Biochem. J. (1980) **190**, 177–182
Printed in Great Britain

Methane Production by the Membranous Fraction of *Methanobacterium thermoautotrophicum*

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(*Received 7 February 1980*)

Intact membrane vesicles are required to synthesize methane from CO_2 and H_2 by disrupted preparations of *Methanobacterium thermoautotrophicum* cells. When membrane vesicles were removed by high-speed centrifugation at 226600 ***g***, the remaining supernatant fraction no longer synthesized methane. Alternatively, if vesicle structure was disrupted by passage through a Ribi cell fractionator at very high pressures (345 MPa), the bacterial cell extract, with all the particulate fraction in it, did not synthesize methane. Methyl-coenzyme M, a new coenzyme first described by McBride & Wolfe [(1971) *Biochemistry* **10**, 2317–2324], was shown to stimulate methane production from CO_2 and H_2, as previously reported, but the methyl group of the coenzyme did not appear to be a precursor of methane in this reaction. No methyl-coenzyme M reductase activity was detected in the cytoplasmic fraction of *M. thermoautotrophicum* cells.

The synthesis of methane from CO_2 by methanogenic bacteria proceeds via a series of reductive steps that are linked to H_2 oxidation. It is currently proposed that CO_2 binds to a coenzyme, coenzyme M (2-mercaptoethanesulphonic acid), before undergoing reduction to methane (Gunsalus *et al.*, 1976; Gunsalus & Wolfe, 1977). Electrons for this reduction are transferred from H_2 via a fluorescent coenzyme, F_{420} (Thauer *et al.*, 1977).

As yet, little is known about the enzymes of methane biosynthesis. This reaction is generally studied with fractions derived from cell-free bacterial extracts that have been centrifuged at 20000–30000 ***g*** for short periods of time.

From these observations it was concluded that methane biosynthesis via the coenzyme M pathway requires soluble enzymes and cofactors. It was recently reported, however, that washed membranes isolated from cell-free extracts of *Methanobacterium ruminantium* synthesized methane from CO_2 and H_2 without the addition of soluble supernatant (Sauer *et al.*, 1979). From this it appeared that *M. ruminantium* may have an alternative pathway of methane biosynthesis that does not involve reductive demethylation of coenzyme M. Obviously, the site of methane biosynthesis in *Methanobacterium* species, i.e. whether in the membranous or cytoplasmic fraction, must be determined before much progress can be made in elucidating details of the methanogenic pathway. In the present paper this question is examined in *Methanobacterium thermoautotrophicum*, an organism that has been used quite extensively to study the mechanism of methanogenesis.

Materials and Methods

Materials

$Na_2{}^{14}CO_3$ (96.8% ^{14}C to total C present) was purchased from Atomic Energy (Ottawa, Ont., Canada) and carefully neutralized before use. [^{14}C]Methyl iodide (18 mCi/mmol) was purchased from New England Nuclear, Lachine, Que., Canada. Percoll (polyvinylpyrrolidone-coated colloidal silica in solution) was purchased from Pharmacia (Canada) Ltd., Dorval, Que., Canada. 2-Mercaptoethanesulphonic acid (ammonium salt) was either prepared by the method of Taylor & Wolfe (1974*a*) or purchased from Pierce Chemical Co., Rockford, IL, U.S.A., as the sodium salt. Unlabelled 2-(methylthio)ethanesulphonate (methyl-coenzyme M) was prepared as described by Taylor & Wolfe (1974*a*). *Me*-^{14}C-labelled 2-(methylthio)ethanesulphonate ([*Me*-^{14}C]methyl-coenzyme M) was prepared by alkylation of 2-mercaptoethanesulphonate with [^{14}C]methyl iodide as described by Gunsalus *et al.* (1978). The purity of the recrystallized compound was checked by high-voltage paper electrophoresis (Whatman 3MM paper) at 1200 V for 1 h in 8% formic acid buffer. In this system [*Me*-^{14}C]methyl-coenzyme M migrated towards the anode and gave a single spot on radioautography.

