

# One-Carbon Metabolism in Methanogenic Bacteria: Analysis of Short-Term Fixation Products of $^{14}\text{CO}_2$ and $^{14}\text{CH}_3\text{OH}$ Incorporated into Whole Cells

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*Methanobacterium thermoautotrophicum*, *M. ruminantium*, and *Methanosarcina barkeri* were labeled with  $^{14}\text{CO}_2$  ( $^{14}\text{CO}_2 + \text{H}^{14}\text{CO}_3^- + ^{14}\text{CO}_3^{2-}$ ) for from 2 to 45 s. Radioactivity was recovered in coenzyme M derivatives, alanine, aspartate, glutamate, and several unidentified compounds. The properties of one important structurally unidentified intermediate (yellow fluorescent compound) displayed UV absorbance maxima at pH 1 of 290 and 335 nm, no absorbance in the visible region, and a fluorescence maximum at 460 nm. Label did not appear in organic phosphates until after 1 min.  $^{14}\text{CH}_3\text{OH}$  was converted by *M. barkeri* primarily into coenzyme M derivatives at 25 s.  $[2-^{14}\text{C}]$ acetate was assimilated by *M. thermoautotrophicum* mainly into alanine and succinate during 2 to 240 s, but not into coenzyme M derivatives or yellow fluorescent compound. Cell-free extracts of *M. thermoautotrophicum* lacked ribulose 1,5-bisphosphate carboxylase activity. The data indicated the absence of the Calvin, serine, and hexulose phosphate paths of  $\text{C}_1$  assimilation in the methanogens examined and indicated that pyruvate was an early intermediate product of net  $\text{CO}_2$  fixation. The in vivo importance of coenzyme M derivatives in methanogenesis was demonstrated.

Methanogenic bacteria catabolize a variety of  $\text{C}_1$  compounds, including  $\text{H}_2/\text{CO}_2$  (no distinctions are made between  $\text{CO}_2$ ,  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  when " $\text{CO}_2$ " is used), CO,  $\text{HCOOH}$ , or  $\text{CH}_3\text{OH}$  (14, 43, 45). The exact paths of  $\text{C}_1$  metabolism into methane or cell carbon have not been determined. It is not known if the anabolic and catabolic pathways used by methanogens involve any common carbon intermediates.

It has been suggested that methane formation involves sequential reduction of carrier-bound  $\text{C}_1$  units (3, 43, 45). The terminal step in methane production involves coenzyme M (2-mercaptoethanesulfonic acid, or CoM), which is apparently unique to methanogens (28, 40). The formation of methane from  $\text{CH}_3\text{-CoM}$  has been described (20, 21, 40), and the involvement of more oxidized CoM derivatives has been suggested (20; J. A. Romesser and R. S. Wolfe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, I2, p. 155).

Although many methanogens are autotrophic, some require the addition of organic substrates. For example, *Methanobacterium ruminantium* requires acetate to supply about 60% of its cell carbon (11). *Methanobacterium thermoautotrophicum* can also assimilate acetate when it is

provided in the medium (41, 47) but is an autotroph (49).

All described methanogens use  $\text{H}_2$  as a source of reducing power and  $\text{CO}_2$  as a methanogenic substrate. *M. ruminantium* can also produce methane from formic acid. *Methanosarcina barkeri* can produce methane from methanol, but not from formic acid. *M. thermoautotrophicum* grows by oxidation of  $\text{H}_2$  or CO as energy sources (14, 49).

Only three fully documented anabolic pathways for growth on  $\text{C}_1$  substrates are known. The Calvin cycle has been demonstrated in many photosynthetic organisms and autotrophic bacteria (29, 35). The serine and hexulose phosphate paths have been demonstrated in a variety of methylotrophic bacteria (23, 35). In addition to these paths, a reductive tricarboxylic acid cycle has been proposed to account for growth on  $\text{CO}_2$  by a photosynthetic bacterium (12, 18), but complete evidence for this pathway has not been established (29). Examination of *M. thermoautotrophicum* for the enzymes of this pathway demonstrated that although many of the necessary oxidoreductase activities were present, the cyclical series of reactions suggested for *Chlorobium thiosulfatophilum* are not supported in this methanogen because of the absence of isocitrate dehydrogenase (46).

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It was the purpose of this investigation to examine the metabolism of C<sub>1</sub> compounds in methanogens by analyzing the intermediates formed during short-term labeling of actively metabolizing whole cells with <sup>14</sup>CO<sub>2</sub> and <sup>14</sup>CH<sub>3</sub>OH. The data support the involvement of CoM derivatives (20) as C<sub>1</sub> carriers in methanogenesis. The intermediates involved in cell carbon synthesis are not those that would be expected if the Calvin, serine, or hexulose phosphate path of one-carbon assimilation were present (6, 13, 23, 25). However, the data are consistent with the synthesis of pyruvate, oxaloacetate, and α-ketoglutarate by carboxylations of acetyl-CoA, phosphoenolpyruvate, and succinyl-CoA (46).

(Preliminary results have been presented elsewhere: L. Daniels and J. G. Zeikus, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, P316, p. 197; L. Daniels and J. G. Zeikus, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, 158, p. 121.)

## MATERIALS AND METHODS

### Chemicals, gases, and radioactive compounds.

All chemicals used were of reagent grade. H<sub>2</sub>/CO<sub>2</sub> (80:20, vol/vol; premixed), N<sub>2</sub> (prepurified), H<sub>2</sub> (extra dry), and CO<sub>2</sub> (bone dry) were purchased from Matheson Scientific, Inc., Joliet, Ill.

