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Sodium Ions and an Energized Membrane Required by *Methanosarcina barkeri* for the Oxidation of Methanol to the Level of Formaldehyde

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Methanogenesis from methanol by cell suspensions of *Methanosarcina barkeri* was inhibited by the uncoupler tetrachlorosalicylanilide. This inhibition was reversed by the addition of formaldehyde. ^{14}C labeling experiments revealed that methanol served exclusively as the electron acceptor, whereas formaldehyde was mainly oxidized to CO_2 under these conditions. These data support the hypothesis (M. Blaut and G. Gottschalk, Eur. J. Biochem. 141: 217–222, 1984) that the first step in methanol oxidation depends on the proton motive force or a product thereof. Cell extracts of *M. barkeri* converted methanol and formaldehyde to methane under an H_2 atmosphere. Under an N_2 atmosphere, however, formaldehyde was disproportionated to CH_4 and CO_2, whereas methanol was metabolized to a very small extent only, irrespective of the presence of ATP. It was concluded that cell extracts of *M. barkeri* are not able to oxidize methanol. In further experiments, the sodium dependence of methanogenesis and ATP formation by whole cells was investigated. Methane formation from methanol alone and the corresponding increase in the intracellular ATP content were strictly dependent on Na^+. If, in contrast, methanol was utilized together with H_2, methane and ATP were synthesized in the absence of Na^+. The same is true for the disproportionation of formaldehyde to methane and carbon dioxide. From these experiments, it is concluded that in *M. barkeri*, Na^+ is involved not in the process of ATP synthesis but in the first step of methanol oxidation.

Methanogenic bacteria are a specialized group of microorganisms which synthesize methane under strictly anaerobic conditions. Whereas many of them are restricted to $H_2 + CO_2$ as a methanogenic substrate, some are additionally able to convert methyl-group-containing substrates. From all methanogens isolated so far, *Methanosarcina barkeri* is metabolically the most versatile organism: besides $H_2 + CO_2$, it can utilize methanol, methylamines, and acetate (1).

In a recent investigation on the mechanism of ATP synthesis in *M. barkeri*, we have shown that methanogenesis from methanol plus hydrogen and ATP synthesis are coupled by a chemiosmotic mechanism (3). It became evident in this study that methanogenesis according to the equation

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O \quad (1)$$

was the simplest to investigate with respect to the mechanism of ATP synthesis. This is reasonable because methanogenesis from methanol alone also involves the oxidation of methanol to CO_2 and, hence, some additional reaction steps:

$$CH_3OH + H_2O \rightarrow CO_2 + 6H \quad (2)$$

$$3CH_3OH + 6H \rightarrow 3CH_4 + 3H_2O \quad (3)$$

The finding that methane formation from methanol alone but not from methanol plus H_2 is subject to inhibition by the uncoupler tetrachlorosalicylanilide (TCS) was attributed to these oxidation steps. It was concluded that the electrons derived from the first step of the methanol oxidation do not flow directly to the final acceptor of the redox level of methanol ($E_0' = 169$ mV) but to a primary acceptor with a more negative redox potential like coenzyme F_{420} ($E_0' = -360$ mV; 5), thus rendering this process energy dependent and, therefore, uncoupler sensitive. In this paper we present experiments which are in agreement with this hypothesis.

It has been shown recently for a number of methanogenic bacteria, including *M. barkeri*, that methane formation is strictly dependent on the presence of sodium ions (14). Furthermore, a stimulation by sodium of ATP synthesis has been demonstrated in *Methanobacterium thermoautotrophicum* (19). As a possible explanation for these observations, the involvement of sodium in a membrane-dependent process of energy conservation was discussed.

The effect of Na^+ on the two types of methane fermentation (equations 1, 2, and 3) was then studied. From the results obtained, it was concluded that Na^+ is not involved in the process of ATP synthesis.

MATERIALS AND METHODS

Organism and cultivation. *M. barkeri* Fusaro (DSM 804) was obtained from the German Collection of Microorganisms, Göttingen, Federal Republic of Germany. The anaerobic techniques for medium preparation and for cultivation were those of Hungate as modified by Bryant (4). The media were prepared under an atmosphere of 80% N_2 and 20% CO_2 as described by Hippe et al. (9); traces of oxygen were removed by passing the gas through a heated copper column. Methanol in a final concentration of 200 mM served as the substrate. The pH was 6.5 to 6.8, and the growth temperature was 37°C. For experiments in which the dependence on sodium ions was investigated, the cells were grown in medium containing the following (per liter of

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glass-distilled water): 40 mmol of imidazole hydrochloride, 0.5 mmol of KH_2PO_4, 0.5 mmol of $K_2HPO_4 \cdot 3H_2O$, 1.7 mmol of $MgCl_2$, 2.0 mmol of $CaCl_2$, 0.1 mmol of $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 10 mmol of ammonium acetate, 2.7 mmol of KCl, 10 ml of vitamin solution (23), 3 ml of trace element solution SL 6 (15), 1 mg of resazurin, 2.6 mmol of K_2S, 1.7 mmol of cysteine hydrochloride, and 2 ml of titanium(III) citrate solution (25); the final pH was 6.8. Growth was initiated by inoculation (10%, vol/vol) with a sample of a stock culture. The stock cultures were grown on the medium described by Scherer and Sahm (17), except that the trace element solution SL 6 (15) was used.