0306-3283/80/070177-08$01.50/1

Methods

Bacterial culture. M. thermoautotrophicum A.T.C.C. 29183 was obtained from the American Type Culture Collection, Rockville, MD, U.S.A. Initial transfers were made into 18mm × 150mm rubber-stoppered culture tubes (Balch & Wolfe, 1976) each containing 10ml of the medium described by Zeikus & Wolfe (1972) under an atmosphere of CO_2/H_2 (1:4), and kept at 65°C. After active growth was established, cultures were transferred anaerobically into 125ml serum bottles (Wheaton Scientific, Millville, NJ, U.S.A.) each containing 100ml of the same medium under CO_2/H_2 (1:4) and kept for 36–48h at 65°C. In the final transfer, 500ml of actively growing culture was inoculated into 14-litre fermentation flasks (Sauer *et al.*, 1979) containing 10 litres of the growth medium described by Zeikus & Wolfe (1972). The flasks were continually stirred at 65°C under CO_2/H_2 (1:4) for 60h. Cells were harvested and stored as described by Sauer *et al.* (1979).

Preparation of cell extracts and incubation procedure. M. thermoautotrophicum cells were disrupted by exposure to ultrafrequency sound as described previously (Sauer *et al.*, 1979) with the following modifications. Total disruption time was limited to 3min and the addition to the buffer of FAD, CoA and pyruvate was omitted. In other experiments cells were broken in a Ribi cell fractionator (model RF-1; Norwalk, CT, U.S.A.) (Bush & Sauer, 1976). The fill valve was not used, but instead the cell suspension was transferred directly into the high-pressure cylinder that had previously been thoroughly flushed with O_2-free H_2. For cell disruption, the Ribi valve assembly was maintained at 0–5°C by continuous adjustment of the cooling gas (H_2). Disrupted cells were always centrifuged at 600*g* for 20min to remove large particles and cell-wall material. Incubations were done in 5ml Erlenmeyer flasks sealed with red-sleeve-type rubber stoppers. The flasks, containing the appropriate incubation mixtures, were gassed for 4min with O_2-free H_2, enzyme protein was added, and gassing was continued for an additional 1min. $NaH^{14}CO_3$ (20μmol, 6.1×10^6d.p.m.) was added last (Sauer *et al.*, 1979). Methane mass and radioactivity were measured as described previously (Sauer *et al.*, 1977).

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

Protein determination. Protein concentration was measured by the method of Miller (1959). Bovine serum albumin (4× crystallized; ICN Nutritional Biochemicals, Cleveland, OH, U.S.A.) was used as standard.

Results

Bacterial pellets of approx. 1g (wet wt.) were stored as described previously (Sauer *et al.*, 1979). Rates of methane production by intact cells from these pellets varied from 64 to 232nmol/min per mg of protein. This variability may reflect the degree to which anaerobiosis was maintained during cell harvesting and storage.

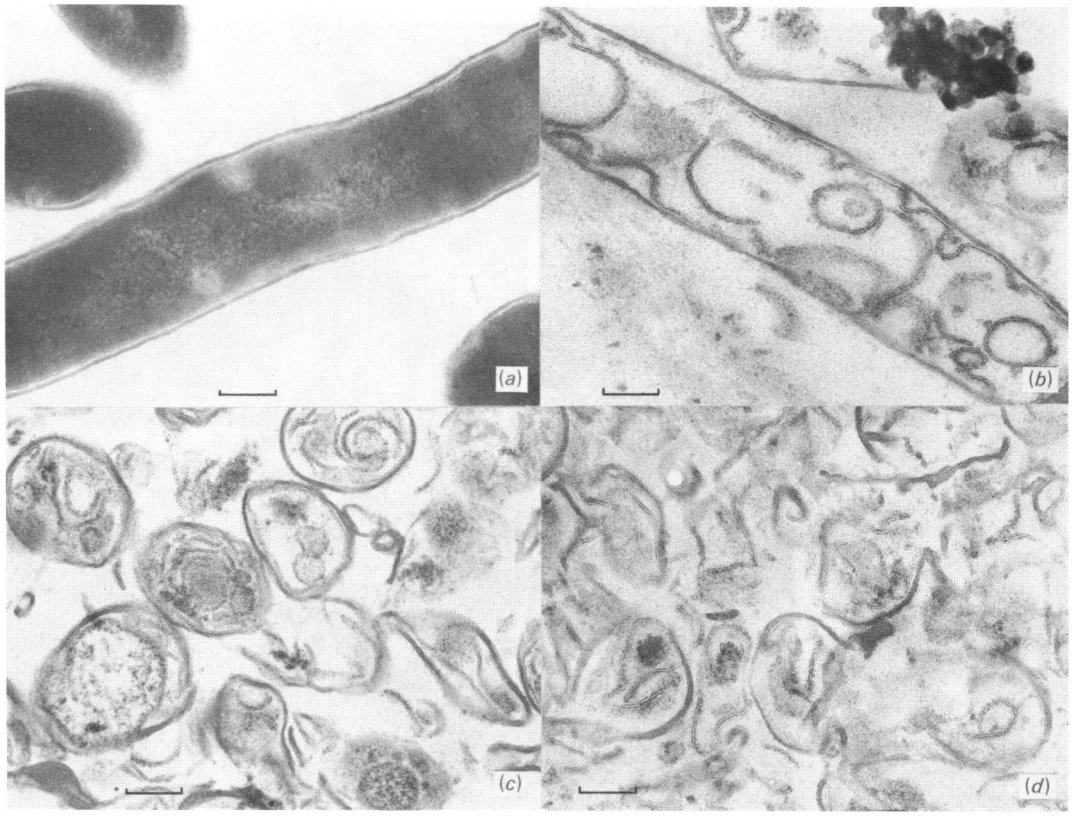
Intact *M. thermoautotrophicum* cells (Plate 1*a*) were readily sedimented by centrifugation at low centrifugal forces (Table 1), with little or no methanogenic activity remaining in the 490*g*-supernatant fraction. After exposure of cells to ultrafrequency sound (which decreased the specific activity to 24%) methane production was associated with intracellular membrane vesicles of different sizes, some of which remained suspended unless very high centrifugal forces were employed (Table 1).

