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# Control of Interspecies Electron Flow during Anaerobic Digestion: Significance of Formate Transfer versus Hydrogen Transfer during Syntrophic Methanogenesis in Flocs

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**Microbial formate production and consumption during syntrophic conversion of ethanol or lactate to methane was examined in purified flocs and digestor contents obtained from a whey-processing digestor. Formate production by digestor contents or purified digestor flocs was dependent on $CO_2$ and either ethanol or lactate but not $H_2$ gas as an electron donor. During syntrophic methanogenesis, flocs were the primary site for formate production via ethanol-dependent $CO_2$ reduction, with a formate production rate and methanogenic turnover constant of 660 μM/h and 0.044/min, respectively. Floc preparations accumulated fourfold-higher levels of formate (40 μM) than digestor contents, and the free flora was the primary site for formate cleavage to $CO_2$ and $H_2$ (90 μM formate per h). Inhibition of methanogenesis by $CHCl_3$ resulted in formate accumulation and suppression of syntrophic ethanol oxidation. $H_2$ gas was an insignificant intermediary metabolite of syntrophic ethanol conversion by flocs, and its exogenous addition neither stimulated methanogenesis nor inhibited the initial rate of ethanol oxidation. These results demonstrated that >90% of the syntrophic ethanol conversion to methane by mixed cultures containing primarily *Desulfovibrio vulgaris* and *Methanobacterium formicicum* was mediated via interspecies formate transfer and that <10% was mediated via interspecies $H_2$ transfer. The results are discussed in relation to biochemical thermodynamics. A model is presented which describes the dynamics of a bicarbonate-formate electron shuttle mechanism for control of carbon and electron flow during syntrophic methanogenesis and provides a novel mechanism for energy conservation by syntrophic acetogens.**

Anaerobic waste treatment is of increasing importance for downstream processing in biotechnological operations (30). High rates of methanogenesis are caused by the close association of different bacteria (species juxtapositioning) in microbial aggregates such as granules, flocs, or biofilms.

The complete conversion of organic matter into methane and $CO_2$ in anaerobic digestion ecosystems requires at least three functionally different trophic groups of bacteria (8, 17, 18, 20): (i) hydrolytic fermentative bacteria, (ii) syntrophic acetogenic bacteria, and (iii) methanogenic bacteria. Trophic group i functions to ferment complex organic matter into simple low-molecular-weight alcohols, organic acids, and $H_2$ gas employing hydrolytic enzymes. The organic fermentation products are further oxidized to acetic acid by trophic group ii, also called obligately syntrophic proton-reducing acetogenic bacteria (4, 13). The syntrophic acetogenic bacteria (syn, Greek: together; trophein, Greek: eat) putatively grow in mixed culture with $H_2$-consuming bacteria such as methanogens because their metabolism can be inhibited by $H_2$ gas (3–5, 23–26). The cytoplasmic pool of oxidized coenzymes is apparently not regenerated in syntrophic acetogenic bacteria under high $H_2$ pressures. Thus, a simultaneous electron transfer from a syntrophic acetogen to an $H_2$-consuming species is putatively essential for growth and metabolism, and this process is called interspecies $H_2$ transfer (5, 16). Methanogenic bacteria finally remove carbon and electrons from the ecosystem by cleavage of acetate to methane and $CO_2$ and reduction of $CO_2$ to methane. $H_2$ gas can be produced in anaerobic ecosystems by hydrolytic fermentative bacteria and syntrophic acetogenic bacteria as a result of proton reduction by hydrogenases, which oxidize reduced intracellular cofactors such as ferredoxins (27).

An alternative equivalent route to $H_2$ production is formate production via pyruvate:formate lyase or ferredoxin:$CO_2$ oxidoreductase (33) and subsequent formate cleavage to $H_2$ and $CO_2$ via formate:hydrogen lyase (33). Many methanogens utilize formate and $H_2$ for $CO_2$ reduction to methane (for a review, see reference 19). Methanogens similar to *Methanobacterium formicicum* or *Methanospirillum hungatii* are among the prevalent methanogens in anaerobic digestors (7, 39), eutrophic lake sediments (22), or syntrophic enrichment cultures (3, 7, 13, 15, 23, 25, 26). These species also produce $H_2$ gas during formate metabolism (31). The apparent turnover of formate in diverse anaerobic digestion ecosystems is usually very rapid (15, 29), and formate and $H_2$ gas pools are interconnected by reversible microbial formate:hydrogen lyases (33, 38). Thus, the control of syntrophic electron flow during methanogenesis could theoretically be achieved by either an interspecies formate transfer or an interspecies $H_2$ transfer coupled process.

Convincing evidence has been presented for the inhibitory effect of $H_2$ gas on the growth and metabolism of syntrophic acetogenic bacteria (3–5, 23–26) without direct evidence for the stoichiometric involvement of $H_2$ gas as the only intermediate in syntrophic methanogenesis. Interspecies $H_2$ transfer has been concluded to be the mechanism which

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controls electron flow in well-studied syntrophic methanogenesis systems including ethanol degradation by the classic "*Methanobacillus omelianskii*" mixed culture (5) and the benzoate-degrading consortium (13). Nonetheless, significant activities of formate metabolism have also been detected in both of these well-studied syntrophic associations (1, 13).

Our laboratory defined the microbial ecophysiology and biochemistry of lactose biomethanation in a steady-state whey-processing chemostat. Lactose was hydrolytically fermented to formate, lactate, and ethanol as major intermediates (6–8). The digestor flora comprised free-living cells and flocs. The syntrophic metabolism of lactate and ethanol was principally performed by *Desulfovibrio vulgaris* and *M. formicicum*, and homoacetogenic bacteria were not significant species in this ecosystem (7). Greater than 75% of syntrophic methanogenesis occurred compartmentalized inside the floc population (35). Purified flocs displayed stoichiometric syntrophic ethanol conversion to acetate and methane, but greater than 95% of the syntrophic methanogenesis was independent of the available $H_2$ pool outside the flocs.

The purpose of this study was threefold. First, to demonstrate the specific significance of $CO_2$ reduction and formate metabolism during the syntrophic conversion of ethanol and lactate to methane. Second, to obtain direct experimental evidence for the role of interspecies formate transfer during syntrophic ethanol conversion to methane and acetate by *D. vulgaris* and *M. formicicum* juxtapositioned in natural flocs from this whey-processing digestor. And finally, to evaluate the quantitative importance of $H_2$ gas as an intermediate of syntrophic ethanol conversion in the purified digestor flocs.

Table 1 provides a comparison of the energetics of key biochemical reactions associated with the overall syntrophic ethanol conversion to acetate and methane (I) via two possible routes (II versus III). Under standard conditions, coupling acetogenic ethanol oxidation via $CO_2$ reduction to formate (III) is nearly equivalent to that of a proton reduction to $H_2$ gas (II). Thus, the energetic difference between these reactions is not large enough thermodynamically to predict or exclude either interspecies $H_2$ transfer or interspecies formate transfer. The major aim of the experiments was to demonstrate and quantitate kinetically the significance of interspecies $H_2$ transfer (II) versus interspecies formate transfer (III) via an ethanol-dependent $CO_2$ reduction.

