Biochem. J. (1979) **178**, 165–172
Printed in Great Britain

Methane Synthesis without the Addition of Adenosine Triphosphate by Cell Membranes Isolated from *Methanobacterium ruminantium*

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(*Received* 21 *June* 1978)

The membrane fraction isolated from broken cells of *Methanobacterium ruminantium* actively synthesized methane from CO_2 and H_2 without the addition of ATP or other cofactors. This activity was lost unless strictly anaerobic conditions were maintained throughout the isolation and incubation procedures. $^3H_2$, but not ^3H_2O, was readily incorporated into methane. This indicates that hydrogen atoms are used in the formation of methane without the prior equilibration of protons with the water phase. Methylenetetrahydrofolate was shown to be converted into methane, but less efficiently than CO_2. The evidence indicates that tetrahydrofolate derivatives may not be of primary importance in the formation of methane from CO_2 and H_2. No requirement for ATP in methanogenesis could be demonstrated. However, chemical reagents that can increase proton conductance in membranes and thereby abolish the membrane electrical potential were also effective inhibitors of methanogenesis. It was postulated that, although the reduction of CO_2 to methane by bacterial membranes may require energy derived from a transmembrane potential, this does not appear to be coupled to the intermediary synthesis of ATP.

Methane can be synthesized by cell-free extracts of *Methanobacterium* M.O.H. (Roberton & Wolfe, 1969), *Methanosarcina barkeri* (Stadtman & Blaylock, 1966) and *Methanobacterium thermoautotrophicum* (Gunsalus & Wolfe, 1977). The precise sequence of reactions involved in the reduction of CO_2 to methane is not well understood, and to date there has been little success in characterizing and purifying the individual enzymes that are involved in this process. Only one enzyme, methylcobalamin–coenzyme M methyltransferase, which catalyses the transfer of methyl groups from coenzyme B_{12} to coenzyme M, and is thought to be part of the reaction sequence, has been purified to any extent (Taylor & Wolfe, 1974). Cell-free extracts have been prepared by disrupting *Methanobacterium* strain M.O.H. cells (Roberton & Wolfe, 1969) and *Methanosarcina barkeri* cells (Stadtman & Blaylock, 1966) with ultrasonic oscillation and pressure-cell treatment, followed by centrifugation at 30000*g* to remove particulate matter. In contrast, in mixed rumen bacteria, which contain *Methanobacterium ruminantium* and *Methanobacterium mobilis* (Smith & Hungate, 1958; Paynter & Hungate, 1968), methanogenic activity was found entirely in the cell fraction that was precipitated at 15000*g* (Sauer *et al.*, 1977).

Although it has been reported that ATP is required in the reduction of CO_2 to methane, its exact role in this process has not been defined (Roberton & Wolfe, 1969). Evidence has been presented for ATP involvement in the final reductive step in methane synthesis (McBride & Wolfe, 1971). We have reported (Sauer *et al.*, 1977) that no requirement could be demonstrated for added ATP. In the present study, whole cells and cell-free extracts of *Methanobacterium ruminantium* were used to confirm and extend the previous studies carried out with mixed rumen microorganisms.

Materials and Methods

Isolation of Methanobacterium ruminantium culture

In all procedures involved in the isolation of *Methanobacterium ruminantium* strict anaerobiosis was maintained throughout. Fresh rumen fluid diluted 10^6–10^8-fold was inoculated into sealed tubes containing 5ml of the reducing media of Paynter & Hungate (1968). The tubes were completely anaerobic and contained a CO_2/H_2 (1:4) atmosphere. Once vigorous growth was established, as indicated by active methanogenesis, samples were streaked on the agar medium of Paynter & Hungate (1968) and incubated in roll tubes. The plating technique was adapted from that described by Holdeman & Moore (1972). The tubes were inoculated spirally (bottom to top) to facilitate the selection of isolated colonies from the top of the tube. Only colonies that had the appearance of those described by Smith & Hungate (1958) were re-inoculated into culture tubes. Those tubes that contained methane-producing organisms were again streaked in roll tubes for colony produc-

tion. This process was repeated at least six times until the roll tubes contained only uniform-appearing methane-producing colonies. When these colonies were inoculated into liquid media, active methanogenesis resulted within a short time.

