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Tetrahydromethanopterin-Dependent Methanogenesis from Non-Physiological C_1 Donors in *Methanobacterium thermoautotrophicum*

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Methanogenesis from the non-physiological C_1 donors thioproline, thiazolidine, hexamethylenetetramine, formaldehyde (HCHO), and $HOCH_2$-S-coenzyme M (CoM) was catalyzed by cell extracts of *Methanobacterium thermoautotrophicum* under a hydrogen atmosphere. Tetrahydromethanopterin (H_4MPT) and HS-CoM were required in the reaction mixture. The non-physiological compounds were found to be in chemical equilibrium with HCHO, which has been shown to react spontaneously with H_4MPT to form methylene-H_4MPT, an intermediate of the methanogenic pathway at the formaldehyde level of oxidation. Highfield (360 MHz) ^1H and ^{13}C nuclear magnetic resonance studies performed on the interaction between HCHO and HS-CoM showed that these compounds are in equilibrium with $HOCH_2$-S-CoM and that the equilibrium is pH dependent. When methanogenesis from the non-physiological donors was followed under a nitrogen atmosphere, the C_1 moiety from each compound underwent a disproportionation, forming methenyl-H_4MPT$^+$ and methane. The compounds tested served as substrates for the enzymatic synthesis of methenyl-H_4MPT$^+$.

Thiazolidine (TAD), thioproline (TPR), and 2-(hydroxymethylthio)ethanesulfonic acid ($HOCH_2$-S-coenzyme M [CoM]) (18) have been previously reported to serve as C_1 donors to the methanogenic pathway in crude cell extracts of methanogenic bacteria. $HOCH_2$-S-CoM was proposed as the intermediate of the pathway at the formaldehyde level of oxidation, whereas it was considered unlikely that the other compounds were actual intermediates (8, 18, 19). In this study, we add to this list of C_1 donors hexamethylenetetramine [$(CH_2)_6N_4$] (HMT) and document that these compounds, including $HOCH_2$-S-CoM, require tetrahydromethanopterin (H_4MPT) (5, 21) and 2-mercaptoethanesulfonic acid (HS-CoM) for conversion of the C_1 unit to CH_4 under H_2. The common link among these compounds is their chemical equilibrium with HCHO. HCHO has been shown to react spontaneously with H_4MPT to generate methylene-H_4MPT, an intermediate of the methanogenic pathway at the formaldehyde level of oxidation (6a). In the case of serine, an H_4MPT-dependent serine transhydroxymethylase is proposed to explain the synthesis of methylene-H_4MPT.

MATERIALS AND METHODS

Preparation of extracts and coenzymes. *Methanobacterium thermoautotrophicum* strain ΔH was mass cultured in a 200-liter fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.); culture conditions and storage of whole cells have been described previously (7). The preparation of cell extracts and cofactor-deficient Sephadex G-25-treated extract was performed as previously reported (9). Boiled-cell extract was prepared as described elsewhere (4); 50 g of cell paste (wet weight) was used for this purpose. Highly purified F_{420} and H_4MPT were prepared as previously described (1, 2, 5).

Reagents. HS-CoM was purchased from Manufacturing Chemists, Inc., Cincinnati, Ohio. $HOCH_2$-S-CoM was synthesized by the method of Romesser and Wolfe (18) and was a gift from J. A. Romesser. HCHO, HMT, TPR, TAD, PIPES buffer [piperazine-N,N′-bis(2-ethanesulfonic acid)], magnesium acetate, and 2-mercaptoethanol were purchased commercially as reagent grade chemicals. Flavin adenine dinucleotide (FAD), cyanocobalamin (CN-Cbl), ATP, and acetylacetone were purchased from Sigma Chemical Co., St. Louis, Mo. [^{13}C]formaldehyde (20% aqueous solution; 90 atom% ^{13}C) was obtained from Merck Sharp & Dohme, Rahway, N.J. ^2H_2O (100 atom% ^2H) was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Paraformaldehyde was obtained from Fisher Chemical Co., Fair Lawn, N.J.

