Methylcobalamin as an Intermediate in Mammalian Methionine Biosynthesis*

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ABSTRACT: Enzyme-bound methylcobalamin is identified as an intermediate in methionine biosynthesis catalyzed by a partially purified N^5-methyltetrahydrofolate:homocysteine methyl transferase obtained from porcine kidney. The enzyme accumulates labeled methyl groups from N^5-methyltetrahydrofolate-^{14}C-*methyl* only in the presence of *S*-adenosylmethionine, although *S*-adenosylmethionine-*methyl-t* can serve as a precursor in the absence of the substrate. In both cases the presence of a very high concentration of the product, methionine, is necessary to cause significant accumulation of labeled methyl on the enzyme. After separation from substrates, only the addition of homocysteine is necessary to demethylate the enzyme. Treatment of the labeled enzyme with hot 80% ethanol releases a labeled compound which by solubility, chromatographic, electrophoretic, and photolytic properties is identical with authentic methylcobalamin.

Studies on the biosynthesis of methionine in animal (Sakami and Ukstins, 1961; Mangum and Scrimgeour, 1962; Buchanan *et al.*, 1964), avian (Dickerman *et al.*, 1964), and bacterial (Weissbach *et al.*, 1963; Stavrianopoulos and Jaenicke, 1967; Taylor and Weissbach, 1969a,b) enzyme preparations have suggested that the reaction

$$N^5\text{-methyltetrahydrofolate} + \text{homocysteine} \longrightarrow \text{tetrahydrofolate} + \text{methionine} \quad (1)$$

is effected by an AdoMet-requiring enzyme, which contains a cobalamin prosthetic group and is dependent on the presence of a reducing system for activity. Taylor and Weissbach (1968) described methods by which transfer of a labeled methyl group to the bacterial enzyme could be effected from methyl-FH_4, *S*-AdoMet,[1] or methyl iodide. The labeled protein transferred its methyl group to homocysteine in an aerobic reaction requiring no cofactors. Upon treatment with hot ethanol (Taylor and Weissbach, 1968) the labeled protein released a labeled compound which displayed the spectral, chromatographic, and chemical properties of methylcobalamin. Taylor and Weissbach (1969b) have formulated a pathway for methyl transfer catalyzed by the bacterial enzyme in which enzyme-bound methylcobalamin is an obligatory intermediate in reaction 1.

Until now, elucidation of the mechanism of the reaction catalyzed by mammalian enzyme has been hindered by the inability to accumulate large quantities of high specific activity enzyme. In this communication, we present direct evidence for enzyme-bound methylcobalamin as an intermediate in mammalian methionine biosynthesis.

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[1] Abbreviations used are: methyl-FH_4, N^5-methyltetrahydrofolate; FH_4, tetrahydrofolate; *S*-AdoMet, *S*-adenosylmethionine; FMN, riboflavin 5′-phosphate.

Materials

N^5-Methyltetrahydrofolate–homocysteine : transmethylase was purified from hog kidneys by protamine sulfate precipitation, ammonium sulfate fractionation, and chromatography on Bio-Gel DM-30 and Sephadex G-200. At all stages of purification, high levels of homocysteine (0.01 M) were required to stabilize the preparation. One unit of enzyme catalyses the synthesis of 1 nmole of Met/mg of protein per hr.

N^5-Methyltetrahydrofolate-*methyl*-^{14}C (61 mCi/mmole) and *S*-AdoMet-*methyl-t* (4200 mCi/mmole) were purchased from Amersham-Searle. *S*-AdoMet-Cl was obtained from Mann Research Laboratories. Homocysteine thiolactone was a product of General Biochemicals. FMN and dithiothreitol were obtained from Calbiochem. Other chemicals were the best commercially obtainable.

Methods

Methylation of homocysteine by methyl-FH_4 was routinely determined by the method of Weissbach *et al.* (1963) with AG 1-X8 resin obtained from Bio-Rad. *S*-AdoMet:homocysteine transmethylase activity was measured essentially by the method of Taylor and Weissbach (1969b) with CG-50 cation-exchange resin purchased from Mallinckrodt Chemical Co., equilibrated with 0.1 M ammonium acetate buffer (pH 5.0).