The organism was a short oval Gram-positive rod usually in chain formation and with no evidence of contamination. No motility could be detected. The structural details of the organism when examined by electron microscopy were exactly as described by Zeikus (1977). Surface colonies in roll-tube cultures were circular, convex, translucent, with entire margins and of pale yellow colour. The organisms required CO_2 and H_2 for methane synthesis, with H_2 serving as electron donor and energy source. The organisms grew in the absence of rumen fluid, and had a requirement for acetate, ammonia, sulphide and branched-chain fatty acids. On the basis of these and the other criteria specified by Bryant (1974), this organism was classified as *Methanobacterium ruminantium*.

To grow the organisms on a larger scale, the cultures were first grown in 500ml flasks containing 300ml of the medium of Paynter & Hungate (1968). When growth was well established, 14-litre flasks were inoculated with 900ml of the actively growing culture. The 14-litre flasks (Labroferm Fermenter Assembly, New Brunswick Scientific Co., Edison, NJ, U.S.A.) were continually stirred and gassed with CO_2/H_2 (1:4). Growth continued for approx. 1 week, as evidenced by the appearance of increasing turbidity and methane production. The cells were harvested when methane production was maximal. The culture was transferred anaerobically to 250ml polyallomer bottles with cap assembly (Beckman Instruments, Toronto, Ont., Canada) and centrifuged at 6000*g* for 10min in a type-19 rotor in a Beckman preparative ultracentrifuge (model L2 65B). After centrifugation, the growth medium was removed by aspiration, and the bottle gassed with CO_2/H_2 (1:4), capped with a rubber stopper, and the bacterial pellet stored at −80°C until used.

Preparation of cell-free extracts and membrane fraction

Bacterial pellets (1.5g wet wt.) were washed and resuspended in 10ml of O_2-free 0.1M-potassium phosphate buffer (pH7.0) containing 2mM-Na_2S. This buffer was stored under H_2. The mixture was transferred to a gassed stoppered 40ml conical glass tube and to this was added: 5mM-sodium pyruvate, 1mM-FAD and 0.1mM-CoA. The tube was gassed with H_2 for 5min. A centre-hole rubber stopper was inserted to allow entry of the ultrasonicator micro-tip (Bronson sonicator model 1856). With continuous N_2 gassing, the cells (0°C) were subjected to ultrafrequency sound (two-thirds of maximum power setting). Cell disruption was carried out for a total of 6min, with 30s of power on followed by 30s of power off. The micro-tip was thoroughly cooled after each 2min interval. After disruption, the sample was regassed for 15min with H_2, transferred to a 12ml Beckman centrifuge tube, capped and centrifuged for 15min at 1600*g*. The supernatant fraction constituted the crude cell extract. The crude cell extract was centrifuged at 106000*g* for 30min. The supernatant was discarded and the pellet resuspended in the potassium phosphate/Na_2S buffer (pH7.0). The centrifugation and washing steps were repeated twice. The final pellet was taken up in half the original volume of buffer, gassed with H_2 and either used fresh or stored at −80°C.

Incubation and gas analyses

All incubations (whole cells, cell extracts or washed membrane) were done in 5ml Erlenmeyer flasks sealed with red-sleeve-type rubber stoppers (14mm × 18mm). Gassing with H_2, and substrate additions were made by injections through the stoppers. All incubation mixtures, unless stated differently, contained the potassium phosphate (pH7.0)/Na_2S buffer, protein and $NaH^{14}CO_3$ (6×10^6 d.p.m., 10μmol). The gas phase was H_2. Incubations were made for specified time periods in a shaking water bath at 38°C. Radioactivity and mass analyses of the gas phase were done as described by Sauer *et al.* (1977). ^3H radioactivity was measured in the effluent gas from the gas partitioner (Fisher model 1200) by passing the gas through CuO and then through a 5mm-internal-diam. tube containing 0.5cm of iron filings at 750°C. The flow rate of the carrier gas (argon) in the gas partitioner was 25ml/min. Hydrogen purge gas (2ml/min) was put into the reduction tube. Propane quench gas entered the counting tube (1ml/min). The transfer lines between the combustion tube and the reduction were maintained at 250–300°C, otherwise condensation resulted in loss of measured radioactivity.

