The Reaction of Multihalogenated Hydrocarbons with Free and Bound Reduced Vitamin B_{12}*

J. M. Wood, F. Scott Kennedy, and R. S. Wolfe

ABSTRACT: Low concentrations of methylene chloride, chloroform, or carbon tetrachloride inhibit cobamide-dependent methyl-transfer reactions leading to the formation of methane in extracts of *Methanobacillus omelianskii* and the N^5-methyl tetrahydrofolate–homocysteine transmethylase of *Escherichia coli* B. In the methane reaction competitive inhibition is observed with chlorinated hydrocarbons when methyl-Co-5,6-dimethylbenzimidazolylcobamide (methylcobalamin) is used as the methyl-donating substrate. However, in the methionine system inhibition of N^5-methyltetrahydrofolate–homocysteine transmethylase is only observed when N^5-methyltetrahydrofolate monoglutamate (5-CH_3-F_4-folate) is used as methyl donor; methionine formation from methylcobalamin is apparently independent of bound cobamide. The halogenated methyl derivatives formed by reacting methylene chloride, chloroform, bromoform, iodoform, carbon tetrachloride, or Freon 12 (CF_2Cl_2) with B_{12-s} have been synthesized and crystallized. The photolysis, under hydrogen, of the derivatives formed from methylene chloride, chloroform, and carbon tetrachloride has been studied in detail. Each chloromethyl derivative yielded methyl chloride as the common gaseous product, but in the case of derivatives formed from chloroform and carbon tetrachloride concomitant liberation of hydrochloric acid was observed. The number of equivalents of hydrochloric acid liberated was shown to be dependent on the number of chlorine atoms present on each respective chloromethyl ligand. Studies concerning the photolysis of the derivative synthesized from [^{14}C]chloroform suggest the initial formation of [^{14}C]chlorocarbene. Aquo-Co-5,6-dimethylbenzimidazolylcobamide (aquocobalamin) was identified as the major cobamide product after exhaustive photolysis followed by aeration of each of the above cobamide derivatives.

The reaction of B_{12-s} with bifunctional reagents has been reported by Smith *et al.* (1964) and Gupta and Huennekens (1964). Dibromethylene was shown to react with B_{12-s} to give the bromovinyl derivative, and when tetramethylene dibromide was used as the reagent the product was shown to be a halogen-free dimer of B_{12} linked by a four-carbon bridge. Both of these reactions are indicative of a mechanism in which the halogen is the leaving group. This report deals with the reaction of B_{12-s} with halogenated reagents which contain more than one halogen atom on the same carbon atom. The products of this reaction when methylene chloride, chloroform, bromoform, iodoform, carbon tetrachloride, or Freon 12 are used as reagents are a group of halomethylcobalamin derivatives. The reactions are summarized in Scheme I. No alkylation was observed when trifluoromethane was used as the reagent, indicating that chlorine, bromine, and iodine will all function as leaving groups in this alkylation, but fluorine will not. These halogenated reagents apparently react readily with protein-bound corrinoid compounds also. Evidence presented below indicates that these reagents competitively inhibit the factor III enzyme which is involved in the final methyl-transfer reaction leading to the formation of methane in *Methanobacillus omelianskii* (Wood and Wolfe, 1966a,b); as well as the cobamide-dependent methyl-transfer reaction leading to the synthesis of methionine from homocysteine in *Escherichia coli* B (Brot and Weissbach, 1965).

Materials and Methods

The culture known as *M. omelianskii* was mass cultured, harvested, and washed as described by Wood *et al.* (1965). Cell-free extracts were prepared by exposing these bacteria, 1 g of cells (wet weight)/ml of 0.5 M potassium phosphate buffer (pH 7.0), to the maximum frequency output of a Branson sonic probe for 2 min at 0°. Cell debris was removed by centrifugation at 23,000*g* for 20 min at 0°.

