Interaction of coenzyme M and formaldehyde in methanogenesis

James A. ROMESSER* and Ralph S. WOLFE†

Department of Microbiology, University of Illinois, Urbana, IL 61801, U.S.A.

(Received 20 January 1981/Accepted 14 April 1981)

Chemical reaction of coenzyme M, sodium 2-mercaptoethanesulphonate (HS-CoM, Na⁺), and formaldehyde formed sodium 2-(hydroxymethylthio)ethanesulphonate (HOCH₂-S-CoM), whereas reaction with the ammonium salt of HS-CoM yielded iminobis-[2-(methylthio)ethanesulphonate], monoammonium salt [NH=(CH₂-S-CoM)₂]. In water, NH=(CH₂-S-CoM)₂ decomposed to 2-(aminomethylthio)ethanesulphonate (NH₂CH₂-S-CoM) and HOCH₂-S-CoM. NH₂CH₂-S-CoM was degraded further to form more HOCH₂-S-CoM. The structures of these coenzyme M derivatives were confirmed by i.r. and n.m.r. spectroscopy and by elemental analysis. When added to cell extracts of *Methanobacterium thermoautotrophicum*, methane was formed from either HOCH₂-S-CoM or NH=(CH₂-S-CoM)₂ at rates comparable with the rate of methane formation from the methanogenic precursor 2-(methylthio)ethanesulphonate (CH₃-S-CoM). Formaldehyde was reduced to methane at similar rates. In addition, certain hemimercaptals, including thiazolidine and thiazolidine-4-carboxylate, were reduced, although at slower rates. The reduction of formaldehyde, thiazolidine, or thiazolidine-4-carboxylate required catalytic amounts of HS-CoM. ATP was required by cell extracts for reduction of each of these methane precursors.

Coenzyme M, identified as 2-mercaptoethanesulphonate (Taylor & Wolfe, 1974), has been shown to be a C₁ carrier in the terminal steps of microbial reduction of CO₂ to methane (McBride & Wolfe, 1971). Taylor & Wolfe (1974) demonstrated that cell extracts of *Methanobacterium bryantii* (formerly strain M.o.H.) reductively demethylated CH₃-S-CoM with hydrogen to yield methane. Gunsalus *et al.* (1978) found that, in addition to CH₃-S-CoM, the methyl-coenzyme M reductase of *Methanobacterium thermoautotrophicum* reductively dealkylated CH₃CH₂-S-CoM but not CH₃CH₂CH₂-S-CoM. Analogues of CH₃-S-CoM modified at the sulphide or sulphonate position, or containing additional methyl-

ene carbon atoms, were not demethylated by the methyl-coenzyme M reductase.

In this paper we present evidence for the involvement of CoM in the activation and reduction of formaldehyde to methane. A preliminary report has appeared (Romesser & Wolfe, 1977).

Materials and methods

Preparation of analogues

Ammonium 2-mercaptoethanesulphonate (HS-CoM) and ammonium 2-(methylthio)ethanesulphonate (CH₃-S-CoM) were prepared as described by Gunsalus *et al.* (1978).

Sodium 2-(hydroxymethylthio)ethanesulphonate (HOCH₂-S-CoM) was prepared by mixing sodium 2-mercaptoethanesulphonate (0.22 g, 1.34 mmol) with 40% (w/v) formalin (1.0 ml, 13.3 mmol) in 7 ml of methanol. The reaction was stirred at room temperature overnight. Acetone was added to precipitate HOCH₂-S-CoM from solution (0.18 g, 69%) (Found: C, 18.5; H, 3.8; Na, 11.6; S, 32.8. Calc. for C₃H₇NaO₄S₂: C, 18.6; H, 3.6; Na, 11.8; S, 33.0%). The n.m.r. spectrum confirmed the synthesis of the hemimercaptal, δ (p.p.m.) [(²H)dimethyl sulphoxide] 2.86 (4H, s, SCH₂CH₂SO₃), 4.63 (2H, d, *J* 6 Hz, SCH₂OH), 5.67 (1 H, t, *J* 6 Hz, OH).

