Structure and Methylation of Coenzyme M (HSCH₂CH₂SO₃)⁺

CRAIG D. TAYLOR and RALPH S. WOLFE

From the Department of Microbiology, University of Illinois, Urbana, Illinois 61801

SUMMARY

Coenzyme M is a recently discovered cofactor which is involved in methyl transfer reactions in Methanobacterium. Information derived from infrared, proton NMR, and ultraviolet spectroscopy as well as from chemical tests and quantitative elemental analysis reveals that the coenzyme is 2,2'-dithiodiethanesulfonic acid. Verification of this structure resides in the comparison of authentic with chemically synthesized 2,2'-dithiodiethanesulfonic acid. Evidence indicates that an active form of this cofactor is 2-mercaptotoethanesulfonic acid which is methylated producing 2-(methylthio)ethanesulfonic acid; this derivative is subsequently reductively demethylated, yielding methane.

Recent studies of methane formation have been devoted to methyltransfer reactions in Methanobacterium (1-3) and Methanosarcina (4-6). When cell extracts of Methanobacterium strain M. o. H. were subjected to anaerobic dialysis, they were resolved for a heat-stable organic cofactor, coenzyme M (CoM), which was required for methane formation from methylcobalamin (1). Two enzymic reactions were defined:

\[
\begin{align*}
\text{anaerobic conditions} & \quad \text{electron donor} \\
\text{methyltransferase} & \quad \text{methyl acceptor} \\
\text{CoM} & \quad \text{CoM} \\
\text{CH}_3\text{Br} & \quad \text{CH}_3\text{Br} \\
\end{align*}
\]

(1)

\[
\begin{align*}
\text{anaerobic conditions} & \quad \text{electron donor} \\
\text{methylreductase} & \quad \text{methyl acceptor} \\
\text{CoM} & \quad \text{CoM} \\
\text{CH}_3\text{H} & \quad \text{CH}_3\text{H} \\
\end{align*}
\]

(2)

Reaction 2, catalyzed by methylreductase, was specifically inhibited by tripolyphosphate and measurement of the radioactivity trapped in [methyl-¹⁴C]CoM served as an assay for CoM (1). Subsequently, sodium borohydride was found to be an excellent electron donor for Reaction 1, and use of 100-fold purified methyltransferase eliminated methyl reductase activity (7). We present here the purification, structural identification, and chemical synthesis of three biologically active forms of CoM. A preliminary report has appeared (8).

EXPERIMENTAL PROCEDURE

Materials—Methylcobalamin was chemically synthesized by the method of Müller and Müller (9); salts were removed by phenol extraction (10). The preparation was eluted from a water-equilibrated column of SP-Sephadex C-25 (ammonium form) with a 0 to 0.1 M linear ammonium acetate gradient. Ammonium acetate was removed by lyophilization. [methyl-¹⁴C]Methylcobalamin was prepared as described by Wood et al. (11) and the preparation was purified by cation exchange chromatography as outlined above. The concentration of each preparation was determined spectrophotometrically (12). Methyl iodide and sodium 2-bromoethanesulfonate were purchased from Aldrich Chemical Co.; [¹³C]methyl iodide from Amersham-Searle; cyanoacetobolin and sodium borohydride from Sigma Chemical Co.; deuterium oxide (99.8% isotopic purity) from Diaprep, Inc.; and sodium 3-trimethylsilyl-propionate 2,2,3,3-d₄ (TSP) from Merck, Sharp, and Dohme of Canada, Ltd. Methylmercaptan and argon were obtained from Union Carbide Corp.; QAE (quaternary aminoethyl)-Sephadex A-25, SP-Sephadex C-25, and Sephadex G-10 from Pharmacia Fine Chemicals, Inc.; and AG 50W-X4 (200 to 400 mesh) cation exchange resin (H⁺ form) from Bio-Rad Laboratories. Methanobacterium strain M. o. H. was mass cultured and cells were stored as previously described (1, 7).