Assay for methane formation. Assays for methane formation were performed in precalibrated vials (20), and methane evolution was monitored as previously reported (7). The standard reaction mixture contained, unless modified where indicated, 19 μmol of PIPES buffer (pH 6.3), 0.8 μmol of ATP, 4 μmol of magnesium acetate, 5 nmol of FAD, 2 nmol of CN-Cbl, 6 nmol of coF_{420}, boiled-cell extract, and enzyme preparations as desired. The final volume was 0.2 ml. The gas atmosphere inside the reaction vial was H_2 or N_2 as indicated.

Assay for H_4MPT. A spectrophotometric assay for H_4MPT has been previously described (6a). The reaction was followed by an increase in A_{340} as methenyl-H_4MPT was formed by the oxidation of methylene-H_4MPT. Apparent K_m values were determined by Lineweaver-Burk double-reciprocal plotting of kinetic data obtained for each C_1 donor.

^{13}C and ^1H spectroscopy. Nuclear magnetic resonance (NMR) spectra were obtained in ^2H_2O on a Nicolet 360-MHz Fourier transform spectrometer equipped with vertical probes for 5-mm tubes for both ^{13}C and ^1H spectroscopy. For ^1H NMR spectra, the observed frequency was 360.061 MHz; the sweep width was 2,000 Hz; the pulse width was 3 μs; and the postacquisition delay was 500 μs. For ^{13}CNMR spectra, the observed frequency was 90.546 MHz; the sweep width was 10,000 Hz; the pulse width was 2 μs; the post-acquisition delay was 1s for broad-band ^1H-decoupled spectra and 1.5 s for single-frequency, off-resonance spectra; and the decoupler frequency was 360.061 MHz for broad-band ^1H-decoupled spectra and 360.058 MHz for single-fre-

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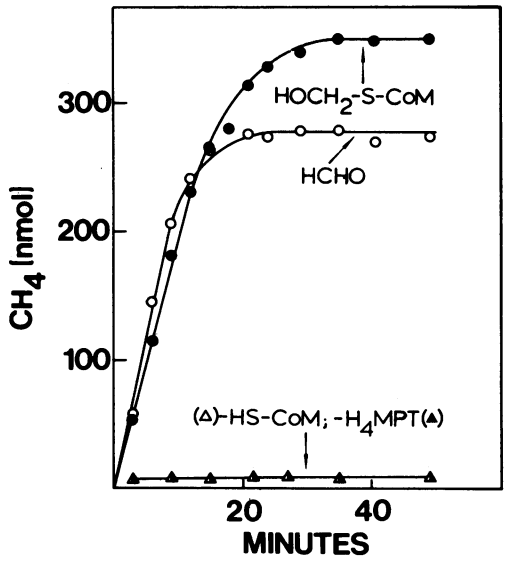

FIG. 1. Methanogenesis under H_2. The reaction mixtures contained, unless omitted. 250 nmol of HCHO, 315 nmol of $HOCH_2$-S-CoM, Sephadex G-25-treated extract, 0.9 mg of protein. 38 nmol of H_4MPT, or 250 nmol of HS-CoM. Other components and conditions are described in the text.

quency, off-resonance spectra. Broad-band ^1H-decoupled spectra were obtained with Levitt-Freeman cycle decoupling. The proton 180° pulse was 180 μs. The chemical shift standards were 3-(trimethylsiyl)propionic acid (Aldrich) (0.0 ppm) for ^1H NMR spectra and 1,4-dioxane (Mallinckrodt, Inc., St. Louis, Mo.) (66.5 ppm) for ^{13}C NMR spectra. The temperature was maintained at 18°C unless otherwise indicated. The p^2H of the sample containing HS-CoM and HCHO (120 μmol each) was 4.8.

Preparation of HS-CoM and HCHO for ^{13}C and ^1H NMR spectroscopy. Three repeated lyophilizations of HS-CoM, Na^+ (120 μmol) in ^2H_2O ensured the exchange of protons for deuterons. Paraformaldehyde was hydrolyzed under vacuum in anoxic ^2H_2O for 24 h at 100°C. The concentration of monomeric, hydrated HCHO was determined to be 4 M by the method of Nash (16). The solution of HCHO in ^2H_2O was maintained under anoxic conditions in a serum vial sealed with a black rubber stopper.