Abbreviations used: HS-CoM, 2-mercaptoethanesulphonate, coenzyme M; CH₃-S-CoM, 2-(methylthio)ethanesulphonate; HOCH₂-S-CoM, 2-(hydroxymethylthio)ethanesulphonate; NH₂CH₂-S-CoM, 2-(aminomethylthio)ethanesulphonate; HN=(CH₂-S-CoM)₂, iminobis-[2-(methylthio)ethanesulphonate]; CH₂=(S-CoM)₂, 2,2'-(methyleneedithio)diethanesulphonate.

* Present address: Experimental Station, E. I. DuPont de Nemours & Co., Wilmington, DE 19898, U.S.A.

† To whom correspondence and requests for reprints should be addressed.

Purity was estimated as greater than 95% as indicated by the lack of an HS-CoM spot when $\text{HOCH}_2\text{--S--CoM}$ was subjected to t.l.c. (see the Results section). Also, no resonance at 2.63 p.p.m. from the sulphhydryl moiety of possibly contaminating HS-CoM was observed in the n.m.r. spectrum of $\text{HOCH}_2\text{--S--CoM}$ in (^2H)dimethyl sulphoxide.

Iminobis-[2-(methylthio)ethanesulphonate], mono-ammonium salt, $[\text{NH}=(\text{CH}_2\text{--S--CoM})_2]$ was prepared by mixing ammonium 2-mercaptoethanesulphonate (0.21 g, 1.32 mmol) with 40% (w/v) formalin (1.0 ml, 13.3 mmol) in 7 ml of methanol. The reaction mixture was stirred overnight at room temperature. $\text{NH}=(\text{CH}_2\text{--S--CoM})_2$ precipitated from solution and was collected by filtration (0.18 g, 79%) (Found: C, 21.2; H, 5.2; N, 8.0; S, 37.3. Calc. for $\text{C}_6\text{H}_{18}\text{N}_2\text{O}_6\text{S}_4$: C, 21.0; H, 5.3; N, 8.2; S, 37.4%). An absorbance in the i.r. was measured for the secondary amine group at 1602 cm^{-1} . The n.m.r. spectrum obtained was δ (p.p.m.) [(^3H) trifluoroacetic acid] 3.42 (8H, multiplet, $\text{SCH}_2\text{CH}_2\text{SO}_3$), 4.41 (4H, t, J 4 Hz, $\text{SCH}_2^+\text{NH}_2\text{CH}_2\text{S}$), 6.50 (4H, t, J 54 Hz, NH_4^+), 7.85 (2H, broad, $\text{CH}_2^+\text{NH}_2\text{CH}_2$).

Ammonium 2,2'-(methyleneedithio)diethanesulphonate $[\text{CH}_2=(\text{S--CoM})_2]$ (203 mg, 66%) was prepared by alkylation of HS-CoM (300 mg, 1.86 mmol) with methylene di-iodide (75 μl , 0.935 mmol) as in the preparation of $\text{CH}_3\text{--S--CoM}$. (Found: C, 18.0; H, 5.6; N, 8.8; S, 38.9. Calc. for $\text{C}_5\text{H}_{18}\text{N}_2\text{O}_6\text{S}_4$: C, 18.2; H, 5.5; N, 8.5; S, 38.8%). The n.m.r. spectrum confirmed the synthesis of the mercaptal, δ (p.p.m.) ($^2\text{H}_2\text{O}$) 3.10 (8H, multiplet, $\text{SCH}_2\text{CH}_2\text{SO}_3$), 3.86 (2H, s, SCH_2S).

The ^1H n.m.r. spectra were recorded on a Varian HA-100 spectrometer or on a Varian EM 390 spectrometer. The i.r. spectra of the KBr pellets were measured on a Beckman model 12 spectrometer.

