JOURNAL OF BACTERIOLOGY, Aug. 1982, p. 932-941
0021-9193/82/080932-10$02.00/0

Vol. 151, No. 2

One-Carbon Metabolism in Methanogens: Evidence for Synthesis of a Two-Carbon Cellular Intermediate and Unification of Catabolism and Anabolism in *Methanosarcina barkeri*

WILLIAM R. KENEALY AND J. G. ZEIKUS*

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Received 12 January 1982/Accepted 14 April 1982

One-carbon metabolic transformations associated with cell carbon synthesis and methanogenesis were analyzed by long- and short-term $^{14}CH_3OH$ or $^{14}CO_2$ incorporation studies during growth and by cell suspensions. $^{14}CH_3OH$ and $^{14}CO_2$ were equivalently incorporated into the major cellular components (i.e., lipids, proteins, and nucleic acids) during growth on H_2-CO_2-methanol. $^{14}CH_3OH$ was selectively incorporated into the C-3 of alanine with decreased amounts fixed in the C-1 and C-2 positions, whereas $^{14}CO_2$ was selectively incorporated into the C_1 moiety with decreasing amounts assimilated into the C-2 and C-3 atoms. Notably, $^{14}CH_4$ and [3-^{14}C]alanine synthesized from $^{14}CH_3OH$ during growth shared a common specific activity distinct from that of CO_2 or methanol. Cell suspensions synthesized acetate and alanine from $^{14}CO_2$. The addition of iodopropane inhibited acetate synthesis but did not decrease the amount of $^{14}CH_3OH$ or $^{14}CO_2$ fixed into one-carbon carriers (i.e., methyl coenzyme M or carboxydihydromethanopterin). Carboxydihydromethanopterin was only labeled from $^{14}CH_3OH$ in the absence of hydrogen. Cell extracts catalyzed the synthesis of acetate from ^{14}CO (~1 nmol/min per mg of protein) and an isotopic exchange between CO_2 or CO and the C-1 of pyruvate. Acetate synthesis from ^{14}CO was stimulated by methyl B_{12} but not by methyl tetrahydrofolate or methyl coenzyme M. Methyl coenzyme M and coenzyme M were inhibitory to acetate synthesis. Cell extracts contained high levels of phosphotransacetylase (>6 μmol/min per mg of protein) and acetate kinase (>0.14 μmol/min per mg of protein). It was not possible to distinguish between acetate and acetyl coenzyme A as the immediate product of two-carbon synthesis with the methods employed.

The cellular characteristics of methanogens are very diverse, yet as a microbial group species they are unified on the basis of unique macromolecular characteristics of archaebacteria (1) and their ability to form methane via growth on one-carbon compounds or acetate (35, 39). *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri* are the only methanogens whose one-carbon metabolism has been examined in detail. Nonetheless, the exact biochemical paths employed for the synthesis of methane or cells from one-carbon compounds remain to be proven (40). Short-term $^{14}CO_2$ fixation studies (4) have established that both species form common intermediary metabolites which include one-carbon carriers (i.e., carboxydihydromethanopterin [YFC], a yellow fluorescent compound identified as a carboxylated pteridine [16], and methyl coenzyme M [methyl CoM] [24, 33]), anabolic products (i.e., alanine, aspartate, and glutamate), methane, and other unidentified immediate fixation products. The mechanism of acetate assimilation into cell carbon involves different portions of the tricarboxylic acid cycle for α-ketoglutarate synthesis in *M. barkeri* and *M. thermoautotrophicum*, but the complete cycle does not function in either species (8, 10, 36, 41). Autotrophic growth of both species on H_2-CO_2 is inhibited by iodopropane, a corrinoid antagonist, but not when acetate is provided in the medium (18). *M. barkeri* and *M. thermoautotrophicum* actively dissimilate CO into CO_2 and CH_4 (3), and both species contain high activity of carbon monoxide dehydrogenase when grown on H_2-CO_2 (3).

The total synthesis of a two-carbon compound (i.e., acetate) from one-carbon metabolism has only been established in *Clostridium thermoaceticum* (12, 21). This anaerobe synthesizes 3 mol of acetic acid per mol of hexose fermented, and one of the acetates produced is derived from the differential metabolism of the C-1 of pyruvate. The biochemical mechanism of this C-2 synthesis involves the reductive carboxylation of a

methyl corrinoid by a C-1 unit (30, 37). Recently, carbon monoxide dehydrogenase was partially purified (5) from a pyruvate-transforming complex in *C. thermoaceticum* (6) and subsequently shown to catalyze $CH_3{}^{14}CO$-S-coenzyme A (CoA) synthesis via the CoA-dependent condensation of methyl tetrahydrofolate and ^{14}CO (15). Although *C. thermoaceticum* is generally regarded as only capable of growth on saccharides, we have been able to grow it with either H_2-CO_2 or CO as the energy source (Kerby and Zeikus, submitted for publication).

The purpose of the present study was to examine whether methane-forming reactions and cell carbon-synthesizing reactions shared common one-carbon transformations and to examine two-carbon synthesis in view of the biochemical activities displayed by *C. thermoaceticum*. *M. barkeri*, but not *M. thermoautotrophicum*, was uniquely suited for this study because this species contains higher levels of corrinoids (20) and carbon monoxide dehydrogenase (19), and it can be grown methylotrophically on methanol, autotrophically on H_2-CO_2, or mixotrophically on H_2-CO_2-methanol (34). Thus, it is possible to study the differential incorporation of ^{14}C-labeled CO, CO_2, or CH_3OH into methane and cell synthesis products. Furthermore, when acetate was assimilated by *M. barkeri* during growth on H_2-CO_2 or methanol, the percentage of the cell carbon derived from either methanol or CO_2 was diminished, suggesting that acetate might be synthesized via a condensation of two one-carbon units (18, 34).

MATERIALS AND METHODS

Chemicals and gases. Substrates for enzymes and coupling enzymes were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals used were of reagent grade. Gases and gas mixtures were purchased from Matheson Scientific, Inc., Joliet, Ill. Sodium [^{14}C]bicarbonate (2 to 10 mCi/mmol), [^{14}C]methanol (20 to 60 mCi/mmol), L-[1-^{14}C]lactate (2 to 10 mCi/mmol), L-[3-^{14}C]lactate (15 to 35 mCi/mmol), [1-^{14}C]acetate (1 to 3 mCi/mmol), [2-^{14}C]acetate (1 to 3 mCi/mmol), and L-[*U*-^{14}C]alanine (>150 mCi/mmol) were purchased from New England Nuclear Corp., Boston, Mass. [1-^{14}C]pyruvate (50 to 20 mCi/mmol) was purchased from Amersham Corp., Arlington Heights, Ill. [^{14}C]carbon monoxide (10 to 50 mCi/mmol) was purchased from ICN, Irvine, Calif. Sodium [^{14}C]bicarbonate was purified before use to remove contaminating labeled compounds. The label was suspended in dilute base, placed in a vial, sealed, and acidified. The evolved CO_2 was trapped in dilute base (95% recovery) and neutralized before use. [^{14}C]methanol was purified from trace contaminants in a Thunberg tube and suspended in 2 ml of dilute base. The contents of the tube were frozen in liquid N_2, and a vacuum was drawn on the tube. The tube was immersed in a 65°C water bath with the bulb immersed in liquid N_2. After approximately one-third of the liquid was distilled and frozen in the bulb, the tube was opened, and the methanol was recovered from the bulb. About 80% recovery of the label was obtained. Methyl CoM was synthesized as described by Gunsalus et al. (13).

