Diffusion of the Interspecies Electron Carriers \( \text{H}_2 \) and Formate in Methanogenic Ecosystems and Its Implications in the Measurement of \( K_m \) for \( \text{H}_2 \) or Formate Uptake

DAVID R. BOONE,* RICHARD L. JOHNSON, AND YITAI LIU
Environmental Science and Engineering, Oregon Graduate Center, 19600 N.W. Von Neumann Drive, Beaverton, Oregon 97006-1999

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We calculated the potential \( \text{H}_2 \) and formate diffusion between microbes and found that at \( \text{H}_2 \) concentrations commonly found in nature, \( \text{H}_2 \) could not diffuse rapidly enough to dispersed methanogenic cells to account for the rate of methane synthesis but formate could. Our calculations were based on individual organisms dispersed in the medium, as supported by microscopic observations of butyrate-degrading cocultures. We isolated an axenic culture of Syntrophomonas wolfei and cultivated it on butyrate in syntrophic coculture with Methanobacterium formicicum; during growth the \( \text{H}_2 \) concentration was 63 nM (10.6 Pa). \( S. \) wolfei contained formate dehydrogenase activity (as does \( M. \) formicicum), which would allow interspecies formate transfer in that coculture. Thus, interspecies formate transfer may be the predominant mechanism of syntrophy. Our diffusion calculations also indicated that \( \text{H}_2 \) concentration at the cell surface of \( \text{H}_2 \)-consuming organisms was low but increased to approximately the bulk-fluid concentration at a distance of about 10 \( \mu \)m from the surface. Thus, routine estimation of kinetic parameters would greatly overestimate the \( K_m \) for \( \text{H}_2 \) or formate.

During the complete degradation of organic matter to \( \text{CH}_4 \) and \( \text{CO}_2 \) in the absence of inorganic electron acceptors other than \( \text{CO}_2 \), acetate accounts for about two-thirds of the methane formed. The catabolism of acetate to \( \text{CH}_4 \) and \( \text{CO}_2 \) is accomplished by methanogens via the aceticlastic reaction, without syntrophic participation by other organisms (30). \( \text{CO}_2 \) reduction to methane accounts for essentially all of the remainder of methane formation (28). In addition to the quantitative importance of \( \text{CO}_2 \)-reducing methanogens, the activity of these organisms is essential for functioning of other metabolic groups (10), especially those which degrade compounds such as propionate (13) and butyrate (32). These compounds are thought to be degraded by interspecies \( \text{H}_2 \) transfer, a process by which \( \text{H}_2 \)-using methanogens maintain low \( \text{H}_2 \) concentration, allowing exergonic \( \text{H}_2 \) production from low-energy electrons (10). However, most of these \( \text{H}_2 \)-using methanogens can also use formate as the electron donor for catabolic \( \text{CO}_2 \) reduction to \( \text{CH}_4 \), and some can also use alcohols, such as ethanol or 2-propanol, as the electron donor. The importance of \( \text{H}_2 \) as a methanogenic substrate relative to formate has been widely assumed, but experimental demonstration is problematic.

The concentrations of \( \text{H}_2 \) and formate in ecosystems such as anaerobic digesters are extremely low because many of the reactions from which they are formed can occur only under these conditions. For instance, propionate and butyrate oxidation (Table 1) is accomplished by syntrophy, with electrons shuttled from fatty acid oxidizing bacteria to methanogens. The propionate-oxidizing bacterium Syntrophobacter wolinii requires the lowest \( \text{H}_2 \) concentration because the free-energy change for propionate oxidation to acetate, \( \text{CO}_2 \), and \( \text{H}_2 \) is negative only when \( \text{H}_2 \) concentration is very low (Fig. 1). The free-energy changes in Fig. 1 were calculated on the basis of reported concentrations in a methanogenic digester (7), with butyrate concentration assumed to be the same as propionate concentration (0.75 mM). If \( \text{H}_2 \) is the interspecies electron carrier, its concentration must be between about 1 and 30 nM in order for propionate oxidation and methanogenesis both to be exergonic, because microbes in that environment use each of these reactions as a source of energy. Thus, the \( \text{H}_2 \) concentration may be about 10 nM. A \( \text{H}_2 \) concentration of 8.8 nM would make reactions i and ii (Table 1) equally exergonic (per mole of propionate or \( \text{CH}_4 \), respectively). A concentration of 11.3 nM would equalize the energy yield per electron pair (i.e., per mole of \( \text{H}_2 \)). Although precise reports of formate concentrations in digesters are scarce, thermodynamic considerations analogous to those discussed above indicate that the concentration of formate is about 2.5 \( \mu \)M (Fig. 1). The low concentrations of these compounds in digesters make reactions that they undergo difficult to quantify.