Preparation of cell extracts

Methanobacterium thermoautotrophicum was grown in a 12-litre New Brunswick fermenter using the general procedures described by Bryant *et al.* (1968). Each 12 litres of growth medium contained 15 g of NH_4Cl , 600 ml of mineral solution no. 2 (Bryant & Robinson, 1961), 60 ml of vitamin solution (Wolin *et al.*, 1963), 60 ml of trace mineral solution (Wolin *et al.*, 1963), 0.5 g of ferrous ammonium sulphate, and 23 g of NaHCO_3 . The cysteine-sulphide reducing agent was prepared separately as a 7.5% (w/v) aqueous solution, autoclaved, and added to the sterile medium. The carbon and energy requirements were supplied as a CO_2/H_2 (1:4) mixture at a flow rate of 700–900 ml/min. The final pH of the CO_2 -equilibrated medium was 7.2–7.4. Growth was initiated with a 1–10 ml inoculum of culture grown overnight in tubes as described by Balch & Wolfe (1976).

Cells of *M. thermoautotrophicum* were harvested

with a continuous flow Sharples centrifuge. The cell paste was diluted with an equal amount (w/v) of 0.1 M-Pipes (1,4-piperazinediethanesulphonic acid), pH 6.9, and gassed with hydrogen. The anaerobic cell slurry was stored frozen at -20°C .

Extracts of *M. thermoautotrophicum* were prepared by passage of the cell slurry through a French pressure cell at 110 400 kPa. Cellular debris was removed by centrifugation of the lysate at 33 000 g (r_{av} , 8.2 cm) for 30 min in capped, stainless steel centrifuge tubes at 4°C . The tubes were maintained anaerobic by filling the gas space above the lysate with H_2 . The dark-brown supernatant solution was decanted into serum vials and stored frozen at -20°C under H_2 at 47 kPa.

Dialysed extract was prepared by dialysing 20 ml of cell extract against two 2-litre volumes of 0.05 M-Pipes buffer, pH 6.9. The dialysis was performed anaerobically by bubbling the buffer with H_2 (50 ml/min) for 1 h before adding the cell extract as well as throughout the dialysis period. The dialysis was performed at 4°C and was allowed to proceed for 12 h in each change of buffer. The dialysed extract was stored frozen at -20°C under H_2 at 47 kPa.

Assay for methanogenesis

Each assay for methanogenesis was performed in a calibrated 5 ml reaction vial as described by Taylor & Wolfe (1974). The standard assay mixture with cell extract contained 75 μmol of Pipes buffer, pH 6.9 (at room temperature), 1.0 μmol of ATP, 1.0 μmol of MgCl_2 , 0.5–1.5 μmol of substrate, and 100 μl of cell extract in a total volume of 0.3–0.4 ml. Each assay with dialysed extract had a similar composition, except that 200 μl of dialysed extract was added. The gas atmosphere was H_2 , and each reaction was performed at 60°C .

To eliminate background methane formed from C_1 units endogenous to the cell extract, a pre-incubation procedure was used. Preincubation was not necessary with dialysed extract. All components except cell extract and substrate were added to each vial, and the vial was flushed with O_2 -free H_2 (10 ml/min) for 15 min. Extract was then added, and the vial was transferred to a shaking water bath at 60°C . The reaction was allowed to proceed for 10 min, and the vial was transferred to an ice bath. Substrate, made anaerobic by bubbling with H_2 (10 ml/min for 10 min), was then added, and the vial was regassed for 5 min with H_2 . The enzymic reduction of substrate to methane was initiated by transferring the vial to the water bath at 60°C . The production of methane was measured in each vial by withdrawing a 50 μl aliquot of the gas phase with a Hamilton syringe and injecting it into a Packard model 7500 series gas chromatograph equipped with a Porapak QS column and hydrogen flame detector.

Chemical determinations

Formaldehyde was determined by the procedure of Brill & Wolfe (1966). Sulphydryl groups were measured with the Ellman (1959) assay. Protein concentration was measured by the method of Lowry *et al.* (1951).

Results

Reaction of formaldehyde with the sodium salt of HS-CoM formed $\text{HOCH}_2\text{-S-CoM}$, whereas reaction of formaldehyde with the ammonium salt yielded $\text{NH}=(\text{CH}_2\text{-S-CoM})_2$. The structure of each of these compounds was confirmed by elemental analysis and by n.m.r. and i.r. spectroscopy (see the Materials and methods section).

