JOURNAL OF BACTERIOLOGY, Jan. 1979, p. 264-273
0021-9193/79/01-0264/10$02.00/0

Vol. 137, No. 1

Transport of Coenzyme M (2-Mercaptoethanesulfonic Acid) in *Methanobacterium ruminantium*

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Received for publication 18 October 1978

A system for transport of coenzyme M, 2-mercaptoethanesulfonic acid (HS—CoM), in *Methanobacterium ruminantium* strain M1 required energy, showed saturation kinetics, and concentrated the coenzyme against a gradient. The process was sensitive to temperature and was maximally active at pH 7.1. Cells took up HS—CoM at a linear rate, with a V_{max} of 312 pmol/min per mg (dry weight) and an apparent K_m of 73 nM. An intracellular pool of up to 5 mM accumulated which was not exchangeable with the medium. Uptake required both hydrogen and carbon dioxide; it was inhibited by O_2. Bromoethanesulfonic acid ($BrCH_2CH_2SO_3^-$), a potent inhibitor of methanogenesis in cell-free extracts, inhibited both uptake and methane production. Results of inhibitor studies with derivatives and analogs of the coenzyme showed that the specificity of the carrier is restricted to a limited range of thioether, thioester, and thiocarbonate derivatives. 2-(Methylthio)ethanesulfonic acid (CH_3—S—CoM) showed an apparent K_i for HS—CoM uptake of 15 nM, being taken up itself with a V_{max} of 320 pmol/min per mg (dry weight) and an apparent K_m of 50 nM. An analysis of intracellular pools after HS—CoM uptake indicated that the predominant forms are a heterodisulfide of unknown composition and CH_3—S—CoM.

The discovery of 2-mercaptoethanesulfonic acid (HS—CoM) as a coenzyme for methyltransfer reactions in methanogens (16) led to the development of a bioassay for the cofactor; *Methanobacterium ruminantium* strain M1 has an obligate growth requirement for the coenzyme (15). The bioassay is both sensitive and quantitative, showing a half-maximal growth yield at 25 nM, a level typical of other vitamin bioassays (1). In a previous communication Balch and Wolfe defined the structural specificity of the coenzyme for growth of strain M1 (2). Of the compounds tested, only a restricted range of thioether, thioester, and thiocarbonate derivatives of the coenzyme M were found to replace the cofactor requirement on an equimolar basis. In addition, $BrCH_2CH_2SO_3^-$, a potent inhibitor of methane production from 2-(methylthio)ethanesulfonic acid (CH_3—S—CoM) in cell extracts of *M. thermoautotrophicum* (8), was found to inhibit the growth response of strain M1 to the coenzyme.

Considerable interest has appeared in the past several years in the uptake and metabolism of micronutrients by bacteria. In particular, studies of vitamin B_6 (9, 10, 12), B_{12} (3, 6), and folate (13) have delineated the range of transport processes available to the cell. The present communication describes a characterization of uptake and metabolism of coenzyme M by *M. ruminantium* M1. These studies represent the first attempt to define the properties of a carrier-mediated process in such strict anaerobes.

MATERIALS AND METHODS

Organisms and growth conditions. *M. ruminantium* M1 was kindly provided by M. P. Bryant, Department of Dairy Science, University of Illinois, Urbana. Other organisms were obtained as described previously (2). Culture medium, anaerobic procedures, and growth conditions were as described by Balch and Wolfe (1, 2). Cells for transport assays were routinely cultured in a 125-ml serum vial (Wheaton Scientific, Millville, N.J.) in 10 ml of medium at 2 atm of pressure of an 80% hydrogen-20% carbon dioxide gas mixture, or in 100 ml of medium in a 1-liter bottle (no. 219760, Wheaton Scientific) modified to accommodate syringe injection (Fig. 1). After sterilization, the atmosphere in the large bottle was pressurized to 2 atm with the gas mixture. The medium in each bottle, when inoculated, was incubated at 39°C in a rotary-shaker water bath at 120 rpm. For safety the large culture bottle was placed inside a cylindrical stainless-steel petridish container which had an opening (3.5 cm in diameter) in the bottom to allow for circulation of water and a similar opening in the top, providing access to the serum top. (The large bottle specified above has a special design which provides the rigidity required for this procedure. We advise that other bottles should not be used for this technique.) Growth was measured at 660 nm with a Bausch & Lomb Spectronic 20 spectrophotometer; an 18-mm light path was used.

Cell water content was determined by the method of Winkler and Wilson (17) with ^{14}C-labeled starch (specific activity, 15.2 mCi/mg).

Measurement of uptake. Cells of a mid-logarithmic culture (A_{660}, 0.4 to 0.8) which had been grown in the presence of 100 ng of HS—CoM per ml (630 nM) were used for transport assays without harvesting and washing. A 2- to 5-ml amount of culture was transferred with a Glaspak disposable syringe (Becton, Dickinson & Co., Rutherford, N.J.) to a 30-ml serum vial which contained an 80% hydrogen-20% carbon dioxide gas mixture at 1 atm of pressure. Prior to initiation of the assay, each cell suspension was preincubated for 15 min at 41°C in a reciprocal shaking water bath (120 strokes/min) to allow for gas equilibration. Uptake was initiated by addition of labeled substrate. At each time period a measured sample of 0.5 to 1.0 ml was removed from the assay vial with a syringe. The sample was added to a membrane filter of 0.22 μm pore size (Millipore Corp., Bedford, Mass.). Cells were vacuum-filtered from the assay mixture and washed twice at room temperature with 2.5-ml portions of a mineral salts buffer (pH 7.1) which contained (g/liter): KH_2PO_4, 2.2; K_2HPO_4, 6.1; $(NH_4)_2SO_4$, 0.45; NaCl, 0.9; $MgSO_4 \cdot 7H_2O$, 0.18; and $CaCl_2 \cdot 2H_2O$, 0.012. This procedure is referred to as the filter assay. For kinetic experiments a rapid-dilution assay was used. A measured 0.5- to 1.0-ml portion was transferred at each time interval (0.5, 1.0, and 1.5 min) to a separate tube which contained 5 ml of mineral salts buffer and 100 μg of HS—CoM per ml (630 μM) at room temperature. The contents of each tube were immediately blended in a Vortex mixer in air for 10 s to terminate transport. Samples were vacuum-filtered as described above. Each membrane filter was placed in a separate scintillation vial which contained 10 ml of Bray's solution (4), and radioactivity was measured with a Packard Tri-Carb model 3375 liquid scintillation spectrometer. Quenching was corrected by the channels ratio method. Transport rates, unless specified otherwise, are expressed as picomoles per minute per milligram (dry weight) of cells, and were calculated from linear plots by the linear least-squares method. Liquid additions to assay vials were not routinely degassed unless more than 25 μl per ml of assay medium was being added.