<sup>14</sup>CH<sub>3</sub>-CoM (570 dpm/μl) and <sup>14</sup>CH<sub>3</sub>-B<sub>12</sub> (methyl vitamin B<sub>12</sub>, 30,000 dpm/μl) were provided by R. S. Wolfe. [<sup>14</sup>C]methenyl tetrahydrofolate (34,000 dpm/μmol) was a gift from F. M. Huennekens. Na<sub>2</sub><sup>14</sup>CO<sub>3</sub> (20 to 60 mCi/mmol); [<sup>14</sup>C]acetic acid, Na salt (55 to 59 mCi/mmol); [<sup>14</sup>C]formic acid, Na salt (59 mCi/mmol); [<sup>14</sup>C]methanol (58 mCi/mmol); [<sup>14</sup>C]pyruvic acid, Na salt (10.5 mCi/mmol); and Na<sub>2</sub><sup>35</sup>S (16.5 mCi/mmol) were purchased from Amersham/Searle, Arlington Heights, Ill. [<sup>14</sup>C]succinic acid (18.5 mCi/mmol) and [<sup>3</sup>H]-acetic acid, Na salt (0.8 mCi/mmol), were purchased from ICN Chemical and Radioisotope Division, Irvine, Calif.

**Organisms and culture methods.** *M. thermoautotrophicum* was the type strain ΔH (49). Cultures of *M. ruminantium* strain PS and *M. barkeri* strain MS were provided by M. P. Bryant. *M. ruminantium* strain M1 was provided by R. S. Wolfe. The routine cultivation methods for methanogens were as described previously (14).

*M. ruminantium* strain PS was grown in a medium (final pH, 7.4) that contained the following constituents per liter of distilled water: KH<sub>2</sub>PO<sub>4</sub>, 0.30 g; Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 2.1 g; NH<sub>4</sub>Cl, 0.5 g; MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.2 g; mineral solution (14), 10 ml; resazurin, 2 mg; vitamin solution (44), 55 ml; yeast extract, 1.0 g; Na<sub>2</sub>S·9H<sub>2</sub>O, 0.96 g; cysteine hydrochloride, 0.96 g; sodium acetate·3H<sub>2</sub>O, 3.0 g; and 0.75 ml of a volatile fatty acid mixture containing the following volumes of acids per 33.2 ml: propionic, 14.8 ml; *n*-butyric, 10.6 ml; isobutyric, 1.8 ml; *n*-valeric, 2.0 ml; DL-2-methylbutyric, 2.0 ml; and isovaleric, 2.0 ml. *M. ruminantium* strain M1 was grown in the same medium with the following

additional components per liter: sodium formate, 1.0 g; Trypticase, 1.0 g; CoM, 33 μg.

*M. barkeri* was mass cultured at 30°C in a 12-liter carboy of the previously described medium (14) without bicarbonate, supplemented with 5 ml of methanol and 10 ml of vitamin solution (44) per liter. The other bacteria were mass cultured and harvested as described previously (14).

*Lactobacillus casei* subsp. *rhamnosus* (ATCC 7469) was obtained from the American Type Culture Collection, Rockville, Md. The culture was grown aerobically in Difco Micro Inoculum broth.

Growth was determined as described previously (14).

**Determination of gases.** CO<sub>2</sub>, CH<sub>4</sub>, and H<sub>2</sub> were measured gas chromatographically on a Becker-Packard gas chromatograph 419 with thermal conductivity detection in combination with a two-channel recorder (Omni-Scribe; Houston Instruments, Austin, Tex.). The operation conditions were as follows: stainless-steel column, 1.3 m by 0.32 cm, filled with Carbosieve B (120/140 mesh; Supelco, Inc., Bellefonte, Pa.); He carrier gas (N<sub>2</sub> when H<sub>2</sub> was to be determined), 5 atm, 60 ml/min; temperatures of column, detector, and injection port, 100, 125, and 45°C, respectively; filament current, 250 (using He as carrier gas) or 150 (using N<sub>2</sub> as carrier gas) mA. The CO<sub>2</sub> concentration in aqueous solution at a given temperature was calculated from solubility data (22).

CH<sub>4</sub> alone was determined more sensitively with a Varian Aerograph gas chromatograph 600-D with a flame ionization detector equipped with a stainless-steel column (1.5 m by 0.32 cm) filled with Porapak N (Waters Associates, Inc., Milford, Mass.) and heated at 75°C. N<sub>2</sub> was used as the carrier gas at a flow rate of 30 ml/min at 1.5 atm.

Gas samples of 0.2 and 0.4 ml were removed and injected by using a 1-ml GlasPak syringe (Becton, Dickinson & Co., Rutherford, N.J.) with a Teflon pressure lock device (Mininert syringe valve; Supelco, Inc.) and a 23-gauge needle, both fitted tightly with Teflon tape.

**Determination of radioactivity.** The radioactivities of <sup>14</sup>CO<sub>2</sub> and <sup>14</sup>CH<sub>4</sub> were determined as described previously (33). The radioactivities of nongaseous samples were determined by scintillation counting in a Packard Tri-Carb scintillation counter. The efficiency of counting for <sup>14</sup>C was 70 to 75%, and for aqueous samples a cocktail was used that contained 0.95 g of *p*-bis[2-(5-phenyloxazolyl)]-benzene, 15.2 g of 2,5-diphenyloxazole, 1,263 ml of Triton X-100, and 3.79 liters of toluene. For dry samples, the same solution without Triton X-100 was used.

**Labeling and extraction of whole cells.** Labeling experiments with <sup>14</sup>CO<sub>2</sub>, <sup>14</sup>CH<sub>3</sub>OH, or [<sup>14</sup>C]acetate were conducted with cells grown to late log phase, harvested in a Sorvall centrifuge equipped with a KSB continuous-flow system (14), and resuspended in the appropriate medium while in a Freter-type glove box (17). The cell suspension was gassed vigorously with H<sub>2</sub>/CO<sub>2</sub> and shaken in an anaerobic culture flask (15) for about 1 h to assure that the cells were rapidly producing CH<sub>4</sub>.

