A New Coenzyme of Methyl Transfer, Coenzyme M*

B. C. McBride† and R. S. Wolfe‡

ABSTRACT: A heat-stable, dialyzable cofactor of methyl transfer has been found in extracts of methane bacteria. The compound is acidic and migrates toward the anode at pH 2.0. It contains phosphate which is released only after prolonged acid hydrolysis. The compound adheres strongly to Dowex 1, is insoluble in nonpolar solvents and absolute ethanol, is ninhydrin negative both before and after treatment with 6 M HCl for 48 hr, and has a single absorption maximum at 260 nm. Extracts of *Methanobacterium* M. O. H. have been resolved for the cofactor by anaerobic dialysis; addition of the cofactor is required for the formation of methane from methylcobalamin. The cofactor is enzymically methylated in an ATP-stimulated reaction from methylcobalamin or from the reduction of CO_2 by cell extracts. The methylated derivative can be isolated and serves as a substrate for methane formation by cell extracts in a reductive, ATP-dependent demethylation reaction. The name coenzyme M is proposed for this cofactor of methyl transfer.

Studies of methyl transfer in *Methanobacterium* have dealt primarily with the terminal transmethylation reaction(s) which accompany methane formation (Wood and Wolfe, 1966a–c). Methyl transfer has been studied in *Methanosarcina barkeri* (Blaylock and Stadtman, 1966; Blaylock, 1968) where cell extracts have been resolved into four components which are required for the transfer of the methyl group of methanol to B_{12s}[1] to form methylcobalamin: (1) a heat-stable cofactor, (2) a cobamide protein, (3) ferredoxin, and (4) and an unknown protein. We now present evidence from studies with hydrogen-grown *Methanobacterium* strain M. O. H. for the existence of a new coenzyme which is involved in transmethylation reactions prior to methane formation. We propose the name, coenzyme M (CoM), as a trivial label for this compound until the precise chemical structure is determined. The isolation, assay, and some biological properties of this compound are presented; a preliminary report has appeared (McBride and Wolfe, 1970).

Experimental Section

Materials. [^{14}C]CH_3-B_{12} was prepared as described previously (Wood *et al.*, 1965). *S*-Adenosylmethionine was obtained from Sigma Chemical Co., DEAE-cellulose, obtained from Brown Co., was treated with 1 N NaOH and 1 N acetic acid prior to use. Dowex 50W-X4 (200–400 mesh, H^+) was obtained from J. T. Baker. Bio-Gel-2 (100–200 mesh) was obtained from Bio-Rad Laboratories. Thin-layer electrophoresis and chromatography were performed on cellulose plates (Eastman Chromagram) or on silica gel plates obtained from Applied Science Laboratories. Protein was determined by the biuret method (Gornall *et al.*, 1949) with bovine serum albumin (Sigma Chemical Co.) as a standard. Total phosphate was determined according to the procedure of Leloir and Cardini (1957). Phosphate on chromatograms was detected by spraying with a solution of ammonium molybdate (1 g), concentrated HCl (3 ml), concentrated perchloric acid (3 ml), water (8 ml), and acetone (86 ml).

Cultural Methods and Preparation of Extracts. Methanobacterium strain M. O. H. was grown as described by Bryant *et al.* (1968). Rumen fluid, volatile fatty acids, and resazurin were omitted from the medium. To each 14-l. fermentor was added 100 ml of vitamin solution (Wolin *et al.*, 1963). The organism also was mass cultured in 230-l. batches in a New Brunswick Fermacell fermentor (Model Special CF 250). Cell extracts were prepared by sonication and stored in 0.05 M potassium phosphate buffer (pH 7.0) as described by Roberton and Wolfe (1970).

CH_4 Assay. CH_4 biosynthesis was followed as described by Wolin *et al.* (1963). CH_4 was measured with a Packard Model 7500 series gas chromatograph which contained a silica gel column connected to a hydrogen flame detector. [^{14}C]CH_4 was measured as described previously (Wood *et al.*, 1965) or by injection of the gas sample into a scintillation vial modified by fitting a rubber septum (Packard 5396730) into a hole drilled through a scintillation vial cap. The vial was agitated vigorously to ensure that [^{14}C]CH_4 was dissolved. Bray's scintillation fluid was used as the counting medium. Assay of the atmosphere over the scintillation fluid revealed that approximately 7% of the [^{14}C]CH_4 was not dissolved and therefore not counted.

Dialyzed Cell Extracts. Cell extract in 0.05 M potassium phosphate buffer was saturated with H_2, poured into No. 8 dialysis tubing (Union Carbide), and quickly placed in anaerobic 0.05 M potassium phosphate buffer (pH 7.0) at 0°. Buffer for anaerobic dialysis was prepared by filling a 3-l. erlenmeyer flask to the neck with buffer and by heating it in a steamer for 2 hr. The hot buffer was removed from the steamer and vigorously sparged with O_2-free H_2 through a Nalgene gas-dispersion tube. The buffer was cooled to 0° in an ice bath. Sparging was continued during cooling and dialysis. After 8-hr dialysis the tubes were transferred to a second erlenmeyer flask of anaerobic buffer which had been connected for 2 hr to the effluent gas from the first dialysis flask. At 16 hr the tubes were transferred to a third anaerobic dialysis flask. After 24 hr the dialyzed extract was dispensed in 3-ml quan-

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† Present address: Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Can.

‡ To whom to address correspondence.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CoM, coenzyme M; CH_3-B_{12}, methylcobalamin, (*Co*-methyl-5,6-dimethylbenzimidazolylcobamide); B_{12r}, 5,6-dimethylbenzimadozolylcob(II)amide; B_{12s}, 5,6-dimethylbenzimadazolylcob(I)amide.

tities into 13 × 100 mm test tubes and was stored at 0° in a reduced state under a H_2 atmosphere.

Assays for Coenzyme M. Assay I was based on the dependence of methane formation upon CoM. To follow the CoM-dependent methane formation of dialyzed cell extract a Warburg flask was fitted with a serum stopper and was gassed with hydrogen as described by Wolin *et al.* (1963). Dialyzed cell extract (17 mg of protein) and $MgSO_4$ (10 μmoles) were added to the main compartment along with the desired quantity of CoM preparation. ATP (10 μmoles) and CH_3-B_{12} (3 μmoles) were placed in the side arm; the reaction was initiated by tipping the contents of the side arm into the main compartment. Methane evolution was measured as described above.