Adenine nucleotide assays

The reactions in the incubation flasks were stopped by the injection of 0.5ml of ice-cold 30% (w/v) $HClO_4$. Samples were kept on ice for 3h, sonicated for 1min at two-thirds of maximum power setting and centrifuged for 10min at 17500*g*. The supernatant was slowly neutralized with 5M-KOH, and the perchlorate salts were removed by centrifugation at 17500*g*. ATP was measured in the supernatant by the technique of Cole *et al.* (1967), as modified by Roberton & Wolfe (1970). ADP plus ATP were measured as described by Roberton & Wolfe (1970), except that the pyruvate kinase incubation step was done in a separate tube and not in the scintillation vial. The combined adenine nucleotides were measured as described by Roberton & Wolfe (1970). Protein was measured by the method of Lowry *et al.* (1951) as modified by Miller (1959).

Chemicals and radioisotopes

^3H_2O (25mCi/g), $^3H_2$ (2.6Ci/ml at s.t.p.) and ^3H_2CO (100mCi/mmol) were purchased from New England Nuclear, Lachine, Que., Canada.

5,10-Methylenetetrahydrofolate (^{14}C- and ^3H-labelled) was prepared as described by Sauer *et al.* (1977).

Carbonyl cyanide *m*-chlorophenylhydrazone and oligomycin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Tetrabutylammonium bromide and triphenylmethylphosphonium bromide were purchased from ICN, Montreal, Que., Canada.

All other chemicals used were of the highest purity commercially available.

Electron-microscope studies

The sectioning and staining of cell fractions for electron-microscope examination were done as described by Sauer *et al.* (1977).

Results and Discussion

Distribution of hydrogenase activity

Hydrogen is the principal energy source for all known methanogenic bacteria. With a few exceptions, methane-producing bacteria can also oxidize formate to supply energy for growth (Thauer *et al.*, 1977). The reaction:

$$CO_2+4H_2 \rightarrow CH_4+2H_2O \quad (1)$$

is exergonic ($\Delta G^{0'} = -131$ kJ/mol) and can supply all the energy necessary for growth (Thauer *et al.*, 1977).

In the utilization of gaseous H_2, the first reaction is believed to be catalysed by a hydrogenase:

$$H_2+\text{oxidized electron carrier} \rightleftarrows \text{reduced electron carrier}+2H^+ \quad (2)$$

Hydrogenase has been isolated from the 22400*g* supernatant of *Methanobacterium ruminantium* (Tzeng *et al.*, 1975*a*), but no attempt was made to determine if the particulate fraction contained hydrogenase activity. The results in Table 1 show that approx. 30% of the total cell hydrogenase activity resides in the particulate fraction. The hydrogenase activity of other anaerobes is known to be associated with the particulate cell fraction (Aspen & Wolin, 1966).

The hydrogenase of *Methanobacterium ruminantium* also catalysed the exchange reaction with water and $^3H_2$ (reaction 3) (Table 2):

$$^3HH+H_2O \rightleftarrows HO^3H+H_2 \quad (3)$$

A similar exchange reaction is catalysed by the hydrogenase isolated from *Desulfovibrio desulfuricans* (Yagi *et al.*, 1968). Table 2 shows that within 5h, less than 1% of the ^3H added at the beginning of the incubation remained. After 18h, the ^3H concentration was barely detectable, but more than 99% was accounted for as ^3H_2O. Small but measurable amounts of $^3H_2$ were present when ^3H_2O was incubated with hydrogenase. The results indicate that under these experimental

Table 1. *Distribution of hydrogenase activity between soluble and particulate fractions of cell extracts of Methanobacterium ruminantium*

Cell free extract of *Methanobacterium ruminantium* was centrifuged at 15000*g* for 30min. The supernatant fraction (soluble fraction) was flushed with H_2 and stored in tightly stoppered tubes. The precipitate was washed, resuspended in buffer and stored as above. Reactions were carried out in 3.0ml Thunberg cuvettes sealed with silicone rubber stoppers. The incubation mixture contained: Tris, pH7.6, 100mM; EDTA, 40mM; $MgCl_2$, 3mM; dithiothreitol, 10mM; $NADP^+$, 2mM; Methyl Viologen, 1.5mM, in a final volume of 1.0ml. The cuvettes were thoroughly flushed with H_2 through the rubber stopper, and then enzyme (75–150μg of protein) was injected through the rubber stopper with a micro-syringe. Reaction rates were measured with a Beckman DK2 recording spectrophotometer at 600nm. A molar absorption coefficient of 8250 litre·mol^{-1}·cm^{-1} was used (Yu & Wolin, 1969). The reduction of 2mol of Methyl Viologen equals the consumption of 1mol of H_2.

