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Formaldehyde Oxidation and Methanogenesis

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Formaldehyde oxidation by cell-free extracts of *Methanobacterium thermoautotrophicum* was shown to drive methanogenesis from CH_3-S-coenzyme M or HCHO under a nonreductive atmosphere of N_2. Under N_2 when HCHO was the sole source of carbon and reducing equivalents in the reaction, it underwent oxidation and reduction events (disproportionation), the sum of the reactions being 3 HCHO + $H_2O \rightarrow CH_4$ + 2 $HCOO^-$ + $2H^+$. This reaction predicts a CH_4/HCHO ratio of 1/3, which is in agreement with the experimental finding of 1/2.9. In extracts of the mesophilic methanogen *Methanococcus voltae* and the extreme thermophile *Methanococcus jannaschii*, which exhibited formate dehydrogenase activity, the CH_4/HCHO ratio was 1/2. NADPH stimulated methane formation from HCHO under N_2. An unidentified, oxygen-labile cofactor, the formaldehyde activation factor, present in boiled-cell extract was discovered. Methanopterin, an oxygen-stable molecule, also substituted for boiled-cell extract.

Under an H_2 gas atmosphere, CH_3-S-coenzyme M (CH_3-5-CoM) [2-(methylthio)ethanesulfonic acid] (6), HCHO (15), and CO_2 (16) are converted to CH_4 by cell-free extracts of *Methanobacterium thermoautrophicum*. In growing cells as well as in cell extracts, reducing equivalents for the reduction of C_1 moieties may be provided by hydrogenase; the precise nature of the electron transport chain is not known, but coenzyme F_{420} and flavin adenine dinucleotide (7, 12) may be involved. In methanogenic bacteria able to grow on formate as the source of carbon and energy, a formate dehydrogenase that uses an $NADP^+$-F_{420} oxidoreductase has been purified and characterized (8). This enzyme system provides the reducing power for CO_2 reduction to CH_4. In this report, we show that the oxidation of HCHO can provide the reducing power for the reduction of HCHO to CH_4.

(A preliminary report of these findings has been presented [J. C. Escalante-Semerena and R. S. Wolfe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, I13, p. 141].)

MATERIALS AND METHODS

Organisms and growth conditions. *Methanobacterium thermoautotrophicum* strain ΔH was cultured in a 200-liter fermentor (New Brunswick Scientific Co., Inc., New Brunswick, N.J.); culture conditions and storage of whole cells have been described before (4). *Methanococcus jannaschii* was grown on a chemically defined medium under an H_2-CO_2 (4:1) gas atmosphere (8a). *Methanococcus voltae* was grown under N_2 with formate as the source of carbon and energy (21).

Preparation of extracts. Cells of *Methanobacterium thermoautotrophicum* were broken by passage of a cell slurry that contained 100 g of wet cells suspended in 100 ml of 20 mM TES buffer [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] at pH 7.1 through a French pressure cell at 1.1×10^5 kPa. The broken-cell suspension was collected under a stream of H_2 in stainless-steel centrifuge tubes. All gases were passed over heated reduced copper at 350°C to remove oxygen. We use the term anoxic to indicate that solutions, glassware, syringes, and cell slurries or extracts were sparged with or handled under a blanket of scrubbed gas. Each tube was sealed, and the suspension was centrifuged at 48,000 × *g* and 4°C. Tubes were transferred into an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.) that contained an atmosphere of 97% N_2–3% H_2, and the supernatant fluid in each tube was decanted into serum vials. Each vial was sealed with a butyl rubber stopper and transferred out of the anaerobic chamber. The contents of each vial were pressurized with H_2 (100 kPa) and stored at −70°C. A cell-free extract of *Methanococcus jannaschii* (32 mg of protein ml^{-1}) or *Methanococcus voltae* (57 mg of protein ml^{-1}) was obtained under anoxic conditions by osmotic shock of 30 g (wet weight) of *Methanococcus jannaschii* cells or 45 g (wet weight) of *Methanococcus voltae* cells in 60 ml of 25 mM TES buffer (pH 6.8) that contained 2 mM 2-mercaptoethanol. A 1-mg amount of DNase and 1 mg of RNase (Sigma Chemical Co., St. Louis, Mo.) were added to the cell suspension and incubated at room temperature for 30 min inside the anaerobic chamber. The broken-cell suspension was dispensed into stainless-steel tubes, which were sealed and centrifuged for 30 min at 48,000 × *g* and 4°C. The supernatant fluid was dispensed and stored as described for *Methanobacterium thermoautotrophicum*. Cell extract of *Methanococcus jannaschii* was a gift from W. J. Jones, and that of *Methanococcus voltae* was a gift from C. W. Pratt.

