JOURNAL OF BACTERIOLOGY, Mar. 1968, p. 1118-1123

Vol. 95, No. 3
Printed in U.S.A.

Hydrogen-oxidizing Methane Bacteria

I. Cultivation and Methanogenesis

M. P. BRYANT, B. C. McBRIDE, AND R. S. WOLFE

Departments of Microbiology and Dairy Science, University Of Illinois, Urbana, Illinois 61801

Received for publication 29 December 1967

A method for the mass culture of hydrogen-oxidizing methane bacteria has been developed; yields of 50 to 60 g (wet weight) of cells per 12 to 14 liters of culture medium were obtained. The methanogenic organism from the culture of *Methanobacillus omelianskii* was grown in a complex medium which was aerated with a gas mixture of hydrogen and carbon dioxide. Extracts prepared from hydrogen-grown cells formed methane from methyl cobalamin, 5-methyl tetrahydrofolate, serine, pyruvate, and carbon dioxide; these substrates have been shown to be precursors of methane in extracts of the ethyl alcohol-grown culture of *M. omelianskii*.

The original culture of *Methanobacillus omelianskii* (*Methanobacterium omelianskii*) (1, 2) has been resolved recently (3*a*) into two distinct organisms, a methanogenic organism (*Methanobacterium* strain M.o.H.) which oxidizes hydrogen and reduces CO_2 to CH_4, and an organism which oxidizes ethyl alcohol to acetic acid with the formation of hydrogen (S organism). With the discovery that strain M.o.H. actually oxidized hydrogen rather than ethyl alcohol, studies on the utilization of hydrogen by this organism (3*a*), as well as by another isolate, *Methanobacterium* strain B.B., were undertaken. We present in this communication a technology for the mass culture of anaerobic bacteria which oxidize H_2 and reduce CO_2 to CH_4, as well as evidence that the same substrates which serve as precursors of CH_4 in extracts of the original ethyl alcohol-grown culture of *M. omelianskii* (6, 7, 8) also are utilized by extracts of hydrogen-grown cells.

MATERIALS AND METHODS

Organisms and culture techniques. The technique described by Hungate (4), as modified by Bryant and Robinson (3), was used in the isolation, maintenance, and subculture of organisms. *Methanobacterium* strain B.B. was isolated by Janet Jeffrey in our laboratory from Boston Bay mud which had been incubated for several days in a shake flask under an atmosphere of 1:1 H_2-CO_2. A medium similar to that of 98-5 described by Bryant and Robinson, with addition of 2.0% rumen fluid, 0.2% Trypticase, 0.2% Na_2CO_3, and with the omission of carbohydrate, was used for the isolation as well as for maintenance of stocks. The cysteine-sulfide solution was added separately before the medium was solidified. A sterile oxygen-free gas atmosphere of 50% H_2 and 50% CO_2 was added to each tube. The hydrogen-oxidizing organism M.o.H. was obtained from the culture of *M. omelianskii*, as described elsewhere (3*a*). Stocks of M.o.H. were maintained in the medium described above with the addition of 30% rumen fluid.

Transfer from a stock was made by use of a platinum loop to 5 ml of medium (without agar and with 10 to 30% rumen fluid), which was contained in a rubber-stoppered, standard test tube (18 × 150 mm). The subculture was incubated at 40 C on a rotary shaker at about 120 rev/min with the tube slanted at an angle of 30 to 40°. The H_2-CO_2 atmosphere was changed twice daily after growth was visible.