Organisms and cultivation. *M. barkeri* neotype strain MS (34) was cultivated in phosphate-buffered basal medium (PBBM) as described previously (18). Cultures were routinely checked for purity by phase-contrast microscopy and by inoculation into heterotrophic media and checking for turbidity as described by Weimer and Zeikus (34).

For labeling studies during growth, *M. barkeri* was cultured in 20-liter carboys containing 5 liters of PBBM with 100 mM methanol and a H_2-CO_2 gas phase. A total of 1 mCi of [^{14}C]methanol or $^{14}CO_2$ was added to the carboys before inoculation with 50 ml of culture. The gas atmosphere was sampled during incubation, and a positive pressure was kept on the carboys with H_2. Methanol specific activity was measured immediately after inoculation. The carboys were incubated with stirring at 37°C until late log phase, and the cells were harvested by centrifugation in polycarbonate bottles (15,000 × *g*) and washed once with 100 mM phosphate buffer (pH 7.0). The cells were then lyophilized for storage until used for extraction.

M. barkeri was mass cultured on H_2-CO_2-methanol in 12 liters of PBBM, supplemented with 100 mM methanol, in a 14-liter Microferm fermentor (New Brunswick Scientific Co., New Brunswick, N.J.). A gas rate of 100 ml of H_2-CO_2 (80:20) per min was employed for growth. *M. barkeri* was grown on H_2-CO_2 or methanol as described by Weimer and Zeikus (34) and on acetate as described by Krzycki et al. (19). Cells used for enzymatic analysis were anaerobically harvested in the late log phase by centrifugation in an RC-5 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) equipped with a KSB continuous flow system. Extracts were prepared as described by Kenealy et al. (17). The protein content of extracts was determined by the method of Lowry et al. (22) with bovine serum albumin in 2 mM dithiothreitol as a standard.

H_2-CO_2-methanol cell suspensions were prepared for short-term labeling by allowing the cells to settle, removing 11.5 liters of medium from the fermentor, and transferring the cells to a holding flask. Cells were labeled, extracted, and analyzed by thin-layer electrophoresis-chromatography-autoradiography as described by Daniels and Zeikus (4).

Purification and degradation of alanine. The $^{14}CO_2$- or $^{14}CH_3OH$-labeled cells (0.5 g) were extracted by the procedure of Roberts et al. (28). The protein-containing cell residue was hydrolyzed with 50% HCl under vacuum in sealed lyophilization ampoules at 121°C for 14 h. The hydrolysate was centrifuged, and the supernatant was brought to dryness, resuspended in 5 ml of distilled water, and neutralized with NaOH. The protein hydrolysate was then applied to an AG1 × 8 acetate (Bio-Rad Laboratories) column (7 by 2.5 cm) and washed with 50 ml of distilled water. The distilled-water wash was brought to dryness and resuspended in 5 ml of distilled water. Alanine was separated from the other amino acids by enzymatic conversion to lactate and purification by the column chromatographic procedures of Fuchs et al. (10). Control samples of [*U*-^{14}C]alanine were treated in the same manner. The lactate content was determined enzymatically (14).

The purity of [^{14}C]lactate was determined by the thin-layer electrophoresis-chromatography-autoradiography procedure of Daniels and Zeikus (4) and by high-performance liquid chromatography (HPLC) by the procedure described below for [^{14}C]acetate.

The lactate formed from alanine was decarboxylated by the bichromate oxidation method described by Fuchs et al. (9). The degradation vessel used in these studies was a Bellco crimp-top anaerobic pressure tube (Bellco Glass, Inc., Vineland, N.J.) sealed with a new pressure bung. After degradation, the CO_2 (C-1 alanine) was trapped in 3 ml of methanol-ethanolamine (4:1) by passing N_2 gas through the degradation vessel and into the CO_2 trap. Samples from the CO_2 trap were counted after first mixing with an equal volume of 1 liter of toluene plus 4 g of 2,5-diphenyloxazole and 0.1 g of *p*-bis-[2-(5-phenyloxazolyl)]benzene. The yield of C-1 label was 85.4% as determined with [1-^{14}C]lactate.

The acetate (C-2 + C-3 of alanine) remaining in the degradation vessel was removed by steam distillation. The acetic acid distillate was neutralized with NaOH and brought to dryness. The salt was suspended with 2 ml of distilled water, and 0.1 ml was removed for radioactivity determination. The rest of the sample was placed in a second pressure tube and brought to dryness by adding 2 ml of 100% ethanol and passing a stream of air over the sample. The tube was cooled to −20°C, 0.3 ml of concentrated H_2SO_4–20% fuming sulfuric acid (5:3) was added, and the tube was cooled to −20°C. Then 30 mg of sodium azide, prepared as described by Sakami (29), was added, and the tube was immediately sealed with a new pressure bung. The tube was incubated at 70 to 75°C for 1 h with frequent mixing and then cooled; the CO_2 produced (i.e., the C-2 of alanine) was trapped and quantified as described above. The yield of the C-1 of acetate was 99.3% as determined with 1-^{14}C standards. The contents of the tube were then made alkaline by the addition of 2 ml of 10 N KOH, and 3 ml of 5% $KMnO_4$ was added. The tubes were placed in a boiling-water bath for 2 h with frequent mixing, cooled, and acidified by the addition of 1.5 ml of 9 N H_2SO_4. The CO_2 liberated (i.e., the C-3 of alanine) was trapped and quantified. The yield of the C-2 of acetate was 70.8% as determined with [2-^{14}C]acetate and [3-^{14}C]lactate standards.