Other procedures. Protein in extracts was estimated by measuring the turbidity at 400 nm in 20% trichloroacetic acid (12). Standard curves for protein determinations were linear between 10 and 50 μg of bovine serum albumin. Formaldehyde was determined by the method of Nash (16), the reaction time being 5 min at 58°C. The rate of formation of 2,6-dimethyl-3,5-diacetyl-1,4-dihydropyridine, the product of the Hantzsch reaction between acetylacetone and ammonium chloride with formaldehyde or $HOCH_2$-S-CoM at 58°C, was followed by an increase in the A_{412} of the solution. The reaction mixture (3 ml) was preincubated for 10 min at 58°C before the addition of a 1-μl sample of HCHO (70 mM) or $HOCH_2$-S-CoM (60 mM).

RESULTS

Methanogenesis with H_2 as electron donor. The H_4MPT-dependent conversion of HCHO and $HOCH_2$-S-CoM to CH_4 by Sephadex G-25-treated extract under H_2 in the presence of cofactors is shown in Fig. 1. No CH_4 evolved in the absence of H_4MPT. Maximal rates of conversion were 31 and 23 nmol of CH_4 per min per mg of protein for HCHO and $HOCH_2$-S-CoM, respectively. In the case of HCHO, HS-CoM also was required for methanogenesis, and in the absence of exogenous FAD, F_{420}, or CN-Cbl, the specific activity was reduced to 18.6, 19.3, and 19.3, respectively. In the case of $HOCH_2$-S-CoM, the omission of FAD, F_{420}, or CN-Cbl reduced the specific activity to 19.8, 19.3, and 19.3, respectively. Conversion of 500 nmol of TPR and 500 nmol of HMT to CH_4 under H_2 showed similar kinetics and also was dependent upon the addition of H_4MPT and HS-CoM to the reaction mixture. Methanogenesis from TPR (specific activity, 5.0) was slightly stimulated by the addition of F_{420} (specific activity, 5.8). In the absence of added F_{420}, a 6-min lag was observed. Methanogenesis from HMT (specific activity, 35) was very slightly stimulated by the addition of F_{420} (specific activity, 38) but not by the addition of FAD (specific activity, 34) or CN-Cbl (specific activity, 35). The addition of H_4MPT to the assay mixture also was required for methanogenesis from TAD.

Methanogenesis under nitrogen. Under an N_2 atmosphere, TAD, TPR, HMT, and $HOCH_2$-S-CoM all underwent HCHO-like disproportionation (6) (Fig. 2). The specific activity for the formation of methane was (nanomoles of CH_4 per minute per milligram of protein): HMT, 2.5; $HOCH_2$-S-CoM, 2.1; TAD, 0.72; and TPR, 0.41. No lag was observed when $HOCH_2$-S-CoM was the substrate; 5-min lags were recorded for methanogenesis from HMT or TAD, and a 9-min lag was observed for methanogenesis from TPR. After 200 min of reaction time, the ratio of CH_4 formed under H_2 to that formed under N_2 was: $HOCH_2$-S-CoM, 0.39; HMT, 0.36; TAD, 0.23; and TPR, 0.17.

Chemical equilibrium of C_1 donors with HCHO. HCHO was found in the solutions of C_1 donors used. The concen-

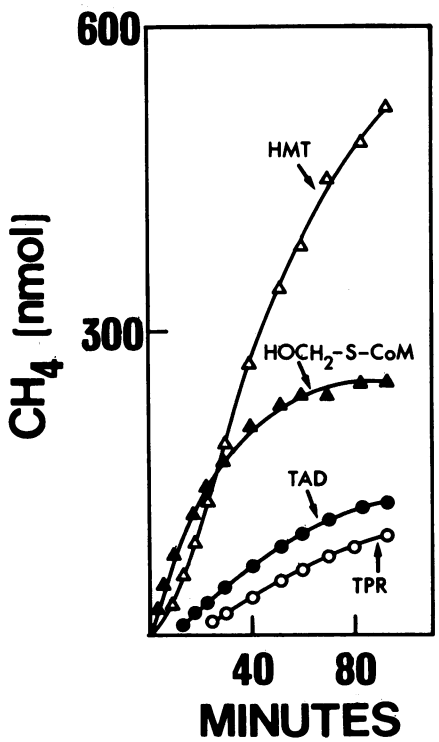

FIG. 2. Methanogenesis under N_2. The reaction mixtures contained, unless omitted, cell extract, 3.6 mg of protein, 500 nmol of TPR, 500 nmol of TAD, 500 nmol of HMT, 600 nmol of $HOCH_2$-S-CoM, or 80 μl of boiled-cell extract. Other components and conditions are described in the text.