The particulate material present in the low-speed supernatant fractions centrifuged at 490, 567 and 630*g* was sedimented, and sections were prepared for electron microscopy. No intact cells were found, although a few large cell fragments such as those shown in Plate 1(*b*) were observed.

Membrane particles in the supernatant fraction ranged in size from small vesicles, which remained suspended after centrifugation at 20400*g*, to large particles of broken cells. Typical vesicle structure consisting of trilaminar membrane is shown at different magnifications in Plate 2. These vesicles were obtained after disruption of cells by ultrafrequency sound and they remained suspended after low-speed centrifugation, i.e. at 500–9000*g*. A small amount of methanogenic activity remained in the 20400*g*-supernatant fraction (Table 1). Because this fraction contained primarily soluble protein and little membrane, its specific activity was very low. The addition of methyl-coenzyme M to the 20400*g*-supernatant fraction did not stimulate methane production. Approx. 55% of the protein in the ultrasonically treated preparation was soluble, i.e. remained in the high-speed 226600*g*-supernatant fraction. This fraction, however, did not synthesize methane from CO_2 and H_2 (Table 1).

Cell particles were separated into three distinct bands by density-gradient centrifugation with polyvinylpyrrolidone-coated colloidal silica (Percoll) (Table 2). The particles in all three bands actively synthesized methane. The highest specific activity was associated with the heaviest particles in band 3. On identical Percoll gradients, intact cells formed a single band distinctly separate from the three bands obtained with disrupted cells.