Metabolic analyses. The radioactivity and content of $^{14}CO_2$ and $^{14}CH_4$ were determined by the gas chromatography-gas proportional counting procedure of Nelson and Zeikus (25). The radioactivity of soluble metabolites was determined by the addition of sample (up to 1 ml) in 5 ml of Packard Instagel and quantification in a Prias PLD scintillation counter (Packard Instrument Co., Inc., Rockville, Md.). Methanol and acetate were quantified by the gas chromatography procedures described by Zeikus et al. (42). The [^{14}C]acetate was purified by HPLC (Perkin-Elmer series III; The Perkin-Elmer Corp., Norwalk, Conn.) with a Bio-Rad organic acids column (Bio-Rad Laboratories, Richmond, Calif.) eluted with 0.015 N H_2SO_4 at a flow rate of 0.8 ml/min. Samples were prepared for HPLC analysis by precipitating protein with 50% methanol, centrifuging, and drying the supernatant. The dried sample was resuspended in a minimal amount of distilled water and injected into the HPLC column. The identity of acetate was verified by the retention time of standards on the column, steam distillation of acetate samples, followed by HPLC analysis, and ensuring acid volatility of the counts. Other volatile compounds, formate and methanol, were well separated from acetate by the organic acids analysis column.

Enzymatic activities. Acetate kinase (EC 2.7.2.1) was measured in the direction of acetyl phosphate synthesis by coupling the activity to pyruvate kinase and lactate dehydrogenase and measuring the oxidation of NADH at 334 nm (ε_{334} = 6.1 $mmol^{-1}$ cm^{-1}). The assay mixture contained: Tris-hydrochloride buffer (pH 7.4; 25°C), 100 mM; phosphoenolpyruvate, 5 mM; $MgCl_2$, 10 mM; adenosine triphosphate, 5 mM; sodium acetate, 20 mM; NADH, 0.3 mM; pyruvate kinase, 0.7 U; lactate dehydrogenase, 1 U; and 10 μl of cell extract (~0.2 mg of protein) in a total volume of 1.0 ml. The assay was initiated by the addition of acetate after first measuring ATPase (EC 3.6.1.3), and activity was dependent on extract, acetate, and ATP. Acetate kinase was also measured in the direction of acetate synthesis by coupling the activity to hexokinase and glucose 6-phosphate dehydrogenase and measuring the reduction of NADP at 334 nm. The assay mixture contained: Tris-hydrochloride buffer (pH 7.4; 25°C), 100 mM; $MgCl_2$, 10 mM; NADP, 1 mM; glucose 6-phosphate dehydrogenase, 2 U/ml; hexokinase, 4 U/ml; ADP, 5 mM; acetyl phosphate, 1 mM; glucose, 5.5 mM; and 10 μl of cell extract in a total volume of 1 ml. The assay was initiated by the addition of acetyl phosphate after the measurement of myokinase activity (EC 2.7.4.3).

Phosphotransacetylase (EC 2.3.1.8) was measured spectrophotometrically by following the formation of acetyl CoA from acetyl phosphate and CoA at 233 nm ($\Delta\varepsilon$ = 4.44 $mmol^{-1}$ cm^{-1}). The reaction mixture contained: Tricine buffer (pH 7.5), 100 mM; acetyl phosphate (lithium salt), 1.0 mM; CoA, 0.3 mM; and 1 μl of cell extract in a total volume of 1.0 ml. The assay was initiated by the addition of acetyl phosphate or extract.

RESULTS

Long-term incorporation of $^{14}CH_3OH$ and $^{14}CO_2$. Cells were cultured mixotrophically on H_2-CO_2-methanol as the carbon and energy sources to compare the incorporation of CO_2 and methanol into methane and cell carbon. Figure 1 compares the specific activity relationships between $^{14}CH_4$ and $^{14}CO_2$ when *M. barkeri* was grown in 20-liter carboys in the presence of either $^{14}CH_3OH$ or $^{14}CO_2$. Significant methane formation began after a 10- to 15-h lag period. The specific activity of $^{14}CO_2$ remained constant until the last two doublings of the methane in the carboy that contained $^{14}CO_2$ as the radiotracer, whereas the specific activity of CO_2 increased during the incubation with $^{14}CH_3OH$ as the radiotracer. The specific activity of methane decreased during the fermentation time course from either ^{14}C tracer, and clearly both methanol and CO_2 were simultaneously transformed to methane.

Cells from each of these carboys (and similar carboys) were harvested in the late log phase of

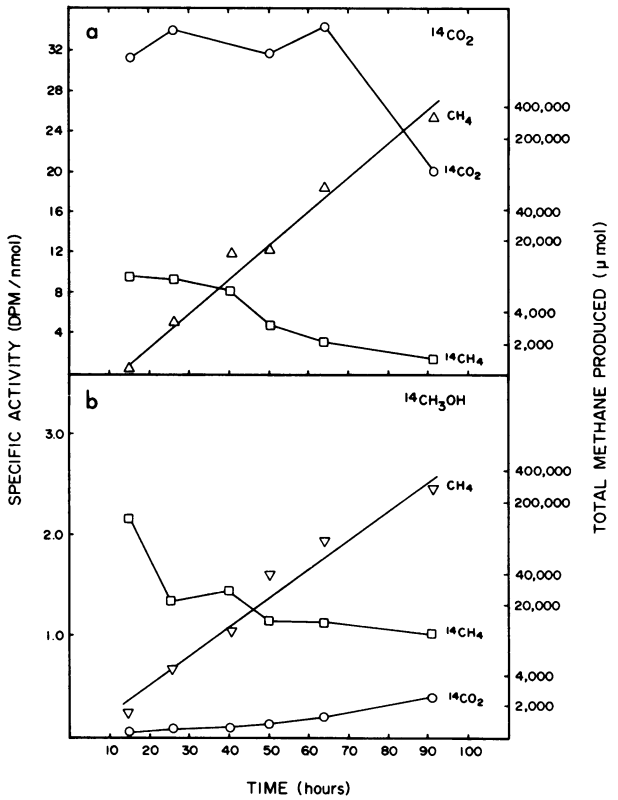

FIG. 1. Relationship between specific activity of $^{14}CO_2$ and $^{14}CH_4$ produced during exponential methanogenesis by *M. barkeri*. Cells were grown in 5 liters of PBBM with H_2-CO_2-methanol, and gas samples were removed and analyzed with time. Each time point represents the average of quadruplicate samples. (A) $Na^{14}CO_3$ (33,000 dpm/μmol) was added as a tracer. (B) $^{14}CH_3OH$ (2,056 dpm/μmol) was added as a tracer.

growth and then fractionated into their different chemical components. The distribution of label into the various cell components is shown in Table 1. The results show that $^{14}CO_2$ and $^{14}CH_3OH$ were equivalently incorporated into the major cell components. The vast majority of the label from either substrate was incorporated into the hydrolyzed residual constituent.