TABLE 1. Energetic and biochemical comparison of dehydrogenation and hydrogenation reactions for syntrophic ethanol conversion to methane by interspecies $H_2$ versus bicarbonate-formate transfer mechanisms

| Reaction type and equation(s) | $\Delta G^{\circ\prime}$ (kJ/reaction) |
|---|---|
| I. Syntrophic ethanol conversion | |
| 2 ethanol + $HCO_3^-$ → 2 acetate$^-$ + $H^+$ + $CH_4$ + $H_2O$ | −116 |
| II. Interspecies $H_2$ transfer | |
| 2 ethanol + $2H_2O$ → 2 acetate$^-$ + $2H^+$ + $4H_2$ | +19.2 |
| $HCO_3^-$ + $H^+$ + $4H_2$ → $CH_4$ + $3H_2O$ | −135 |
| III. Interspecies bicarbonate-formate transfer | |
| 2 ethanol + $4HCO_3^-$ → 2 acetate$^-$ + $2H^+$ + $4HCO_2^-$ + $2H_2O$ | +14 |
| $4HCO_2^-$ + $H_2O$ + $H^+$ → $CH_4$ + $3HCO_3^-$ | −130 |
| IV. Biochemical redox[a] | |
| Formate: hydrogen lyase | |
| $H_2$ + $HCO_3^-$ → $HCO_2^-$ + $H_2O$ | −1.3 |
| Hydrogenase | |
| $2Fd^{red}$ + $2H^+$ → 2 $Fd^{ox}$ + $H_2$ | +3.08 |
| $CO_2$ reductase | |
| $2Fd^{red}$ + $2H^+$ + $HCO_3^-$ → $2Fd^{ox}$ + $HCO_2^-$ + $H_2O$ | +1.77 |
| Alcohol-acetaldehyde dehydrogenase | |
| $CH_3CH_2OH$ + $4Fd^{ox}$ + $H_2O$ → $CH_3COO^-$ + $4Fd^{red}$ + $5H^+$ | +3.44 |

[a] $Fd^{red}$, Reduced ferredoxin; $Fd^{ox}$, oxidized ferredoxin.

## MATERIALS AND METHODS

**Chemicals, media, cultivation techniques, and biocatalyst preparation.** Nonradioactive chemicals, media, and cultivation techniques were as described previously (35). Sodium bicarbonate ($^{14}C$, 8 Ci/mol), sodium formate ($^{14}C$, 44 Ci/mol), and sodium acetate ($^3H$, 6,000 Ci/mol) were purchased from ICN Radiochemicals (Irvine, Calif.). Sodium lactate (DL, 1-$^{14}C$, 54 Ci/mol) was from Amersham Corp. (Arlington Heights, Ill.), and sodium pyruvate (1-$^{14}C$, 8.1 Ci/mol) was from New England Nuclear Research Products (Boston, Mass.). Conditions for operation of a steady-state whey-processing chemostat were as described previously (8, 35). Freshly purified washed flocs were prepared by fractionation and cultivation procedures described elsewhere (35). Phosphate-buffered basal (PBB) medium was prepared as described previously (21).

**$H_2$ inhibition studies.** Gas chromatographic analysis of gaseous and dissolved nonradioactive fermentation products and substrates was performed as described previously (35). Purified flocs in PBB medium (45 ml) were incubated in 158-ml vials under 2 atm (202.6 kPa) of $N_2$-$CO_2$ (95:5, vol/vol) in a gyratory water bath at 35°C and 100 rpm. As indicated in the figures, different amounts of ethanol, acetate, and $H_2$ gas were added to adjust the respective concentrations in liquid and headspace phases. Acetate was added to achieve constant zero-order rates of acetoclastic methanogenesis at acetate concentrations above 12 mM (35). To verify the concentration of dissolved $H_2$ gas in the liquid phase, 5-ml liquid samples from each inhibition experiment (45-ml total volume) were withdrawn with a sterile glass syringe and injected into a 158-ml pressure vial containing $H_2$-free $N_2$ at 1 atm (101.3 kPa) pressure. The vials were vigorously shaken. Extracted $H_2$ gas during the first 0.5 min was taken as the dissolved $H_2$ in the 5-ml liquid sample. Controls without added $H_2$ gas were extracted in parallel to determine the $H_2$ background from the culture. The background-corrected values obtained for the different $H_2$ concentrations were slightly higher than predicted from the Bunsen coefficient at 37°C.

**$CO_2$ incorporation assays.** Samples from the whey laboratory digestor contents or purified flocs from the same digestor were anaerobically transferred into autoclaved serum bottles with an $N_2$-$CO_2$ (95:5, vol/vol) headspace. Purified flocs were suspended in 40 ml of PBB medium (21) and were incubated in 158-ml bottles. In all other experiments, undiluted complete digestor contents (7 ml) were incubated in stoppered 8.6-ml serum vials with 0.1 ml of 2.5% $Na_2S$ and 0.1 ml of 10% $Na_2CO_3$. All serum vials were incubated at atmospheric pressure in a gyrotory water bath (G76; New Brunswick Scientific Co., Inc., Edison, N.J.) at 100 rpm and 37°C. Ethanol, sodium-D,L-lactate, or sodium acetate was added from 1 M neutralized stock solutions in PBB medium to the indicated final concentrations, and 0.1 ml of an $NaH^{14}CO_3$ solution in $H_2O$ (2 mCi/ml) was added to give final $^{14}CO_2$ specific activities between 0.63 and 2.2 Ci/mol. In

experiments with complete digestor contents, the acetate amendment to acetate levels above 12 mM was achieved by an organic shock load to the digestor 24 h before the experiments. Radioactive experiments were run at least in duplicate with one nonradioactive control. The total $CO_2$ content in the vials was determined for each experiment by acid extraction and an analysis of the headspace $CO_2$ content from the nonradioactive parallel control. Samples from floc experiments (3 ml) or digestor contents (0.2 ml) were taken anaerobically and immediately processed. Gas samples were taken from the headspace with pressure-lock syringes. Equivalent volumes of anaerobic $N_2$ were added after each sampling to compensate for the volume losses, and the appropriate corrections were applied in the concentration calculations.

**Sample pretreatments for HPLC analysis.** Samples for analysis of dissolved $^{14}CO_2$ incorporation products were pretreated as follows. A 3-ml sample from the experimental bottle was injected into a 158-ml serum bottle sealed with a black butyl stopper and containing an $N_2$ atmosphere and 100 μl of 6.5 N HCl. Radioactive $CO_2$ was stripped by extensive shaking, and the fluid was removed with a tuberculin syringe. NaOH (100 μl; 6.5 N) was added to the fluid, and the stripped sample was stored at −20°C or analyzed immediately. For analysis, samples (200 μl) were acidified with 20 μl of 10 N $H_2SO_4$ in capped 400-μl plastic vials, and 20 μl of [$^3$H]acetate (9 mCi/liter) was added. The sample was mixed and centrifuged at 10,000 × *g* for 5 min. Clear supernatant samples (200 μl) were used for the high-pressure liquid chromatographic (HPLC) analysis. Alternatively, for low sample volumes, samples (200 μl) from the experimental bottle were directly acidified with 20 μl of 10 N $H_2SO_4$ and 20 μl of [$^3$H]acetate was added and processed as described above. Both pretreatments gave identical results, and the carryover of radioactive $CO_2$ in the HPLC analysis system was negligible.