Direct approaches for measurement of the degradation rate of \( \text{H}_2 \) or formate in anaerobic digesters, for instance, by using isotopes to measure turnover (38), have been unsuccessful for several reasons. The most important problem is that there are many exchange reactions which occur, leading to the formation of tritiated water from \( ^3\text{H}_2 \) or \( ^3\text{CO}_2 \) or \( ^3\text{CO}_2 \) from \( ^2\text{H}_2 \) in the absence of net reactions. Also, the concentrations of \( \text{H}_2 \) and formate are very small, and because the rates of production and consumption of these molecules may be very rapid (2), the average lifetimes of such molecules are very short. Thus, concentrations of these molecules may change rapidly during collection of samples for analysis. For example, the rate of \( \text{CO}_2 \) reduction to methane in the digester mentioned above (7) was 181 nmol of methane per liter per s. Assuming that \( \text{H}_2 \) was used in that reaction, the \( \text{H}_2 \) utilization rate was 722 nmol/liter per s and the turnover rate of \( \text{H}_2 \) was 72/s, giving an average lifetime of 0.014 s for \( \text{H}_2 \). Because of these difficulties, the direct measurement of \( \text{H}_2 \) or formate turnover has not been accomplished. This rapid turnover also raises questions relating to mass transfer of the \( \text{H}_2 \) between producers and consumers and suggests that some other mechanism may be operating.

* Corresponding author.
The first major, experimental evidence indicating that formate may be an important extracellular intermediate in methanogenic systems was provided by Thiele and Zeikus (42). We present an investigation of a butyrate-oxidizing bacterium in coculture with \( \text{H}_2 \) and formate-consuming organisms and an analysis of \( \text{H}_2 \) and formate diffusion between such microbes and show that interspecies \( \text{H}_2 \) transfer alone probably does not account for observed reactions in methanogenic ecosystems. Further, we show that previous determinations of \( K_w \) for hydrogenases reflect physical factors of diffusion rather than characteristics of the enzyme.

**MATERIALS AND METHODS**

**Source of cultures and growth conditions.** *Methanobacterium formicicum* MF (OGC 55) and *Desulfovibrio* sp. strain G11 (OGC 18) were obtained from the Oregon Graduate Center culture collection, Beaverton, Oreg. The isolation of *Syntrophomonas wolfei* LYB (OGC 65) and strain 8508 (OGC 60) is described herein. Rumen-fluid-based medium (32) was used early in the study. Later, cultures were grown in MS medium, which contained (per liter of Milli-Q-deionized water, 5.9 mS/m [Millipore Corp., Bedford, Mass.]) 4.0 g of NaOH, 2.0 g of yeast extract (Difco Laboratories, Detroit, Mich.), 2.0 g of Trypticase peptones (BBL Microbiology Systems, Cockeysville, Md.), 1.0 g of \( \text{NH}_4\text{Cl}, 1.0 \text{ g of MgCl}_2 \cdot 6\text{H}_2\text{O}, 0.2 \text{ g of 2-mercaptoethanesulfonic acid, 0.4 } \text{ g of K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O, 0.4 } \text{ g of CaCl}_2 \cdot 2\text{H}_2\text{O, 250 mg of Na}_2\text{S} \cdot 9\text{H}_2\text{O, 5.0 mg of disodium EDTA \cdot 2H}_2\text{O, 1.5 mg of CoCl}_2 \cdot 6\text{H}_2\text{O, 1.0 mg of resazurin, 1.0 mg of MnCl}_2 \cdot 4\text{H}_2\text{O, 1.0 mg of FeSO}_4 \cdot 7\text{H}_2\text{O, 1.0 mg of ZnCl}_2, 0.4 \text{ mg of AlCl}_3 \cdot 6\text{H}_2\text{O, 0.3 mg of Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O, 0.2 mg of CuCl}_2 \cdot 2\text{H}_2\text{O, 0.2 mg of NiSO}_4 \cdot 6\text{H}_2\text{O, 0.1 mg of H}_2\text{SeO}_3, 0.1 \text{ mg of H}_2\text{BO}_3, and 0.1 mg of NaMoO}_4 \cdot 2\text{H}_2\text{O.} \) Medium was prepared by adding all ingredients other than 2-mercaptoethanesulfonic acid and sulfide and allowing the solution to come to equilibrium with a gas mixture of \( \text{N}_2 \) and \( \text{CO}_2 \) (7:3) by bubbling for at least 1 h. The 2-mercaptoethanesulfonic acid was added, and the medium was dispensed into 126-ml serum bottles (nominal volume, 100 ml) and sealed with butyl rubber stoppers while air was excluded. The medium was autoclaved (121°C, 20 min). Sulfide was added 1 h before use from sterile, \( \text{O}_2 \)-free stock solutions. Culture techniques were the syringe modifications of the Hungate technique (23). Cultures were incubated at 37°C, and liquid