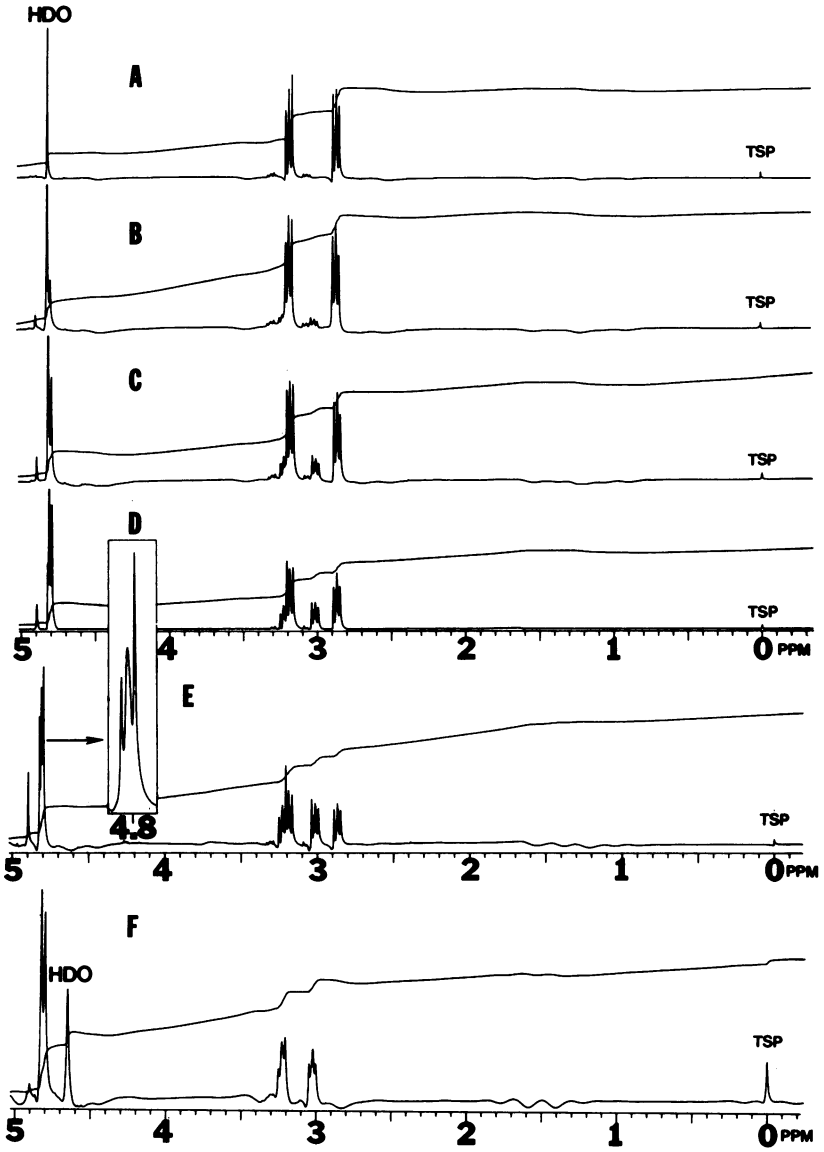

FIG. 3. ^1H NMR studies on $HOCH_2$-S-CoM. The spectra were obtained in ^2H_2O after 100 scans under the conditions described in the text. Spectra A through E were obtained at 18°C, and spectrum F was obtained at 30°C after 30 min of temperature equilibration time. The sample contained HS-CoM (120 μmol) and various amounts of HCHO (0, 20, 40, 60, or 120 μmol, respectively, for A through E). The inset shows an expanded close-up of the region between 4.75 and 4.85 ppm of spectrum E. The sample used to obtain spectrum F contained 360 μmol of HCHO.

