METHANE PRODUCTION BY CELL-FREE PARTICULATE FRACTION OF RUMEN BACTERIA

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SUMMARY: Rumen bacteria retained methanogenic activity when stored at -60° under H_2. This activity, which resides in Methanobacterium ruminantium and Methanobacterium mobilis, is not lost when the cells are broken, as has been suggested. Unlike in Methanosarcina barkerii and Methanobacterium M.o.H., in rumen bacteria methanogenic enzymes are not soluble but readily precipitated at 15,000 g. Methane was synthesized from tetrahydrofolate derivatives but at slower rates than from CO_2. From the data, it was not possible to determine if methyl- and methylene tetrahydrofolate were oxidized to CO_2 prior to reduction to CH_4. In room light, CH_3-B_{12} was reduced to CH_4 non-enzymatically in the presence of protein. When the reactions were carried out in the dark, very little CH_4 was formed from CH_3-B_{12} by rumen bacterial enzymes. The cell-free particulate fraction did not require added ATP for methanogenesis but showed an absolute requirement for H_2.

INTRODUCTION: Most methane bacteria have simple nutritional requirements with hydrogen and carbon dioxide supplying both energy and carbon (1). In addition, Methanosarcina barkerii can utilize methanol and acetate for growth and methane production (2). Much of the work dealing with the pathway of methane formation has been carried out with Methanobacterium M.o.H. and M. barkerii. Cell-free extracts are generally prepared by sonic oscillation (3) or by combined sonic oscillation and French pressure cell disruption (4) followed by centrifugation at 30,000 g for 25 to 60 min.

Methanobacterium ruminantium and Methanobacterium mobilis are present in the rumen flora (approx. 4×10^8 cells per ml) and together account for total methane production (5, 6). We have observed that rumen bacterial pellets, prepared and stored as described previously (7), actively synthesize methane. In this report we show that methanogenic activity is entirely in the particulate fraction after complete cell disruption. The role of intermediates in the production of methane by the membrane fraction of rumen bacterial cells was examined.

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MATERIALS AND METHODS:

Preparation of cell-free extract. Rumen bacterial pellets were prepared as described (7) but stored at -60° in clarified rumen fluid under H_2. To thawed 10 ml pellets (200 to 250 mg protein) was added with continuous H_2 gassing: FAD (1 mM); CoA, (0.1 mM); pyruvate, (5 mM); and pyruvate lyase isolated from Clostridium pasteurianum (8) (≈ 2 mg). These additions kept the bacterial cell fractions fully reduced through subsequent steps provided that extreme care was used to exclude O_2 and maintain a constant N_2 or H_2 atmosphere. Cells were disrupted by 3 min sonication in a Bronson sonifier cell disruptor (90 to 100 watts) in cycles of 15 s on and 45 s off. The partially disrupted cell suspension was then put through an R-1 Ribi cell fractionator at 25,000 p.s.i. This was followed by an additional 3 min sonication as before. The cell extract was then centrifuged 30 min at 2380 g (unless specified otherwise) under H_2 in tightly stoppered tubes. A one ml aliquot of this cell extract was centrifuged at 100,000 g. The resulting pellet was fixed in glutaraldehyde/osmium tetraoxide and stained with uranyl acetate/lead citrate for examination in a Phillips 300 electron microscope. No intact bacterial cells were found.

Incubation and assay procedure. Cell fractions (0.5 ml) were incubated at 38° in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM $Na_2S.9H_2O$ (final vol. 1.0 ml) in 5 ml Erlenmeyer flasks sealed with serum bottle rubber stoppers. Additions were made as specified in legends to Figures and Tables. Before addition of enzyme, each flask was evacuated and flushed with H_2 for three 1 min periods. Substrate and enzyme were added by microsyringe to H_2 filled flasks before incubation. Gas samples were withdrawn at timed intervals with a gas syringe (Precision Sampling Corp., Baton Rouge, La.) and assayed on a gas partitioner (Fisher Model 1200) coupled to a recording integrator (Infotronics CRS 110A). The effluent gas, after partitioning, was passed through copper tubing into a combustion furnace and then into a radioactivity monitor (Barber-Colman Series 500). For measurement of $^{14}CO_2$ column 2 of the gas partitioner was by-passed. Alternately, $^{14}CO_2$ was measured by injecting a gas sample into a sealed flask containing Hyamine hydroxide in the center well as described previously (9).

