JOURNAL OF BACTERIOLOGY, Jan. 1979, p. 420-432
0021-9193/79/01-0420/13$02.00/0

Vol. 137, No. 1

Methane Formation and Methane Oxidation by Methanogenic Bacteria

A. J. B. ZEHNDER* AND T. D. BROCK

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

Received for publication 25 July 1978

Methanogenic bacteria were found to form and oxidize methane at the same time. As compared to the quantity of methane formed, the amount of methane simultaneously oxidized varied between 0.3 and 0.001%, depending on the strain used. All the nine tested strains of methane producers (*Methanobacterium ruminantium, Methanobacterium* strain M.o.H., *M. formicicum, M. thermoautotrophicum, M. arbophilicum, Methanobacterium* strain AZ, *Methanosarcina barkeri, Methanospirillum hungatii,* and the "acetate organism") reoxidized methane to carbon dioxide. In addition, they assimilated a small part of the methane supplied into cell material. Methanol and acetate also occurred as oxidation products in *M. barkeri* cultures. Acetate was also formed by the "acetate organism," a methane bacterium unable to use methanogenic substrates other than acetate. Methane was the precursor of the methyl group of the acetate synthesized in the course of methane oxidation. Methane formation and its oxidation were inhibited equally by 2-bromoethanesulfonic acid. Short-term labeling experiments with *M. thermoautotrophicum* and *M. hungatii* clearly suggest that the pathway of methane oxidation is not identical with a simple back reaction of the methane formation process.

Aerobic methane oxidizers utilize methane as a source of energy and carbon (1). They attack methane by means of an oxygenase, an enzyme which needs molecular oxygen to carry out this process (12). Under anaerobic conditions, methane is believed to be inert and therefore not of further use for microorganisms. However, evidence has been reported by geologists and geochemists that methane might nevertheless be metabolized at a very low rate in the oxygen-free part of marine waters and sediments. This conclusion has been based on measurements of methane profiles in anoxic sediments from Santa Barbara Basin (4), Cariaco Trench (21) and Long Island Sound (15) which suggested consumption of methane in the zone of active sulfate reduction. A laboratory study by Davis and Yarbrough (8) indicated that *Desulfovibrio desulfuricans* may oxidize very small amounts of methane while growing on lactate. Sorokin (24), however, could not detect methane utilization by sulfate reducers when it was present as sole energy and carbon source. Wertlieb and Vishniac (26) described a *Rhodopseudomonas gelatinosa* strain which anaerobically showed a light-dependent incorporation of a small amount of methane into cell material and also a minor, light-independent oxidation of methane to carbon dioxide. Quayle stated in his review (19) that anaerobic bacteria capable of utilizing methane as sole carbon source are unknown. This communication describes results demonstrating methane utilization by methanogenic bacteria and presents some evidence of a specific pathway for methane oxidation in these organisms.

MATERIALS AND METHODS

Organisms. The following strains of hydrogen-oxidizing methanogens were used in this study: *Methanobacterium ruminantium* sludge strain (23), *Methanobacterium* strain M.o.H. (6), *M. formicicum* (13, 16), *M. thermoautotrophicum* (35), *Methanobacterium arbophilicum* (34), *Methanobacterium* strain AZ (32), *Methanosarcina barkeri* strain MS (13; C. G. T. P. Schnellen, Dissertation, Delft University of Technology, De Maasstad, Rotterdam, The Netherlands, 1947), and *Methanospirillum hungatii* strain JF1 (9).

A methanogen which produces methane exclusively from the methyl group of acetate was also studied. This organism, for which a name has not yet been proposed, will be referred to in this paper as the "acetate organism." The paramount difference from the other known methanogens consists in its inability to oxidize hydrogen for methane formation (B. Huser et al. and A. J. B. Zehnder et al., in preparation). Strains of a similar organism in mixed cultures have been described by various authors (3, 10, 16, 18, 25).

Media and cultivation. To prepare sterile media for subculture and maintenance of the organisms, the following stock solutions were used (chemicals in grams per liter of distilled water): (A) KH_2PO_4, 27.2;

(B) Na_2HPO_4, 28.4; (C, mineral salt solution) NH_4Cl, 24; NaCl, 24; $CaCl_2 \cdot 2H_2O$, 8.8; and $MgCl_2 \cdot 6H_2O$, 8; (D) $NaHCO_3$, 80; (E, trace metal solution) $FeCl_2 \cdot 4H_2O$, 2; H_3BO_3, 0.05; $ZnCl_2$, 0.05; $CuCl_2$, 0.03; $MnCl_2 \cdot 4H_2O$, 0.5; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.05; $AlCl_3$, 0.05; $CoCl_2 \cdot 6H_2O$, 0.05; $NiCl_2$, 0.05; Na_2SeO_3, 0.1; ethylenediaminetetraacetate, 0.5; and 1 ml of concentrated HCl; (F) Vitamin mixture (28); (G) $Na_2S \cdot 9H_2O$, 240.2; (H, fatty acids) a mixture of equal amounts of isobutyric, α-methylbutyric, isovaleric, and valeric acids; (I) resazurin, 1.

The basal medium for the hydrogen-oxidizing methanogens had the following composition: 10 ml of solution A, 23.5 ml of solution B, 1 ml of solution I, and 2.5 g of yeast extract were made up with distilled water to 900 ml and sterilized at 121°C; 1 ml of trace metal solution E, 1 ml of vitamin solution F, and 12.5 ml of solution C in 35.5 ml of water were added aseptically to the autoclaved medium by using a Millex microsyringe filter holder with a 0.2-μm membrane filter (Millipore Corp., Bedford, Mass.). Next, 0.5 g of cysteine-hydrochloride and 1 ml of sulfide solution G in 50 ml of solution D were added in the same way. Immediately after, the medium was gassed (32) with an oxygen-free gas mixture of 80% H_2 and 20% CO_2 to remove all the oxygen and to supply the substrates for the growth of the methanogens. When substrates such as formate (*Methanobacterium ruminantium, M. formicicum, Methoanospirillum hungatii*), methanol (*Methanosarcina barkeri*), and acetate (*M. barkeri*) were used, the gas phase in the culture vessel was 80% N_2 and 20% CO_2. In these cases, the substrate concentration was 5 g/liter.

Some strains require, besides the basal medium, an additional supply of organic compounds for growth: *M. ruminantium* and *Methanobacterium* M.o.H. require 2.5 g of sodium acetate/liter and 1 ml of solution H/liter; *M. hungatii* requires 2.5 g of Trypticase/liter.

The basal medium for the "acetate organism" was prepared in the same way as that for the hydrogen-oxidizing methanogens, with the exception that the medium contained *no* yeast extract and *no* cysteine hydrochloride. As a supplementary reducing agent, $Na_2S_2O_4$ was added from a freshly prepared anaerobically filter-sterilized solution, to obtain a final concentration of 0.03 g/liter. For this organism 4.5 g of sodium acetate/liter served as substrate, and the gas phase was 80% N_2 and 20% CO_2.

All methanogens were routinely cultivated in 150-ml serum vials closed with black lip rubber stoppers (2) and sealed with an aluminum seal (2). The amount of medium was normally 20 ml. Experiments were performed (if not otherwise specified) in 35-ml sealed serum vials containing 20 ml of medium. The gas phase was brought to 1.8 atm. When necessary, it was replaced in order to maintain a constant pH of 7.0 (32).