tration of HCHO as measured at 58°C after 5 min of reaction time (as described above) in freshly prepared solutions was indicative of the degree and rate of equilibrium between the parent molecule and free HCHO. Our findings were as follows and are expressed as C_1 donor, millimolar concentration of C_1 donor, millimolar concentration of HCHO found. $HOCH_2$-S-CoM, 60, 60; HMT, 50, 24; TAD, 50, 11; TPR, 50, 5. The rate of formation of the product of the Nash reaction (2,6-dimethyl-3,5-diacetyl-1,4-dihydropyridine) was very similar with HCHO (225 nmol/min) and $HOCH_2$-S-CoM (201 nmol/min).

^1H NMR spectroscopy. Figure 3 (panels A to E) shows the ^1H NMR spectra of a sample containing HS-CoM (120 μmol) and increasing amounts of HCHO (0, 20, 40, 60, and 120 μmol). The p^2H of the sample containing a 1:1 ratio of HS-CoM:HCHO was 4.8. The chemical shifts of HDO and HCHO were assigned in a control experiment to be 4.81 and 4.82 ppm, respectively. In situ formation of $HOCH_2$-S-CoM was followed by a downfield change of 0.15 ppm in the chemical shift of the multiplet between 2.84 and 2.99 ppm to 3.01 and 3.16 ppm. In all instances, the sum of the integrals of both multiplets was equal to the integral of the multiplet between 3.19 and 3.23 ppm. The chemical shift of the hydroxymethyl group was assigned to be 4.79 ppm. The inset shows an expanded close-up of the region between 4.75 and 4.85 ppm of spectrum E. Figure 3F shows the spectrum of the sample containing 360 μmol of HCHO and 120 μmol of HS-CoM at 30°C. It should be noted that free, monomeric hydrated HCHO was present even when HS-CoM was present in a twofold excess over HCHO. An unidentified contaminant was also observed at 4.89 ppm. Increasing the temperature to 30°C induced an upfield change of 0.17 ppm in the chemical shift of HDO from 4.81 to 4.65 ppm (Fig. 3F), and the signals for HCHO (4.82 ppm) and $HOCH_2$-S-CoM

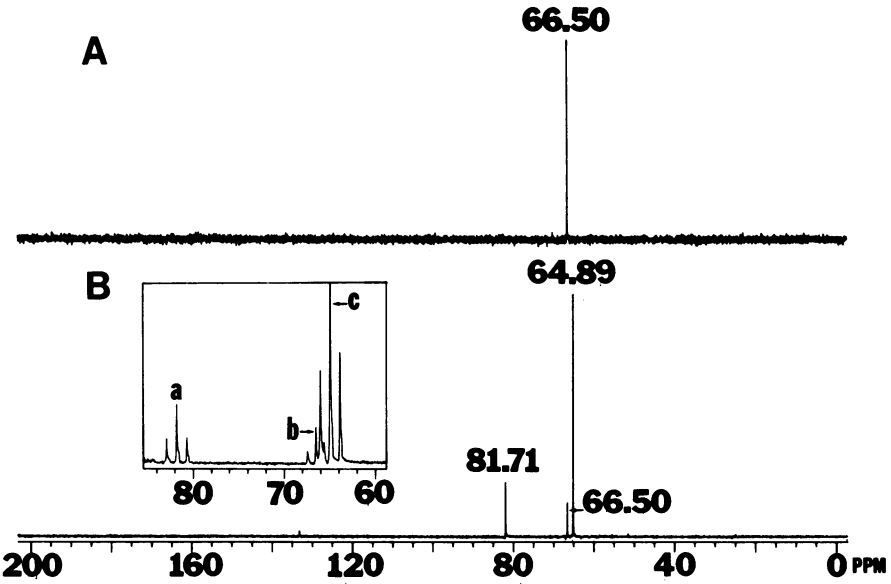

FIG. 4. ^{13}C NMR studies on $HOCH_2$-S-CoM. Broad-band ^1H-decoupled spectrum of a sample containing HS-CoM (120 μmol) and 1,4-dioxane (227 μmol) (A) and of the same sample plus $H^{13}CHO$ (120 μmol) (B). Spectra A and B were obtained after 100 scans. The inset shows the single-frequency off-resonance spectrum of the sample used for spectrum B. Lowercase lettering indicates the central signal of each triplet, with the triplet for 1,4-dioxane (b) and [$HOCH_2$-^{13}C]$HOCH_2$-S-CoM (c) overlapping.