Cell fraction	Activity (% of total)	Specific activity (nmol of H_2/min per mg of protein)
Soluble	73	151
Particulate	27	67

Table 2. *Conversion of $^3H_2$ and ^3H_2O into C^3H_4 and exchange with H_2 and H_2O by crude cell extracts of Methanobacterium ruminantium*

In Expt. 1, 0.9μCi of $^3H_2$ and 1.2μCi of ^3H_2O were added to each incubation. Each flask contained 4.7mg of protein. Gas phase was 1ml of H_2, the remainder argon. In Expt. 2, 115μCi of $^3H_2$ and 110μCi of ^3H_2O were added to each incubation. Gas phase was as in Expt. 1. Each flask contained 4.2mg of protein.

Expt. no.	Tracer added	Percentage of added tracer recovered after 5h in: H_2	H_2O	CH_4
1	$^3H_2$	0.57	98.46	0.87
	^3H_2O	0.17	—	0
		Percentage of added tracer recovered after 18h in: H_2	H_2O	CH_4
2	$^3H_2$	0.003	99.10	0.640
	^3H_2O	0.019	—	0.004

conditions, the incorporation of $^3H_2$ into ^3H_2O is essentially irreversible.

Incorporation of ^3H into methane

^3H was incorporated into C^3H_4 by crude cell extracts of *Methanobacterium ruminantium* (Table 2). C^3H_4 production did not increase after 5h, probably because by then excess $^3H_2$ had largely been incorporated into ^3H_2O. In contrast with these results, no ^3H_2O conversion into C^3H_4 was detectable at 5h. Only by increasing the concentration of ^3H_2O 100-fold (Expt. 2, Table 2) was it possible to detect any C^3H_4 formation after 18h. These results clearly show that the protons used in the formation of CH_4 from CO_2 are derived from H_2 and that these do not first equilibrate with protons from water.

Conversion of 5,10-methylenetetrahydrofolate into methane

In a previous report (Sauer *et al.*, 1977) we found that 5,10-methylenetetrahydrofolate was converted into methane by cell extracts of rumen bacteria. It was observed that these intermediates were converted into CO_2 as well as methane. These results did not prove conclusively that the tetrahydrofolate derivatives were reduced to methane. The possibility remained that the methyl and methylene groups were reoxidized to CO_2 and then reduced to methane by a pathway other than the tetrahydrofolate one.

The ambiguous results obtained with 5,10-[^{14}C]-methylenetetrahydrofolate (Sauer *et al.*, 1977) can be resolved by labelling the methyl groups with ^3H (Table 3). Table 3 shows conclusively that 5,10-[^{3}H]-methylenetetrahydrofolate undergoes direct reduction to yield methane. In this experiment, oxidation of 5,10-[^{3}H]methylenetetrahydrofolate to CO_2 would have resulted in the loss of ^3H atoms, which would then have equilibrated with the protons of water. The results in Table 2 show that no radioactivity from ^3H_2O is recovered in methane even after 5h.

Localization of methane-synthesizing activity in membrane fractions of Methanobacterium ruminantium

To date, the enzymes of methanogenesis have not been purified. Blaylock & Stadtman (1966) reported methane synthesis with a soluble supernatant fraction (30000*g* for 60min) obtained from *Methanosarcina barkeri*. Roberton & Wolfe (1969) used supernatant solutions (30000*g* for 25min) from cell-free extracts of *Methanobacterium* M.O.H. for methane synthesis. Similarly, active preparations were isolated from the supernatant fraction (33000*g* for 30min) of cell extracts from *Methanobacterium thermoautotrophicum* (Gunsalus & Wolfe, 1977).

It has been reported that cell-free extracts prepared from either fresh cells of *Methanobacterium ruminantium* or from cells stored at −20°C under H_2 failed to synthesize methane (Bryant *et al.*, 1968; Tzeng *et al.*, 1975*b*). We had previously reported (Sauer *et al.*, 1977) that the methane-synthesizing activity of rumen micro-organisms resides in the particulate and not the supernatant fraction and that the failure by others to obtain active cell-free preparations was probably because excessively high *g* values were used in the preparation of extracts. We reported that most of the methanogenic activity precipitated after 30min at 15000*g*.