5-CH_3-H_4-Folate homocysteine transmethylase[1] was

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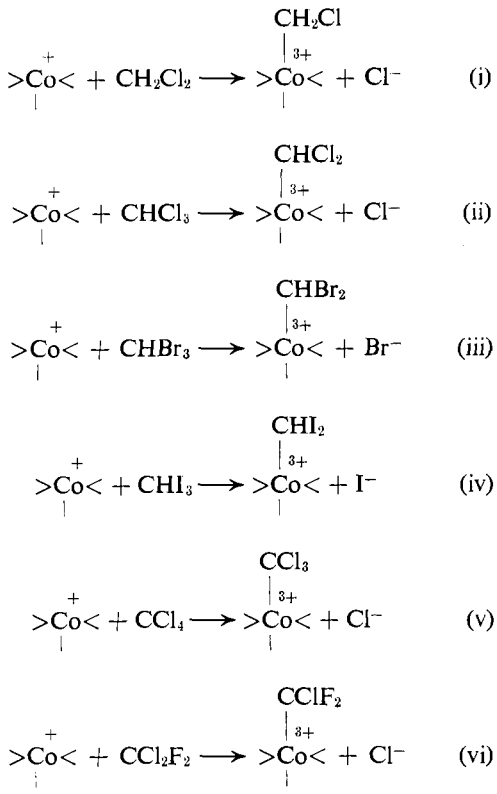
[1] Abbreviations used in this work that are not listed in *Biochemistry 5*, 1445 (1966), are: cyanocobalamin, CN-Co-5,6-dimethylbenzimidazolylcobamide; aquocobalamin, OH_2-Co-5,6-dimethylbenzimidazolylcobamide; P_{12-s}, Co^+-5,6-dimethylbenzimadazolylcobamide; cyano-factor III, CN-Co-5-hydroxybenzimadazolylcobamide; chloromethylcobalamin, CH_2Cl-Co-dimethylbenzimadazolylcobamide; dichloromethylcobalamin, $CHCl_2$-Co-dimethylbenzimadazolylcobamide; dibromoethylcobalamin, $CHBr_2$-Co-dimethylbenzimidazolylcobamide; diiodomethylcobalamin, CHI_2-Co-dimethylbenzimidazolylcobamide; trichloromethylcobalamin, CCl_3-Co-dimethylbenzimidazolylcobamide; difluorochloromethylcobalamin, CF_2Cl-Co-dimethylbenzimidazolylcobamide; 5-CH_3-H_4-folate, *dl*-N^5-methyltetrahydrofolate monoglutamate; SAM, *S*-adenosylmethionine iodide.

Scheme I



purified by Drs. H. Weissbach and R. T. Taylor, Laboratory of Clinical Biochemistry, National Institutes of Health, Bethesda, Md., by the method outlined by Taylor and Weissbach (1967). Protein was determined by the method of Lowry *et al.* (1951).

Chemical and Analytical Methods. Methylcobalamin was prepared by the method of Müller and Müller (1962). *dl*-5-CH_3-H_4-Folate was prepared and standardized by the method described by Wood *et al.* (1965). Methane was assayed by gas chromatography on a silica gel column by the method of Wolin *et al.* (1963). Methionine formation from methylcobalamin was assayed by the method of Weissbach *et al.* (1965). Methionine formation from 5-CH_3-H_4-folate was assayed by the method of Brot *et al.* (1966). Methyl chloride was assayed by a modification of the gas chromatographic technique of Usone *et al.* (1962). For this assay a Beckman GC 2 gas chromatograph containing Apiezon L in a Chromosorb column (35–80 mesh) was used at a column temperature of 100° and with a helium carrier gas pressure of 25 lb. Standard assay conditions were attained by injecting 0.4 cc of gas with a calibrated syringe from a standard photolysis reaction mixture. This gas chromatographic technique was found to be excellent for the separation of methyl chloride (retention time 69.0 sec), methylene chloride (retention time 122 sec), chloroform (retention time 243 sec), and carbon tetrachloride (retention time 380 sec). Methyl chloride was distinguished from methane (retention time 68 sec) by simultaneously injecting samples into both the chromosorb and silica gel column systems; methyl chloride was not detected on the silica gel column system, whereas methane was detectable on both systems. [^{14}C]Methyl chloride was assayed using the gas chromatographic technique described by Wood *et al.* (1965).

Photolysis of halomethyl ligands from halomethylcobalamin derivatives was accomplished by dissolving a standard amount of each analog in water and by placing a 3.0-ml sample of each solution in a separate scintillation vial fitted with a serum stopper. Each vial was flushed with a continuous stream of O_2-free H_2 for 15 min prior to illumination with a 200-W tungsten filament lamp from a distance of 15 cm. The cold air blast from a hair dryer was used to prevent heating during the photolysis process. For carbene-trapping experiments photolysis reactions were conducted in methanol under N_2 in the presence of diphenylacetylene or *trans*-stilbene.

The hydrochloric acid liberated during the photolysis of halomethylcobalamin derivatives was estimated by titration with 0.002 N sodium hydroxide, under N_2, with the aid of a Metrohm automatic titration apparatus.