**Radioactive fermentation products analysis.** Radioactive dissolved organic $^{14}CO_2$ incorporation products were measured by HPLC with a Perkin-Elmer series 3 liquid chromatograph with an Aminex HP-X87 column (300 by 8.7 mm; Bio-Rad Laboratories, Richmond, Calif.). The column was operated at 10 to 13 lb/in$^2$ and 25°C, and the flow rate was 0.6 ml/min with 0.0144 N sulfuric acid used as the mobile phase. Fractions of 0.3 ml were collected with an FC-100 microfractionator (Gilson Medical Electronics, Middleton, Wis.), and the radioactivity was determined by scintillation counting in a Packard PRIAS Tri-Carb scintillation counter. Instagel (Packard) was used as scintillation liquid. A computerized dual-channel, dual-label analysis was performed on an Apple II computer by the channels ratio method by standard techniques. [$^{14}$C]formate and [$^3$H]acetate dissolved in mobile phase were used to determine the channel cross talk coefficients. The counting efficiency for $^{14}$C was 44% at optimized channel widths and gains.

Gaseous radioactive products were analyzed on a Packard model 417 gas chromatograph with a thermal conductivity detector in series with a Packard model 894 gas proportional counter. The separation of $CH_4$, $CO_2$, and CO was performed at 95°C on a 4-foot (122-cm), 2-mm inner diameter, stainless-steel column with Spherocarb 60/80 (Analabs-Foxboro Co., North Haven, Conn.). The system was operated with He as the carrier gas at 50 ml/min. Radioactive peaks were standardized with $^{14}CO_2$ (0.82 mCi/mol). Dissolved radioactive $CO_2$ in the experimental sample was determined by injecting 1 ml into a stoppered 10-ml pressure vial containing 1 ml of 1 N HCl. Radioactive methane and $CO_2$ in the gas phase were determined by direct analysis of the headspace in the experimental bottles.

Figure 1 demonstrates the quality of the HPLC-radioactive tracer analysis methods used. The system completely separated pyruvate, lactate, formate, acetate, propionate, and butyrate (data not shown). Ethanol eluted at completely different elution times. Elution time reproducibility was better than 97% with respect to the internal standard ([$^3$H]acetate). [$^{14}$C]formate and [$^{14}$C]acetate were readily separated. The base line fluctuated usually between 50 and 150 cpm. A correction of possible [$^{14}$C]acetate counts per minute in the formate peak region was accomplished, when necessary, by multiplication of the respective $^3$H counts per minute in the formate region with the $^{14}$C/$^3$H ratio in the acetate peak region. Acetate carryover into the formate peak was always less than 10% of the [$^{14}$C]formate counts per minute. The achieved separation was comparable with the results for similar methods published elsewhere showing elution of simple carbohydrates and amino compounds before the organic acids (14).

## RESULTS

**Formate as a key intermediate of syntrophic methanogenesis.** Experiments were designed to examine whether for-

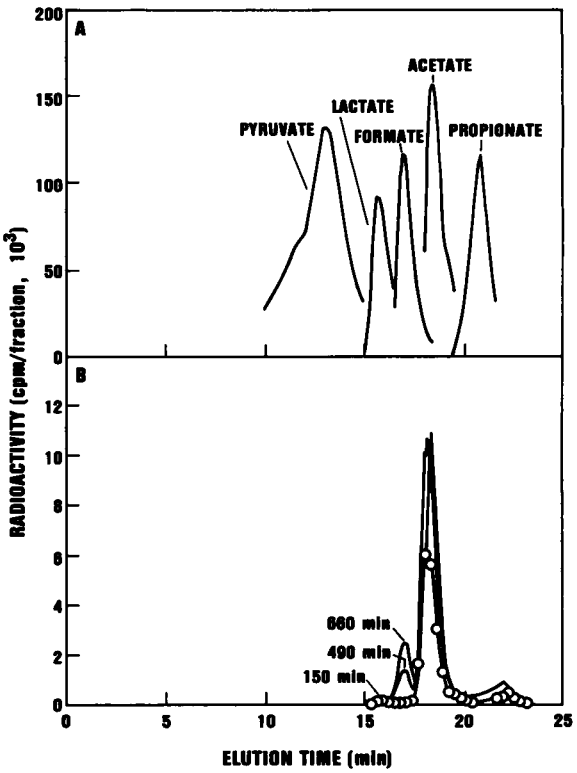


FIG. 1. HPLC-radiotracer methods performance. (A) Calibration of the HPLC chromatograph with various $^{14}$C-labeled organic acid standards. Each compound was analyzed in a single run with [$^3$H]acetate as the internal standard. The elution times shown are relative to the internal standard. Each compound showed a single peak. (B) Example for the separation and quantification quality of HPLC analysis of syntrophic $^{14}CO_2$ incorporation products. Three typical traces of $^{14}$C counts per minute from the same experiment in the presence of 0.04% $CHCl_3$ are shown superimposed and relative to the internal [$^3$H]acetate standard. The base line outside the peaks was 50 to 150 cpm. Symbol: ○, initial trace at 150 min.

mate was a significant product of $^{14}CO_2$ reduction in association with syntrophic methanogenesis from ethanol or lactate. Figure 2 compares the effect of $H_2$ and ethanol additions on the $^{14}CO_2$ incorporation into the formate pool by whey digestor contents. [$^{14}$C]formate levels were always below 10 μM in the presence either of exogenous $H_2$ (16,000 Pa) alone or ethanol (1.5 mM) alone. Formate was measured as a significant $CO_2$ incorporation product only in the presence of ethanol plus $H_2$, and the [$^{14}$C]formate levels were then concentration dependent on ethanol (Fig. 2). The [$^{14}$C]formate levels should have been identical under $H_2$ atmosphere (16,000 Pa) alone or $H_2$ (16,000 Pa) plus ethanol (1.5 mM) if $H_2$ was a physiological electron donor for $CO_2$ reduction. Therefore, these results indicate that radioactive formate was not formed as a product of $CO_2$ reduction by $H_2$ gas. Similar experimental results were obtained when lactate was substituted for ethanol (data not shown).