Alanine was purified from the hydrolyzed cell residues and degraded to compare the distribution of ^{14}C tracer among its carbon atoms. Table 2 shows the distribution of label in alanine from $^{14}CH_3OH$ or $^{14}CO_2$ incorporation when *M. barkeri* was grown on H_2-CO_2-methanol. [^{14}C]methanol was preferentially incorporated into the C-3 of alanine. The remainder of the label was found at equal percentages in the C-1 and C-2 of alanine. Cells assimilated $^{14}CO_2$ into alanine quite differently. Here the majority of the $^{14}CO_2$ was incorporated into the C-1 of alanine, with decreasing percentages in the C-2 and C-3 atoms.

Table 3 compares the specific activity of alanine and its individual carbon atoms with those of methane, methanol, and CO_2 during growth

TABLE 1. Distribution of $^{14}CH_3OH$ or $^{14}CO_2$ incorporated into cells of *M. barkeri*[a]

Extraction step	Radioactivity incorporated (% of total)	
	$^{14}CO_2$	$^{14}CH_3OH$
Cold 5% trichloroacetic acid soluble	6.73 ± 6.31	5.87 ± 3.71
75% Ethanol soluble	4.17 ± 1.46	4.10 ± 0.20
75% Ethanol–ether (1:1) soluble	0.77 ± 0.21	1.50 ± 0.40
Hot 5% trichloroacetic acid soluble	11.90 ± 1.44	11.03 ± 2.07
Hydrolyzed residue	76.40 ± 6.32	77.37 ± 2.06

[a] Cells were grown in 20-liter carboys on H_2-CO_2-methanol with either $^{14}CO_2$ or $^{14}CH_3OH$ as the radiotracer; they were harvested and extracted as described in the text. Freeze-dried cells from three separate labelings for each label source were extracted. The values represent the mean ± standard deviation for three separate cell batches. The total dpm extracted ranged from 1.1×10^6 to 6.6×10^7.

TABLE 2. Distribution of ^{14}C label in the alanine synthesized during growth of *M. barkeri* on H_2-CO_2-methanol[a]

Tracer assimilated	Sample size (dpm)	Isotopic distribution in alanine carbon atoms (% of label)[b]		
		C-1	C-2	C-3
[^{14}C]methanol	2,200	23.2 ± 6.2	23.1 ± 3.71	53.6 ± 3.00
[^{14}C]CO_2	12,500	50.2 ± 5.1	32.0 ± 3.04	15.7 ± 1.90

[a] *M. barkeri* was grown in the presence of label, and alanine was purified and degraded as described in the text. The values represent the mean ± standard deviation for 12 degradations of [^{14}C]methanol-derived alanine and 20 determinations for the [^{14}C]CO_2-derived lactate. No carryover of [^{14}C]lactate was detected in the acetate distilled from [1-^{14}C]lactate degradation. Controls with L-[*U*-^{14}C]alanine indicated a distribution of 33.6, 27.7, and 33.5 for carbon atoms 1, 2, and 3, respectively. A small spillover of 1% of the [1-^{14}C]acetate recovered as [2-^{14}C]acetate and 0.1% of the [2-^{14}C]acetate recovered as [1-^{14}C]acetate was detected.

[b] Percent label in the C-1, C-2, and C-3 of alanine was calculated by the following equations:

$$\text{C-1 (\%)} = \frac{\text{dpm recovered as } CO_2}{\text{Total dpm degraded}} \times \frac{1}{\text{yield}} \times 100$$

$$\text{C-2 (\%)} = (100 - \text{\% C-1}) \times \frac{\text{dpm of acetate recovered as C-1}}{\text{dpm of acetate degraded}} \times \frac{1}{\text{yield}}$$

$$\text{C-3 (\%)} = (100 - \text{\% C-1}) \times \frac{\text{dpm of acetate recovered as C-2}}{\text{dpm of acetate degraded}} \times \frac{1}{\text{yield}}$$

The yield values for determination of percent label were 0.854, 0.993, and 0.708 for C-1, C-2, and C-3, respectively.

on H_2-CO_2-methanol. These data are the results of six separate radiotracer experiments. The specific activity values reported were determined in the late exponential phase before the last doubling of methane. Notably, the specific activity of C-1 alanine compared directly with that of CO_2 in experiments with $^{14}CO_2$ as the label, whereas the specific activity of C-3 alanine was directly comparable to that of methane in experiments with $^{14}CH_3OH$ as the label.

Short-term assimilation of $^{14}CO_2$ and $^{14}CH_3OH$. The short-term assimilation of $^{14}CO_2$ and $^{14}CH_3OH$ into intermediary metabolites was examined in tracer-labeled cell suspensions of *M. barkeri* previously cultured on H_2-CO_2-methanol and harvested in the exponential growth phase. A time course for $^{14}CO_2$ incorporation into acetate and alanine is shown in Table 4. Notably, the addition of iodopropane, a corrinoid antagonist, after 0.5 min of incubation completely inhibited acetate synthesis by 2.5 min, whereas $^{14}CO_2$ incorporation continued into alanine. In addition to alanine and acetate, methane and other intermediary metabolites described by

TABLE 3. Specific activity comparisons of one-carbon metabolites of alanine formed during growth of *M. barkeri* on H_2-CO_2-methanol[a]

Source of label	Expt	Sp act (dpm/nmol)						
		One-carbon metabolites				Carbon atoms of alanine[b]		
		CO_2	Methane	Methanol	Alanine	C-1	C-2	C-3
CO_2	I	3.59	0.1		6.97	3.50	2.23	1.09
	II	23.25	1.85		49.45	24.82	15.82	7.76
	III	7.66	0.27		12.73	6.39	4.07	2.00
CH_3OH	IV	0.31	1.56	2.92	3.27	0.76	0.76	1.75
	V	0.31	1.05	2.06	2.20	0.51	0.51	1.18
	VI	1.20	2.99	7.70	4.22	0.98	0.97	2.26

[a] *M. barkeri* was cultured with either $^{14}CH_3OH$ or $^{14}CO_2$, and the specific activities of methanol, CO_2, CH_4, and alanine were determined as described in the text. The specific activities of methanol and alanine represent the mean of more than four determinations. The specific activities of CO_2 and CH_4 are expressed as the values present at the beginning of the last doubling of cells (see Fig. 1) from when the cells were harvested.

[b] The specific activity of alanine carbon atoms was determined by multiplying the specific activity of alanine by the fraction of radioactivity present in that carbon atom (see Table 2).