Phosphocellulose (Cellex P) was obtained from Bio-Rad and was generated by the method of Lezius and Barker (1965). Gel filtration was performed on Bio-Gel P10. Columns (50 × 0.5 cm) were prepared and components of photolysis reaction mixtures were eluted in 2.0-ml fractions with water. [^{14}C]Chloroform (specific activity 2.2 mCi/mmole) was obtained from New England Nuclear Corp.

Results

Properties of the Halomethylcobalamins. Chloromethylcobalamin, dichloromethylcobalamin, dibromomethylcobalamin, diiodomethylcobalamin, trichloromethylcobalamin, and difluorochloromethylcobalamin were prepared by reacting an excess of methylene chloride, chloroform, bromoform, iodoform, carbon tetrachloride, and Freon 12, respectively, with six individual batches of $B_{12\text{-}s}$, each batch being prepared with 100 mg of cyanocobalamin and 8.0 g of zinc dust in 150 ml of 10% (w/v) ammonium chloride. After phenol extraction of each batch by the method of Johnson *et al.* (1963), the resulting aqueous solution, which contained the respective halomethylcobalamin derivative, was adjusted to pH 3.0 with 6 N acetic acid and was applied to a separate phosphocellulose column (20 × 3 cm). Each halomethylcobalamin derivative remained red in color and was eluted from this column with 0.01 N acetic acid. Methylcobalamin and other impurities changed from red to yellow and adhered strongly to the column at this low pH. The pH of each solution of the halogenated derivative was adjusted to 7.0 with 6.0 N ammonium hydroxide prior to lyophilization and crystallization from aqueous acetone. Yields of greater than 50% of each derivative were obtained routinely. When $B_{12\text{-}s}$ was prepared by the borohydride reduction method of Johnson *et al.* (1963), halomethylcobalamin derivatives were

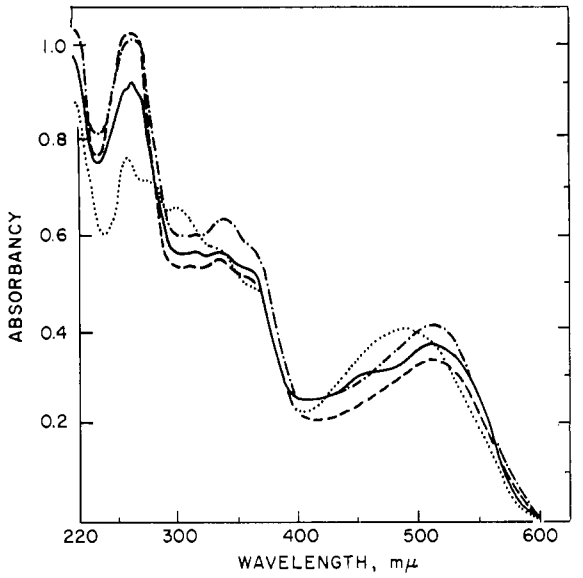


FIGURE 1: Comparison of the absorption spectra of the chloromethylcobalamins with that of methylcobalamin in 0.1 N acetic acid. (····) Methycobalamin, (——) chloromethylcobalamin, (—·—·) dichloromethylcobalamin, and (– – –) trichloromethylcobalamin.

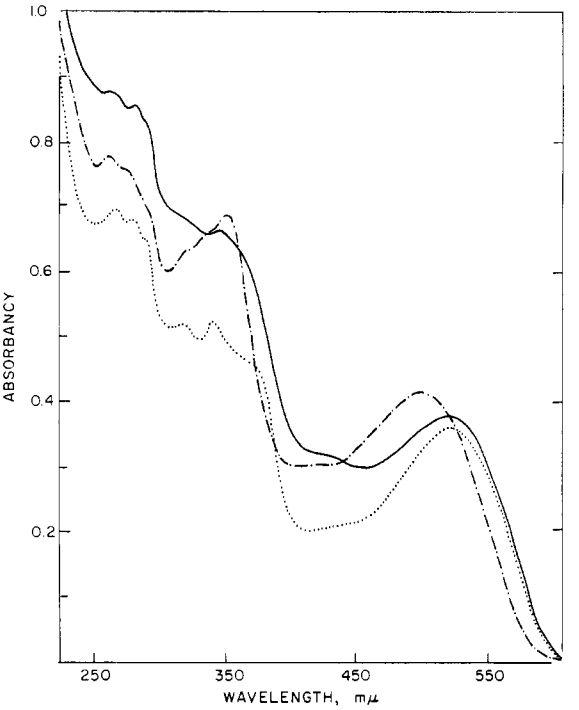


FIGURE 2: Absorption spectra of fluorine-, bromine-, and iodine-containing halomethylcobalamins recorded in water. (·····) Diiodomethylcobalamin, (—·—·) difluorochloromethylcobalamin, and (——) dibromomethylcobalamin.

obtained in much lower yields, and under these strong reducing conditions the percentage of methylcobalamin impurity in reaction mixtures increased considerably, particularly when methylene chloride was used as reagent.