(4.79 ppm) could be readily observed. No change in the chemical shift of any signal other than HDO was observed under these conditions. A further increase in the temperature to 60°C shifted the signal for HDO to 4.32 ppm with the concomitant broadening of it and the HCHO and $HOCH_2$-S-CoM peaks as well (spectrum not shown), but all integrals remained unchanged.

In a separate experiment performed at p^2H 6.8 (100 mM potassium phosphate), the ^1H NMR spectrum of a sample containing equimolar amounts of HS-CoM and HCHO (120 μmol) at 18°C showed resonance signals at 4.81 and 4.79 ppm corresponding to HDO and $HOCH_2$-S-CoM, respectively (spectrum not shown). The chemical shift of the β-methylene of HS-CoM changed as described above due to formation of the adduct. No evidence of unreacted HS-CoM was observed. When the temperature of the sample was increased to 30°C, a change in the chemical shift of HDO was noticed, and then a signal corresponding to HCHO (4.82 ppm) became evident.

^{13}C NMR spectroscopy. ^{13}C NMR spectra of [$HOCH_2$-^{13}C]$HOCH_2$-S-CoM was obtained by reacting HS-CoM (120 μmol) with $H^{13}CHO$ (120 μmol). Figure 4A shows the spectrum of HS-CoM and 1,4-dioxane (227 μmol). Only the latter was observed after 100 scans. Figure 4B shows the location of $H^{13}CHO$ (81.71 ppm), 1,4-dioxane (66.5 ppm, internal standard), and [$HOCH_2$-^{13}C]$HOCH_2$-S-CoM (64.89 ppm) in the broad-band ^1H-decoupled spectrum of the sample. The inset shows the single-frequency off-resonance spectrum of the sample with the expected multiplicities (triplets) for the three different kinds of methylene groups. Lowercase lettering in the inset shows the central signal of each triplet with the triplets of 1,4-dioxane (b) and [$HOCH_2$-^{13}C]$HOCH_2$-S-CoM (c) overlapping.

Synthesis of methenyl-H_4MPT^+ from C_1 donors. The methanogenic compounds tested substituted for HCHO as the C_1 donors in the synthesis of methenyl-H_4MPT^+ catalyzed by a methylene-H_4MPT oxidoreductase present in cell extracts of *M. thermoautotrophicum* (6a, 7). The apparent K_m for H_4MPT was 0.04 mM, and those for the C_1 donors were (millimolar): HCHO, 0.07; $HOCH_2$-S-CoM, 0.12; TAD, 0.36; TPR, 0.59; and HMT, 1.25.

DISCUSSION

Figure 5 illustrates the relationship of the results presented in this study to the central pathway of methanogenesis. Of the compounds tested, the chemical equilibrium with HCHO is displaced toward HCHO as the latter reacts chemically with H_4MPT to form methylene-H_4MPT, which then is reduced to methyl-H_4MPT. A proposed transmethylation reaction generates CH_3-S-CoM, and a final reduction of this compound by the methylreductase system yields CH_4. Figure 5 also explains how CH_4 is formed under a nonreductive atmosphere of N_2. The reducing potential required for the synthesis of CH_4 is generated by the oxidation of methylene-H_4MPT to methenyl-H_4MPT^+ (7). For methanogenesis under N_2, the model predicts a ratio of CH_4 to substrate of 0.33, which agrees with our results for $HOCH_2$-S-CoM and HMT. This is reminiscent of the disproportionation of HCHO reported elsewhere (7). The results obtained with TAD and TPR can be explained by suboptimal concentrations of HCHO due to limited dissociation of the substrates. Serine is not in chemical equilibrium with HCHO, but an H_4MPT-dependent serine transhydroxymethylase has been previously isolated and characterized (A. Oren, J. C. Escalante-Semerena, and R. S. Wolfe, unpublished data). The scheme proposed in Fig. 5 is supported further by the finding that HMT, TAD, TPR, and $HOCH_2$-S-CoM all substituted for HCHO in the spectrophotometric assay for the methylene-H_4MPT oxidoreductase. The fact that HCHO could not be detected as an intermediate, when serine served as a substrate for the synthesis of methenyl-H_4MPT^+, indicated that serine transhydroxymethylase was coupled to methylene-H_4MPT oxidoreductase (Oren et al., unpublished data). Our results predict that methanogenesis would occur from any compound in chemical equilibrium with HCHO, and CH_4 formation from such compounds would be dependent upon the addition of H_4MPT and HS-CoM to the reaction mixture.

