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Mass-Spectrometric Studies of the Interrelations among Hydrogenase, Carbon Monoxide Dehydrogenase, and Methane-Forming Activities in Pure and Mixed Cultures of *Desulfovibrio vulgaris*, *Desulfovibrio desulfuricans*, and *Methanosarcina barkeri*

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The activities of pure and mixed cultures of *Desulfovibrio vulgaris* and *Methanosarcina barkeri* in the exponential growth phase were monitored by measuring changes in dissolved-gas concentration by membrane-inlet mass spectrometry. *M. barkeri* grown under H_2-CO_2 or methanol produced limited amounts of methane and practically no hydrogen from either substrate. The addition of CO resulted in a transient H_2 production concomitant with CO consumption. Hydrogen was then taken up, and CH_4 production increased. All these events were suppressed by KCN, which inhibited carbon monoxide dehydrogenase activity. Therefore, with both substrates, H_2 appeared to be an intermediate in CO reduction to CH_4. The cells grown on H_2-CO_2 consumed 4 mol of CO and produced 1 mol of CH_4. Methanol-grown cells reduced CH_3OH with H_2 resulting from carbon monoxide dehydrogenase activity, and the ratio was then 1 mol of CH_4 to 1 mol of CO. Only $^{12}CH_4$ and no $^{13}CH_4$ was obtained from ^{13}CO, indicating that CO could not be the direct precursor of CH_4. In mixed cultures of *D. vulgaris* and *M. barkeri* on lactate, an initial burst of H_2 was observed, followed by a lower level of production, whereas methane synthesis was linear with time. Addition of CO to the mixed culture also resulted in transient extra H_2 production but had no inhibitory effect upon CH_4 formation, even when the sulfate reducer was *D. vulgaris* Hildenborough, whose periplasmic iron hydrogenase is very sensitive to CO. The hydrogen transfer is therefore probably mediated by a less CO-sensitive nickel-iron hydrogenase from either of both species.

Methanogenic bacteria are able to utilize a great variety of substrates, and the pathways toward methane synthesis are beginning to be well documented (14, 24, 42, 54). Nonetheless, some puzzling questions remain unanswered concerning the role of enzymes such as hydrogenases and carbon monoxide dehydrogenase in the different steps of methanogenesis.

Methanosarcina barkeri can live on and produce methane from different substrates, such as H_2-CO_2, methanol, methylamines, or acetate (49). Hydrogen can also be utilized in mixotrophy with methanol, and the dismutation of CH_3OH to CO_2 and CH_4 is replaced by the more efficient reduction of 1 mol of methanol by 1 mol of H_2 (35). Hydrogen is also a possible intermediate in methane production from methanol alone, since it has been shown to accumulate, in particular when methane production is inhibited by 2-bromoethane sulfonate (5).

Under natural conditions, hydrogen may be an intermediate in the catabolism of methane precursors or may originate from the activity of associated organisms through an interspecific hydrogen transfer. The importance of this transfer has been demonstrated by using mixed cultures of sulfate-reducing and methanogenic bacteria (9, 34, 50, 51), the latter acting as an electron acceptor for H_2 utilization in low-sulfate medium (9). The interspecific transfer of hydrogen and its utilization in methanogenesis require enzyme systems, among which hydrogenases must have a prominent role (24).

The existence of nickel-containing hydrogenases in methanogenic bacteria has been recognized (1). *M. barkeri* DSM 800 contains at least one soluble nickel iron hydrogenase (18), which is active mostly in H_2 uptake (G. Fauque, *FEMS Symposium 49*, in press), but multiple hydrogenase activities are likely (31, 48). In the genus *Desulfovibrio*, some species such as *Desulfovibrio gigas* or *D. multispirans* possess only one hydrogenase, whereas three hydrogenases of different types have been purified from *D. vulgaris* Hildenborough (32). In mixed cultures, the question arises of which hydrogenase from either of the organisms mediates the hydrogen transfer between the sulfate reducer and the methanogen.