In parallel control experiments, $^{14}CO_2$ incorporation into the acetate pool was also examined (data not shown). These experiments demonstrated that $CO_2$ incorporation into [$^{14}$C]acetate pools was dependent on ethanol and independent of $H_2$ concentration. Hence, this excluded acetogenesis from $H_2$ and $CO_2$ and suggested that an isotopic exchange occurred between the carboxyl group of acetate and [$^{14}$C]formate. Low formate levels could have been a product of rapid unspecific $CO_2$ reduction combined with a rapid formate turnover rate. Thus, experiments were designed to eliminate the possibility that ethanol additions inhibited formate turnover. Figure 3 shows the [$^{14}$C]formate turnover in whey digestor contents in the absence (0.02 mM) or presence (1.4 mM) of ethanol conversion to acetate. Kinetic analysis revealed first-order kinetics and identical, rapid turnover constants of 0.15/min. The ethanol-dependent formate levels were thus only due to higher rates of $CO_2$ reduction to formate and not to an ethanol-dependent inhibition of the formate turnover. Methane was an insignificant end product of the [$^{14}$C]formate turnover. Formate:hydrogen lyase activity or an isotope exchange between [$^{14}$C]formate and $HCO_3^-$ was probably responsible for the turnover, because more than 99.5% of the labeled gaseous turnover products were recovered as $^{14}CO_2$ after acidification of the reaction mixture. The formate:hydrogen lyase reaction was thermodynamically favorable at the measured [$HCO_3^-$] and [$H_2$] levels (17 mM and 0.1%, respectively). The possibility of an active isotope exchange between formate and bicarbonate does not question measurements of ethanol-dependent $CO_2$ reduction, because formate from $CO_2$ reduction has the same specific radioactivity as the bicarbonate-$CO_2$ pool.

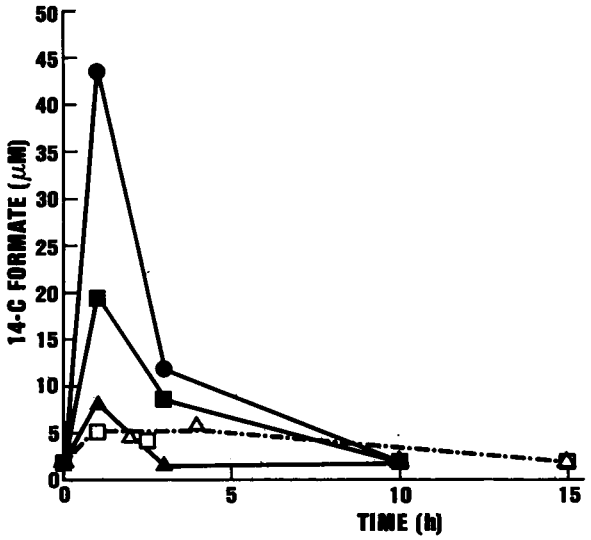


FIG. 2. Dependence of syntrophic formate production by anaerobic digestor contents on ethanol versus hydrogen. Anaerobic serum vials contained 7 ml of digestor contents, 1.4 ml of $N_2$-$Co_2$ (95:5, vol/vol), 0.1 ml of 10% $NaHCO_3$, 0.1 ml of 2.5% $Na_2S$, 14 mM acetate, 200 μCi of $NaH^{14}CO_3$, and the millimolar amounts of ethanol and/or pascals of $H_2$ indicated below. The control was without ethanol or $H_2$ additions. Symbols: ●, 3 mM ethanol, 32,000 Pa of $H_2$; ■, 1.5 mM ethanol, 16,000 Pa of $H_2$; ▲, no ethanol, 16,000 Pa of $H_2$; △, 1.5 mM ethanol, no $H_2$; □, control.

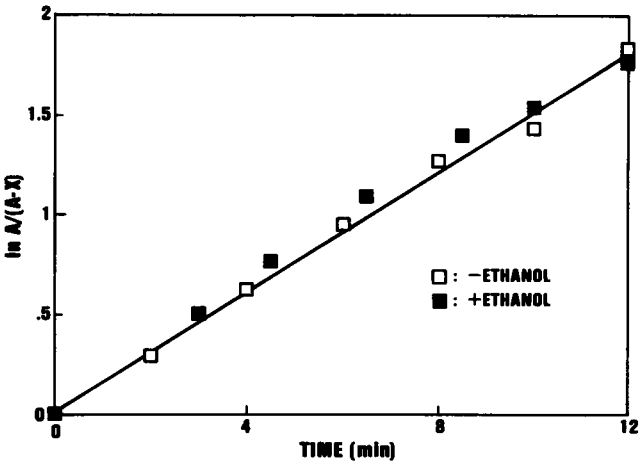


FIG. 3. Kinetic comparison of [$^{14}$C]formate turnover by the digestor contents with endogenous (0.02 mM) versus exogenous (1.4 mM) ethanol levels. Anaerobic serum vials contained 7 ml of digestor contents, 1.4 ml of $N_2$-$CO_2$ (95:5, vol/vol), 0.1 ml of 10% $NaHCO_3$, 0.1 ml of 2.5% $Na_2S$, 15 mM acetate, and the amounts of ethanol indicated in the text. [$^{14}$C]sodium formate (2.5 μCi) was added at 0 min. *A*, Initial formate radioactivity (cpm/0.2 ml); *X*, formate radioactivity removed (cpm/0.2 ml).

To demonstrate (see Fig. 4 and Table 2) that formate was an intermediate of syntrophic methanogenesis from ethanol, we blocked formate consumption with chloroform, a potent inhibitor of methanogens (2). The gas phase contained 50% $H_2$, 30% $CO_2$, and 20% $N_2$ to reduce formate decomposition via formate:hydrogen lyase activity in the aqueous phase. Immediately after $CHCl_3$ (0.04%, final concentration) and 50% $H_2$ addition at 330 min, ethanol consumption (Fig. 4B) and methane production (Table 2) stopped and [$^{14}$C]formate accumulated with time, reaching 300 μM. In the absence of exogenous $CHCl_3$, formate levels remained between 10 and 20 μM and the ethanol oxidation continued. This suggested that the prevalent formate-utilizing methanogenic bacteria rapidly utilized formate in the absence of $CHCl_3$ despite the presence of 50% $H_2$ gas and thus maintained the [$^{14}$C] formate levels below 30 μM. The rates of ethanol consumption were basically identical in parallel experiments with digestor contents incubated without $H_2$, and $H_2$ did not inhibit the syntrophic ethanol consumption in the digestor contents in three separate preparations (data not shown).

To test whether acetogenic ethanol consumption was actually inhibited by the accumulated formate, we added 16 mM sulfate at 720 min to experimental vials that were $CHCl_3$ inhibited since the ethanol-oxidizing species (*D. vulgaris*) could use sulfate as an electron acceptor alternate to using