The 200-ml culture. When the absorbancy at 660 mμ as recorded in an Evelyn colorimeter reached about 0.6, the test-tube culture was added to 200 ml of the same medium, contained in a flask like that illustrated in Fig. 1. The gas was filtered through sterile cotton in a glass tube (A). This tube was attached with rubber tubing to a piece of (Inconel 600) nickel-chrome, 0.25-inch (0.6 cm) diameter tubing (B) which was inserted in a rubber stopper. The exit tube (C) also was Inconel 600. A 500-ml or 1-liter baffle-bottomed flask (G), Bellco no. 603 (Bellco Glass, Inc., Vineland, N.J.), was used. The stopper assembly was covered by a Morton closure (D) (Bellco) which had two 0.25-inch holes drilled in the top to form a tight fit with the two metal tubes (B, C). Each of two pressure fingers (E) of the Morton closure was bent outward and was used as a hook to which a rather strong spring (F) was attached. The other end of the spring was attached to a rigid wire which encircled the flask and which was bent to form a hook. To provide a simple bubble-indicator for gas passage through the flask, a standard test tube (J), 16 × 150 mm, was wired to the metal exit tube and

was half filled with water. A one-hole stopper was inserted into the tube and a 6.5-inch (16.5-cm) no. 18 gauge hypodermic needle (I) was placed loosely in the hole. The barrel of a 0.5-ml disposable glass syringe (Hypak no. 701S, Becton, Dickinson and Co., Rutherford, N.J.) was broken about 3 cm from the end, filled with cotton (H), and attached to the exit tube with rubber tubing. The flask with medium was sterilized with I detached from H but with the top assembly held in place by the springs (F). After removal from the sterilizer, gas was added through A, and H was attached to I so that the rate of gas exit was visible. The gas flow rate was increased to 300 to 400 cc per min when the flask was first attached, so as to sweep out air as quickly as possible. Additions to the flask were made aseptically by raising the stopper assembly after unhooking the springs while the high rate of gas flow was maintained. The flask was incubated in a water-bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at about 240 rev/min, and with a gas flow of about 35 cc per min.

The apparatus for supplying a gas mixture of 80% H_2 and 20% CO_2 to culture flasks is shown in Fig.. 2 To remove traces of oxygen, the gas mixture (A)

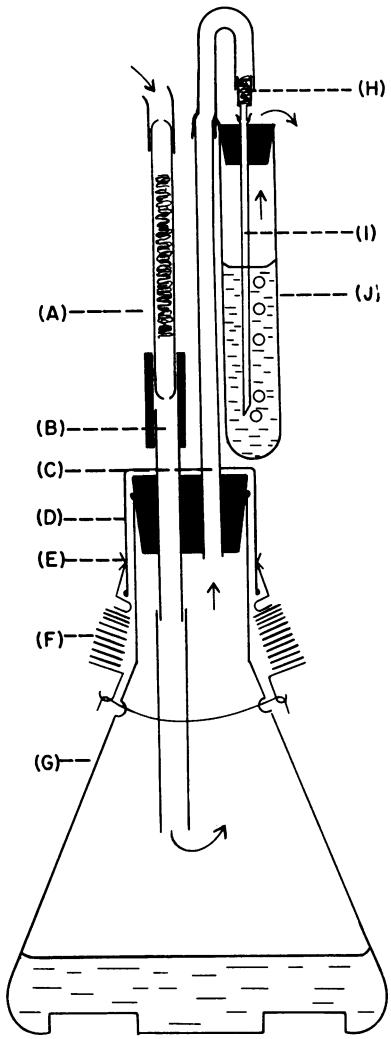

FIG. 1. *Anaerobic shake flask for cultivation of bacteria in a H_2 + CO_2 atmosphere. (A) Glass tubing with sterile cotton filter; (B) 0.25-inch nickel-chrome tube (Inconel 600); (C) exit tube (Inconel 600); (D) Morton closure; (E) pressure finger of Morton closure bent outward; (F) spring; (G) baffle-bottom flask (Bellco no. 603); (H) end of 0.5-ml disposable glass syringe with cotton; (I) 6.5-inch no. 18 gauge hypodermic needle placed loosely in one-hole rubber stopper; (J) standard 16 × 150 mm test tube.*