<table>
<thead>
<tr>
<th>Reactions</th>
<th>Equation</th>
<th>( \text{kJ/reaction} )</th>
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<tbody>
<tr>
<td>( \text{H}_2 ) transfer</td>
<td>(i) Methanogenesis ( 4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} )</td>
<td>-130.747</td>
</tr>
<tr>
<td>(ii) Propionate oxidation ( \text{CH}_3\text{CH}_2\text{COO}^- + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{CO}_2 + 3\text{H}_2 )</td>
<td>71.667</td>
<td></td>
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<tr>
<td>(iii) Butyrate oxidation ( \text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^- + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2 )</td>
<td>48.296</td>
<td></td>
</tr>
<tr>
<td>Formate transfer</td>
<td>(iv) Methanogenesis ( 4\text{HCO}_3^- + 4\text{H}^+ \rightarrow \text{CH}_4 + 3\text{CO}_2 + 2\text{H}_2\text{O} )</td>
<td>-144.543</td>
</tr>
<tr>
<td>(v) Propionate oxidation ( \text{CH}_3\text{CH}_2\text{COO}^- + 2\text{H}_2\text{O} + 2\text{CO}_2 \rightarrow \text{CH}_3\text{COO}^- + 3\text{HCO}_3^- + 3\text{H}^+ )</td>
<td>82.014</td>
<td></td>
</tr>
<tr>
<td>(vi) Butyrate oxidation ( \text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^- + 2\text{HCO}_3^- \rightarrow 2\text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{HCO}_3^- )</td>
<td>55.194</td>
<td></td>
</tr>
<tr>
<td>Net</td>
<td>( \text{i + ii or iv + v (Methanogenesis from propionate)} ) ( \text{CH}_3\text{CH}_2\text{COO}^- + \frac{1}{2}\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \frac{1}{4}\text{CH}_4 + \frac{3}{4}\text{CO}_2 )</td>
<td>-26.393</td>
</tr>
<tr>
<td>( \text{i + iii or iv + vi (Methanogenesis from butyrate)} ) ( \text{CH}_3\text{CH}_2\text{COO}^- + \text{H}_2 + \frac{3}{4}\text{CO}_2 \rightarrow 2\text{CH}_3\text{COO}^- + \text{H}^+ + \frac{3}{4}\text{CH}_4 )</td>
<td>-17.078</td>
<td></td>
</tr>
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*Calculated for a temperature of 37°C from free energies of formation from the elements (40).*

**FIG. 1.** Free-energy change of propionate oxidation, butyrate oxidation, and methanogenesis at concentrations measured in an anaerobic digester (7, 8, 9) except formate and \( \text{H}_2 \) concentrations. (A) With \( \text{H}_2 \) as the interspecies intermediate. Methanogenesis, methane production from \( \text{H}_2 \) and \( \text{CO}_2 \) (reaction i of Table 1); Propionate, propionate oxidation to \( \text{H}_2 \), \( \text{CO}_2 \), and acetate (reaction ii of Table 1); Butyrate, butyrate oxidation to acetate (reaction iii of Table 1). \( \text{H}_2 \) concentration was calculated from its partial pressure (43). (B) With formate as intermediate. Methanogenesis, methane production from formate (reaction iv of Table 1); Propionate, propionate oxidation to acetate and formate (reaction v of Table 1); Butyrate, butyrate oxidation to acetate and formate (reaction vi of Table 1).
cultures were incubated on a shaker. Medium was solidified for roll tube isolations by including 20 g of agar per liter.