Preparation of substrates. d,l-Tetrahydrofolate (d,l-H_4-folate) was prepared by hydrogenation of d,l-folic acid over platinum oxide (10). N^5,N^{10},^{14}C-Methylenetetrahydrofolate (5,10-$^{14}CH_2$-H_4-folate) was prepared from H_4-folate and ^{14}C-formaldehyde (New England Nuclear Corp.) (10 mCi per mmole) (11). N^5-^{14}C-Methyltetrahydrofolate (5-$^{14}CH_3$-H_4-folate) was prepared by reduction of 5,10-$^{14}CH_2$-H_4-folate with sodium borohydride (12).

Unlabeled methylcobalamin (methyl-Co-5,6-dimethylbenzimidazolcobamide) was prepared by the procedure of Müller and Müller (13). ^{14}C-Methylcobalamin was prepared by the conversion of cyanocobalamin to hydroxocobalamin (14) and reacting this with ^{14}C-methyl iodide (10 mc per mmole) (15). All prepared substrates were stored at -20° in an inert atmosphere in sealed glass ampoules and shielded from light.

RESULTS AND DISCUSSION: Rates of CH_4 production by stored, frozen, mixed rumen bacterial cells are shown in Table 1. Approximately 50% of the activity was lost when the cells were disrupted by pressure and sonication. Changing the gas phase from H_2 to CO_2:H_2 (20:80) stimulated CH_4 production by intact cells but not by disrupted cells. Disrupted cells made no CH_4 in the absence of H_2.

Table 1. Rates of Methane Production by Intact and Disrupted Rumen Bacterial Cells.

Gas Phase	Intact Cells	Disrupted Cells
	(millimicromoles CH_4/mg protein/h)	
H_2	237	108
H_2:CO_2 (80:20)	271	98
CO_2 I*	42	0
CO_2 II	27	

Intact cells (0.5 ml of cell suspension before disruption) and cell fractions were incubated as described in the text. Incubations continued for 6 h and rates were calculated from the linear slopes following the initial 30-60 min lag caused by "oxygen shock" to the system.

* I is the initial rate of CH_4 production (30 min to 2 h); II is the final rate (2 h to 6 h).

The intact bacterial cells produced some H_2 (0.6 µmole in the first h) which permitted a low rate of CH_4 production (Table 1). CH_4 production did not continue after cessation of H_2 synthesis.

An initial (0-60 min) lag in CH_4 production was observed both with intact and disrupted cells (Fig. 1). This was probably the result of minute traces of O_2 dissolved in buffers. The high sensitivity to O_2 of CH_4 producing bacteria is known (1). The rate of CH_4 synthesis was decreased by approximately 40% after 3 min sonication and decreased slightly more after high pressure disruption in the Ribi press and an additional 3 min sonication (Fig. 1). The activity resided entirely in the particulate fraction. The supernatant that remained after a 30 min centrifugation at 15,000 g was devoid of activity (Fig. 1). This shows that, unlike in other CH_4 producers, the CH_4 producing enzyme(s) in M. ruminantium and M. mobilis are membrane bound and not solubilized by sonication and pressure disruption. These findings explain previous reports (16, 17) that cell-free