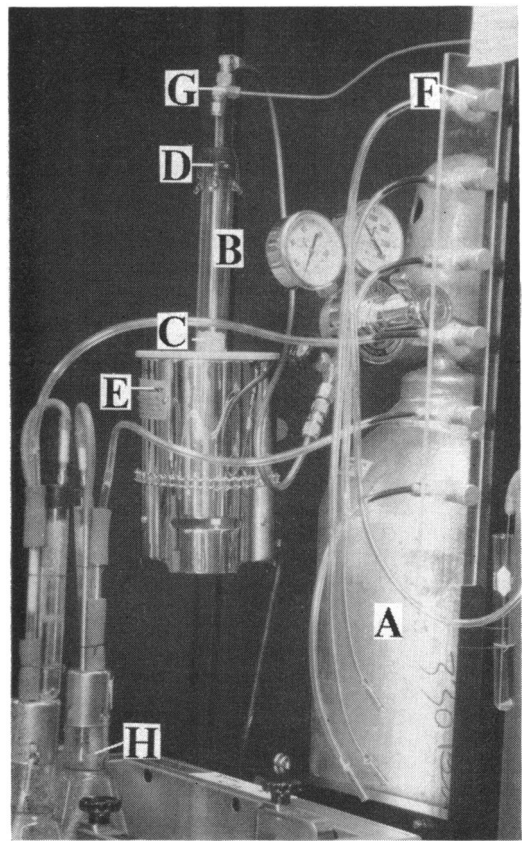

FIG. 2. *Apparatus for supplying an oxygen-free gas atmosphere to culture flasks. (A) gas cylinder of H_2-CO_2 (80:20); (B) Vycor glass tube; (C) reduced copper oxide wire; (D) rubber stopper wired in place; (E) electric furnace; (F) metering valve; (G) Swagelok connectors, for inlet tube [which opens at bottom of reduced copper column (C)] and for exit tube of scrubbed gas which connects to manifold of metering valves (F) from which gas is supplied to shake flasks (H) in water bath.*

(Matheson Co., Inc., Joliet, Ill.) was passed through a column of heated copper wire (C). The Vycor glass tube, 3 × 30 cm (B), was filled to a height of about 15 cm with pieces of copper oxide wire (C) and was fitted with a rubber stopper (D) which was wired in place. A flexible copper tube conducted gas from the cylinder to the bottom of the Vycor tube; a shorter tube served as an exit through the stopper. The glass tube was heated in an electric furnace (E) (Sargent, Chicago, Ill.). Initially, the copper oxide wire was reduced by passing hydrogen slowly through the heated tube. (To avoid condensation in lines, it was found necessary, when generating the copper, to continue gassing slowly for several days to reduce the copper completely and to remove all water vapor.) Swagelok connectors (G) (Matheson) were used to attach the Nupro very fine metering valves (F) (Matheson) to the gas line. Gas from the manifold was delivered to each growth flask (H).

The 12-liter culture. The 200-ml culture was shaken at 40 C, and when an absorbancy of about 0.6 was reached it was used to inoculate a 14-liter fermentor. At this stage, the amount of rumen fluid in the medium was reduced to 2%. To mass culture the organism, a 14-liter fermentor (New Brunswick Microferm) was modified as illustrated in Fig. 3. Gas was provided from a cylinder of hydrogen (welding grade) and from a cylinder of carbon dioxide, each of which was connected with rubber pressure tubing to a gas proportioner (A) (Matheson). A pressure of about 10 lb was maintained in the tubing on the cylinder side of the proportioner. From the proportioner, the gas mixture (usually 80% H_2 and 20% CO_2) was passed through a column of heated copper (C) as described for Fig. 2, then through a sterile cotton filter in a stainless-steel holder (E) (New Brunswick Co.), and into the fermentor. The exit port (H) was vented in a hood. Two other ports were provided. One was an inoculation probe (F) through which an inoculum could be passed aseptically to another fermentor or through which samples could be withdrawn from the fermentor. This probe was a piece of glass tubing inserted in a rubber stopper which was placed in a small suction flask. The arm of the suction flask was filled with cotton. The other port consisted of an amber rubber tube which was rolled at the open end so as to receive easily a glass inoculation probe from another fermentor. This port was kept in a test tube with cotton plug as illustrated (G). The entire fermentor assembly, which contained 12 liters of medium, was steam-sterilized with a paper cover over the vent tube (H), with the filter (E) disconnected from the copper column (D), and with clamps 1, 2, and 3 closed. Upon removal from the sterilizer, the filter (E) was immediately connected to the exit tube from the copper column (D). Clamp 3 was opened, and gassing was initiated at a flow rate of 600 cc per min with a stirring rate of 400 rev/min. When the medium was reduced and had reached a temperature of 40 C, it was inoculated from the growth flask through port I; after inoculation, the gas flow was reduced to 200 cc per min.