The results in Table 4 confirm the previous report (Sauer *et al.*, 1977) that the particulate fraction of

Table 3. *Conversion of radioactive CO_2 and radioactive 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4folate) into CH_4 by membrane fraction of Methanobacterium ruminantium*

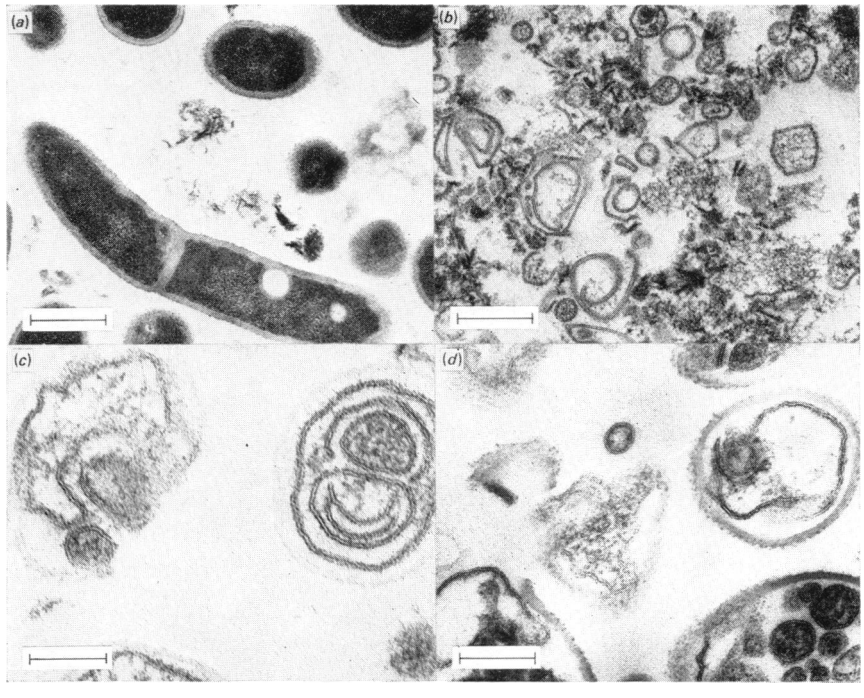
Membrane protein (3.2mg) was added to each incubation. Substrates added to each incubation flask were as follows: $NaH^{14}CO_3$, 15μmol, 8×10^6d.p.m.; DL-5,10-$^{14}CH_2$-H_4folate, 0.28μmol, 4.1×10^6d.p.m.; DL-5,10-C^3H_2-H_4folate, 0.26μmol, 24.6×10^6d.p.m. All incubations were made in an H_2 atmosphere at 38°C. Numbers in parentheses represent μmol of labelled derivative converted into methane.

Substrate added	Percentage of added tracer recovered in CH_4	
	2.5h	18h
$^{14}CO_2$	6.4 (0.96)	60.0 (9.01)
5,10-$^{14}CH_2$-H_4folate	8.9 (0.02)	26.1 (0.04)
5,10-C^3H_2-H_4folate	7.4 (0.01)	12.9 (0.02)

Table 4. *Distribution of methanogenic activity between the soluble and the particulate fraction of cell-free extracts of Methanobacterium ruminantium*

The 20 K pellet refers to the pellet obtained when the cell-free extract prepared as described in the Materials and Methods section was centrifuged for 30min at 26700*g*. The 20 K supernatant is the corresponding supernatant fraction. The 40 K pellet refers to the pellet obtained when the 20 K supernatant fraction described above was centrifuged for an additional 30min at 106500*g*. The 40 K supernatant is the corresponding supernatant fraction. The conditions of incubation were as described in the text.

Cell fraction	Total CH_4 (d.p.m.)	CH_4 (d.p.m./mg of total protein)	CH_4 (d.p.m./mg of membrane protein)
20 K pellet	195000	57700	57700
20 K supernatant	61000	19900	51200
40 K pellet	82800	58300	58300
40 K supernatant	3050	1360	—



EXPLANATION OF PLATE 1

Ultrastructure of Methanobacterium ruminantium

(*a*) Cross-section and longitudinal section of intact *Methanobacterium ruminantium* cells. One cell is undergoing division. The cell wall is triple layered as described by Zeikus (1977). The cytoplasm has a granular appearance. Some membranes and vacuoles can be seen in the cytoplasm (bar indicates 0.39 μm). (*b*) Washed membranes used for the incubations. The membranes appear heavily folded, and, in most cases, have formed closed vesicles (bar indicates 0.39 μm). (*c*) Higher magnification of the cell membrane. A faint outline of cell-wall material is still visible. The membrane appears to be triple layered, tightly folded and has formed vesicular structures (bar indicates 0.19 μm). (*d*) Densely coiled intracytoplasmic membrane bodies that are sometimes isolated from *Methanobacterium ruminantium*. Some cell-wall material is still adherent to the membranes (bar indicates 0.24 μm).