TABLE 4. Effects of iodopropane on the identified metabolic intermediates of short-term $^{14}CO_2$ assimilation by *M. barkeri*[a]

Iodopropane	Time (s)	Incorporation into components (dpm/mg of cells)						
		Acetate	Alanine	Glutamate	Aspartate	Methyl CoM	YFC	Total
Minus	15	82	24				20	881
	30	171	36			52	18	1,260
	60	423	123			54	95	2,514
	150	914	486		94	267	58	5,000
	300	1,583	726	62	176	278	49	8,545
Plus	15	97	32					1,034
	30	219	48			73	18	1,697
	60	381	141		10	132	22	2,145
	150	461	262		65	75	57	3,125
	300	478	447	52	134	92	26	4,142

[a] Cells grown on H_2-CO_2-methanol were suspended in PBBM plus 100 mM methanol and exposed to $^{14}CO_2$ in the presence of hydrogen gas. Iodopropane (final concentration, 1 mM) was added at 35 s. The $^{14}CO_2$ specific activity was 200 and 120 dpm/nmol for experiments with and without iodopropane, respectively. Acetate was determined by HPLC analysis, and other products were determined by thin-layer electrophoresis-chromatography-autoradiography as described in the text. The deviation in all repeat experiments was less than ±10% of the dpm reported.

Daniels and Zeikus (4) were labeled at early times. CoM derivatives and the pteridine coenzyme YFC were labeled at 30 s. Aspartate and glutamate were also labeled, but only after 5 min. Iodopropane addition did not drastically affect the apparent rate or amount of $^{14}CO_2$ incorporation into these intermediary metabolites. It was not possible to definitively identify [^{14}C]acetyl phosphate or [^{14}C]acetyl CoA with the methods used for analysis.

Cell suspensions assimilated $^{14}CH_3OH$ differently than $^{14}CO_2$ in short-term experiments. Methane, acetate, and CoM derivatives were rapidly labeled (<30 s), but $^{14}CH_3OH$ incorporation into these metabolites was neither increased at 5 min nor significantly decreased by iodopropane addition. Label was incorporated into acetate at a level of 3,700 dpm/mg of cells with $^{14}CH_3OH$ at a specific activity of 360 dpm/nmol. The rate of $^{14}CH_3OH$ conversion to $^{14}CO_2$ was <0.1 that of $^{14}CH_4$ formation. Notably, YFC was not labeled by $^{14}CH_3OH$ unless H_2 was removed from the experimental conditions. In addition, label was also detected in an autoradiographic spot that migrated near a [^{14}C]methyl B_{12} standard. Although the structural identity of this spot was not verified, a similar intermediate was labeled when methanogens were exposed to $^{14}CH_4$ (38).

Enzymes of two-carbon synthesis. The synthesis of a two-carbon intermediate from one-carbon compounds by cell extracts of *M. barkeri* was examined in view of the acetate-forming enzymes present in *C. thermoaceticum*. Table 5 shows that [^{14}C]acetate was a significant and major product of ^{14}CO assimilation by cell extracts. The CO-assimilating activity of *M. barkeri* extracts varied considerably with regard to the methyl donor employed and the presence of CO_2 or $Fe(NH_4)_2SO_4$. Acetate synthesis from ^{14}CO was stimulated by methyl B_{12} and $Fe(NH_4)_2SO_4$. Methyl tetrahydrofolate or meth-

TABLE 5. CO assimilating activities of *M. barkeri*[a]

Assay conditions[b]	^{14}CO incorporated (dpm)		Acetate synthesis (nmol/min per mg of protein)
	Total	Acetate	
Complete	2,524	2,175	0.38
− Methyl B_{12}	879	630	0.11
− MgATP	2,851	2,404	0.42
− H_2 + N_2	2,221	1,774	0.31
− Methyl B_{12} + Methyl THF	1,409	1,030	0.18
+ PYR + CoA	4,916	2,175	0.38
− Methyl B_{12} + Methyl CoM	357	286	0.05
+ CoM	677	286	0.05
+ CO_2	1,021	916	0.16
+ $Fe(NH_4)_2SO_4$	6,723	5,781	1.01

[a] Cell extracts were from H_2-CO_2-grown *M. barkeri*. Complete conditions were: *N*-tris(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid (pH 7.2), 100 mM; $MgCl_2$, 10 mM; ATP, 5 mM; methyl B_{12}, 2.5 mM; H_2 gas phase and extract, 0.1 ml (0.9 mg of protein). Concentrations of additions as indicated were: methyl CoM, methyl-N_5-tetrahydrofolate (THF), and CoM, 2.5 mM; CO_2, 20% gas phase; $Fe(NH_4)_2SO_4$, 1.5 mM; pyruvate (PYR), 1 mM; CoA, 0.1 mM. The reaction was initiated by the addition of extract and incubated for 1 h at 37°C in 5.7-ml anaerobic vials that contained 1.0 ml of ^{14}CO (106 dpm/nmol). The reaction was stopped by the addition of methanol (1 ml) and prepared for HPLC analysis as described in the text. The values represent the mean of duplicate experimental vials.

yl CoM were unable to replace methyl B_{12}. The presence of CoM or methyl CoM stimulated methane formation sixfold; however, CoM was highly inhibitory to acetate synthesis. The addition of pyruvate and CoA enhanced the total ^{14}CO incorporated but had no effect on [^{14}C]acetate synthesis. Removal of ATP and $MgCl_2$ appeared stimulatory for acetate synthesis. Carbon dioxide was inhibitory to ^{14}CO assimilation into acetate. The specific activity of $^{14}CO_2$ present in the vials with CO_2 added was 1.3 dpm/nmol, whereas it was 50 dpm/nmol with the complete assay condition.

Acetate was also synthesized by *M. barkeri* extracts in reaction mixtures containing either [1-^{14}C]pyruvate or $^{14}CO_2$ as label and the same general conditions shown in Table 5. An exchange reaction with [1-^{14}C]pyruvate and added CO_2 or CO was present at rates of 2.5 and 0.1 nmol/min per mg for CO_2 and CO, respectively. [^{14}C]acetate was only a minor product (1 to 5%) when the label incorporated by reaction mixtures was [1-^{14}C]pyruvate or $^{14}CO_2$.

The analysis of acetate activation enzyme activities was initiated because of the detection of acetate as a product of one-carbon assimilation by cell suspensions or extracts. Table 6 compares the activity of acetate-transforming enzymes in relationship to the growth substrates used. Acetate kinase levels were noticeably lower in cells grown with methanol as a substrate, and nearly an order of magnitude greater activity was present in cells grown on acetate or H_2-CO_2 alone. Cell extracts contained ATPase, which was subtracted as a background activity when acetate kinase was assayed in the direction of acetyl phosphate synthesis. Thus, the value reported is that dependent on acetate addition alone. An approximate K_m for acetate of 3 mM was determined for the activity. Myokinase activity was also present, and this value was subtracted as a background when acetate kinase was measured in the direction of acetate synthesis. A very high level of phosphotransacetylase activity for acetyl CoA synthesis from CoA and acetyl phosphate was present in all cell extracts examined. However, the activity of phosphotransacetylase measured in the reverse direction was much lower (50 to 100 nmol/min per mg of protein). The acetate activation enzyme activities were not inhibited by oxygen but were completely destroyed by boiling cell extracts.