The utilization of CO in methanogenesis was suspected (26) long before carbon monoxide dehydrogenase had been purified from *M. barkeri* (30). In this species, as well as in most other methanogens, carbon monoxide dehydrogenase activity (22, 30) results in either CO oxidation (12, 26) or CO production (7, 10, 16). Actually, the main role of carbon monoxide dehydrogenase is in the reversible incorporation of CO or of CO_2 into the carbonyl group of acetyl coenzyme A (29, 53), and hydrogen would thus be a by-product (5), which can be utilized as an electron donor in methane formation (37).

The purpose of the present work was to ascertain the roles of hydrogenase and carbon monoxide dehydrogenase activities in pure and mixed cultures of diverse *Desulfovibrio* and *Methanosarcina* strains in methanogenesis. Since all these activities were dealing with gas uptake or production, mass

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spectrometry associated with a membrane gas inlet technique (3) was particularly well suited to monitoring the short-term kinetics of transient intermediates. It was expected that the specific inhibitory effect of carbon monoxide upon the activity of different hydrogenases would help in the characterization of the enzyme responsible for the interspecific hydrogen transfer, but the H_2-producing activity of carbon monoxide dehydrogenase complicated the pattern. The latter activity proved to have a nonnegligible role in methanogenesis, because the yield of methane was increased by the reducing equivalent provided via intermediary dihydrogen production.

MATERIALS AND METHODS

Chemicals and gases. All reagents and chemicals were of analytical grade (obtained from E. Merck AG, Darmstadt, Federal Republic of Germany, or Prolabo). Gases (grade N45) were from l'Air Liquide, Paris, France, and were freed from oxygen by passage through BASF Catalyst (BASF, Ludwigshafen, Federal Republic of Germany) or a solution of photoreduced methyl viologen. Deuterium gas (99.8% ^2H) and ^{13}CO (99.2% ^{13}C) were purchased from Oris Saclay, Gif sur Yvette, France.

Organisms and culture media. The bacterial strains used were *D. vulgaris* Hildenborough NCIB 8303; *D. desulfuricans* ATCC 27774; and *M. barkeri* DSM 800, DSM 1311, and DSM 1538.

All the cultures were grown in anaerobic culture tubes or serum bottles; strict anaerobic techniques were used (2, 11). Pure cultures of sulfate-reducing bacteria were grown in a medium consisting of the following components (in grams per liter): KH_2PO_4, 0.4; K_2HPO_4, 0.2; NaCl, 0.6; NH_4Cl, 0.5; $MgCl_2 \cdot 6H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.05; yeast extract, 1.0; Na_2SO_4, 2.1; and resazurin, 0.001. Trace elements solution (8) and vitamin mix (52) were also added (at 10 ml/liter each). The pH was adjusted to 6.8 to 6.9 with Na_2CO_3 while the medium was bubbled with N_2-CO_2 (80:20, vol/vol). After sterilization, the medium was reduced with $Na_2S \cdot 9H_2O$ (final concentration, 0.5 mM) and inoculated (5% inoculum). The cells were grown at 37°C (32°C for *D. vulgaris*). Depending to the experiments, H_2–CO_2–5 mM acetate or lactate alone (20 mM) were used as energy and carbon sources. *D. vulgaris* was also grown without sulfate on 30 mM pyruvate. Pure cultures of *M. barkeri* were grown in the same medium as for *Desulfovibrio* spp. but lacking Na_2SO_4 and lactate, with H_2 or methanol (100 mM) as the source of energy.

Mixed cultures of either *D. vulgaris* Hildenborough or *D. desulfuricans* ATCC 27774 plus *M. barkeri* DSM 1311 were grown in a medium supplemented with 20 mM lactate, but without Na_2SO_4, under an atmosphere of N_2-CO_2 (80:20, vol/vol).

Metabolic activities. The cultures were transferred anaerobically without further treatment from the culture flask to an airtight reaction vessel connected via a vacuum line and a cold trap to the ion source of the mass spectrometer (model 8-80; VG Instruments) (3, 25). The gases initially present were first eliminated by sparging the culture for 4 to 5 min with pure nitrogen or argon. The vessel was then closed by lowering a plunger down to the liquid level so that no headspace was left and only dissolved gases were involved in the reactions. This avoided diffusion problems at the interface between the liquid and the atmosphere and, moreover, allowed a better precision in the measurement of initial velocities (25).