Preparation of cell suspensions and cell extracts. Fresh cell suspensions of *M. barkeri* were prepared for each experiment. Cells in the late-logarithmic growth phase were harvested by centrifugation and washed once with 100 mM sodium piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (Na-PIPES) buffer (pH 6.7) containing 1 mg of resazurin and 2 ml of titanium(III) citrate solution (25) per liter. The cell pellet was resuspended in the same buffer to a concentration of 10 to 20 mg of protein per ml and stored on ice until used. This suspension was used directly for experiments with whole cells, or the cells were disrupted by one passage through a French press (Aminco, Silver Spring, Md.) previously flushed with N_2. The ruptured cells were collected in a stoppered anaerobic centrifuge tube which was connected to the pressure cell via a tube and a hypodermic needle and were then centrifuged at 20,000 × *g* for 15 min. The supernatant was transferred into a Hungate tube and used directly for the assay. In experiments in which the sodium dependence was tested, freshly harvested cells from the early-logarithmic growth phase were used; they were washed twice with 50 mM potassium phosphate buffer (pH 6.8) containing 2 mM $MgCl_2$ and 5 mM dithiothreitol. NaCl was added as indicated.

The protein concentration of the cell suspensions was determined by the method of Schmidt et al. (18) and that of the cell extracts was determined by the method of Beisenherz et al. (2), with bovine serum albumin as the standard.

Experimental conditions. The experiments with cell suspensions were carried out in 58-ml bottles containing 9 or 4.5 ml of Na-PIPES buffer. The gas atmosphere was oxygen-free nitrogen or hydrogen. A portion (1.0 or 0.5 ml, respectively) of the cell suspension was added to the buffer to give a final concentration of 1 to 2 mg of protein per ml. The suspension was then preincubated for 10 min on a rotary shaker at 37°C. Additions were made as indicated for each experiment. TCS was added as an ethanolic solution; the control received ethanol only. The formaldehyde solution was freshly prepared by heating paraformaldehyde to 180°C and collecting the formaldehyde gas formed by passage through distilled water containing 10% ethanol. To study the effect of sodium ions, the experiments were done in 58-ml bottles containing 10 ml of 5 mM potassium phosphate buffer (pH 6.8) with 2 mM $MgSO_4$ and 5 mM dithiothreitol. The final concentration of protein was 0.05 to 0.2 mg/ml.

The experiments with cell extracts were carried out in 5.5-ml Hungate tubes containing, in 1 ml of assay mixture, 950 μl of the cell extract (see above), 25 μl of 0.2 M ATP, and 25 μl of 0.2 M $MgCl_2$

Methane determination. Methane was determined by gas chromatography on a Perkin-Elmer model 900 gas chromatograph (Bodenseewerk, Überlingen, Federal Republic of Germany) equipped with a flame ionization detector. A glass column (2 m by 2 mm) packed with Porapak QS (Riedel de Häen, Seelze, Federal Republic of Germany) was used. The injector, oven, and detector temperatures were 200, 100, and 250°C, respectively. Nitrogen was the carrier gas and the flow rate was 24 ml/min. Gas samples (5 μl) were directly removed from the gas phase above the cell suspension or the extract and injected into the gas chromatograph. Methane formation is given in millimoles per liter of medium.

ATP content. The intracellular ATP content was determined as described previously (3).

Labeling studies. [^{14}C]formaldehyde (specific radioactivity, 30 nCi/μmol) or [^{14}C]methanol (specific radioactivity, 33 nCi/μmol) was added to cell suspensions or cell extracts with a 10-μl syringe. For determination of the added radioactivity, a 0.5-ml sample was withdrawn with the syringe, diluted with 0.5 ml of H_2O followed by the addition of 5 ml of Quickszint 212 (Zinsser, Frankfurt, Federal Republic of Germany), and counted in a liquid scintillation counter (model LS 7500; Beckman Instruments, Inc., Fullerton, Calif.).

After the addition of the substrates, the production of methane was monitored by gas chromatography. When methanogenesis had ceased, the cell suspension or the extract was acidified by the addition of 0.1 ml of 2 M HCl per ml of medium. $^{14}CH_4$ and $^{14}CO_2$ were determined by liquid scintillation counting by a modification of the method of Zehnder et al. (24): to remove the CO_2 from the gas phase, a 1-ml gas sample was removed directly from the gas phase above the acidified medium and injected into a 2.3-ml, rubber-sealed glass vial which contained 1 ml of 0.3 M NaOH. A 1-ml sample of the CO_2-free gas phase was withdrawn and injected into the headspace of a specially manufactured scintillation vial (14 ml) containing 11.5 ml of Quickszint 501 (Zinsser). The scintillation vial was closed with a gas tight butyl rubber septum fixed with a screw cap. Of the CH_4 added into the vial, 70% was adsorbed into the scintillation fluid within 1 h. The radioactivity was then determined in the scintillation counter. For $^{14}CO_2$ determination, 0.9 ml of the 0.3 M NaOH solution mentioned above containing the adsorbed CO_2 was placed in a scintillation vial. After the addition of 5 ml of Quickszint 212, the radioactivity was counted. The amount of $^{14}CO_2$ dissolved in the liquid phase was calculated by using Bunsen adsorption coefficients (20).