Methanobacterium ruminantium contains the methanogenic activity. Approx. 70% of the activity was in the fraction that precipitated at 26700*g*. The remaining activity was in the pellet that precipitated after centrifugation at 106500*g* for 30min. Less than 2% of the original methanogenic activity remained in the 106500*g* supernatant fraction (Table 4). As shown, methane synthesis calculated per mg of cell membrane protein remained constant. These results show that, at least in *Methanobacterium ruminantium*, the membranous fraction is required for methane production from CO_2 and H_2. There appears to be no requirement for the high-speed-supernatant fraction or the soluble cofactors contained therein.

Ultrastructure of Methanobacterium ruminantium cells and membranes

Plate 1(*a*) shows the ultrastructural appearance of intact *Methanobacterium ruminantium* cells in longitudinal and cross section. Plates 1(*b*), 1(*c*) and 1(*d*) are photomicrographs taken at different magnifications of the washed membrane fraction used in this study. Shown are typical large, heavily folded trilaminar membrane particles with some cell-wall material still attached. As shown in Plates 1(*b*) and 1(*c*), the preponderance of membrane particles are in the form of closed vesicles. Tightly folded intracytoplasmic membrane bodies, shown here in Plate 1(*d*) have been reported in a number of methane-producing micro-organisms (Zeikus, 1977). From the large size of the membrane particles and the presence of adherent cell-wall material, it is clear that methane-synthesizing activity would be sedimented by subjecting crude cell extracts to even moderately high *g* values.

Effect of ATP on methane synthesis

There are published reports that indicate a requirement for ATP in a variety of reactions that have been proposed as intermediary steps in methane biosynthesis. Cell-free extracts of *Methanosarcina barkeri* have an ATP requirement for methanogenesis when methylcobalamin or methanol are substrates; however, this requirement is not specific for ATP, and other nucleotides can be substituted (Blaylock & Stadtman, 1966). The addition of ATP also has been reported to stimulate methane production from CO_2 and H_2 in cell-free extracts of *Methanobacterium* M.O.H. (Roberton & Wolfe, 1969). The most likely site of ATP action with these cell extracts appears to be at the level of methyl-group transfer and reductive demethylation. Thus ATP appeared to be required for the methylcobalamin–coenzyme M methyltransferase reaction (McBride & Wolfe, 1971), although later studies indicated that the purified enzyme may not require ATP (Taylor & Wolfe, 1974). The conclusion then appears to be that ATP and H_2 are required in *Methanobacterium* M.O.H. for the reductive demethylation of methyl-coenzyme M (Wolfe, 1971).

In contrast with these systems, with washed membrane fractions isolated from *Methanobacterium ruminantium*, the addition of ATP did not stimulate methane production (Table 5). In confirmation of our previous results obtained with cell-free extracts of mixed rumen micro-organisms (Sauer *et al.*, 1977), ATP can inhibit methane synthesis at concentrations of 5–10mM. These results, of course, do not rule out the possibility that crude cell extract or washed membrane contained sufficient quantities of ATP (stored or generated during incubation) to allow methane synthesis to proceed. Therefore adenine nucleotide concentration in *Methanobacterium ruminantium* cells and cell-free extracts were measured at different times of incubation.

Changes in adenine nucleotide concentration during incubation of intact cells, crude cell extract and washed membrane

The results in Table 6 show the changes in adenine nucleotide concentration when intact cells of *Methanobacterium ruminantium* that were stored frozen (−80°C) under H_2 are thawed and incubated. Concentrations of ATP and ADP rapidly increased during methanogenesis, whereas AMP underwent a concomitant decrease. These results (Table 6) are similar to those obtained by Roberton & Wolfe (1969) with cells freshly harvested from actively growing cultures of *Methanobacterium* M.O.H. When adenine nucleotide concentrations were measured in intact cells synthesizing methane, the values, expressed as energy charge (Atkinson, 1968), ([ATP] + 0.5 [ADP])/([ATP] + [ADP] + [AMP]), increased from less than 0.1 to 0.59 (Table 6). For *Methanobacterium* M.O.H. during maximal methane production, an energy charge of 0.67 has been reported (Chapman *et al.*, 1971). These results show that the reduction of CO_2 to methane (reaction 1), which is exergonic (Thauer *et al.*, 1977), is coupled to the

Table 5. *Effect of added ATP on rate of methane synthesis by washed membrane fraction from Methanobacterium ruminantium*

Preparation of membrane fraction and conditions of incubation were as described in the text.