The flavin reducing system was prepared fresh daily and contained 1 mg of FMN and 4 mg of PtO_2 per ml of water. It was reduced under a stream of hydrogen. Spectrophotometric examination showed that 70% of the flavin was adsorbed to the platinum oxide under these conditions. The effective concentration of reduced flavin is thus 30% of that indicated in the appropriate legends.

Incubations for Protein Labeling. The enzyme accumulated labeled methyl groups on incubating 8–24 mg of protein with the labeled substrate in the presence of 0.1–0.15 M L-Met and the flavin reducing system. Specific details are given in the appropriate legends. Incubations were for 15 min in the dark under a hydrogen atmosphere. The labeled

TABLE I: Conditions for Synthesis of Methionine from Methyl-FH_4.

Incubation	nmoles of ^{14}C as Met/mg of Protein per hr
Complete system, 37°[a]	6487
−Methyl-FH_4-^{14}C	0
−*S*-AdoMet	807
−Homocysteine	511
−Dithiothreitol	5773
−$FMNH_2$	46
Complete system, 0°	15

[a] Complete system (1.0 ml) contains enzyme (0.029 mg), methyl-FH_4-^{14}C (0.346 μmole at 1.725×10^6 dpm/μmole), *S*-AdoMet (50 nmoles), homocysteine (10 μmoles), dithiothreitol (10 μmoles), potassium phosphate buffer (pH 7.4, 100 μmoles), $FMNH_2$ (195 nmoles), and PtO_2 (0.4 mg). Incubations were for 15 min at 37° under positive H_2 pressure in the dark. Methionine was determined as described in Materials and Methods.

TABLE II: Adenosylmethionine: Homocysteine Transmethylase Activity of *N*-Methyltetrahydrofolate:Homocysteine Transmethylase.

	nmoles of ^3H as Met/mg per hr
Complete[a]	23.5
−$FMNH_2$	2.83
−Homocysteine	12.9
+Unlabeled methyl-FH_4 (0.34 μmole)	5.4
0° incubation	0.39

[a] Complete system (1.0 ml) contains enzyme, methyl-FH_4: homocysteine transmethylase (specific activity 2000, 0.55 mg), *S*-AdoMet-*methyl-t* (800 mCi/mmole, 15.6 nmoles), potassium phosphate buffer (pH 7.4, 100 μmoles), homocysteine (10 μmoles), dithiothreitol (10 μmoles), FMNH (195 nmoles), and PtO_2 (0.4 mg). Methionine was determined as described in the text. Paper electrophoresis at pH 1.9 showed that at least 95% of the radioactivity was distributed between methionine, and the sulfone and sulfoxide of methionine.

protein was separated from low molecular weight material by gel filtration on columns of Sephadex G-25 medium (2 × 20 cm), equilibrated with 0.01 M potassium phosphate buffer (pH 7.4) and 10^{-3} M Met. Columns were run at 4° in the dark.

Transfer of Labeled Methyl Groups from Enzyme to Homocysteine. The ability of the labeled protein to transfer methyl groups to homocysteine was tested by incubating portions of the Sephadex effluent above with the various components of standard transmethylase assays. After incubation, the protein was precipitated with trichloroacetic acid containing carrier methionine, and the supernatant solutions were applied to columns of Dowex 50-X8 (0.7 × 5 cm). After washing the columns with water, Met was eluted with 3 N NH_4OH (Taylor and Weissbach, 1967a). The identity of the labeled methionine was confirmed by paper electrophoresis at pH 1.8.

Extraction and Characterization of the Radioactive Protein-Bound Species. For characterization of the labeled species bound to the enzyme, portions of the Sephadex effluent above were treated as follows: 1.0–4.0 μmoles of unlabeled substrate (methyl-FH_4 or *S*-AdoMet) and 0.5 μmole of carrier methyl-B_{12} were added to 8–15 mg of labeled enzyme. This mixture was made 80% in ethanol and refluxed on a steam bath for 30 min. The ethanol supernatant was taken to dryness under reduced pressure, and was then purified through a standard phenol extraction (Barker *et al.*, 1960). The material was subjected to paper electrophoresis on Whatman No. 1 at 45 V/cm, pH 1.8 for 1.5–3 hr. Strips of electrophoretograms were scanned in a Packard 7201 radiochromatogram scanner.