Preparation of $^{14}CH_4$. A 12-ml amount of bicarbonate-free basal medium was prepared anaerobically in the pressure tube (2). The tube was pressurized with oxygen-free hydrogen and inoculated with *Methanobacterium thermoautotrophicum* and 1 mCi of $NaH^{14}CO_3$ (specific activity, 50 mCi/mmol). To maintain the pH around neutrality despite the fact that no CO_2 was added with the gas phase, this modified medium was buffered with a 20 mM phosphate buffer. Hence, the buffer capacity of the medium was high enough to continuously protonize the bicarbonate to replace the CO_2 converted to methane. After a 3-day incubation at 60°C, all bicarbonate was converted to methane. Then the medium was made alkaline with sodium hydroxide to trap traces of CO_2 left. The headspace of the pressure tube was transferred into a sealed and evacuated 7-ml serum vial, with a 10-ml Pressure Lok gas syringe (Precision Sampling Corp., Baton Rouge, La.). The part of the headspace removed from the tube was simultaneously replaced with water. The 7-ml serum vial was subsequently tipped halfway into liquid nitrogen to freeze out the methane. To allow the rest of the hydrogen to escape, the vial was then opened, sealed again, and removed from the liquid nitrogen. The loss of $^{14}CH_4$ during these manipulations was between 2 and 4%. To trap oxygen, which may have contaminated the radioactive methane during transfer and removal of hydrogen, titanium(III) solution (31) was injected into the 7-ml serum vial.

The "acetate organism" was also successfully used for the production of $^{14}CH_4$ from [2-^{14}C]acetate. In this case no modified medium was necessary and no hydrogen was present. The use of a methanogenic culture to produce $^{14}CH_4$ was first suggested by Lacy Daniels (personal communication).

Radiochemical purity of the biologically synthesized $^{14}CH_4$. The $^{14}CH_4$ obtained from the biological source was tested for contamination by injecting subsamples (around 10 μCi) into rubber septum-sealed scintillation vials containing 2 ml of various solvents. After equilibration, samples of the liquid were analyzed for radioactivity. One vial contained phenethylamine mixed with methanol (1:1) to check for radioactive molecules such as carbon dioxide or methane thiol. Acid-soluble, but otherwise volatile, compounds (e.g., some amines) were trapped in a 1 N H_2SO_4 solution. Methanol and toluene acted as solvents for possible organic compounds not soluble in water. The vials were vigorously shaken; then the seal was removed and the liquid was sparged with air for 5 min to eliminate the methane remaining in the liquid. The liquid phase was then analyzed for radioactivity. To determine the radioactivity of the acid samples, the solution was mixed with Aquasol (New England Nuclear Corp., Boston, Mass.). All other vials received 10 ml of a toluene-based liquid scintillation counting solution containing 0.375 g of 2,5-diphenyloxazole (PPO, Beckman Instruments, Inc., Fullerton, Calif.) and 0.1 g of 1,4-bis-2(4-methyl-5-phenyloxazolyl)benzene (dimethyl-POPOP, Packard Instrument Co., Inc., Downers Grove, Ill.) per 1,000 ml of toluene. The radioactivity was counted with a Tri-Carb 3375 liquid scintillation spectrometer (Packard Instrumen Co., Inc.) with the window set at 4 to 1,000 and the gain at 12%. Quench corrections were made by the channels-ratio method. There was no difference between the background activity and the values obtained from analyses of the different solvents, at the 95% confidence interval.

In addition to the above controls, samples of the radioactive gas were injected into a gas chromatograph-gas proportion counter (17) set at the highest possible sensitivity, so that any possible contaminants

would be seen. We detected no material other than $^{14}CH_4$, within a 30-min time, when we used the column packing and gas chromatograph settings described by Nelson and Zeikus except that the column temperature was 90°C instead of 190°C (17); the well-defined $^{14}CH_4$ peak was seen within 30 s of injection, and no other radioactive signal was obtained.

Gas measurements. CH_4 and CO_2 were quantified with a Poropak QS (100/120 mesh) column in a gas chromatograph (model 419; Packard Instrument Co., Inc.) equipped with a thermal conductivity detector.

Determination of methane oxidation. To examine the amount of methane oxidized by methanogens, we incubated these organisms at 35°C in 35-ml serum vials containing 20 ml of medium and their corresponding substrates; 30 μCi of radioactive methane was injected into the headspace. In the course of the growth, the organism produced methane and therefore continuously diluted the [^{14}C]methane. For each time point the specific activity of methane was determined by the gas chromatographic proportion counter method described by Nelson and Zeikus (17). To analyze for methane oxidation products and methane assimilated, the following fractionation procedure was used for each time point. Two 1-ml liquid samples were taken aseptically with a Pressure Lok syringe. One sample was injected through a rubber septum into a scintillation vial which contained 2 ml of phenethylamine (scintillation grade) and 2 ml of methanol. Subsequently, the scintillation vial was vigorously shaken to absorb all CO_2. The rubber septum was then removed, and the liquid was sparged with air during 5 min by use of a Pasteur pipette to eliminate the methane remaining in the liquid. Then the scintillation vial received 10 ml of a toluene–fluor mixture. To dissolve totally the 1 ml of water in this scintillation mixture, another 5 ml of methanol had to be added. The radioactivity in this vial represented the total amount of free and bound "$^{14}CO_2$" formed by oxidation of $^{14}CH_4$. (Note: No distinction is made between CO_2 (gaseous), CO_2 (aqueous), HCO_3^- and CO_3^{2-} when the symbol "CO_2" is used.)

The second 1-ml sample was acidified with one drop of concentrated HCl. After being sparged with air for 5 min to remove both CO_2 and CH_4, the sample was filtered through a 0.45-μm membrane filter (Gelman Instrument Co., Ann Arbor, Mich.) and washed twice with phosphate buffer. The filters were dried and counted in the toluene-based scintillation cocktail. The counts on the filter were considered to be the assimilated $^{14}CH_4$. The filtrate, which contained the acid-soluble compounds, was mixed with Aquasol.

For the calculation of the total amount of CH_4 oxidized, it was assumed that the organisms do not discriminate between $^{14}CH_4$ and $^{12}CH_4$. This is probably not true, but the exact discriminations and isotopic effects are not yet known. Furthermore, as the specific activity of methane and carbon dioxide is changing continuously, the average specific activity was used for a given time period. The average specific activity was calculated as the arithmetic mean of the values obtained for the two time points which span that period.

Products of methane oxidation. A 1-ml sample from a culture incubated under H_2, CO_2, and $^{14}CH_4$ was transferred with a Pressure Lok syringe into a sealed 7-ml serum vial of which the exact volume was determined. The vial contained enough acidified water (pH 0) so that the headspace volume amounted to 1 ml after injection of the sample. The headspace was analyzed by the gas chromatographic proportion counter method (17). For $^{14}CO_2$ the amount measured by this method after adjusting for the liquid was equal to the difference of counts between the acidified unfiltered sample and the basic sample. Therefore, the $^{14}CO_2$ (aqueous) + $H^{14}CO_3^-$ formed was calculated by subtracting from the basic sample the counts on the filter plus those in the filtrate. These values were then corrected for the amount of gaseous $^{14}CO_2$ in the headspace, taking in account the volume of the headspace, the actual pH, the bicarbonate concentration, and the salinity of the medium (32).

To analyze the acid-soluble compounds, a sparged acidified sample was brought back to pH 6 with 1 M phosphate buffer and the sample applied to a Dowex-1-formate column (14). The eluate was collected in 5-ml portions of which 1 ml was mixed with 10 ml of Aquasol and counted. As a marker, tritium-labeled acetate was added to the sample prior to the application onto the column.

A gas chromatograph (Packard, model 419) with a flame ionization detector was also used for the identification of radioactive products of the anaerobic methane oxidation. The gas chromatograph was equipped with a 1.5-m-long column packed with Poropak QS (100/120 mesh). Three scintillation vials in a row containing 2 ml of phenethylamine, 2 ml of methanol, and 10 ml of the scintillation cocktail were connected to the flame ionization detector (Fig. 1). In the detector all organic compounds are oxidized to CO_2, and the CO_2 can consequently be trapped by the phenethylamine. When a peak started to appear on the recorder, a set of scintillation vials was joined to the detector, and at the end of the peak they were disconnected. For each peak, a new set was applied.