The purity of these halomethylcobalamin derivatives was ascertained by subjecting them to high-voltage electrophoresis in 4% formic acid for 3 hr at a potential gradient of 30 V/cm and a current of 1.25 mA/cm on Whatman No. 3MM chromatography paper. Although halomethylcobalamins were not separable from each other by this technique (mobility 12.5 cm), they were separable from methylcobalamin (mobility 14.7 cm). No marked spectral differences are apparent between halomethylcobalamins and methylcobalamin at neutral pH. However, in acid solution these halogenated analogs remain red in color; methylcobalamin changes from red to yellow, and considerable differences in both visible and ultraviolet regions are observed (Figures 1 and 2).

Photolysis of the Chloromethylcobalamins. Photolysis, under H_2, of each chloromethylcobalamin derivative gave methyl chloride as the common gaseous product. The rates of methyl chloride formation were shown to be dependent on the number of chlorine atoms present on the chloromethyl ligand of the analog tested (Figure 3). Extraction of the photolysis products formed from each chloromethyl analog into toluene followed by gas chromatography of the organic phase failed to detect the transient appearance of methylene chloride or chloroform. The number of equivalents of hydrochloric acid liberated during the photolysis process was dependent on the number of chlorine atoms present on each chloromethyl ligand. The stoichiometry of the above photolysis process for each derivative tested is documented in Table I.

From the above data it is clear that during the photolysis of dichloromethylcobalamin or trichloromethylcobalamin, other products in addition to methyl chloride and hydrochloric acid must be produced, since equilibrium is reached before stoichiometry for methyl chloride production is attained.

Mechanism of the Photolysis of Dichloromethylcobalamin. To facilitate the determination of the photolytic mechanism for dichloromethylcobalamin this derivative was synthesized from [^{14}C]chloroform. Studies on the photolysis of [^{14}C]methylcobalamin, under argon, demonstrated that this homolytic cleavage of the carbon–cobalt bond resulted in the liberation of $\cdot{}^{14}CH_3$ which could either react with protons to give

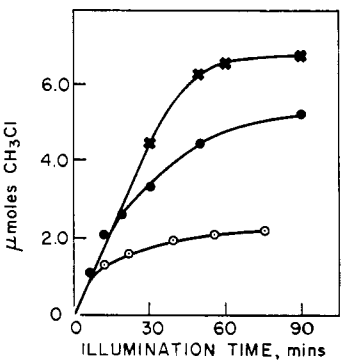


FIGURE 3: Rates of photolytic cleavage of methyl chloride, under H_2, from 8.0 mg of chloromethylcobalamin (✖—✖), dichloromethylcobalamin (●—●), and trichloromethylcobalamin (⊙—⊙). Each derivative was dissolved in 3.0 ml of water.

TABLE I: Formation of Hydrochloric Acid and Methyl Chloride from Chloromethylcobalamin Derivatives by Photolysis under H_2.[a]

Compound (μmoles)	CH_3Cl (μmoles)	HCl (μmoles)
Chloromethylcobalamin (20.0)	16.20	0.00
Dichloromethylcobalamin (20.0)	11.20	11.60
Trichloromethylcobalamin (20.0)	4.23	7.72

[a] Reaction time, 2.5 hr; gas phase, H_2; incubation temperature, 25°. Photolysis conditions as described in text.

TABLE II: Photolysis of [^{14}C]Dichloromethylcobalamin in the Presence of Diphenylacetylene.[a]

Reaction Product	Total Counts Incorp (cpm)	% Counts Incorp
Methylchloride	5.03×10^6	48.9
Aquocobalamin	4.0×10^6	38.8
Diphenylacetylene	1.02×10^6	9.9

[a] Photolysis reaction mixture contained [^{14}C]-dichloromethylcobalamin (4.47 μmoles; specific activity 2.3×10^6 cpm/μmole) in 2.1 ml of methanol with 4.5 μmoles of diphenylacetylene. This reaction mixture was photolysed for 3 hr at 25° under N_2.