The 150-ml labeling vessel (Fig. 1) and all hoses were gassed well with H<sub>2</sub>/CO<sub>2</sub> to assure anaerobic

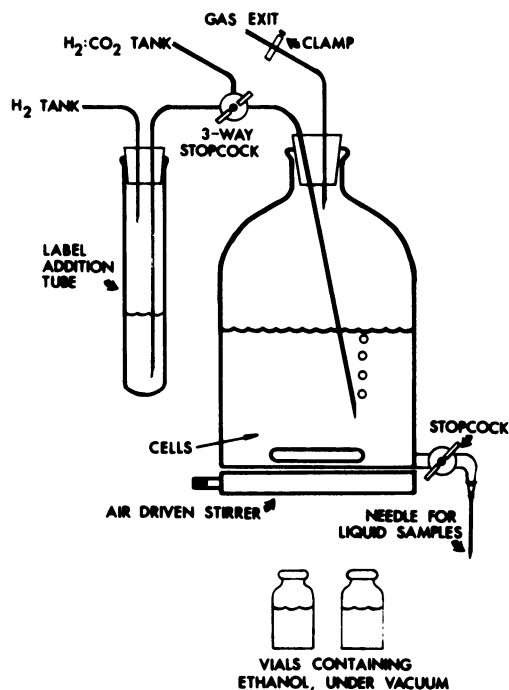


FIG. 1. Time course labeling apparatus.

conditions. Harvested and resuspended cells (80 ml) at the appropriate temperature were added by syringe to the vigorously gassed labeling vessel. For experiments with thermophiles, the vessel was adjusted to the appropriate temperature by submersion in a water bath and insulated upon its removal with foam rubber to keep the temperature constant. Before the addition of label, the vessel was firmly fixed on an air-driven stirrer. Label was equilibrated for at least 15 min at the temperature of the cell suspension in a crimp-top pressure tube that contained 10 ml of the specified medium. The tube allowed rapid pressurization with H<sub>2</sub> through an 18-gauge needle and a rapid exit of the liquid through a stainless-steel tube (ID, 2.5 mm) into the labeling vessel. The H<sub>2</sub>/CO<sub>2</sub> tank overpressure was 0.5 atm, and the H<sub>2</sub> tank overpressure was 1.5 atm.

The gas exit on the vessel was closed, and the three-way stopcock was turned to simultaneously add label and pressurize the system to 1.5 atm. Liquid samples (about 11 ml) were collected into sealed and evacuated vials of 100% ethanol (the final ethanol concentration was 40 to 60%). The exit stopcock was attached to a 14-gauge needle, which allowed penetration into the vials and a fast sampling rate. The vials filled automatically in <1 s.

For incorporation of Na<sub>2</sub><sup>35</sup>S into whole cells, *M. thermoautotrophicum* was grown in 28-ml pressure tubes (1) in the presence of 700 μCi of Na<sub>2</sub><sup>35</sup>S (16.5 mCi/mmol). After 2 days of growth (absorbance at 660 nm, > 0.5), ethanol was added to a final concentration of 35%.

The 40 to 60% ethanol suspensions of cells that were labeled with either <sup>35</sup>S or <sup>14</sup>C were mixed for 2 h and then centrifuged at 18,000 × *g* for 20 min. The cell pellet was suspended in 80% ethanol, mixed for 2 h,

and centrifuged as above. The cell extraction procedure was repeated three times in 20% ethanol. Supernatants from all the steps above were pooled. It was assured that >95% of the label was removed from the cells by monitoring each step for radioactivity. The pooled supernatants were evaporated to dryness on a rotary evaporator at 30°C, suspended in a small volume of water (1.0 or 2.0 ml), and centrifuged for 20 min at 18,000 × *g* to remove any precipitate. The concentrated labeled extracts were routinely stored under N<sub>2</sub> at -15°C.

**Separation and autoradiography of labeled intermediates.** Thin-layer electrophoresis (TLE) was used as the first dimension in all two-dimensional separations described. Extract (1 to 3 μl) was applied to a plastic-backed Cel MN-300 plate (0.1-mm cellulose, 20 by 20 cm; Machery-Nagel) in the lower left corner 5 cm from the side and 3.5 cm from the bottom. The plate was sprayed evenly with a freshly made buffer modified from Schurmann (37) (3.2 ml of pyridine, 12.0 ml of acetic acid, 250 ml of distilled water, pH 4.0), and placed in the TLE chamber (Desaga-Brinkman, Brinkman Instruments, Des Plaines, Ill.) with the origin closest to the negative pole. Saturated Whatman no. 1 chromatography paper connected the plate with the two buffer troughs. Separation was conducted at a constant current of 12 or 14 mA for 50 min. The plate was then thoroughly dried at room temperature with a hair dryer before further analysis.

Thin-layer chromatography (TLC) was conducted with freshly made solvent A: 90 ml of secondary butanol, 15 ml of 88% formic acid, 30 ml of water (31, 37). After TLE, thoroughly dried plates were placed in the tank at right angles to the electrophoretic separation. Plates were developed once to 14 cm, removed, dried, and placed in again to develop to 17 cm.

Positions of labeled compounds on thin-layer plates were determined by autoradiography. Thoroughly dried plates were placed in X-ray exposure holders (36 by 43 cm), their corners were marked with radioactive ink, and X-ray film (Kodak SB-5) was placed over them. After exposure for about 10 days, films were developed with Kodak developer and fixer. Radioactivity of specific compounds on thin-layer plates was determined by carefully scraping the cellulose off and counting it in the nonaqueous scintillation cocktail.