Areas of electrophoretograms exhibiting both red color due to carrier methyl-B_{12} and radioactive peaks were eluted with H_2O and tested for photolability. Aliquots of the eluates were photolyzed at 0° for 15 min at a distance of 10 cm from a 150-W tungsten floodlamp, while identical aliquots were kept dark. Eluates were then applied to 0.5 × 3 cm columns of Dowex 50-X8 (H^+) in the dark, and the columns were eluted with water. The major photolysis product, labeled formaldehyde (Hogenkamp, 1966) was washed through the columns with water, while the corrinoids were retained on the columns. Photolability is expressed as the per cent of the applied radioactivity which is eluted with H_2O.

Results

Requirements for Methionine Synthesis. Table I illustrates the requirements for the transfer of labeled methyl from methyl-FH_4 to homocysteine by the porcine kidney enzyme. These are similar to those reported for a partially purified liver preparation (Loughlin *et al.*, 1964) and for the bacterial system (Taylor and Weissbach, 1967b; Stavrianopoulos and Jaenicke, 1967). The stimulation by *S*-AdoMet of methyl-FH_4:homocysteine transmethylase activity was variable from one enzyme preparation to another, with limits of approximately 3- to 22-fold. Indeed, sequential fractions from a single chromatographic separation showed different degrees of *S*-AdoMet stimulation, as if there had been partial fractionation with respect to this property (*cf.* Rüdiger and Jaenicke, 1969).

In common with the bacterial enzyme (Taylor and Weissbach, 1967b; Stavrianopoulos and Jaenicke, 1967) the porcine kidney enzyme catalyzes the transfer of methyl from *S*-AdoMet to Met. The conditions for optimal transfer are given in Table II. The addition of unlabeled methyl-FH_4, the normal methyl donor for methionine synthesis, decreases the incorporation of label from *S*-AdoMet into Met.

Isolation of Enzyme-Bound Label. The porcine kidney transmethylase preparation was stabilized by maintaining high concentrations of homocysteine throughout the purifi-

TABLE III: Transfer of Protein Label to Homocysteine.[a]

	% of Label Recovd as Met		
	Expt 1	Expt 2	Expt 3
Additions to basic system			
None, 0°	15		
None, 37°	15	13	14
+Homocysteine, 0°		12	
+Homocysteine, 37°	89	91	54
+*S*-AdoMet, 37°			14.5
+*S*-AdoMet + $FMNH_2$, 37°			14.6
+Methyl-FH_4 + *S*-AdoMet + $FMNH_2$, 37°		15	

[a] Basic system contains (1.0 ml; pH 7.4) 100 μmoles of potassium phosphate buffer (pH 7.4), plus labeled protein from Sephadex column, 5107 dpm of ^{14}C in expt 1, and 1080 dpm of ^{14}C in expt 2, both derived from methyl-FH_4-*methyl*-^{14}C, and 87,000 dpm of ^{3}H derived from *S*-AdoMet-*methyl-t* in expt 3. When indicated, homocysteine was added as 10 μmoles, methyl-FH_4 as 0.36 μmole, *S*-AdoMet as 0.05 μmole, and $FMNH_2$ as 0.1 ml of a solution containing 1 mg of FMN and 4 mg of platinum oxide per ml, prereduced as described in the text. Incubations were for 15 min in the dark. Paper electrophoresis at pH 1.8 indicated that at least 80% of the radioactivity eluted from Dowex 50 was methionine.

cation. Attempts to remove the homocysteine prior to use of the enzyme, either by dialysis or gel filtration, resulted in rapid loss of catalytic activity. For this reason, all incubations contain endogenous homocysteine. This is reflected in the non-zero level of catalysis in the absence of added homocysteine in Tables I and II.