To follow H_2, CO_2, and CH_4 levels in the effluent gas, the total effluent flow rate was measured in a bubble flow cylinder. CO_2 was determined as the difference in flow rate after passing the effluent

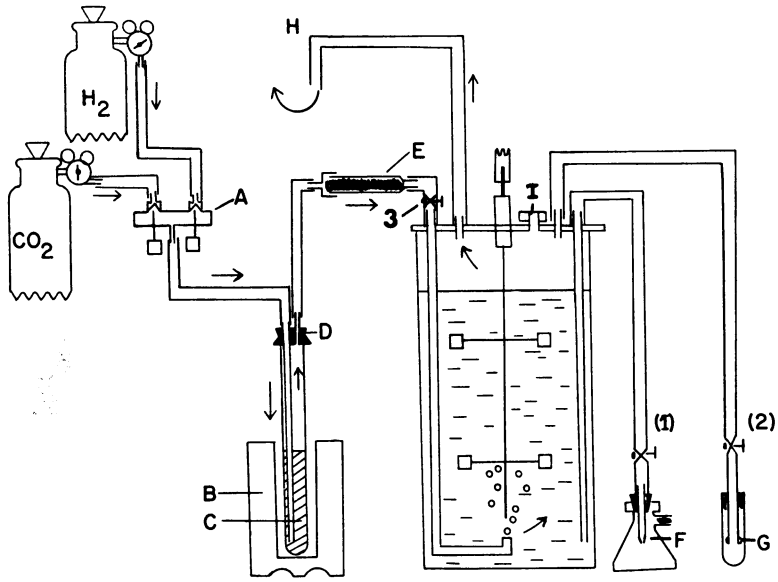

FIG. 3. *Apparatus for providing a gas mixture of H_2 and CO_2 to a 12-liter fermentor. (A) Gas proportioner; (B) electric furnace; (C) reduced copper; (D) rubber stopper wired in place; (E) stainless-steel holder for sterile filter; (F) port for removing samples; (G) port for receiving inoculation from another fermentor; (H) effluent vent to hood; (I) inoculation port; (1, 2, and 3) screw-clamp valves.*

through a trap which contained 20% KOH. CH_4 was determined by gas chromatography. H_2 was identified by gas chromatography and was measured as the difference after subtraction of the amounts of CH_4 and CO_2.

To follow growth by absorbancy, a sample was removed aseptically from the fermentor through port F, the volume of the channel (about 30 ml) being discarded before aseptic collection of 10 ml in a colorimeter tube. About 5 mg of sodium dithionite was added to each sample and mixed to insure a consistent reduction of resazurin in all measurements of absorbancy.

Chemicals. Methyl cobalamin was synthesized as described previously (7). CH_4, CO_2, $^{14}CH_4$, and $^{14}CO_2$ were determined with a Packard gas chromatograph and scintillation counter as described previously (6, 7). 5-Methyl tetrahydrofolate was prepared as described by Wood et al. (6).