Three vials were used to ensure that no loss of radioactive CO_2 occurred. In fact, about 85% of the counts were recovered in the first vial and the rest were recovered in the second one. The third vial rarely showed radioactivity above the background level. The

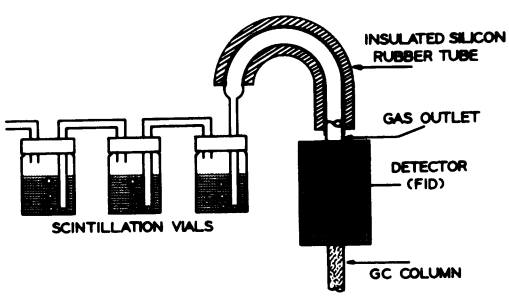

FIG. 1. *Apparatus for the detection of ^{14}C-labeled organic compounds. The compounds were separated in the column of the gas chromatograph (GC), totally oxidized in the flame ionization detector (FID), and subsequently trapped as $^{14}CO_2$ in liquid scintillation fluid containing phenethylamine.*

conditions for a run were chosen so as to get the peaks far enough from each other so that cross-contamination was avoided. Measurements were also taken between the peaks to see if products were formed in concentrations too low for detection with the flame ionization detector.

Determination of the position of labeled carbon in acetate. (i) Chemical method. In the course of the Schmidt degradation (22), acetate is split into methylamine and carbon dioxide by means of azide in sulfuric acid. The methyl group is the precursor of methylamine, and the carbon dioxide is derived from the carboxyl group.

(ii) Biological method. Medium containing acid-soluble radioactivity was neutralized, made anaerobic, and injected through a 0.22-μm Millex filter into a vial containing the acetate organism and organic carbon-free basal medium. During incubation, acetate from the sample is decarboxylated, and methane and carbon dioxide are formed. Methane is exclusively formed from the methyl group of acetate. Carbon dioxide, on the contrary, comes not only from the carboxyl group but a very small amount comes also from the methyl group (A. J. B. Zehnder et al., in preparation).

The biological method is simpler and the yields are nearly 100%. The Schmidt degradation, besides requiring a greater expenditure of time, also gives much lower yields: 50 to 60% when the degradation is performed with medium without a prior extraction of acetate.

Short-term labeling of methanogenic bacteria. Methanogens were precultured in their growth medium. After growth was completed, the cells were harvested as follows. The cultures were injected anaerobically and aseptically into pressure tubes and centrifuged for 30 min at 1,500 × *g*. The supernatant fluid was removed with a syringe, and the pellets were washed with oxygen-free phosphate buffer (pH 7) and centrifuged again. This time the pellet was taken up in a volume of organic carbon-free basal medium sufficient to give about 10^{10} cells per ml. Each of three 10-ml serum vials received 2 ml of concentrated cell suspension. To one vial 2 mCi (50 mCi/mmol) of sodium [^{14}C]bicarbonate was added. The nitrogen headspace contained enough hydrogen to allow the organism to reduce all "CO_2" to methane. This vial was also buffered with triple the amount of phosphate normally present in the basal medium, to prevent a drastic pH change which occurs during the utilization of CO_2 to form methane. The second vial had 10 times more bicarbonate, which was unlabeled, and the corresponding amount of CO_2 in the headspace to keep the pH during the experiment around neutrality. The idea was to provide the system with a large enough sink or buffer for the "$^{14}CO_2$" formed from $^{14}CH_4$. This prevents intermediates from being labeled with "$^{14}CO_2$" which might falsely appear to be labeled from $^{14}CH_4$. The partial pressure of hydrogen was 10 times higher than in the first vial and was brought back to its initial pressure every 2 h. In addition, the headspace contained [^{14}C]methane (50 mCi/mmol). The third vial contained nonradioactive bicarbonate and labeled methane, just as the second, but no hydrogen. The first vial was incubated for 20 min, and the second and third were incubated for 360 min. The incubation was stopped by injecting the cell suspension into an equal amount of ethanol. The cells were extracted by the procedure of Daniels and Zeikus (7).

Thin-layer chromatographic analysis. The extracts from the short-term labeling were analyzed by two-dimensional chromatography with thin-layer electrophoresis as the first and thin-layer chromatography as the second dimension, as described by Daniels and Zeikus (7).

Chemicals, gases, and radioisotopes used. 2-Bromoethane sulfonic acid sodium salt was obtained from Eastman Kodak Co., Rochester, N.Y. All chemcials were of reagent grade. Gases and gas mixtures were purchased from Matheson Gas Products, Joliet, Ill., in anaerobe purity, which means an oxygen content of less than 5 ppm. The following radiochemicals were obtained from New England Nuclear Corp.: sodium [^{14}C]bicarbonate (50 mCi/mmol) and sodium [2-^{14}C]acetate (2 mCi/mmol). Sodium [^{3}H]acetate (2.6 Ci/mmol) was purchased from Amersham Searle, Arlington Heights, Ill.

RESULTS

Methane formation, growth, and methane oxidation by methane bacteria. All methanogenic bacteria tested were able to oxidize methane to a certain extent (Table 1) while simultaneously growing and producing methane. The most common end product was carbon dioxide, but methanol and acetate were also formed (*Methanosarcina barkeri* and the "acetate organism"). Besides these three identified products some other acid-soluble compounds carrying label from methane were released into the medium. Compared with the major products, only a minor amount of methane found its way into those molecules which could be, at least partially, amino acids. This assumption is based on the observation that *Methanobacterium* strain AZ excretes amino acids during growth (32). The biomass also contained carbon from methane, and the relatively high portion of radioactivity suggests a direct assimilation. If exclusively "CO_2" were incorporated, and therefore also only the formed "$^{14}CO_2$" were used for biosynthesis, the label would have been considerably diluted and its part should be orders of magnitude smaller in the biomass than actually measured. *M. ruminantium* and the slow-growing *Methanobacterium* strain M.o.H. were relatively less efficient methane oxidizers. *M. formicicum, M. arbophilicum,* and *Methanobacterium* strain AZ oxidized methane fairly well, but were far from the oxidation capability of *M. thermoautotrophicum, Methanosarcina barkeri,* and *Methanospirillum hungatii.* In the case of the organisms able to use either H_2 and CO_2 or formate, the species-specific ratio between methane formed and oxidized was not affected whether they were cultivated on one

TABLE 1. *Methane formation, growth, and the products of methane oxidation from various methane bacteria*

Organism and substrate	CH_4 formed (μmol)[a]	Yield[b]	CH_4 oxidized (nmol)[a, c]	"CO_2" (%)[d]	Methanol (%)[d]	Acetate (%)[d]	Acid-soluble (%)[d, e]	Biomass (%)[d]	CH_4 oxidized/CH_4 formed (%)
Methanobacterium ruminantium									
$H_2 + CO_2$	700.9	7.84×10^6	68.6	80.2			9.7	10.1	0.001
Formate	383.9	7.59×10^6	34.2	48.3			42.1	9.6	0.001
Methanobacterium M.o.H.									
$H_2 + CO_2$	525.0	7.09×10^6	79.1	86.3			11.7	1.9	0.0015
M. formicicum									
$H_2 + CO_2$	700.9	7.62×10^{6f}	658.4	92.8			2.1	5.2	0.094
Formate	549.1	7.35×10^{6f}	549.8	90.7			6.5	2.8	0.1
M. thermoautotrophicum									
$H_2 + CO_2$	971.0	6.80×10^6	2,827.6	96.0			2.3	1.8	0.29
M. arbophilicum									
$H_2 + CO_2$	725.9	8.03×10^6	331.5	83.0			4.6	12.4	0.046
Methanobacterium AZ									
$H_2 + CO_2$	725.9	8.65×10^6	390.9	90.7			3.3	6.0	0.054
Methanosarcina barkeri									
$H_2 + CO_2$	581.3	—[f]	1,293.3	81.8			14.5	3.6	0.22
Methanol	1,205.4	—[f]	3,900.0	79.8	6.4	2.4	1.5	9.9	0.32
Acetate	200.9	—[f]	400.8	71.0			28.7	0.3	0.20
Methanospirillum hungatii									
$H_2 + CO_2$	760.7	7.59×10^6	1,780.7	99.0			0.5	0.4	0.23
Formate	586.5	7.15×10^6	896.0	97.7			2.0	0.3	0.15
"Acetate organism"									
Acetate	568.0	—[g]	795.0	62.5		35.0	0.0	3.1	0.14

[a] By a 20-ml culture in 4 days.