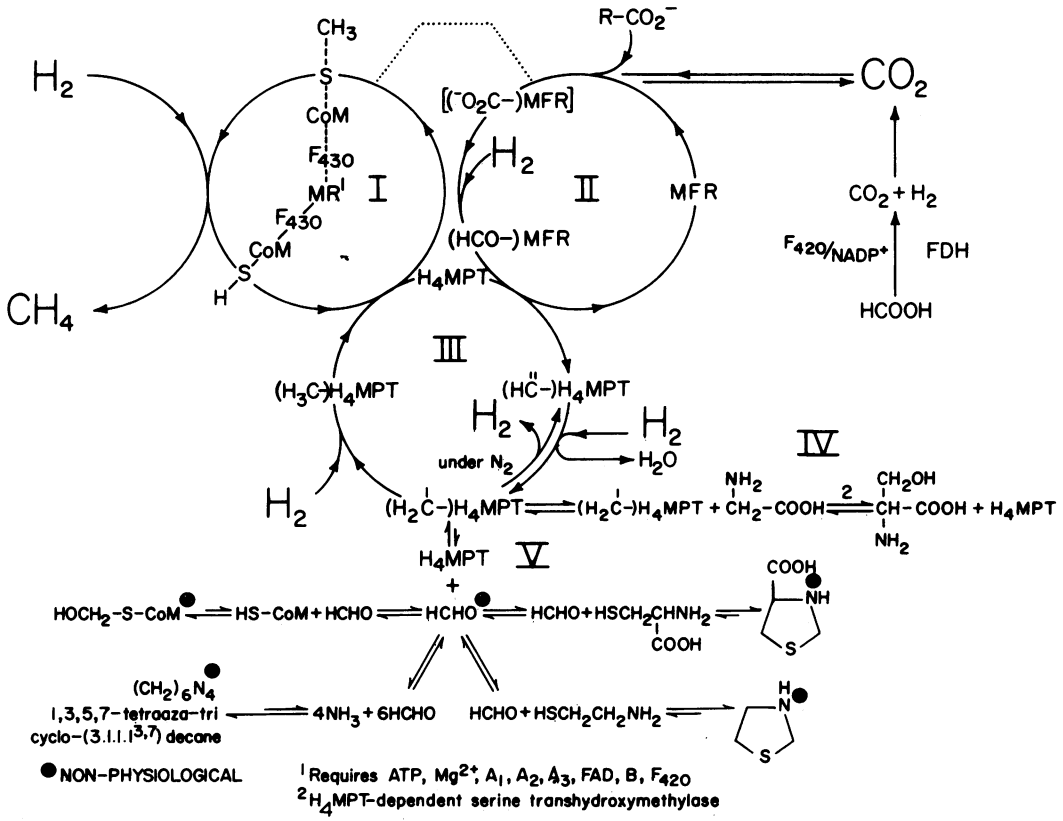

FIG. 5. Relationship of non-physiological C_1 donors to the methanogenic pathway. (I) Cycle I represents the CH_3-S-CoM, 2-(methylthio)ethanesulfonic acid, methylreductase system (MR) (20). Protein components C(3), A1, A2, and A3 (15) are required for reduction of the methyl groups to CH_4, with molecular hydrogen serving as the electron donor. In addition, ATP, Mg^{2+}, FAD, and component B (a coenzyme of unknown structure) have been previously shown to be required (9, 15). CoF_{430}, a nickel tetrapyrrole, is the chromophore of component C (3, 17). (II) Cycle II indicates the activation and reduction of CO_2 to the formyl level with methanofuran (MFR) (13, 14) as the C_1 carrier (J. A. Leigh, Ph.D. dissertation, University of Illinois, 1983). (III) Cycle III shows the role of H_4MPT (5, 21) as the C_1 carrier at the methine, methylene, and methyl levels of oxidation. (IV) Serine transhydroxymethylase has been shown to transfer the hydroxymethyl groups of serine to H_4MPT to form methylene-H_4MPT. (V) The non-physiological compounds TAD, TPR, and HMT as well as hydroxymethyl-CoM are hydrolyzed to HCHO which reacts chemically with H_4MPT to form methylene-H_4MPT. (Modified from J. C. Escalante-Semerena, Ph.D. dissertation, University of Illinois, 1983, and Escalante-Semerena et al. [6].)