RESULTS

Growth of the 12-liter culture. Cell yields of about 60 g (wet weight) per 12-liter fermentor were obtained for strain M.o.H. at a stirring rate of 400 rev/min. The flow rate of the gas mixture (H_2-CO_2, 80:20) was maintained at 200 cc per min for the first 24 hr and then was increased at a rate proportional to consumption of the individual gases; at the time of harvest (70 hr), the flow rate into the fermentor was 650 cc per minute. An excess of hydrogen and CO_2 was continually detected in the effluent, indicating that growth rate was not limited by a dearth of gases. Results of a typical growth response of the organism in a 12-liter culture are shown in Fig. 4. An average, maximal absorbance of 0.58 was obtained routinely at about 65 to 70 hr after inoculation with 200 ml of an actively growing culture. The ratio of hydrogen consumed to methane formed approached 3.6:1 during the linear portion of the growth curve, the theoretical ratio of H_2-CH_4 being 4:1. In the final portion of the growth response, the ratio was lower, suggesting that methane was being formed from carbon moieties more reduced than CO_2. Similar results were obtained for strain B.B., although this culture was generally more difficult to grow.

Dependence on hydrogen. Methane formation by a 12-liter culture of strain M.o.H. was dependent upon hydrogen, as shown in Fig. 5. In this experiment, the input of CO_2 to the culture was maintained at 5.8 mmoles per min, whereas the H_2 input was increased stepwise as shown. At 43 hr, when the ratio of H_2 consumed to CH_4 formed approached 4:1, the input of H_2 was drastically reduced; a sharp decrease in the rate of CH_4 formation followed. At 48 hr, the H_2 input was raised again, and an increase in H_2 consumption and in CH_4 formation followed. The plotted lines for CH_4 and H_2 between 48 and 57 hr are presented for continuity and do not represent measured rates. Results similar to those just described were obtained when CO_2 input was limited in the presence of excess H_2.

That methane formation by strain B.B. has a similar dependence upon H_2 and CO_2 is shown in Table 1. In this experiment, resting cells were used; harvested cells were resuspended in 0.05 M potassium phosphate buffer at *p*H 7.0. The

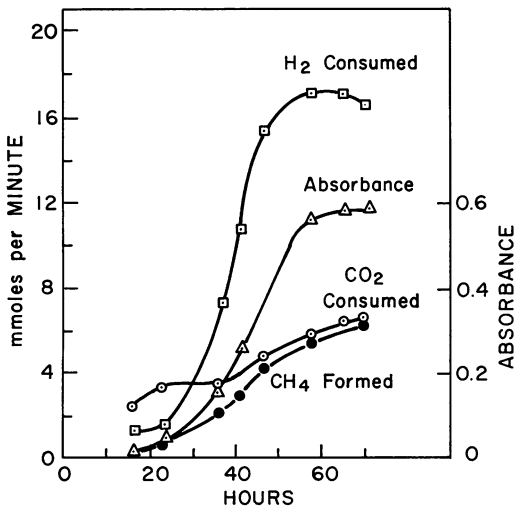

FIG. 4. *Growth of Methanobacterium strain M.o.H. in a 12-liter fermentor.*

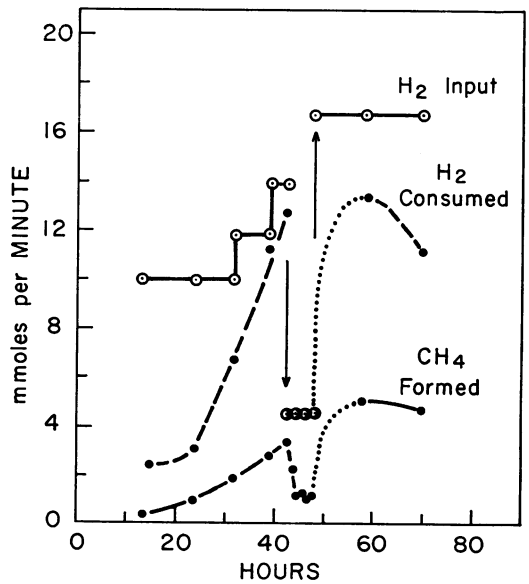

FIG. 5. *Effect of limiting hydrogen on the formation of methane by a 12-liter culture of Methanobacterium strain M.o.H.*