Anoxic column chromatography of cell-free extracts was performed as previously described (5). About 30 ml of extract was loaded onto a Sephadex G25 (medium grade) column (2.5 by 102 cm) previously equilibrated with 30 mM Tris-chloride buffer at pH 7.0 containing 10 mM 2-mercaptoethanol. Fractions of the eluate that were free of low-molecular-weight factors (as determined by their inability to catalyze methanogenesis from CO_2, CH_3-S-CoM, or HCHO without addition of boiled-cell extract [BCE]) were pooled, pressurized with N_2 (100 kPa), and stored at −70°C. This extract is referred to as Sephadex G25-treated extract.

To prepare BCE, a slurry of cells in water of *Methanobacterium thermoautotrophicum* (50 g [wet weight] in 100 ml), *Methanococcus jannaschii* (10 g [wet weight] in 20 ml), or *Methanococcus voltae* (10 g [wet weight] in 20 ml) was heated in a boiling-water bath under a stream of N_2 with occasional stirring for 30 min. This suspension was cooled under N_2, sealed, and transferred into an anaerobic chamber

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where it was dispensed into stainless-steel centrifuge tubes. Each tube was sealed, and the suspension was centrifuged for 30 min at 48,000 × *g* and 4°C. After centrifugation the supernatant fluid was sealed in serum vials inside an anaerobic chamber. The contents of each vial were pressurized with N_2 (100 kPa) and stored at −20°C. BCE of *Methanococcus jannaschii* and *Methanococcus voltae* were a gift from W. J. Jones.

Preparation and assay of component B. Component B, a required component of the CH_3-S-CoM methylreductase system, was purified by anoxic anion-exchange chromatography and gel filtration and was assayed as described by R. S. Tanner (Ph.D. thesis, University of Illinois, Urbana, 1982). A typical assay mixture contained: TES buffer at pH 6.9, 12 μmol; ATP, 0.6 μmol; $MgCl_2 \cdot 6H_2O$, 4 μmol; CH_3-S-CoM, 200 nmol; and double-pass Sephadex G25-treated extract, 1.0 mg of protein. Preparations containing component B were added as desired. The assay volume was 120 μl and the gas atmosphere was H_2. For the purification of component B the BCE from 118 g of wet cells was acidified with 6 N HCl to pH 4.8, centrifuged at 10,000 × *g* and 25°C to remove insoluble material, and loaded on a bed volume (5 by 5 cm) of DEAE-Sephadex A25 anion-exchange resin equilibrated with 30 mM sodium acetate (pH 4.8) that contained 1 mM dithiothreitol (Sigma Chemical Co.). The column was then washed with 1 bed volume of equilibrating buffer followed by 1 bed volume of equilibrating buffer containing 160 mM NaCl. Then a threefold bed volume linear gradient (160 to 300 mM NaCl) was applied to the resin to elute bound material. Fractions containing component B were pooled, lyophilized, resuspended in anoxic water, and loaded onto a column (2.5 by 98 cm) of Sephadex G15 (superfine) equilibrated with water. The flow rate of elution was 30 ml h^{-1}. Fractions that contained component B were pooled, lyophilized, and resuspended in 10 ml of anoxic water. A 10-μl sample of this solution stimulated the conversion of CH_3-S-CoM to CH_4 under H_2 by Sephadex G25-treated extract at a rate of 1 μmol of CH_4 h^{-1} mg of protein^{-1}. In the absence of added component B CH_4 evolved at a rate of 0.2 μmol of CH_4 h^{-1} mg of protein^{-1}. This preparation of component B was used throughout the experiments.

Preparation of FAF and methanopterin. Formaldehyde activation factor (FAF) was purified from crude BCE by anoxic anion-exchange chromatography on DEAE-Sephadex A25 at pH 4.8. The conditions for the purification step are identical to those described in the purification of component B. Methanopterin was purified by the method described by Leigh and Wolfe (11) and was a gift from John A. Leigh.