The different activities were measured by monitoring peak height variations corresponding to the appearance or consumption of the different dissolved gases (H_2, CO, and CH_4) involved in the metabolic reactions. The mass peaks of interest were successively scanned by a peak-jumping system monitored by an Apple II data acquisition system. A complete acquisition cycle lasted about 20 s.

Hydrogen production and uptake were monitored on mass peak 2 (H_2^+). The H^+-D_2 exchange activity was determined by the appearance of HD (mass peak 3) and H_2 (mass peak 2) in the presence of 20% D_2. To discriminate an eventual methane formation coming directly from CO reduction, we used ^{13}CO; this had the further advantage that CO consumption could be monitored on peak 29 ($^{13}CO^+$) instead of peak 28 ($^{12}CO^+$), which suffers a possible N_2^+ interference. In the ion source, the methane molecule splits into several fragments in definite proportions, which give different peaks, the most important being peaks 16 (CH_4^+) and 15 (CH_3^+). With [^{13}C]methane, the corresponding mass peaks are 17 ($^{13}CH_4^+$) and 16 ($^{13}CH_3^+$). Since mass peak 16 is common to both isotopic species, the calculations were preferentially made from mass peak 15 for $^{12}CH_4$ and from mass peak 17 for $^{13}CH_4$.

Carbon dioxide production could also be measured by monitoring mass peaks 44 ($^{12}CO_2^+$) and 45 ($^{13}CO_2^+$). To condense H_2O vapor but not CO_2, liquid nitrogen (−190°C) was replaced by ethanol-dry ice (−80°C) in the cold trap. Peak 32 was also monitored to check anaerobiosis.

The sensitivities (ratios of concentration to corresponding peak height) were determined by using pure gases added as small volumes of saturated aqueous solutions at a known temperature. The solubilities were calculated from Bunsen coefficients (32a). The results were calculated on a molar basis and normalized to the total protein content of the cell, determined by a modified Lowry method (33), after ultrasonication.

RESULTS AND DISCUSSION

Results for *D. vulgaris* Hildenborough grown without sulfate. In the absence of CO, the rate of hydrogen production mediated by the hydrogenase activity of *D. vulgaris* with pyruvate as the electron donor was ca. 30 nmol/min per mg of protein (Fig. 1a). After addition of 10 μM CO, the cells exhibited a carbon monoxide dehydrogenase activity (ca. 4.7 nmol of CO consumed per min per mg of protein), but H_2 production was nearly unchanged. This can be explained by an apparent compensation between two opposite effects of CO: on the one hand the inhibition of hydrogenase activity, and on the other hand the production of hydrogen via carbon monoxide dehydrogenase. In the presence of 40 μM KCN, hydrogenase-mediated H_2 production was not affected until CO was added (Fig. 1b). Then, as carbon monoxide dehydrogenase was inhibited by KCN, added CO was not consumed and consequently exerted a certain inhibitory effect (about 25%) upon hydrogenase activity (Fig. 1b). The inhibition by CO of the in vivo H^+-D_2 exchange activity, measured with a smaller volume of cells, was only 30% with 1 μM CO and 70% with 10 μM CO. The presence in *D. vulgaris* Hildenborough of two Ni-containing membrane-bound hydrogenases (32) could explain the observation that the inhibitory effect of CO in vivo was far lower than the inhibition rate previously found with the very sensitive purified periplasmic iron hydrogenase of that strain (4).

Results for *M. barkeri* grown with H_2-CO_2. The data in Fig. 2 refer to a typical experiment made with growing cells of *M.*