**Analytical techniques.** Methane was quantified by gas chromatography with flame ionization detection (7), and H$_2$ was measured by the mercury vapor method (13). H$_2$ concentration was calculated from its partial pressure, with 1 Pa equal to 5.94 nM (43). During growth of cocultures, H$_2$ production and degradation may be rapid, but because the average H$_2$ concentration in the aqueous phase is not expected to change significantly during growth, mass transfer between the gas and aqueous phases should be small and the concentrations should be in equilibrium. Thus, we measured H$_2$ partial pressure in the gas phase and from it calculated (43) the dissolved H$_2$.

**Calculations of interspecies diffusion of H$_2$ and formate.** For cell concentrations of $10^6$ to $10^7$ organisms per ml, the average distance between cells is approximately 100 µm. This distance is large compared with the size of the organisms, so it was anticipated that concentrations of extracellular metabolites would only deviate from bulk phase in the immediate vicinity of the organisms. This assumption allowed mass transfer to or from a cell to be modeled as radial diffusion from a sphere into a semi-infinite medium. The diffusion equation in spherical coordinates is:

$$\frac{\partial C}{\partial t} = D \left( \frac{\partial^2 C}{\partial r^2} + \frac{2}{r} \frac{\partial C}{\partial r} \right)$$  \hspace{1cm} (1)

where $C$ is the concentration at any point in time and space, $D$ is the diffusion coefficient for the metabolite of interest (H$_2$ or formate), and $r$ is the distance from the organism.

By assuming constant bulk-phase and cell surface concentrations, the solution to equation 1 is (19):

$$\frac{C - C_0}{C_1 - C_0} = -\frac{a}{r} \text{erfc} \left( \frac{r - a}{2\sqrt{D}t} \right)$$  \hspace{1cm} (2)

where $C_0$ is the bulk-phase concentration, $C_1$ is the cell surface concentration, $a$ is the radius of the cell, and $\text{erfc}(\cdot)$ is the complimentary error function. A cell diameter of 1 µm was used in the simulations.

A useful algorithm for the complementary error function [erfc($x$)] is given by Press et al. (34): $Z = ab(x), T = 1/(1 + 0.5 \times Z), \text{erfcc} = T \times \exp(-T^2 - 1.26551223 + T \times (0.00002368 + T \times (0.37409196 + T \times (0.99678418 + T \times (-0.18628806 + T \times (0.27886807 + T \times (-1.13520398 + T \times (1.48851587 + T \times (-0.82215223 + T \times 0.17087277))))))))$. If $x \geq 0$, then erfc($x$) = erfcc; if $x < 0$, then erfc($x$) = 2 - erfcc.

Diffusion coefficients for H$_2$ and formate in water at 25°C are 0.045 and 0.15 mm$^2$/s, respectively (20). In contrast, the diffusion coefficient for the organisms themselves is 0.00001 mm$^2$/s.

We have developed a Lotus (Lotus Development Corp., Cambridge, Mass.) spreadsheet (IBM-compatible format) for these calculations which can be obtained by sending us a blank diskette.

**RESULTS**

**Isolation of S. Wolfei LYB.** Our inoculum was 1 ml of effluent (i.e., 1 ml of a $10^{-6}$ dilution) from an anaerobic digestor treating bean curd (tofu) processing wastes. This inoculum was added to 5 ml of a rumen-fluid-based medium (32) with 20 mM butyrate as the substrate. The culture degraded butyrate to methane and acetate, and it was maintained by periodic transfer to fresh medium. An H$_2$-using, coccoid methanogen was isolated from the enrich-

![FIG. 2. Growth of S. Wolfei and M. formicicum in a dispersed, syntrophic coculture on butyrate.](http://aem.asm.org/)

ment culture by using roll tubes containing the rumen-fluid-based medium with H$_2$-CO$_2$ as the substrate. The methanogen was purified by three successive colony picks, and purity was ascertained by microscopic examination of cultures grown in liquid medium with or without substrate (H$_2$) and in thioglycolate broth (Difco). We named the methanogen strain 8508 and deposited it in the Oregon Graduate Center culture collection (OGC 60).