$\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ was found to decompose in water. When an aqueous solution of $\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ was chromatographed on Eastman 6064 cellulose plates in acetone/water (16:3, v/v), two spots (R_F 0.11 and R_F 0.36) appeared upon spraying the chromatogram with silver fluoresceinate, a reagent which yields a yellow colour with sulphonates. In the same solvent system single spots were obtained from HS-CoM (R_F 0.58), from the disulphide $(\text{S-CoM})_2$ (R_F 0.26), or from $\text{HOCH}_2\text{-S-CoM}$ (R_F 0.36). These results indicate the hydrolysis of the disulphonate $\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ to an unidentified monosulphonate. Both spots on the $\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ thin-layer chromatogram gave positive reactions with Ellman reagent [5,5'-dithiobis-(2-nitrobenzoic acid), 'DTNB'] and with dinitrophenylhydrazine indicating that sulphydryl and aldehyde groups were chemically available in each compound. Assay of $\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ with Ellman reagent and with dinitrophenylhydrazine showed that each mol of $\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ yielded 2.06 mol of sulphydryl and 1.94 mol of aldehyde.

To study the hydrolysis of $\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ n.m.r. spectroscopy was used (Table 1). Shortly after being dissolved in $^2\text{H}_2\text{O}$, three singlet peaks, δ 4.69, 4.27 and 4.10 p.p.m., were observed in addition to a multiplet at 3.13 p.p.m. ($-\text{SCH}_2\text{CH}_2\text{SO}_3^-$) (spectrum 4). With time, the ratio of the integrations of the singlet peaks changed (spectrum 5) but the sum of the integrations of the singlet resonances always gave a ratio of two protons to the four protons in the $-\text{SCH}_2\text{CH}_2\text{SO}_3^-$ resonance. As $\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ was hydrolysed the 4.69 p.p.m. resonance increased in intensity at the expense of the 4.27 p.p.m. resonance. At the same time, the 3.13 p.p.m. multiplet shifted to 3.04 p.p.m. and became broader, the ethylene proton resonance then appearing very similar to the resonance observed for $\text{HOCH}_2\text{-S-CoM}$ (spectrum 3). Comparison of the spectrum of

Table 1. *N.m.r. spectra of coenzyme M derivatives in aqueous solution*
Chemical shifts are shown relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate in $^2\text{H}_2\text{O}$. Compositions of aqueous solutions are calculated as percentage of the total integration of $\text{R-CH}_2\text{-S-CoM}$ protons in each sample.

Spectrum	Sample	Composition	Molar ratio	Observed chemical shifts (δ , p.p.m.)			Composition of aqueous solution (%)		
				4.72			$\text{HOCH}_2\text{-S-CoM}$ ($\delta = 4.7$ p.p.m.)	$\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ ($\delta = 4.27$ p.p.m.)	$\text{NH}_2\text{CH}_2\text{-S-CoM}$ ($\delta = 4.10$ p.p.m.)
1	$\text{H}_2\text{C(OH)}_2$								0
2	HS-CoM					2.97	100	0	0
3	$\text{HOCH}_2\text{-S-CoM, Na}^+$			4.70		3.06		75	20
4	$\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ (10 min)			4.69	4.27	4.10	5	19	20
5	$\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ (2 h)			4.69	4.27	4.10	61	10	13
6	HS-CoM, Na^+ + NH_4Cl + $\text{H}_2\text{C(OH)}_2$		1:1:2	4.70	4.27	4.10	77	14	63
7	$\text{HOCH}_2\text{-S-CoM, Na}^+$ + NH_4Cl		1:10	4.70	4.27	4.10	23	16	61
8	$\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ + NH_4Cl		1:7	4.69	4.27	4.10	23		