Assay II employed the enzymic methylation of CoM from [^{14}C]CH_3-B_{12}. In this assay the amount of [^{14}C]CH_3-CoM formed was calculated from the specific activity of the [^{14}C]CH_3-B_{12} added, the assumption being made that only one site on the CoM molecule was methylated. The reaction vessel was a Warburg flask fitted with a rubber serum cap which was gassed as described above. After all oxygen was flushed from the flask, 0.3 ml of 0.05 M potassium phosphate buffer at pH 7.0, 0.1 ml of 0.1 M $MgSO_4$, and 0.1 ml of 0.1 M tripolyphosphate (an inhibitor of methane biosynthesis) were added to the main compartment. Three minutes were allowed for O_2 to diffuse out of the aqueous components prior to the addition of 0.2 ml (5.6 mg of protein) of dialyzed cell extract. To the side arm were added 0.1 ml of 0.1 M ATP, 0.02 ml of 0.01 M [^{14}C]CH_3-B_{12} (0.5 μCi/μmole) (2×10^5 cpm), and 0.1 ml of CoM preparation. The flask was sealed by fitting a serum stopper onto the side arm. To ensure that completely anaerobic conditions were attained a No. 22 syringe needle was pushed through the serum stopper on the side arm; gassing was continued for an additional 3 min. Because the assay is dependent on the total volume of the reaction mixture all flasks were gassed at the same rate and for the same period of time. Gas flow was approximately 150 cm^3/min. The flask was sealed by removal of the effluent needle followed by the influent needle. The flask was incubated at 40° with shaking.

Methylation was followed by tipping the contents of the main compartment into the side arm of the Warburg flask and by removing 0.1 ml of the reaction mixture with a 0.1-ml Hamilton syringe. This material was squirted into a slurry of Dowex 50W-X4 (200–400 mesh, H^+) and rapidly mixed. The reaction was stopped by oxidation, the rapid drop in pH, and absorption of all B_{12} compounds onto Dowex 50; [^{14}C]CH_3-CoM was not absorbed. Large quantities of Dowex 50 H^+ slurry were made at one time and the H_2O content of this mixture was determined. The slurry was made just dilute enough to facilitate easy pipeting. The slurry (1 ml) was then mixed with 2 ml of distilled H_2O for assay purposes. The Dowex 50 beads which contained the absorbed B_{12} were allowed to settle out; 0.4 ml of the supernatant solution was placed in a scintillation vial together with 2 ml of 0.1 N NaOH and 15 ml of Bray's scintillation fluid. The samples were counted in a Mark I liquid scintillation system (Nuclear-Chicago, Chicago, Ill.).

Partial Purification of CoM. Frozen cells (1000 g) were mixed with 2 l. of hot water at 60–80° and the slurry was poured into a 5-l. florence flask. The mixture was heated to 90° in a steamer and then was maintained at 90° with stirring for 30 min on a heated magnetic stirrer. The solution was cooled to 4° in an ice bath after which it was centrifuged at 8000*g* for 10 min. The supernatant was collected and adjusted to 80% ethanol concentration with 95% ethanol. This solution was heated to 75° in a steamer and then cooled to 4°. The precipitate was removed by centrifugation at 4000*g* for 10 min. The yellow supernatant solution was suction filtered through Whatman No. 1 filter paper by use of a Büchner funnel. The filtrate was concentrated to a reddish brown viscous syrup by flash evaporation. The syrup was diluted to 100 ml with distilled water. To recover additional coenzyme the cell pellet which remained after the first water extraction was resuspended in water, and the hot water extraction was repeated. The supernatant solution was concentrated by flash evaporation to 100 ml without ethanol treatment; both extracts were combined.

The combined extracts were adjusted to pH 3.7 with 6 N HCl. (The use of phosphoric or sulfuric acids to adjust pH was avoided, since their use resulted in approximately 60% loss of coenzyme activity.) The precipitate which formed was removed by centrifugation. The dark brownish-red supernatant was applied to a Dowex 50W-X4 (200–400 mesh, H^+ column, 5.5 × 30 cm). A precipitate which slowed the flow rate formed at the surface of the Dowex beads; the top of the column was stirred frequently to resuspend the precipitate. The coenzyme was eluted with distilled water. Collection of CoM was begun when the effluent became acidic and was continued until the first yellow band to move down the column was 5 cm from the bottom of the Dowex resin. This fraction, which contained all CoM activity, was light yellow in color and exhibited a blue fluorescence due to impurities when exposed to long-wave ultraviolet light. The fraction was concentrated to 50 ml by flash evaporation and was neutralized with 1 N NaOH. The solution was cooled to 4° and 1 M barium acetate was added until no further precipitation could be detected; barium-insoluble salts were removed by centrifugation and discarded. The supernatant solution was adjusted to pH 7.0 with 1 N NaOH and more barium acetate was added. This solution was stored at 0° for 10 hr. After removal of the precipitated barium salts by centrifugation the supernatant was treated batchwise with Dowex 50 H^+ to remove excess barium. Dowex resin was removed by filtration. The solution was considered to be free of barium when no precipitate formed as it was mixed with sodium tripolyphosphate. The acidic filtrate was freed of the majority of acetic and hydrochloric acids by flash evaporation. The concentrated solution (about 40 ml) was adjusted to pH 7.0 and stored at −20°; this stage of purification of CoM is referred to as preparation A.

Additional Purification of CoM. Preparation A (15 ml) was diluted to 200 ml and was applied to a large DEAE-cellulose (acetate) column (4 × 45 cm) which had been equilibrated with water. The column was washed successively with three column volumes of water, two column volumes of 0.1 M ammonium acetate (pH 7.0), two column volumes of 0.3 M ammonium acetate (pH 7.0), and two column volumes of 0.5 M ammonium acetate. Only the 0.5 M eluate was retained and concentrated; ammonium acetate was removed by lyophilization. The coenzyme was dissolved in water and stored at −20°. This material was used for physiological studies and is referred to as preparation B.