Synthesis of [2-^{35}S]ammonium 2-mercaptoethanesulfonate ($H^{35}S$—CoM). $H^{35}S$—CoM was synthesized by the procedure of Gunsalus et al. (8) with the following modifications: 2.7 mg of [^{35}S]thiourea (283 mCi/mmol; New England Nuclear Corp., Boston, Mass.) was dissolved in 130 μl of absolute ethanol and added to a 0.3-ml Reacti-Vial (Pierce Chemical Co., Rockford, Ill.) which contained 32.4 mg of sodium bromoethanesulfonate and 7.5 mg of unlabeled thiourea in the solid state. The mixture was refluxed for 4 h at 83°C in the sealed vial (Miniert Teflon valve, Pierce Chemical Co.) with frequent mixing until an observable precipitate formed. The hot ethanol was removed with a 1-ml syringe fitted with a 25-gauge needle. The precipitate was washed with three separate 100-μl portions of hot ethanol (80°C). The remaining residue was dissolved in 92 μl of concentrated NH_4OH and refluxed for 2 h at 105°C. The residue was transferred to a small column, 4 by 80 mm (a Pasteur pipette), which contained Sephadex SP C-25 (NH_4^+) (Pharmacia Fine Chemicals, Inc., N.J.) equilibrated with water. The sample was eluted with 2 ml of water and lyophilized to dryness. The residue was dissolved in 100 μl of water and applied to a cellulose powder (CF-11) column (8 mm by 25 cm; Whatman, Clifton, N.J.) which had been equilibrated with acetone–water (16:3). Fractions which gave a positive reaction to Ellman's reagent (7) were pooled. Acetone was removed by gentle evaporation under nitrogen, and the sample was lyophilized to dryness. The residue was dissolved in 200 μl of water and recrystallized by the addition of 3 ml of diethylether. The precipitate was collected on a Nucleopore filter (1.2 μm; Nucleopore, Pleasanton, Calif.). Identity and purity of the product was confirmed by thin-layer chromatography (TLC) on cellulose sheets (no. 13255, Eastman, Rochester, N.Y.) in two separate solvent systems of acetone–water (16:3) and acetone–water–concentrated NH_4OH (16:2:1) as well as by thin-layer electrophoresis (TLE) at pH 2.0 with formic acid–acetic

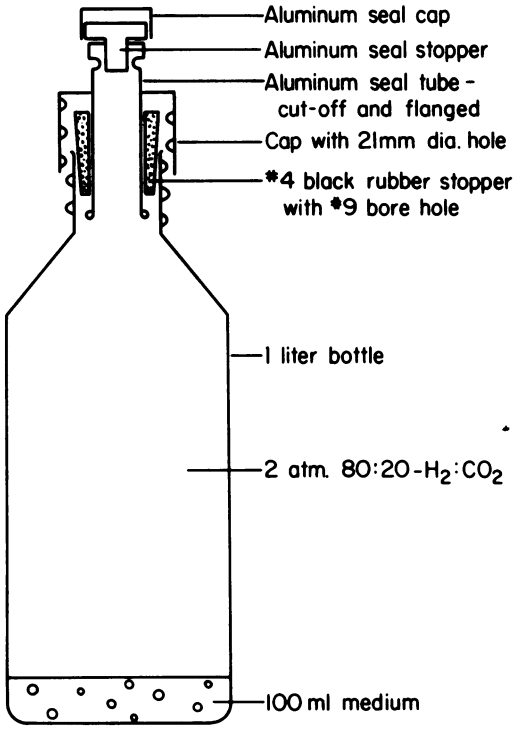

FIG. 1. *Modified 1-liter storage bottle used for growing cells in 100- to 200-ml liquid volumes at 2 atm of pressure. The cap has a 21-mm diameter hole to allow serum tube (no. 2048-00150, Bellco Glass, Vineland, N.J.), which has been cut and flanged, to protrude from the bottle. The tube is sealed in the bottle with a no. 4 black rubber stopper with a no. 9 size hole. The tube and stopper are held in place by screwing the bottle cap down. A black rubber stopper (no. 2048-11800, Bellco Glass) is crimped into place with a one-piece aluminum seal (no. 224183, Wheaton Scientific, Millville, N.J.).*

acid-water (13:58:1,000) for 45 min at 12 mA, 400 V in a Desaga-Brinkman apparatus. A 50% yield was obtained (10 mg) with a specific activity of 80 mCi/mmol.

Synthesis of [2,2'-^{35}S]ammonium 2,2'-dithiodiethanesulfonate (^{35}S—CoM)$_2$ and [2-^{35}S]ammonium 2-(methylthio)ethanesulfonate (CH_3—^{35}S—CoM). (^{35}S—CoM)$_2$ and CH_3—^{35}S—CoM were synthesized from H^{35}S—CoM as described by Gunsalus et al. (8) and were purified by CF-11 column chromatography as described above. Other coenzyme M derivatives and analogs used in inhibitor studies were obtained as described previously (2, 8).

Extraction of the intracellular ^{35}S pool from cells. To determine the composition of the intracellular ^{35}S pool, cells which had taken up H^{35}S—CoM were harvested and washed as described in each experiment. The cells were extracted at 60°C in 10 ml of methanol-water (66:33) which contained 100 μl of 0.1 M potassium phosphate buffer (pH 7.5) and 50 μl of 100 mM *N*-ethylmaleimide (NEM). HS—CoM was quantitatively derivatized by NEM under these conditions. After 5 min at 60°C, the precipitate, which was devoid of radioactivity, was removed by centrifugation and discarded. The supernatant solution was flash-evaporated to dryness at 30°C. The residue was resuspended in a minimal volume of methanol-water (66:33) and spotted on an Eastman cellulose thin-layer sheet along with the appropriate nonradiolabeled carrier (20 μg of each/spot). Plates were developed with acetone-water-concentrated NH_4OH (16:2.5:0.5) in the first dimension. Where indicated, plates were dried and subjected in the second dimension to TLE at pH 2.0 with formic acid-acetic acid-water (13:58:1,000) for 45 min at 12 mA, 400 V. Silver-fluoresceinate reagent (14), Ellman's reagent (7), or potassium ferricyanide-ferric chloride reagent (14) was used to visualize appropriate compounds. To reduce disulfide bonds, extracts were treated with dithiothreitol (100 mM) for 4 h; under these conditions (^{35}S—CoM)$_2$ was quantitatively reduced to H^{35}S—CoM. After TLC or TLE each plate was thoroughly dried, and an autoradiogram was obtained by exposing Eastman Kodak NS-2T X-ray film to the sheet for 1 to 10 days. To quantitate radioactivity the chromatogram was cut into segments, each segment being placed in a separate scintillation vial which contained 2 ml of methanol. After 15 min, 10 ml of Bray's solution (4) was added to each vial, and radioactivity was determined as described above.