Synthesis of Ammonium 2-Mercaptoethanesulfonate (HS-CoM)—Sodium 2-bromoethanesulfonate monohydrate (4.1 g) was dissolved in a 40-mole excess of concentrated ammonium hydroxide, and the solution was rapidly saturated with hydrogen sulfide gas. The mixture was stirred for 3 hours with a slow stream of hydrogen sulfide bubbling through the mixture. Precautions were taken to prevent entrance of air into the reaction mixture. The following steps were performed as quickly as possible to prevent autooxidation of the thiol. The ammonium hydroxide and volatile ammonium sulfide were removed by flash evaporation. The solid residue was dissolved in water and applied to a QAE-Sephadex A-25 (acetate form) column (5 × 20 cm) that had been equilibrated with water. HS-CoM was separated from the bromide salts by elution with a linear 0 to 3 M ammonium acetate gradient. The fractions which contained the thiol were located by reacting small samples with nitrous acid; HS-CoM was detected by the formation of the red S-nitroso derivative. The desired fractions were pooled and flash-evaporated to remove ammonium acetate. HS-CoM which may have oxidized to the corresponding disulfide was removed by precipitation with aqueous acetone. HS-CoM remained in solution. The acetone solution was removed by flash evaporation. HS-CoM was crystallized from methanol upon addition of diethyl ether and was recrystallized two times from the same solvent mixture. A 50% yield was obtained. Data from quantitative elemental analysis are provided in Table I.

Synthesis of Ammonium 2,2'-Dithiodiethanesulfonate (S-CoM) (0.5 g) was dissolved in 30 ml of 30% aqueous ammonium hydroxide, and the solution was bubbled with oxygen until the thiol could no longer be detected with nitrous acid. (S-CoM) was crystallized from water upon addition of acetone. The compound was recrystallized two times from the same solvents. A 20% yield
was obtained. Data from quantitative elemental analysis are presented in Table I. For certain studies the ammonium ions of synthetic (S-CoM)$_2$ were replaced by sodium ions by passage of an aqueous solution of the coenzyme through an SP-Sephadex C-25 column (sodium form) that had been equilibrated with water. Sodium (S-CoM)$_2$ was crystallized twice from aqueous acetone.  

**Synthesis of Ammonium $\alpha$-(Methylthio)ethanesulfonate**—The procedure for the synthesis of CH$_2$-S-CoM was identical with the synthesis of HS-CoM except that thymemercaptan was used instead of hydrogen sulfide. CH$_2$-S-CoM was recrystallized three times from aqueous acetone. A 20% yield was obtained. Results of quantitative elemental analysis are shown in Table I.

A large scale enzymic methylation of HS-CoM was performed in a 125-ml double sidearm Erlemeyer flask. The reaction mixture (25 ml) contained 1.25 mmoles of potassium phosphate buffer at pH 7.1, 154 mmoles of HS-CoM, 31 mmoles of [methyl-$^{14}$C]methylcobalamin (specific activity, 1020 cpm per nmole), 415 mmoles of ammonium acetate, and 100-fold purified methyltransferase (1.6 mg of protein). The reaction flask was made anaerobic, and the reaction was initiated by tipping in [methyl-$^{14}$C]methylcobalamin from one of the sidearms. The reaction was allowed to proceed until all of the [methyl-$^{14}$C]methylcobalamin had reacted. The excess nonradioactive methylcobalamin was tipped in from the other sidearm, and the reaction was allowed to proceed until all of the HS-CoM was methylated. The reaction mixture was passed through a water-equilibrated Bio-Rad AG 50W-X4 (H$^+$ form) column (2.5 X 90 cm). Ninety-eight percent of the recovered radioactivity was obtained. Results of quantitative elemental analysis are shown in Table I. 2-methoxyacetate was used, methylcobalamin and (S-CoM)$_2$ were omitted.

**Quantitative Assay of (S-CoM)$_2$ and HS-CoM**—The enzymic assay for (S-CoM)$_2$ or HS-CoM was a modification of the method of McBride as described by Taylor and Wolfe (7). The upper limit of the assay was 300 mmoles for HS-CoM and 150 mmoles for (S-CoM)$_2$. A 100-fold purified preparation of methylcobalamin-CoM methyltransferase was employed and sodium borohydride was used as the electron donor when (S-CoM)$_2$ was assayed. For assay of column eluates, the procedure was the same as described above except that the reaction was initiated by injection of a freshly prepared anaerobic solution of [methyl-$^{14}$C]methylcobalamin and sodium borohydride to the reaction mixture which contained enzyme as well as (S-CoM)$_2$ (0.05 or 0.1 ml of the column eluate). Ammonium acetate did not interfere with the assay. The reaction was terminated after 20 to 30 min of incubation by addition of the cation exchange resin directly to the entire reaction mixture. The supernatant solution (0.5 ml) was counted for radioactivity.