[^{14}C]methane or could abstract methyl groups from the corrin ring to give [^{14}C]ethane of half the specific activity of the [^{14}C]methane (Hogenkamp, 1965). If the mechanism of cleavage of the [^{14}C]dichloromethyl ligand from [^{14}C]dichloromethylcobalamin is analogous to the above reaction then methylene chloride would be a transient intermediate in the formation of [^{14}C]methyl chloride from this substrate. The reaction path is visualized in Scheme II. When the kinetics of [^{14}C]methyl chloride formation from [^{14}C]dichloromethylcobalamin were studied in the presence and absence of an excess of methylene chloride, no significant difference in the rates of [^{14}C]methyl chloride formation in the two reactions was observed (Figure 4). This would indicate that $\cdot{}^{14}CHCl_2$ is not the initial product of the homolytic cleavage of [^{14}C]-dichloromethylcobalamin. These data prompted us to examine the possibility of cleavage of the chlorine–carbon bond in addition to the homolytic cleavage of the carbon–cobalt bond to generate the diradical [^{14}C]chlorocarbene. To trap [^{14}C]chlorocarbene, photolysis reactions were conducted in absolute methanol under nitrogen in the presence of the carbene-trapping reagent diphenylacetylene (Seyferth *et al.*, 1965). Residual diphenylacetylene together with any [^{14}C]-cyclopropene derivative, formed by the addition of [^{14}C]chlorocarbene to the acetylene moiety, was readily extracted into ether leaving the cobalamin product in aqueous methanol. The distribution of radioactivity in various components of the system after exhaustive photolysis is demonstrated in Table II. The radioactive cobamide was separated from compounds of low molecular weight by column chromatography on Bio-Gel P10. This cobamide product exhibited an identical spectrum to aquocobalamin, and when this material was treated with an excess of potassium cyanide at pH 7.8 the product was shown to have an identical spectrum to cyanocobalamin. Purification of this cyano derivative by chromatography on Bio-Gel P10 indicated that alkaline cyanolysis did not cause any loss of radioactivity. This observation indicated that either the [^{14}C]dichloromethyl ligand is covalently linked to the corrin ring or is still coordinated to the cobalt. X-Ray crystallographic studies are in progress to determine the position(s) of chloroalkyl addition to this cobamide.

Scheme II

$$>Co^{3+}(^{14}CHCl_2) \xrightarrow{h\nu} >Co^{3+} + \cdot{}^{14}CHCl_2$$

$$\cdot{}^{14}CHCl_2 + H_2 \longrightarrow {}^{14}CH_2Cl_2 + H\cdot$$

$$\cdot H + {}^{14}CH_2Cl_2 \longrightarrow HCl + \cdot{}^{14}CH_2Cl$$

$$\cdot{}^{14}CH_2Cl + H_2 \longrightarrow {}^{14}CH_3Cl + H\cdot$$

$$H\cdot \longrightarrow 0.5H_2$$

$$>Co^{3+}(^{14}CHCl_2) + 1.5H_2 \xrightarrow{h\nu} >Co^{3+} + CH_3Cl + HCl$$

Enzymic Studies. With the discovery that a factor III enzyme is involved in the final methyl-transfer reaction leading to the formation of methane from methylcobalamin in cell-free extracts of *M. omelianskii* (Wood and Wolfe, 1966a,b), the possibility of using halogenated hydrocarbons to competitively inhibit this system was examined. Bauchop (1967) has already demonstrated that chloroform, carbon tetrachloride, or Freon inhibit methane formation, with concomitant stimulation of H_2 formation, in the rumen of the sheep.

Low levels of methylene chloride, chloroform, and carbon tetrachloride exhibit inhibition of a competitive nature in the methane system (Figure 5). The possibility of the methane enzyme utilizing chloromethylcobalamin as substrate for the biological formation of methyl chloride was tested. The results of this experiment showed that no methyl chloride was formed, there being specificity for the methyl ligand only.

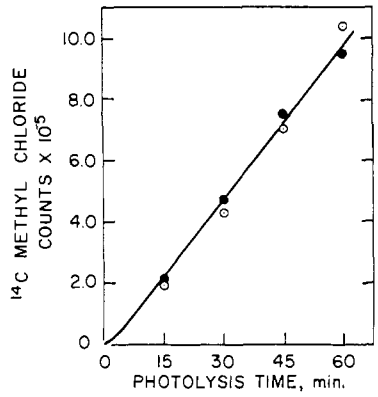


FIGURE 4: The effect of methylene chloride (200 μmoles) on the kinetics of [^{14}C]methyl chloride formation from [^{14}C]-dichloromethylcobalamin (2.88 μmoles in 3.0 ml of water). (●) + CH_2Cl_2; (○) − CH_2Cl_2.