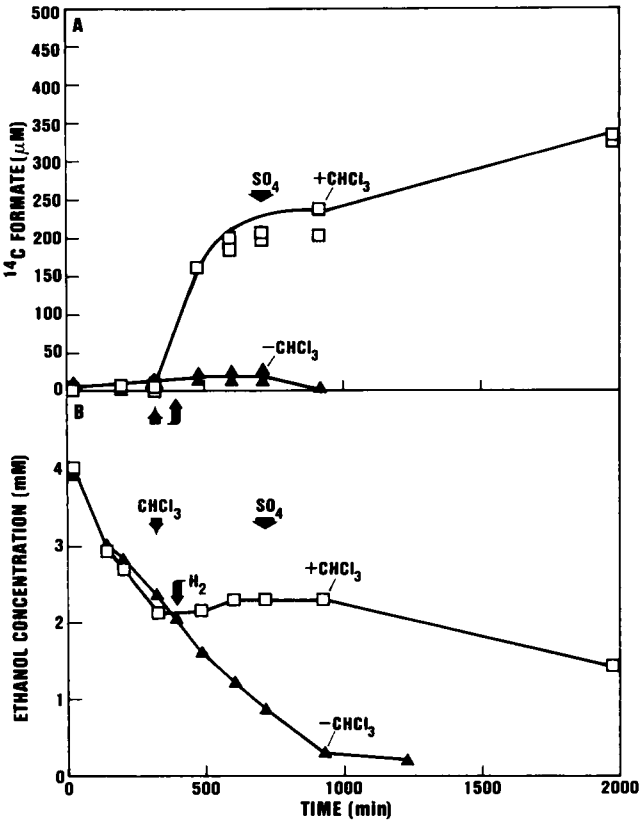


FIG. 4. Influence of $H_2$ and sulfate addition on syntrophic conversion of ethanol to formate in the presence versus the absence of the methanogenesis inhibitor $CHCl_3$. Serum vials contained 7 ml of digestor contents, 1.4 ml of $H_2$-$N_2$-$CO_2$ (50:20:30, vol/vol/vol), 0.1 ml of 10% $NaHCO_3$, 0.1 ml of 2.5% $Na_2S$, 3 mM ethanol, 30 mM acetate, and 800 μCi of $NaH^{14}CO_3$. $H_2$, (50%), 16 mM $Na_2SO_4$, or 0.04% $CHCl_3$ was added as indicated by the arrows.

*M. formicicum*. After a lag, ethanol consumption reinitiated and formate levels increased (Fig. 4; Table 2). The results in Fig. 4 and Table 2 showed that (i) $CHCl_3$ did not inactivate ethanol-oxidizing acetogens, (ii) formate was synthesized from bicarbonate and ethanol, and (iii) the lack of electron acceptors inhibited ethanol oxidation in the presence of high formate concentrations.

The free energy for ethanol dehydrogenation to $H_2$ was +15.4 kJ per reaction in the presence of 50,000 Pa of $H_2$ versus −14.0 kJ per reaction in the presence of $N_2$; however, ethanol oxidation continued in the presence of $H_2$ (Table 2).

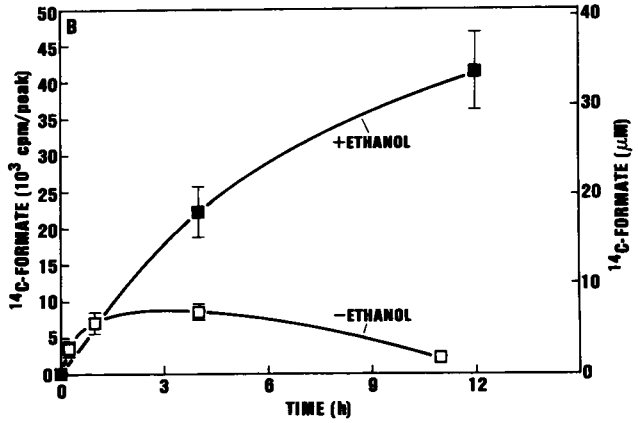


FIG. 5. Dependence of syntrophic $CO_2$ reduction to formate by purified digestor flocs on ethanol. Anaerobic serum vials contained 0.35 mg of protein from purified digestor flocs, 40 ml of PBB medium, 118 ml of $N_2$-$CO_2$ (95:5, vol/vol), 100 μCi of $NaH^{14}CO_3$, 13 mM acetate, and 6.9 mM ethanol (+ethanol) or 0.02 mM ethanol (−ethanol). The flocs were preincubated in PBB medium in the absence of [$^{14}$C]bicarbonate with 10 mM ethanol and 8 mM acetate, and 40-ml samples were taken for $CO_2$ incorporation assays with 100 μCi of $NaH^{14}CO_3$ after 50% ethanol conversion (+ethanol) and when ethanol was depleted (−ethanol).

This showed that $H_2$ could not be the reducing equivalents that mediated control of syntrophic ethanol oxidation because ethanol oxidation proceeded despite positive free energy changes. On the other hand, the free energy for ethanol dehydrogenation to formate was −34.5 versus −19.7 kJ per reaction in the presence versus the absence of 50,000 Pa of $H_2$. Although the initial rate of ethanol consumption was the same in the $H_2$ as in the $N_2$ gas phase, the measured rate at 3 to 5 h decreased by 40% (i.e., from 5 to 3 μM/min), which correlated with the predicted differences in thermodynamic driving force owing to the formate accumulation (i.e., from −34.5 to −19.7 kJ per reaction).

**Interspecies electron transfer in flocs.** Previous studies showed that bacterial flocs in the digestor contents were the active site of syntrophic ethanol metabolism (35). To demonstrate the importance of $CO_2$ reduction to formate during syntrophic ethanol conversion, we placed purified flocs (0.35 mg of protein) into serum vials that contained 10 mM ethanol and 8 mM acetate. Subsamples were taken when 50 and 100% of the ethanol was converted to methane and acetate; these subsamples were incubated with $H^{14}CO_3^-$ in an $N_2$-$CO_2$ atmosphere (95:5, vol/vol). Figure 5 compares the time course of formate production by these flocs in the presence or absence of ethanol. In the presence of ethanol,

TABLE 2. Bioenergetic analysis of $H_2$ versus formate as the intermediary reducing equivalents which control syntrophic methanogenesis from ethanol and bicarbonate by anaerobic digestor contents[a]

| Condition | Metabolite concn | | | | | Ethanol consumption (μM/min) | Driving force (kJ/reaction) | |
|---|---|---|---|---|---|---|---|---|
| | Ethanol (mM) | Acetate (mM) | Formate (μM) | $CH_4$ (μmol/vial) | $CO_2$ (mM) | | $\Delta G'_{H_2}$ | $\Delta G'_{formate}$ |
| $N_2$-$CO_2$ | 2.36 | 28.4 | 5 | ND | 21.7 | 5 | −14.0 | −34.5 |
| $H_2$-$CO_2$ | 0.86 | 32.6 | 20 | 5.4 | 21.7 | 2.9 | +15.4 | −19.7 |
| $H_2$-$CO_2$ + $CHCl_3$ | 1.7 | 30.6 | 250 | 2.1 | 21.7 | 0 | +13.5 | −8.6 |

[a] Experimental conditions were as described in the legend to Fig. 4. The measurements and calculations were determined 400 min after initiation of the time course experiments. The driving force (34) was calculated with the following equations: $\Delta G'_{H_2} = 9.6 + 5.94 \log ([acetate]\,[H_2]^2/[ethanol])$ and $\Delta G'_{formate} = 7.0 + 5.94 \log ([acetate]\,[formate]^2/[ethanol]\,[HCO_3^-]^2)$.

[b] ND, Not determined.

[$^{14}$C]formate levels reached 30 to 40 μM ($n$ = 4), whereas in the absence of syntrophic ethanol metabolism, [$^{14}$C]formate levels were below 15 μM. Thus, formate was a direct product of ethanol-dependent syntrophic $CO_2$ reduction. The formate levels measured were comparable to the values obtained with complete digestor contents at 30,000 to 50,000 Pa of $H_2$ (Fig. 2 and 4).