Assay methods. Assays for methane formation were performed in stoppered precalibrated vials (6). The standard reaction contained 16 μmol of TES buffer (pH 6.9), 4 μmol of $MgCl_2 \cdot 6H_2O$, and 0.8 μmol of ATP. Enzyme preparations and cofactors were added as desired. The final assay volume was 200 μl. The gas atmosphere was H_2 or N_2 as indicated. The reaction was initiated by transfer of the vials into a water bath at 60°C (*Methanobacterium thermoautotrophicum*), 37°C (*Methanococcus voltae*), or 80°C (*Methanococcus jannaschii*). The assay mixture was prepared at room temperature before starting the reaction by shifting to the desired assay temperature. When cell-free extract of *Methanococcus voltae* was used, the reaction mixture was maintained on ice before transferring to a 37°C water bath. Methane formation was followed by sampling the headspace in the vial with a Hamilton gas-tight syringe (Hamilton Co., Reno, Nev.). A 20-μl sample was injected into a Packard gas chromatograph equipped with a flame ionization detector and a Porapak Q column. Specific activity is defined in nanomoles of CH_4 per minute per milligram of protein. Formate dehydrogenase (FDH) was assayed as reported previously (17). The assay was performed at 60°C when cell-free extracts of *Methanobacterium thermoautotrophicum* were tested for FDH activity.

Chemicals. CH_3-S-CoM was synthesized as previously described (14); HS-CoM (2-mercaptoethanesulfonic acid) was purchased from MC/B Manufacturing Chemist, Inc., Cincinnati, Ohio. All other reagents were reagent grade and were used without further purification.

Analytical procedures. Protein in extracts was estimated by measuring the turbidity at 400 nm in 20% trichloroacetic acid. Standard curves for protein determinations were linear between 10 and 50 μg of bovine serum albumin (10). Free formate was determined as described previously (18). HCHO was determined by the procedure of Nash (13) at 58°C for 5 min.

RESULTS

When HCHO was incubated under H_2 in the presence of Sephadex G25-treated extract, it was quantitatively converted to CH_4 (Fig. 1). The specific activity for CH_4 formation was 19 nmol of CH_4 min^{-1} mg of protein^{-1}. When H_2 was replaced by N_2, HCHO was readily oxidized to generate reducing equivalents for reduction of HCHO to CH_4 (disproportionation events). The specific activity for methanogenesis from HCHO under N_2 was 6.5 nmol of CH_4 min^{-1} mg of protein^{-1}, and the yield of CH_4 obtained was 35% of that obtained under H_2 (Fig. 1).

In the presence of CH_3-S-CoM the oxidation of HCHO provided the methylreductase system with the reducing equivalents needed to reduce CH_3-S-CoM to CH_4. Whenev-

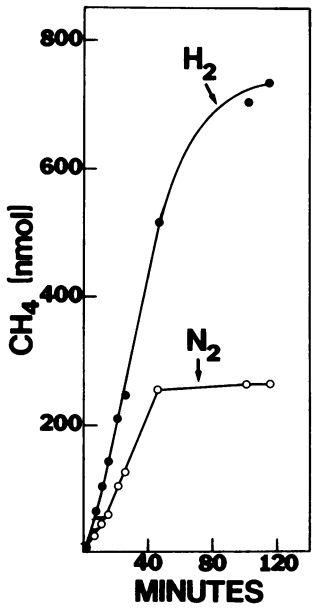

FIG. 1. Time course of methanogenesis from HCHO. The reaction mixture contained the following: HCHO, 850 nmol; HS-CoM, 250 nmol; Sephadex G25-treated extract, 1.0 mg of protein; BCE, 20 μl. The atmosphere in the headspace of the reaction vials was H_2 or N_2 as indicated. Other components of the reaction mixture are described in the text. No CH_4 evolved in the absence of added HCHO.