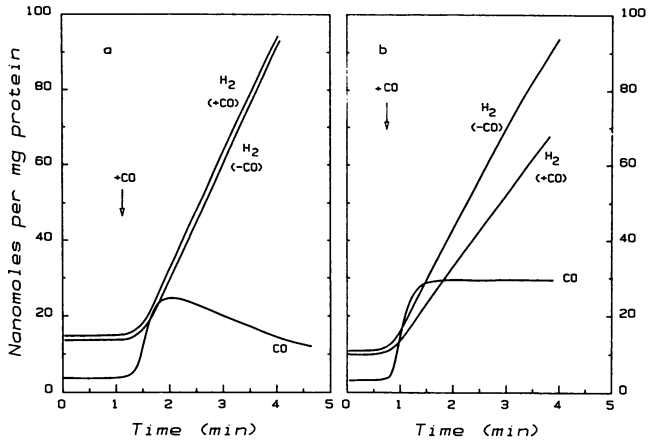

FIG. 1. CO consumption and effect of CO on H_2 evolution in cells of *D. vulgaris* Hildenborough (2.36 mg of protein in 10 ml) with pyruvate as the substrate in the absence of KCN (a) and after addition of 40 μM KCN (b). At the times marked by the arrows, the vessel was closed and a CO-saturated solution was (or was not) injected into the reaction vessel (final concentration, 10 μM).

barkeri DSM 1538, and similar patterns were obtained with strains DSM 1311 and DSM 800. Acetate, which was an additional carbon source during the growth period, is known to be little utilized or not utilized in methane production in the presence of H_2 and CO_2 (19, 28, 37).

After elimination of dissolved gases (H_2 and CH_4) initially present in the culture, the cells evolved practically no hydrogen (less than 0.1 nmol/min per mg of protein). Although most of initial CO_2 was also eliminated, a certain amount of bicarbonate was probably present in the culture at pH 6.9, and catabolic CO_2 production could also occur, but the absence of H_2 prevented any production of methane from CO_2. A very low level of endogenous methane production was nonetheless observed, probably originating from a residual acetate metabolism. When a small amount of labeled carbon monoxide (final concentration, 25 μM ^{13}CO) was injected into the culture, that gas was consumed and dihydrogen was concomitantly evolved (Fig. 2a). As the CO concentration decreased, the rate of H_2 evolution became lower, and eventually H_2 disappeared again. The rate of methane production increased with time, in comparison with the rate in the control without CO; then, as H_2 was exhausted, methanogenesis ceased (not shown in Fig. 2a). The ratio between H_2 or CO consumed and CH_4 produced (Table 1, line 4) corresponded fairly well to the stoichiometry of 4:1 normally found in methane synthesis from H_2-CO_2.

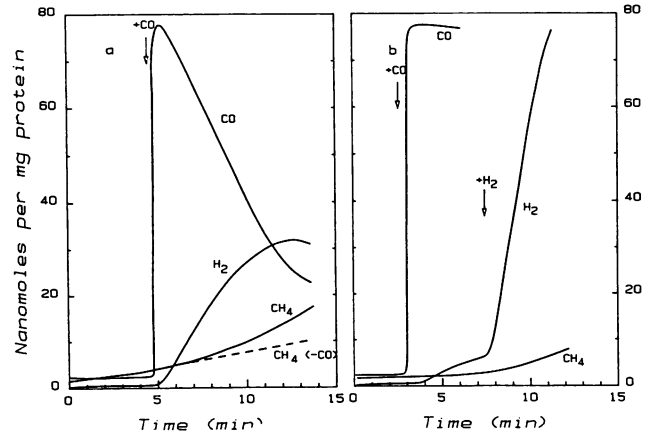

FIG. 2. CO consumption and H_2 and CH_4 production in cells of *M. barkeri* DSM 1538 (3.5 mg of protein in 10 ml) after growth with H_2-CO_2-acetate in the absence of KCN (a) and after addition of 40 μM KCN (b). The first arrow (in panels a and b) indicates injection of CO (final concentration, 25 μM). The second arrow (in panel b) indicates injection of 50 μM H_2. ----, CH_4 production in a control without CO. H_2 and CH_4 were eliminated by a nitrogen flow, but acetate was still present in the culture.

TABLE 1. Rates of CO, H_2, and CH_4 production or consumption in cells of *M. barkeri* DSM 1538 after growth with H_2-CO_2,-acetate[a]

Compound	Rate[b] at following time (min) after CO addition:				
	0.5	1.5	4.0	5.0	7.0
CO[c]	−9.7	−8.4	−7.3	−6.6	−2.9
H_2	6.5	5.9	3.2	2.0	−0.1
CH_4[d]	0.6	0.7	1.0	1.2	0.9
$(H_2 + CO)/CH_4$[e]	5.3	3.6	4.1	3.8	3.3

[a] Calculated from the data in Fig. 2a.
[b] The rate is expressed as nanomoles per minute per milligram of protein.
[c] At time zero, CO was added (final concentration, 25 μM). No substrate (except acetate) was present initially.
[d] CH_4 corresponds to CO-dependent CH_4 formation (the initial rate before CO addition was 0.6 nmol/min per mg of protein).
[e] $(H_2 + CO)/CH_4$ is the sum of production or disappearance rates of H_2 and CO divided by the CO-dependent rate of CH_4 production.