**Purification of YFC.** *M. thermoautotrophicum* cells (40 g, wet wt) obtained from a fermentor culture harvested in the middle to late log phase (absorbance at 660 nm, 1.0) and grown as previously described (14) were extracted with aqueous ethanol solutions as described above and flash evaporated to 5 ml. A small amount of extract prepared from cells labeled with <sup>14</sup>CO<sub>2</sub> that contained <sup>14</sup>C-labeled yellow fluorescent compound (YFC) was added to the extract as a marker for YFC. YFC was purified by sequential column chromatography. Purity of all steps described below was monitored by absorbance at 280 nm, yellow fluorescence, and radioactivity. The extract was applied to a short G-10 Sephadex column (2.5 by 16.5 cm) and eluted with 100 mM ammonium formate, pH 4.0. Two distinct radioactivity peaks were observed, but only the first peak was coincident with yellow fluorescence. The yellow fluorescent fractions (first peak) were combined, evaporated, and applied to a second G-10 column (1.5 by 115 cm) and eluted with 100 mM ammo-

nium acetate, pH 4.0. Fractions in the first peak which contained both the major absorbance (first of five peaks) and radioactivity (first of three peaks) were coincident with yellow fluorescence, as determined by analysis of samples on TLC plates and by observing them under UV light. These fractions were combined, flash evaporated to dryness, suspended in water, applied to a diethylaminoethyl-Sephadex (A-25) column (1 by 60 cm), and eluted with a linear gradient of 200 ml of water versus 200 ml of 2 N ammonium acetate, pH 4.0. YFC eluted as a radioactive peak at 100 ml that was coincident with yellow fluorescence. TLE-TLC and autoradiography showed that the only  $^{14}\text{C}$ -labeled compound in this peak was YFC. The major YFC-containing fractions were combined, evaporated, suspended in water, applied to a G-25 column (1 by 209 cm), and eluted with 50 mM ammonium acetate, pH 4.0. YFC eluted in the major absorbance peak (first of three peaks), and these fractions were concentrated and desalted by G-10 Sephadex column chromatography (1.5 by 115 cm), using water as the eluant. Bioassays for folic acid were conducted with samples further purified by TLC in solvent A.

**Identification and characterization of labeled intermediates.** Labeled compounds were identified by their migration positions in TLE-TLC relative to several known compounds and by their coincidence with added labeled standards, using solvent A described above and also the following solvent systems. Solvent B consisted of 75 g of redistilled phenol and 25 g of water (7). Solvent C consisted of 90 ml of water-saturated isopropyl ether and 30 ml of 88% formic acid (42). Solvent D consisted of mixture A (125 ml of *n*-butanol and 8.4 ml of water) and mixture B (62 ml of propionic acid and 79 ml of water); equal volumes of mixtures A and B were mixed to form solvent D (7). Solvent E consisted of 70 ml of isoamyl alcohol-35 ml of 5 M formic acid (34). This mixture formed two phases; the top layer was used for chromatography. Ninhydrin spray was used to detect amino acids, and a UV lamp (Mineral light, UVS-11) was used to locate fluorescent compounds. Sugar phosphates were identified on chromatograms by the method of Bandurski and Axelrod (2).

Formate was determined by Dowex AG-1-formate (24) and by silicic acid (32) column chromatography. Formate was routinely identified in the trapped volatile compounds obtained during flash evaporation of ethanol extracts of cells as follows. Samples (0.5 ml) adjusted to pH 6 were applied to Dowex Ag-1-formate columns (0.4 by 15 cm) and eluted with a gradient of 120 ml of 2 N formate versus 125 ml of water. [ $^{14}\text{C}$ ]-formate eluted as a radioactive peak at 17 to 22 ml. Recoveries of [ $^{14}\text{C}$ ]-formate from experimental samples or commercial standards were greater than 95%.

UV and visible spectroscopic measurements were made on a Gilford 240 spectrophotometer. Spectra were determined using a Gilford wavelength scanner attachment. Fluorescence spectra were determined with an Aminco-Bowman spectrofluorometer.

Acid phosphatase (wheat germ, Sigma type 1; EC 3.1.3.2) was used to treat short-term-labeling extracts to observe if phosphatase caused modifications in TLE-TLC patterns. Each reaction contained 60  $\mu\text{mol}$  sodium acetate, pH 5.0, 10  $\mu\text{l}$  of concentrated extract,

and 10  $\mu\text{g}$  of phosphatase in a total volume of 0.6 ml. The reaction was run for 5 h at 37°C and stopped by the addition of 0.4 ml of 95% ethanol. The reaction mixture was then evaporated at room temperature to 10  $\mu\text{l}$  under a stream of  $\text{N}_2$ , and 1  $\mu\text{l}$  was used for TLE-TLC analysis.

CoM derivatives were analyzed for by the method of Balch and Wolfe (1, 39). Radioactive samples were scraped off TLC plates and added to pressure tubes of *M. ruminantium* M1 medium lacking CoM. Cells grown to an absorbance at 660 nm of 0.4 on a medium originally containing 0.2 nmol of CoM per ml were inoculated (0.25 ml) into the tubes. Absorbance readings were taken at intervals by allowing the cellulose to settle out during 1 h of standing upright. CoM, as the sodium salt, was purchased from Pierce Chemical Co., Rockford, Ill.

*L. casei* (ATCC 7469) was used to assay for folic acid derivatives. Tubes that contained 10 ml of folic acid casei medium (Difco) and varying amounts of folic acid or YFC (0.2 to 2.0  $\mu\text{g}$  per assay if YFC has extinction coefficients similar to those of folate derivatives [8]) were inoculated with 2 drops of a cell suspension prepared according to the *Difco Manual* (16).

**Preparation of crude enzymes and enzyme assays.** Crude enzymes were prepared from cells as described previously (14). Ribulose 1,5-bisphosphate carboxylase was assayed as described by McFadden and Denend (30) but modified for anaerobic conditions. The protein content of extracts was determined by the method of Lowry et al. (26).