**Assay for Methane**—A small scale modification of the method of McBride and Wolfe (1) was performed in a test tube (13 X 55 mm) which contained 12.5 mmoles of potassium phosphate buffer at pH 7.1, 1.2 mmoles of magnesium sulfate, 1.5 mmoles of (S-CoM)$_2$, 1.3 mmoles of ATP, 3.7 mmoles of methylecobalamin, diazide crude extract (2 to 4 mg protein), and water. When CH$_2$-S-CoM (3 nmol) was used, methylecobalamin and (S-CoM)$_2$ were omitted. The volume of the reaction mixture was 0.25 ml. Each constituent except enzyme was added to the tube which then was sealed with a serum stopper. A 24-gauge hypodermic needle and a 22-gauge exit needle were inserted in the serum cap, and the tube was flushed for 10 min at a rate of 10 cc per min with hydrogen which had been scrubbed of oxygen by passage over heated copper wire. The gassing needles were removed, and the reaction was initiated by injecting enzyme. The enzyme preparation was stored prior to use in a separate tube under a hydrogen atmosphere. Each gas sample (100 ml) was withdrawn by syringe and analyzed for methane by gas chromatography as described previously (1).

**Instruments**—A Cary model 14 spectrophotometer was used for absorption spectra in the ultraviolet and visible ranges. A Beckman model DU spectrophotometer was used to follow the absorption of fractions at 260 and 400 nm. A Perkin-Elmer 521 infrared spectrometer, Varian HA-100 and A-60 NMR spectrometers, a Nuclear-Chicago Mark I liquid scintillation system, a Desaga-Brinkmann TLE system, and a Packard gas chromatograph were used for appropriate analyses.

### Results

**Purification of CoM**—Whole cells were suspended in distilled water (1:1, w/v) and stirred for 30 min at 80-90°. The cell residue from this extraction was subjected to a second extraction by the same procedure. The supernatant solution was lyophilized, ground into a fine powder, and stored in a sealed bottle at room temperature. CoM was extracted by stirring the powder in anhydrous methanol (1:10, w/v) for 30 to 40 min. The insoluble residue was removed by filtration. Two additional extractions were performed by this procedure. The clear red-orange supernatant solution was flash-evaporated to yield a red-brown oil-like residue. This fraction, obtained from the extraction of 1.5 to 2 kg of wet cells, was taken up in 100 ml of water and applied to a QAE-Sephadex A-25 column (5 X 30 cm) (acetate form) that had been equilibrated with water. The sample was eluted with 3 liters of a 0 to 3 M ammonium acetate linear gradient (Fig. 1). CoM activity was always located at two positions, Peak A and Peak B. Peak B co-eluted with coenzyme F$_{490}$, a fluorescent electron carrier found in high amounts.
The elution profile of (S-CoM)$_2$ on QAE-Sephadex A-50 is shown in Fig. 1 (left). The elution profile was determined by directly measuring the absorbance of the fraction (3 ml) in each tube (18 x 150 mm) at 400 nm. (S-CoM)$_2$ activity was determined by assaying a 0.05-ml sample from each tube in a reaction mixture (0.25 ml) which contained 12.5 μmoles of potassium phosphate buffer at pH 7.1, 2.5 μmoles of sodium tripolyphosphate, 550 nmoles of sodium borohydride, 127 nmoles of [methyl-14C]methylcobalamin (specific activity, 4470 cpm per n mole), and 100-fold purified methylcobalamin-CoM methyltransferase (85 μg of protein). Reaction time, 15 min.

The elution profile from a Sephadex G-10 separation of the components of Peak B is shown in Fig. 2 (right). The elution profile was determined by measuring the absorbance at 260 nm with a Beckman model DU spectrophotometer. Rectangular quartz cells with a 1-cm light path were used. (S-CoM)$_2$ activity was assayed as described in Fig. 1.

The recovery of CoM from this purification scheme was 65 mg of crystalline CoM per kg of wet packed cells. Accurate determination of the recovery from this scheme was not possible as inhibitors of the methyltransferase reaction were present in both the initial, lyophilized water extract and the methanol-soluble residue. For example, the total activity found in Peak B of the anion exchange eluate (Table I) increased nearly 3-fold over that observed in the methanol-soluble residue.