Results

Purification of [^{14}C]CH_3-*CoM.* Partially purified CoM (preparation A) was enzymically methylated on a preparative scale from [^{14}C]CH_3-B_{12}. During purification [^{14}C]CH_3-CoM was detected by use of standard scintillation techniques for

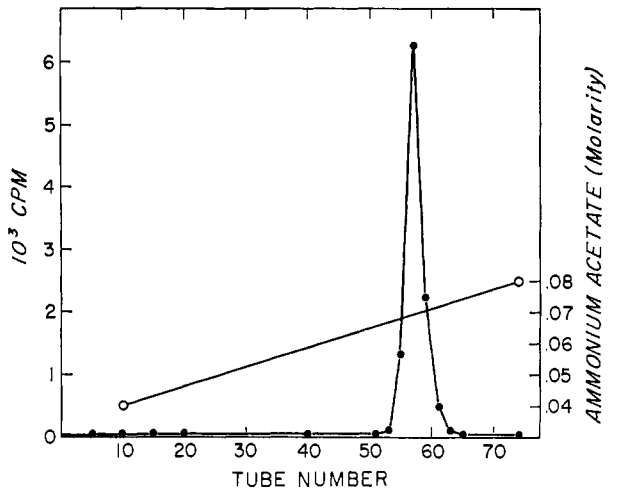

FIGURE 1: Chromatography of [^{14}C]CH_3-CoM on DEAE-cellulose acetate. (○) Molarity of ammonium acetate (●) counts per minute.

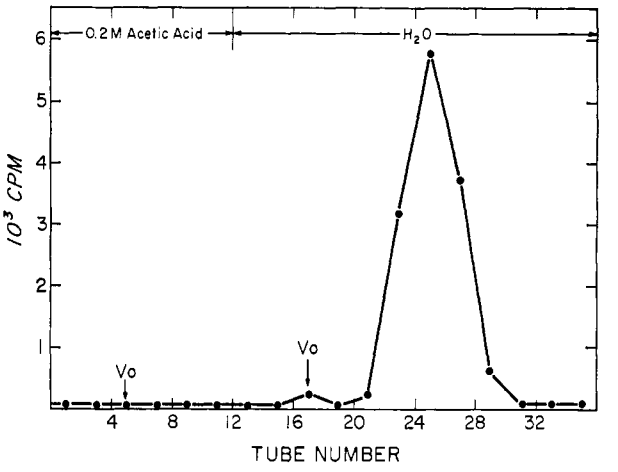

FIGURE 2: Chromatography of [^{14}C]CH_3-CoM on Bio-Gel P-2.

^{14}C. To methylate larger amounts of CoM the reaction mixture was scaled up as follows. Each of 6, 125-ml erlenmeyer flasks was sealed with a no. 5 black rubber stopper. Each flasks was gassed by extending a syringe needle to a point just above the surface of the reaction mixture which contained 20.0 ml of 0.05 M potassium phosphate buffer at pH 7.0, 2.0 ml of 0.1 M $MgSO_4$, 1.0 ml of 0.1 M ATP, 2.0 ml of 0.1 M sodium tripolyphosphate, and 5.0 ml of CoM preparation A. After thoroughly gassing the reaction mixture with H_2, 0.4 ml of 0.05 M CH_3-B_{12} and 4.0 ml of undialyzed cell extract (35 mg of protein/ml) were added. The flask was sealed anaerobically. A seventh reaction flask consisted of a 150-ml double-armed Warburg flask. The reaction mixture for this flask was the same as for the other six flasks except that the CH_3-B_{12} was placed in one side arm, and in addition 0.15 ml of [^{14}C]CH_3-B_{12} (0.01 M, 0.5 μCi/μmole) was added to the other side arm. The radioactive cobamide was tipped into the reaction mixture first, and after allowing 8 min for all of the isotope to be incorporated, the CH_3-B_{12} was added. The flasks were incubated on a shaker in the dark for 35 min at 37°. The reaction was stopped by pouring the flask contents into a slurry of Dowex 50 H^+ beads.

The methylated coenzyme was separated from Dowex beads by filtration. The acidic filtrate was centrifuged to remove precipitated protein. The supernatant was neutralized with 6 N NaOH and concentrated to 30 ml by flash evaporation. Barium acetate (1 M) was added until no further precipitation occurred; the barium-insoluble salts were removed by centrifugation and discarded. The supernatant was adjusted to pH 7.0 and more barium acetate was added. This solution was frozen and then thawed at 0°, the precipitate was removed, and the procedure was repeated until no further precipitation occurred. The light pink solution was concentrated to 20 ml and applied to a Dowex 50 H^+ column (5 × 40 cm). [^{14}C]CH_3-CoM was eluted with water and collected in 5-ml fractions. The ^{14}C peak tubes were pooled and acetic acid was removed by flash evaporation. The [^{14}C]coenzyme solution was concentrated to 40 ml, neutralized, and if stored, was kept at −20°.

To continue purification 20 ml of the Dowex 50 eluate was diluted to 80 ml and applied to a large DEAE-cellulose (acetate) column (5 × 45 cm). The column was washed with four column volumes of distilled water followed by two column volumes of 0.1 M ammonium acetate (pH 7.0); 10-ml fractions were collected. [^{14}C]CH_3-CoM eluted as a symmetrical peak with 0.1 M ammonium acetate. The radioactive fractions were pooled and the ammonium acetate was removed by lyophilization. The dried [^{14}C]CH_3-CoM was dissolved in 20 ml of distilled water and applied to a DEAE-cellulose (acetate) column (2.5 × 42 cm) which had been equilibrated with distilled water. The resin was washed with two column volumes of water. The coenzyme was eluted in a linear gradient of ammonium acetate: 250 ml of 0.04 M ammonium acetate (pH 7.0) was placed in the mixing chamber, and 250 ml of 0.08 M ammonium acetate pH 7.0 was placed in the reservoir of the gradient making device. The flow rate was adjusted to 90 ml/hr; 10-ml fractions were collected. CoM eluted at 0.06 M ammonium acetate as shown in Figure 1. Radioactive fractions were pooled and ammonium acetate was removed by lyophilization.