RESULTS

Growth of organism. *M. ruminantium* M1 was easily cultivated in 100- to 200-ml amounts in the pressurized atmosphere of the 1-liter bottle described in Fig. 1. Under these conditions, cells had an average generation time of 12 to 14 h. Cells that were harvested and washed anaerobically once in a mineral salts buffer and resuspended in coenzyme-free medium showed rates of uptake identical to that of unwashed cells. When resuspended only in mineral salts buffer, cells showed a depressed rate (70%) of uptake. Identical rates of uptake were observed in cells for periods up to 2 h, the rate of proliferation being too low to affect uptake significantly. One absorbance unit corresponded to 0.45 mg (dry weight)/ml when mid-logarithmic cells were used. Cell water content was found to be 3.2 (± 0.2) μl of cell water/mg of cell dry weight.

Time course of HS—CoM uptake. Uptake rates of radiolabeled substrate were identical as measured by the filter assay or the rapid-dilution assay. Uptake of H^{35}S—CoM was found to occur at an optimal rate in cells from the mid-logarithmic growth phase which were being cultivated in the presence of HS—CoM at levels between 0.63 and 630 μM (Table 1). The rate of uptake was found to be proportional to cell concentration in the range tested (50 to 1,000 μg of dry weight/ml).

Figure 2 shows a typical time course of coenzyme uptake when cells were incubated at 41°C in the presence of excess coenzyme (50 μM). Uptake was linear for up to 40 min, with an average rate of 250 to 300 pmol/min per mg (dry weight) and a range of 200 to 400 pmol/min per mg (dry weight) for different cell preparations. This corresponds to an average rate of 225 molecules of HS—CoM/cell per s (Table 2). Under these conditions cells accumulated HS—CoM to form an intracellular ^{35}S pool against a concentration gradient at least 10^6-fold higher than substrate detectable in the medium. The ^{35}S pool was not reversibly exchangeable with the medium or the mineral salts buffer in the presence or absence of excess unlabeled HS—CoM (500 μM).

Kinetics of HS—CoM uptake. Uptake was concentration dependent and demonstrated saturation kinetics. A linear least-squares fit of a double reciprocal plot of uptake velocity over the concentration range of 20 to 1,000 μM for

TABLE 1. *Effect of increasing concentrations of HS—CoM in the growth medium on uptake of H^{35}S—CoM by cell suspensions of M. ruminantium M1*[a]

HS—CoM in medium		H^{35}S—CoM uptake (pmol/min per mg, dry wt)
Amt (μg/ml)	Concn (μM)	
0.001	0.0063	195
0.01	0.0630	250
0.1	0.630	400
1.0	6.30	370
10.0	63.00	400
100.0	630.00	360

[a] Cells were grown for 60 h in 10 ml of medium supplemented with the indicated amounts of HS—CoM. Harvested cells were washed anaerobically with 3 volumes of coenzyme-free medium and resuspended in 4 ml of the same medium. H^{35}S—CoM uptake was assayed by the rapid-dilution assay described in Materials and Methods.

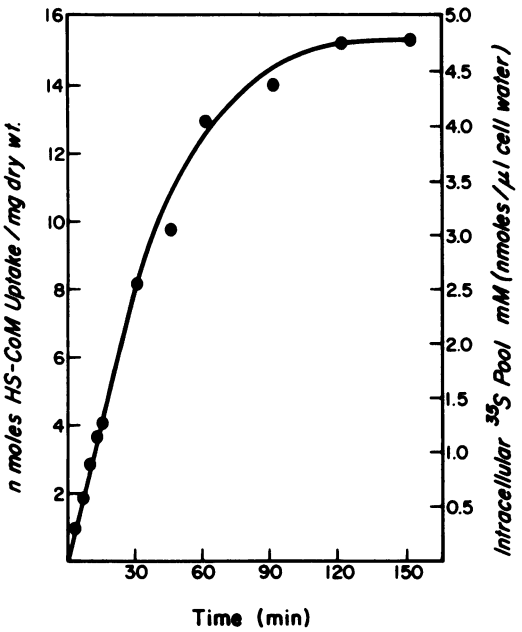

FIG. 2. *Time course of HS—CoM uptake. Cells were incubated in the presence of 50 μM $H^{35}S$—CoM, uptake being followed by the filter assay. Samples were removed for assay at the indicated times.*

TABLE 2. *Uptake of $H^{35}S$—CoM by strain M1 in the presence of limiting substrate*[a]

Time (min)	A Extracellular $H^{35}S$—CoM[a] (nmol/ml of filtrate)	B Uptake of $H^{35}S$—CoM (nmol/ml of filtered cell suspension)	C Intracellular ^{35}S pool[b] (μM)	*Ratio* C/A
0	1.18	—	—	—
6	0.90	0.28	410	455
12	0.55	0.63	930	1,690
18	0.27	0.93	1,360	5,037
24	<0.0005[c]	1.19	1,750	>10^6
30	<0.0005	1.20	1,760	>10^6

[a] Cells (426 μg [dry weight]/ml, 2.5 × 10^9 cells/ml by Petroff-Hausser chamber count) were pulsed with 5.88 nmol of $H^{35}S$—CoM. A 1-ml amount of cell suspension was transferred to a 0.22-μm membrane filter at the indicated time intervals and washed three times. The filtrate was collected directly into a scintillation vial mounted beneath the filter; 5.95 nmol of ^{35}S was recovered in cells.

[b] Cell water: 3.2 μl/mg (dry weight).

[c] No radioactivity detectable above background.

three separate experiments yielded an apparent K_m of 73 nM and a V_{max} of 312 pmol/min per mg of dry weight (correlation coefficient = 0.98).

Effect of pH on uptake. HS—CoM uptake was pH dependent, demonstrating optimal activity over a broad range (pH 6.2 to 8.0), when short-term (3 min) preincubation conditions were used (Fig. 3). When longer preincubation times were used to allow for complete equilibration of the HCO_3^-/CO_2 buffer (15 min), a narrower pH optimum was observed from pH 6.9 to 7.8. A pH range of 7.0 to 7.2 was used routinely.