Freshly purified floc preparations were incubated in PBB medium with 12 mM ethanol and 14 mM acetate and pulsed with ethanol and different amounts of $H_2$ to test whether $H_2$ gas was important as an intermediate of syntrophic metabolism inside the flocs. Syntrophic ethanol conversion to acetate and methane proceeded in the presence of various hydrogen levels for more than 4 h before a significant inhibition occurred at 21,000 Pa of added $H_2$ (Fig. 6). The thermodynamic driving force for acetogenic $H_2$ production at 21,000 Pa of $H_2$ was calculated to be +1.61 kJ per reaction, but the initial reaction rate was not inhibited. A delayed transient inhibition of ethanol consumption was observed at 10,800 Pa. Lower $H_2$ partial pressures (1,800 Pa) did not significantly inhibit ethanol oxidation. The addition of exogenous $H_2$ did not stimulate the total rate of methane

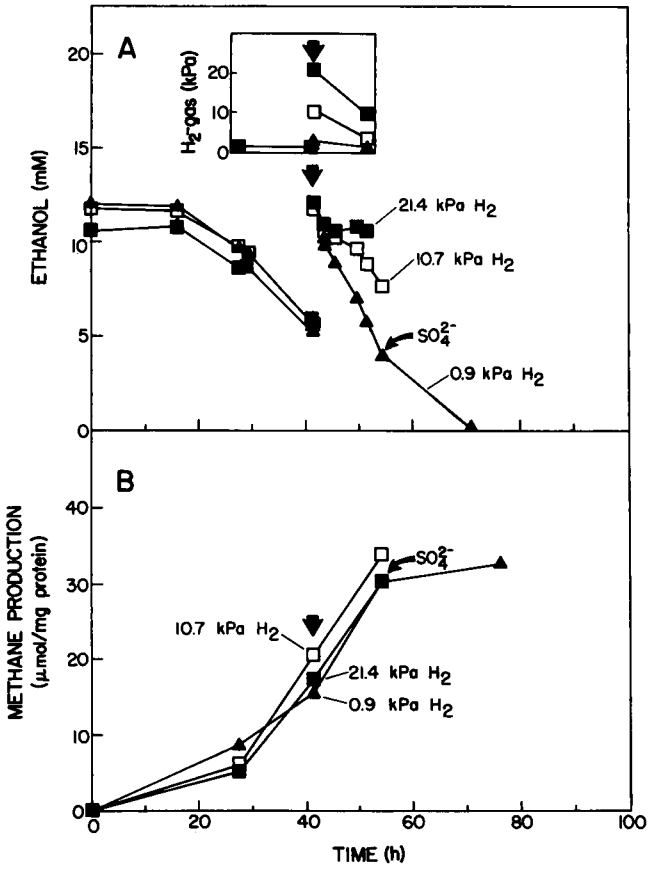


FIG. 6. Influence of exogenous $H_2$ levels and sulfate addition on syntrophic conversion of ethanol to methane. Freshly prepared flocs (19.5 mg per vial) were incubated with 11 mM ethanol and 14 mM acetate in three parallel 158-ml serum vials that contained 45 ml of PBB medium and an $N_2$-$CO_2$ (95:5, vol/vol) atmosphere. At 41 h, 14 mM ethanol and the $H_2$ gas levels indicated were added. The $H_2$ partial pressure in the vials before the additions was 300 to 400 Pa. Where indicated, 10 mM $Na_2SO_4$ was added. The non-sulfate-containing control (data not shown) reached 38 μM methane per mg of protein at 70 h.

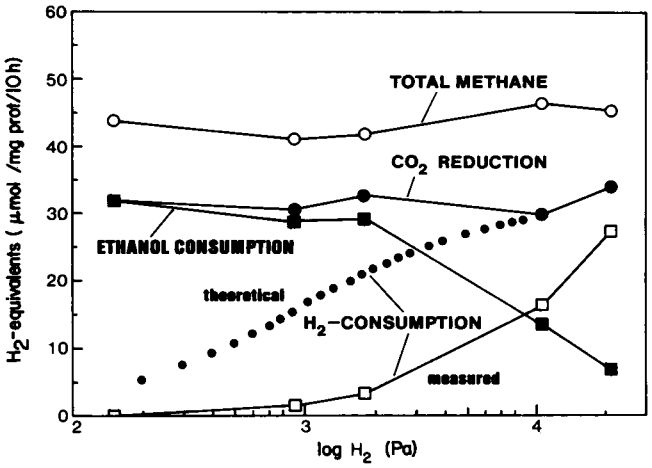


FIG. 7. Influence of $H_2$ partial pressure on the electron balance of syntrophic ethanol conversion to methane. The data were calculated from the 41- to 51-h conditions shown in Fig. 7. The nonconnected dots (●) represent predicted theoretical values for $H_2$ consumption by the prevalent $H_2$-consuming methanogen *M. formicicum* in this digestor ecosystem with a $K_s$ of $H_2$ consumption (35) of 6.5 μM dissolved $H_2$ (1,000 Pa) and a $V_{max}$ of 3.5 μM $H_2$ per mg of floc protein per h.

formation from $H_2$. This indicated that $CO_2$-reducing methanogens in the flocs were already quantitatively saturated with hydrogen equivalents during active ethanol oxidation at low endogenous $H_2$ partial pressures (200 to 400 Pa). A phase transfer limitation of $H_2$ into the flocs can be excluded because $CO_2$ reduction to methane was independent of the $H_2$ concentration in the headspace and the $H_2$ flux into the flocs was large enough to inhibit the ethanol-oxidizing acetogens. It would take less than 120 s for the $H_2$ pools within the floc phase and the digestor liquid phase to reach equilibrium (28). The addition of $Na_2SO_4$ to floc samples in the presence of 15 mM acetate blocked methane formation, but not ethanol consumption, showing that *D. vulgaris* was an ethanol-oxidizing organism in the flocs.

Figure 7 compares the $H_2$ equivalents associated with syntrophic ethanol conversion to methane and the consumed $H_2$ gas as a function of the experimental $H_2$ concentration. $CO_2$-reducing methanogens were apparently saturated with reducing equivalents in the flocs even at low $H_2$ concentrations because the total amount of electrons used for $CO_2$ reduction remained constant and independent of $H_2$ up to 21,000 Pa. This indicates that an $H_2$-independent syntrophic mechanism provided the reducing equivalents for methanogenic $CO_2$ reduction.

## DISCUSSION

These results demonstrate several new ecophysiological features of the anaerobic digestion process. First, formate is an important intermediary metabolite and a key source of reducing equivalents which control electron flow during syntrophic methanogenesis from complex organic matter. Second, interspecies formate transfer between syntrophic acetogens and methanogens functions to couple electron flow to methanogenic $CO_2$ reduction, when either ethanol or lactate is oxidized to acetate. Finally, interspecies formate transfer inside microbial flocs can function to separate syntrophic electron flow from the $H_2$ metabolism in anaero-

bic ecosystems. This is a possible explanation for the observed compartmentalization or separation of syntrophic methanogenesis in digestors and lake sediments from the dissolved $H_2$ pool (9, 10).