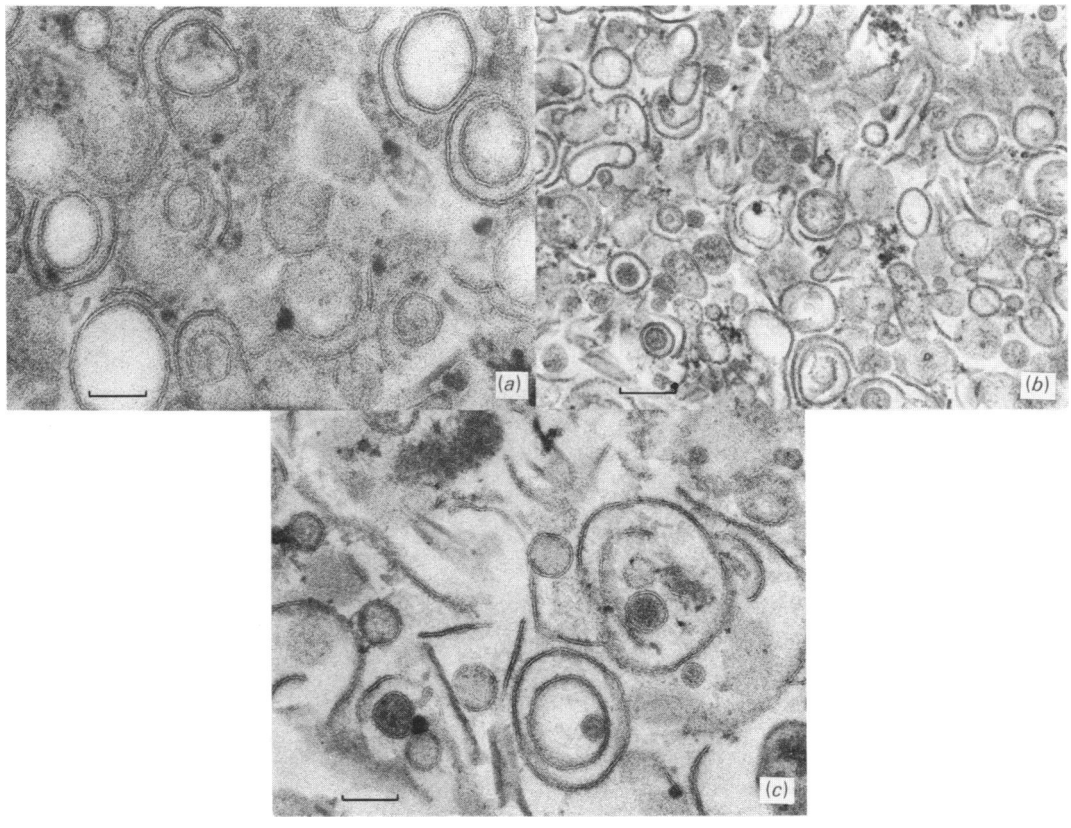
Intact vesicles apparently were required in order for methane production to proceed. Whole cells disrupted in a Ribi cell fractionator at 172MPa



EXPLANATION OF PLATE 1

Ultrastructure of Methanobacterium thermoautotrophicum

(*a*) Intact cells of *M. thermoautotrophicum* in longitudinal section and cross-section. The internal membranes described by Doddema *et al.* (1979) are shown (bar indicates 0.12 μm). (*b*) Large cell particles of *M. thermoautotrophicum* after disruption with ultrafrequency sound as described in the text. These particles sedimented and formed a pellet at 870 ***g***. The cytoplasm has washed out. Cell wall and intracellular membranes are clearly visible (bar indicates 0.12 μm). (*c*) *M. thermoautotrophicum* cells after disruption in Ribi cell fractionator at 172 MPa as described in the text. The cytoplasm has washed out but cell-wall material remains. The membrane appears in sheet or vesicle formation (bar indicates 0.15 μm). (*d*) *M. thermoautotrophicum* cells after disruption in Ribi cell fractionator at 345 MPa. No intact vesicles can be seen. Only membrane sheets remain. Cell-wall material is still present (bar indicates 0.12 μm).



EXPLANATION OF PLATE 2

Ultrastructure of Methanobacterium thermoautotrophicum

(*a*) Large vesicles present in the 870**g**-supernatant fraction from disrupted *M. thermoautotrophicum* cells. The trilaminar membrane is similar to that found in *M. ruminantium* (Sauer *et al.*, 1979). There are no large cell-wall particles in this fraction (bar indicates 0.10μm). (*b*) Different-size vesicles present in 5800**g** supernatant of *M. thermoautotrophicum* cell extract. A few membrane sheets are present, but most membranes are in vesicular form (bar indicates 0.24μm). (*c*) Higher magnification of the same 5800**g**-supernatant fraction. Trilaminar membrane sheets and vesicles are shown. The membrane vesicles shown in (*a*), (*b*) and (*c*) actively synthesize methane from CO_2 and H_2 without the addition of cytoplasm (bar indicates 0.12μm).