Effect of temperature on uptake. A sharp temperature optimum for HS—CoM uptake was observed at 51°C (Fig. 4). The activation energy was calculated to be 9 kcal/mol. The transport assay was routinely carried out at 41°C, this being the optimal growth temperature for *M. ruminantium* M1.

Effect of gas phase on uptake. Uptake of HS—CoM by strain M1 required both H_2 and CO_2 (Fig. 5). Cells incubated in the presence of H_2 alone or N_2-CO_2 (80:20) showed no uptake activity. Uptake was potently inhibited by the addition of O_2 to cells that were being incubated in the presence of H_2-CO_2 (80:20). Cells that were heat-treated at 100°C for 5 min exhibited no uptake of label. Results of preliminary experiments indicated that formate replaced the hydrogen and carbon dioxide requirement for the transport system.

Effect of metabolic inhibitors. Table 3 lists the effects of metabolic inhibitors on HS—CoM uptake. Addition of sodium azide, 2,4-dinitrophenol, iodoacetate, or $BrCH_2CH_2SO_3^-$ (inhibitors of methanogenesis in whole cells, as well as methane production from CH_3—S—CoM by methyl-coenzyme M reductase in cell extracts of

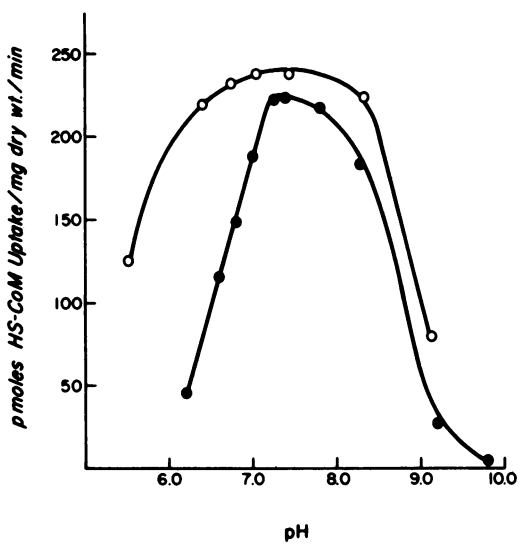

FIG. 3. *Effect of pH on uptake. Each cell suspension was adjusted to the indicated pH with NaOH or HCl and preincubated for 3 min* (○) *or 15 min* (●) *prior to addition of labeled substrate (0.5 μM). Uptake was followed with the rapid dilution assay. The pH also was measured immediately upon completion of each assay.*

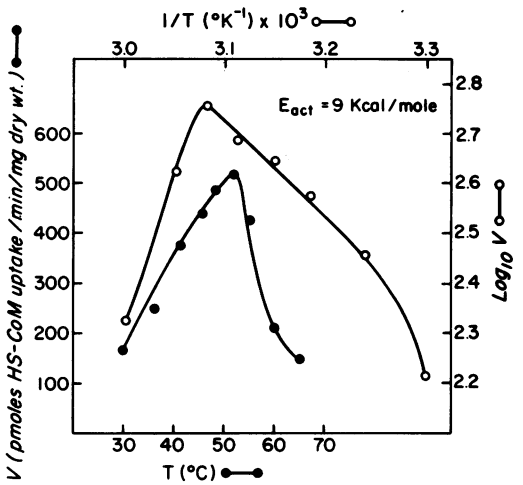

FIG. 4. *Effect of temperature on uptake. Cells were preincubated for 3 min at the indicated temperature prior to addition of labeled substrate (0.5 μM). Uptake was followed with the rapid dilution assay.*

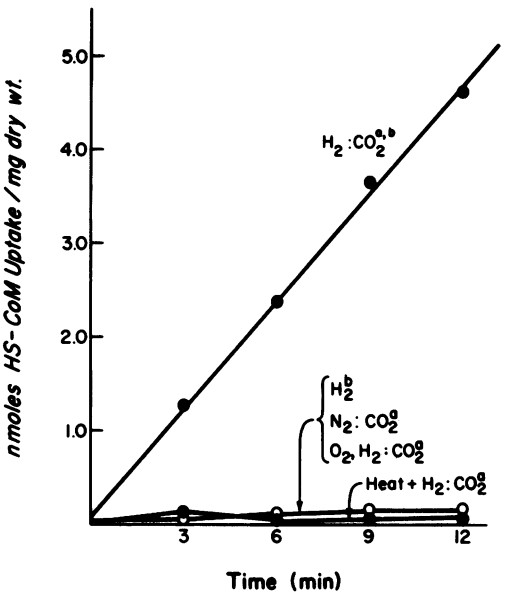

FIG. 5. *Effect of gas phase on HS—CoM uptake. Cells were harvested and resuspended in coenzyme-free medium (without formate) which contained the indicated buffer: (b) 50 mM potassium phosphate at pH 7.1; (a) 50 mM $NaHCO_3$ at pH 7.1 under an H_2-CO_2 (80:20) gas mixture. Cells were transferred to assay vials and preincubated for 15 min under the indicated gas phase prior to addition of labeled substrate (5 μM). O_2 was added to the H_2-CO_2 (80:20) gas phase at 0.25 atm over-pressure. Heat-killed cells were prepared by heating cells for 5 min at 100°C. Uptake was followed with the filter assay.*

M. thermoautotrophicum [8, 11]) resulted in marked inhibition of uptake. Neither carbonyl cyanide-*m*-chlorophenol hydrazone (CCCP), a compound which renders membranes permeable to protons, nor *N,N'*-dicyclohexylcarbodiimide (DCCD), an adenosine triphosphatase inhibitor, affected coenzyme uptake when added under the conditions shown. Valinomycin, a compound known to collapse membrane potentials, showed slight inhibition of uptake.

Inhibition of uptake by derivatives and analogs of HS—CoM. A number of derivatives and analogs of HS—CoM were examined for their effect on uptake when added at a 100-fold molar excess over that of HS—CoM prior to the addition of labeled substrate. The results were as follows.

(i) The indicated percent inhibition of uptake was observed upon addition of the following derivatives: $^-OOCCH_2$—S—CoM, 66%; CH_3CH_2—S—CoM, 78%; CH_3OCO—S—CoM, 84%; CH_3—S—CoM, 98%; CH_3CO—S—CoM, 98%; $HOCH_2$—S—CoM, 99%; and (S—CoM)$_2$, 99%.

(ii) No inhibition was observed with $(CH_3)_2\overset{+}{S}$—CoM, $^-O_3SCH_2CH_2$—S—CoM, or $^-O_3SCH_2CH_2SCH_2$—S—CoM.