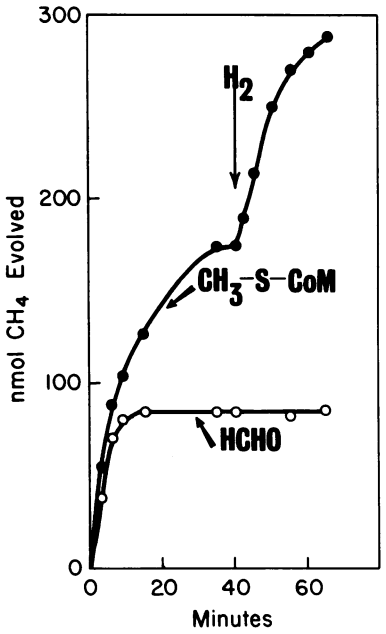

FIG. 2. Addition of H_2 to an HCHO-depleted reaction drives methanogenesis from unreacted CH_3-S-CoM. Each reaction mixture contained the following: cell-free extract, 3 mg of protein; HCHO, 270 nmol; BCE, 10 μl. The reaction was performed under N_2. One of the reaction mixtures (●) also contained 300 nmol of CH_2-S-CoM; the other one (○) contained only HCHO. Other components are described in the text. At completion, 179 μmol of H_2 was injected into the reaction vials. Each experiment was performed in duplicate. CH_4 was not formed in the absence of HCHO.

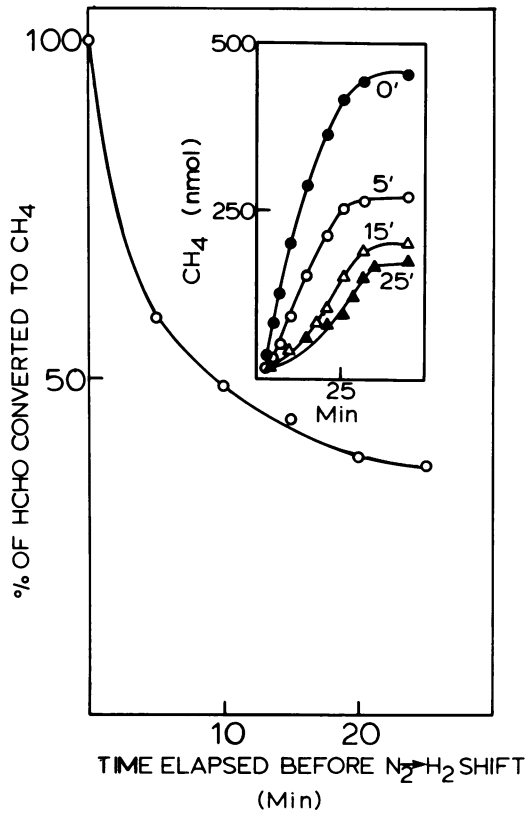

FIG. 3. Availability of HCHO to the methanogenic pathway under H_2 after preincubation of the reaction mixture under N_2. The reaction mixtures contained the following: BCE, 50 μl; Sephadex G25-treated extract, 1.1 mg of protein; HCHO, 440 nmol. Other components are described in the text. The reaction vials were incubated at 60°C under N_2. An excess of H_2 (42 μmol) was introduced into the vials at the times indicated in the inset. Final yields of CH_4 evolved were then divided by the starting amount of substrate added and plotted as percentage of HCHO converted to CH_4. Each point represents a separate experiment. The inset shows the time course of CH_4 evolution for four of the experiments.

er CH_3-S-CoM was added in excess over HCHO in the reaction mixture, some of the CH_3-S-CoM added was not converted to CH_4 due to a dearth of reducing power. The addition of H_2 gas to the system at the end of the reaction stimulated the reduction of remaining CH_3-S-CoM (Fig. 2). More oxidized or reduced compounds than HCHO, i.e., $HCOO^-$ or CH_3OH, did not substitute for HCHO in the reaction. When HCHO was present in the reaction as the sole source of carbon and reducing power, no additional CH_4 was evolved after the introduction of H_2 to the reaction vial. At completion, the ratio of CH_4 produced to HCHO added was 1/2.9. Results presented in Fig. 3 show that the percentage of HCHO converted to CH_4 under H_2 was related to the length of time the system was incubated under N_2 before introduction of an excess of H_2 into the system. The percentage of HCHO oxidized was limited to approximately 66% of the substrate added. In a control experiment in which no H_2 was added, only 35% of the substrate was converted to CH_4 (data not shown).