After the determination of $^{14}CH_4$ and $^{14}CO_2$, the acidified medium was sparged with N_2 for 10 min. From this CO_2-free liquid, a 0.5-ml sample was taken and diluted with 0.5 ml of H_2O; its radioactivity was determined after the addition of 5 ml of Quickszint 212. This radioactivity is referred to as unconverted or assimilated.

Na^+ concentration. For determination of the Na^+ concentration, a portion of the cell suspension was freed of cells by centrifugation and diluted to a final Na^+ concentration of 0.1 to 2 mM. The concentration was measured in a flame photometer (model 700; Eppendorf-Gerätebau, Hamburg, Federal Republic of Germany).

Chemicals, gases, and radioisotopes. All chemicals were reagent grade and were purchased from E. Merck AG, Darmstadt, Federal Republic of Germany, except for PIPES which was purchased from Sigma Chemical Co., Taufkirchen, Federal Republic of Germany. ATP was obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany. [^{14}C]methanol and [^{14}C]formaldehyde were purchased from New England Nuclear Corp., Dreieich, Federal Republic of Germany. TCS was a gift from I. Booth, Aberdeen, United Kingdom. Gases were obtained from Messer Griesheim, Kassel, Federal Republic of Germany.

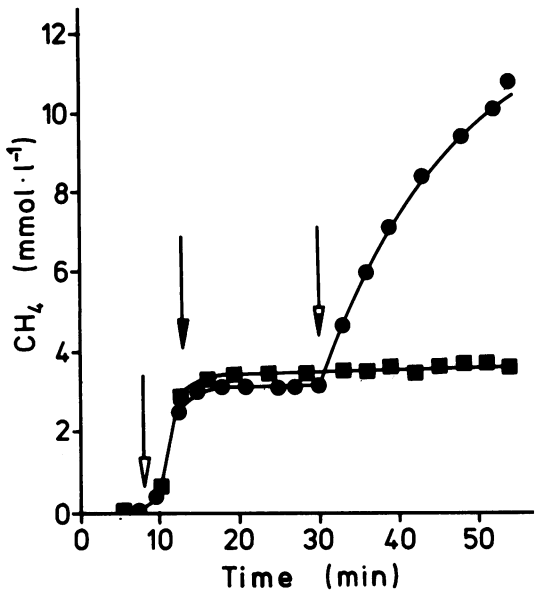

FIG. 1. Inhibition of methane formation from methanol under N_2 by TCS and reversal of this inhibition by the addition of formaldehyde. Methanol (20 mM, final concentration) (open arrow), TCS (10 μM) (closed arrow), and formaldehyde (4 mM) (semiopen arrow) were added to a 5-ml cell suspension of *M. barkeri* (1.7 mg of protein per ml) as indicated. Symbols: ■, methanol added, followed by the addition of TCS; ●, methanol added, followed by the addition of TCS and formaldehyde.

RESULTS

Methane formation from methanol plus formaldehyde in the presence of a protonophore. The inhibition of methane formation from methanol by *M. barkeri* as effected by the uncoupler TCS could result from an energy requirement of the first step of methanol oxidation; if so, it should be possible to overcome this inhibition by formaldehyde. This compound is readily convertible into methylenetetrahydromethanopterin by cell extracts of *Methanobacterium thermoautotrophicum* (7) and could function as the electron donor under uncoupled conditions.

To test this idea, we performed the experiment shown in Fig. 1. The addition of methanol to two identical resting-cell suspensions under a gas atmosphere of N_2 led to the formation of methane. As described previously (3), methanogenesis was rapidly and completely inhibited by the addition of the uncoupler TCS. After 15 min of inhibition, formaldehyde was added to one of the cell suspensions, resulting in the restoration of methanogenesis. In controls that had not received any substrate, no methane was formed by the cell suspensions used in these experiments.

This experiment suggested that methanol is reduced by electrons derived from the oxidation of formaldehyde in the presence of TCS. This assumption was confirmed by labeling experiments (Table 1). The formation of $^{14}CH_4$ and of $^{14}CO_2$ from [^{14}C]methanol or [^{14}C]formaldehyde in the presence of either unlabeled formaldehyde or unlabeled methanol was studied. Experiments 1 and 2 (Table 1) were the controls; the $^{14}CH_4/^{14}CO_2$ ratios found were close to the theoretical values, which are 3 for methanol and 1 for formaldehyde. These ratios were shifted toward methane when [^{14}C]methanol was added with unlabeled formaldehyde (Table 1, experiment 3). The reverse was true for the combination of [^{14}C]formaldehyde plus unlabeled methanol (Table 1, experiment 4). The results obtained clearly indicate that methanol was preferentially reduced to methane and formaldehyde was preferentially oxidized to CO_2. This preference for methanol as the electron acceptor was even more pronounced in the presence of TCS (Table 1, experiments 5 and 6). Very little $^{14}CO_2$ was formed from [^{14}C]methanol, and relatively little $^{14}CH_4$was formed from [^{14}C]formaldehyde.