Hemithioacetal formation from HCHO and RSH groups is a base-catalyzed reaction with negligible adduct formation at pH 3 (10). The formation of hemithioacetals from HCHO has been studied with 2-mercaptoethanol as a model compound (10). The equilibrium constant for hemithioacetal formation from HCHO and 2-mercaptoethanol was estimated to be 620 M^{-1} at pH 6.57 (10). The reported pK_a values for the sulfhydryl groups of 2-mercaptoethanol and HS-CoM are very similar, 9.57 and 9.37, respectively (11).

Our findings indicate that HCHO and HS-CoM react spontaneously to form $HOCH_2$-S-CoM, and as predicted, the formation of $HOCH_2$-S-CoM is pH dependent. As shown in Fig. 3A to E, at pH 4.8 free monomeric, hydrated HCHO (4.82 ppm) was present in solution even when HS-CoM was in a twofold excess (Fig. 3D), reflecting the reduced reactivity of the thiolate anion at that p^2H; both HCHO and $HOCH_2$-S-CoM as well as unreacted HS-CoM were observed in the ^1H and ^{13}C NMR spectra. The results obtained at p^2H 6.8 correlate with the increased reactivity of the SH groups upon deprotonation, with the concomitant shift of the equilibrium toward the formation of $HOCH_2$-S-CoM; however, at 30°C the presence of a small amount of free HCHO suggests that at physiological pH, HCHO is available for reaction with H_4MPT. This idea is supported further by the fact that the non-physiological compounds served as donors of HCHO for the enzymatic synthesis of methenyl-H_4MPT^+ at pH 7 and at rates that most likely reflect the rate of equilibrium of the parent compound with HCHO.

As reported previously (18), we also found that solutions of HS-CoM and $HOCH_2$-S-CoM of equivalent concentrations reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman reagent) at pH 8.1 at rates that were too fast to be accurately measured with conventional techniques (M. I. Donnelly, personal communication). Nevertheless, this observation strongly indicates that in the solution of $HOCH_2$-S-CoM, the sulfhydryl groups are almost as available as they are in a solution of HS-CoM.

Even though HS-CoM (1.25 mM) and 2-mercaptoethanol (10 mM) are routinely present in the methanogenic assay (pH 6.3) with HCHO as the substrate (5), they seem to have little effect on the conversion of HCHO to CH_4, since equimolar amounts of CH_3-S-CoM and HCHO under the same experimental conditions show very similar reaction rates (6a). This suggests that the rate of equilibrium of HCHO and HS-CoM with $HOCH_2$-S-CoM may be faster than the enzymatic reactions involved in the disproportionation of HCHO under N_2 or in its reduction to CH_4 under H_2, thus maintaining a steady level of HCHO available to react

with H_4MPT. Alternatively, the association constant for the formation of methylene-H_4MPT may be much larger than the one for the formation of $HOCH_2$-S-CoM. Based on the data presented in this communication and elsewhere (6a) we have documented that HS-CoM is not the carrier at the formaldehyde level of oxidation.

The stimulatory effect of FAD and F_{420} on the rate of conversion of HCHO or $HOCH_2$-S-CoM may be explained by subsaturating levels of these coenzymes previously reported as part of component A of the methylreductase system (15). Stimulation of the reaction rate of methanogenesis from the non-physiological substrates by CN-Cbl is not understood but is less dramatic than the four- to fivefold stimulation observed in the methylreductase system (W. B. Whitman and R. S. Wolfe, Fed. Proc. **41**:5157, 1982).

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