[^{14}C]CH_3-CoM was dissolved in 10 ml of distilled water and treated batchwise with Dowex 50 to remove cations. The acidic solution was concentrated to 4 ml and applied to a Bio-Gel P-2 (100–200 mesh, column 0.5 × 90 cm) which was equilibrated with 0.2 M acetic acid. Three void volumes of 0.2 M acetic acid were passed through the column, this was followed by three void volumes of water as shown in Figure 2. Fractions of 3 ml each were collected at a flow rate of 90 ml/hr. The Bio-Gel column was operated at 4°; all other columns were operated at room temperature.

The combined radioactive eluates from Bio-Gel P-2 were evaporated to dryness and resuspended in 1 ml of water. The coenzyme was then crystallized from a water–ethanol (10:90) solvent pair. Approximately 40 mg of crystallized [^{14}C]CH_3-CoM was obtained from 1 kg of whole cells (wet weight). This represents 45% of the coenzyme found in the hot water extracts of whole cells.

Criteria of Purity. The purity of the methylated coenzyme was determined by electrophoretic and chromatographic analysis. When [^{14}C]CH_3-CoM was electrophoresed at 400 V on silicic acid plates in formic acid–acetic acid–water (50:156:1794, v/v) followed by chromatography in 1-butanol–acetic acid–water (2:1:1, v/v), a single radioactive spot was observed, which had an R_{ATP} of 1.25 in the former system and an R_F of 0.52 in the latter system. Charring with H_2SO_4 at 100° revealed that this was the only area which contained organic material. Other electrophoretic and chromatographic systems which yielded similar results were: electrophoresis

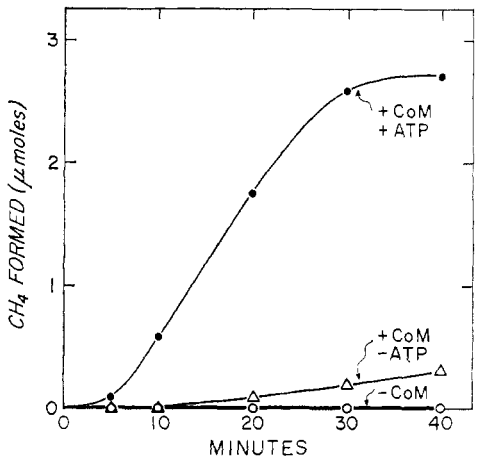

FIGURE 3: Effect of CoM on CH_4 biosynthesis by dialyzed cell extracts. Each flask contained potassium phosphate buffer (pH 7.0, 100 μmoles), magnesium sulfate (10 μmoles), CH_3-B_{12} (3 μmoles), ATP where indicated (10 μmoles), CoM preparation B where indicated (0.05 ml), and dialyzed cell extract (17 mg of protein). H_2 atmosphere. Temperature 40°. Total liquid volume 1.2 ml.

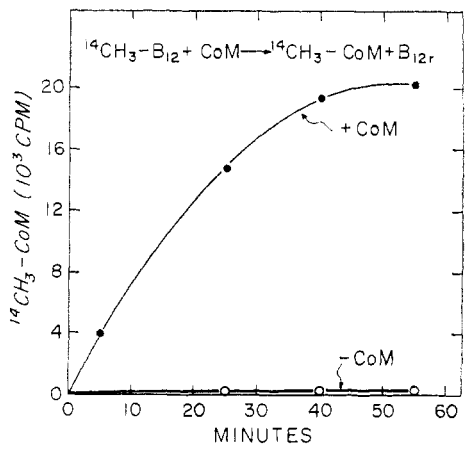

FIGURE 4: Methylation of CoM. Each flask contained potassium phosphate buffer (pH 7.0, 100 μmoles), ATP (10 μmoles), magnesium sulfate (10 μmoles), CoM preparation B where indicated (0.05 ml), [^{14}C]CH_3-B_{12} (0.5 μCi/μmole, 0.2 μmole), sodium tripolyphosphate (10 μmoles), and dialyzed cell extract (5 mg of protein). H_2 atmosphere. Temperature 40°. Total liquid volume 1.7 ml.

in 0.05 M potassium phosphate buffer (pH 7.4) and chromatography in isobutyric acid–concentrated ammonium hydroxide–water (66:1:33, v/v).

Spectra. CoM methylated or nonmethylated is a colorless, nonfluorescing compound. Spectral studies performed on a Cary 14 recording spectrophotometer show a single ultraviolet-absorbing peak at 260 nm in phosphate buffer at pH 7.0.

Phosphate. The coenzyme contains an acid-stable phosphate which was removed only when treated with 2 N H_2SO_4 at 100° for 2 hr. Phosphate could be detected on chromatograms sprayed with molybdate reagent only after incubation of plates for 24 hr. The stability of the phosphoric bond suggests that phosphate is bonded to an alcohol and precludes bonding to a carboxyl or an amino group.

Functional Groups. CoM does not form a colored complex when treated with ninhydrin. Treatment of the compound with 1 N HCl for 48 hr at 100°, did not release any ninhydrin-positive material. Sulfide could not be detected. The compound was not inactivated by treatment with 1×10^{-3} M *p*-chloromercuribenzoate. A standard reaction mixture was placed in a double side-arm reaction flask. In one side arm was placed [^{14}C]CH_3-B_{12}, in the other side arm was placed CoM plus 0.1 ml of 2×10^{-3} M *p*-chloromercuribenzoate, and in the center well was placed cell extract, buffer, Mg^{2+}, and ATP. Thus, CoM was exposed to 1×10^{-3} M *p*-chloromercuribenzoate and both compounds were tipped into a reaction mixture where the final concentration of *p*-chloromercuribenzoate was 5×10^{-5} M, a concentration which did not inhibit the enzymic methylation of CoM.

Electrophoretic Properties. [^{14}C]CH_3-CoM migrates rapidly toward the anode at pH 2.5. The compound has an R_{ATP} of 1.25 when electrophoresed in formic–acetic–water (50:156:1794, v/v) at 400 V.