No free formate was found when assayed by endpoint determination with the formyl-tetrahydrofolate synthetase assay. Formate was quantitatively recovered when it was added to the reaction mixture as an internal standard. FDH activity was not detected in cell-free extracts of *Methanobacterium thermoautotrophicum*. Addition of increasing amounts of NADPH to the reaction mixture containing Sephadex G25-treated extract enhanced the rate of methane formation from HCHO under N_2 at a linear rate, from 132 nmol of CH_4 h^{-1} with no NADPH added to the system to 495 nmol of CH_4 h^{-1} when the final concentration of NADPH added was raised to 4 mM, showing a 3.75-fold increase in the rate of the reaction.

FAF. When component B of the methylreductase system was added instead of BCE to a reaction mixture containing Sephadex G25-treated extracts, no methanogenesis occurred from HCHO under N_2, whereas under H_2 component B promoted the reduction of HCHO to CH_4 under an H_2 atmosphere (Fig. 4). This indicated the presence of an unidentified factor in BCE required for the reaction. Single additions or combinations of flavin adenine dinucleotide, flavin mononucleotide, NAD^+, $NADP^+$, coenzyme F_{420}, or factor F_{430} in the presence of component B did not substitute for BCE. Figure 5 shows the location of the methanogenesis-stimulating fractions along the elution profile of a DEAE-Sephadex A25 anion-exchange column at pH 4.8 to which acidified BCE was applied under anaerobic conditions. The peak of activity eluted at 200 mM NaCl. These fractions stimulated methanogenesis from CH_3-S-CoM under N_2, with HCHO serving as the source of reducing equivalents. This new activity was referred to as the FAF. Methanopterin substituted for BCE in the reaction (Fig. 6); saturation of the reaction mixture was reached at a final concentration of 130 μM. The maximum rate attained was 710 nmol of CH_4 h^{-1}. Addition of the cofactor also decreased the lag observed before the onset of CH_4 evolution, from 14.5 min in the

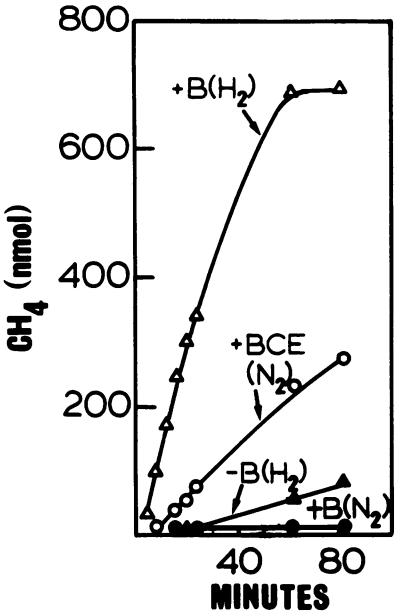

FIG. 4. Effect of component B of the methylreductase system on disproportionation of HCHO under N_2. The reaction mixture contained the following: HS-CoM, 250 nmol; HCHO, 850 nmol; Sephadex G25-treated extract, 1.5 mg of protein. Other components are described in the text. Symbols: Under N_2, with component B (10 μl) (●) or BCE (○); under H_2, with (△) or without (▲) component B.

absence of methanopterin to 10 min at saturation. Final yields were taken after 52 min of reaction time. At this point all of the C_1 units from CH_3-S-CoM were accounted for as CH_4 (data not shown). When methanopterin was omitted, no CH_4 was evolved.

Activity in other methanogens. Cell-free extract of *Methanococcus voltae* grown on $HCOO^-$ was used to study the

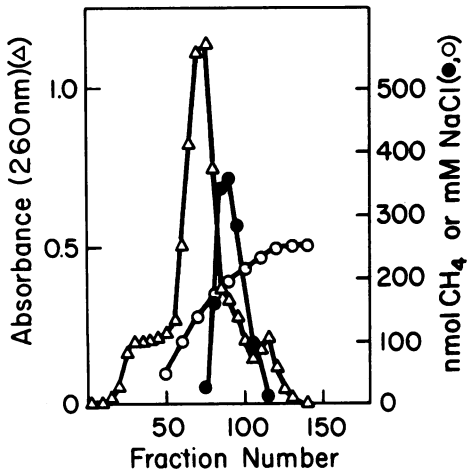

FIG. 5. Anoxic anion-exchange chromatography of BCE at pH 4.8. The assay mixtures for methanogenesis under N_2 contained the following: component B, 10 μl; HCHO, 580 nmol; HS-CoM, 250 nmol; Sephadex G25-treated extract, 1.0 mg of protein; column fraction, 20 μl. Other components are described in the text. Symbols: Location of methanogenesis-stimulating fractions (●); absorbance at 260 nm (△); molarity of NaCl (○). A 20-μl sample from each fraction was used in the methanogenic assay.