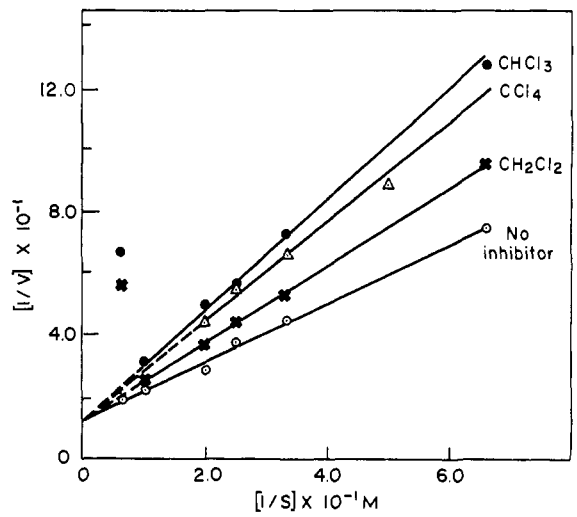


FIGURE 5: Competitive inhibition of methane formation from methylcobalamin by chlorinated hydrocarbons. Reaction mixtures contained: extract, 47.2 mg of protein; methylene chloride and chloroform, 0.2 μmole, respectively; carbon tetrachloride, approximately 50 mμmoles; ATP, 10 μmoles; potassium phosphate buffer (pH 7.0), 760 μmoles; and variable levels of methylcobalamin. Total liquid volume 2.25 ml, gas phase H_2, incubated at 40° for 15 min.

When the methane enzyme was inhibited by alkylation with methylene chloride, chloroform, or carbon tetrachloride this inhibition was shown to be reversed by photolytic cleavage of each respective chloroalkyl ligand from the enzyme–inhibitor complex (Figure 6). These data are consistent with our earlier observation that propyl iodide strongly inhibits this enzyme (Wood and Wolfe, 1966a,b). Photolytic cleavage of the propyl ligand from the enzyme–inhibitor complex caused significant reactivation of this methyl-transfer reaction. Lower rates for this photoreactivated enzyme are observed when chloroform and particularly carbon tetrachloride are used as inhibitors. It would appear that the possible addition of very reactive free radicals such as chlorocarbene or dichlorocarbene to the corrin ring or to amino acid residues close to the catalytic site for methyl transfer could interfere with the formation of the enzyme–substrate complex.

Studies with homogeneous 5-CH_3-H_4-folate homocysteine transmethylase demonstrated that chlorinated hydrocarbons are potent competitive inhibitors of methyl transfer when 5-CH_3-H_4-folate is used as methyl-donating substrate (Figure 7). When methylene chloride (10.0 mμmoles) or carbon tetrachloride (5.4 mμmoles) was used as the inhibitor of 5-CH_3-H_4-folate homocysteine transmethylase, under precisely the same reaction conditions employed with chloroform, competitive inhibition was observed and K_I values of 3.74 and 3.44 $\times$ 10^{-5} were calculated for each respective chlorinated hydrocarbon. However, no inhibition of methyl transfer by these compounds is observed when methylcobalamin is used as methyl donor (Table III).

These data support the view of Brot *et al.* (1966) that the transfer of the methyl group from methylcobalamin to homocysteine is independent of bound cobamide, but the transfer of the methyl group from 5-CH_3-H_4-folate to homocysteine proceeds *via* bound reduced cobamide.

Discussion

The synthesis and characterization of a new group of halogenated cobalamin derivatives has been carried out. The authenticity of these derivatives is substantiated by spectral studies and by photolytic cleavage of haloalkyl ligands, from each derivative, under H_2. A detailed study of the photolysis of the derivative formed from [^{14}C]chloroform has been undertaken. These experiments demonstrate that the mechanism of photolytic cleavage of the [^{14}C]dichloromethyl ligand is not analogous to that observed for the homolytic cleavage of the [^{14}C]methyl ligand from [^{14}C]-methylcobalamin (Hogenkamp, 1965). The results of experiments in which free-radical trapping agents were

TABLE III: Bound Reduced Cobamide Independence of Methionine Formation from Methylcobalamin.[a]

mμmoles of [^{14}C]Methyl-cobalamin (580 cpm/ mμmole) Added to Reaction Mixture	Counts $\times$ 10^{-3} Methionine	Counts $\times$ 10^{-3} Methionine in Presence of 20 mμmoles of Chloroform
25	0.29	0.32
50	0.79	0.82
75	1.38	1.43
100	1.80	1.92

[a] Reaction mixture contained: $^{5}CH_3$-H_4-folate homocysteine transmethylase; 4.5 μg of protein; *dl*-homocysteine, 250 mμmoles; potassium phosphate buffer (pH 7.0), 20.0 μmoles; [^{14}C]methylcobalamin; and chloroform (as indicated). Total reaction volume 0.2 ml, under H_2, incubated at 37° for 30 min.