ATP added (mM)	Rate of CH_4 synthesis (nmol/min per mg of protein)
0	15.60
2	13.50
5	11.40
10	9.30

Table 6. *Adenine nucleotide concentrations in intact cells of Methanobacterium ruminantium synthesizing methane*
Conditions of incubation are described in the text.

Time (min)	CH_4 (nmol/mg of protein)	Adenine nucleotide concentration (nmol/mg of protein)			
		ATP	ADP	AMP	Energy charge
0	0	0.31	2.86	15.90	0.09
2	0.99	0.37	2.42	14.10	0.09
5	12.90	0.25	3.55	12.90	0.12
10	51.30	0.70	3.67	13.00	0.15
20	387.00	2.08	4.47	7.54	0.31
30	985.00	2.04	4.15	3.15	0.44
60	2718.00*	2.98	4.49	2.35	0.53
90	2355.00*	2.07	5.62	0.86	0.59

* Methane production stopped because most of the CO_2 had been converted into CH_4.

synthesis of ATP in intact cells of *Methanobacterium ruminantium*.

No ATP synthesis can be demonstrated in these bacterial cells after disruption by ultrasonication (Table 7). That the ATP concentration at zero time was greater in the crude cell extract (Table 7) than in the intact cells (Table 6) is fortuitous, since ATP concentrations decrease with time (even in cells stored at −80°C) and these values therefore merely reflect the length of time the cells were stored before use.

It is nevertheless clear from data in Table 7 that methanogenesis is not coupled to adenine nucleotide phosphorylation in either crude cell extracts or washed membranes. The presence of ATP phosphohydrolase (ATPase, EC 3.6.1.3) in crude cell extracts is indicated by the progressive decrease in ATP concentration with time. The washed membrane fraction actively synthesized methane in spite of the fact that the ATP concentration was exceedingly low and did not increase during incubation (Table 7). These results indicate that the reduction of CO_2 to methane by H_2 can proceed in 'uncoupled' membrane fractions and involves neither the utilization nor the production of ATP.

Table 7. *ATP concentration and methane synthesis in crude cell extracts and in twice-washed membrane fraction isolated from Methanobacterium ruminantium*
Bacterial membrane preparation and conditions of incubation are described in the text.

Time (min)	CH_4 synthesis (nmol/mg of protein)		ATP concentration (nmol/mg of protein)	
	Crude cell extract	Washed membrane	Crude cell extract	Washed membrane
0	0	0	2.36	0.11
2	0.90	0	2.05	0.10
5	7.22	1.80	1.99	<0.05
10	35.60	10.90	1.70	0.08
20	153.00	46.50	1.39	0.07
30	260.00	112.00	1.07	0.07
60	871.00	343.00	0.67	0.05
90	1210.00	652.00	0.49	0.07

These results put in question the role of tetrahydrofolate intermediates in methanogenesis from CO_2 and H_2. The first step in this reaction is the reduction of CO_2 to formate by the enzyme formate dehydrogenase (EC 1.2.1.2) present in *Methanobacterium ruminantium* (Tzeng *et al.*, 1975*b*). In cell extracts of *Methanobacterium ruminantium* producing methane from $^{14}CO_2$, 75% of the water-soluble radioactivity is present as formate (F. D. Sauer, unpublished work). The second step is the activation of formate according to reaction 4, catalysed by 10-formyltetrahydrofolate synthetase (EC 6.3.4.3):

$$H_4\text{folate} + ATP + HCO_2H \rightleftarrows 10\text{-CHO-}H_4\text{folate} + P_i + ADP + H_2O \quad (4)$$

The active substrate for this reaction is the magnesium chelate of ATP (Curthoys & Rabinowitz, 1971) with a K_m of 86 μM (Paukert *et al.*, 1976). In the experiment with washed membrane from *Methanobacterium ruminantium* cells reported in Table 7, the maximum ATP concentration present at any time was 0.3 μM, i.e. 280-fold lower than the reported K_m for the enzyme. This makes it unlikely that in *Methanobacterium ruminantium*, the tetrahydrofolate intermediates have the major role in methanogenesis that they may have in *Methanobacterium* M.O.H. (Wood & Wolfe, 1965). More likely, these intermediates are primarily required for C_1-transfer reactions in purine biosynthesis, as has been suggested (Thauer *et al.*, 1977).