The conclusion can be drawn from these data that the oxidation of methanol to the level of formaldehyde by whole cells requires the proton motive force or a product thereof. In further studies we investigated whether the energy requirement of this reaction could be fulfilled by the hydrolysis of ATP. This was done by experiments with cell extracts.

Methane formation from methanol and formaldehyde by cell extracts. Cell extracts of *M. barkeri* were able to synthesize methane from methanol under H_2 and from formaldehyde under both H_2 and N_2 at a linear rate until the substrate was exhausted (Fig. 2). Under N_2, however, conversion of methanol to methane occurred only at a very low rate and was incomplete even though ATP was present. This was confirmed by labeling experiments (Table 2). [^{14}C]methanol and [^{14}C]formaldehyde were reduced to $^{14}CH_4$ under H_2 as well by whole cells as by cell extracts (Table 2, experiments 1, 3, 5, and 7). Under N_2, both substrates were disproportionated by whole cells (Table 2, experiments 2 and

TABLE 1. Methanogenesis from [^{14}C]methanol or [^{14}C]formaldehyde in the presence of unlabeled formaldehyde or methanol by whole cells of *M. barkeri* under a gas atmosphere of N_2 and effect of TCS[a]

Expt no.	10 μM TCS	Concn (mmol liter^{-1}) of:			Radioactivity (Mdpm)[b]				Product (Mdpm)[b]		
		Labeled substrate	Unlabeled substrate	CH_4 formed	Added	Unconverted or assimilated	Converted[c]	Recovered[d]	$^{14}CH_4$	$^{14}CO_2$	$^{14}CH_4/^{14}CO_2$
1	−	4.7 (CH_3OH)		3.5	3.500 (100)	0.154 (4.4)	3.232 (92.4)	3.386 (96.8)	2.501 (71.5)	0.731 (20.9)	3.42
2	−	3.3 (HCHO)		1.6	2.157 (100)	0.055 (2.6)	2.205 (101.8)	2.260 (104.8)	1.087 (50.4)	1.118 (51.4)	0.98
3	−	4.8 (CH_3OH)	2.8 (HCHO)	5.4	3.593 (100)	0.153 (4.3)	3.463 (96.4)	3.616 (100.7)	2.941 (81.9)	0.522 (14.5)	5.65
4	−	4.7 (HCHO)	9.2 (CH_3OH)	7.6	3.097 (100)	0.080 (2.6)	2.923 (94.3)	3.001 (96.9)	0.918 (29.6)	2.005 (64.7)	0.46
5	+	5.0 (CH_3OH)	2.8 (HCHO)	5.7	3.756 (100)	0.042 (1.1)	3.613 (96.2)	3.655 (97.3)	3.517 (93.6)	0.096 (2.6)	35.6
6	+	4.8 (HCHO)	9.2 (CH_3OH)	8.1	3.155 (100)	0.0 (0.0)	3.066 (97.1)	3.066 (97.1)	0.389 (12.3)	2.677 (84.8)	0.15

[a] The assays were started by the addition of the substrate(s) to 10-ml cell suspensions (1 mg of protein per ml). After the cessation of methanogenesis, the distribution of the radioactivity was determined.
[b] Values in parentheses indicate percentages.
[c] Calculated from the sum of the radioactivity found in $^{14}CH_4$ and $^{14}CO_2$.
[d] Calculated from the sum of the converted radioactivity and from the unconverted or assimilated radioactivity.

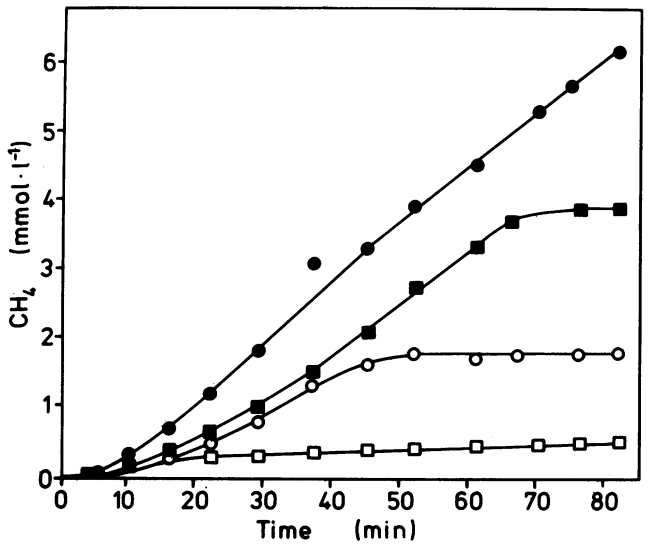

FIG. 2. Methanogenesis from methanol or formaldehyde under H_2 or under N_2 by cell extracts of *M. barkeri*. The reaction mixture, which consisted of 5 mM ATP and 5 mM $MgCl_2$ in 1 ml of cell extract (7.1 mg of protein per ml), was preincubated for 20 min at 37°C under H_2 or N_2 as indicated. At 0 min, formaldehyde or methanol was added to a final concentration of 4 or 20 mM, respectively. Symbols: ●, CH_3OH and H_2; ■, HCHO and H_2; □, CH_3OH and N_2; ○, HCHO and N_2.