In the presence of 40 μM potassium cyanide (Fig. 2b), carbon monoxide dehydrogenase activity and related hydrogen evolution were severely inhibited and the rate of methane production was significantly diminished. Since CN^- is known not to inhibit methanogenesis from H_2-CO_2 in *Methanosarcina* spp. (43), the decrease in the rate of CH_4 production could be assigned to the lack of dihydrogen resulting from the inhibition of carbon monoxide dehydrogenase by KCN. When H_2 was added directly in the presence of KCN, CH_4 production slightly increased but was not fully restored, which could indicate an inhibitory effect of KCN upon an intermediate in the electron transfer cycle during methanogenesis.

From the preceding results, it can be concluded that CO-dependent H_2-evolving activity was responsible for the increased methane production. It has indeed been clearly established with cell extracts from different acetotrophic *Methanosarcina* species that CO supports, as well as or even better than H_2, the methylreductase activity which is responsible for the last step in methane formation (36). Moreover, the existence of a ferredoxin-dependent electron transport from the carbon monoxide dehydrogenase complex to a membrane-bound hydrogenase in acetate-grown *Methanosarcina* spp. has been established (47). Therefore, in CO-dependent CH_4 production, CO serves as an indirect electron donor, but the origin of the C atom of CH_4 must be considered.

In the present experiments, the methane isotopic species appearing from ^{13}CO was $^{12}CH_4$ (seen as $^{12}CH_3^+$ in mass peak 15) and not $^{13}CH_4$ (no mass peak 17 was detected). No $^{13}CO_2$ was observed in the presence of ^{13}CO. In contrast, when $^{13}CO_2$ and H_2 were added directly, $^{13}CH_4$ was produced in a proportion corresponding to the ratio of $^{13}CO_2$ to $^{12}CO_2$ (data not shown). Therefore, the C atom of CO cannot be the direct precursor of CH_4.

Results of *M. barkeri* grown with methanol. The data

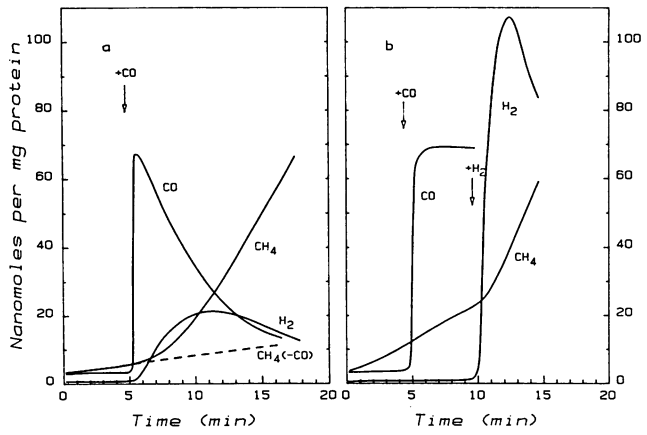

FIG. 3. CO consumption and H_2 and CH_4 production in cells of *M. barkeri* DSM 1538 (4.17 mg of protein in 10 ml) grown with methanol in the absence of KCN (a) and after addition of 40 μM KCN (b). The first arrow (in panels a and b) indicates injection of CO (final concentration, 25 μM). The second arrow (in panel b) indicates injection of 50 μM H_2. ----, CH_4 production in a control without CO. Methanol was still present in the culture.