In Methanobacterium (13). From preparation to preparation, the per cent of the total activity found in Peak A varied in a reciprocal manner to that found in Peak B. Both derivatives possessed a strong negative charge, requiring 1.8 and 2.5 m ammonium acetate to elute Peaks A and B, respectively, from the column. Since Peak B contained from 70 to 88% of the total activity, this fraction was used for further purification. Ammonium acetate was removed by flash evaporation at 40°. The CoM-containing eluate from the equivalent of 4 kg of wet cells was dissolved in 8 to 10 ml of water and fractionated on a Sephadex G-10 column (2.5 x 90 cm) that had been equilibrated with water. The elution fluid was water. As shown in Fig. 2, CoM eluted in a single peak and was separated from much contaminating material that absorbed at 260 nm, the bulk of which was coenzyme Fe. The pooled active fractions from the G-10 eluate were reduced to 10 ml by flash evaporation. The sample was applied to a continuous electrophoresis apparatus, and separation was effected on a paper curtain at 400 volts (8 to 10 ma) in a pH 4, 0.02 m ammonium acetate buffer. CoM migrated 10 to 13 cm toward the positive pole while descending 7 cm and was separated from fluorescent compounds. The active fractions were flash-evaporated at 40° to remove the ammonium acetate buffer. The solid residue was taken up in a small amount of water and recrystallized three times from aqueous acetone, yielding colorless platelets with a melting point greater than 250°.

The recovery of CoM from this purification scheme was 65 mg of crystalline CoM per kg of wet packed cells. Accurate determination of the recovery from this scheme was not possible as inhibitors of the methyltransferase reaction were present in both the initial, lyophilized water extract and the methanol-soluble fraction. For example, the total activity found in Peak B of the anion exchange eluate (Table I) increased nearly 3-fold over that observed in the methanol-soluble residue.

Structure of CoM—Evidence obtained from infrared, proton NMR, and ultraviolet spectroscopy as well as from chemical tests and quantitative elemental analyses revealed that CoM as isolated is the ammonium salt of 2,2'-dithiodiethanesulfonic acid (S-CoM)$_2$. This compound possesses the following structure:

\[ \text{H}_2\text{N}^-\text{O}\text{SCH}_2\text{CH}_2\text{SSCH}_2\text{CH}_2\text{SO}_3^-\]NH$_3^+$

Fig. 3 shows that the infrared spectra of authentic and synthetic (S-CoM)$_2$ are identical. Each spectrum is largely dominated by the absorptions of the sulfate and ammonium ions. The strong absorptions at Peaks d and e (1170 and 1035 cm$^{-1}$) represent, respectively, the asymmetric and symmetric SO$_3^-$ stretching modes characteristic of sulfonic acid salts. The shoulders on the left of Peak d are probably the wag motion of the methylene groups attached to sulfur. The ammonium ion stretch and deformation modes appear at a and c, respectively (3200, 3060, and 1435 cm$^{-1}$); these absorptions disappear revealing the methylene stretch (2970 and 2930 cm$^{-1}$) and deformation (1420 cm$^{-1}$) modes when the ammonium ion is replaced by sodium ion as shown in the inset of Fig. 3. The fact that untreated aqueous solutions of both authentic and synthetic (S-CoM)$_2$ gave a positive reaction with Nessler's reagent provides additional chemical evidence for the presence of the ammonium ion. Evidence suggests that the peaks located at g (590 and 530 cm$^{-1}$) may involve the sulfonate group; Palmer (14), working with potassium dithionate, assigned the sharp peaks observed at 577 and 516 cm$^{-1}$ to be fundamental absorptions of the SO$_3^-$ deformation modes.

The absorptions observed with a variety of CoM derivatives which possess an intact sulfonate group as well as with sodium ethanesulfonate suggest that the peaks located at f and g involve the sulfonate moiety. All absorptions in this region as well as in the SO$_3^-$ stretch region were lost in the spectrum of ammonium 3,3'-dithiodipropionate where the sulfonate group is replaced by a carboxyl group. Data provided by Bellamy (15) and Colthup et al. (16) also were used to aid in peak assignments. The proton NMR spectra of deuterium oxide solutions of authentic and synthetic (S-CoM)$_2$ are identical in all respects as shown in Fig. 4. There are no additional resonances downfield.
FIG. 3. Infrared spectra of authentic and synthetic (S-CoM). One milligram of crystalline (S-CoM)₄ (ammonium salt) was mixed in 200 mg of anhydrous spectral grade potassium bromide. The mixture was heated over phosphorous pentoxide at 90° for 1 hour.