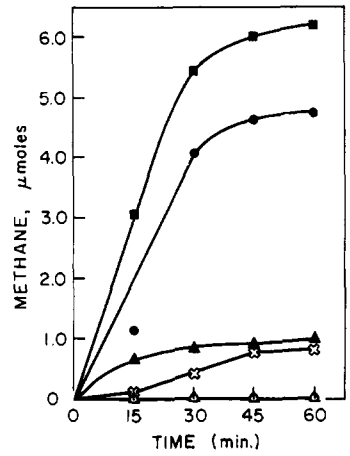


FIGURE 6: Photoreactivation of the inhibited factor III enzyme involved in the final methyl-transfer reaction leading to the formation of methane. Inhibited reaction mixtures contained: extract, 25.0 mg of protein; methylene chloride, chloroform, and carbon tetrachloride, 1.0 μmole, respectively; methylcobalamin, 5.0 μmoles; ATP, 10.0 μmoles; and potassium phosphate buffer (pH 7.0), 500 μmoles. Total liquid volume, 1.3 ml, gas phase H_2, incubated at 40°. Photoreactivated reaction mixtures were duplicates of the above except that extracts and inhibitor were photolysed for 1 hr at 25° prior to the addition of substrate and ATP. Inhibited: methylene chloride (⊠—⊠), chloroform (⊙—⊙), and carbon tetrachloride (△—△). Photoreactivated: methylene chloride (■—■), chloroform (●—●), and carbon tetrachloride (▲—▲).

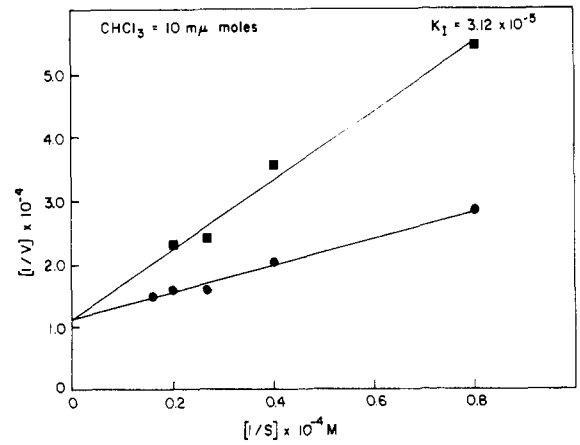


FIGURE 7: Competitive inhibition of methionine formation from 5-CH_3-H_4-folate by chloroform. Each reaction mixture contained; 5-CH_3-H_4-folate homocysteine transmethylase, 4.5 μg of protein; *S*-adenosylmethionine, 10 mμmoles; *dl*-homocysteine, 125 mμmoles; potassium phosphate buffer (pH 7.4), 20.0 μmoles; mercaptoethanol, 30 mμmoles; *dl*-[^{14}C] CH_3-H_4-folate, 16,435 cpm/mμmole, where indicated; and cyanocobalamin, 10.0 mμmoles. Total reaction volume 0.9 ml, under H_2, incubated at 37° for 15 min.

used suggest that cleavage of the [^{14}C]dichloromethyl ligand yields the highly reactive diradical chlorocarbene. The large percentage of ^{14}C trapped by the corrin ring when [^{14}C]dichloromethylcobalamin is the substrate for photolysis compared with the small percentage of ^{14}C trapped when [^{14}C]methylcobalamin was employed is indicative of a different mechanism. This view also is supported by our inability to implicate [^{14}C]-methylene chloride as an intermediate in the reaction sequence, and by our inability to demonstrate ^{14}C incorporation into diphenylacetylene from [^{14}C]-methylcobalamin. From the experimental data obtained the reaction sequence shown in Scheme III is suggested for the cleavage of [^{14}C]dichloromethylcobalamin under H_2.