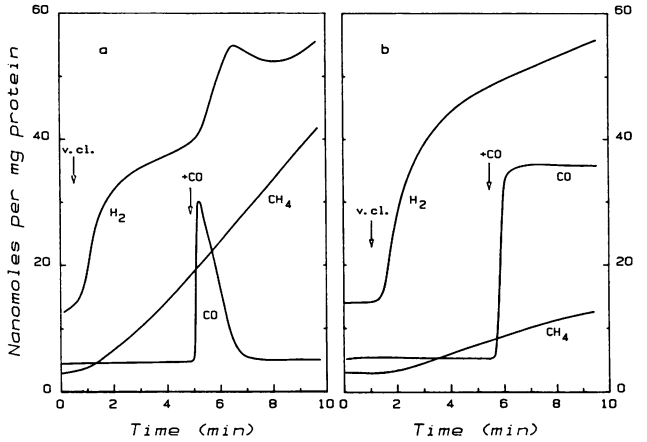

FIG. 4. CO consumption and H_2 and CH_4 production in a mixed culture of *D. desulfuricans* ATCC 27774 and *M. barkeri* DSM 1311 (3.32 mg of protein in 10 ml) grown with lactate in the absence of KCN (a) and after addition of 40 μM KCN (b). The first arrow (in panels a and b) indicates when the vessel was closed (v.cl.). The second arrow (in panels a and b) indicates injection of CO (final concentration, 10 μM).

presented in Fig. 3 were again obtained with *M. barkeri* DSM 1538 cells. Similar data were found with cells of the other two strains. Although methanol was still present, these cells showed the same general behavior as those grown with H_2-CO_2-acetate. Hydrogen evolution was less than 0.02 nmol/min per mg of protein, and a certain amount of methane formation could be related to methanol metabolism, which does not require the energetic participation of H_2 (Fig. 3a). The carbon monoxide dehydrogenase activity of methanol-grown cells, measured as CO consumption in the presence of 25 μM CO, was not very different from that recorded with cells grown with H_2-CO_2-acetate (Fig. 2a and 3a; Tables 1 and 2). CO consumption resulted in transient H_2 production and then uptake, whereas a progressive and large increase in methane synthesis was observed (Fig. 3a).

The main difference between cells grown with H_2-CO_2 or methanol was in the ratio of the sum of CO plus H_2 consumed to CH_4 produced. When H_2-CO_2 was used, this ratio remained around 4 (Table 1, line 4), whereas with methanol it shifted to a value of 1 a few minutes after CO addition (Table 2, line 4). This result points to the occurrence of a progressive switch from the dismutation of CH_3OH to its reduction by CO-dependent H_2. Hydrogen production by methanogens grown with methanol had previously been observed only when CO concentrations were higher than 20%, whereas with lower concentrations, the H_2 present was taken up (37). The present results show that even with low CO pressures, H_2 is involved as an intermediate in methanogenesis and is the electron donor in the reduction of methanol to methane. It is obvious that this methane cannot be labeled from ^{13}CO, and, indeed, only $^{12}CH_4$ was found (27).

In the presence of 40 μM KCN, added carbon monoxide was no longer consumed, related H_2 evolution ceased, and methanogenesis was diminished, although the level was still significantly higher than before CO addition (Fig. 3b). Direct methane production from added H_2 (Fig. 3b) was, in contrast, unaffected by KCN and CO, and a ratio of 1:1 between H_2 consumption and CH_4 formation was also observed.

TABLE 2. Rates of CO, H_2, and CH_4 production or consumption in cells of *M. barkeri* DSM 1538 after growth with methanol[a]

Compound	Rate[b] at following time (min) after CO addition:				
	1.0	2.7	4.3	5.5	7.5
CO[c]	−9.2	−9.6	−5.6	−4.9	−3.0
H_2	6.8	4.1	1.3	0	−1.1
CH_4[d]	0.4	2.7	3.9	4.6	5.5
$(H_2 + CO)/CH_4$[e]	6.0	2.0	1.1	1.1	0.8

[a] Calculated from the data in Fig. 3a.
[b] The rate is expressed as nanomoles per minute per milligram of protein.
[c] At time zero, CO was added (final concentration, 25 μM). Methanol (100 mM) was present initially.
[d] CH_4 corresponds to CO-dependent CH_4 formation (the initial rate before CO addition was 0.7 nmol/min per mg of protein).
[e] $(H_2 + CO)/CH_4$ is the sum of production or disappearance rates of H_2 and CO divided by the CO-dependent rate of CH_4 production.