of 5 ppm. The observed symmetrical resonances at 3.28 and 3.11 ppm are of a 4-proton system of the type AB₂ characteristic of an aliphatic compound of the configuration XCH₂CH₂Y. The possibility of having an AA'BB' system of the configuration of X(CH₂CH₂)₂Y is eliminated on the basis of quantitative elemental analysis, and particularly the synthesis of (S-CoM)₄. The assignments made were based on observing the chemical shifts of the ethylene resonances of a variety of aliphatic sulfonate, sulfide, and disulfide compounds (17, 18). The presence of both a sulfonate and disulfide group in (S-CoM)₄ displaces the resonances of both methylene groups approximately 0.4 ppm downfield relative to the resonances observed in aliphatic compounds which contain only one of the functional groups of interest. Resonances of methylene groups next to the sulfonate functional group were in general located downfield of those methylene groups next to sulfide or disulfide moieties.

Ultraviolet spectra of authentic and synthetic (S-CoM) exhibit absorption maxima at 191 to 193 and 245 nm. The extinction coefficients which were determined for the short wavelength band for both preparations ranged between 5800 and 6400 liters mole⁻¹ cm⁻¹. Because these measurements were made at the extreme lower end of the useful wavelength range of the Cary model 14 instrument, attention should be drawn only to the order of magnitude. The extinction coefficient at 245 nm, however, may be determined with accuracy and for synthetic (S-CoM)₄ was found to be 380 liters mole⁻¹ cm⁻¹, respectively (19). The elemental compositions of authentic and synthetic (S-CoM)₄ are shown in Table I; neither differs from that calculated by greater than 0.46%.

Authentic and synthetic (S-CoM)₄ were identical in their biological behavior. When 1.5 μmoles of synthetic (S-CoM)₄ were added to a standard reaction mixture which contained 4.9 μmoles of [methyl-¹⁴C]methylcobalamin and 100-fold purified methylcobalamin-CoM methyltransferase (8 μg of protein), a perfectly linear reaction rate was produced, with 400 nmoles of CH₄S-CoM being produced in 10 min. The reaction rate of authentic (S-CoM)₄ was identical. In a separate experiment where the reaction was allowed to proceed to completion, the reaction mixture contained 52 μg of 100-fold purified methyltransferase and 2.5 μmoles of [methyl-¹⁴C]methylcobalamin. Two levels of synthetic (S-CoM)₄, 152 and 304 nmoles, and two levels of authentic (S-CoM)₄, 148 and 296 nmoles, were tested. For each mole of (S-CoM)₄ added 1.98 ± 0.10 moles of methyl groups were bound. The ratio remained within the above described limits when either 100-fold purified methyltransferase was used with sodium borohydride as the electron donor or when crude extracts were used with sodium borohydride or hydrogen as the electron source.

Reduction of (S-CoM)₄—The function of the electron requirement in the methyltransferase reaction was found to be that of reducing the disulfide bond of (S-CoM)₄ prior to methylation. HS-CoM functions as a methyl acceptor in the absence of an electron donor as shown by the open and closed triangles, in Fig. 5. Comparison of the open and closed circles shows that methylation of (S-CoM)₄ requires an electron donor. There was a negligible chemical methylation of HS-CoM from methyl-
cobalamin. HS-CoM was stoichiometrically methylated when the methyltransferase-catalyzed reaction was allowed to proceed to completion.

2-(Methylthio)ethanesulfonic Acid—The results presented above indicate that two methyl groups are bound for each mole of (S-CoM). The methyl derivative of the coenzyme retained a strong negative charge, suggesting that methyl-CoM could be 2-(methylthio)ethanesulfonic acid.