Effect of ATP phosphohydrolase inhibitors and membrane-modifying agents

The finding that specific ATP phosphohydrolase inhibitors did not impair methane production in cell-free extracts of *Methanobacterium ruminantium* (Table 8) is further evidence that ATP is not required for this process. Neither oligomycin nor *NN'*-dicyclohexylcarbodi-imide, both of which are inhibitors of membrane-bound bacterial ATP phosphohydrolase (Haddock & Jones, 1977; Melandri *et al.*, 1977), were effective in inhibiting methane synthesis (Table 8).

Although there does not appear to be an ATP requirement for methane biosynthesis in *Methanobacterium ruminantium* membranes, this in itself does not rule out the possiblity that energy may be required to initiate the reaction. There are a number of energy-dependent reactions, that is reactions coupled to

Table 8. *Effects of membrane-modifying agents and ATP phosphohydrolase inhibitors on methane sythesis in crude cell extracts of Methanobacterium ruminantium*

Where necessary, inhibitors were dissolved in ethanol. The equivalent volume of ethanol (10 μl) without inhibitor was then added to the control. Concentrations of inhibitors are given in parentheses. Incubations were as described in the text.

Additions	CH_4 synthesis	
	(nmol/min per mg of protein)	(% of control)
None	47.31	100
Carbonyl cyanide *m*-chlorophenylhydrazone (50 μM)	1.25	2.6
2,4-Dinitrophenol (0.1 mM)	0.10	<1
Triphenylmethylphosphonium bromide (2 mM)	5.93	12.5
Tetrabutylammonium bromide (8 mM)	6.59	13.9
Deoxycholate (0.25%, w/v)	1.73	3.7
Oligomycin (2 μg)	38.89	82.2
Dicyclohexylcarbodi-imide (40 μM)	69.26	146.4

electron flow, which do not appear to involve ATP hydrolysis. These include the energy-dependent transport against concentration gradients of solutes such as amino acids, sugars and cations. It is generally accepted that the energy required for these processes is linked to the movement of protons across membranes and is described by the relationship:

$$\Delta\bar{\mu}_{H^+} = \Delta\psi - \left(\frac{2.3RT}{F}\right)\Delta pH$$

where $\Delta\bar{\mu}_{H^+}$ is the electrochemical potential of protons across the membrane, $\Delta\psi$ the electrical potential, and ΔpH the chemical difference in proton concentration across the membrane (Mitchell, 1966, 1967).

Table 8 shows that chemical reagents that decrease the protonmotive force, $\Delta\bar{\mu}_{H^+}$, also decreased methane synthesis. 2,4-Dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone, which are proton conductors that probably abolish both electrical and pH gradients across bacterial membranes (Ramos & Kaback, 1977; MacDonald & Lanyi, 1975), were also powerful inhibitors of methanogenesis in *Methanobacterium ruminantium* membranes (Table 8).

Triphenylmethylphosphonium bromide and tetrabutylammonium bromide are cations of strong bases capable of penetrating phospholipid (Grinius *et al.*, 1970) and bacterial membranes (MacDonald & Lanyi, 1975). These cations have been shown to abolish electrical but not pH gradients across synthetic and bacterial membranes (Grinius *et al.*, 1970; MacDonald & Lanyi, 1975). These compounds too were effective inhibitors of methane synthesis (Table 8). Similarly, sodium deoxycholate, a membrane-solubilizing agent, also totally abolished methane production (Table 8). These results suggest that electrical (and pH) gradients across *Methanobacterium ruminantium* membranes (i.e. 'energized' membranes) may be a prerequisite for methane production from CO_2. If this is so, then this energy must be used directly and without the prior intermediary formation of ATP.

We acknowledge the expert technical assistance of Mr. W. Cantwell. The assistance of Mr. W. Addison in growing bacterial cultures is acknowledged. This is Animal Research Institute Contribution no. 765, Ottawa, Ont. K1A 0C6, Canada.

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