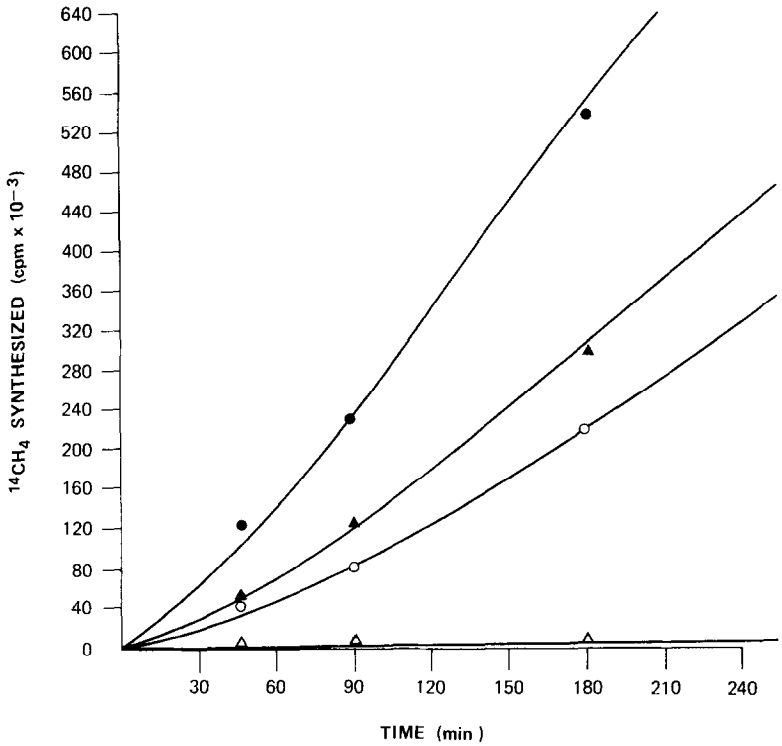

Fig. 1. Bacterial cells (intact and disrupted) were incubated in an H_2 atmosphere as described in text. Reaction mixtures contained 1 μcurie $KH^{14}CO_3$ per flask and were started with protein addition (12.5 mg protein per flask). $^{14}CH_4$ synthesis by intact cells, ●——●; after 3 min sonication, ▲——▲; after Ribi cell disruption and an additional 3 min sonication, o——o; after 15,000 g centrifugation, △——△.

extracts (22,000 g supernatant) of M. ruminantium show no methanogenic activity. The 15,000 g pellet contained almost all the methanogenic activity.

In the reduction of CO_2 to CH_4 some ATP is required, presumably in the initial reduction of CO_2 to CH_2O (18). The requirement however, is not stoichiometric and less than 1 mole of ATP is used per mole of CH_4 formed (3). In the present study, the rumen bacterial fraction did not require the addition of ATP to form CH_4 from CO_2, in fact, the addition of ATP was slightly inhibitory.

Earlier experiments in this laboratory had indicated that high speed (100,000 g) supernatant of rumen bacteria stimulated CH_4 production when added to the particulate fraction. Fractionation of this supernatant fraction showed

Table 2. $^{14}CH_4$ Synthesis from $^{14}CO_2$ or $^{14}CH_3OH$ by Intact Cells and Cell-free Particles of Rumen Bacteria.

Added to Incubation	Substrate	
	$KH^{14}CO_3$	$^{14}CH_3OH$
	(cpm/mg protein/hr)	
Intact Cells		
Expt. I	121,600	36,000
Expt. II	162,600	28,200
Cell-free Particles		
Expt. I	123,915	37,917
Expt. II	27,400	4,100

To the incubation mixture described in the text, was added 1 μCi $KH^{14}CO_3$ (10 mCi/mmole) or 1 μCi $^{14}CH_3OH$ (3.3 mCi/mmole). Endogenous CO_2 in whole cells was approx. 5 mM and in cell-free particles approx. 2 mM. In Expt. I cell-free particles were used fresh; in Expt. II cell-free particles had been stored frozen.

that the stimulatory activity was entirely in the flavin fraction and could be completely replaced by the addition of FAD to the incubation mixtures.