Solubility. As expected of such a polar compound the coenzyme is soluble in water, 85% ethanol, or 50% acetone. It is insoluble in absolute alcohol, acetone, chloroform, or ether.

Stability. CoM and CH_3-CoM are stable to heat treatment. A solution of CoM was divided into three portions. One portion was placed in 0.05 M phosphate buffer (pH 7.0), a second portion was placed in 1 M NaOH, and a third portion was placed in 1 M HCl. Each mixture was heated at 100° for 1 hr after which time the pH was adjusted to 7.0, and the compound was tested in the methylation reaction. CH_3-CoM was similarly treated and then was tested in the demethylation assay. Both compounds were fully active after heat treatment at pH 7.0 and in 0.1 M HCl but exhibited a slight loss of activity after heat treatment in 0.1 M NaOH. The compounds pass through an Amicon ultrafiltration membrane with an exclusion size of 500.

Stimulation of CH_4 Biosynthesis. Anaerobic dialysis resolves cell-free extracts of strain M. O. H. for a low molecular weight cofactor required for methane formation (assay I). As shown in Figure 3 addition of CoM (preparation B) restores CH_4-forming ability; ATP also is required. The reaction was found to be inhibited by high concentrations of CoM and by a contaminant in impure coenzyme preparations. For these reasons and because of variations in the activity of the dialyzed extracts this reaction was used only for qualitative determinations of CoM activity.

Methylation Assay. The results of a transmethylation assay performed as described in the Experimental Section (Assay II) is illustrated in Figure 4. B_{12r} (Wolin *et al.*, 1964) is a product of the methylation of CoM. Anaerobic dialysis removes a compound which is methylated when incubated with CH_3-B_{12} and cell extract. Dowex 50 quantitatively removes unreacted [^{14}C]CH_3-B_{12}. The validity of the assay and its relevance to the biosynthesis of CH_4 were proven in the following experiments. (i) All the ^{14}C migrated as a homogeneous entity in both chromatographic and electrophoretic analysis as mentioned previously. This was true for both crude and purified coenzyme preparations, indicating homogeneity of [^{14}C]CH_3-CoM. (ii) The results of using the methylated coenzymes as a substrate for CH_4 formation are illustrated in Figure 5. [^{14}C]CH_3-CoM was prepared by transmethylation from [^{14}C]CH_3-B_{12}, and then was partially purified by hot 80% ethanol extraction followed by passage through a Dowex 50 H^+ column. The radioactive effluent was neutralized, concentrated, and then used as a substrate in the standard CH_4 assay. The reaction was more rapid (0.78 μmole of [^{14}C]CH_4/hr per mg of protein) than the reductive demethylation of CH_3-B_{12} to form CH_4 (0.24

TABLE I: Requirements for the Methylation of Coenzyme M.[a]

Reaction Conditions	[^{14}C]CH_3-CoM
Omissions	$\times 10^3$ cpm
None	3.8
None (boiled cell extract)	0.0
−Mg	1.9
−ATP	2.2
−MgATP	1.2
−CoM	0.1
−Cell extract	0.0
$-H_2$	0.0
$-H_2$ + NADPH	4.3
$-H_2$ + NADH	2.1
$-H_2$ + FADH	0.2
$-H_2$ + FMNH	0.2

[a] Complete reaction mixture contained: potassium phosphate buffer (pH 7.0, 100 μmoles), ATP (10 μmoles), magnesium sulfate (10 μmoles), [^{14}C]CH_3-B_{12} (0.5 μCi/μmole, 0.2 μmole), dialyzed cell extract (5.2 mg of protein), tripolyphosphate (10 μmoles), and where indicated NADPH (1.0 μmole), NADH (1.0 μmole), FADH (1.0 μmole), and FMNH (1.0 μmole). H_2 atmosphere, argon replaced H_2 where indicated. Temperature 40°. Total liquid volume 1.7 ml. Reaction time 5 min.

μmole of CH_4/hr per mg of protein). ATP is required. There was a stoichiometric relationship between the amount of isotope added and the amount found in methane. (iii) The methylation of CoM is quantitative. In an experiment where CoM was methylated from [^{14}C]CH_3-B_{12} the relationship between the amount of CoM added and the amount of [^{14}C]CH_3-CoM detected was linear. CoM concentration was increased from 0.1 to 0.4 ml; the [^{14}C]CH_3-CoM formed increased from 5×10^3 to 23×10^3 cpm. The rate of methylation is slowed by higher concentrations of CoM.

Requirements for Methylation. The requirements for the enzymatic methylation of CoM are described in Table I. Methylation was stimulated when ATP and $MgSO_4$ were added. Cell extract was essential, and boiled extract was inactive. Strict, anaerobic conditions were required. Replacement of hydrogen with O_2-free argon caused 80–90% inhibition of methylation; the small endogenous activity was attributed to reduced compounds present in the cell extracts. In additional experiments it was found that if undialyzed cell extracts were used as a source of enzyme, [^{14}C]CO_2 was reduced, forming [^{14}C]CH_3-CoM. Dialysis apparently removes or destroys a component required for the activation or reduction of CO_2. A number of electron donors were tried in place of hydrogen. In these experiments argon replaced H_2 as the gas atmosphere in the reaction flasks. NADPH was a better source of electrons than hydrogen, NADH had approximately half the activity and the reduced flavins were inactive. NADPH was not oxidized to H_2. The rate of methylation of CoM is dependent on the concentration of NADPH as shown in Figure 6.