[b] Expressed as the number of cells formed per micromole of methane produced.

[c] We assumed arbitrarily that there is no discrimination between [^{12}C]- and [^{14}C]methane.

[d] As a percentage of the total amount of CH_4 oxidized.

[e] Radioactivity remaining in solution after acidification (pH < 2), degassing, and filtering through a 0.45-μm membrane filter.

[f] *M. formicicum* forms clumps, but 0.5 h of intensive shaking breaks the agglomerations into very small pieces which allow cell counting. The clumps of *M. barkeri*, however, are very resistant, and a meaningful count of the cells is therefore not possible.

[g] The acetate organism forms very long filaments which do not allow a satisfactory cell count. Moreover, 4 days of incubation is not long enough for significant growth of this organism because of its long generation time ($t_d \sim 9$ days).

substrate or the other. However, *M. barkeri* clearly oxidized more methane when grown on methanol than on H_2 and CO_2 or acetate. As described below the "acetate organism" reutilized its synthesized acetate. Therefore, with increasing incubation time more "$^{14}CO_2$" was recovered than acetate. The values for "CO_2" and acetate in Table 1 represent a time point where acetate had already become limiting (see Fig. 3b). After 4 days of growth in a sealed serum vial, there was the same kind of limiting conditions for the other methane bacteria and their substrates, except methanol. That the oxidation observed was strictly anaerobic is shown by the fact that active methane formation also occurred simultaneously, and the indicator resazurin remained reduced throughout the experiment.

Control experiments. An ideal control would be a culture medium inoculated with an inactive microorganism. The inactivation of microorganisms is commonly done by the addition of chemicals such as formaldehyde or mercury chloride. These additives, however, modify the chemical composition of the medium, and the control is thus not strictly a real control. Another approach is the heat sterilization of the inoculated medium, but such an extreme treatment changes the characteristics of the organisms. Proteins are denatured, cell walls are destroyed, and the organization of the cell is lost. Moreover, bicarbonate decomposes at temperatures above 100°C rather rapidly, causing a shift to higher pH ranges which will consequently influence the solubility of organic and inorganic compounds.

Based on these considerations, we decided to run at least two different controls for each experiment. One control vial, incubated at 35°C, contained medium but no organisms. Methane oxidation in this vial would be due exclusively to a chemical or physical process. The second control vial contained the organisms and was incubated at 70°C in the case of mesophilic organisms and at 90°C with *M. thermoautotrophicum*. This more gentle heat treatment represents a compromise and is intended to show any nonmetabolic effects by only the presence

of the microbes. The uninoculated vials and those incubated at higher temperatures never showed labeled compounds other than methane (Table 2). To examine further whether the observed methane oxidation might be an artifact, *M. hungatii* was grown in vials containing 2 ml of methane of increasing specific radioactivity. Hydrogen and carbon dioxide acted as growth substrates. The results (Table 3) show that the measured radioactivity in the carbon dioxide fraction paralleled the radioactivity in methane. However, the total amount of carbon dioxide, calculated from the mean specific activity of methane and the $^{14}CO_2$ formed, remained relatively constant. Between 0.2 and 0.23% of the methane formed was reoxidized to carbon dioxide. Together with the other controls just described, this experiment clearly shows that the anaerobic oxidation of methane was actively carried out by the growing culture of *M. hungatii*.

Acetate and methanol as products of anaerobic methane oxidation. During the growth on basal medium with $^{14}CH_4$ added to the headspace, the "acetate organism" produced labeled carbon dioxide and labeled acid-soluble compounds. The analysis of the medium by use of a Dowex-1-formate column (Fig. 2) showed very clearly that all ^{14}C activity was in one peak, which coincided with the marker peak of tritium-labeled acetate. The gas chromatographic test (Fig. 1) confirmed that the acetate organism was able to form acetate from methane. The

TABLE 2. *Methane formation and oxidation by a growing culture of Methanospirillum hungatii compared to the control experiments*

Conditions	$^{14}CH_4$ added (μCi)	CH_4 formed (ml)	$^{14}CO_2$ formed (dpm)
Medium inoculated and incubated at 35°C	21.3	15.421	206,400
Medium *not* inoculated but incubated at 35°C	19.3	None	25
Medium inoculated and incubated at 70°C	20.4	0.01	136

TABLE 3. *Methane formation and oxidation by Methanospirillum hungatii with hydrogen and carbon dioxide as substrates*[a]

$^{14}CH_4$ added (μCi/mmol)	CH_4 formed (mmol)	Avg sp act of the $^{14}CH_4$ (μCi/mmol)	$^{14}CO_2$ formed (μCi)	CH_4 oxidized (μmol)	CH_4 oxidized/CH_4 formed (%)
10.41	0.732	2.04	0.0031	1.491	0.20
127.63	0.682	27.14	0.041	1.500	0.22
1,155.70	0.704	234.01	0.373	1.593	0.23

[a] At the start, 2-ml amounts of methane of different specific activities were added to the corresponding vials.

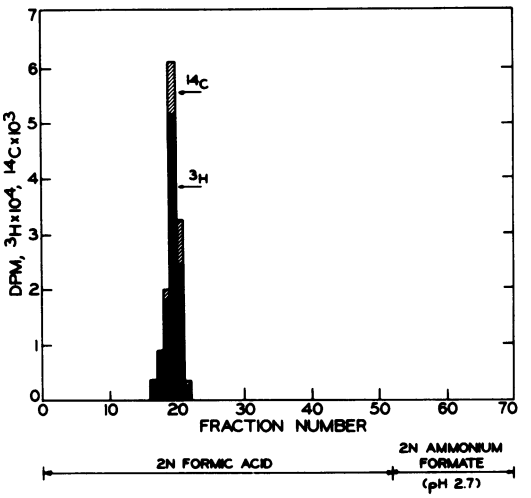

FIG. 2. *Distribution of the acid-soluble ^{14}C radioactivity in the medium on which the "acetate organism" was grown under an atmosphere containing $^{14}CH_4$. This analysis was done with a Dowex-1-formate column and [^{3}H]acetate served as marker.*

recovery of radioactivity was 95% and 93% for the Dowex column and the gas chromatography, respectively. The Schmidt degradation and the injection of the radioactive medium into a culture of actively growing unlabeled acetate organism showed that methane is the precursor of the methyl group in the synthesized acetate (Table 4). In the biological test, a small amount of radioactivity was recovered in the carbon dioxide fraction. The reasons are that: (i) the "acetate organism" oxidizes methane also to carbon dioxide which may be utilized for the formation of the carboxyl group of acetate and (ii) the acetate organism needs electrons for its biomass production. Therefore, the organism has to oxidize some acetate by producing carbon dioxide from the methyl group in order to fulfill its requirements (no oxidation of inorganic compounds such as sulfide or iron was observed). This second point is responsible for the "CO_2" formation in the biological acetate degradation and accounts for the labeled "CO_2" found in the control. The recovery of labeled carbon dioxide from the acid-soluble fraction is thus a consequence of processes one and two.

