCHEME I: Reaction Scheme for the Photolysis of Alkylcobalamins.[a]



[a] Adapted from a reaction scheme proposed by Heicklen and Johnston (1962a,b).

oxidation of vitamin B_{12r} by alkyl peroxide and hydroxyl radicals. The oxidation of vitamin B_{12r} by hydroxyl radicals would be expected to be fast, because the hydroxyl radical is particularly reactive. Reaction 5 has been suggested by Pratt (1964) to account for the fast oxidation of vitamin B_{12r} during photolysis.

The results presented in Table I show that when the photolysis is carried out in the presence of oxygen, formaldehyde and aquocobalamin are the major products, while methanol, formic acid, and carbon

dioxide are minor products. In addition to reaction 3, six reactions, presented in Scheme I, would yield formaldehyde. However, the low yield of methanol indicates that reactions 10 and 13 are minor reactions because they yield equimolar quantities of formaldehyde and methanol. If reactions 8 and 11 were major reactions, the concentration of the hydroxyl radicals or alkyl peroxide radicals available for oxidizing vitamin B_{12r} would be greatly reduced. This necessitates oxidation of vitamin B_{12r} by oxygen. However, the very fast oxidation rate of vitamin B_{12r} during photolysis indicates that oxygen is not the oxidizing agent. Thus this observation suggests that reactions 8 and 11 are minor ones. By the same argument, reaction 9, which describes the decomposition of the alkyl peroxide formed in reaction 8, would be eliminated as a major reaction yielding formaldehyde.

When the photodecomposition of methylcobalamin is carried out in the presence of only a trace of oxygen (expt 4) the major products are formaldehyde, methane, ethane, and vitamin B_{12r}. The relatively large yield (0.13 μmole, 23%) of formaldehyde is surprising because less than 0.01 μmole of oxygen is present in solution/μmole of methylcobalamin. Since formaldehyde is formed only in the presence of oxygen (approximately 0.1 μmole of oxygen is required for the oxidation of 0.13 μmole of methyl radicals to formaldehyde) an additional source of oxygen must be available. Jaselskis and Diehl (1958) reported that aquocobalamin in solution binds oxygen; if similar binding between oxygen and methylcobalamin takes place and if this bound oxygen is not removed by repeated evacuation at 30 mm, sufficient oxygen would be available for the oxidation of methyl radicals to formaldehyde. Indeed oxygen binding may also explain the observation of Dolphin *et al.* (1963) that the alkylcobalamins are insensitive to light only after all the oxygen has been removed from the solution at a high vacuum (10^{-6} mm). At 10^{-4} mm photolysis of the alkylcobalamin was reported to yield olefins and paraffins; however, the yield of aldehyde was not indicated. The formation of formaldehyde could be best explained by reactions 2–4 and 11. At low oxygen concentration reaction 12 would be expected to be a major one because insufficient oxygen would be available to react with all the alkyl radicals. Radical coupling between an alkyl radical and an alkyl peroxide radical would yield dialkyl peroxide. The very low yield of methanol indicates that this dialkyl peroxide radical, if formed, does not decompose to an aldehyde and an alcohol (reaction 13). The identification of methane as a product of the photodecomposition of methylcobalamin suggests that the methyl radical has abstracted a hydrogen atom, probably from the corrin nucleus of vitamin B_{12r} or methylcobalamin. The finding that the specific activity of methane is identical with that of ^{14}C-methylcobalamin indicates that no free ^{12}C-methyl radicals are formed. Hydrogen abstraction from the corrin nucleus is most likely to occur at C-3, C-8, C-13, and C-19 (Figure 1) because of the low carbon–hydrogen bond strengths (77 kcal/mole) (Morrison and Boyd, 1961). Such a reaction would yield a vitamin B_{12r} bi-

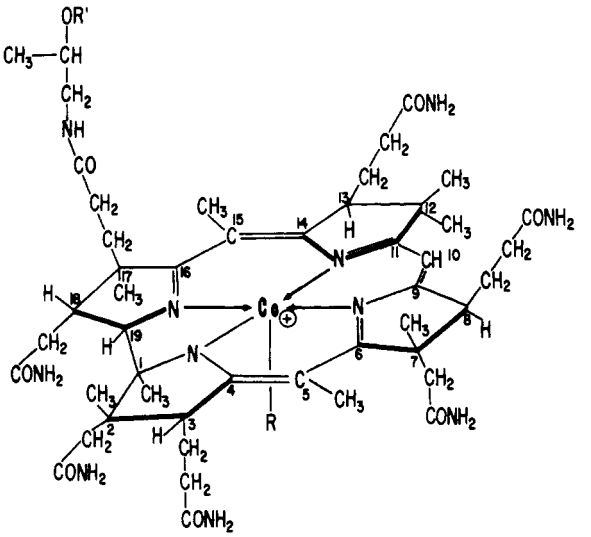


FIGURE 1: Structure of the corrin ring of the corrinoids. Methylcobalamin: R = methyl, R′ = 1-α-D-ribofuranosyl-(5,6-dimethylbenzimidazole)-3′-phosphoryl. The propionamide side chain of ring A is located above the plane of the corrin ring.

radical or a methylcobalamin radical, which in turn could react with methyl radical to yield vitamin B_{12r} or methylcobalamin, methylated at these carbon atoms (reactions 15 and 16). The results of expt 4 indicate that the corrinoid fraction indeed contains considerable radioactivity.