M. barkerii can grow in 1% methanol and produce CH_4 from CH_3OH with retention of the hydrogen atoms and without equilibration with CO_2 (19). Methanol, however, is not a good substrate for other methane producing bacteria including M. M.o.H. (1). In rumen bacteria, ^{14}C-methanol was converted to $^{14}CH_4$ both by intact cells and by cell-free particles (Table 2) but at a lower rate than $^{14}CO_2$. The rate of CO_2 reduction to CH_4 was probably greater than indicated since the radioactivity of the $^{14}CO_2$ undergoes dilution with endogenous CO_2 of cell extracts. Cell-free particles when stored, lose some activity (Table 2).

In cell-free extracts of rumen bacteria the addition of up to 30 mM methanol did not decrease the rate of $^{14}CO_2$ reduction to $^{14}CH_4$ (data not shown). This suggests that methanol and CO_2 may not be reduced to CH_4 by a common pathway in rumen bacteria.

Table 3. $^{14}CH_4$ and $^{14}CO_2$ Synthesis by Cell-Free Particulate Fraction from N^5,-N^{10}-^{14}C-Methylenetetrahydrofolate, N^5-^{14}C-Methyltetrahydrofolate and ^{14}C-Methylcobalamin.

Substrate	Rates of production (cpm/mg protein/h)		
	$^{14}CH_4$	$^{14}CO_2$ (initial 30 min)	$^{14}CO_2$ (0.5 to 3 h)
5,10-$^{14}CH_2$-H_4-folate	1,740	7,060	2,310
5,10-$^{14}CH_2$-H_4-folate plus HCO_3^- (50 mM)	403	3,070	1,840
5-$^{14}CH_3$-H_4-folate	1,070	4,440	610
$^{14}CH_3$-B_{12}	60	--	--

To the incubation mixture described in the text were added: radioactive substrate (0.5 µmole, 5 x 10^5 dpm); ATP (10 mM); $MgSO_4$ (5 mM). Reaction mixtures were incubated for 6 h in the dark and rates measured as in Table 1.

It has been shown that 5,10-CH_2-H_4-folate, 5-CH_3-H_4-folate and CH_3-B_{12} are converted to CH_4 by cell-free extracts of M. barkerii and M. M.o.H. (4, 12). Rumen bacterial cell-free particulate fraction also synthesized CH_4 from 5,10-CH_2-H_4-folate and 5-CH_3-H_4-folate (Table 3). At the same time, with these intermediates there was an initial high rate of $^{14}CO_2$ formation followed after 30 min by a decreased rate of $^{14}CO_2$ release. To yield CO_2, 5-CH_3-H_4-folate and 5,10-CH_2-H_4-folate presumably are oxidized through methyl- and formyl-tetrahydrofolate to yield formate. Formate may be oxidized to CO_2 by formate dehydrogenase (ferredoxin dependent) which is present in M. M.o.H. (12). From the results in Table 3, it is impossible to state if the methyl and methylene groups of the H_4-folate derivatives first undergo oxidation to CO_2 before reduction to CH_4. The possibility that H_4-folate derivatives equilibrate with CO_2 before undergoing reduction to CH_4 is supported by the observation that $^{14}CH_4$ radioactivity is diluted by the addition of HCO_3^- to the incubation mixture (Table 3). Although