DISCUSSION

These data extend general knowledge on one-carbon metabolism in methanogenic bacteria. Most notably, the results provide evidence in support of previous suggestions (18, 40) that: (i) methane and cell carbon precursors share a common intermediate(s) (i.e., initial one-carbon transformation reactions of cell carbon and energy metabolism are unified in methanogens); (ii) the synthesis of a two-carbon cell intermediate is derived from a C-1 + C-1 condensation reaction involving a methyl corrinoid; (iii) *M. barkeri* synthesizes acetate from CO_2 or methanol or both, and it is readily interconvertible with acetyl CoA; and (iv) carbon assimilation mechanisms of methylotrophy and autotrophy in *M. barkeri* show a common pathway involving one-carbon carrier-bound intermediates for the synthesis of methane and a two-carbon cell precursor.

Figure 2 provides a scheme that incorporates the present evidence with previous studies on one-carbon metabolism in *M. barkeri*. The radiotracer analysis of Daniels and Zeikus (4) demonstrated that predominantly YFC, now characterized as carboxydihydromethanopterin (16), but also methyl CoM, alanine, and aspartate were the first identifiable short-term incorporation products of $^{14}CO_2$ fixation, whereas $^{14}CH_3OH$ was fixed into the same intermediates but with most of the label appearing as [^{14}C]methyl CoM. The finding here that methanol labeled YFC only in the absence of hydrogen

TABLE 6. Relationship of carbon and energy source for growth and acetate transformation enzyme activities of *M. barkeri*[a]

Carbon and energy source	Sp act (μmol/min per mg of protein at 37°C)				
	Acetate kinase[b]		Phosphotransacetylase	Myokinase	ATPase
	Acetyl phosphate formation	Acetate formation			
H_2-CO_2	0.82	1.08	6.98	0.25	0.02
Methanol	0.11	0.14	ND	0.18	0.01
Acetate	0.56	1.67	8.07	0.09	0.03
H_2-CO_2-methanol	0.07	0.18	6.95	0.18	0.01

[a] The values represent the mean determinations for more than three extracts prepared from different batches of cells that were harvested in the late exponential growth phase. Assay conditions are described in the text. ND, Not determined. The deviation observed in all repeat experiments was less than ±10% of the activity reported.

[b] Values reported are already corrected for the background myokinase and ATPase activities.

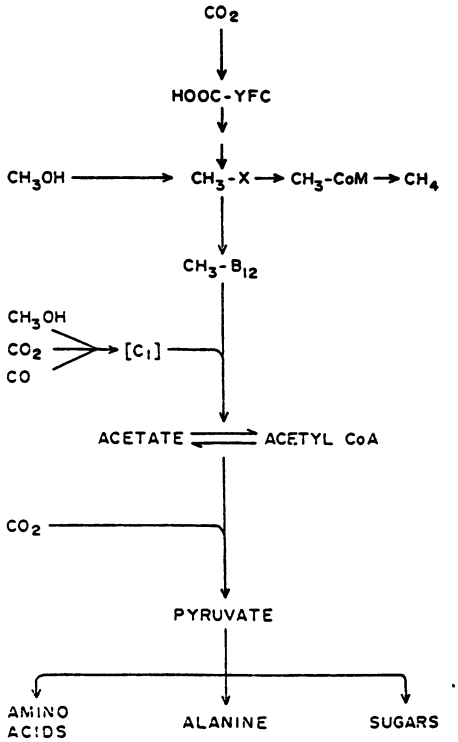

FIG. 2. Schematic relationship between synthesis of a two-carbon intermediate from one-carbon assimilation reactions reported here and the previously described one-carbon transformations of *M. barkeri*. One-carbon compounds are initially transformed via oxidoreduction of carrier-bound intermediates which include carboxyl YFC (4, 16) and methyl CoM (4, 24). Synthesis of a two-carbon cell precursor (i.e., acetate or acetyl CoA) occurs via the condensation of a C-1 intermediate with a methyl corrinoid. Pyruvate is synthesized via reductive carboxylation of acetyl CoA (35) and is subsequently aminated to alanine (17). The exact identity of methyl X is not established (i.e., methyl B_{12} [32] or methyl CoM [31]).

supports its function in CO_2 reduction to methane and methanol oxidation to CO_2. This also explains why *M. barkeri* cannot grow on hydrogen and methanol without CO_2 present (34). Methanol is shown to enter the pathway as methyl X because Wood and co-workers (J. M. Wood and J. Legall, personal communication) have purified a corrinoid enzyme from *M. barkeri* and obtained kinetic data suggesting its use in direct methylation of CoM. Shapiro and Wolfe (31) reported that synthesis of methyl CoM was dependent only on methanol and CoM. The path of one-carbon metabolism presented is consistent with: methanol labeling acetate and methyl CoM at very early times; methanol being specifically incorporated into the C-2 of acetate or acetyl CoA and hence the C-3 of alanine; and a similar specific activity for [3-^{14}C]alanine and the $^{14}CH_4$ derived during growth on H_2-CO_2-$^{14}CH_3OH$. Considerably more work is required to prove this path of one-carbon metabolism, especially enzyme purification-characterization and analysis of specific metabolic mutants.

The methyl donor for acetate synthesis is shown as methyl B_{12}, since acetate synthesis in cell extracts was stimulated by this methyl donor and not methyl tetrahydrofolate or methyl CoM. Free methyl B_{12} may not be the actual methyl donor but rather an enzyme-bound methyl corrinoid. This would be consistent with the previous data on iodopropane inhibition of acetate synthesis in *M. barkeri* (18) and the inability to demonstrate [^{14}C]methyl B_{12} as an early one-carbon fixation product (4).

Radiotracer evidence was provided here for CO_2 or CO as the C-1 acetate precursor; however, during growth on methanol alone, *M. barkeri* would synthesize this precursor from a methanol-derived C-1 unit (34). The incorporation of ^{14}CO into acetate or acetyl CoA (the direct precursor to pyruvate) is accounted for by the active carbon monoxide dehydrogenase activity present in *M. barkeri* (3, 19). It was not possible to distinguish whether acetate or acetyl CoA was the immediate product because of the very active phosphotransacetylase and acetate kinase activities present in cell extracts. *M. barkeri* neither produces acetate as a catabolic product (~7 to 12 nmoles of acetate per mg of cells) nor catabolizes it when the cells are maintained on H_2-CO_2-CH_3OH metabolism (18, 35). Thus, the use of phosphotransacetylase and acetate kinase to produce ATP is unlikely. The data suggest that acetate is the first anabolic product of C-1 metabolism, and it is then activated by acetate kinase and phosphotransacetylase.