Our early attempts to demonstrate the accumulation of an enzyme-bound methylated intermediate with this enzyme preparation were all unsuccessful. We concluded that either there was no kinetically competent, enzyme-bound methylated intermediate, or transmethylation from that species to endogenous homocysteine was much faster than the transfer from substrate to intermediate. Preliminary kinetic studies (G. T. Burke, J. H. Mangum, and J. D. Brodie, in preparation) revealed that product inhibition by methionine was uncompetitive with respect to methyl-FH_4. Double-reciprocal plots of velocity *vs.* methyl-FH_4 concentration in the presence of methionine, resulted in a set of parallel lines when different amounts of methionine were added to the methyl-FH_4: homocysteine transmethylase assay system (Cleland, 1963). This result suggested that addition of high concentrations of methionine might inhibit transfer of a methyl group from an intermediate to homocysteine without inhibiting transfer from the substrate to the proposed intermediate. Indeed, upon incubating the enzyme with labeled substrate in the presence of high levels of methionine (0.1–0.15 M), we were able to isolate a labeled protein from the incubation mixture.

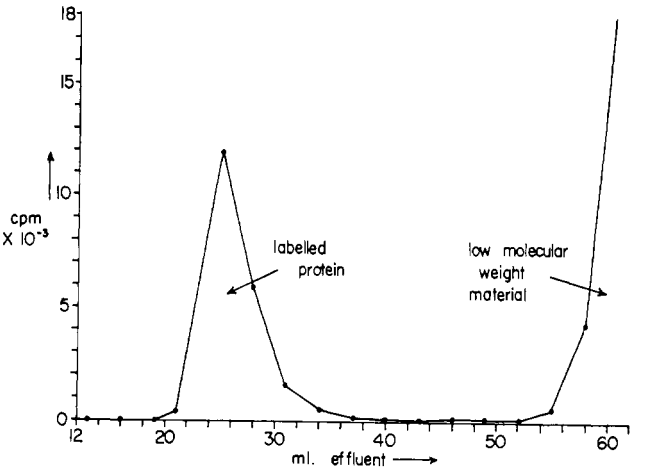


FIGURE 1: Typical elution profile of protein-labeling incubation mixture on Sephadex G-25 medium. The column (2 × 20 cm) was run at 4° in the dark, with a flow rate of 0.5 ml/min. The incubation mixture (1.85 ml) contained enzyme (17.2 mg), potassium phosphate buffer (pH 7.4, 100 μmoles), *S*-Ado-Met-*methyl-t* (0.025 μmole) 5.76 × 10^7 dpm), $FMNH_2$ (0.195 μmole), platinum oxide (0.4 mg), and L-Met (250 μmoles).

After separation of labeled protein from other components of the incubation mixture by Sephadex chromatography, the protein was reincubated in attempts to effect transfer of the label to homocysteine. A typical elution profile, in Figure 1, shows that labeled protein was readily separated from low molecular weight materials. It must be emphasized that labeled protein could only be isolated in significant quantities when the incubation was carried out in the presence of very high concentrations of methionine. In the absence of methionine, the protein failed to accumulate label, and any loosely bound label which was not separated from the protein by Sephadex was removed when the mixture was made 8 M in urea and subjected to gel filtration. The conditions under which the label on the protein could be recovered as methionine are shown in Table III. Whether the enzyme had been labeled by *S*-AdoMet or by methyl-FH_4, only homocysteine was necessary to demethylate the enzyme, and no component tested could replace homocysteine. The identification of the product as methionine was verified by paper electrophoresis.

The various lines of evidence suggesting the participation of a B_{12}-coenzyme as a methyl-carrying intermediate in mammalian methionine biosynthesis were confirmed by the isolation and characterization of the labeled species which accumulated on the porcine kidney enzyme in the presence of high concentrations of methionine. The protein was extracted with hot 80% ethanol containing excess unlabeled substrates and carrier methyl-B_{12}, the ethanol was removed and the residue purified through phenol extraction as described in Methods.