A butyrate-oxidizing bacterium was isolated in monoclonic coculture from the butyrate-degrading enrichment culture by using strain 8508 as a lawn in the rumen-fluid-based medium with 20 mM butyrate. We picked an epifluorescent colony from a roll tube inoculated with 10 nl of a $10^{-4}$ dilution of the enrichment culture. Two additional (sequential) times a colony was picked, diluted, and reincubated into roll tube medium with a lawn of strain 8508. Each time a single colony type was evident. The last time, a colony was picked and inoculated into liquid medium, and the butyrate-degrading partner was named strain LYB. Microscopic examination revealed a coculture of a curved rod similar to S. Wolfei (strain LYB) and an epifluorescent, coccoid methanogen (strain 8508). No growth occurred when this culture was used to inoculate thioglycolate broth or butyrate-free MS medium in the absence or presence of air. We inoculated the culture in MS medium with 10 mM butyrate and 20 mM crotonate. After growth, the culture was maintained by three sequential transfers in this medium. We then inoculated dilutions into roll tubes containing MS medium with 20 mM crotonate. A colony was picked, diluted, and reincubated into roll tube MS medium with 20 mM crotonate. After three successive transfers, colonies of the culture of S. Wolfei (strain LYB; OGC 65) were pure.

**Measurement of H$_2$ concentrations in a syntrophic culture.** We selected a coculture of M. formicicum and S. Wolfei as a methanogenic ecosystem in which to study interspecies electron transfer. Microscopic examinations indicated that cells of M. formicicum and S. Wolfei remained dispersed during growth. The H$_2$ concentration was constant (63 nM), and CH$_4$ production proceeded exponentially, as expected (Fig. 2). Because butyrate was the source of H$_2$, net H$_2$ production ceased when butyrate was exhausted. At this time the methanogens continued to catabolize H$_2$ until its concentration dropped to 35 nM. This latter concentration we took to be the lowest at which methanogens can catabolize H$_2$. This was also similar to the final H$_2$ concentration found by Lovely (27) after growth of M. formicicum FJ-1.

We also measured the H$_2$ concentration which could be formed by S. Wolfei in the absence of H$_2$ uptake. We grew S.
H₂ diffusion from the bulk aqueous phase to methanogenic cells. We calculated the rate of diffusion to methanogens of H₂ from solution with H₂ concentration of 63 nM (the concentration measured in a syntrophic, butyrate-degrading coculture; Fig. 2). We used a spherical diffusion model and initial conditions of a 1-μm cell with a cell surface concentration of 0, 17.5, or 35 nM. Figure 3 shows the concentration gradient of H₂ which would rapidly develop (within 0.1 s) near a methanogen maintaining the given cell surface concentration. The H₂ concentration approaches that of the bulk aqueous phase at distances of 10 μm or greater from the cell. Thus, in dispersed cultures, each cell can be considered independently with respect to uptake of H₂ from solutions of low concentration. Figure 4 demonstrates the effect of cell surface H₂ concentration and cell size on the mass transfer rate from a 63 nM solution. It shows that 0.71 amol/s would diffuse to a methanogen with a diameter of 1 μm if it maintained a cell surface concentration of 35 nM, the lowest concentration M. formicicum was able to attain in our experiments. That translates to formation of 55 ml of CH₄ per liter of a digestor each day if it contained 10⁷ H₂-producing methanogens per ml. Further, Fig. 4 shows that further lowering the cell surface concentration of H₂ would have a limited effect on mass transfer to methanogens, resulting in a maximum of 125 ml of CH₄ per liter of digestor each day if methanogens could maintain a cell surface concentration of 0.