The acetate synthesis activity level reported here is a minimal value because it does not account for the acetate further transformed (i.e., converted to acetyl CoA and other intermediary metabolites), and it relies on a substrate (CO) which may not be encountered during the growth of the organism. The inhibition of acetate synthesis by CoM and CO_2 can be explained by the methylation of CoM by the same intermediate that donates the methyl group to acetate and CO_2 entering the same intermediate as CO. More studies are required to optimize the acetate synthesis activity of *M. barkeri*, especially purification and characterization of carbon monoxide dehydrogenase. CO_2 inhibited the synthesis of acetate, but probably not by dilution of the CO_2 produced from CO. The specific activity of CO_2 was 50-fold less when CO_2 was present, yet the acetate synthesis was only lessened twofold.

In general, it has become apparent from increased investigation that the biochemistry of

one-carbon metabolism differs in *M. barkeri* and *M. thermoautotrophicum*. Thus, it is worth noting here that acetate synthesis, acetate kinase, and phosphotransacetylase are not considered of significance in the autotrophic growth of *M. thermoautotrophicum* (26). The presence of acetate kinase was first noted in *M. barkeri* by Stadtman (32). These species also differ in the portions of the tricarboxylic acid cycle used to synthesize α-keto acids (10, 35, 41) and their amination to alanine, aspartate, and glutamate (17). Perhaps major differences exist in the specific carrier-bound one-carbon transformations.

However, both *M. barkeri* and *M. thermoautotrophicum* may use a common biochemical mechanism for the synthesis of a two-carbon intermediate from H_2-CO_2 because acetate addition relieved the inhibition of autotrophic growth caused by iodopropane (18). It should be noted here that studies of carbon isotope fractionations in *M. thermoautotrophicum* (11) indicate that a major portion of the CO_2 fixed into cells is derived from non-methanogenic intermediates. This is to be expected, but these studies do not imply that common metabolic intermediates are not shared in the synthesis of methane and cell carbon from H_2-CO_2 (R. K. Thauer, personal communication).

Methanogens are considered by Balch et al. (1) to be of ancient origin and to be placed in a phylogenetic kingdom separate from other described anaerobic bacteria. It is interesting to cite a trend that suggests metabolic similarity between *M. barkeri* and certain sporeforming anaerobes such as *C. thermoaceticum* and *Butyribacterium methylotrophicum* (21, 23, 43). These include synthesis of acetic acid from one-carbon metabolism, the presence of high cellular levels of corrinoids, and high carbon monoxide dehydrogenase activity. If future studies can prove a common two-carbon synthesis reaction in both homoacetogens and methanogens, then other molecular taxonomic approaches, such as protein sequence homologies and nucleic acid hybridization studies, should compare the relatedness between these two distinct microbial groups, both of which can metabolize H_2-CO_2, methanol, or CO as carbon and energy sources.

The biochemical mechanism that accounts for autotrophy and methylotrophy in *M. barkeri* appears quite distinct from that described for aerobic methylotrophs (27) and phototrophic microbes (2, 7, 9). The photoautotrophs assimilate CO_2 either via the reductive pentose phosphate pathway or the reverse tricarboxylic acid cycle, whereas methylotrophs primarily assimilate formaldehyde via the hexulose phosphate or serine pathways. *M. barkeri*, like the aerobic methylotrophs, displays high metabolic efficiency in cell carbon synthesis because common biochemical intermediates are used in both catabolic and anabolic one-carbon transformations. Evidence is provided here that the C-3 atoms of alanine and methane, when derived from $^{14}CH_3OH$, share a common intermediate. Thus, the methyl group of acetate when *M. barkeri* was grown on H_2-CO_2-methanol is derived from intermediates directly exchangeable with methanogenic intermediates. Also, evidence is provided which supports a unique method of carbon synthesis in the microbial world in that cell carbon is primarily synthesized via the assimilation of methyl-level carbon and a more oxidized C-1 unit into a two-carbon intermediate which is carboxylated to form other cellular metabolites. Finally, analysis of one-carbon metabolism in *M. barkeri* provides an explanation for the relationships between heterotrophy, methylotrophy, and autotrophy in that growth on one-carbon compounds necessitates formation of the same cell carbon precursor that is provided when acetate is the carbon source.

ACKNOWLEDGMENTS

This work was supported by the College of Agricultural and Life Sciences, University of Wisconsin, by grant DE-AC02-80ER1075 from the Department of Energy Basic Sciences Division. W.R.K. was supported by Public Health Service training grant T32 GM07215-04 from the National Institutes of Health.

We thank Jill O'Brien for excellent technical assistance and J. Krzycki for valuable discussions.

LITERATURE CITED

1. **Balch, W. E., G. E. Fox, L. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260–296.
2. **Bassham, J. A., and M. Calvin.** 1957. The path of carbon in photosynthesis. Prentice-Hall, Inc., Englewood Cliffs, N.J.
3. **Daniels, L., G. Fuchs, R. K. Thauer, and J. G. Zeikus.** 1977. Carbon monoxide oxidation by methanogenic bacteria. J. Bacteriol. **132**:118–126.
4. **Daniels, L., and J. G. Zeikus.** 1978. One-carbon metabolism in methanogenic bacteria: analysis of short-term fixation products of $^{14}CO_2$ and $^{14}CH_3OH$ incorporated into whole cells. J. Bacteriol. **136**:75–84.
5. **Drake, H. L., S. I. Hu, and H. G. Wood.** 1980. The purification of carbon monoxide dehydrogenase, a nickel enzyme from *Clostridium thermoaceticum*. J. Biol. Chem. **255**:7174–7180.
6. **Drake, H. L., S. Hu, and H. G. Wood.** 1981. Purification of five components from *Clostridium thermoaceticum* which catalyze synthesis of acetate from pyruvate and methyl tetrahydrofolate. J. Biol. Chem. **256**:11137–11144.
7. **Evans, N. C. W., B. B. Buchanan, and D. I. Arnon.** 1966. A new ferredoxin dependent carbon reduction cycle in a photosynthetic bacterium. Proc. Natl. Acad. Sci. U.S.A. **55**:928–934.
8. **Fuchs, G., and E. Stupperich.** 1978. Evidence for an incomplete reductive carboxylic acid cycle in *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **118**:121–126.
9. **Fuchs, G., E. Stupperich, and G. Epen.** 1980. Autotrophic CO_2 fixation in *Chlorobium limicola*. Evidence for the operation of a reductive tricarboxylic acid cycle in growing cells. Arch. Microbiol. **128**:64–71.
10. **Fuchs, G., E. Stupperich, and R. K. Thauer.** 1978. Acetate

assimilation and the synthesis of alanine, aspartate, and glutamate by *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **117**:61–66.