A purification procedure was worked out which gave optimum resolution of labeled methyl-B_{12} from other labeled species in the reaction mixture, namely, Met, methyl-FH_4, and *S*-AdoMet. In control experiments, phenol extraction removed over 80% of Met, about 97% of *S*-AdoMet, and about 90% of methyl-FH_4. Electrophoresis in formic acid–acetic acid (pH 1.8) gave a good separation of all components with *S*-AdoMet displaying the highest mobility, followed by

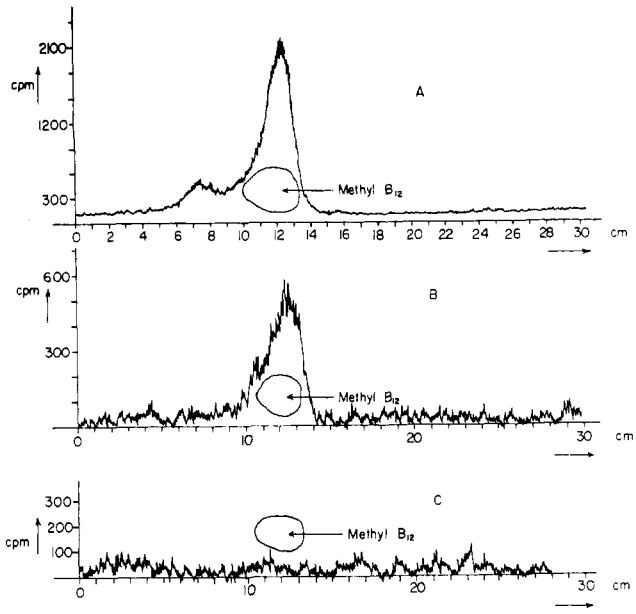


FIGURE 2: Electrophoresis of labeled compounds released from methionine synthetase by ethanol extraction following incubation with labeled substrates. (A) *S*-Ado-Met-*methyl-t*. Incubation contained in 1.9 ml, enzyme (specific activity 1600, 11.2 mg), *S*-Ado-Met-*methyl-t* (0.025 μmole, 5.76×10^7 dpm, buffer pH 7.4, 100 μmoles), $FMNH_2$ (0.195 μmoles), platinum oxide (0.4 mg), and L-Met (225 μmoles). (B) Methyl-FH_4-*methyl*-^{14}C plus *S*-Ado-Met. Incubation contained in 1.95 ml, enzyme (specific activity 1600, 9.3 mg), methyl-FH_4-*methyl*-^{14}C (0.328 μmole and 4.4×10^7 dpm), unlabeled *S*-Ado-Met (0.05 μmole, buffer pH 7.4, 100 μmoles), FMNH (0.195 μmole) platinum oxide (0.4 mg), and L-Met (250 μmoles). (C) Methyl-FH_4-*methyl*-^{14}C minus *S*-Ado-Met. Incubation contained in 1.95 ml, enzyme (specific activity 1600, 9.3 mg), methyl-FH_4-*methyl*-^{14}C (0.328 μmole and 4.4×10^7 dpm; buffer pH 7.4, 100 μmoles), $FMNH_2$ (0.195 μmole), platinum oxide (0.4 mg), and L-Met (250 μmoles). Incubations were for 15 min in the dark at 37°. Following incubation, protein was separated from low molecular weight on Sephadex G-25 medium, extracted with ethanol, purified, and electrophoresed as described in the text. Electrophoresis was for 2 hr at 2500 V in the dark.

TABLE IV: Photolability of the Labeled Extract.[a]

Source of Label	% Eluted from Dowex 50 H^+ by H_2O	
	Light	Dark
Methyl-FH_4-*methyl*-^{14}C	92	20
S-AdoMet-*methyl-t*	95	3
Authentic methyl-B_{12}-*methyl*-^{14}C	99	3

[a] Labeled material was eluted from areas of electrophotograms displaying red color due to carrier methyl-B_{12}, and divided into aliquots for photolysis as described in Methods. Following treatment, the degree of carrier photolysis was checked spectrally, and samples were applied to Dowex 50 columns in the dark. Results are expressed as percentage of total label eluted with H_2O.

methionine, methyl-FH_4, and methyl-B_{12}. Carrier recoveries of 10–35% through the total purification procedure were typical. Unlabeled substrates (methyl-FH_4 or *S*-AdoMet) were added at the ethanol extraction step to dilute the specific radioactivity of any substrate which had not been removed by the gel filtration. The resulting dilution enabled sufficient removal of substrate label at the phenol extraction step that the radiochromatogram scanner detected only methyl-B_{12} on the electrophoresis strip.