(iii) Addition of either one or two methylene carbons to the ethylene bridge between the sulfide and sulfonate moieties of the coenzyme resulted in the following inhibition: $HSCH_2CH_2CH_2SO_3^-$, 46%; $HSCH_2 \cdot CH_2CH_2CH_2SO_3^-$, 83%.

(iv) Replacement of the sulfide moiety by

TABLE 3. *Effect of metabolic inhibitors[a] and uncoupling agents on HS—CoM uptake*

Inhibitor	Final concn	Inhibition (%)
Sodium azide	10 mM	95
Potassium cyanide	10 mM	0
2,4-Dinitrophenol	1 mM	69
Iodoacetate	100 μM	88
$BrCH_2CH_2SO_3^-$	1 μM	100
$Br(CH_2)_3SO_3^-$	10 μM	0
DCCD[b]	1 mM	0
CCCP[b]	10 μM	0
Valinomycin	10 μM	20

[a] Inhibitor was added to cells 3 min prior to addition of 0.5 μM $H^{35}S$—CoM. Uptake rate was determined from 0.5-, 1.0-, and 1.5- min time points. Rate is expressed relative to a control which contained no inhibitor.

[b] DCCD (*N,N'*-dicyclohexylcarbodiimide) and CCCP (carbonyl cyanide-*m*-chlorophenyl hydrazone) dissolved in methanol. A 10-μl portion was added to 1 ml of cell suspension to give the final concentration indicated. No inhibition was observed by addition of methanol alone.

hydroxyl, amino, hydrogen, or sulfonate functional group resulted in a decreasing range of inhibition: 49, 20, 10, and 0%, respectively.

(v) Replacement of the sulfonate moiety by a carboxyl, sulfhydryl, or amino functional group resulted in a decreasing range of inhibition: 61, 18, and 0%, respectively.

The effect of increasing inhibitor concentrations on uptake was examined for some of the above compounds (Fig. 6). Here, 50% inhibition of uptake occurred at a molar ratio (inhibitor to HS—CoM) of 0.1 for $BrCH_2CH_2SO_3^-$; 1.0 for $(S—CoM)_2$, $CH_3—S—CoM$, and $CH_3CO—S—CoM$; and greater than 10 for the other derivatives and analogs tested.

Since $CH_3—S—CoM$ replaced HS—CoM on an equimolar basis in the bioassay (1) and is an important intermediate in methanogenesis (16), the kinetics of its interaction with the carrier protein were examined. As shown in Fig. 7, $CH_3—S—CoM$ was a strong competitive inhibitor of the HS—CoM transport system (K_i = 15 nM). In addition, uptake of $CH_3—^{35}S—CoM$ was found to be concentration dependent and showed saturation kinetics. A double reciprocal plot of uptake velocity over the concentration range of 15 to 1,000 μM yielded an apparent K_m of 50 nM and a V_{max} of 320 pmol/min per mg of dry weight (correlation coefficient = 0.99). The $CH_3—S—CoM$ transport system showed a requirement for hydrogen and carbon dioxide identical to that observed for HS—CoM. Uptake of $CH_3—S—CoM$ was not inhibited by the analogs $CH_3SCH_2CH_2NH_2$, $CH_3SCH_2CH_2OH$, $CH_3S(CH_2)_3SO_3^-$, or $CH_3S(CH_2)_4SO_3^-$ when each compound was added at a 100-fold molar excess prior to the addition of labeled substrate. $(^{35}S—CoM)_2$, which also replaced the growth requirement for HS—CoM at one-half molar concentration (1), was found to be taken up at a rate one-half that of the reduced coenzyme. Such results are in keeping with the observation that $(S—CoM)_2$ was unstable in the assay medium which contained 0.05% cysteine, the disulfide being converted predominantly to the reduced form of the coenzyme and a heterodisulfide of cysteine and HS—CoM (2).

$BrCH_2CH_2SO_3^-$ was found to be a potent inhibitor of growth (2) and uptake when it was incubated with cells prior to the addition of HS—CoM (Fig. 6). When HS—CoM was injected 10 s prior to addition of $BrCH_2CH_2SO_3^-$, nearly a 100-fold molar excess of the inhibitor was required to terminate uptake over a 10-min period. A similar effect on growth was observed (2). Since $BrCH_2CH_2SO_3^-$ irreversibly inactivates CH_4 production from $CH_3—S—CoM$ by the intracellular methyl-coenzyme M reductase

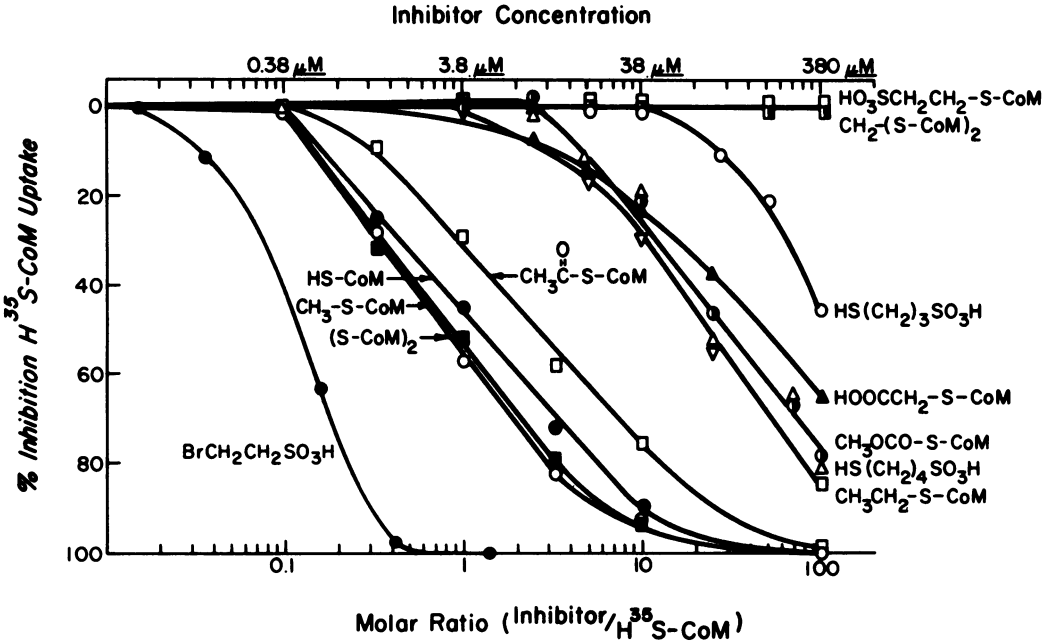

FIG. 6. *Inhibition of uptake by derivatives and analogs of HS—CoM. Cells were incubated for 3 min with the indicated level of inhibitor prior to addition of $H^{35}S—CoM$ (3.8 μM). Uptake was followed by taking samples at 3, 6, 9, and 12 min. The filter assay was used and rates were determined from the linear portion of curve. Rates are expressed relative to a control which contained no additions.*