Another factor which affects mass transfer is cell size. The cells of M. formicicum were not spheres but rods 0.5 to 0.8 μm wide and 2.5 to 5 μm long (measured during growth with S. wofei on butyrate). However, the rate of diffusion of H₂ to spheres even as large as 4 μm in diameter could not account for rapid methanogenesis (Fig. 4). Therefore, even with the most optimistic assumptions of a large cell surface and low surface concentration of H₂, the calculated rates of H₂ diffusion are much lower than the observed rates of CH₄ synthesis.

Calculation of interspecies diffusion of H₂. Figure 4 also shows that by maintaining surface H₂ concentrations higher than 63 nM, H₂-producing bacteria can maintain a mass transfer of H₂ into solution; thus, the model can also be used for estimation of H₂ diffusion from H₂-producing microbes. Unless microbes are within about 10 μm of each other, the presence of H₂-producing microbes does not affect H₂ uptake except as it affects the concentrations in bulk solution. However, the mass transfer from the producing organisms into bulk solution must equal the mass transfer of H₂ from

FIG. 3. Diffusion of H₂ or formate to a coccus 1 μm in diameter. Initial boundary conditions are an infinite aqueous phase, the indicated concentration of that phase, and the indicated concentration at the cell surface. (A) Steady-state concentration profile of H₂ near a H₂-consuming cell with a cell surface concentration of 0, 17.5, or 35 nM and a bulk-phase concentration of 63 nM. (B) Steady-state concentration profile of formate near a formate-consuming cell with a cell surface concentration of 0, 4.5, or 9.1 μM and bulk-phase concentration of 16.4 μM.

FIG. 4. Effect of cell surface concentration on mass transfer of H₂ and formate to various sizes of spherical cells. (A) Mass transfer from a bulk-solution concentration of 63 nM H₂ to an H₂-consuming cell. (B) Mass transfer from a bulk-solution concentration of 16.4 μM formate to a formate-consuming cell. The upper abscissa indicates the cell surface formate concentration; the lower abscissa shows the cell-surface concentration of H₂ which would be in equilibrium with formate (for comparison to Fig. 4A).
bulk solution to the methanogens in order to maintain a constant \( \text{H}_2 \) concentration in solution.

**Interspecies electron carriers other than \( \text{H}_2 \).** The diffusional limitations of \( \text{H}_2 \) as an interspecies electron carrier indicated that another substance might be responsible for rapid syntrophic degradation of compounds such as propionate and butyrate in methanogenic ecosystems. Although the diffusion coefficient of formate is about one-fifth that of \( \text{H}_2 \), the calculated concentration of formate in our culture medium was 260-fold higher than that of \( \text{H}_2 \) (based on thermodynamic equilibrium between formate and \( \text{H}_2 \)). If such equilibrium exists, 63 nM \( \text{H}_2 \) (equivalent to 10.6 Pa) is at equilibrium with 16.4 \( \mu \text{M} \) formate (conditions of 0.3 atm \( \text{CO}_2 \) [30.4 kPa], \( \text{pH} \) 7.3). We used our spherical diffusion model to calculate formate diffusion to a 1-\( \mu \text{m} \)-diameter spherical bacterium (Fig. 3B). Assuming the cell surface formate concentration was in equilibrium with the lowest measured \( \text{H}_2 \) concentration for the methanogen (35 nM \( \text{H}_2 = 9.1 \mu \text{M} \) formate), the mass transfer rate of formate was 69.3 amol/s per bacterium, 98-fold faster than that of \( \text{H}_2 \). Formate diffusion from a bulk concentration of 16.4 \( \mu \text{M} \) to a population of 10\(^7\) methanogens per ml, with a diameter of 1 \( \mu \text{m} \) and surface formate concentration of 9.1 \( \mu \text{M} \), would give a methanogenic rate of 5.4 liters of \( \text{CH}_4 \) per liter per day (from \( \text{CO}_2 \) reduction).