Table 1. *Methane production by intact cells and disrupted cell preparation of M. thermoautotrophicum after 20 min centrifugation with different centrifugal forces*

Cells were disrupted by ultrafrequency-sound treatment for 3 min. The cell preparations were transferred under a constant flow of H_2 to thick-walled 12 ml glass centrifuge tubes, and sealed with black rubber stoppers (Bellco Glass Co., Vineland, NJ, U.S.A.). Samples were centrifuged sequentially at indicated centrifugal forces (up to 630 ***g***) in an International centrifuge (model SBV). After each centrifugation, some of the supernatant was withdrawn by syringe through the rubber stopper without disturbing the underlying pellet, and used in the methane-assay system. When higher centrifugal forces were used the centrifugations were done in 13 ml capped polyallomer tubes (Beckman Instrument Co., Toronto, Ont., Canada) with a Beckman preparative ultracentrifuge (model L2 65B). These samples were each centrifuged separately. The cell preparations were maintained at 0–4°C throughout. For the incubations, the reaction mixture contained: potassium phosphate buffer, pH 7.0, 100 mM; Na_2S, 2 mM; ATP, 4 mM; $MgCl_2$, 20 mM; protein, 1.5–2.6 mg; $NaH^{14}CO_3$, 20 mM (6.1×10^6 d.p.m.); in a final volume of 1.0 ml. Reaction flasks were thoroughly flushed with O_2-free H_2 (passed through a heated copper column) before additions of enzyme protein. Incubations were done at 65°C under H_2, and 0.1 ml gas samples were analysed at 15 min intervals to establish rates. Longer intervals were used when samples had low activity. Numbers in parentheses are the same values recalculated on the basis of membrane protein alone without including the soluble or cytoplasmic protein.

20 min centrifugation (*g* value)	Methane production rate (nmol/min per mg of protein)	
	Whole cells	Activity in the supernatant after centrifugation of disrupted cell preparation
1	232.0	55.1 (191.1)
490	0.3	50.4 (241.2)
567	0.2	37.4 (105.8)
630	0.2	26.7 (190.9)
5800		9.5
9100		11.0
20400		0.055
20400 + 5 μmol of methyl-coenzyme M		0.054
226600		0

Table 2. *Separation into fractions of M. thermoautotrophicum cell extract on a density gradient of Percoll*

Fractions were obtained as described in the text. Incubation procedure was as described in Table 1.

Fraction	Methane production rate (nmol/min per mg of protein)
Soluble top layer	0.6
Band 1	4.8
Band 2	39.0
Band 3	52.0

retained 49% of the original specific activity (Table 3). At this pressure the disrupted cell preparation contained intact vesicles (Plate 1*c*), many of which were still within the fragmented cell wall. When whole cells were disrupted at higher pressure (345 MPa), methane production was almost totally abolished (Table 3), with less than 0.01% of the original activity remaining. In these same preparations, hydrogenase activity decreased by only 20% when Ribi-cell-fractionator pressures were increased from 172 MPa to 345 MPa. Appreciably less hydrogenase activity was detectable in the cells before disruption (Table 3). The possibility of O_2 shock to the enzymes of methane synthesis was minimized because H_2 gas, purified over a hot copper column, was used to flush and cool the Ribi valve assembly during cell disruption. As seen in Plate 1(*d*), no intact vesicles appear to be present when *M. thermoautotrophicum* cells were disrupted at very high pressures.

Methane was synthesized by membrane-vesicle preparations of *M. thermoautotrophicum* without added ATP (Table 4), but the addition of 4 mM-ATP significantly stimulated the rate of methane production. In contrast, the 33000 ***g***-supernatant fraction derived from disrupted *M. thermoautotrophicum* cells synthesized no methane without added ATP (Gunsalus & Wolfe, 1977). In agreement with results obtained by Gunsalus & Wolfe (1977), the addition of methyl-coenzyme M stimulated methane biosynthesis from CO_2 and H_2 (Table 4), although the maximum stimulation of 84% reported in the present paper is far less than the 30-fold stimulation that those authors observed when methyl-coenzyme M was added to a 33000 ***g***-supernatant fraction derived from disrupted *M. thermoautotrophicum* cells.

Methyl-coenzyme M stimulated methane synthesis from CO_2 and H_2 without donating its methyl

Table 3. *Effects of pressures used in disrupting M. thermoautotrophicum cells on methane production*
Intact cells from a stored pellet (approx. 1.5 g wet wt.) were suspended in 12 ml of 0.1 M-potassium phosphate buffer, pH 7.0, containing 2 mM-Na_2S (Sauer *et al.*, 1979), and divided into two equal 6 ml fractions. One fraction was then put through the Ribi cell fractionator at 172 MPa, the other fraction at 345 MPa, exactly as described in the text. Incubation procedure was as described in Table 1. The hydrogenase assay method of Chen & Mortenson (1974) was used except that H_2 evolution was measured with a gas partitioner (Sauer *et al.*, 1977).