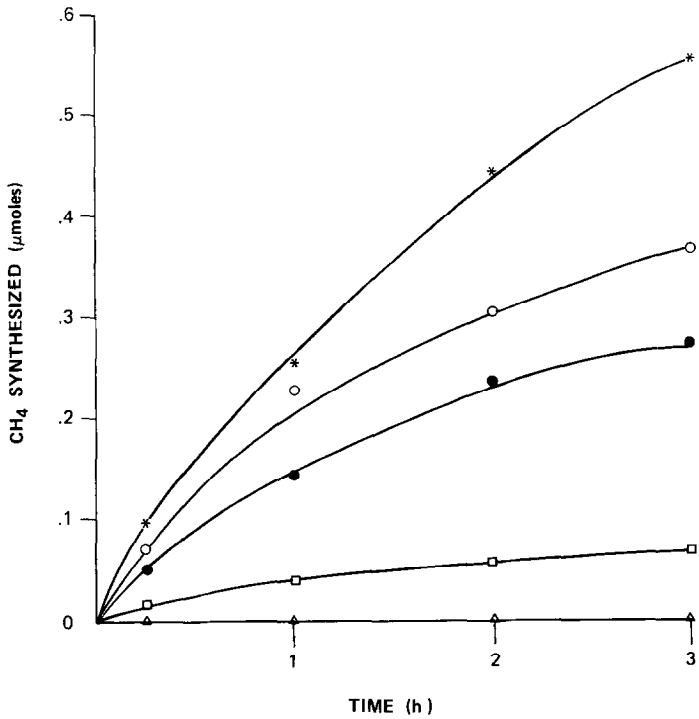

Fig. 2. CH_4 release from CH_3-B_{12} by cell-free extract (12 mg) of C. pasteurianum. The reaction mixture contained: potassium phosphate, (pH 7.0), 0.1 M; $MgSO_4$, 10 mM; ATP, 10 mM; CH_3B_{12}, □——□, 0.1 mM; ●——●, 0.5 mM; o——o, 1 mM; *——*, 3 mM; and were incubated under H_2 in room light. Lower line (Δ——Δ) represents the same incubation mixture with 3 mM CH_3-B_{12} incubated in the dark.

the conversion of the H_4-folate derivatives to CH_4 was totally dependent on the particulate fraction, CH_3-H_4-folate and CH_2-H_4-folate were readily oxidized to CO_2 by the 15,000 g supernatant fraction of rumen bacteria (unpublished data). No appreciable CH_4 was produced from methylcobalamin when precautions were taken to exclude light totally from the incubation mixtures (Table 3). If CH_3-B_{12} containing reaction mixtures are incubated under H_2 in room light, non-specific CH_4 release takes place (Fig. 2). In this experiment, more than 0.5 μmole of CH_4 was formed by soluble extracts of C. pasteurianum, a non-methane producing bacteria. There was no CH_4 release in the absence of light (Fig. 2). This emphasizes the fact that unless such experiments are carried out in the total absence of light, spurious CH_4 release from CH_3-B_{12} may result. From these

results it does not appear that free methylcobalamin is an intermediate in CH_4 synthesis by rumen bacteria. Possibly, in these bacteria, methyl group transfer can be catalyzed by B_{12} only when in the protein bound form.

To date, it had not been possible to prepare cell-free extracts of M. ruminantium that can synthesize CH_4 (16, 17). The results of the present investigation show that cell-free extracts of rumen bacteria which include M. ruminantium and M. mobilis do synthesize CH_4 but the activity resides entirely in the particulate or membrane fraction and not in the supernatant (15,000 g) fraction. Since active methanogenic cell extracts from M. M.o.H. and M. barkerii are routinely isolated in the 30,000 g supernatant (3, 4) it is possible that the methanogenic activity in cell-free extracts of rumen bacteria has been overlooked because excessively high g forces may have been used in the preparation of these extracts. It is known that M. ruminantium cells are difficult to break whereas cells from M. M.o.H. and M. barkerii are easily disrupted by sonication (1). Therefore, the methanogenic enzymes in the rumen bacteria may be much more tightly membrane bound than those in M. M.o.H. or M. barkerii. The enzyme system that reduces CO_2 to CH_4 has not been purified in M. barkerii or M. M.o.H. beyond the 30,000 g centrifugation step. It cannot be ruled out therefore, that in these organisms too, the methanogenic activity is associated with the membrane fraction.

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