## RESULTS

**Early intermediates formed in whole cells labeled with  $^{14}\text{CO}_2$  and  $^{14}\text{CH}_3\text{OH}$ .** *M. thermoautotrophicum*, *M. barkeri*, and *M. ruminantium* labeled with  $^{14}\text{CO}_2$  formed similar radioactive products. Figure 2 shows the positions after two-dimensional separation of all the major nonvolatile labeled components of the water- and aqueous ethanol-soluble fractions of suspensions of *M. thermoautotrophicum* incubated with  $^{14}\text{CO}_2$  for 60 s. Table 1 compares the relative contributions of these labeled products formed after 25 s by *M. thermoautotrophicum* and *M. ruminantium* incubated with  $^{14}\text{CO}_2$  and *M. barkeri* incubated with  $^{14}\text{CO}_2$  or  $^{14}\text{CH}_3\text{OH}$ . The radioactive intermediates formed during  $\text{CO}_2$  metabolism were similar in all three methanogens and included  $\text{CH}_3\text{-CoM}$ , alanine, aspartate, and several structurally unidentified compounds ( $\text{C}_1\text{-X-T}$ , YFC, UNF-1, and UNF-2). Glutamate was labeled in *M. thermoautotrophicum* and *M. ruminantium* at 25 s, but not in *M. barkeri*. However, glutamate was labeled at 35 s in *M. barkeri*. When *M. barkeri* was labeled with  $^{14}\text{CH}_3\text{OH}$  for 25 s, radioactivity was observed in  $\text{CH}_3\text{-CoM}$  and  $\text{C}_1\text{-X-T}$ , but not in alanine, glutamate, or aspartate. Relative percentages varied with the experiments. Methane and formic acid were volatile products of  $\text{CO}_2$  metabolism

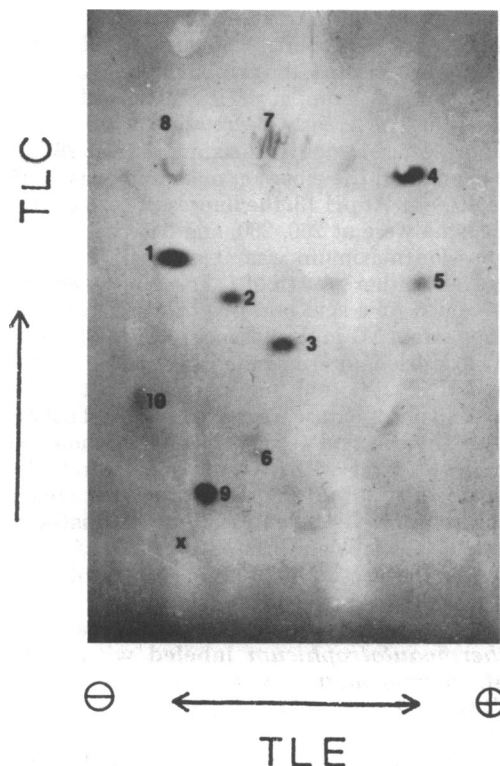


FIG. 2. Autoradiogram showing the positions of labeled intermediates formed by *M. thermoautotrophicum* labeled with <sup>14</sup>C<sub>2</sub> for 60 s. The respective electrophoretic poles are indicated as + or -; x refers to the origin. The numbers refer to the following compounds: 1, alanine; 2, glutamate; 3, aspartate; 4, C<sub>1</sub>-X-T (structure not identified, a CoM derivative); 5, CH<sub>3</sub>-CoM; 6, UNF-1 (structure not identified); 7, UNF-2 (structure not identified); 8, amino acids (methionine, leucine, isoleucine, and valine); 9, YFC (structure not identified); 10, yellow fluorescent spot (structure not identified).

in *M. thermoautotrophicum*. After 10 s of labeling, formate accounted for 10 to 15% of the total label assimilated. Results similar to these were obtained with *M. barkeri*. Methanogens incubated with <sup>14</sup>CO<sub>2</sub> or <sup>14</sup>CH<sub>3</sub>OH for 25 s did not produce labeled 3-phosphoglyceric acid, other organic phosphates, serine, CH<sub>3</sub>-B<sub>12</sub> (even when precautions were taken at all steps of analysis to protect the sample from light), or methenyltetrahydrofolate.

**Properties of the unidentified intermediates of C<sub>1</sub> metabolism.** The compound designated C<sub>1</sub>-X-T was demonstrated to be a CoM derivative. Table 2 shows that C<sub>1</sub>-X-T and CH<sub>3</sub>-CoM replaced CoM in supporting growth by the CoM-requiring strain M1 of *M. ruminantium*. Additional evidence concerning its CoM nature was obtained by labeling growing cells with Na<sub>2</sub><sup>35</sup>S for several generations. Figure 3 compares the products of <sup>35</sup>S-labeled cells in the presence or absence of a <sup>14</sup>C-labeled added extract that contained <sup>14</sup>CH<sub>3</sub>-CoM, <sup>14</sup>C<sub>1</sub>-X-T, and [<sup>14</sup>C]YFC. The two major <sup>35</sup>S spots were coincident with added <sup>14</sup>CH<sub>3</sub>-CoM and <sup>14</sup>C<sub>1</sub>-X-T, indicating that C<sub>1</sub>-X-T was a sulfur-containing compound. It was also discovered that C<sub>1</sub>-X-T

TABLE 2. Bioassay of labeled compounds for CoM activity

Substrate	Maximal growth (absorbance at 660 nm)
None	0.11
CoM <sup>a</sup>	0.67
C <sub>1</sub> -X-T	0.65
CH <sub>3</sub> -CoM	0.64
YFC	0.02
Origin	0.05

<sup>a</sup> CoM, 1.8 nmol per tube.