In the digestor ecosystem studied, formate was the important intermediary metabolite of syntrophic ethanol conversion to methane. Flocs were the active site for syntrophic ethanol conversion because >75% of this activity was floc associated (35) and formate accumulated to fourfold-higher levels (40 μM) in floc suspensions than in the complete digestor contents (10 μM). Formate was the important reduced intermediary metabolite because $CHCl_3$ addition stimulated formate levels from 40 to 250 μM and the formate turnover was very rapid. A methanogenic formate turnover constant within the floc of 0.275/min was calculated during active syntrophic ethanol conversion.

The measured formate pool concentration (10 μM) and turnover constants outside the flocs (0.15/min) yielded a maximum formate flux to $H_2$ and $CO_2$ of 90 μM/h. It can be computed from the simultaneously measured syntrophic ethanol flux in these experiments (440 μM/h) and a stoichiometry of 2 mol of formate per mol of ethanol that maximally 10% of the formate appeared in the external pool of the digestor contents where it could have been converted to $H_2$ gas by formate:hydrogen lyase activity. However, formate turnover to methane in the flocs was approximately 50 times faster than formate turnover to $H_2$ in the environment.

Several lines of biochemical thermodynamic analysis support the quantitative insignificance of $H_2$ as the source of the reducing equivalents which controlled interspecies electron transfer during syntrophic ethanol conversion to methane in the ecosystem we studied and raise queries about previous studies. The ratio of [formate]/[$H_2$] in the liquid phase depends on the bicarbonate concentration in the presence of a reversible formate:hydrogen lyase activity (Fig. 8A). At 30% $CO_2$, this ratio would be as high as 0.9999 in our system. The molar ratio of formate/hydrogen in a complete test system, however, is strongly dependent on the liquid/headspace ratio, because $H_2$ is poorly soluble. It should be noted that many previous studies on interspecies $H_2$ transfer were based on mass balances at the end of the experiments (3, 4, 13, 23–25, 40) with standard test tubes with liquid/headspace ratios as small as 0.3 and in which the physiological dissolved molar formate/$H_2$ ratio of nearly 195 was altered to nearly 1.0. Under these conditions, $H_2$ and not formate would be the preferred electron donor.

Figure 8B compares the relative rates of methanogenesis from formate versus $H_2$ in the presence of formate:hydrogen lyase activity based on the existing kinetic data from pure cultures of $H_2$ and formate-utilizing methanogens (31, 32). Methanogenic utilization of formate instead of $H_2$ could show rates of methane formation three to four times higher at physiological $H_2$ partial pressures. This has important consequences in bacterial flocs or granules ($10^{10}$ to $10^{12}$ bacteria per ml), with high volumetric reaction rates and without internal diffusion limitations (28). Thus, formate could be a better source of reducing equivalents for methanogenic $CO_2$ reduction, and this has been observed in digestor granules (11, 12).

Figure 8C compares the theoretical free energy difference for acetogenic excretion of reducing equivalents as either $H_2$ or formate. It would be energetically advantageous for syntrophic acetogens to excrete formate between 10 and 200 μM when $H_2$ partial pressures are $\geq 10^{-3}$ atm (≥0.1 kPa) and even at [$H_2$] below $10^{-3}$ atm when formate levels are ≤10 μM. These concentrations are within the reported range for

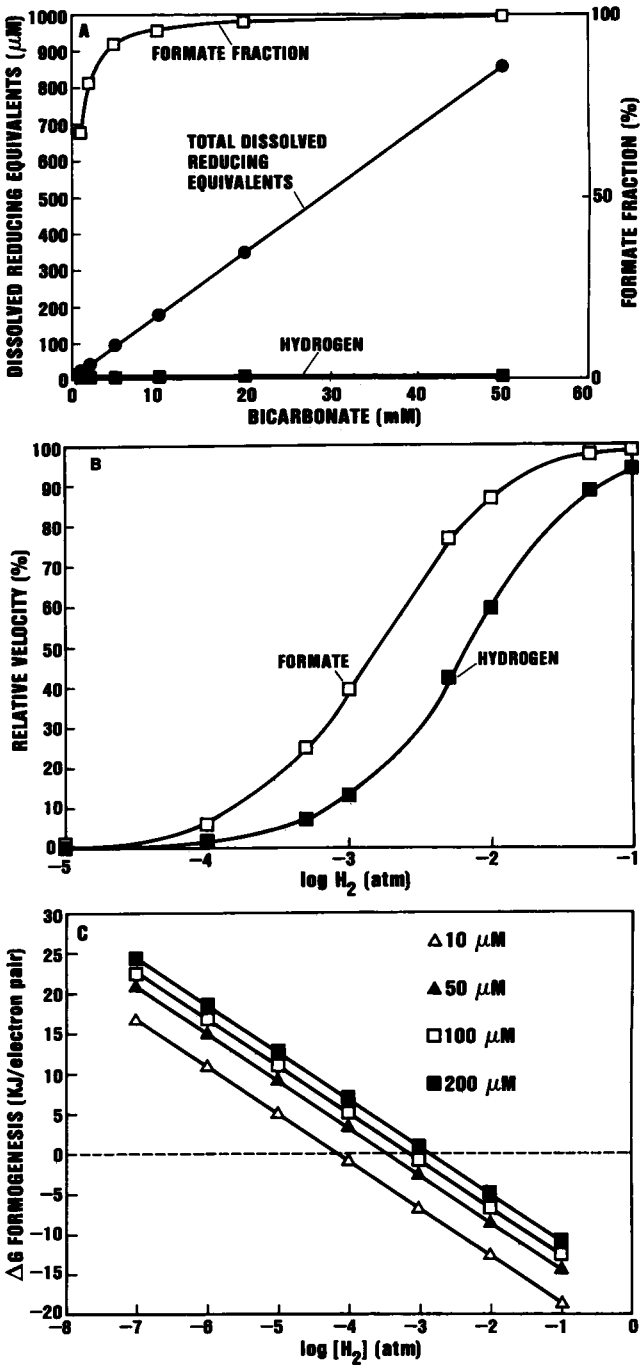


FIG. 8. Analysis of physical and bioenergetic factors controlling formate and $H_2$ metabolism during anaerobic digestion. (A) Dependence of the dissolved reducing equivalents on the bicarbonate concentration. The data were calculated for an $H_2$ partial pressure of 1,000 Pa. (B) Relative rates of methanogenesis via formate or $H_2$ as a function of the $H_2$ partial pressure. It was assumed that $H_2$, bicarbonate, $H_2O$, and formate were at chemical equilibrium at 35°C ($K_{assoc}$ = 1.66). The concentration of bicarbonate used was 80 mM. A $K_m$ for $H_2$ of 5 μM and for formate of 220 μM were used (31, 32). $V_{max}$ was assumed to be identical for both substrates. (C) Dependence of the bioenergetic advantage of $CO_2$ reduction to formate on the $H_2$ partial pressure. The free energy difference for acetogenic formate versus $H_2$ production (ΔG FORMOGENESIS) was calculated according to reference 34 and Table 2. A bicarbonate concentration of 21 mM and equal ethanol and acetate concentrations were used.

the whey digestor (6), the rumen (15), and lake sediments (29).