hydrolysed $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$ with that of $\text{HOCH}_2-\text{S}-\text{CoM}$ suggests that $\text{HOCH}_2-\text{S}-\text{CoM}$ was a major decomposition product of $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$ and that the 4.69 p.p.m. resonance in the $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$ spectrum was due to $-\text{S}-\text{CH}_2\text{OH}$. Addition of formaldehyde to hydrolysed $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$ resulted in the appearance of a resonance at 4.72 p.p.m. (results not shown). The lack of a 4.72 p.p.m. resonance in the spectrum of $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$ and the dissimilarity of the $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$ spectrum from the combined individual spectra of formaldehyde and $\text{HS}-\text{CoM}$ (spectra 1 and 2) suggests that free formaldehyde and $\text{HS}-\text{CoM}$ were not decomposition products of $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$. Also, the 4.69 p.p.m. resonance was not likely to be due to solvent effects or to interaction of formaldehyde with the SO_3^- or NH_4^+ moieties, since in a separate experiment (results not shown) mixing of formaldehyde with ammonium bromoethanesulphonate did not alter the original spectrum of bromoethanesulphonate. When $\text{HS}-\text{CoM}$ (Na^+ salt), NH_4Cl , and formaldehyde were added to $^2\text{H}_2\text{O}$, a spectrum nearly identical to that of hydrolysed $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$ was obtained (spectrum 6). Addition of excess formaldehyde to this mixture resulted in the appearance of a separate singlet at 4.72 p.p.m. Clearly, $\text{HS}-\text{CoM}$, NH_4^+ , and formaldehyde react in water to form $\text{HOCH}_2-\text{S}-\text{CoM}$ and $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$.

The large peak area of the 4.27 p.p.m. resonance upon first dissolving $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$ in $^2\text{H}_2\text{O}$ would suggest that this resonance was due to the $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$ protons. The 4.10 p.p.m. resonance, therefore, was due to the methylene protons of $\text{NH}_2\text{CH}_2-\text{S}-\text{CoM}$. Not only were $\text{NH}_2\text{CH}_2-\text{S}-\text{CoM}$ and $\text{HOCH}_2-\text{S}-\text{CoM}$ hydrolysis products from $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$ but they also were interconverted via the reaction:

Indeed, as a solution of $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$ (25 mg/ml) was hydrolysed, the pH of the solution increased from 2.8 to 3.6. When excess NH_4Cl was added to an aqueous solution of $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$, the 4.10 p.p.m. resonance increased in intensity proportional to a decrease in intensity of the 4.7 p.p.m. resonance (spectrum 8). Similarly, addition of NH_4Cl to a solution of $\text{HOCH}_2-\text{S}-\text{CoM}$ (Na^+ salt) resulted in a decrease in the 4.7 p.p.m. resonance and the appearance of resonances at 4.10 and 4.27 p.p.m. (spectrum 7). The concentration of NH_4^+ ion in the medium, therefore, modulates the relative abundance of $\text{HOCH}_2-\text{S}-\text{CoM}$, $\text{NH}_2\text{CH}_2-\text{S}-\text{CoM}$ and $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$. All the data (n.m.r., chromatographic, and chemical) suggest the facile interconversion of these species, although it would appear that $\text{HOCH}_2-\text{S}-\text{CoM}$ is normally the predominant species.

When either $\text{HOCH}_2-\text{S}-\text{CoM}$ or $\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$ was added to cell extracts of *M. thermoautotrophicum*, methane was formed at rates comparable with the rate of $\text{CH}_3-\text{S}-\text{CoM}$ reduction (Table 2). Methane was not formed from the mercaptal, $\text{CH}_2=(\text{S}-\text{CoM})_2$, nor could cleavage of the mercaptal to form $\text{HS}-\text{CoM}$ be demonstrated. Addition of formaldehyde to cell extracts resulted in methane production at rates equal to the CoM methanogenic precursors, whereas hemimercaptals such as thiazolidine and thiazolidine-4-carboxylate were reduced at much slower rates.