Results for mixed *Desulfovibrio* and *Methanosarcina* cultures. The data summarized in Fig. 4 refer to an experiment performed with a mixed culture of *D. desulfuricans* ATCC 27774 and *M. barkeri* DSM 1311. With this mixed culture, an initial but transient H_2 production was always observed (Fig. 4a), probably related to the hydrogenase activity of the sulfate reducer with lactate as the electron donor (38). The rate of H_2 production decreased within 2 min, and linear methane production was observed at the same time. The ratio between the production of CH_4 and the decrease in H_2 evolution was 1:4, i.e., the same as observed with *M. barkeri* grown on H_2-CO_2, indicating that most of the hydrogen produced by the sulfate reducer was transferred to the methanogen and used in methane formation. A CO injection resulted in another H_2 burst but no increase in the rate of CH_4 production, which had probably reached a maximal level by using H_2 from the sulfate reducer. After CO was exhausted, net H_2 evolution ceased and then returned to the rate observed before CO addition.

In mixed cultures, potassium cyanide also suppressed carbon monoxide dehydrogenase activity and the CO-dependent burst of H_2 production (Fig. 4b). Although there were similar levels of hydrogen production, the rate of methane synthesis was lower in the presence (Fig. 4b) than in the absence (Fig. 4a) of KCN. This indicates a possible inhibi-

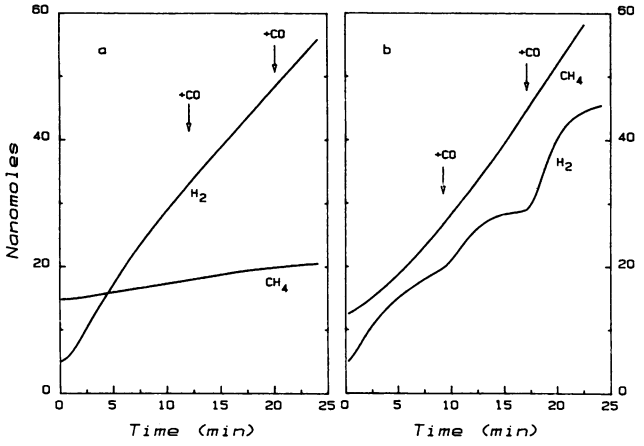

FIG. 5. H_2 and CH_4 production in a mixed culture of *D. vulgaris* Hildenborough and *M. barkeri* DSM 1311 grown with lactate by the supernatant *D. vulgaris* cells after decantation of *M. barkeri* (a) and after replacement of 2 ml of the preceding by 2 ml of the resuspended mixed culture (b). The first arrow (in panels a and b) indicates injection of 2 μM CO. The second arrow (in panels a and b) indicates injection of CO (final concentration, 10 μM). Results are given as micromoles of gas in 10 ml of medium. (The initial mixed culture contained 0.28 mg of protein per ml.)

tory effect of KCN on some unidentified electron carrier involved in the pathway of methane synthesis from H_2 and CO_2, because this was also postulated for *M. barkeri* on H_2-CO_2 (Fig. 2b). H_2 production and CH_4 synthesis were not further inhibited when CO was added, although CO did not disappear in the presence of KCN.

Figure 5a shows results for a mixed culture of *D. vulgaris* Hildenborough plus *M. barkeri* DSM 1311 from which most of the methanogen aggregates were eliminated through decanting, leaving mainly the sulfate reducer. With that culture (Fig. 5a), H_2 evolution could be observed; its rate decreased slightly with time. A very low rate of production of methane indicated that a few methanogen cells were still present in the supernatant. Carbon monoxide appeared to have little effect upon the overall H_2 production, in agreement with the results of the experiment with the pure culture (Fig. 1a). At the end of this experiment, 2 ml was removed from the reaction vessel (from a total volume of 10 ml) and replaced by 2 ml of the initial coculture (Fig. 5b). This was done to stress the specific effects of the mixed culture upon the balance of the different gases involved. In the reconstituted coculture (Fig. 5b), the initial H_2 evolution rate was followed by a lower steady-state rate, which was compensated by an increasing methane production. Successive CO additions (Fig. 5b, arrows) resulted in increased H_2 production, which lasted until all added CO was consumed but did not significantly affect the CH_4 synthesis. Thus, practically no inhibitory effect of CO upon H_2 utilization in methanogenesis was observed. A CO concentration of 10 μM, as used here, should inhibit most of the H_2 uptake activity of *D. vulgaris* Hildenborough periplasmic iron hydrogenase (4). This is an indication that the hydrogen transfer to the methanogenic process was channeled not via the periplasmic iron hydrogenase from the latter species but via the less CO-sensitive nickel-containing hydrogenases from one or both organisms.