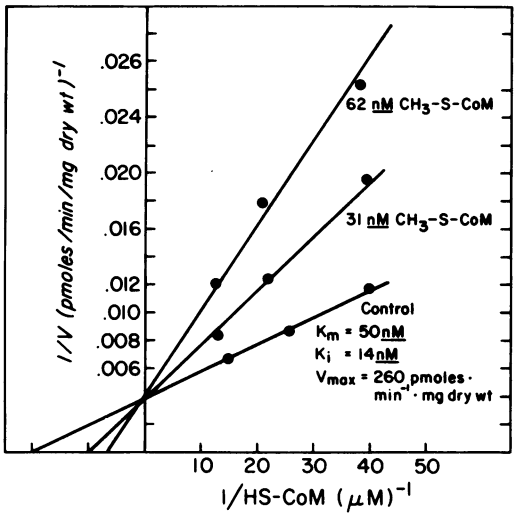

FIG. 7. *Kinetics of CH_3—S—CoM inhibition of HS—CoM uptake. CH_3—S—CoM was premixed with HS—CoM prior to initiation of the experiment. Uptake was initiated by addition of $H^{35}S$—CoM (± CH_3—S—CoM) to give the final concentrations indicated. Uptake was followed with the rapid-dilution assay.*

(8), these data suggest that the primary site of inhibition is intracellular. As shown in Fig. 8, preincubation of $BrCH_2CH_2SO_3^-$ with cells for 15 min before addition of 100-fold molar excess substrate terminated both methane production and uptake; when the order of addition of $BrCH_2CH_2SO_3^-$ and HS—CoM was reversed, no effect on uptake or CH_4 production was observed.

Composition of the ^{35}S intracellular pool. Cells that had been incubated for 3, 10, 20, and 40 min in the presence of excess $H^{35}S$—CoM were filtered rapidly and extracted in the presence of NEM, a sulfhydryl trapping reagent, to determine the distribution of ^{35}S in the oxidized and reduced forms of the coenzyme as well as other metabolites (Fig. 9). Four major radioactive peaks were observed. Peaks II, IV, and V co-chromatographed with $(S—CoM)_2$, CH_3—S—CoM, and NEM—S—CoM, respectively. CH_3—S—CoM and peak I (Fig. 9) constituted the major metabolites, NEM—S—CoM represented less than 1% of the total radiolabel present at 40 min. The distribution of radiolabel in extracts obtained from cells pulsed for 10 min with CH_3—^{35}S—CoM was very similar to that of $H^{35}S$—CoM pulsed cells (Fig. 10A and B). Treatment of these extracts with 100 mM dithiothreitol (Fig. 10A and B) quantitatively reduced peak I to a radiolabeled compound which comigrated with HS—CoM, suggesting that peak I was a heterodisulfide. Results of two-dimensional analysis of these extracts by TLC and TLE (Table 4) may be summarized as follows. (i) In extracts pulsed with $H^{35}S$—CoM, 12% of the total label was detectable as the reduced form (NEM—S—CoM). The remaining 78% was found as a heterodisulfide, CH_3—S—CoM, or $(S—CoM)_2$. Reduction of the heterodisulfide (peak I) resulted in at least two electrophoretically distinct, strongly anionic compounds (AC-1 and AC-2, Table 4) in addition to HS—CoM which were not previously detected by TLC (Fig. 10A). (ii) In extracts pulsed with CH_3—^{35}S—CoM, both NEM—S—CoM and CH_3—S—CoM were found at nearly twice the level found in $H^{35}S$—CoM pulsed cells and accounted for greater than 50% of the total label (Table 4, Fig. 10B). The heterodisulfide (peak I) was quantitatively reduced by dithiothreitol to a compound comigrating in both dimensions with HS—CoM (Table 4).

Uptake of HS—CoM by other methanogens. The uptake activity of methanogens avail-

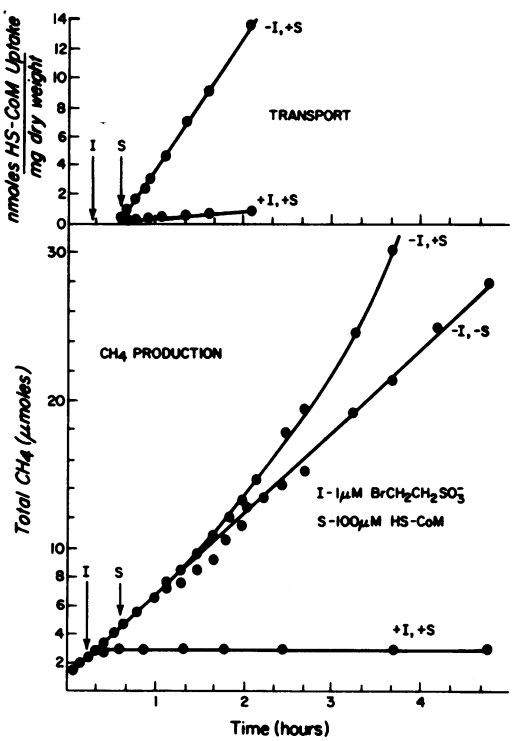

FIG. 8. *Inhibition of methane production and uptake by $BrCH_2CH_2SO_3^-$. Mid-logarithmic cells were incubated with inhibitor prior to addition of a 100-fold molar excess of HS—CoM. Uptake was followed with the filter assay. CH_4 was determined with a Poropak QS column in a Packard gas chromatograph (model no. 871) equipped with hydrogen flame detector.*