We developed a model which explains the results quantitatively and propose a new hypothetical mechanism of energy conservation for syntrophic acetogens. Figure 9 illustrates this bicarbonate-formate electron shuttle model, which is quite different from interspecies $H_2$ transfer since bicarbonate is a substrate for the acetogens, not for the methanogens. *D. vulgaris* and *M. formicicum* were the prevalent syntrophic acetogen and $CO_2$-reducing methanogen, respectively, in the anaerobic digestion ecosystem examined here (7). The acetogens transport four molecules of bicarbonate into their cytoplasm. $CO_2$ and $OH^-$ are then produced intracellularly by putative carbonic anhydrase. $CO_2$ is the physiological substrate of a putative ferredoxin:$CO_2$ oxidoreductase (33), which oxidizes reduced ferredoxin molecules from the acetogenic metabolism of two molecules of ethanol. Four molecules of formic acid are produced, which dissociate into formate and protons at the physiological pH of the ecosystem. Methanogenic bacteria utilize four molecules of formate, one proton, and $H_2O$ to produce $CH_4$ and three molecules of bicarbonate. One molecule of $HCO_3^-$ is provided by acetoclastic methanogenesis in this ecosystem. Thus, acetate, a product of the syntrophic metabolism, is recycled via bicarbonate into formate, an intermediate of syntrophic methanogenesis. The kinetic coupling of adjacent metabolic partners creates a syntrophic microniche within a floc (36) and results in the postulated formation of a cyclic bicarbonate-formate electron shuttle mechanism accounting for syntrophic methanogenesis.

At least three different bacterial species are required for efficient syntrophic methanogenesis in anaerobic digestion ecosystems according to this model: formogenic acetogens, formate-utilizing methanogens, and acetoclastic methanogens. Formogenic acetogens produce acetate and formate which are consumed by acetoclastic and formate-utilizing methanogens. In return for energy substrates, the methanogens provide conditions for an energetically favorable acetogenesis by formate removal, bicarbonate production, and by maintaining the molar bicarbonate/formate ratio at 1,000 to 10,000. This huge ratio functions as thermodynamic battery for the syntrophic acetogens, and it may provide, under in situ conditions, −5 to −10 kJ per electron pair more free energy than a mechanism dependent on interspecies $H_2$ transfer. Thus, the acetogens could obtain a larger fraction of the overall free energy change of the syntrophic conversion if they communicated via interspecies formate transfer in lieu of interspecies $H_2$ transfer. The bicarbonate-formate electron shuttle mechanism can explain syntrophic acetogenic energy conservation under conditions in which the bioenergetics of the acetogenic catabolism would not permit substrate level phosphorylation (35, 37; D. F. Dwyer, D. R. Shelton, and J. M. Tiedje, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, I27, p. 126). If the putative $CO_2$ reductase was connected to the exterior membrane surface and carbonic anhydrase was in the cytoplasm of the acetogenic bacteria, then catabolic $CO_2$ reduction would lead to water cleavage, charge separation, acidification of the periplasmic space, and generation of a proton motive force since formic acid is a much stronger acid than bicarbonate. Thus, acetogenic $CO_2$ reduction contains the theoretical possibility for ATP generation by electron transport-coupled phosphorylation.

Finally, it appears that interspecies $H_2$ transfer and bicarbonate-formate transfer are both plausible mechanisms for coupling syntrophic methanogenesis, but more detailed studies are needed to assess their relative quantitative significance in natural and applied ecosystems. The formate-bicarbonate electron shuttle model proposed here will be

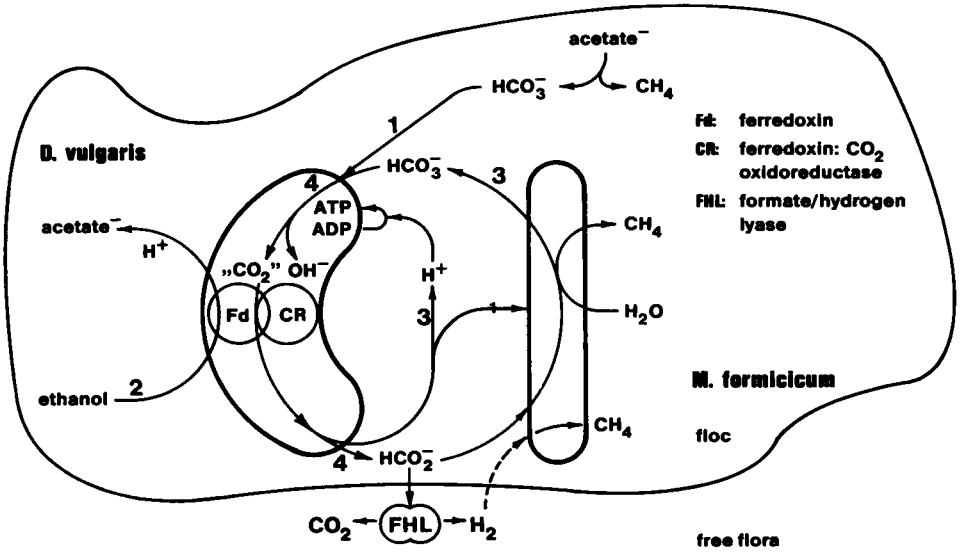


FIG. 9. Hypothetical model illustrating the principles of the bicarbonate-formate electron shuttle mechanism for explaining both control of interspecies electron flow during syntrophic ethanol conversion to methane by anaerobic digestor flocs and energy conservation by electron transport-mediated phosphorylation in syntrophic acetogens. The juxtapositioning of *D. vulgaris* and *M. formicicum* in flocs enables methanogenic $CO_2$ regeneration to be coupled with acetogenic $CO_2$ reduction to formate which itself serves as the mediator of interspecies electron flow. Energy conservation in the acetogen is postulated to occur as a consequence of putative cytoplasmic carbonic anhydrase and a membrane-linked $CO_2$ reduction which leads to generation of an alkaline cell interior, acidic exterior, and a proton motive force to drive membrane-coupled ATP synthesis. The model shows the dynamics of formate metabolism in the digestor ecosystem. More than 90% of the $CO_2$-dependent syntrophic ethanol conversion to methane occurs via interspecies formate transfer within flocs, whereas <10% occurs via interspecies $H_2$ transfer caused by cleavage of floc-excreted formate in the soluble digestor phase to $H_2$ and $CO_2$.

further tested with pure and mixed cultures to examine the significance of interspecies bicarbonate-formate transfer in other ecosystems and to obtain evidence for the proposed electron transport coupled phosphorylation mechanism of syntrophic acetogenic energy conservation. Clearly, interspecies $H_2$ transfer is the only plausible mechanism in syntrophic cocultures whose partners lack formate metabolism. However, the significance of interspecies bicarbonate-formate transfer should be reevaluated in other previously studied mixed cultures including the original "*M. omelianskii*" system (1), the benzoate consortium (13), and butyrate-degrading systems containing *Syntrophomonas wolfei* (37).

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