4), but only formaldehyde was disproportionated by cell extracts (Table 2, experiment 8). When cell extracts were incubated with [^{14}C]methanol under N_2 (Table 2, experiment 6), very little substrate was utilized (5.1%), and a negligible amount of radioactivity was encountered as $^{14}CO_2$. This demonstrated that cell extracts of *M. barkeri* were not able to oxidize methanol.

Methanogenesis and ATP synthesis from methanol in the absence of Na^+. *M. barkeri* cells which had been harvested in the exponential growth phase were suspended in buffer containing various concentrations of Na^+ and incubated under atmospheres of N_2 or H_2. Methanol was then added. Methanogenesis from methanol depended on Na^+ if N_2 was the gas phase (Fig. 3A). This has already been reported by Perski et al. (14). In contrast, such a strict Na^+ dependence of methanogenesis was not observed under an H_2 atmosphere (Fig. 3B).

The independence of methane formation from Na^+ did not necessarily exclude the requirement of this ion for ATP synthesis. Even though methane formation was not affected by Na^+, it was conceivable that under these conditions an uncoupling between methanogenesis and ATP synthesis had occurred due to a lack of Na^+. To test this possibility, the experiments shown in Fig. 4 were performed.

Under H_2, the intracellular ATP content of resting cells increased to about the same degree upon the addition of methanol, irrespective of the sodium concentration (Fig. 4A). Under N_2, the addition of methanol led to an increase of the intracellular ATP content in the presence of 5 mM Na^+ (Fig. 4B). In the absence of Na^+, however, there was only a comparatively small increase of the intracellular ATP, paralleled by a low rate of methane production. That the small ATP content and the low rate of methanogenesis were indeed due to a lack of Na^+ is apparent from the increase in ATP content and the stimulation of methanogenesis after the addition of NaCl. A similar stimulation was achieved by Na_2SO_4.

Methane formation from formaldehyde in the absence of Na^+. Formaldehyde was disproportionated to CH_4 and CO_2 by whole cells and cell extracts of *M. barkeri* (Tables 1 and 2). The effect of Na^+ on this fermentation was also studied. The conversion of formaldehyde to methane and CO_2 did not depend on Na^+; the same rate of methane formation was observed at 0.3 and 6.3 mM Na^+ (Fig. 5). The increase of the ATP content of the cells also was not affected by Na^+ (Fig. 5).

These results indicate that a role for Na^+ exists in the oxidation of methanol, a conclusion which is supported by the experiments shown in Fig. 6. Under an N_2 atmosphere and at a low Na^+ concentration, methane formation from methanol proceeded at a low rate; it was greatly stimulated by formaldehyde.

Labeling experiments revealed that *M. barkeri* cells converted less than 3.8% of added methanol (0.3 mM) when the Na^+ concentration was below 0.3 mM. The addition of unlabeled formaldehyde resulted in an 88% consumption of the [^{14}C]methanol added under these conditions; 83% of the label was recovered as $^{14}CH_4$, and only 5% was recovered as $^{14}CO_2$. This proves the preferential use of methanol as the electron acceptor at low sodium concentrations.

TABLE 2. Conversion of [^{14}C]methanol or [^{14}C]formaldehyde under H_2 or under N_2 by whole cells or by cell extracts of *M. barkeri*[a]

Expt no.	Substrate (gas phase)	Prepn[b]	Radioactivity (kdpm)[c]				Product (kdpm)[c]		
			Added	Unconverted or assimilated	Converted[d]	Recovered[e]	$^{14}CH_4$	$^{14}CO_2$	$^{14}CH_4/^{14}CO_2$
1	$^{14}CH_3OH$ (H_2)	C	668.4 (100)	50.8 (7.6)	540.2 (80.8)	591.0 (88.4)	533.4 (79.8)	6.8 (1.0)	89.2
2	$^{14}CH_3OH$ (N_2)	C	668.4 (100)	23.0 (3.4)	642.1 (96.1)	665.1 (99.5)	522.6 (78.2)	119.5 (17.9)	4.4
3	$^{14}CH_2O$ (H_2)	C	312.0 (100)	73.5 (23.6)	204.0 (65.3)	277.5 (88.9)	198.7 (63.6)	5.3 (1.7)	37.6
4	$^{14}CH_2O$ (N_2)	C	312.0 (100)	33.1 (10.6)	252.2 (80.8)	285.3 (91.4)	139.0 (44.5)	113.2 (36.3)	1.2
5	$^{14}CH_3OH$ (H_2)	E	668.4 (100)	7.9 (1.2)	658.9 (98.6)	666.8 (99.8)	649.0 (97.1)	9.9 (1.5)	64.9
6	$^{14}CH_3OH$ (N_2)	E	668.4 (100)	629.5 (94.2)	33.8 (5.1)	663.3 (99.3)	31.1 (4.7)	2.7 (0.4)	11.8
7	$^{14}CH_2O$ (H_2)	E	312.0 (100)	57.6 (18.5)	246.1 (78.9)	303.7 (97.4)	223.8 (71.8)	22.3 (7.1)	10.0
8	$^{14}CH_2O$ (N_2)	E	312.0 (100)	31.3 (10.0)	265.9 (85.2)	297.2 (95.2)	150.7 (48.3)	115.2 (36.9)	1.3