Results presented in Fig. 6 show that the methylated coenzyme which was isolated from a large scale reaction mixture (see “Experimental Procedure”) possesses an NMR spectrum identical with that of ammonium 2-(methylthio)ethanesulfonate which was chemically synthesized. The resonances of protons a and b appear, respectively, at 3.15 ppm (relative intensity, 1.8) and 2.88 ppm (relative intensity, 2.0). The large singlet labeled c at 2.17 ppm (relative intensity, 3.1) is typical of the proton resonances of a methyl group attached to sulfide. The small singlet at 2.24 ppm resulted from a contaminant in the solvent used for crystallization. The presence of the methyl group shifts resonances a and b upfield, with that of b being shifted to a greater extent. This lends additional support to the proton assignments made. No evidence was obtained to support the possibility that the methylated derivative of CoM is the methylsulfonate ester. Such a CoM derivative would be a neutral molecule and possess physical properties considerably different from those of the coenzyme. The NMR spectra of (S-CoM) in deuterium oxide (99.8% isotopic purity) were obtained with a Varian HA-100 NMR spectrometer. A NMR tube (2-mm inside diameter X 5-mm outside diameter X 17.7 mm) was used. The spectra were obtained under the following conditions (where the conditions are different, the value for authentic (S-CoM) is given before that for synthetic (S-CoM)): concentration, 3 mg per 0.05 ml and 10 mg per 0.07 ml; temperature, 28°C; frequency response, 1 and 5 Hz; radio frequency attenuator, 20 db; sweep time, 1000 s; sweep width, 500 Hz; sweep offset, 0 and 473 Hz; spectrum amplitude, 10,000; lock signal, sodium 3-trimethylsilyl-propionate-2,2,3,3-d₄ (TSP) and hydrogen deuterium oxide (HOD); field milligauss (manual oscillator frequency), 0.1 and 0.05 mG; field milligauss (sweep frequency), 0.05 and 0.1 mG; reference compound, TSP.

FIG. 4. NMR spectra of authentic and synthetic (S-CoM). The NMR spectra of (S-CoM) in deuterium oxide (99.8% isotopic purity) were obtained with a Varian HA-100 NMR spectrometer. A NMR tube (2-mm inside diameter x 5-mm outside diameter x 17.7 mm) was used. The spectra were obtained under the following conditions (where the conditions are different, the value for authentic (S-CoM) is given before that for synthetic (S-CoM)): concentration, 9 mg per 0.07 ml and 9 mg per 0.06 ml; temperature, 28°C; frequency response, 2 and 20 Hz; radio frequency attenuator, 26 db; sweep time, 500 s; sweep width, 506 Hz (inset, 250 Hz); sweep offset, 0 and 472 Hz; spectrum amplitude, 10,090; lock signal, sodium 3-trimethylsilyl-propionate-2,2,3,3-d₄ (TSP) and hydrogen deuterium oxide (HOD); field milligauss (manual oscillator frequency), 0.1 mG; field milligauss (sweep frequency), 0.04 and 0.1 mG; reference compound, TSP.

FIG. 5. Comparison of the reductive requirement of the methylcobalamin-CoM methyltransferase reaction when HS-CoM or (S-CoM) was used as the methyl acceptor. The reaction mixtures (0.25 ml) contained 12.5 pmoles of potassium phosphate buffer at pH 7.1; where indicated, 1.50 pmoles of (S-CoM) or 3.01 pmoles of HS-CoM; where indicated, 550 nmoles of sodium borohydride; 3.7 pmoles of [methylv-¹C]methylocobalamin (specific activity, 1730 cpm per n mole); and 100-fold purified methylreductase (13 µg of protein).

FIG. 6. Comparison of the NMR spectra of authentic and synthetic CH₃-S-CoM. The NMR spectra of CH₃-S-CoM in deuterium oxide (99.8% isotopic purity) were obtained with a Varian HA-100 NMR spectrometer. A NMR tube (2-mm inside diameter x 5-mm outside diameter x 17.7 mm) was used. The spectra were obtained under the following conditions (where the conditions are different, the value for the enzymically synthesized, authentic CH₃-S-CoM is given before that for synthetic CH₃-S-CoM): concentration, 3 mg per 0.05 ml and 10 mg per 0.07 ml; temperature, 28°C; frequency response 1 and 5 Hz; radio frequency attenuator, 20 db; sweep time, 1000 s and 500 s; sweep width, 500 Hz; sweep offset, -473 and 0 Hz; spectrum amplitude, 10,000 (inset, 10 X gain), and 6,000; lock signal, sodium 3-trimethylsilyl-propionate-2,2,3,3-d₄ (TSP) and hydrogen deuterium oxide (HOD); field milligauss (manual oscillator frequency), 0.1 and 0.05 mG; field milligauss (sweep frequency), 0.05 and 0.1 mG; reference compound, TSP.
different than those observed. The methyl proton resonances of an aliphatic methylsulfonate ester lie very close to 3.9 ppm, a position very easily distinguishable from the resonances observed.