Scheme III

$$^{14}CHCl_2 \quad ^{14}CHCl\cdot$$
$$\mid^{3+} \qquad \mid$$
$$>Co< \xrightarrow{h\nu} >Co< + Cl\cdot$$
$$\mid \qquad \mid$$

$$^{14}CHCl\cdot$$
$$\mid$$
$$>Co< \xrightarrow{h\nu} >\overset{3+}{C}o< + H\dot{^{14}C}Cl$$
$$\mid \qquad \mid$$

$$Cl\cdot + H_2 \longrightarrow HCl + H\cdot$$

$$H\dot{^{14}C}Cl + H_2 \longrightarrow {}^{14}CH_3Cl$$

$$H\cdot \longrightarrow 0.5H_2$$

$$^{14}CHCl_2$$
$$\mid^{3+}$$
$$>Co< + 1.5H_2 \xrightarrow{h\nu} >\overset{3+}{C}o< + {}^{14}CH_3Cl + HCl$$
$$\mid \qquad \mid$$

The ease with which multihalogenated hydrocarbons react with both free and bound reduced cobamide may indicate the mechanism of toxicity of these compounds in biological systems. The discovery that chlorinated hydrocarbons inhibit cobamide-dependent methyl-transfer reactions is pertinent here, especially the observed inhibition of methionine biosynthesis in *E. coli* B.

Haloalkyl derivatives of vitamin B_{12} may be used as substrates for the synthesis of new derivatives. Of particular interest is the possible use of difluorochloromethylcobalamin as a substrate for the synthesis of a fluorine-containing coenzyme analog 5′-difluoroadenosyl 5,6-dimethylbenzimidazolylcobamide.

Acknowledgments

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Disproportionation of Vitamin B_{12r} under Various Mild Conditions*

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ABSTRACT: When vitamin B_{12r} dissolved in 1 M NaCl aqueous solution was allowed to stand in the presence of methyl iodide under anaerobic conditions in the dark overnight at room temperature, B_{12r} was converted into an almost equimolar mixture of methylcobalamin and aquocobalamin (vitamin B_{12a}). Under the experimental conditions mentioned above, the change did not occur in the absence of the salt. Other electrolytes, such as KCl, Na_2SO_4, $NaNO_3$, and $MgCl_2$, showed a similar effect. In the case of KCN or Na_2SO_3, cyanocobalamin or cobalamin sulfonate was formed instead of B_{12a}, respectively. Other alkylating agents, such as dimethyl sulfate, also yielded methylcobalamin. An equimolar mixture of B_{12r} and cobinamide$_r$, more easily reducible than B_{12r}, gave methylcobalamin and methylcobinamide in the yields corresponding to 16 and 80% of the initial amounts of B_{12r} and cobinamide$_r$, respectively. A comparative study on the effects of NaCl, NaI, and CsCl showed that the order of their effectiveness accorded with Hofmeister's lyotropic series. When a very high concentration of B_{12r} solution, where a portion of solid B_{12r} remained undissolved, was allowed to react with methyl iodide for 2 days in nitrogen atmosphere, methylcobalamin was obtained in about 20% yield without the help of electrolytes. In the visible absorption spectrum of B_{12r} in 1 M NaCl solution, no change was observed which demonstrated the occurrence of B_{12s} and B_{12a}. A comparative study on the alkylating actions of various monohaloacetates with different reactivity toward B_{12r} in 1 M NaCl revealed that the reactivity of alkylating agent had very marked influence on the reaction. These results would be explained as follows: a very small portion of B_{12r} disproportionates to B_{12s} and B_{12a}; B_{12s} thus formed is not only a very small amount but is also short lived; the formation of alkylcobalamin depends on whether an alkylating agent can trap this short-lived B_{12s} or not. When these reactions of B_{12r} were carried out in hydrogen atmosphere, the yield of alkylcobalamin was higher than that in nitrogen atmosphere.

The important role of reduced forms of vitamin B_{12} in biological systems has been increasingly suggested. Of the two reduced forms of the vitamin, B_{12s}, a two-electron reduction product, is highly reactive and yields alkylcobalamin by reacting with alkyl halide. This fact has led to the supposition that B_{12s} is an active species in several biochemical reactions, such as methane formation (Blaylock and Stadtman, 1964; Wood and Wolfe, 1966), methionine biosynthesis (Weissbach *et al.*, 1965), and conversion of cobalamin into its coenzyme form (Vitols *et al.*, 1964; Weissbach *et al.*, 1966). However, the appearance of B_{12s} has not been detected spectrophotometrically during the enzyme reactions. On the other hand, the absorption spectrum of B_{12r}, a one-elec-

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