Methylation of CoM from [^{14}C]CH_3-B_{12}. The rate of methylation of CoM is dependent on the concentration of [^{14}C]CH_3-B_{12} as shown in Figure 7. Under conditions of the assay 0.4 μmole of [^{14}C]CH_3-B_{12} supported a maximum

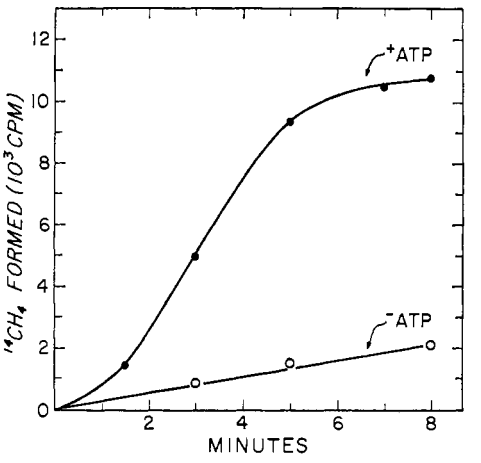

FIGURE 5: [^{14}C]CH_4 biosynthesis from [^{14}C]CH_3–CoM. Each flask contained potassium phosphate buffer (pH 7.0, 100 μmoles), ATP where indicated (10 μmoles), crude cell extract (5.7 mg of protein), [^{14}C]CH_3–CoM (11,850 cpm, 0.1 ml), and magnesium sulfate (10 μmoles). H_2 atmosphere. Temperature 40°. Total liquid volume 1.7 ml.

rate of methylation. The reaction is not inhibited by high concentrations of substrate.

pH. As shown in Figure 8 the methylation reaction has a clearly defined pH optimum in the region of 7.0–7.2.

Temperature. As shown in Figure 9 methylation occurs over a wide temperature range, being optimal near 40°, the optimal growth temperature of the organism.

Protein. The rate of methylation is directly proportional to the amount of dialyzed cell extract added. The methylation reaction does not show the extreme dilution effects characteristic of CH_4 biosynthesis by cell extracts. When the amount of cell extract was increased over a range of 1–5 mg of protein/flask, the amount of [^{14}C]CH_3-CoM detected increased linearly from 0.9×10^3 to 5.2×10^3 cpm.

Effect of Oxygen on Cell Extracts. An anaerobic cell extract was divided into two portions. Portion a was main-

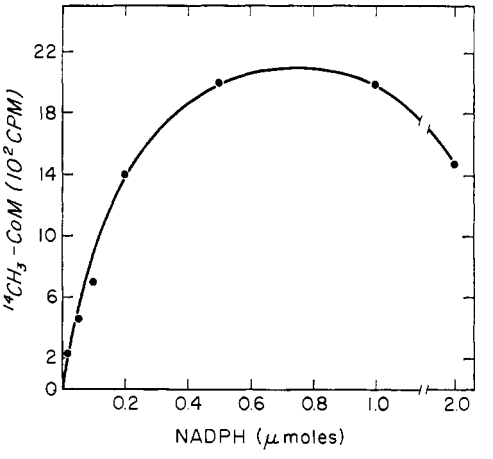

FIGURE 6: Effect of NADPH concentration on the methylation of CoM. Each reaction flask contained potassium phosphate buffer (pH 7.0, 100 μmoles), ATP (10 μmoles), magnesium sulfate (10 μmoles), [^{14}C]CH_3–B_{12} (0.5 μCi/μmole, 0.2 μmole), dialyzed cell extract (5.0 mg of protein), NADPH (as indicated), (coenzyme M preparation B (0.1 ml), and sodium tripolyphosphate (10 μmoles). Argon atmosphere. Temperature 40°. Total liquid volume, 1.7 ml. Reaction time 10 min.

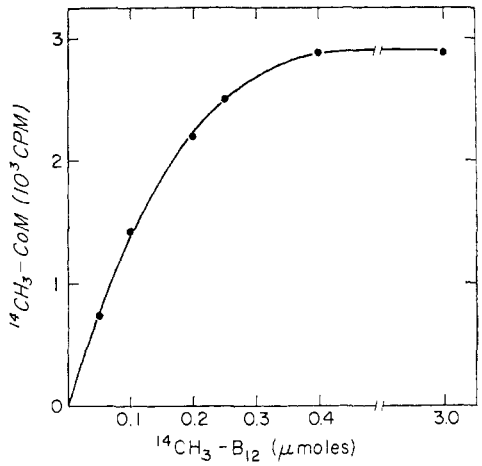

FIGURE 7: Effect of [^{14}C]CH_3-B_{12} concentration on the methylation of CoM. Each flask contained potassium phosphate buffer (pH 7.0, 100 μmoles), ATP (10 μmoles) magnesium sulfate (10 μmoles), dialyzed cell extract (6.3 mg of protein), CoM preparation B (0.4 ml), tripolyphosphate (10 μmoles), [^{14}C]CH_3-B_{12} (0.5 μCi/μmole, as indicated). H_2 atmosphere. Temperature 40°. Total liquid volume 1.7 ml. Reaction time 10 min.

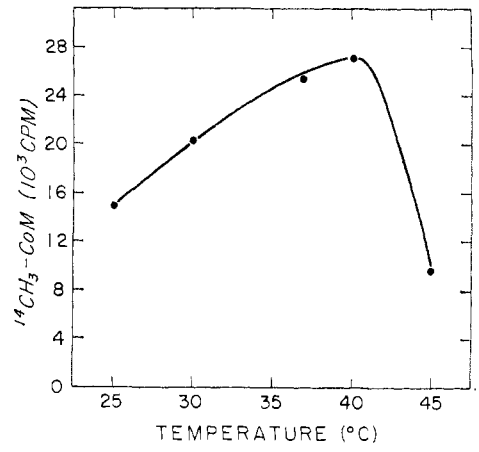

FIGURE 9: Effect of temperature on the methylation of CoM. Each reaction flask contained: potassium phosphate buffer (pH 7.0, 100 μmoles), ATP (10 μmoles), magnesium sulfate (10 μmoles), tripolyphosphate (10 μmoles), coenzyme M preparation B (0.1 ml), [^{14}C]CH_3-B_{12} (0.5 μCi/μmole), 0.2 μmole), and dialyzed cell extract (5.8 mg of protein). H_2 atmosphere. Temperature as indicated. Total liquid volume, 1.7 ml. Reaction time 5 min.

tained in a H_2 atmosphere; portion b was vigorously shaken in air for 5 min. Portion a was then distributed equally into three Warburg flasks, and portion b was similarly placed in another set of three flasks. One flask in each set was gassed with H_2 and the other two with argon. NADPH was added as an electron donor to one of the argon flasks in each set. The ability of these reaction mixtures to utilize H_2 or NADPH as an electron donor is described in Table II. Cell extracts which were oxidized and then made anaerobic were unable to use H_2 as a source of electrons, however, they retained their ability to use electrons originating from NADPH. The decreased activity compared to the unoxidized control may be due to incomplete removal of O_2 after oxidation. These results suggest that the labile enzymes are those involved in the initial oxidation of hydrogen.