By use of dialysed extracts it was shown that the reduction of formaldehyde to methane required $\text{HS}-\text{CoM}$. In the absence of added $\text{HS}-\text{CoM}$, the rate of methane production from $1\mu\text{mol}$ of formaldehyde was $27\text{nmol}\cdot\text{h}^{-1}\cdot(\text{mg of protein})^{-1}$. In the presence of $1\mu\text{mol}$ of $\text{HS}-\text{CoM}$ this rate was increased to $550\text{nmol}\cdot\text{h}^{-1}\cdot(\text{mg of protein})^{-1}$. The residual activity observed in the absence of

Table 2. Methane formation from potential methanogenic precursors

Each assay was performed with cell extract and $0.5\mu\text{mol}$ of substrate as described in the Materials and methods section.

Substrate	Rate of methane formation [$\text{nmol}\cdot\text{h}^{-1}\cdot(\text{mg of protein})^{-1}$]	Yield of methane (mol formed/mol of substrate)
$\text{CH}_3-\text{S}-\text{CoM}$	550	0.99
$\text{HOCH}_2-\text{S}-\text{CoM}$	567	1.03
$\text{NH}=(\text{CH}_2-\text{S}-\text{CoM})_2$	533	1.98
$\text{CH}_2=(\text{S}-\text{CoM})_2$	0	0
HCHO	540	1.04
Thiazolidine	291	0.98
Thiazolidine-4-carboxylate*	41	0.80

* Reaction incomplete after 100 min of incubation.

Fig. 1. Effect of HS-CoM on the reduction of formaldehyde to methane

Each reaction mixture contained dialysed extract. Formaldehyde was added where indicated (500, 1000, or 1500 nmol) as described in the Materials and methods section. HS-CoM was added to each reaction mixture to give the ratio of formaldehyde:HS-CoM indicated.

Fig. 2. Effect of ATP on the reduction of $\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ to methane

Each reaction mixture contained dialysed extract and 500 nmol of $\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ as described in the Materials and methods section. Where indicated ATP was added (10, 25, or 75 nmol) to the reaction mixture.

HS-CoM probably reflected the slow release of HS-CoM bound to protein as mixed disulphides. In bioassays performed by Dr. W. E. Balch it was determined that HS-CoM was present at a level of 100 $\mu\text{g}/\text{ml}$ (0.63 mM) in cell extracts. This level was decreased only 25-fold (4 $\mu\text{g}/\text{ml}$, 0.025 mM) in dialysed extracts, even though the dialysis dilution factor was 1:10000.

Maximal rates of methanogenesis from formaldehyde were observed when the ratio of formaldehyde to HS-CoM concentrations was approx. 1 (Fig. 1). At ratios much greater than 1, inhibition of methane formation was observed. This inhibition was related to at least two factors. First, excess formaldehyde inhibited the system. For example, at a ratio of formaldehyde:HS-CoM of 3, much slower rates of methanogenesis were observed when 1500 nmol of formaldehyde served as substrate (a 1000 nmol excess of formaldehyde) than when 500 nmol HCHO was used (a 330 nmol excess of formaldehyde). Second, at very high formaldehyde:HS-CoM ratios, the HS-CoM concentration itself became limiting. This was shown by the large inhibition in the rate of reduction of 500 nmol of formaldehyde at a formaldehyde:HS-CoM ratio of 20. Here, although the formaldehyde excess was only 475 nmol, the 25 nmol of HS-CoM present was insufficient to allow for a fast rate of methane formation. ATP stimulated the reduction of $\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ (Fig. 2). This stimulation was observed as an increase in the rate of methane

formation from $\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ and as a decrease in the lag before methanogenesis began. As with the reduction of $\text{CH}_3\text{-S-CoM}$ (Gunsalus & Wolfe, 1978) this stimulation was catalytic. Similar observations were made when $\text{HOCH}_2\text{-S-CoM}$, formaldehyde or thiazolidine were tested as substrate for methanogenesis in dialysed extract.