As shown in Figure 2, the only significant radioactivity remaining had the electrophoretic mobility of methyl-B_{12}. In Figure 2A, the minor peak of lower mobility was shown by photolysis to be made up of about 50% methyl-B_{12}. The identity of the remaining label was not determined. After correcting for carrier recovery, a range of 62–80 pmoles of methyl-B_{12}/mg of protein (specific activity 1600) was observed with *S*-AdoMet as the methyl donor. When methyl-FH_4 was the methyl group donor, the enzyme accumulated 21–35 pmoles of methyl-B_{12}/mg. As shown in Figure 2C, methyl-FH_4 does not transfer significant radioactivity to methyl-B_{12} in the absence of unlabeled *S*-AdoMet. Even under conditions of severe product inhibition, small but significant amounts of label are recovered as methionine from the protein-labeling preincubation; therefore, it may be that these levels of methyl-B_{12} recovery reflect the relative rate of methylation of the protein by methyl-FH_4 and *S*-AdoMet, and demethylation by endogenous homocysteine.

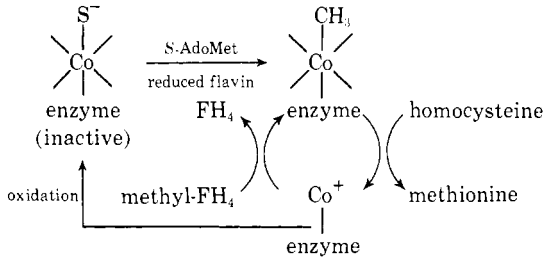
Photolability of the Labeled Extract. After electrophoresis, areas of the papers displaying color due to methyl-B_{12} carrier were eluted with water, and the eluates were subjected to the photolability check described in Methods. Table IV shows the results of these experiments, as well as the behavior of authentic methyl-B_{12} treated in the same manner. Before photolysis, both extracts and authentic methyl-B_{12}-*methyl*-^{14}C were held on Dowex 50 in the dark. Following photolysis at 0°, however, more than 90% of the applied label was eluted from the columns with water. Thus the photolytic properties of the labeled compounds released from the labeled enzyme by ethanol extraction are identical with those of authentic methyl-B_{12}-*methyl*-^{14}C.

Discussion

Previous studies with animal methyl-FH_4:homocysteine transmethylase have been limited by the unavailability of large amounts of highly purified enzyme. Nevertheless, several lines of evidence suggested that a corrinoid prosthetic group was an intermediate methyl group acceptor. These included the isolation of a somewhat inactive though highly processed enzyme from pig liver in which the ratio of B_{12} content to specific activity was relatively constant (Loughlin *et al.*, 1964), and the more convincing copurification of methyl-FH_4:homocysteine transmethylase, methyl-B_{12}:homocysteine transmethylase, and ^{60}Co-labeled protein activities from ^{60}Co-labeled vitamin B_{12} injected chickens (Dickerman *et al.*, 1964). Recent studies (Kerwar *et al.*, 1966; Brodie, 1967) have been inconclusive because of technical difficulties and competing reactions in the crude enzyme systems used by these workers.