[a] The assay mixtures contained 950 μl of cell extract (7.5 mg of protein per ml) or cells, 25μl of $MgCl_2$ (0.2 M), and 25μl of ATP (0.2 M). The assays were initiated by the addition of the label. After the cessation of methanogenesis, the distribution of the radioactivity in the products was determined.

[b] C, Whole cells; E, cell extract.

[c] Values in parentheses indicate percentages.

[d] Calculated from the sum of the radioactivity found in $^{14}CH_4$ and $^{14}CO_2$.

[e] Calculated from the sum of the converted radioactivity and from the unconverted or assimilated radioactivity.

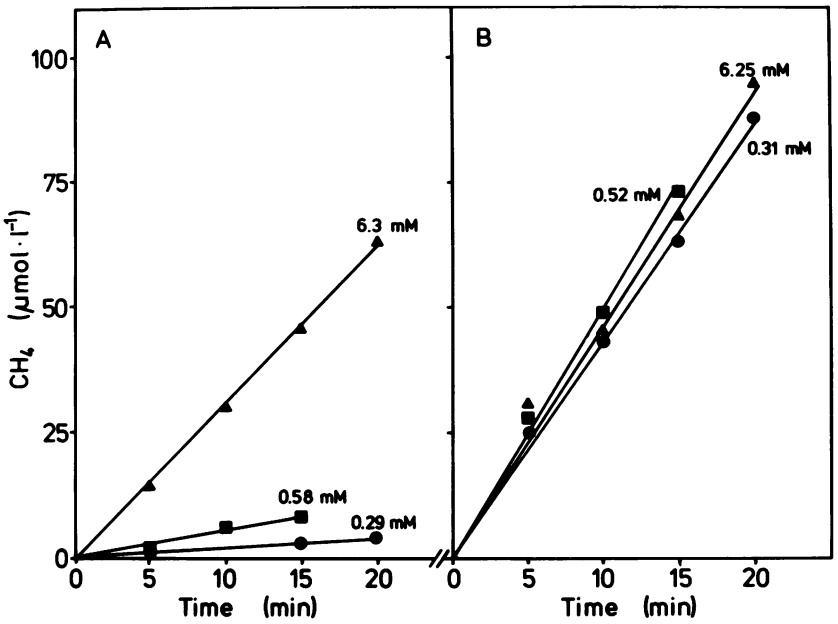

FIG. 3. Sodium dependence of methane formation from methanol by whole cells of *M. barkeri* under either N_2 (A) or H_2 (B). The cell suspensions contained various concentrations of Na^+ (added as NaCl) which are indicated in the graph. The final protein concentration was 0.06 mg/ml (A) or 0.04 mg/ml (B). At 0 min, methanol was added to a final concentration of 20 mM.

DISCUSSION

The data presented in this study allow some conclusions to be made concerning the role of sodium ions in the metabolism of methanogenic bacteria and the specific needs of the oxidation of methanol to the level of formaldehyde in *M. barkeri*. Na^+ has been shown to be required for the growth of all methanogenic bacteria that have been tested (14). This is also true for the growth of *M. barkeri* on methanol, both in the presence and in the absence of H_2 (V. Müller, unpublished data). However, methane formation by resting-cell suspensions according to equations 1 and 4 was not Na^+ dependent:

$$2HCHO \rightarrow CH_4 + CO_2 \quad (4)$$

In addition, the increased intracellular ATP content that was associated with these fermentations did not require Na^+. This finding rules out a role for Na^+ in the coupling of methane and ATP formation. It also rules out an involvement of Na^+ in the two reduction reactions that lead from the level of formaldehyde to methane and in the two oxidation reactions by which formaldehyde is converted to CO_2