Discussion

Early studies by Schnellen (1947) demonstrated that cell suspensions of some methanogens were unable to reduce formic acid or methanol to methane. Stadtman (1949) found that although copious amounts of [^{14}C]methane were formed from $^{14}\text{CO}_2$ by cell extracts of *Methanococcus vannielii*, formaldehyde and methanol could not be detected. These observations prompted Barker (1956) to propose that the C_1 intermediates in methanogenesis were bound to one or more carriers. More recently, Taylor *et al.* (1976) have confirmed that pools of free formic acid, formaldehyde, and methanol are not formed during methanogenesis. Work by McBride & Wolfe (1971) and Taylor & Wolfe (1974) has established that coenzyme M is the methanogenic C_1 carrier at the methyl level of oxidation. The results presented here suggest that

coenzyme M also serves as the C_1 carrier at the formaldehyde level of oxidation.

Fig. 3 summarizes the observed chemical reactions of coenzyme M with formaldehyde or formaldehyde equivalents. Data from n.m.r. analysis indicate that $\text{HOCH}_2\text{-S-CoM}$ is readily formed from formaldehyde and HS-CoM in aqueous solution. In the presence of NH_4^+ , $\text{NH}_2\text{CH}_2\text{-S-CoM}$ and $\text{NH}=(\text{CH}_2\text{-S-CoM})_2$ also may form, though at low NH_4^+ concentration $\text{HOCH}_2\text{-S-CoM}$ is the predominant species present in solution. Free HS-CoM and formaldehyde are not observed unless one is present in excess over the other. Normally formaldehyde is greater than 99.9% hydrated in aqueous solution. Kallen & Jencks (1966) have measured molar equilibrium constants for the reaction:

and found constants of 300 to 3000 for $\text{X} = \text{H}_2\text{NR}$ and 34000 for $\text{X} = \text{HSR}$. These data support the assertion that $\text{HOCH}_2\text{-S-CoM}$ is the predominant formaldehyde species in aqueous solution.

$\text{HOCH}_2\text{-S-CoM}$ was found to be readily reduced to methane by cell extracts of *M. thermoautotrophicum*. That CoM may be a C_1 carrier at the formaldehyde level of oxidation is supported by the following observations. (i) $\text{HOCH}_2\text{-S-CoM}$ was the predominant species present when formaldehyde and HS-CoM were added in aqueous solution. (ii) HS-CoM was required for formaldehyde reduction. (iii) Maximal rates of methanogenesis were obtained when the formaldehyde: HS-CoM ratio was approx. 1. Observations (i) and (iii) tend to argue against the possibility that the HS-CoM requirement for the reduction of formaldehyde may solely reflect the role of CoM in the $\text{CH}_3\text{-S-CoM}$ methylreductase.

Since methanol is not a methanogenic substrate in *M. thermoautotrophicum*, formaldehyde cannot be directly reduced to methanol. If formaldehyde is reduced on a carrier other than coenzyme M, then this carrier must be of relatively large molecular weight (>5000) or associated with large molecular weight components of the cell extract, since dialysed extract retained activity with formaldehyde as substrate.

The mechanism by which $\text{HOCH}_2\text{-S-CoM}$ is reduced has not been determined, but it is clear that $\text{HOCH}_2\text{-S-CoM}$ is not reduced via mercaptal formation with HS-CoM , since $\text{CH}_2=(\text{S-CoM})_2$ was not enzymically reduced to methane. A mercaptal might be formed, however, with a sulphhydryl of an enzyme. Similarly, the hemimercaptal could react with an amino group to yield an aminosulphide. Both mercaptal and aminosulphide formation would separate the dehydration and reduction reactions required to convert $\text{HOCH}_2\text{-S-CoM}$ to $\text{CH}_3\text{-S-CoM}$ into two steps. Synthesis and enzymic characterization of $\text{HOCH}_2\text{-S-CoM}$ derivatives such as $\text{CH}_3\text{OCH}_2\text{-S-CoM}$ may be useful in characterizing the importance of mercaptal or aminosulphide formation in the reduction of $\text{HOCH}_2\text{-S-CoM}$.