Despite the literature specification, some methylamine was converted to CO_2 during the Schmidt degradation. That is why the $^{14}CO_2$ fraction was rather high in the chemical tests. When the results for the unknown are compared with the control (added [2-^{14}C]acetate), it seems reasonable to conclude that labeled methane goes virtually completely into the methyl group of acetate.

M. barkeri also formed both carbon dioxide

and acid-soluble compounds. The analysis of the medium demonstrated that this organism was able to form methanol and acetate from methane (Table 1). This acetate also was predominantly labeled in the methyl position.

Time course for methane oxidation by the acetate organism, *M. barkeri*, and *M. hungatii*. While producing methane from acetate, the "acetate organism" reoxidized up to approximately 0.5% of the formed methane to either "CO_2" or acetate. Figure 3a represents a typical time course of methane formation and

TABLE 4. *Determination of the label position in acetate with the "acetate organism" (acetate decarboxylated) in the biological method and the Schmidt degradation, a chemical method*

Conditions	dpm in the fractions of the sample[a]				dpm in the fractions of the control			
	CH_3COOH	CO_2	CH_4	CH_3NH_2	CH_3COOH[b]	CO_2	CH_4	CH_3NH_2
Before degradation								
Biological method	71,799				40,870			
	(100)[c]				(100)			
Schmidt degradation	35,200				40,867			
	(100)				(100)			
After degradation								
Biological method	650	2,998	68,335		2,380	200	37,260	
	(0.9)	(4.2)	(95.2)		(5.8)	(0.5)	(91.2)	
Schmidt degradation	12,548	5,182		18,300	19,702	2,150		21,450
	(35.6)	(14.7)		(52)	(48.2)	(5.3)		(52.5)

[a] A 10-ml sample (5 ml for Schmidt degradation) of the medium in which the acetate organism was grown on unlabeled acetate and under a nitrogen-carbon dioxide atmosphere containing [^{14}C]methane.

[b] [2-^{14}C]acetate.

[c] Values in parentheses represent the percentage.

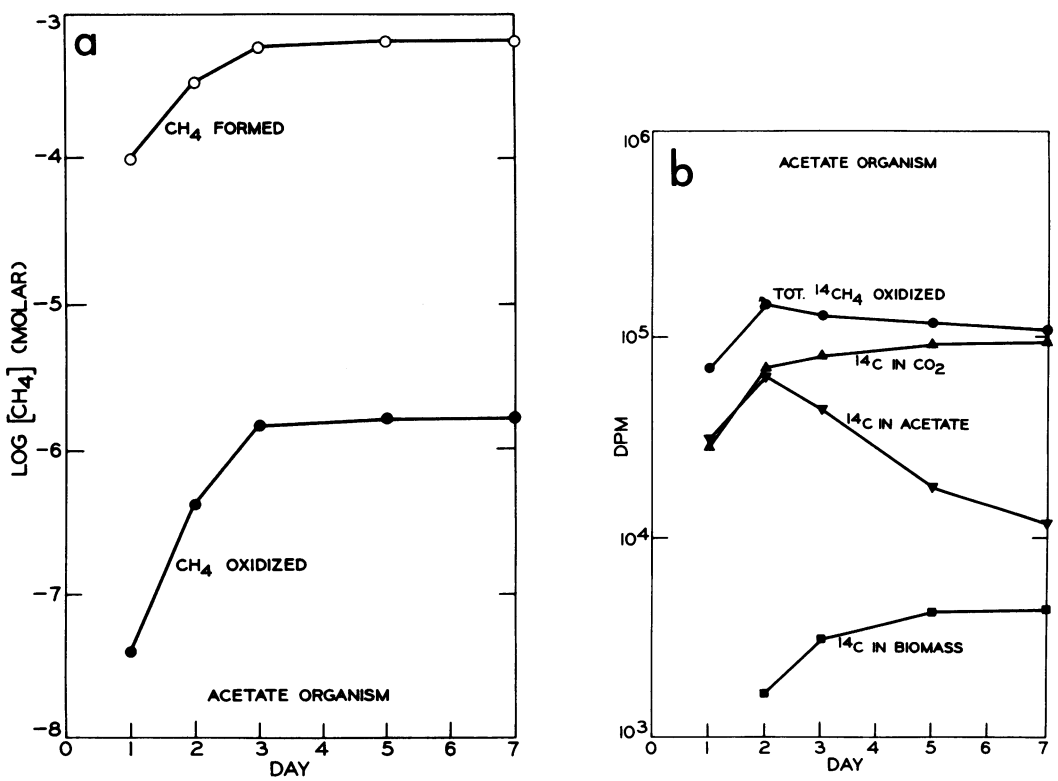

FIG. 3. *(a) Time course of methane formation and oxidation by the "acetate organism." (b) Time course of the occurrence of the different oxidation products.*

methane oxidation during growth in a serum vial. In Fig. 3b the oxidation products are individually shown. Up to 2 days of incubation, carbon dioxide and acetate were formed in relatively equal mounts. Then the nonlabeled acetate, added as substrate, started to become limiting, and the synthesized acetate was reutilized for methane formation although carbon dioxide was still produced at a very reduced rate. The incorporation rate of ^{14}C into the biomass does not represent growth since the acetate organism has a doubling time of approximately 9 days.

Figure 4a and 4b give the time courses for *M. barkeri* grown on 5 g of methanol per liter. In our medium, *M. barkeri* stopped growing after having used up half of the substrate. A possible pH effect on the growth can be excluded because the bicarbonate buffer in the medium was strong enough to outbalance the additional CO_2 input from the disproportionation of methanol during methane formation (33). Yeast extract is stimulatory but is not necessary for growth (P. J. Weimer and J. G. Zeikus, Arch. Microbiol., in press), and its depletion therefore should not account for growth cessation. One possible explanation is that *M. barkeri* stopped growing because of an ammonium limitation, if it was unable to assimilate any of the nitrogen-containing compounds of yeast extract. The calculation of ammonia limitation is based on a molar growth yield of Y_{CH_4} of 7.2 g/mol (Weimer and Zeikus, in press) and a cell nitrogen content of 12%. Because methanol was always present in a considerable amount, hence providing a large pool for the dilution of the specific activity of the methanol formed from methane, no substantial decrease in radioactivity in methanol could be detected, due to a reutilization of the synthesized methanol. The same is also true for acetate, since the yeast extract we used contains approximately 0.5 mmol of acetate per g. In the case of *M. barkeri*, carbon dioxide played a much more important role as oxidation product because 10 times more methane was converted to carbon dioxide than to acetate and methanol. In Fig. 5, the relationship between cell growth, methane formation, and methane oxidation are shown for a time course with *M. hungatii*. The data show that under our experimental conditions the increase of methane oxidized paralleled the increase of methane formation and growth and further support the findings that methane bacteria oxidize methane at the same time they form it.