TABLE 1. Distribution of radioactivity among the stable, nonvolatile labeled components of methanogens incubated with <sup>14</sup>CO<sub>2</sub> or <sup>14</sup>CH<sub>3</sub>OH for 25 s<sup>a</sup>

Organism	Labeled substrate	% of total cpm recovered on TLC plate								
		Ala-nine	Gluta-mate	Aspar-tate	CH <sub>3</sub> -CoM	C <sub>1</sub> -X-T	YFC	UNF-1	UNF-2	Others
<i>M. thermoautotrophicum</i>	CO <sub>2</sub>	22.2	8.2	3.2	9.5	27.9	2.3	18.6	2.7	5.4
<i>M. ruminantium</i> strain PS	CO <sub>2</sub>	4.8	7.1	0.9	20.2	11.8	33.4	4.4	9.8	7.7
<i>M. barkeri</i>	CO <sub>2</sub>	7.1	0	2.8	12.4	6.2	25.1	20.1	3.7	22.7
<i>M. barkeri</i>	CH <sub>3</sub> OH	0	0	0	46.3	29.4	4.3	0	0	20.0

<sup>a</sup> Conditions: *M. thermoautotrophicum*: 5.3 mg (dry weight)/ml; specific activity of label, 2,500 dpm/nmol; medium, lacking resazurin and containing 28 mM NH<sub>4</sub>Cl; temperature, 50°C; 2,766 cpm recovered from plate (80% recovery). *M. ruminantium*: 20.0 mg (dry weight)/ml; specific activity of label, 2,200 dpm/nmol; medium, all components double strength (except PO<sub>4</sub>, which was 1/5 normal), lacking resazurin and containing 10 mM acetate; temperature, 28°C; 1,843 cpm recovered from plate (57% recovery). *M. barkeri* (CO<sub>2</sub>): 16.2 mg (dry weight)/ml; specific activity of label, 2,200 dpm/nmol; medium, lacking resazurin; temperature, 28°C; 1,655 cpm recovered from plate (34% recovery). *M. barkeri* (methanol): 19 mg (dry weight)/ml; specific activity of label, 700 dpm/nmol; medium, lacking resazurin; temperature, 22°C; 1,872 cpm recovered from plate (51% recovery).

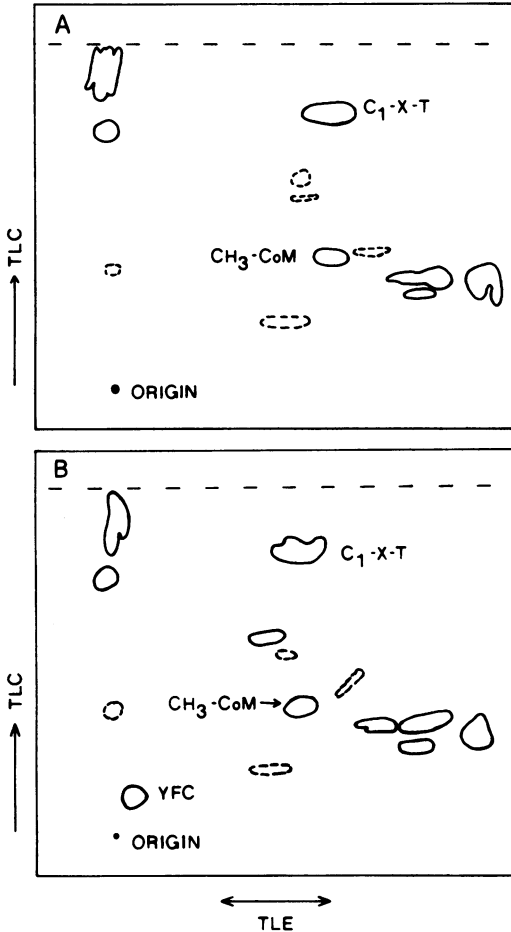


FIG. 3. Sketches of autoradiograms showing the positions of labeled intermediates obtained from analysis of *M. thermoautotrophicum* labeled with  $\text{Na}_2^{35}\text{S}$  for 2 days. (A)  $^{35}\text{S}$ -labeled compounds only. (B)  $^{35}\text{S}$ -labeled compounds plus  $^{14}\text{C}$ -labeled YFC,  $\text{CH}_3\text{-CoM}$  and  $\text{C}_1\text{-X-T}$ . The areas enclosed by dashed lines indicate faintly labeled spots.

converted slowly to  $\text{CH}_3\text{-CoM}$  when stored as a dry solid on a TLC plate at room temperature.

The labeled intermediate YFC on chromatograms was coincident with a yellow fluorescence under UV light. YFC did not react with ninhydrin, its electrophoretic mobility was not altered by acid phosphatase treatment, and it did not display electrophoretic mobility at pH 4.0. YFC was labeled in whole cells by  $^{14}\text{CO}_2$  or  $^{14}\text{CH}_3\text{OH}$ , but not by  $^{35}\text{S}$  (Fig. 3). YFC was stable as a dry solid on TLC plates and was not affected by light. Some additional, positively charged, chromatographic forms of YFC were observed in extracts that had been heated ( $100^\circ\text{C}$ ) or exposed to dilute acid (0.5 M HCl). Yellow fluorescent spots were also seen as faintly labeled

spots in normally treated extracts (see Fig. 2). The spectral properties of purified YFC were examined. At pH 1, the absorbance maxima were at 290 and 335 nm; at pH 13 the absorbance at 335 nm was greatly decreased. At pH 1, the fluorescence excitation maxima were at 287 and 338 nm, and the emission maximum was at 455 to 460 nm. At pH 13, the fluorescence excitation maxima were at 260, 280, and 365 nm, and the emission maximum was at 450 nm. YFC supported neither growth of *L. casei* in the absence of added folic acid nor growth of *M. ruminantium* strain M1 in the absence of CoM (Table 2). The labeled intermediates designated UNF-1 and UNF-2 did not fluoresce, were not altered by acid phosphatase treatment, were not colored by ninhydrin, and were not labeled in whole cells by  $^{35}\text{S}$ . Controls with [ $^{14}\text{C}$ ]-methenyl tetrahydrofolate and  $^{14}\text{CH}_3\text{-B}_{12}$  demonstrated that none of the observed unidentified intermediates of whole cells labeled with  $^{14}\text{CO}_2$  or  $^{14}\text{CH}_3\text{OH}$  displayed the electrophoretic and chromatographic properties of these compounds.