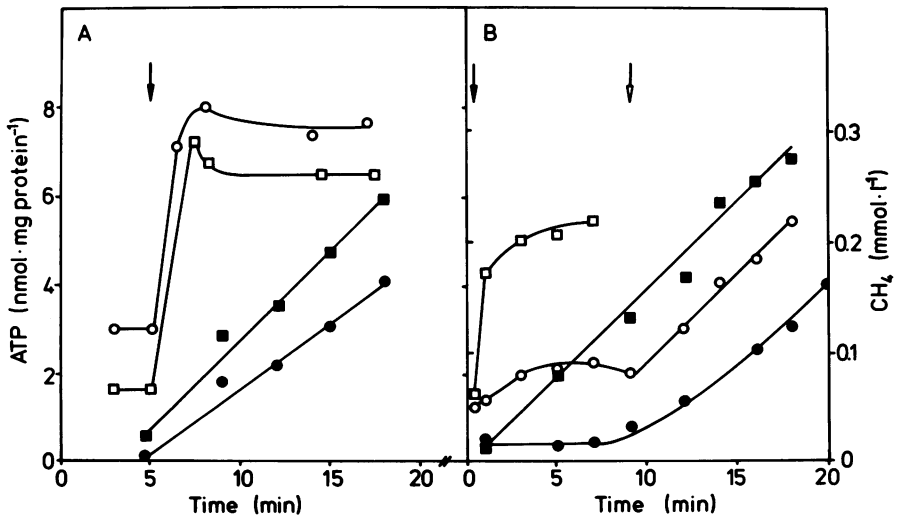

FIG. 4. Sodium dependence of methanogenesis and intracellular ATP content in whole cells of *M. barkeri* during methanol conversion under either H_2 (A) or N_2 (B). At the times indicated (closed arrows), methanol was added to 10-ml cell suspensions to a final concentration of 20 mM. Symbols: ●, CH_4 and ○, ATP at an Na^+ concentration of 0.3 mM; ■, CH_4 and □, ATP at an Na^+ concentration of 6.3 mM. At the time indicated by the open arrow, the Na^+ concentration in one of the cell suspensions was increased from 0.3 to 5 mM by the addition of NaCl. The final protein concentration was 0.08 mg/ml (A) or 0.12 mg/ml (B).

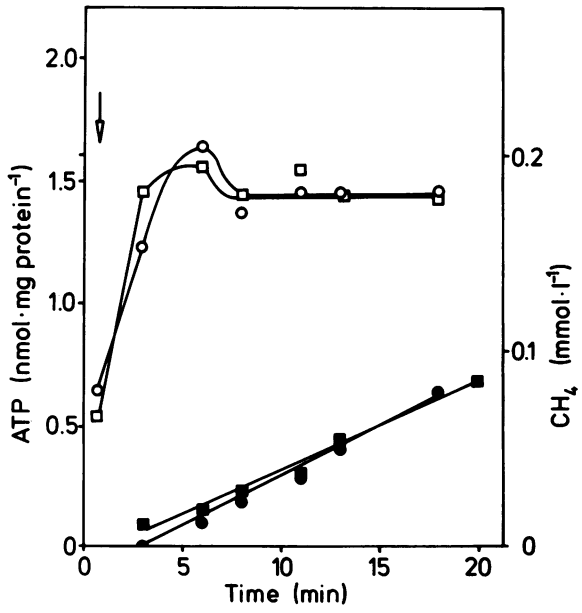

FIG. 5. Sodium dependence of methanogenesis and intracellular ATP content in whole cells of *M. barkeri* during formaldehyde conversion under N_2. At the time indicated by the arrow, formaldehyde was added to 10-ml cell suspensions to a final concentration of 1 mM. The final protein concentration was 0.2 mg/ml. Symbols: ●, CH_4 and ○, ATP at an Na^+ concentration of 0.3 mM; ■, CH_4 and □, ATP at an Na^+ concentration of 6.3 mM.

by *M. barkeri*. Methane formation from methanol with formaldehyde as the electron donor was also not Na^+ dependent. Therefore, Na^+ cannot be involved in the reductive activation of the methyltransferase by which methanol is converted to methyl-coenzyme M (21).

It has been observed that ATP synthesis driven by a potassium diffusion potential in *Methanobacterium thermoautotrophicum* is dependent on Na^+ (19). An Na^+-dependent ATP synthase was not detectable, and the operation of an Na^+-H^+ antiporter was proposed, the presence of which has now been demonstrated (P. Schönheit, personal communication). This antiporter apparently plays a role in the regulation of the pH gradient (ΔpH) across the cytoplasmic membrane of this organism. Since Na^+ is also required for methane formation at a ΔpH of 0 (P. Schönheit, personal communication), additional functions must be exerted by Na^+ in the methanobacterial cells. One additional function can be described on the basis of the results presented here. Na^+ is required for the first step of methanol oxidation in *M. barkeri*. The evidence is as follows: (i) methanol plus H_2, but not methanol alone, is converted to methane by whole cells in the absence of Na^+; (ii) methanol plus formaldehyde and formaldehyde alone yield methane and carbon dioxide in the absence of Na^+ in the expected ratio; (iii) the addition of Na^+ restores the ability of the cells to form methane and carbon dioxide from methanol alone. Hence, the oxidation of methanol to the level of formaldehyde is the only reaction in which Na^+ can be involved.