Conclusion. The main conclusion from this work concerns the involvement of hydrogenases and carbon monoxide dehydrogenase in the anabolic and catabolic cycles associated with methane synthesis by *M. barkeri*.

Both H_2-CO_2- and methanol-grown cells of *M. barkeri* utilized added H_2 or H_2 produced via carbon monoxide dehydrogenase activity as a source of electrons for CH_4 formation. In methanol-grown cells, H_2 resulting from carbon monoxide dehydrogenase activity was used to reduce methanol to methane (35), a more efficient reaction than the simple dismutation of methanol. Owing to the presence of one (18) or more (5) H_2-oxidizing hydrogenases, it is very likely that in cells grown with H_2-CO_2, the hydrogen atoms of CH_4 originate from protons and not directly from hydrogen (13, 41, 44). Moreover, the nickel-iron hydrogenase from *M. barkeri* is not very sensitive to CO, since 25 μM CO inhibited only 20% of the in vivo H^+-D_2 exchange activity (parallel experiment; results not shown).

The experiments with ^{13}C-labeled CO prove that in autotrophic growth with H_2-CO_2, the C atom of the CH_4 molecule does not come directly from CO, apparently not even via CO oxidation to CO_2. A key role for carbon monoxide dehydrogenase is in the synthesis of acetyl coenzyme A from the methyl and carbonyl groups (53) first demonstrated in acetogenic bacteria (15, 23, 40, 46). In methanogens, acetyl coenzyme A is a central intermediate in catabolic methane production by acetotrophic cells (6, 17, 20, 27), as well as in anabolic acetate synthesis by autotrophic cells (10, 16, 21, 45). In that case, the carbon from CO or CO_2 enters the carbonyl group of acetyl coenzyme A, whereas the methyl group is transferred via a corrinoid enzyme. Moreover, a reversible isotopic exchange between CO (or CO_2) and the carbonyl group of acetyl coenzyme A has been observed in *M. barkeri* (17). It is therefore likely that the CO carbon atom with its ^{13}C label is incorporated directly, via carbon monoxide dehydrogenase and the anabolic acetyl coenzyme A pathway, into acetate and other cell constituents, whereas H_2, a by-product of the reaction, is used to reduce endogenous unlabeled CO_2. With methanol-grown cells, no labeling of methane is due to occur from ^{13}CO, since the methyl group of methanol is incorporated directly into methyl coenzyme M and further reduced to methane by CO-dependent H_2. The methyl group from methanol is also used, together with the carbon from CO, in the anabolic acetyl coenzyme A pathway (53).

With mixed *Desulfovibrio* and *Methanosarcina* cultures, an equilibrium was maintained between H_2 production by the sulfate reducer and utilization by the methanogen. When comparing Fig. 2a and 4a, it is clear that methane production, in terms of total protein content, is higher in the mixed culture than in the pure culture of methanogens grown with H_2-CO_2. From the rather low inhibitory effect of carbon monoxide upon the transfer of hydrogen to the methanogenic bacteria, even when the sulfate reducer is *D. vulgaris* Hildenborough, it can be concluded that this transfer is not mediated by the CO-sensitive periplasmic iron hydrogenase from the latter species but is more probably mediated via the relatively less CO-sensitive, nickel-containing hydrogenases from *M. barkeri*. The specific role of the membrane-bound hydrogenases isolated from *D. vulgaris* Hildenborough (39) should also be examined.

This work is a first approach to the elucidation of in vivo gas metabolism in methanogenic bacteria, in pure cultures or in association with sulfate-reducing bacteria, by membrane-inlet mass spectrometry together with the use of stable isotypes. This short-term, on-line technique can prove an excellent tool for studying the different physiological or biochemical aspects of methanogenesis.

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