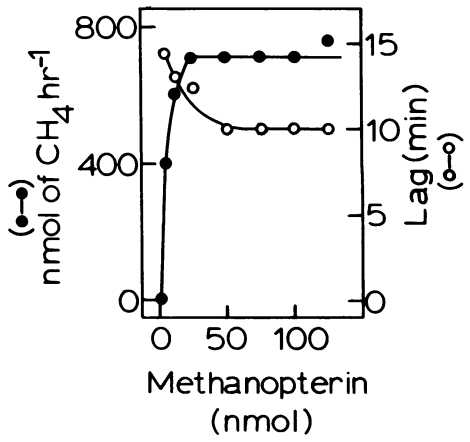

FIG. 6. Stimulatory effect of methanopterin on the HCHO-driven conversion of CH_3-S-CoM to CH_4. The reaction mixture contained the following: Sephadex G25-treated extract, 1.9 mg of protein; CH_3-S-CoM, 340 nmol; HCHO, 730 nmol; methanopterin, as indicated. The reaction was performed under an N_2 atmosphere. Other components of the reaction mixture are described in the text. Final yields were taken after 52 min of reaction time. At this point all of the C-1 units from CH_3-S-CoM were accounted for as CH_4. The extract was free of methanopterin. After 118 min of reaction time, no CH_4 was formed when methanopterin was not added to the reaction mixture.

disproportionation of HCHO. The ratio of $CH_4(N_2)/CH_4(H_2)$ was 0.497 at 92% completion of the reaction. When cell-free extract of *Methanococcus jannaschii* grown on H_2-CO_2 was used for the reaction, the ratio of $CH_4(N_2)/CH_4(H_2)$ was 0.49, also at 92% completion of the reaction (Table 1).

DISCUSSION

In each experiment in which HCHO underwent disproportionation events, a CH_4/HCHO ratio of 1/2.9 was observed. This stoichiometry indicates that the oxidation of HCHO stops at the formyl level of oxidation, 2 mol of HCHO being oxidized to generate enough reducing power to reduce 1 mol of HCHO to CH_4. Other results support this concept. (i) Formate did not substitute for HCHO in the reaction. (ii) FDH activity has been described only in methanogens able to grow on formate: *Methanobacterium formicicum* (17), *Methanococcus vannielii* (8), *Methanococcus voltae* (C. W. Pratt, personal communication), and *Methanococcus jan-*

TABLE 1. Disproportionation of HCHO by cell-free extracts of *Methanococcus voltae* and *Methanococcus jannaschii*

Organism	HCHO added (nmol)	nmol of CH_4 formed		CH_4 formed (N_2)/ CH_4 formed (H_2)
		Under N_2	Under H_2	
M. jannaschii	500	224	460[b]	0.487
M. voltae	750	343	690[d]	0.497

[a] Cells were grown on H_2-CO_2 (4:1) as the source of energy and carbon. Growth temperature, 80°C.

[b] Yields of CH_4 were obtained after 38 min of reaction time (92% completion). The assay mixture contained 2.9 mg of cell-free extract and 50 μl of BCE. Other components are described in the text. The assay temperature was 37°C.

[c] Cells were grown on $HCOO^-$ as the source of carbon and energy. Growth temperature, 37°C.

[d] Yields of CH_4 were obtained after 13 min of reaction time (92% completion). The assay mixture contained 4.8 mg of cell-free extract and 50 μl of BCE. The assay temperature was 80°C.

naschii (W. J. Jones, personal communication). FDH was reported to be absent in *Methanosarcina barkeri* grown on CH_3OH or CH_3NH_2 (20). *Methanobacterium thermoautotrophicum* only grows on H_2 and CO_2, and an FDH has not been reported. No FDH activity was found in cell-free extracts of *Methanobacterium thermautotrophicum*. Although the experimental results suggested that formate was the product of the reaction, free formate was not detected after completion of the reaction, indicating possibly a bound formyl moiety.