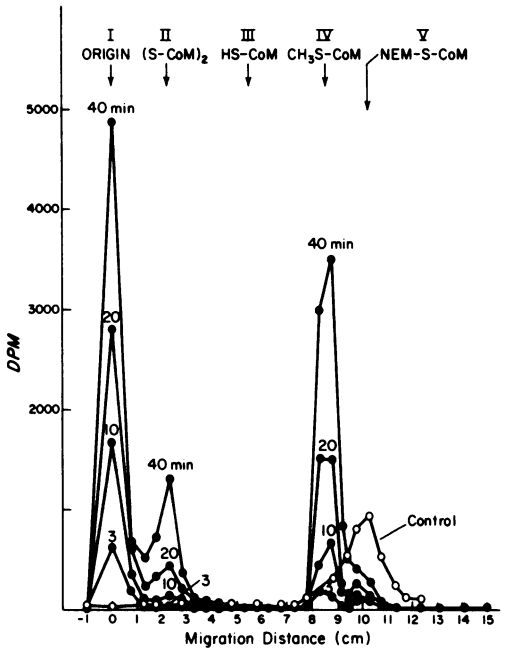

FIG. 9. *Distribution of intracellular ^{35}S in $H^{35}S$—CoM pulsed cells. Portions of 10 ml of cells (350 μg[dry weight]/ml) which were incubating with $H^{35}S$—CoM (5 μM) were removed at the indicated times by use of a GasPak 10-cm³ disposable syringe. The sample was rapidly filtered (15 s), and cells were washed (<30 s) with two 5-ml volumes of mineral salts buffer by use of a Swinnex 47-mm disk filter holder (Millipore Corp., Bedfore, Mass.) fitted with a 47-mm Nucleopore membrane (0.4 μm pore size). Filtered cells were extracted immediately, and a 20-μl portion was chromatographed in acetone-water-concentrated NH_4OH (16:2.5:0.5) and counted as described in Materials and Methods. Prior to addition of $H^{35}S$—CoM, control cells were washed and added to extraction buffer; then a known amount of $H^{35}S$—CoM was added to insure complete recovery of the N-ethylmaleimide (NEM) derivative.*

able in pure culture was examined. Actively growing cells of *Methanobacterium* strain M.o.H., *M. ruminantium* PS, *M. formicicum*, *M. thermoautotrophicum*, *M. mobile*, *Methanospirillum hungatii*, *Methanosarcina barkeri*, *Methanococcus* strain PS, and *Methanococcus vannielii* were incubated in the presence of 5 μM $H^{35}S$—CoM, uptake being followed with the filter assay. Of the 10 species examined, only *Methanospirillum hungatii* demonstrated a significant level of uptake, the rate being approximately 30% of that observed for strain M1. *Methanobacterium mobile*, a methanogen which requires a growth factor found in rumen fluid, and *M. ruminantium* PS, a methanogen which is morphologically similar to strain M1 but which

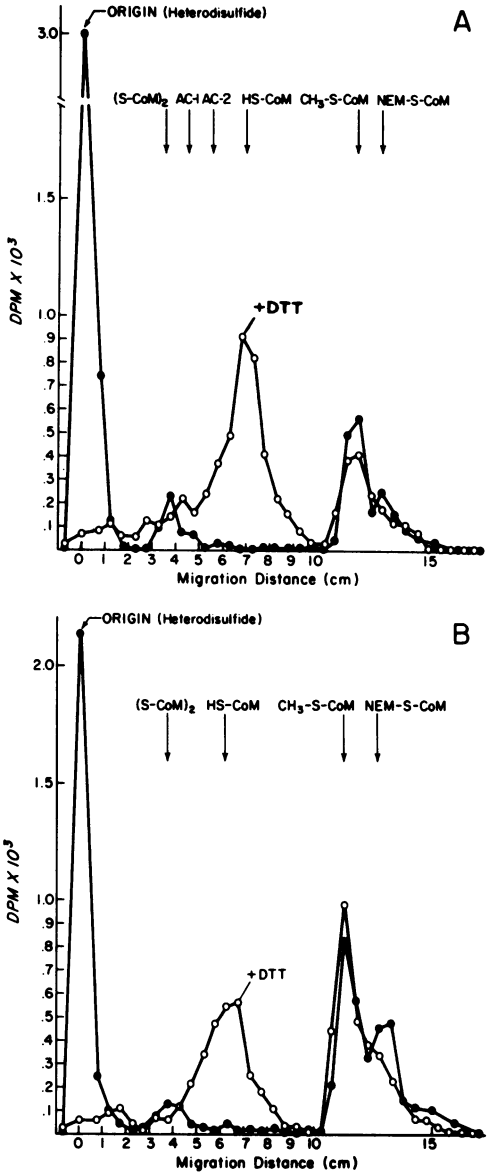

FIG. 10. *Effect of dithiothreitol (DTT) on extracts from cells pulsed for 10 min with $H^{35}S$—CoM (A) or CH_3—^{35}S—CoM (B). Amounts of 100 ml of cells (310 μg[dry weight]/ml) were incubated with limiting labeled substrate (0.5 μM) to insure complete uptake in 10 min. Cells were pelleted anaerobically (15,000 × g for 3 min) and extracted under an N_2 atmosphere. A 20-μl portion of the extract was chromatographed and counted as described in Materials and Methods. Where indicated, extracts were incubated with 100 mM DTT prior to chromatography. The chromatogram was developed with acetone-water-concentrated NH_4OH (16:2.5:0.5). AC-1 and AC-2 are unidentified anionic compounds comigrating with HS—CoM (see Table 4).*

TABLE 4. *Analysis of extracts prepared from cells pulsed with CH_3—^{35}S—CoM or H^{35}S—CoM by thin-layer chromatography (TLC) and thin-layer electrophoresis (TLE)*[a]

Compound[b]	R_f[c]		H^{35}S—CoM				CH_3—^{35}S—CoM			
			−DTT		+DTT		−DTT		+DTT	
	TLC	TLE	dpm	% of total[d]	dpm	% of total	dpm	% of total	dpm	% of total
NEM—S—CoM	1.00	0.37	499	12	415	11	1,002	22	670	18
CH_3—S—CoM	0.90	0.59	728	17	750	19	1,562	34	1,408	38
HS—CoM	0.74	0.78	—[e]	—	205	6	—	—	1,581	43
AC-2	0.61	1.00	—	—	1,741	45	—	—	—	—
AC-1	0.43	1.00	—	—	725	19	—	—	—	—
(S—CoM)$_2$	0.30	0.70	404	9	—	—	275	6	—	—
Origin	0.02	0.70	2,645	62	—	—	1,717	38	—	—
Total			4,276		3,836		4,556		3659	

[a] A 100-ml amount of cells (300 μg [dry weight]/ml) was incubated with a limiting amount of labeled substrate (0.5 μM) to insure complete uptake in 10 min. Cells were pelleted anaerobically (15,000 × *g*, 3 min) and extracted under an N_2 atmosphere in the presence of 5 mM *N*-ethylmaleimide (NEM). A 20-μl portion of concentrated extract (± dithiothreitol [DDT]) was spotted on a cellulose sheet and developed in the first dimension in acetone-water-concentrated NH_4OH (16:2.5:0.5) and subjected to electrophoresis in the second dimension at pH 2.0 formic:acetic:water (13:58:1,000) for 45 min at 12 mA, 400 V. Spots were located; radioactivity was extracted and counted as described in Materials and Methods.