Pressure for cell disruption in Ribi cell fractionator	Methane production rate (nmol/min per mg of protein)	Hydrogenase activity (nmol/min per mg of protein)
Whole cells	63.7	40.3
After disruption at 172 MPa	31.4	328.5
After disruption at 345 MPa	<0.01	263.9

Table 4. *Cofactor requirements for methane production by disrupted cell preparation of M. thermoautotrophicum*
Different cell preparations were used in each experiment. For Expts. 1, 2 and 3, the 600 *g*-supernatant fractions, prepared as described in the text, were given an additional centrifugation at 15000–20000 *g* for 15 min to sediment membrane vesicles. The resulting pellets were resuspended in one-third of the original volume with O_2-free 0.1 M-potassium phosphate buffer, pH 7.0, containing 2 mM-Na_2S. This step removed most of the soluble cofactors. In Expt. 4, the 600 *g* supernatant was used directly. The incubation procedure was as described in Table 1 except for the omission of ATP in Expt. 1 and the addition of methyl-coenzyme M in Expts. 2, 3 and 4. The specific radioactivity of [*Me*-^{14}C]methyl-coenzyme M (Expts. 3 and 4) was 24000 d.p.m./μmol.

Expt. no.	Cofactor added (mM)	Methane production rate (nmol/min per mg of protein)	Methane derived from methyl-coenzyme M (% of total)
1	None	102.5	—
	ATP (4)	169.6	—
2	ATP (4)	36.8	—
	Methyl-coenzyme M (0.8) + ATP (4)	53.8	—
3	ATP (4)	14.0	—
	[*Me*-^{14}C]Methyl-coenzyme M (5) + ATP (4)	25.8	0
4	ATP (4)	37.4	—
	[*Me*-^{14}C]Methyl-coenzyme M (5) + ATP (4)	42.0	2.73

group (Table 4). In Expt. 4 (Table 4) a total of 23.77 μmol of methane was produced in 180 min. Of this total, only 0.65 μmol of methane was derived from the 5 μmol of [*Me*-^{14}C]methyl-coenzyme M in the incubation mixture. In Expt. 3 (Table 4) no radioactive methane was detectable after 18 h of incubation; this indicates that methane biosynthesis was stimulated by methyl-coenzyme M but that the methyl group thereof was not a precursor of the methane formed. Intact cells of *M. thermoautotrophicum* were no more effective than disrupted cells in synthesizing ^{14}C-labelled methane from [*Me*-^{14}C]methyl-coenzyme M. In separate experiments with cell extracts of *M. ruminantium*, 89% of the added methyl-coenzyme M was recovered intact after a 60 min incubation period (F. D. Sauer, unpublished work). This supports the conclusion that methyl-coenzyme M is not readily degraded by the methanogenic bacteria used in these studies.

Discussion

It has been assumed that methane production is catalysed by soluble enzymes (Wood *et al.*, 1965; Blaylock & Stadtman, 1966; Roberton & Wolfe, 1969; Gunsalus & Wolfe, 1977). This assumption was based on the observation that, when cell extracts of methanogenic organisms are centrifuged at 23000–30000 *g*, some activity remained in the supernatant fraction. A few of the enzymes that are believed to have a role in methane biosynthesis have been partially purified. These include methylcobalamin–coenzyme M methyltransferase (Taylor & Wolfe, 1974*b*), an ATP- and CO_2-requiring methyl-coenzyme M reductase (Gunsalus *et al.*, 1978; Wolfe, 1979) and an O_2-sensitive hydrogenase (Wolfe, 1979). It has also been reported that there is a requirement for soluble cofactors, which include methyl-coenzyme M (McBride & Wolfe, 1971), ATP (Roberton & Wolfe, 1969) and an FMN

derivative, coenzyme F_{420} (Tzeng *et al.*, 1975), which participates in two-electron transfer reactions (Wolfe, 1979).

A recent paper (Sauer *et al.*, 1979) suggests that, in *M. ruminantium*, methane was synthesized by the membrane fraction, and there appeared to be no requirement for high-speed soluble supernatant provided that O_2 was strictly excluded from the system. It was noted that washed membrane synthesized methane at 50% of the rate observed with crude cell extracts (i.e. 10.3 nmol/min per mg of protein compared with 20.4 nmol/min per mg of protein) and that there was no requirement for ATP. Some energy, however, seemed to be required, since chemical reagents that abolish proton gradients across membranes were also powerful inhibitors of methane synthesis (Sauer *et al.*, 1979). In agreement with this, the present work indicates that in *M. thermoautotrophicum*, as in *M. ruminantium*, intact membrane vesicles are required for methane biosynthesis.