The observation that thiazolidine-4-carboxylate and thiazolidine are methane precursors suggests that any hemimercaptal can function as a C_1 donor. Each hemimercaptal would be expected to participate in an equilibrium much as that described for $\text{HOCH}_2\text{-S-CoM}$. That these hemimercaptals were reduced at rates significantly lower than was formaldehyde indicates that the rate at which formaldehyde is released in the aldehyde-hemimercaptal equilibrium is a rate-limiting step. In the case of these two hemimercaptals, interaction of the $-\text{S-CH}_2\text{OH}$ group with the amino group, resulting

Fig. 3. Reactions of coenzyme M with formaldehyde or formaldehyde equivalents

in cyclization to the thiazolidine, is an additional component of the equilibrium.

It is not clear why $\text{HOCH}_2\text{-S-CoM}$ was reduced, after a 40 min lag, without the addition of ATP. Possibly $\text{HOCH}_2\text{-S-CoM}$ reduction to $\text{CH}_3\text{-S-CoM}$ was coupled to ATP synthesis. The ΔG° for the reduction of formaldehyde to methanol with H_2 is about -42 kJ/mol (-10 kcal/mol) (Stadtman, 1967). This, however, is barely sufficient energy to allow for ATP synthesis. Also, Robertson & Wolfe (1969) showed that the rate of ATP resynthesis in extracts of *M. bryantii* with H_2/CO_2 as substrate was too slow to account for the observed catalysis by ATP. The mechanism of the catalytic role of ATP in methanogenesis by cell extracts remains to be elucidated.

The excellent technical assistance of Deborah Szurgot, Robert Mangoff, Paquita Erazo, and Victor Gabriel is gratefully acknowledged. J. A. R. was the recipient of a traineeship from the U.S. Public Health Service GM07-283(603). This work was supported by the National Science Foundation Grant NSF PCM 76-02652 and U.S. Public Health Service Grant AI 12277.

References

- Balch, W. E. & Wolfe, R. S. (1976) *Appl. Environ. Microbiol.* **32**, 781-791
- Barker, H. A. (1956) *Bacterial Fermentations*, pp. 1-27, John Wiley and Sons, New York
- Brill, W. J. & Wolfe, R. S. (1966) *Nature (London)* **212**, 253-255
- Bryant, M. P. & Robinson, J. M. (1961) *J. Dairy Sci.* **44**, 1446-1456
- Bryant, M. P., McBride, B. C. & Wolfe, R. S. (1968) *J. Bacteriol.* **95**, 1118-1123
- Ellman, G. (1959) *Arch. Biochem. Biophys.* **82**, 70-77
- Gunsalus, R. P. & Wolfe, R. S. (1978) *J. Bacteriol.* **135**, 851-857
- Gunsalus, R. P., Romesser, J. A. & Wolfe, R. S. (1978) *Biochemistry* **17**, 2374-2377
- Kallen, R. G. & Jencks, W. P. (1966) *J. Biol. Chem.* **241**, 5864-5878
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- McBride, B. C. & Wolfe, R. S. (1971) *Biochemistry* **10**, 2317-2324
- Robertson, A. M. & Wolfe, R. S. (1969) *Biochim. Biophys. Acta* **192**, 420-429
- Romesser, J. A. & Wolfe, R. S. (1977) *Proc. Annu. Meet. Am. Soc. Microbiol.* **12**, 155
- Schnellen, C. G. T. P. (1947) *Onderzoekingen over de Methaangisting*, De Maasstad, Rotterdam
- Stadtman, T. C. (1949) *Studies on the Methane Fermentation*, Ph.D. Thesis, University of California, Berkeley
- Stadtman, T. C. (1967) *Annu. Rev. Microbiol.* **25**, 121-142
- Taylor, C. D. & Wolfe, R. S. (1974) *J. Biol. Chem.* **249**, 4879-4885
- Taylor, G. T., Kelley, D. P. & Pirt, S. J. (1976) in *Microbial Production and Utilization of Gases (H_2 , CH_4 , CO)* (Schlegel, H. G., Gottschalk, G. & Pfennig, N., eds.), pp. 173-180, E. Goltze, Göttingen
- Wolin, E. A., Wolin, M. J. & Wolfe, R. S. (1963) *J. Biol. Chem.* **238**, 2882-2886