[b] NEM—S—CoM, *N*-ethylmaleimide derivatized HS—CoM; AC, anionic compound.

[c] R_f values relative to compound with highest mobility as measured from origin.

[d] Percentage of total disintegrations per minute recovered from the plate.

[e] Not detectable.

synthesizes HS—CoM intracellularly, showed low levels of uptake, the rate being less than 10% of that found for strain M1. No uptake was observed for the other species examined.

DISCUSSION

HS—CoM uptake by *M. ruminantium* M1 shows characteristics of a carrier-mediated, active process. Uptake is dependent on energy, pH, and temperature; it shows saturation kinetics and is maintained against a concentration gradient. An analysis of the intracellular ^{35}S pool of cells pulsed with H^{35}S—CoM indicates that the coenzyme is found predominantly in the heterodisulfide, CH_3—S—CoM, and (S—CoM)$_2$ forms, only 10% being trapped by NEM after 10 min. Reduction of the heterodisulfide with dithiothreitol reveals two electrophoretically distinct, strongly anionic compounds (AC-1 and AC-2) in addition to HS—CoM. In contrast, CH_3—^{35}S—CoM pulsed cells show greater than 50% of the pool to be the NEM and methylated forms of the coenzyme, the heterodisulfide being quantitatively reduced to CoM. None of the intracellular forms is exchangeable with the medium. It is not clear whether these forms are a result of intracellular metabolism or a product of group translocation, in which an energy-requiring process mediates cell entry through chemical modification. If direct energy coupling to the porter occurs, it would most likely drive CH_3—S—CoM uptake. CH_3—S—CoM is transported with high efficiency; it is a potent competitive inhibitor of the HS—CoM carrier and is a major stable intracellular form of the coenzyme. Other derivatives such as CH_3CO—S—CoM may be transported with equal facility. Use of intact vesicles of strain M1 will be necessary to define the relationship of energy coupling to the transported form of the coenzyme.

The carrier-mediated uptake of HS—CoM provides a molecular basis for study of the specificity and sensitivity of the bioassay. The ability of strain M1 to grow in the presence of trace levels of coenzyme M is reflected in the high affinity of the uptake system for HS—CoM (K_m, 73 nM). This value is characteristic of other vitamin transport systems (7, 12, 13). CH_3—S—CoM, which replaces the cofactor requirement quantitatively in the bioassay, is readily taken up by strain M1. The inhibition of HS—CoM uptake by other derivatives of coenzyme M closely parallels their activity in the bioassay (2). The thioether, thioester, and thiocarbonate derivatives (CH_3—S—CoM, CH_3CH_2—S—CoM, $HOCH_2$—S—CoM, CH_3CO—S—CoM, and CH_3OCO—S—CoM) are inhibitors of HS—CoM uptake and stoichiometrically replace the coenzyme requirement in the bioassay. In contrast $(CH_3)_2$—S^+—CoM, CH_2—(S—CoM)$_2$, and $^-O_3SCH_2CH_2$—S—CoM have no cofactor activity and were found not to inhibit uptake at a 100-fold molar excess. The exception, $^-OOCCH_2$—S—CoM, inhibits uptake significantly but lacks cofactor activity. This may reflect the inability of intracellular enzymes to cleave these derivatives to yield HS—CoM (J. A. Romesser, Ph.D. thesis, University of Illinois, Urbana, 1978).

Although no structural analogs were found to

replace coenzyme M activity in the bioassay (2), several showed notable inhibition of uptake. Results restrict the structural specificity of the carrier to compounds with a terminal sulfonate linked by an ethylene bridge to a sulfhydryl; the latter moiety can be modified to a restricted range of thioether, thioester, and thiocarbonate derivatives.

$BrCH_2CH_2SO_3^-$ is a potent inhibitor of growth (2), uptake, and methane production by methylcoenzyme M reductase (8), an enzyme system active in the terminal steps of methane production from hydrogen and carbon dioxide (R. P. Gunsalus, Ph.D. Thesis, University of Illinois, Urbana, 1977). However, addition of HS—CoM prior to addition of the inhibitor substantially negates the inhibition of uptake by whole cells. These results suggest a low affinity of the carrier for $BrCH_2CH_2SO_3^-$, its primary mode of inhibition being inactivation of the intracellular methylcoenzyme M reductase. Attempts to demonstrate a K_i for $BrCH_2CH_2SO_3^-$ inhibition of HS—CoM transport were not successful, but did support the notion that the carrier has a high binding constant for the halogenated analog. That translocation requires a functional methylreductase is further supported by the observations that removal of either substrate, hydrogen or carbon dioxide, or addition of iodoacetate or 2,4-dinitrophenol, also inhibits uptake. Whether the enzyme system plays a role in energy transduction or is required for other reasons remains to be clarified.

Only one other methanogen exhibited a significant level of uptake, when compared to strain M1. Considering that all methanogens tested have a significant intracellular coenzyme M pool (2), when grown in HS—CoM-free medium, it is not surprising that the uptake capability is not universally expressed. Strain M1, however, is dependent on the exogenous coenzyme M supply found in the rumen, presumably provided by other methanogens. Since coenzyme M plays a central role in the energy metabolism of these organisms, the survival of M1 is reflected in its unique ability to elaborate a specific, high-affinity uptake system.

The culture vessel diagrammed in Fig. 1 represents a significant advance for the culture of methanogens in 100- to 200-ml amounts. The previously described technique (5) employed an open system through which a sterile, anaerobic gas atmosphere was passed. Loss of sulfide from the culture medium introduced a variable, producing erratic growth responses for certain methanogens. In addition, the medium in the flask was exposed to air during sterilization and required reduction before it could be inoculated. When the technique which employed a pressurized atmosphere (1) was extended to the liter bottle, all of the advantages of the serum tube were found to apply to the bottle. This procedure renders the previously used culture vessel obsolete (5).

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Debbie Szurgot and Victor Gabriel. We thank J. A. Romesser and R. P. Gunsalus for kindly providing the coenzyme M derivatives used in the inhibitor studies.

This work was supported by Public Health Service grant AI 12277 from the National Institute of Allergy and Infectious Diseases and by grant PCM 76-02652 from the National Science Foundation.

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