Methyl-FH_4:homocysteine methyl transferase activities isolated from *E. coli*, however, have been purified to essential homogeneity and methyl-B_{12} has clearly been shown to be

an intermediate in the reaction. Taylor and Weissbach (1969b) have postulated the following scheme for the interrelationships of *S*-AdoMet, methyl-FH_4, and cobalamin in the bacterial methionine synthetase. The key point for the



present discussion is that both *S*-AdoMet and methyl-FH_4 can donate a methyl group to the same site on the enzyme, generating enzyme-bound methyl-B_{12}, which has the properties of an obligatory intermediate for methionine synthesis. These authors present data indicating that once methylated by *S*-AdoMet, the enzyme can oscillate between methylated and demethylated reduced forms transferring methyl from methyl-FH_4 to homocysteine without further need for *S*-AdoMet, provided that reducing conditions can be maintained to protect the nucleophilic Co^{+1} which they depict as a participant in the cycle. Rüdiger and Jaenicke (1968) presented kinetic data which support the concept of methyl transfer from methyl-FH_4 to homocysteine *via* an enzyme-bound, methylated intermediate, presumably methyl-B_{12}. Recently, Rüdiger and Jaenicke (1969) reported a purification procedure from *E. coli*, yielding an enzyme which was independent of *S*-AdoMet in the presence of a reducing system. The enzyme became highly dependent on *S*-AdoMet if incubated with homocysteine and reisolated by gel filtration, suggesting that their enzyme had been obtained in the methylated form. Taylor and Weissbach (1969a) obtained a similarly *S*-AdoMet-independent preparation by preincubating the bacterial enzyme with *S*-AdoMet and isolating the methylated protein on Sephadex. They showed that this methylated protein was capable of catalyzing methyl-FH_4:homocysteine transmethylation in the absence of added *S*-AdoMet, and that the amount of activity seen was directly dependent on the strength of the reducing conditions.

A procedure (J. H. Mangum and J. A. North, in preparation) for purifying B_{12} transmethylase from hog kidney which yielded enzyme preparations with specific activities in excess of 16,000 was effected through the use of high concentrations of homocysteine throughout the procedure. Kinetic studies (G. T. Burke, J. H. Mangum, and J. D. Brodie, in preparation) with the purified transmethylase showed that methionine was an inhibitor of its own synthesis; this inhibition was uncompetitive with respect to methyl-FH_4 and noncompetitive with homocysteine. This suggested that a sufficiently large amount of methionine in an incubation mixture might diminish methyl transfer from a proposed intermediate and allow that intermediate to accumulate. We found that this was indeed the case; labeled methyl from both *S*-AdoMet and methyl-FH_4 did accumulate in methyl-B_{12} under conditions of severe product inhibition. Once isolated, this methyl-B_{12} displayed the expected physical and chemical properties while the labeled protein was shown to transfer its label to homocysteine without added cofactors.

We have observed that *S*-AdoMet donates its methyl to methylcobalamin at a faster rate than does methyl-FH_4; yet the overall rate of transfer from *S*-AdoMet to homocysteine is much slower than methyl-FH_4:homocysteine transmethylase. Although this finding is consistent with the early results found in the bacterial system (Taylor and Weissbach, 1968) it is at variance with later work in the same system (Taylor and Weissbach, 1969a). The resolution of this problem must await further kinetic analysis.

With the exception of methionine, the requirements for the synthesis of methyl-B_{12} are similar to those reported by Taylor and Weissbach (1969a) for the bacterial system. Due to the high concentration of homocysteine, small but significant amounts of label are transferred into methionine by both *S*-AdoMet and by methyl-FH_4 even in the presence of greater than 0.1 M methionine. The amount of synthesis is so low that an absolute requirement for *S*-AdoMet to effect methyl-FH_4:homocysteine transmethylation could not be demonstrated, but we observed that labeled methyl-B_{12} was not detected in incubations containing labeled methyl-FH_4 in the absence of *S*-AdoMet.

We have thus directly shown that enzyme-bound methyl-B_{12} has the properties required of an intermediate in mammalian methionine biosynthesis. Although mechanistic aspects of the participation of B_{12} and the detailed interrelationship of methyl-FH_4 and *S*-AdoMet are not the subjects of this communication, preliminary results suggest that the proposed *E. coli* pathway (Taylor and Weissbach, 1969b) is consistent with observations in porcine kidney.