**Short-term time course products of *M. thermoautotrophicum* labeled with  $^{14}\text{CO}_2$ .** *M. thermoautotrophicum* was labeled with  $^{14}\text{CO}_2$  for 2 to 45 s. The kinetics of  $^{14}\text{CO}_2$  uptake into cells over this time period was linear for 35 s, and the rate of incorporation decreased slowly after this time. Table 3 shows the nonvolatile labeled intermediates formed with time. The percentage which any of these intermediates contributed to the total radioactivity as a function of time is shown in Fig. 4. Alanine was highly labeled at the earliest time point and exhibited a negative slope with time, whereas glutamate had a positive slope. YFC was labeled at all time points and had a slight negative slope; it was frequently observed to contribute more to the total label than it did in this experiment. The percentage of label in CoM derivatives increased with time.

The absence of label in 3-phosphoglyceric acid (the immediate product of the Calvin cycle) indicated the absence of ribulose 1,5-bisphosphate carboxylase. This was confirmed by assays for this enzyme under a wide variety of conditions. It was assured that extracts of *M. thermoautotrophicum* did not contain an inhibitor for ribulose 1,5-bisphosphate carboxylase in controls performed with *Anabaena cylindrica* extracts. Controls with *Rhodospirillum rubrum* demonstrated that 3-phosphoglyceric acid was an immediate product of  $^{14}\text{CO}_2$  incorporation into whole cells, using the procedures described above. Organic phosphates became labeled and increased in contribution to the total label with time when *M. thermoautotrophicum* was labeled

TABLE 3. Distribution of radioactivity among the stable, nonvolatile labeled components of *M. thermoautotrophicum* labeled with <sup>14</sup>CO<sub>2</sub><sup>a</sup>

Time (s)	Radioactivity (cpm/spot on TLC plate)								
	Alanine	Gluta- mate	Aspar- tate	CH <sub>3</sub> - CoM	C <sub>1</sub> -X-T	YFC	UNF-1	UNF-2	Others
2	194	11	52	0	59	11	59	48	0
8	401	68	75	104	330	50	265	67	46
15	487	148	66	179	475	58	328	89	112
25	614	226	88	263	772	64	515	75	149
35	884	312	61	246	870	63	376	127	190
45	692	246	40	147	1,031	45	377	112	193

<sup>a</sup> Conditions were as described in the footnote to Table 1. Recoveries were 60 to 80% of the counts per minute applied to the plates.

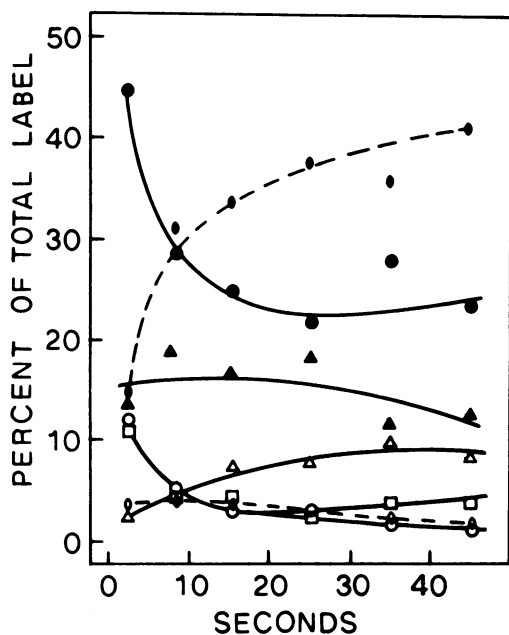


FIG. 4. Short-term time course labeling of *M. thermoautotrophicum* with <sup>14</sup>CO<sub>2</sub>. Variation with time of the percentage a compound contributes toward the total radioactivity recovered during analysis. Symbols: ●, alanine; ○, aspartate; △, glutamate; ▲, UNF-1; □, UNF-2; ●, CoM derivatives; ○, YFC. Conditions are described in the footnote to Table 1. Recovery from a TLC plate was about 60 to 80% of the counts per minute applied.

with <sup>14</sup>CO<sub>2</sub> for longer periods (1 to 5.5 min).

**Short-term time course of *M. thermoautotrophicum* labeled with [2-<sup>14</sup>C]acetate.** *M. thermoautotrophicum* assimilates significant amounts of acetate into cell carbon during growth on H<sub>2</sub>/CO<sub>2</sub> (41, 47). Thus, the labeled intermediates formed by incubation of whole cells with this C<sub>2</sub> compound were compared with the products of CO<sub>2</sub> fixation. Uptake of [2-<sup>14</sup>C]-acetate into cells was linear with time. Table 4 shows the results of analysis of the samples.

TABLE 4. Distribution of radioactivity among the stable, nonvolatile labeled components of *M. thermoautotrophicum* incubated with <sup>14</sup>CH<sub>3</sub>COOH<sup>a</sup>

Time (s)	Radioactivity (cpm/spot on TLC plate)				
	Ala- nine	Succi- nate	C-2	C-3	Others
2	0	130	70	30	7
8	0	193	136	51	7
20	8	248	124	54	10
45	27	294	160	71	5
90	40	280	146	62	7
180	519	372	156	123	140
240	493	217	146	81	73

<sup>a</sup> Conditions: <sup>14</sup>CH<sub>3</sub>COOH (375 μCi; 121,000 dpm/nmol) in 10 ml of modified medium (minus res-azurin and containing 28 mM NH<sub>4</sub>Cl) was added at zero time to an 80-ml suspension of rapidly stirring cells (5.3 mg [dry weight]/ml) in the same modified medium; incubation temperature was 50°C.

Only four compounds were significantly labeled: alanine, succinate, and two unidentified compounds. After 90 s, some other amino acids were lightly labeled. The unidentified compounds, C-2 and C-3, were not amino acids or sugar phosphates and displayed electrophoretic and chromatographic mobilities similar to those of carboxylic acids. Succinate and C-2 and C-3 contributed >95% of the label at 2 s, but alanine increased greatly with time, accounting for almost 50% at 240 s. YFC, CH<sub>3</sub>-CoM, C<sub>1</sub>-X-T, UNF-1, or UNF-2 was not labeled.