The first step of methanol oxidation seems to be considerably complex. This reaction is also responsible for the complete inhibition of methanogenesis from methanol by the protonophore TCS. This conclusion must be drawn from experiments with formaldehyde. When mixtures of methanol and formaldehyde were administered to the cells together with TCS, methanol was exclusively reduced to methane, whereas formaldehyde was predominantly oxidized to CO_2.

In correspondence with this finding, cell extracts of *M. barkeri* did not catalyze the disproportionation of methanol at all. The rate of methanogenesis was also negligible in the presence of ATP, so that it apparently depended directly on the proton motive force ($\Delta\tilde{\mu}_{H^+}$). A slow conversion of methanol to methane under N_2 catalyzed by cell extracts of *M. barkeri* was observed by Hutten et al. (10). From the stoichiometric increases of CH_4 and CO_2, which were determined by gas chromatography, the authors concluded that methanol underwent a disproportionation reaction; but since no labeling experiments were performed, they did not explicitly demonstrate that the CO_2 produced actually originated from methanol. In agreement with the experiments of Hutten et al. (10), we also observed the formation of CO_2 to a certain extent (K. Fiebig, unpublished data), but when labeled methanol was used no labeled CO_2 was found, indicating that this CO_2 originated from other sources present in the extract. The protein concentration in the experiments of Hutten et al. (10) was 10 mg/ml, so other metabolites present in the extract were possibly the source of the CO_2 produced.

Methanogenesis from formaldehyde was intensely studied with *Methanobacterium thermoautotrophicum*. In the presence of H_2 formaldehyde has been shown to be readily converted to methane by cell extracts (16). Under an N_2 atmosphere, it undergoes a disproportionation to methane and methenyltetrahydromethanopterin, methenyltetrahydromethanopterin being the compound that is formed from formaldehyde and tetrahydromethanopterin and that is actually subject to the disproportionation reactions (6–8). A

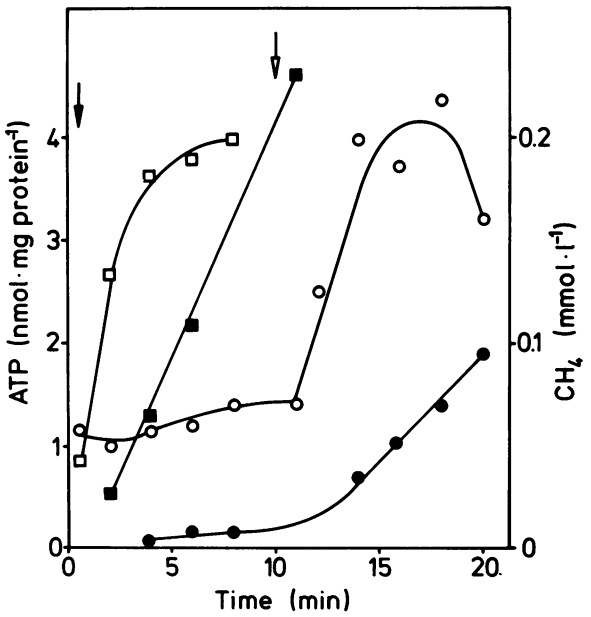

FIG. 6. Stimulation of Na^+-dependent methane formation from methanol under N_2 and of the intracellular ATP level by the addition of formaldehyde. At the time indicated by the closed arrow, methanol was added to 10-ml cell suspensions to a final concentration of 20 mM. At the time indicated by the open arrow, formaldehyde (0.5 mM, final concentration) was added to the suspension which contained 0.3 mM of Na^+. The final protein concentration was 0.19 mg/ml. Symbols: ●, CH_4 and ○, ATP at an Na^+ concentration of 0.3 mM; ■, CH_4 and □, ATP at an Na^+ concentration of 6.3 mM.

CH_4/HCHO ratio of 1/2 was observed when cell extracts of *Methanococcus jannaschii* and *Methanococcus voltae* were incubated with formaldehyde (7), suggesting that CO_2 was the oxidation product. This kind of disproportionation to methane and CO_2 has been proven with cell extracts and whole cells of *M. barkeri* by the labeling experiments presented in this paper. Formate is apparently not an intermediate in the oxidation of formaldehyde to CO_2, because *M. barkeri* was reported not to contain formate dehydrogenase (22).

In connection with the results presented it is of interest that cytochromes were found in the methanogenic bacteria that grow on methyl-group-containing substrates (11). Their redox potential is in the same range as that of the CH_3OH/HCHO couple (12). Taking all of these findings into consideration, the requirements for methanol oxidation can be defined as follows:

$$CH_3OH + X - H \xrightarrow{Na^+,\ \Delta\tilde{\mu}_{H^+},\ \text{cytochromes}} X - CH_2OH + 2e^- + 2H^+$$

Finally, it should be mentioned that a new methanogenic organism, "*Methanosphaera stadtmaniae*," has been recently described as exhibiting a very restricted substrate spectrum (13). This methanogen grows exclusively on methanol plus molecular hydrogen but not on methanol alone. "*Methanosphaera stadtmaniae*" does not contain cytochromes and is obviously unable to oxidize methanol.

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