A number of reports indicate that methane can be synthesized from CO_2 and H_2 by the 30000*g*-supernatant fractions from *Methanosarcina barkeri*, *Methanobacterium* M.O.H. and *M. thermoautotrophicum* (Wood *et al.*, 1965; Blaylock & Stadtman, 1966; Roberton & Wolfe, 1969; Gunsalus & Wolfe, 1977). This is difficult to reconcile with the present findings that intact membrane vesicles are required for methane synthesis and that no methane is synthesized if these vesicles are removed by centrifugation or broken at very high pressures in the Ribi cell fractionator. The observation by Doddema *et al.* (1979) that, in *M. thermoautotrophicum*, hydrogenase activity is located entirely on internal membrane structures virtually rules out the possibility that the reduction of CO_2 to methane can be catalysed entirely by the soluble supernatant fraction, since hydrogenase is a key enzyme in methane biosynthesis.

Rather concentrated cell suspensions have been used in many of the studies that have shown methanogenic activity in the 30000*g*-supernatant fraction. Wood *et al.* (1965), Roberton & Wolfe (1969), McBride & Wolfe (1971) and Gunsalus & Wolfe (1977) routinely used a 50% (w/v) cell suspension in the preparation of cell extracts, whereas in the present work cell suspensions of 10–15% (w/v) concentration were used. Doddema *et al.* (1978) observed that membranes could not be completely removed from *M. thermoautotrophicum* cell extracts (20%, w/v) even after a 1h centrifugation at 100000*g*. It therefore seems probable that, with the heavier cell suspensions, centrifugation at 30000*g* for 25–30 min was not sufficient to remove all membrane particles. This residual membrane fraction may have accounted for the methanogenic activity that remained in the 30000*g*-supernatant fraction.

Coenzyme M (free or methylated) is required for growth of *M. ruminantium* strain M1, and is present in all methanogenic bacteria isolated to date (Balch & Wolfe, 1979*a*). *M. ruminantium* strain M1 has an energy-dependent transport system specific for coenzyme M (Balch & Wolfe, 1979*b*). It has also been shown that methane-producing bacteria, when exposed to $^{14}CO_2$, synthesize [*Me*-^{14}C]methyl-coenzyme M (Daniels & Zeikus, 1978). Nevertheless the exact role of coenzyme M in methanogenesis is not clear. In crude cell extracts, the rate of methane production from methyl-coenzyme M is lower than that from CO_2 and H_2. The following rates of methyl-coenzyme M reductase activity (in nmol/min per mg of protein) have been reported: 13.0 (McBride & Wolfe, 1971); 3.6 (Taylor & Wolfe, 1974*a*); 3.8 (Gunsalus & Wolfe, 1977); 2.1 (Gunsalus & Wolfe, 1978). Further reports indicate that methyl-coenzyme M stimulates the reduction of CO_2 to methane (Gunsalus & Wolfe, 1977; Wolfe, 1979). Gunsalus & Wolfe (1977) reported that, with the 30000*g* supernatant fraction from crude cell extracts of *M. thermoautotrophicum*, methane production was 1.5 nmol/min per mg of protein from CO_2 and H_2. With the addition of methyl-coenzyme M, this rate increased to 44.5 nmol/min per mg of protein, a 30-fold stimulation (Wolfe, 1979). Stimulation of methane production from CO_2 and H_2 by methyl-coenzyme M was also observed in the present experiments, with one important difference. The stimulatory effect did not depend on a concomitant reduction of methyl-coenzyme M to methane. Furthermore, high rates of methanogenesis (up to 170 nmol/min per mg of protein) were obtained with intact membrane vesicles from *M. thermoautotrophicum*, without the addition of methyl-coenzyme M. Clearly further studies are required in order to elucidate the exact role of methyl-coenzyme M in methane production from CO_2 and H_2.

We acknowledge the expert technical assistance of Mr. Wm. Cantwell. This paper is Contribution no. 887 from the Animal Research Institute, Agriculture Canada.

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