**Formate dehydrogenase in \( \text{S. wolfei} \).** In order for interspecies formate transfer to be effective for butyrate oxidation, the butyrate-oxidizing bacterium must be able to produce formate and methanogens must catalyze it. Formate utilization by \( \text{M. formicicum} \) has been reported (3, 14), but the ability of \( \text{S. wolfei} \) to produce formate has not. To find out whether \( \text{S. wolfei} \) LYB has formate dehydrogenase activity, we grew it in MS medium with 20 mM crotonate, and after growth was complete we added 50 mM formate and measured the \( \text{H}_2 \) partial pressure and incubated for 1 h at 37°C. During this time, the \( \text{H}_2 \) partial pressure increased from 35 Pa (208 nM) to 8.7 kPa (52 \( \mu \text{M} \)). Thus, \( \text{S. wolfei} \) cells could metabolize formate, apparently oxidizing it and producing \( \text{H}_2 \). During growth on crotonate, the major catabolic reactions of \( \text{S. wolfei} \) are oxidation of crotonate to butyrate and reduction of an equal quantity of crotonate to acetate. Formate (or \( \text{H}_2 \)) production or consumption is not required for these reactions (6), so formate dehydrogenase activity may be constitutive and could function during butyrate catabolism in syntrophic cultures. If formate dehydrogenase and hydrogenase are both in equilibrium with an intracellular electron carrier such as \( \text{NAD} \), the concentration of formate may be calculated from the concentrations of \( \text{H}_2 \) and \( \text{HCO}_3^- \) and the free-energy change of the reaction; this is consistent with the concentrations of \( \text{H}_2 \) measured above.

**DISCUSSION**

The extremely high turnover rates of \( \text{H}_2 \) which would be necessary if \( \text{CO}_2 \) reduction in digestors were due solely to \( \text{H}_2 \) oxidation was one of the factors leading to speculation about other mechanisms of electron transfer. Previously the problem of excessively high \( \text{H}_2 \) turnover rate has been explained by close physical associations or transfer within microenvironments (9, 10, 18). Boone (9, 10) proposed that particles of fermentable biomass may contain microenvironments with higher \( \text{H}_2 \) concentrations than that of the bulk aqueous phase because of rapid fermentation reactions. \( \text{H}_2 \) in such micro-environments could be degraded by methanogens before it diffused out of the microenvironments. Later, studies in the laboratory of Zeikus (18, 41) proposed that close physical associations of syntrophic \( \text{H}_2 \)-producing and \( \text{H}_2 \)-consuming bacteria allowed rapid interspecies \( \text{H}_2 \) transfer. However, such microenvironments may reduce the turnover rate of \( \text{H}_2 \) only if the microenvironments contain significantly higher \( \text{H}_2 \) concentrations than is present in the bulk solution. Thus, the significance of either of these models in explaining \( \text{H}_2 \) turnover in digestors is limited to only those substrates whose oxidation is relatively favorable, such as carbohydrates or amino acids. \( \text{H}_2 \) transfer from propionate and butyrate still requires microenvironments of low \( \text{H}_2 \) concentration, and a rapid turnover rate is still required.

A number of cocultures have been reported which carry out syntrophic catabolism by interspecies transfer of reducing equivalents to methanogens (4, 11, 15, 22, 29, 31, 32, 33, 37, 44); these illustrate the importance of interspecies electron transfer in methanogenic ecosystems. In most of these cocultures, the methanogenic partner is able to use either formate or \( \text{H}_2 \) as the electron acceptor, so it is not clear which one is the interspecies electron carrier. In two reported cocultures, \( \text{H}_2 \) is the only possible interspecies electron carrier because the methanogen is unable to use formate.

**Methanobacterium bryantii** (Methanobacterium sp. strain M.O.H.) is unable to use formate, and it is the methanogenic partner in the \( "\text{Methanobacillus omelianskii}" \) coculture (15) and in a coculture with \( \text{S. wolfei} \) (31). However, \( \text{S. wolfei} \), the nonmethanogenic partner in \( "\text{Methanobacillus omelianskii}" \), produces \( \text{H}_2 \) at concentrations substantially higher than the fatty-acid oxidizers (15), so \( \text{H}_2 \) diffusion is less likely to limit metabolism in this case. The \( \text{S. wolfei} \) organism contains a formate hydrogenlyase system, either when grown axenically (35) or when in coculture (i.e., \( "\text{Methanobacillus omelianskii}" \) (5). The growth of the \( \text{S. wolfei} \) coculture with \( \text{M. bryantii} \) was slow, perhaps because of slow diffusion of \( \text{H}_2 \). When \( \text{S. wolfei} \) is cultured with \( \text{Methanospirillum hungatei} \), which can catalyze formate, growth is much more rapid (31). Further, the \( \text{S. wolfei-M. bryantii} \) coculture grows rapidly as slant cultures (31), suggesting that the more intimate contact relieves problems of interspecies \( \text{H}_2 \) diffusion.