If ethane were produced by radical coupling of methyl radicals (reaction 14), its specific activity would be twice that of ^{14}C-methylcobalamin. The low specific activity of ethane (0.50 μcurie/μmole) suggests that radical coupling does not occur to an appreciable extent. This finding is in accord with the data of Dolphin *et al.* (1963) who reported that photolysis of ethylcobalamin at very low oxygen concentration (10^{-4} mm) yields mainly ethylene and no appreciable amounts of butane. The low specific activity of the ethane produced in expt 4 suggests methyl abstraction from the corrin ring by a methyl radical to yield ethane. Methyl abstraction is most likely to occur at C-7, C-12, and C-17 (ΔH = 50 kcal/mole) (Morrison and Boyd, 1961). Analogous to the hydrogen abstraction, methyl abstraction would yield a vitamin B_{12r} biradical or a methylcobalamin radical. Because the specific activity of ethane is lower than that of ^{14}C-methylcobalamin, isotope dilution by ^{12}C-ethane must have taken place. No specific reaction for the formation of ^{12}C-ethane can be formulated, but it seems possible that this alkane could be derived from the side chains of the corrin ring.

The nonparticipation of water in the photolysis reaction is reflected in the low yield of methanol and the formation of vitamin B_{12r} under anaerobic conditions. If hydrogen abstraction from the solvent had taken place, the hydroxyl radicals formed would be expected to react with methyl radicals to form methanol, or oxidize vitamin B_{12r} to aquocobalamin.

The results of the titration experiments indicate that 1 equiv of base is produced/mole of methylcobalamin photolyzed, when at least an equimolar amount of oxygen is present. When the photolysis is carried out in the presence of limiting quantities of oxygen, less base is produced. These results suggest that under these conditions secondary reactions take place which change the nature of the corrin ring and affect the oxidation state of the cobalt atom.

It is clear that photolysis of methylcobalamin in the presence of only a trace of oxygen is very complex. At moderate light intensities the rate of photolysis is very low, probably due to the recombination of vitamin B_{12r} and the methyl radical. The aqueous solvent may affect the photolysis by providing a "solvent cage" around the photolyzed molecule and thus favor recombination of the radicals (Hammond and Turro, 1963). At high light intensity many side reactions occur, and more than 30% of the radioactivity cannot be accounted for. The results presented above indicate that anaerobic photolysis of the alkylcobalamins does not only yield vitamin B_{12r} but several alkylated vitamin B_{12} derivatives.

Acknowledgments

Some of the preliminary experiments in this investigation were done at the Technological Research Laboratory of the Fisheries Research Board of Canada in Vancouver with the assistance of Mrs. T. G. Oikawa. I am grateful to Drs. J. M. Wood and R. S. Wolfe, of the University of Illinois, for doing the gas analyses and to Dr. K. Uyeda, University of California, for the enzymic determination of formic acid.

References

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Dolphin, D., Johnson, A. W., Rodrigo, R., and Shaw, N. (1963), *Pure Appl. Chem. 7*, 539.

Hammond, G. S., and Turro, N. J. (1963), *Science 142*, 1541.

Hayes, F. N. (1963), *Packard Tech. Bull. 1*, 4.

Heicklen, J., and Johnston, H. S. (1962a), *J. Am. Chem. Soc. 84*, 4030.

Heicklen, J., and Johnston, H. S. (1962b), *J. Am. Chem. Soc. 84*, 4394.

Hogenkamp, H. P. C. (1964), *Ann. N. Y. Acad. Sci. 112*, 552.

Hogenkamp, H. P. C., Barker, H. A., and Mason, H. S. (1963), *Arch. Biochem. Biophys. 100*, 353.

Hogenkamp, H. P. C., and Oikawa, T. G. (1964), *J. Biol. Chem. 239*, 1911.

Hogenkamp, H. P. C., Rush, J. E., and Swenson, C. A. (1965), *J. Biol. Chem. 240*, 3641.

Jaselskis, B., and Diehl, H. (1958), *J. Am. Chem. Soc. 80*, 2147.

Johnson, A. W., Merwyn, L., Shaw, N., and Smith, E. L. (1963a), *J. Chem. Soc.*, 4146.

Johnson, A. W., Shaw, N., and Wagner, F. (1963b), *Biochim. Biophys. Acta 72*, 107.

Loomis, A. G., (1928), International Critical Tables, Vol. 3, New York, McGraw-Hill, p 258.

MacFayden, D. A. (1945), *J. Biol. Chem. 158*, 107.

Morrison, R. T., and Boyd, R. N. (1961), Organic Chemistry, Boston, Mass., Allyn and Bacon, p 39.

Pratt, J. M. (1964), *J. Chem. Soc.*, 5154.

Rabinowitz, J. C., and Pricer, W. E. (1957), *J. Biol. Chem. 229*, 321.

Wagner, F., and Bernhauer, K. (1964), *Ann. N. Y. Acad. Sci. 112*, 580.

Weissbach, H., Peterkofsky, A., Redfield, B. G., and Dickerman, H. (1963), *J. Biol. Chem. 238*, 3318.

Wolin, E. A., Wolin, M. J., and Wolfe, R. S. (1963), *J. Biol. Chem. 238*, 2882.