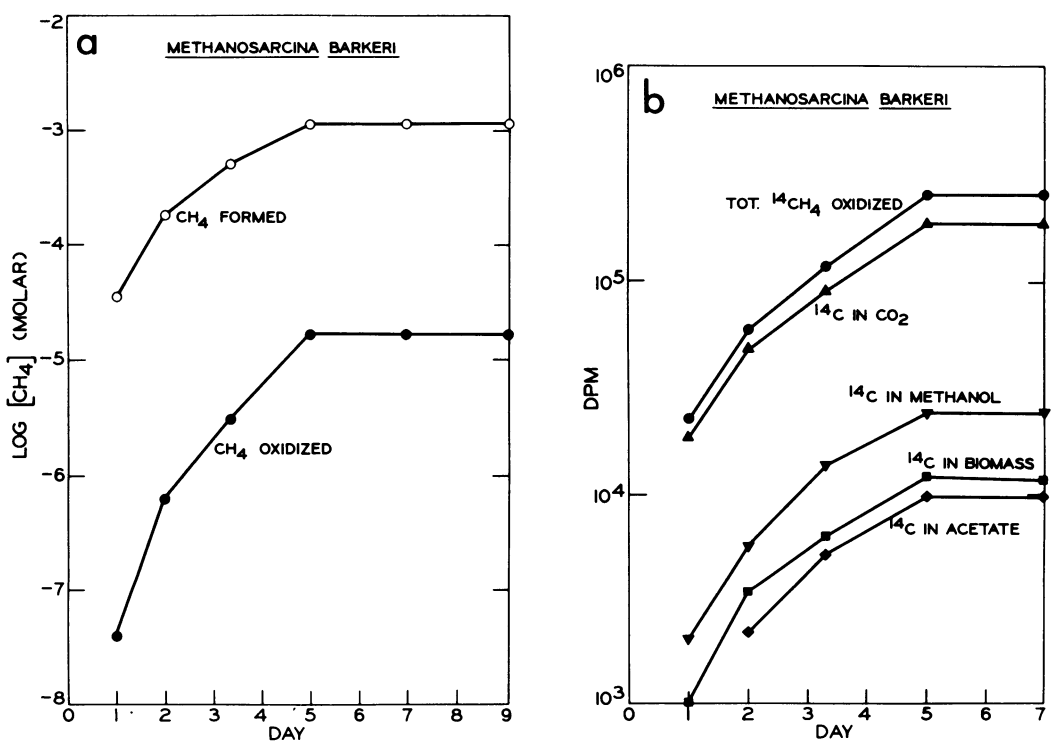

FIG. 4. *(a) Time course of methane formation and oxidation by Methanosarcina barkeri. (b) Time course of the occurrence of the different oxidation products.*

Inhibition of methane oxidation by 2-bromoethanesulfonic acid. The formation of methane in methanogenic bacteria is specifically inhibited by 2-bromoethanesulfonic acid, a coenzyme M analog (11). This inhibitor also has a negative effect on methane oxidation. Moreover, both processes are identically affected. As an example, Fig. 6 represents the concentration-inhibition diagram for the acetate organism. The values for the inhibition were obtained from consecutive measurements over a 5-day incubation period.

Assimilation products of *M. thermoautotrophicum* and *M. hungatii* labeled with "$^{14}CO_2$" and $^{14}CH_4$. Methane bacteria are able to assimilate CH_4 to a certain extent (Table 1). Table 5 shows the amount of radioactive methane formed and oxidized and the incorporated label in a short-term labeling experiment with *M. thermoautotrophicum* and *M. hungatii*. Because of the significant amount of ^{14}C incorporated into biomass, it is possible to examine whether the same intermediates are involved in "CO_2" and methane fixation. We chose these two organisms for study of methane assimilation for the following reasons. Both organisms are good methane oxidizers. They excrete only minor amounts of acid-soluble radioactivity, and their oxidation end product is only "CO_2" (Table 1). In addition, they represent a mesophilic and a thermophilic methane producer. Moreover,

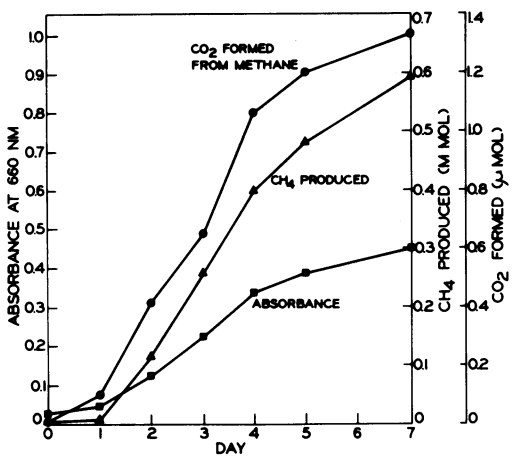

FIG. 5. *Time course of growth, methane formation, and methane oxidation by Methanospirillum hungatii. The total amount of "CO_2" formed was calculated for each time point from the mean specific activity of methane and from the "$^{14}CO_2$" formed. The corresponding activities for "$^{14}CO_2$" are (in dpm): 69,032 (day 1), 79,021 (day 2), 86,713 (day 3), 89,623 (day 4), 91,082 (day 5), and 93,548 (day 7).*

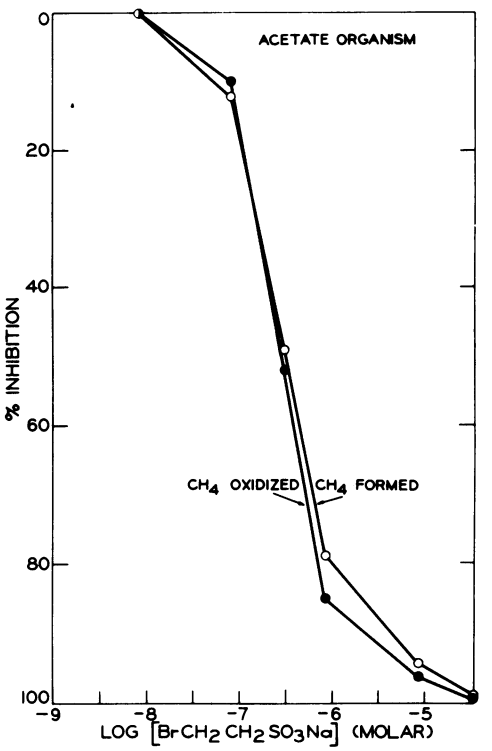

FIG. 6. *Effect of 2-bromoethanesulfonic acid on methane formation and oxidation by the "acetate organism."*

TABLE 5. *Formation and oxidation of methane and incorporation of the labeled substrate into biomass in a short-term labeling experiment*

Organism	Vial[a]	Addition	$^{14}CH_4$ formed (μCi)	"$^{14}CO_2$" formed (μCi)	Added label incorporated into biomass (μCi) ($\times 10^{-3}$)
Methanobacterium thermoautotrophicum	A	"$^{14}CO_2$," 2,000 μCi	29.13		53.24
	B	$^{14}CH_4$, 90.54 μCi		10.59	28.13
	C	$^{14}CH_4$, 81.62 μCi		4.35	16.0
Methanospirillum hungatii	A	"$^{14}CO_2$," 2,000 μCi	16.34		29.28
	B	$^{14}CH_4$, 35.45 μCi		2.48	6.89
	C	$^{14}CH_4$, 33.21 μCi		0.98	3.24

[a] Each vial contained 2×10^{10} cells. (A) Vial containing $^{14}CO_2 + H_2$; incubation time, 20 min. (B) Vial containing $CO_2 + H_2 + {}^{14}CH_4$; incubation time, 360 min. (C) Vial containing $CO_2 + {}^{14}CH_4$; incubation time, 360 min.

the labeled intermediates of "CO_2" assimilation in *M. thermoautotrophicum* have been well characterized by Daniels and Zeikus (7). *M. thermoautotrophicum* and *M. hungatii* formed similar radioactive products when labeled with "$^{14}CO_2$" (Fig. 7). Figure 7 shows very clearly that no methyl-coenzyme M was formed from methane. In contrast to "CO_2," only a few very distinct compounds were labeled and remained stationary during thin-layer electrophoresis, suggesting that they are electrically neutral at pH 4. These compounds labeled by methane did not fluoresce or react with ninhydrin. Further, they appeared only very weakly, if at all, on the "CO_2"-labeled plate. There was no obvious, qualitative difference in the autoradiograms of the extracts whether the cells were incubated with methane or with methane and hydrogen.

DISCUSSION

Methanogenic bacteria were found to be able to oxidize methane to a certain extent while simultaneously producing methane. The major oxidation product was carbon dioxide, but in addition the "acetate organism" formed acetate and *M. barkeri* formed acetate and methanol. Interestingly, the only microbes able to form acetate and methanol were those which could use these compounds as methane precursor.