Requirements for Demethylation of CH_3-CoM. The requirements for demethylation, shown in Table III are identical with those found for CH_4 biosynthesis from CO_2 or CH_3-B_{12}.

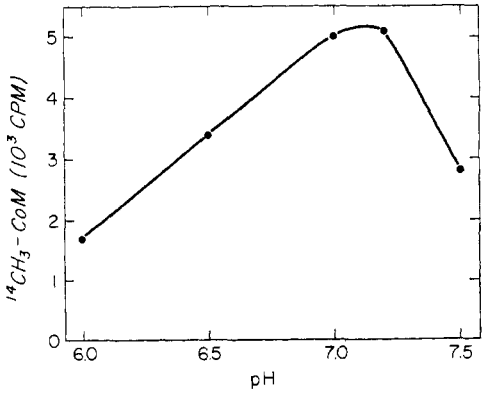

FIGURE 8: Effect of pH on the methylation of CoM. Each reaction flask contained potassium phosphate buffer (pH as indicated, 10 μmoles), ATP (10 μmoles), magnesium sulfate (10 μmoles), [^{14}C]CH_3-B_{12} (0.5 μCi/μmole, 0.2 μmole), CoM preparation B (0.05 ml), sodium tripolyphosphate (10 μmoles), and dialyzed cell extract (6.4 mg of protein). H_2 atmosphere. Temperature 40°. Total liquid volume 1.7 ml. Reaction time 5 min.

Mg^{2+} is required only when the cell extract is diluted or when dialyzed cell extract is used. Strict anaerobic conditions are required for enzyme catalysis. In additional experiments, chloroform, chloramphenicol, and methylviologen were found to inhibit the reaction. Homocysteine, which inhibits CH_4 biosynthesis from CH_3-B_{12} but not from CO_2, does not inhibit methane formation from CH_3-CoM (unpublished results), indicating that CoM is not a transmethylation factor involved in methionine biosynthesis but rather is a component of the methane biosynthetic pathway.

ATP. The effects of increasing concentrations of ATP on demethylation are shown in Figure 10. The requirement for ATP is catalytic (40); however, the extracts have high levels of ATPase activity, necessitating the use of larger quantities of ATP; as shown, high concentrations of ATP inhibit the reaction. The optimal ATP concentration varies; the greater the amount of protein the more ATP is required.

TABLE II: Effect of Electron Donors on Methylation of CoM in Treated Cell Extracts.[a]

Enzyme	Gas Atmosphere	Source of Electrons	[^{14}C]CH_3-CoM
Reduced (a)[b]	Argon	None added	254
	H_2	H_2	1070
	Argon	NADPH	1750
Oxidized (b)[b]	Argon	None added	135
	H_2	H_2	129
	Argon	NADPH	967

[a] Each reaction flask contained potassium phosphate buffer (pH 7.0, 100 μmoles), ATP (10 μmoles), magnesium sulfate (10 μmoles), coenzyme M preparation B (0.05 ml), [^{14}C]CH_3-B_{12} (0.5 μCi/μmole, 0.2 μmole), sodium tripolyphosphate (10 μmoles), and dialyzed cell extract (5.7 mg of protein). NADPH where indicated, 1.0 μmole. Gas atmosphere as indicated. Temperature 40°. Total liquid volume, 1.7 ml. Reaction time 10 min. [b] See text.

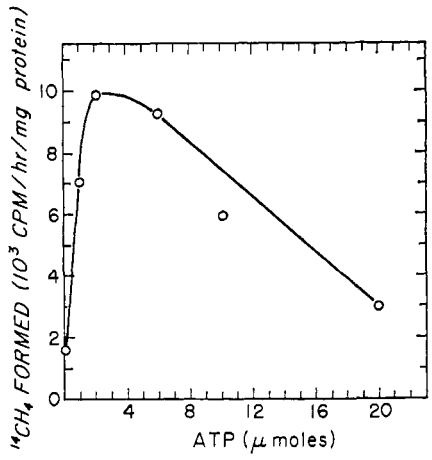

FIGURE 10: Effect of ATP concentration on [^{14}C]CH_4 formation from [^{14}C]CH_3-CoM. Each reaction flask contained potassium phosphate buffer (pH 7.0, 100 μmoles), ATP (10 μmoles), magnesium sulfate (10 μmoles), [^{14}C]CH_3-CoM (2.1 $\times$ 10^4 cpm), and crude cell extract (10.5 mg of protein). H_2 atmosphere. Temperature 40°. Total liquid volume, 1.25 ml. Reaction time 3 min.

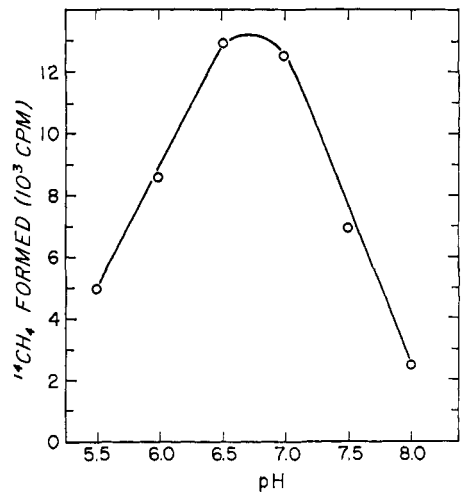

FIGURE 11: Effect of pH on [^{14}C]CH_4 formation from [^{14}C]CH_3-CoM. Each reaction flask contained potassium phosphate buffer (pH as indicated, 100 μmoles), ATP (10 μmoles) magnesium sulfate (10 μmoles), crude cell extract (9.9 mg of protein), and [^{14}C]CH_3-CoM (3 $\times$ 10^4 cpm). H_2 atmosphere. Temperature 40°. Total liquid volume, 1.25 ml. Reaction time 6 min.