TABLE 1. *Conversion of $^{14}CO_2$ to $^{14}CH_4$ by cell suspensions of Methanobacterium strain B.B. grown on H_2-CO_2 (1:1)*[a]

Flask	Gas atmosphere	$^{14}CO_2$ added		$^{14}CH_4$ formed	
		Counts	Amt (μmoles)	Counts	Amt (μmoles)
1	H_2	2,881	50	1,759	41
2	H_2	—	—	—	2
3	N_2	2,881	50	0	0

[a] Reaction mixture contained washed cells, 100 mg, dry weight (cells were harvested at an optical density of 0.4 and were suspended in 0.05 M potassium phosphate buffer at *p*H 7.0); $NaH^{14}CO_3$ as indicated; gas atmosphere as indicated. Total liquid volume, 1.5 ml. Reaction time, 15 min at 40 C.

TABLE 2. *Formation of CH_4 from various substrates by extracts of Methanobacterium strains M.o.H. and B.B.*[a]

Substrate added (μmoles)	CH_4 formed in 20 min (μmoles)	
	M.o.H.	B.B.
CH_3B_{12} (5)	4.7	3.4
$5CH_3H_4$ folate (5)	1.2	—
Serine (100)	6.0	6.0
Pyruvate (100)	1.8	3.1
Formate (100)	0.0	1.8
CO_2-H_2 (50:50)	0.3	3.6

[a] Each reaction mixture contained: potassium phosphate buffer, *p*H 7.0, 700 μmoles; adenosine triphosphate, 10 μmoles; substrate as indicated; crude extract from strain M.o.H. where indicated, 40 mg of protein; crude extract from strain B.B. where indicated, 70 mg of protein; H_2 atmosphere (20 cc at 1 atm) except where indicated. Total liquid volume, 1.7 ml. An endogenous level of CH_4 formation (0.2 μmole per 20 min) was subtracted from the values presented for M.o.H.; endogenous was negligible for strain B.B.

reaction vessel was a modified Warburg flask, as described previously (7). There is an absolute dependence of CH_4 formation upon H_2, indicating that an endogenous source of electrons is not available for the reduction of CO_2. There is, however, a slight endogenous formation of CH_4 in a H_2 atmosphere in the absence of added CO_2, indicating that CH_4 is formed from carbon precursors in the cells or from residual CO_2 in the reaction mixture.

Cell extracts from hydrogen-grown cells. Methyl cobalamin, 5-methyl tetrahydrofolate, serine, pyruvate, and CO_2 have been reported to serve as substrates for the formation of methane by cell-free extracts of the ethyl alcohol-grown culture of *M. omelianskii* (6–8). These same substrates were tested in extracts from cells of strain M.o.H. and of strain B.B. As shown in Table 2, extracts of strain M.o.H. readily form methane from methyl cobalamin; 5-methyl tetrahydrofolate is somewhat less effective as a methyl donor. Serine served as an excellent substrate, whereas pyruvate and CO_2 were poor substrates. Pyruvate, serine, and formate were added in large amounts, since these substrates may enter a variety of pathways. The slow utilization of CO_2 by extracts may be related to labile components of the enzyme system involved in the activation and reduction of CO_2 to the methyl level. We have not detected formation of methane from formate by cell extracts of the original, ethyl alcohol-grown culture of *M. omelianskii* or by extracts of strain M.o.H. In contrast, extracts of strain B.B. formed significant amounts of methane from formate, as well as from the other substrates tested, the rate from CO_2 being about 20 times higher than that by extracts of strain M.o.H. On the basis of cell morphology, colony type, and electron donors for methane formation, both strain B.B. and M.o.H. appear closely related to *Methanobacterium formicicum*. However, strain M.o.H. does not utilize formate.