\( \text{CO}_2 \) reduction to methane is the predominant source of methane in the rumen ecosystem, but as in anaerobic digestors, the \( \text{CO}_2 \) concentration of \( \text{H}_2 \) and formate in the rumen make turnover rates difficult to measure (17, 24). Hungate and his co-workers first recognized the potential importance of formate as a methanogenic substrate in the rumen. Carroll and Hungate (17) investigated formate dissimilation in the rumen fermentation and showed that rumen contents rapidly convert formate to \( \text{H}_2 \) and \( \text{CO}_2 \). Later, Hungate and co-workers (24) showed that extracellular formate may be cleaved by nonmethanogens and that the apparent \( K_m \) of methanogens for formate is too low to account for the rate of methanogenesis. The methanogenic rate in the rumen correlates more closely with the \( \text{H}_2 \) concentration than the formate concentration, suggesting that \( \text{H}_2 \) is a more important substrate in that environment (24). Bryant and Wolin (16) suggested that formate produced by fermentative bacteria in the rumen may diffuse to formate-decomposing methanogens or may be split to \( \text{H}_2 \) and \( \text{CO}_2 \) extracellularly in the rumen.

Electron transfer from \( \text{NADH} \) of fatty acid-oxidizing bacteria to \( \text{F}_{240} \) of methanogens requires several steps (Fig. 5): the reduction of an extracellular electron carrier (\( \text{H}^+ \) or \( \text{HCO}_3^- \) to \( \text{H}_2 \) or \( \text{HCOO}^- \)), the diffusion of that carrier into the bulk aqueous phase, the diffusion from the bulk aqueous phase to a methanogenic bacterium, and the oxidation of that electron carrier by a methanogen, forming reduced \( \text{F}_{240} \).
showed that *S. wofei* can form H₂ from formate, suggesting that it may have an NAD-linked formate dehydrogenase as well as an NAD-linked hydrogenase.

Our diffusion calculations comparing H₂ and formate were based on an assumption that interspecies electron transfer is not kinetically limited by enzymatic formation or consumption of H₂ or formate at the cell surface but that that concentration is determined by the thermodynamics of the producing and consuming reactions. Our calculations of interspecies H₂ transfer based on measured concentrations indicates that interspecies H₂ diffusion cannot occur at a sufficient rate to account for syntrophic catabolism of propionate and butyrate in digesters or cocultures. Even if methanogens could maintain their cell surfaces with a H₂ concentration of 0, H₂ could not diffuse to their surfaces fast enough to account for the rate of methanogenesis. On the basis of the assumption that interspecies diffusion of H₂ and formate is the rate-limiting step, formate diffusion accounted for 98-fold more interspecies electron transfer than did H₂ diffusion, and interspecies formate transfer could account for the measured methanogenic rate. The importance of formate as a methanogenic substrate is also reflected by the widespread capability for formate dissimilation among the *Methanobacterium* and *Methanoregula* (12).

An important implication of our diffusion calculations is that rapid diffusion of electron carriers between cell surfaces and the bulk aqueous phase requires steep concentration gradients, so diffusion must be considered when kinetic parameters such as Kₘ are measured for cell-bound enzymes. Kinetic parameters of hydrogenases of methanogens have been determined by using whole cells and measuring the depletion of H₂ from solution (1, 25, 26, 36, 39). However, Michaelis-Menton kinetics are based on the assumption that enzymes, substrates, and products are each at uniform concentrations throughout the solution. The data presented in this report indicate that the H₂ or formate concentration is not uniform during such experiments and that those experiments measured reaction rates of hydrogenases at cell surfaces where the enzymes were exposed to much lower concentrations of H₂ than were present in the bulk phase (Fig. 3). Thus, determinations of kinetic constants for enzymes such as hydrogenases must consider the steep concentration gradients close to the cell surfaces.

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**LITERATURE CITED**