Nucleotides. The effect of a variety of nucleotides on CH_4 formation from CH_3-CoM is described in Table IV. The trinucleotides GTP, CTP, and UTP stimulate the reaction but are less efficient than ATP; ITP has no effect. ADP is slightly stimulatory; AMP, IDP inhibit the reaction. It is likely that one of the latter two compounds is formed from ATP in the reaction mixture, and this could explain why ATP is inhibitory in high concentrations.

Relationships between [^{14}C]CH_4 Formed and [^{14}C]CH_3-CoM Added. The amount of [^{14}C]CH_4 formed is directly proportional to the amount of [^{14}C]CH_3-CoM added. The total amount of methane formed increased linearly from 11 $\times$ 10^3 to 43 $\times$ 10^3 cpm when the concentration of [^{14}C]CH_3-CoM was increased over a 4-fold range.

Demethylation Rate as a Function of Protein Concentration. The rate of demethylation is directly related to the protein concentration of the reaction mixture. When the extract concentration was increased over a range of 5–20 mg of protein per flask, the amount of [^{14}C]CH_4 produced increased linearly from 2.5 $\times$ 10^3 to 9 $\times$ 10^3 cpm.

pH. The demethylation reaction occurs optimally between pH 6.5 and 7.0 as shown in Figure 11.

Growth Assay. Methanobacterium ruminantium strain M-1 has an obligate growth requirement for an unknown factor found in rumen fluid by M. P. Bryant (1970). Bryant has found (personal communication) that this factor can be replaced with purified preparations of CoM. Results of the microbial assay will be published elsewhere, but we note here that the assay is extremely sensitive and could form the basis of a technique to detect much smaller quantities of CoM than the transmethylation assay.

Organisms Assayed for CoM. A number of methanogenic and nonmethanogenic organisms were investigated to determine if they contained measurable quantities of CoM.

TABLE III: Requirements for the Demethylation of [^{14}C]CH_3-CoM.[a]

Reaction Conditions	[^{14}C]CH_4 Formed after 5 min (cpm)
Omissions	
—None	10,000
—Mg	5,300
—ATP	1,640
—MgATP	1,260
—H_2	250
—Enzyme	0

[a] Complete reaction mixtures contained potassium phosphate buffer (pH 7.0, 100 μmoles), ATP (5 μmoles), magnesium sulfate (10 μmoles), crude cell extract (9.9 mg of protein), and [^{14}C]CH_3-CoM (6.1 $\times$ 10^4 cpm). H_2 atmosphere, when H_2 was omitted it was replaced with air. Temperature 40°. Total liquid volume 1.2 ml. Reaction time 5 min.

TABLE IV: Effect of Various Nucleotides on CH_4 Formation.[a]

Addition	[^{14}C]CH_4 Formed (cpm)	% of ATP Control
ATP	5250	
ADP	1470	28
AMP	670	13
GTP	3080	59
UTP	3200	61
CTP	4060	77
ITP	975	19
IDP	350	7
None	1040	20

[a] Each reaction flask contained potassium phosphate buffer (pH 7.0, 100 μmoles), nucleotide as indicated (5 μmoles), magnesium sulfate (10 μmoles), crude cell extract (9.9 mg of protein), [^{14}C]CH_3-coenzyme M (2.2 $\times$ 10^4 cpm). H_2 atmosphere. Temperature 40°. Total liquid volume, 12. ml. Reaction time 3 min.

Crude factor preparations were made by extracting whole cells with 80% ethanol, followed by flash evaporation to remove ethanol and to concentrate the solution. The cofactor preparations were assayed by the methylation assay as all preparations inhibited CH_4 biosynthesis. *Methanobacterium formicicum* strain BB, *Methanobacterium ruminantium* strain P. S., and *Methanosarcina barkeri* contain CoM in amounts comparable to *Methanobacterium* strain M. O. H. The methylated product was extracted from the reaction mixture and was found to coelectrophorese with a known sample of [^{14}C]CH_3-CoM. *Desulfovibrio vulgaris 8303*, an organism reported to form small amounts of CH_4 (Postgate, 1969) did not have CoM. CoM was not detected in *Clostridium thermoaceticum*, *Clostridium sticklandii*, *Butyrivibrio* sp., or *Clostridium kluyveri*. It was not found in liver, heart, spinach, or yeast extract. *S*-Adenosylmethionine, tetrahydrofolic acid, choline, betaine, NAD, NADP, FAD, aquo-B_{12}, UTP, DDP, homocysteine, glutamine, trimethylsulfonium iodide, *N'*-methylmicotinamide, 1-methylnicotinamideprocarbazine, or *NN*-dimethylaniline did not replace CoM in the methane biosynthesis or methylation assay.

Discussion

CoM is a heat-stable, dialyzable compound which appears to exhibit a classical growth factor, coenzyme relationship. CH_3-CoM is an intermediate in the biosynthesis of methane from CH_3-B_{12} and from CO_2. This statement is supported by results of studies on the rapid kinetics of the demethylation of CH_3-CoM to CH_4 and by the finding of [^{14}C]CH_3-CoM when extracts or whole cells were incubated with [^{14}C]CO_2.

The presence of CoM in all the methanogenic organisms examined suggests that it is probably an integral component of those organisms which synthesize CH_4 as a primary metabolic end product. It is not an intermediate in methionine biosynthesis as homocysteine does not compete for methyl groups in the reductive cleavage of the CH_3-CoM to methane.

The function of CoM as indicated by results of the transmethylation assays is to accept a methyl group and to donate it for methane biosynthesis. It is conceivable that CoM could acquire a C-1 unit more oxidized than a methyl group, and that reduction of the C_1 moiety could occur in a manner analogous to the biosynthesis of N^{10}-formyltetrahydrofolate and its subsequent reduction to 5-CH_3THFA.

The methyl-accepting atom on CoM is unknown, but it must be a strong nucleophile; the most obvious choices being nitrogen or sulfur as both are strong nucleophiles which function in known biological methylation reactions. Attempts at implicating a sulfur atom have been negative, leaving nitrogen as the most likely candidate.

The inability to find CoM activity in nonmethanogenic tissues may simply be a reflection of the insensitivity of the assay procedures. The quantities in other tissues might be expected to be very low, but should be detectable by use of the sensitive microbial growth assay of Bryant *et al.* (1971).

Acknowledgments

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