## DISCUSSION

Unlike other autotrophs or methylotrophs, methanogenic bacteria metabolize C<sub>1</sub> compounds into both methane and cell carbon. Thus, the intermediates formed during short-term labeling with <sup>14</sup>CO<sub>2</sub> or <sup>14</sup>CH<sub>3</sub>OH must be examined to understand both catabolic and anabolic processes in methanogens. It is clear from the labeling patterns observed that methanogens are quite different from other autotrophs

or methylotrophs and that the three diverse species investigated use similar intermediates in  $C_1$  metabolism. It is also clear that the structure and function of some components of  $C_1$  metabolism in methanogens are unknown and warrant further study.

The data presented here demonstrate the *in vivo* importance of CoM in methanogens. CoM derivatives were major products of all species labeled with  $^{14}CO_2$ . They also accounted for the vast majority of the total intermediates formed when *M. barkeri* was labeled with  $^{14}CH_3OH$ , which suggests that  $CH_3OH$  reacts directly with CoM, as reported by Gunsalus et al. in cell-free extracts (20). Some workers have suggested that folate derivatives or  $CH_3-B_{12}$  is involved in the production of methane (4, 5, 9, 10, 27, 28, 38, 40, 44). The labeling data presented here do not demonstrate the involvement of  $CH_3-B_{12}$  or methyl or formyl tetrahydrofolate in methanogenesis from  $CO_2$  or  $CH_3OH$ . It is possible that methanogens contain  $CH_3-B_{12}$  and methenyl tetrahydrofolate. However, the techniques used here would only detect these  $C_1$  carriers if they were significantly labeled. If  $CH_3-B_{12}$  and methenyl tetrahydrofolate were involved in a major metabolic pathway, one might expect the pool sizes of these carriers to be similar to those of CoM derivatives. The absence of these labeled folate derivatives is consistent with the enzyme data (19; unpublished data) showing that the levels of formyl tetrahydrofolate synthase and methylene tetrahydrofolate dehydrogenase were too low to be involved in a major pathway of  $CO_2$  reduction.

The labeling of a CoM derivative chromatographically distinct from  $CH_3-CoM$  does not demonstrate the involvement of a more oxidized CoM derivative in methanogenesis, since its structure and function are not known. However, the appearance of [ $^{14}C$ ]formate as a product of  $^{14}CO_2$  labeling, where formate is not a free intermediate (3, 20, 43), suggests that formate may be a breakdown product of some unstable  $C_1$  carrier. The formyl CoM described by Romesser and Wolfe (J. A. Romesser and R. S. Wolfe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, 12, p. 155) is a possibility for such a compound, since it decomposes to formate and HS-CoM.

The labeling data from short-term time courses with *M. thermoautotrophicum* support the activities of the pyruvate synthase and  $\alpha$ -ketoglutarate synthase demonstrated in cell-free extracts (46). The negative slope obtained for alanine in relation to the other products formed (Fig. 4) suggests that pyruvate is an early net  $CO_2$  fixation product and supports the presence of pyruvate synthase and alanine dehydrogenase activities in *M. thermoautotrophicum* (46). The

synthesis of pyruvate by this reaction is also supported by the labeling of alanine by [ $2-^{14}C$ ]acetate. The negative slope of aspartate in relation to the other products formed is consistent with it being an early intermediate, and the positive glutamate slope suggests it is a later intermediate of  $CO_2$  metabolism. The presence of pyruvate synthase, phosphoenolpyruvate carboxylase, and  $\alpha$ -ketoglutarate synthase activities in *M. thermoautotrophicum* is also supported by the position of label found in alanine, aspartate, and glutamate obtained from cells grown in the presence of [ $^{14}C$ ]acetate (R. Thauer, personal communication). The evidence presented here for  $CO_2$  reduction in methanogens does not allow any hypothesis concerning the involvement of these carboxylations in a reductive tricarboxylic acid cycle. Indeed, the enzymes necessary for the conversion of  $\alpha$ -ketoglutarate into acetyl-CoA and oxaloacetate, as reported for the reductive tricarboxylic acid cycle (12, 18), were not demonstrated in *M. thermoautotrophicum* (46). However, acetyl-CoA must be produced for pyruvate synthesis to occur. Acetate could be synthesized by a  $C_1 + C_1$  condensation, as reported in some *Clostridium* species (36), or it may be generated by the splitting of a  $C_4$ ,  $C_5$ , or  $C_6$  compound. An experimental approach that should distinguish between the two possibilities would be to degrade the [ $^{14}C$ ]alanine formed during short-term  $^{14}CO_2$  labeling experiments. If a  $C_1 + C_1$  condensation occurred, label would be distributed at early times among all carbons of alanine, but if acetate comes from another source, only the carboxyl should be labeled.

Analysis of the products formed when *M. barkeri* was incubated with  $^{14}CH_3OH$  shows that methanol labels primarily CoM intermediates, indicating that the cell carbon intermediates may arise only from carbon at a more oxidized level. It is not known if  $CH_3OH$  must be oxidized fully to  $CO_2$  before its incorporation into cell carbon.

The labeling data demonstrate the absence of the expected intermediates of the Calvin (6, 13), serine (25, 35), or hexulose phosphate (23, 25) path of  $C_1$  assimilation. Additional evidence for the absence of the Calvin cycle was provided by our inability to detect ribulose 1,5-bisphosphate carboxylase in *M. thermoautotrophicum*. The absence of the serine and hexulose phosphate paths is also supported by investigators' inability to demonstrate the key enzymes of each of these pathways (41; unpublished data).

YFC was found in all three methanogens examined. At 2- to 45-s incorporation times with  $^{14}CO_2$  or  $^{14}CH_3OH$ , the percentage of total label in YFC often exceeds the contribution of label recovered in CoM derivatives. A compound with



the properties described for YFC has not been reported in methylotrophic or autotrophic bacteria or in eucaryotic autotrophs (6, 13, 18, 23, 25, 29, 35). The structural identity and role of YFC in C<sub>1</sub> metabolism of methanogens warrants further attention.

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