11. **Fuchs, G., R. K. Thauer, H. Ziegler, and W. Stichler.** 1979. Carbon isotope fractionation by *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **120**:135–139.
12. **Ghambeer, R. K., H. G. Wood, M. Schulman, and L. Ljungdahl.** 1971. Total synthesis of acetate from CO_2. III. Inhibition by alkyhalides of the synthesis from CO_2, methyltetrahydrofolate and methyl-B_{12} by *Clostridium thermoaceticum*. Arch. Biochem. Biophys. **143**:471–484.
13. **Gunsalus, R. P., J. A. Romesser, and R. S. Wolfe.** 1978. Preparation of coenzyme M analogues and their activity in the methyl coenzyme M reductase of *Methanobacterium thermoautotrophicum*. Biochemistry **17**:2374–2377.
14. **Hohorst, H.-J.** 1965. L(+)-lactate. Determination with lactic dehydrogenase, p. 266–270. *In* H. Bergmeyer (ed.), Methods of enzymatic analysis, 2nd ed. Academic Press, Inc., New York.
15. **Hu, S., H. L. Drake, and H. G. Wood.** 1982. Synthesis of acetyl coenzyme A from carbon monoxide, methyl tetrahydrofolate and coenzyme A by enzymes from *Clostridium thermocellum*. J. Bacteriol. **149**:440–448.
16. **Keltjens, J. T., and G. D. Vogels.** 1981. Novel coenzymes of methanogens, p. 120–129. Proceedings of the 3rd International Symposium on Microbial Growth on C_1 Compounds. Heyden and Sons, Ltd., London.
17. **Kenealy, W. R., T. E. Thompson, K. R. Schubert, and J. G. Zeikus.** 1982. Ammonia assimilation and synthesis of alanine, aspartate, and glutamate in *Methanosarcina barkeri* and *Methanobacterium thermoautotrophicum*. J. Bacteriol. **150**:1357–1365.
18. **Kenealy, W., and J. G. Zeikus.** 1981. Influence of corrinoid antagonists on methanogen metabolism. J. Bacteriol. **146**:133–140.
19. **Krzycki, J. A., R. H. Wolkin, and J. G. Zeikus.** 1982. Comparison of unitrophic and mixotrophic substrate metabolism by an acetate-adapted strain of *Methanosarcina barkeri*. J. Bacteriol. **149**:247–254.
20. **Krzycki, J. A., and J. G. Zeikus.** 1980. Quantification of corrinoids in methanogenic bacteria. Curr. Microbiol. **3**:243–245.
21. **Ljungdahl, L. G., and H. G. Wood.** 1982. Acetate biosynthesis, p. 1–46. *In* D. Dolphin, (ed.). Wiley Interscience, New York.
22. **Lowry, O. H., N. J. Rosebrough, A. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
23. **Lynd, L., R. Kerby, and J. G. Zeikus.** 1982. Carbon monoxide metabolism of the methylotrophic acidogen *Butyribacterium methylotrophicum*. J. Bacteriol. **149**:255–263.
24. **McBride, B. C., and R. S. Wolfe.** 1971. A new coenzyme of methyl transfer, coenzyme M. Biochemistry **10**:2317–2324.
25. **Nelson, D. R., and J. G. Zeikus.** 1974. Rapid method for the radioisotopic analysis of gaseous end products of anaerobic metabolism. Appl. Microbiol. **28**:258–261.
26. **Oberlies, G., G. Fuchs, and R. K. Thauer.** 1980. Acetate thiokinase and the assimilation of acetate in *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **128**:248–252.
27. **Quayle, J. R.** 1972. The metabolism of one-carbon compounds in microorganisms. Adv. Microb. Physiol. **7**:119–203.
28. **Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britton.** 1957. Studies of biosynthesis in *Escherichia coli*. Carnegie Institute, Washington, D.C.
29. **Sakami, W.** 1955. Handbook of isotope tracer methods, p. 63–70. Department of Biochemistry, Case Western Reserve School of Medicine, Cleveland, Ohio.
30. **Schulman, M., R. K. Ghambeer, L. G. Ljungdahl, and H. G. Wood.** 1973. Total synthesis of acetate from CO_2. VII. Evidence with *Clostridium thermoaceticum* that the carboxyl of acetate is derived from the carboxyl of pyruvate by transcarboxylation and not by fixation of CO_2. J. Biol. Chem. **248**:6255–6261.
31. **Shapiro, S., and R. S. Wolfe.** 1980. Methyl-coenzyme M, an intermediate in the methanogenic dissimilation of C_1 compounds by *Methanosarcina barkeri*. J. Bacteriol. **141**:728–734.
32. **Stadtman, T. C.** 1967. Methane fermentation. Annu. Rev. Microbiol. **21**:212–242.
33. **Taylor, C. D., and R. S. Wolfe.** 1974. Structure and methylation of coenzyme M ($HSCH_2CH_2SO_3$). J. Biol. Chem. **249**:4879–4885.
34. **Weimer, P. J., and J. G. Zeikus.** 1978. One carbon metabolism in methanogenic bacteria. Cellular characterization and growth of *Methanosarcina barkeri*. Arch. Microbiol. **119**:49–57.
35. **Weimer, P. J., and J. G. Zeikus.** 1978. Acetate metabolism in *Methanosarcina barkeri*. Arch. Microbiol. **119**:175-182.
36. **Weimer, P. J., and J. G. Zeikus.** 1979. Acetate assimilation pathway of *Methanosarcina barkeri*. J. Bacteriol. **137**:332–339.
37. **Welty, F. K., and H. G. Wood.** 1978. Purification of the "corrinoid" enzyme involved in the synthesis of acetate by *Clostridium thermoaceticum*. J. Biol. Chem. **253**:5832–5838.
38. **Zehnder, A. J. B., and T. D. Brock.** 1979. Methane formation and methane oxidation by methanogenic bacteria. J. Bacteriol. **137**:420–432.
39. **Zeikus, J. G.** 1977. The biology of methanogenic bacteria. Bacteriol. Rev. **41**:514–541.
40. **Zeikus, J. G.** 1980. Chemical and fuel production by anaerobic bacteria. Annu. Rev. Mirobiol. **34**:423–464.
41. **Zeikus, J. G., G. Fuchs, W. Kenealy, and R. K. Thauer.** 1977. Oxidoreductases involved in cell carbon synthesis of *Methanobacterium thermoautotrophicum*. J. Bacteriol. **132**:604–613.
42. **Zeikus, J. G., P. W. Hegge, and M. A. Anderson.** 1979. *Thermoanaerobium brockii* gen. nov. spec. nov. A new caldoactive anaerobic bacterium. Arch. Microbiol. **122**:41–47.
43. **Zeikus, J. G., L. H. Lynd, T. E. Thompson, J. A. Krzycki, P. W. Weimer, and P. W. Hegge.** 1980. Isolation and characterization of a new, methylotrophic, acidogenic anaerobe, the Marburg strain. Curr. Microbiol. **3**:381–388.