These results suggest that the disproportionation of HCHO under N_2 proceeds as indicated by the following favorable reactions (19). It must be emphasized that HCHO, $HCOO^-$, and CH_3OH represent oxidation states only and should not be interpreted as the actual intermediates:

(i) $2\ HCHO + 2\ H_2O \rightarrow 2\ HCOO^- + 2\ H_2 + 2\ H^+$
$\Delta G^{\circ\prime} = 2(-23.4\ kJ\ mol^{-1})$

(ii) $HCHO + H_2 \rightarrow CH_3OH$
$\Delta G^{\circ\prime} = -44.8\ kJ\ mol^{-1}$

(iii) $CH_3OH + H_2 \rightarrow CH_4 + H_2O$
$\Delta G^{\circ\prime} = -112.5\ kJ\ mol^{-1}$

(iv) $3\ HCHO + H_2O \rightarrow CH_4 + 2\ HCOO^- + 2H^+$
$\Delta G^{\circ\prime} = -204.1\ kJ\ mol^{-1}$

Our results indicated that the oxidized C-1 units generated during the disproportionation of HCHO were not converted to CH_4 when an excess of H_2 was introduced into the system. This may be explained by the generation of a product that cannot donate the C-1 unit to the methanogenic pathway, or alternatively by the inability of the enzyme system to reduce the C-1 unit to CH_4 under H_2 after prolonged incubation under N_2. This problem is currently under investigation. In cell-free extracts of methanogens that are able to grow on formate as the carbon and energy source, the predicted set of reactions would be:

(i) $HCHO + H_2O \rightarrow HCOO^- + H_2 + H^+$
$\Delta G^{\circ\prime} = -23.4\ kJ\ mol^{-1}$

(ii) $HCOO^- + H_2O \rightarrow HCO_3^- + H_2$
$\Delta G^{\circ\prime} = +1.3\ kJ\ mol^{-1}$

(iii) $HCHO + H_2 \rightarrow CH_3OH$
$\Delta G^{\circ\prime} = -44.8\ kJ\ mol^{-1}$

(iv) $CH_3OH + H_2 \rightarrow CH_4 + H_2O$
$\Delta G^{\circ\prime} = -122.5\ kJ\ mol^{-1}$

(v) $2\ HCHO + H_2O \rightarrow CH_4 + HCO^{3-} + H^+$
$\Delta G^{\circ\prime} = -179.4\ kJ\ mol^{-1}$

This scheme predicts a CH_4/HCHO ratio of 1/2. When cell-free extract of *Methanococcus voltae* was used, CH_4/HCHO = 1/2.01, whereas in the case of *Methanococcus jannaschii* a CH_4/HCHO ratio of 1/2.05 was observed.

Although crude and Sephadex G25-treated cell-free extracts of *Methanobacterium thermoautotrophicum* oxidized HCHO under a nonreductive atmosphere of N_2 and drove methanogenesis from CH_3-S-CoM or HCHO, the rate of methanogenesis under an N_2 atmosphere was slower than that under H_2. A simple explanation for these results is that under N_2 the concentration of reducing equivalents available to the enzymatic system is limited. Due to the inhibitory effect of HCHO on methanogenesis from HCHO under H_2 (J. C. Escalante-Semerena, Ph.D. thesis, University of Illinois, Urbana, 1983), it was not possible to add an excess of substrate. NADPH stimulated the rate of methanogenesis under N_2 with HCHO as the source of reducing equivalents and carbon. NADPH reduces CH_3-S-CoM to CH_4 under N_2 with no other source of reducing power present, and an NADPH-coenzyme F_{420} oxidoreductase was postulated to be responsible for the transfer of electrons to the methylreductase system (3). However, $NADP^+$ has not been identified as the electron carrier involved in the oxidation of HCHO. A factor in BCE was required for the conversion of formaldehyde to methane by cell extracts. We referred to this factor as the FAF. Component B (6) of the methylreductase system did not substitute for the FAF; however, methanopterin (9, 11) did substitute, suggesting that FAF and methanopterin may be related.

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