It has to be emphasized that our experiments never showed a net methane oxidation. Under

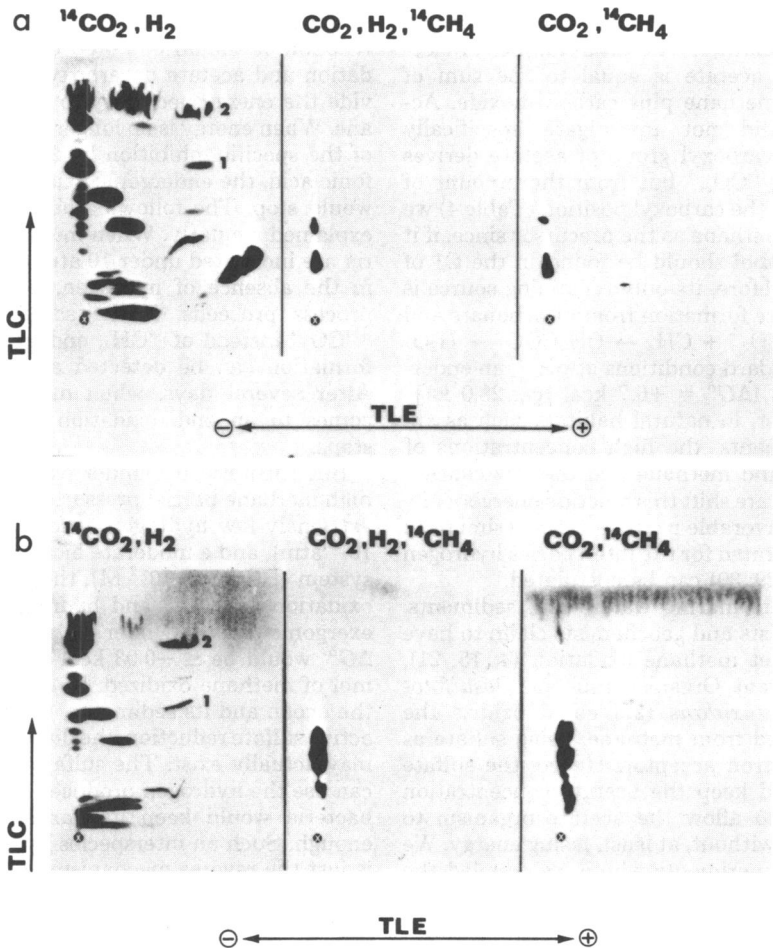

FIG. 7. *Autoradiograms showing the position of the labeled intermediates formed by Methanobacterium thermoautotrophicum (a) and Methanospirillum hungatii (b) from "$^{14}CO_2$" or $^{14}CH_4$. The respective electrophoretic poles are indicated as + or −, and ⊕ refers to the origin. TLC, thin-layer chromatography; TLE, thin-layer electrophoresis. The numbers refer to the following compounds: 1, methylcoenzyme M (CH_3CoM); 2, CoM derivative. For identification of the other spots, see Daniels and Zeikus (7).*

the laboratory conditions employed, the ratio of methane oxidized to methane formed barely exceeded 0.3% (*M. barkeri* with methanol as substrate); it could even be as low as 0.001% (*M. ruminantium*). Despite the small extent of methane oxidation, the results show clearly that methane is not completely inert anaerobically and that some biochemical mechanism for anaerobic methane oxidation must exist. It is of further interest that a group of microorganisms is able to use in some way one of the end products of their own energy metabolism.

Anaerobic oxidation processes always raise the question of what are the electron acceptors and the free energy changes in these reactions. The simplest product of methane oxidation is acetate, and its formation from methane and carbon dioxide requires no redox reaction in which electrons have to be transferred from one molecule to another. The total number of electrons in the acetate is equal to the sum of electrons in methane plus carbon dioxide. Actually, we did not investigate specifically whether the carboxyl group of acetate derives directly from "CO_2," but from the amount of label found in the carboxyl position (Table 4) we can exclude methane as the precursor since, if it were, more label should be found in the C1 of acetate. Therefore, its only remaining source is "CO_2." Acetate formation from bicarbonate and methane ($HCO_3^- + CH_4 \rightarrow CH_3COO^- + H_2O$) is, under standard conditions at pH 7, an endergonic process ($\Delta G^\circ = +6.7$ kcal [ca. 28.0 kJ]/mol). However, in natural habitats such as anaerobic sediments, the high concentrations of bicarbonate and methane and the low concentration of acetate shift this reaction energetically into a more favorable range; a process similar to that demonstrated for the interspecies hydrogen transfer (20, 29, 30) can be postulated.

In anaerobic marine waters and sediments, where geologists and geochemists claim to have observed a net methane oxidation (4, 15, 21), sulfate is present. Organisms such as *Desulfotomaculum acetoxidans* (27) could oxidize the acetate formed from methane, using sulfate as terminal electron acceptor. Hence, the sulfate reducer would keep the acetate concentration low enough to allow the acetate organism to form acetate without, at least, losing energy. We did several experiments where we coupled the acetate organism with *D. acetoxidans* in a medium high in "CO_2" and under 10 atm of methane, but a clear unquestionable net oxidation could never be demonstrated. This does not mean that the above process does not take place, but rather that our conditions might not imitate completely the natural environment.

Most of our observations indicate that methane oxidation is not simply a back or exchange reaction which takes place in the course of methane formation. Since the "acetate organism" produces both acetate and carbon dioxide, but is not able to convert carbon dioxide to methane (Zehnder et al., in preparation), its conversion of methane to CO_2 cannot be a simple back reaction. Also, studies with hydrogen-oxidizing methanogens (Fig. 7) strongly suggest that methane oxidation is clearly separated from the methane production site. In the case of a back reaction, hydrogen together with unlabeled carbon dioxide should prevent, to a certain extent, the labeling of the intermediates with methane. No such effect has been observed (Fig. 7). On the contrary, the presence of hydrogen rather stimulated the overall methane oxidation.

Although the inhibition experiment with 2-bromoethanesulfonic acid might suggest a back reaction, it should be noted that hydrogen oxidation and acetate decarboxylation might provide the energy necessary to oxidize the methane. When energy is no longer produced because of the specific inhibition by 2-bromoethanesulfonic acid, the endergonic oxidation of methane would stop. The following observation may be explained similarly. When methanogenic bacteria are incubated under 10 atm of methane, but in the absence of hydrogen, a slow oxidation process proceeds. If the same vials contain "$^{14}CO_2$" instead of $^{14}CH_4$, endogenous methane formation can be detected at the same time. After several days, when methane formation comes to an end, oxidation of methane also stops.

But theoretically, under extreme conditions, high methane partial pressure ($p_{CH_4} \geq 100$ atm), extremely low hydrogen concentrations ($p_{H_2} \leq 10^{-5}$ atm), and a moderate bicarbonate-buffered system ($HCO_3^- \sim 10^{-2}$ M), the overall methane oxidation to "CO_2" and hydrogen becomes an exergonic process. Under the above assumption, $\Delta G^{\circ\prime}$ would be ≤ -0.03 kcal (ca. 0.125 kJ) per mol of methane oxidized. In the deeper part of the ocean and its sediments, in the presence of active sulfate reduction, the discussed conditions may actually exist. The sulfate reducers which can use the hydrogen produced by the methane bacteria would keep its partial pressure low enough. Such an interspecies hydrogen transfer is just the reverse mechanism of that described by Bryant et al. (5) where the sulfate reducer forms hydrogen in the absence of sulfate and the methanogens play the role of an electron acceptor.

The short-term labeling experiment is in accordance with our observations with whole cells (Table 1) that methane can be directly assimilated. A detour via carbon dioxide formation

with its subsequent assimilation seems not to be responsible for the radioactivity incorporated into the biomass of methane bacteria.