We examined the possibility that (S-CoM)₂ was methylated to yield 2-(dimethylsulfonium)ethanesulfonate ((CH₃)₂-S-CoM) which decomposed to CH₃-S-CoM prior to analysis. The NMR spectrum of chemically synthesized (¹⁴CH₃)₂-S-CoM is presented in Fig. 7. The spectrum in deuterium oxide revealed symmetrical ethylene proton resonances of the type a,b at 3.65 and 3.46 ppm (cumulative relative intensity, 4.0). A singlet corresponding to the methyl proton resonances was located at 3.00 ppm (relative intensity, 5.5). CH₃-S-CoM and (CH₃)₂-S-CoM were readily separable by anion exchange chromatography and thin layer electrophoresis. (CH₃)₂-S-CoM forms an internal salt between the sulfonium and sulfonate moieties. This imparts a charge considerably different from that of CH₃-S-CoM. (CH₃)₂-S-CoM was found to be completely stable under the conditions used for analysis. Reaction mixtures which contained methyltransferase were directly fractionated by anion exchange chromatography (Fig. 8A). When limiting amounts of [methyl-¹⁴C]methylcobalamin were added to a reaction mixture, 92 and 190 nmoles of methane were produced in 25 min. In a separate experiment, the reaction was allowed to proceed to completion with the methyl group from 101 and 202 nmoles of synthetic CH₃-S-CoM being completely converted to methane. From 101 and 202 nmoles of authentic CH₃-S-CoM, 92 and 190 nmoles of methane, respectively, were detected.

As shown in Table II, (CH₃)₂-S-CoM was found to be completely inert as a methyl donor in the methylreductase-catalyzed reaction in incubation periods up to 70 min. No methane production was observed when (CH₃)₂-S-CoM and HS-CoM were present together in the reaction mixture; (CH₃)₂-S-CoM would not methylate HS-CoM to yield biologically active CH₃-S-CoM. Results from a separate experiment showed that CH₃-S-CoM could not be further methylated by methyltransferase.

**DISCUSSION**

The fact that CoM was isolated as the disulfide possibly reflects an artifact of isolation. Thiol derivatives such as HS-CoM are, under neutral or alkaline conditions, easily oxidized to disulfides. Anaerobic precautions were not taken when CoM was isolated. Early work on the isolation of CoA revealed that much of it was in the form of a disulfide (20). Mild hydrolysis of partially purified CoA released 2,2'-dithiodiethylamine. Disulfide formation may likely explain the presence of more than one active derivative of CoM in crude preparations. The component of Peak A in the QAE-Sephadex A-25 eluate may be a heterodisulfide possessing only one sulfonate moiety RSSCH₄CH₂SO₄⁻ (R-S-S-CoM). The sulfonate group will significantly govern the behavior of such a CoM derivative on an anion exchange column. Thus, (S-CoM)₂, possessing two sulfonate moieties would be expected to elute at a higher ionic strength than a derivative possessing only one sulfonate group. This is in fact the case. (S-CoM)₂ elutes from a QAE-Sephadex A-25 column at approximately 2.5 m ammonium acetate whereas CH₃-S-CoM and HS-CoM both elute at a concentration less than 2 m. The observation that the ratios of CoM activity in Peaks A and B vary in a reciprocal manner from preparation to preparation may simply reflect the proportions of (S-CoM)₂ and R-S-S-CoM present.
It is one of the smallest and simplest of the known coenzymes, is indicated by \( X \).

Radioactivity was determined by counting sections (1.0 min. The known samples were located by staining with iodine vapors. Radioactivity was determined by counting sections (1.0 x 1.5 cm) of the thin layer in Bray's solution. The origin is indicated by \( \times \).

HS-CoM, 2-mercaptopropanesulfonate, has unique properties. It is one of the smallest and simplest of the known coenzymes, is highly acidic, and has an unusually high concentration of sulfur for its size. When the mercapto group is methylated, the coenzyme does not employ an "onium" ion as the active site of methyl transfer. This latter property stands in contrast to methyltransfer compounds such as S-adenosylmethionine, choline, betaine, dimethylthetin, or dimethylpropionin. The coenzyme has similarities to CoA and lipoic acid but so far appears to have a completely different role in biochemistry. It will be interesting to see if the coenzyme can handle a C₁ moiety more oxidized than a methyl group.

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