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Chemical and Biological Studies with Fluoroalkylcobalamins*

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ABSTRACT: A new homologous series of fluoromethylcobalamins has been synthesized by reacting a variety of Freons with Cob(I)alamin (B_{12-s}). A comparative study of the physical and chemical properties of the nephelauxetic series $CF_3 > CF_2Cl > CF_2H > CH_3$ shows that there is a linear relationship between the electronegativity of each ligand and the stability of the Co–C σ bond to light. Also, a linear relationship exists between the stability of the 5,6-dimethylbenzimidazole (Bz)–Co complex and the electronegativity of each ligand. However, ligands which contain more than one chlorine atom (*e.g.*, $CFCl_2$) are more photolabile than any of the above derivatives, and evidence is available that these *gem*-dihalides generate carbenes upon photolysis. The authenticity of these fluoromethylcobalamins was rigorously confirmed by fluorine nuclear magnetic resonance and 220-MHz proton nuclear magnetic resonance. Cobalamin analogs containing $CFCl_2$, CF_2Cl, and CF_3 in place of CH_3 were shown to be competitive inhibitors for methylcobalamin in enzymatic methane formation by cell extracts of the methanogenic bacterium (MOH). Ligands containing more than one chlorine atom were shown to be more potent inhibitors than those which contained one or zero chlorine atom. Difluoromethylcobalamin replaces methylcobalamin as a substrate in the methane system; in the presence of ATP and hydrogen as a source of electrons, this analog yields methane. The physical and chemical properties of these fluoromethylcobalamins allow us to predict why the difluoromethyl analog is a substrate for the methane enzyme when all the other fluorine-containing cobalamins are inhibitors. The significance of the lack of specificity of the methane enzyme system for methylcobinamide, methyl factor III, methylcobalamin, and the abiogenic methylcobaloximes is discussed.

Bauchop (1967) made the initial observation that Antifoam A causes inhibition of methane formation in the rumen of sheep. Inhibition of methanogenesis was shown to be accompanied by immediate concomitant evolution of hydrogen. Methane formation in the rumen may be considered by the following general reaction

$$CO_2 + 4H_2 \rightleftharpoons CH_4 + 2H_2O$$

Clearly this equilibrium in the rumen is affected by Antifoam A. It was discovered subsequently that Freon 12 (CF_2Cl_2), which is present as a propellant in Antifoam A, was responsible for the inhibition of methane formation in the rumen ecosystem. The involvement of vitamin B_{12} in methane formation prompted us to examine whether strongly nucleophilic Cob(I)alamin could be the site for this inhibition (Wood and Wolfe, 1966; Wood *et al.*, 1968).

Freons are not generally regarded as alkylating agents, but Cob(I)alamin is such a strong nucleophile that a homologous series of fluoromethylcobalamins can be synthesized in good yield by standard procedures used for the synthesis of alkylcobalamins (Pailes and Hogenkamp, 1968; Buckman *et al.*, 1969). Details of the synthesis of this homologous series are outlined in Scheme I.

This homologous series of compounds has been used to examine some of the parameters which determine the stability of the Co–C σ bond, and how these parameters relate to our interpretation of both the ultraviolet–visible spectra and the nuclear magnetic resonance spectra of alkylcobalamins in general.

We have already reported the details of the synthesis of a series of halomethylcobalamins which contain chlorine, bromine, and iodine (Wood *et al.*, 1968). One interesting feature of the properties of these halomethylcobalamins is that the *gem*-dihalides photolyze to give carbenes, whereas ligands containing only one halogen atom (excluding fluorine) give radicals by homolytic cleavage (Scheme II) (Hogenkamp, 1966; Schrauzer *et al.*, 1968; Kennedy *et al.*, 1969).

A study of the first-order photolysis rates of these fluoromethyl derivatives provides an ideal model system for studying the effect of electron withdrawal from cobalt on the stability of the Co–C σ bond. A comparison of the ligands CF_3, CF_2H, and CH_3 is of particular interest since fluorine has a similar Van der Waals radius to hydrogen and strain introduced by bulky electron-withdrawing substituents need not be considered (Hogenkamp *et al.*, 1965). The present study helps to confirm the different photolysis mechanisms which are proposed for mono- and dichloromethylcobalamins (Scheme II).

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