Our experiments demonstrate that methane oxidation cannot be followed with tracers in pure culture without simultaneously observing methane formation. This is also true for studies in sewage sludge and lake sediments which indicate that methane bacteria are indeed involved in the anaerobic methane oxidation process (Zehnder and Brock, in preparation). A discussion of the oxidation rates calculated by geologists and geochemists from diffusion parameters is inappropriate at this stage, as we do not know for sure whether one group or a consortium of different groups of bacteria is able, probably only under certain stress conditions, to carry out a net anaerobic methane oxidation.

ACKNOWLEDGMENTS

Financial support was provided by the Department of Energy (contract EY-76-S-2-2161), the College of Agricultural and Life Sciences, University of Wisconsin, and the Wisconsin Alumni Research Foundation. A.J.B.Z. was supported in part by a postdoctoral fellowship from the Swiss National Foundation for Scientific Research.

We thank J. G. Zeikus and L. Daniels for valuable discussion and technical assistance. We also thank M. P. Bryant, B. Huser, R. S. Wolfe, K. Wuhrmann, and J. G. Zeikus for providing pure cultures of methane bacteria.

LITERATURE CITED

1. **Anthony, C.** 1975. The biochemistry of methylotrophic micro-organisms. Sci. Prog. (London) **62**:167–206.
2. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. **32**:781–791.
3. **Barker, H. A.** 1936. Studies upon the methane producing bacteria. Arch. Microbiol. **7**:420–438.
4. **Barnes, R. O., and E. D. Goldberg.** 1976. Methane production and consumption in anoxic marine sediments. Geology **4**:297–300.
5. **Bryant, M. P., L. L. Campbell, C. A. Reddy, and M. R. Crabill.** 1977. Growth of Desulfovibrio in lactate or ethanol media low in sulfate in association with H_2-utilizing methanogenic bacteria. Appl. Environ. Microbiol. **33**:1162–1169.
6. **Bryant, M. P., E. A. Wolin, M. J. Wolin, and R. S. Wolfe.** 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. Arch. Mikrobiol. **59**:20–31.
7. **Daniels, L., and J. G. Zeikus.** 1978. One-carbon metabolism in methanogens: analysis of short-term fixation products of $^{14}CO_2$ and $^{14}CH_3OH$ incorporated into whole cells. J. Bacteriol. **136**:75–84.
8. **Davis, J. B., and H. F. Yarbrough.** 1966. Anaerobic oxidation of hydrocarbons by *Desulfovibrio desulfuricans*. Chem. Geol. **1**:137–144.
9. **Ferry, J. G., P. H. Smith, and R. S. Wolfe.** 1974. *Methanospirillum*, a new genus of methanogenic bacteria and characterization of *Methanospirillum hungatii* sp. nov. Int. J. Syst. Bacteriol. **24**:465–469.
10. **Ferry, J. G., and R. S. Wolfe.** 1976. Anaerobic degradation of benzoate to methane by a microbial consortium. Arch. Microbiol. **107**:33–40.
11. **Gunsalus, R., D. Eirich, J. Romesser, W. Balch, S. Shapiro, and R. S. Wolfe.** 1976. Methyl transfer and methane formation, p. 191–197. *In* H. G. Schlegel, G. Gottschalk, and N. Pfennig (ed.), Microbial production and utilization of gases (H_2, CH_4, CO). E. Goltze, Goettingen.
12. **Higgins, I. J., and J. R. Quayle.** 1970. Oxygenation of methane by methane-grown *Pseudomonas methanica* and *Methanomonas methanooxidans*. Biochem. J. **118**: 201–208.
13. **Kluyver, A. J., and C. G. T. P. Schnellen.** 1947. Fermentation of carbon monoxide by pure cultures of methane bacteria. Arch. Biochem. **14**:57–70.
14. **LaNoue, K., W. J. Nicklas, and J. R. Williamson.** 1970. Control of citric acid cycle activity in rat heart mitochondria. J. Biol. Chem. **245**:102–111.
15. **Martens, C. S., and R. A. Berner.** 1977. Interstitial water chemistry of anoxic Long Island Sound sediments. I. Dissolved gases. Limnol. Oceanogr. **22**:10–25.
16. **Mylroie, R. L., and R. E. Hungate.** 1954. Experiments on the methane bacteria in sludge. Can. J. Microbiol. **1**: 55–64.
17. **Nelson, D. R., and J. G. Zeikus.** 1974. Rapid method for radioisotopic analysis of gaseous end products of anaerobic metabolism. Appl. Microbiol. **28**:258–261.
18. **Pretorius, W. A.** 1972. The effect of formate on the growth of acetate utilizing methanogenic bacteria. Water Res. **6**:1213–1217.
19. **Quayle, J. R.** 1972. The metabolism of one-carbon compounds by microorganisms. Adv. Microb. Physiol. **7**: 119–203.
20. **Reddy, C. A., M. P. Bryant, and M. J. Wolin.** 1972. Characteristics of S organism isolated from *Methanobacillus omelianskii*. J. Bacteriol. **109**:539–545.
21. **Reeburgh, W. S.** 1976. Methane consumption in Cariaco Trench waters and sediments. Earth Planet. Sci. Lett. **15**:334–337.
22. **Sakami, W.** 1955. Handbook of isotope tracers methods. School of Medicine, Western Reserve University, Cleveland.
23. **Smith, P. H., and R. E. Hungate.** 1958. Isolation and characterization of *Methanobacterium ruminantium* n. sp. J. Bacteriol. **75**:713–718.
24. **Sorokin, Y. I.** 1957. Ability of sulfate reducing bacteria to utilize methane for reduction of sulfate to hydrogen sulfide. Dokl. Akad. Nauk SSSR **115**:816–818.
25. **van den Berg, L., G. B. Patel, D. S. Clark, and C. P. Lentz.** 1976. Factors affecting rate of methane formation from acetic acid by enriched methanogenic cultures. Can. J. Microbiol. **22**:1312–1319.
26. **Wertlieb, D., and W. Vishniac.** 1967. Methane utilization by a strain of *Rhodopseudomonas gelatinosa*. J. Bacteriol. **93**:1722–1724.
27. **Widdel, F., and N. Pfennig.** 1977. A new anaerobic sporing acetate-oxidizing, sulfate-reducing bacterium: *Desulfotomaculum* (emend.) *acetoxidans*. Arch. Microbiol. **112**:119–122.
28. **Wolin, E. A., M. J. Wolin, and R. S. Wolfe.** 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238**:2882–2886.
29. **Wolin, M. J.** 1976. Interactions between H_2-producing and methane-producing species, p. 141–150. *In* H. G. Schlegel, G. Gottschalk, and N. Pfennig (ed.), Microbial production and utilization of gases (H_2, CH_4, CO). E. Goltze, Goettingen.
30. **Zehnder, A. J. B.** 1978. Ecology of methane formation, p. 349–376. *In* R. Mitchell (ed.), Water pollution microbiology, vol. 2. John Wiley & Sons, Inc., New York.
31. **Zehnder, A. J. B., and K. Wuhrmann.** 1976. Titanium (III) citrate as a nontoxic oxidation-reduction buffering system for the culture of obligate anaerobes. Science **194**:1165–1166.
32. **Zehnder, A. J. B., and K. Wuhrmann.** 1977. Physiology of a *Methanobacterium* strain AZ. Arch. Microbiol.

111:199–205.
33. **Zeikus, J. G.** 1977. The biology of methanogenic bacteria. Bacteriol. Rev. **41**:514–541.
34. **Zeikus, J. G., and D. L. Henning.** 1975. *Methanobacterium arbophilicum* sp. n., an obligate anaerobe isolated from wetwood in trees. Antonie von Leeuwenhoek J. Microbiol. Serol. **41**:543–552.
35. **Zeikus, J. G., and R. S. Wolfe.** 1972. *Methanobacterium thermoautotrophicum* sp. n., an anaerobic, autotrophic, extreme thermophile. J. Bacteriol. **109**:707–713.