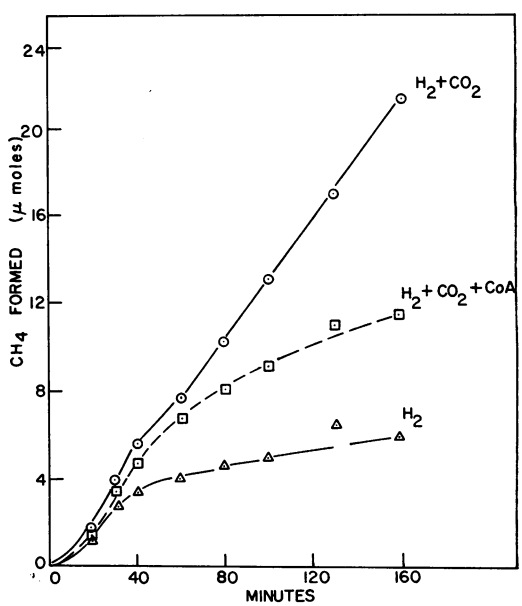

FIG. 6. *Formation of methane from CO_2 and H_2 by cell-free extracts of Methanobacterium strain M.o.H. Each reaction mixture contained: potassium phosphate buffer, pH 7.0, 700 μmoles; adenosine triphosphate, 10 μmoles; CoA where indicated, 0.01 μmole; crude extract, 45 mg of protein; gas atmosphere where indicated, 80% H_2 and 20% CO_2 or 100% H_2. Total liquid volume, 1.7 ml. Temperature, 40 C.*

The effect of coenzyme A (CoA) on a long-term incubation of cell extracts in a H_2-CO_2 (80:20) atmosphere is shown in Fig. 6. In these crude extracts of hydrogen-grown cells, it is probable that CoA inhibits methane formation by diverting CO_2 or the carboxylation product of CO_2 fixation to compounds other than those used for methane formation. This is supported by the fact that the initial rate of methane formation is independent of the presence of CoA. It was found that extracts were most active when prepared from that phase of the culture when growth was most rapid; as shown in Fig. 6, such extracts have a significant endogenous formation of methane in a hydrogen atmosphere.

DISCUSSION

Our initial efforts to grow methanogenic organisms on H_2 and CO_4 were concerned with an attempt to adapt the techniques of Repaske (5) for *Hydrogenomonas* to hydrogen-oxidizing anaerobes. Following a personal communication (Repaske, 1965), a gas mixture of H_2 and CO_2 was pumped by a diaphragm pump through a sparger in the culture medium; from the reservoir above the medium, the gas was passed over a heated copper column to remove traces of oxygen and then was returned to the medium again. Our first success was obtained with this closed system, but growth was erratic. The rate of aeration was limiting for large cultures, and moisture condensation in the lines proved difficult to control. Exploratory experiments with the flow-through system described in this communication were successful, and these techniques have been used routinely for the past year. Cell yields of the methanogenic bacterium, strain M.o.H., are about sixfold higher when grown on H_2 than the cell yields obtained with the culture of *M. omelianskii* grown in the ethyl alcohol-carbonate medium (7). Exploratory experiments with *Methanobacterium ruminantium* have yielded about 27 g (wet weight) of cells per 12 liters of medium, but so far we have been unable to prepare active extracts from this organism. We believe that these culture techniques should be applicable to the growth of other hydrogen-oxidizing anaerobes, which may or may not use an electron acceptor system other than CO_2.

Since the methanogenic organism in the culture of *M. omelianskii* was shown to oxidize hydrogen rather than ethyl alcohol (3*a*), it was pertinent to know whether the substrates which had been found to serve as precursors of methane in extracts of the symbiotic culture (6–8) also were active in extracts of the hydrogen-grown organism, strain M.o.H. It now is clear that the methane-forming system is complete in strain M.o.H. and that its formation or function is not dependent on association with the S organism. On the basis of results reported elsewhere (3*a*), as well as from the present study, the role of hydrogen as a substrate for methane bacteria in nature assumes major importance.

ACKNOWLEDGMENTS

We thank Janet Jeffrey, Kenneth Holmer, and Norman Ryckman for assistance, and Roy Repaske for helpful discussions. This investigation was supported by Public Health Service grant UI-SW00045-01 and by National Science Foundation grant GB-4481X.

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